UNIVERSITAT POLITÈCNICA DE VALÈNCIA

INVOLVEMENT OF THE MELANOCORTIN SYSTEM IN THE REGULATION OF CIRCADIAN AND BEHAVIOURAL MECHANISMS IN ZEBRAFISH

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Involvement of the melanocortin system in the regulation of circadian and behavioural mechanisms in zebrafish

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Informa que la Tesis Doctoral titulada `Involvement of the melanocortin system in the regulation of circadian and behavioural mechanisms in zebrafish' ha sido realizada por Alejandra Godino Gimeno en el Instituto de Acuicultura de Torre de la Sal (IATS) bajo su dirección y que, una vez revisado y comprobado el trabajo, considera que reúne los requisitos necesarios para la obtención del grado de Doctor, por lo que autoriza su presentación.

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Esta tesis ha sido realizada por **Alejandra Godino Gimeno** contratada predoctoral, a través del programa de 'Ayudas para la formación de personal investigador' (FPI) BES-2017-082424 de la Agencia Estatal de Investigación, en Instituto de Acuicultura Torre de la Sal (IATS) del Consejo Superior de Investigaciones Científicas (CSIC) en el Grupo de investigación de Control de la Ingesta en Peces dirigido por José Miguel Cerdá Reverter, director de esta tesis. Los trabajos llevados a cabo en esta tesis han sido financiados por Ministerio de Ciencia, Innovación y Universidades (MICIU) a través de los siguientes proyectos:

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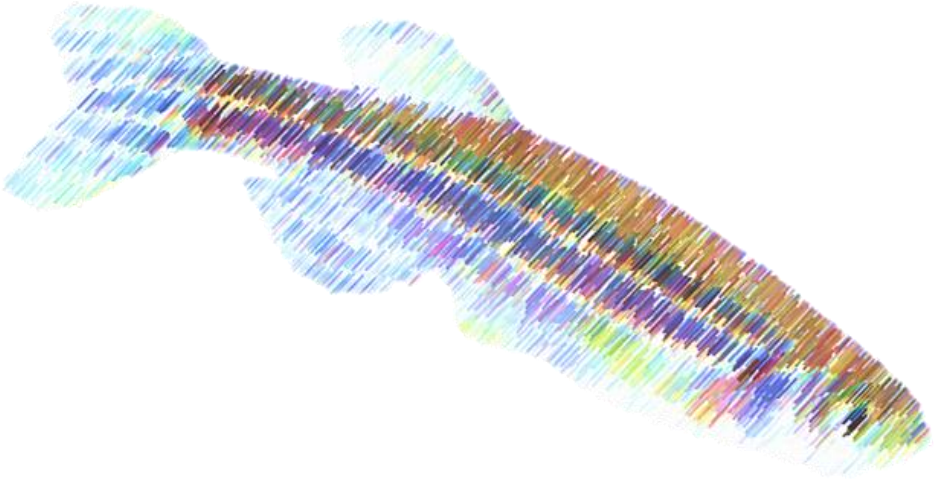

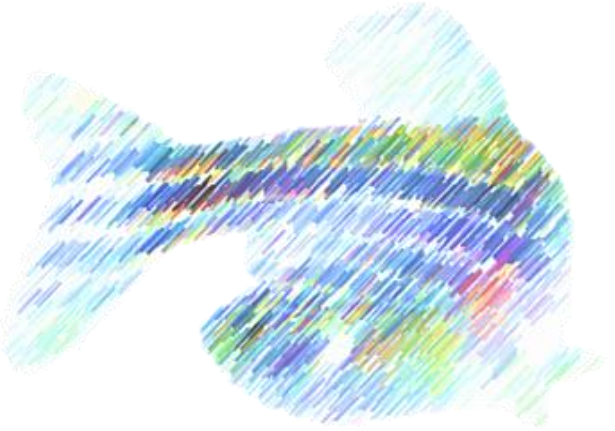

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General abbreviations

5HIAA: 5-hydroxy indoleacetic acid

5HT: 5-hidroxitriptamina, serotonin

5HTP: 5-hydroxytryptophan

ACTH: adrenocorticotropin hormone

AGRP: agouti-related protein

ASIP: agouti signalling protein

CRH: corticotropin-releasing hormone

DA: dopamine

DEG: Database of Essential Genes

DOPAC: dihydroxyphenylacetic acid

DRN: dorsal raphe nucleus

FDR: false discovery rate

GO: Gene ontology

HPA: hypothalamic–pituitary–adrenal axis

HPI: hypothalamic–pituitary–interrenal axis

HPLC: high performance liquid chromatography

ICV: intra-cerebro-ventricular

KEGG: Kyoto Encyclopedia of Genes and Genomes

LA: locomotor activity

L-dopa: L-dihydroxyphenylalanine

LTM: long-term memory

MC1R-MC5R: melanocortin receptors 1 to 5

mPFC: medial prefrontal cortex

MRAP: melanocortin accessory protein

MSH: melanocyte-stimulating hormone

NA: noradrenaline

NAc: nucleus acumens

NOT: novel object test

NTDT: novel tank diving test

OFT: open field test

PC: prohormone convertase

PI: pars intermedia

POA: preoptic area

POMC: proopiomelanocortin precursor

PVN: paraventricular nucleus

qPCR: quantitative polymerase chain reaction

RPD: rostal pars distalis

SCN: suprachiasmatic nucleus

STM: short-term memory

RESUMEN

El sistema de melanocortina es una estructura clave en la regulación de una amplia gama de funciones fisiológicas que incluyen la melanogénesis, la respuesta al estrés y el equilibrio energético, mediante la unión a una familia de receptores acoplados a la proteína G específicos (MC1R-MC5R). La sobreexpresión de agonistas inversos, la proteína de señalización agutí (Asip) y la proteína relacionada con agutí (Agrp) da como resultado un aumento de la ingesta de alimentos, de crecimiento lineal y de peso corporal. Asip regula la polaridad de pigmentación dorsoventral a través del MC1R, y la sobreexpresión induce obesidad en ratones al unirse al Mc4r central. La sobreexpresión de *asip1* en el pez cebra transgénico (*asip1-Tg*) mejora el crecimiento, sin afectar la acumulación lipídica (obesidad), incluso cuando se alimentan bajo regímenes inductores severos. Los peces *asip1-Tg* no necesitan comer más para crecer más y más rápido, lo que sugiere una mayor eficiencia alimentaria. Además, los peces *asip1-Tg* criados en alta densidad son capaces de crecer mucho más que los peces de tipo salvaje (WT) criados en baja densidad, aunque los peces *asip1-Tg* parecen ser más sensibles al estrés por hacinamiento que los peces WT.

El análisis transcriptómico comparativo del intestino de *asip1-Tg* refleja una expresión diferencial de transportadores aminoácidicos, monocarboxilatos, transportadores iónicos y de vitaminas. La sobreexpresión reduce la integridad del epitelio intestinal aumentando su permeabilidad paracelular y potencia el transporte electrogénico de aminoácidos. Así, la combinación de resultados transcriptómicos y electrofisiológicos sugiere que los peces transgénicos poseen mayor capacidad para la absorción de nutrientes y, por extensión una mejora en la eficiencia alimenticia que podría explicar, en parte, ese crecimiento diferencial bajo tasas de ingesta similares.

Esta tesis tuvo también como objetivo investigar el fenotipo comportamental de los animales *asip1-Tg*, específicamente si mantienen un fenotipo dominante

asociado con una mayor tasa de alimentación. Los resultados experimentales de luchas diádicas y el test de reflejo especular muestran, por el contrario, un carácter reactivo/subordinado en los *asip1-Tg* que aboga directamente por una participación del sistema de melanocortinas en la regulación del comportamiento de peces. Mejorar la motivación alimentaria sin promover la agresividad en los peces, evitando la amenaza a las poblaciones nativas en caso de un escape de las instalaciones de cultivo, hace que la inhibición del sistema de melanocortinas, a través de la sobreexpresión de *asip1*, sea un objetivo factible para el desarrollo de líneas genéticamente modificadas, cuya comercialización está autorizada por la agencia gubernamental estadounidense de alimentos y medicamentos (FDA).

El perfil subordinado de los animales *asip1-Tg*, junto con una activación del eje del estrés, sugiere que estos animales pueden mostrar un comportamiento de ansiedad. Con el fin de analizarlo, utilizamos diversos tests comportamentales ya estandarizados que incluyen el test de "campo abierto" (OF), "nuevo objeto (NO)" y "nuevo tanque (NTDT)". En todos los test los resultados indicaron que los peces *asip1-Tg* muestran un comportamiento de ansiedad que además está íntimamente relacionado con una severa disminución de los niveles centrales de serotonina (5HT) y dopamina y elevación de su recaptación neuronal y degradación. La administración de fluoxetina, un inhibidor de la recaptación de serotonina (ISRS), es capaz de recuperar el fenotipo comportamental salvaje, mitigando el comportamiento de ansiedad en los peces transgénicos y rescatando los niveles centrales de 5HT.

Este comportamiento de ansiedad podría repercutir en una alteración del comportamiento locomotor de los animales, por ello estudiamos las diferencias potenciales en los ritmos circadianos de actividad locomotora (RCAL). Los resultados muestran que los animales *asip1-Tg* exhiben una disrupción completa del ritmo de actividad, con una actividad muy elevada durante todo el ciclo diario, pero especialmente durante la noche, periodo en el cual los animales WT reducen a niveles basales

su actividad. Esta disrupción es concomitante con una desaparición del ritmo diario de serotonina y melatonina, hormona secretada desde la pineal durante el periodo nocturno que gobierna los periodos de sueño/vigilia, debido a la inhibición de su secreción nocturna. Además, los resultados muestran una pérdida de ritmos de expresión de genes reloj (*per1a* y *clock1a*) mostrando también una disrupción del reloj molecular. La incubación, in vitro, de glándulas pineales con Asip1 produjo una inhibición de la secreción de melatonina replicando los resultados obtenidos in vivo y demostrando un efecto directo de Asip1, a través de receptores específicos, sobre la fisiología de la pineal.

El pez cebra se ha convertido en un organismo modelo importante para estudios de los efectos metabólicos sobre los procesos emocionales y cognitivos. En esta tesis doctoral, se utilizó como modelo para investigar los efectos de la obesidad inducida por sobrealimentación sobre el comportamiento de ansiedad y sobre los procesos cognitivos (aprendizaje y memoria). La obesidad no tuvo ningún efecto sobre la ansiedad, pero produjo una disminución de la memoria a corto plazo, estudiada mediante test de condicionamiento aversivo. Este estudio proporciona, además, un protocolo fiable para evaluar el efecto de las enfermedades metabólicas en la función cognitiva y conductual, lo que respalda al pez cebra como modelo para la neurociencia cognitiva y conductual.

RESUM

El sistema de melanocortina és una estructura clau en la regulació d'una ampla gamma de funcions fisiològiques que inclouen la melanogènesi, la resposta a l'estrès i l'equilibri energètic, mitjançant la unió a una família de receptors acoblats a la proteïna G específics (MC1R-MC5R). La sobreexpressió d'agonistes inversos, la proteïna de senyalització agutí (Asip) y la proteïna relacionada con agutí (Agrp) dona com a resultat un augment de la ingesta d'aliments, de creixement lineal i de pes corporal. Asip regula la polaritat de pigmentació dors-ventral a través del MC1R, y la sobreexpressió indueix obesitat en ratolins en unir-se al MC4R central. La sobreexpressió de asip1 en el peix zebra transgènic (*asip1-Tg*) millora el creixement, sense afectar l'acumulació lipídica (obesitat), inclús quan s'alimenten sota règims inductors severs. Los peces *asip1-Tg* no necessiten menjar més per a créixer més i més ràpid, lo qual suggereix una major eficiència alimentària. A més a més, els peixos *asip1-Tg* criats en alta densitat són capaces de créixer molt més que els peixos de tipus salvatge (WT) criats en baixa densitat, malgrat que els peixos *asip1-Tg* semblen ser més sensibles a l'estrès per amuntegament que els peixos WT.

L'anàlisi transcriptòmic comparatiu de l'intestí de *asip1-Tg* reflecteix una expressió diferencial de transportadors aminoacídics, monocarboxilats, transportadors iònics i de vitamines. La sobreexpressió redueix la integritat de l'epiteli intestinal augmentant la seua permeabilitat paracel·lular i potencia el transport electrogènic d'aminoàcids.

Per tant, la combinació de resultats transcriptòmics i electrofisiològics suggereix que els peixos transgènics posseeixen major capacitat per l'absorció de nutrients i, per extensió una millora en la eficiència alimentària que podria explicar, en part, eixe creixement diferencial sota taxes d'ingesta similars.

Aquesta tesi tingué també com a objectiu investigar el fenotip comportamental dels animals *asip1-Tg*, específicament si mantenien un fenotip dominant associat amb una major taxa d'alimentació. Els resultats experimentals utilitzant lluites diàdiques i el test de reflex especular mostren, pel contrari, un caràcter reactiu/subordinat en los *asip1-Tg* que advoca directament per una participació del sistema de melanocortines en la regulació del comportament de peixos. Millorar la motivació alimentària sense promoure l'agressivitat en los peces, evitant l'amenaça a les poblacions natives en cas d'un escapament de les instal·lacions de cultiu, fa que la inhibició del sistema de melanocortines, a través de la sobreexpressió de asip1, siga un objectiu factible per al desenvolupament de línies genèticament modificats, la qual comercialització està autoritzada per l'agencia governamental estatunidenca d'aliments i medicaments (FDA).

El perfil subordinat dels animals *asip1-Tg*, junt amb una activació de l'eix de l'estrès, suggereix que aquests animals poden mostrar un comportament d'ansietat. Amb el fi d'analitzar-lo, utilitzem diversos test comportamentals ja estandaritzats que inclouen el test de "campo obert" (OF), "nou objecte (NO)" i "nou tanc (NTDT)". A tots els test els resultats indicaren que els peixos *asip1-Tg* mostren un comportament d'ansietat que a més a més està íntimament relacionat amb una severa disminució dels nivells centrals de serotonina (5HT) i dopamina i elevació de la seua recaptació neuronal i degradació. L'administració de fluoxetina, un inhibidor de la recaptació de serotonina (ISRS), es capaç de recuperar el fenotip comportamental salvatge, mitigant el comportament d'ansietat en els peixos transgènics i rescatant els nivells centrals de 5HT.

Este comportament d'ansietat podria repercutir en una alteració del comportament locomotor dels animals, per la qual cosa vam estudiar les diferències potencials en els ritmes circadians d'activitat locomotora (RCAL). Els resultats mostren que els animals *asip1-Tg* exhibeixen una disrupció completa del ritme

d'activitat, amb una activitat molt elevada durant tot el cicle diari, però especialment durant la nit, període al qual els animals WT redueixen a nivells basals la seua activitat. Esta disrupció es concomitant amb una desaparició del ritme diari de serotonina i melatonina, hormona secretada des de la pineal durant el període nocturn que governa els períodes de somni/vigília, a causa de la inhibició de la seua secreció nocturna. A més a més, els resultats mostren una pèrdua de ritmes de expressió de gens rellotge (*per1a* y *clock1a*) mostrant també una disrupció del rellotge molecular. La incubació, in vitro, de glàndules pineals con Asip1 va produir una inhibició de la secreció de melatonina replicant els resultats obtinguts in vivo y demostrant un efecte directe de Asip1, a través de receptors específics, sobre la fisiologia de la pineal.

El peix zebra s'han convertit en un organisme model important per a estudis dels efectes metabòlics sobre els processos emocionals i cognitius. En esta tesi doctoral, se va utilitzar com a model per investigar els efectes de la obesitat induïda per sobrealimentació sobre el comportament d'ansietat i sobre els processos cognitius (aprenentatge i memòria). L'obesitat no va tindre cap efecte sobre l'ansietat, però va produir una disminució de la memòria a curt termini, estudiada mitjançant tests de condicionament aversiu. Aquest estudi proporciona, a més a més, un protocol fiable per a avaluar l'efecte de les malalties metabòliques en la funció cognitiva i conductual, lo que recolza al peix zebra com a model per a la neurociència cognitiva i conductual.

SUMMARY

The melanocortin system plays a key role in the regulation of a wide range of physiological functions including melanogenesis, stress response and energy balance, through binding to a family of specific G protein-coupled receptors (MC1R-MC5R). Overexpression of inverse agonists, agouti-signalling protein (Asip) and agouti-related protein (Agrp) results in increased food intake, linear growth and body weight. Asip regulates dorso-ventral pigmentation polarity through MC1R, and overexpression induces obesity in mice by binding to the central MC4R. Overexpression of *asip1* in transgenic zebrafish (*asip1-Tg*) enhances growth, without affecting lipid accumulation (obesity), even when fed under severe inducing regimens. The *asip1-Tg* fish do not need to eat more to grow bigger and faster, suggesting increased feed efficiency. In addition, *asip1-Tg* fish reared at high density are able to grow much larger than wild-type (WT) fish reared at low density, although *asip1-Tg* fish appear to be more sensitive to overcrowding stress than WT fish.

Comparative transcriptomic analysis of *asip1-Tg* gut reflects differential expression of amino acid, monocarboxylate, ionic and vitamin transporters. Overexpression reduces the integrity of the intestinal epithelium by increasing its paracellular permeability and enhances electrogenic amino acid transport. Thus, the combination of transcriptomic and electrophysiological results suggests that transgenic fish possess a greater capacity for nutrient absorption and, by extension, an improvement in feed efficiency that could explain, in part, this differential growth under similar intake rates.

This thesis also aimed to investigate the behavioural phenotype of *asip1-Tg* animals, specifically whether they maintain a dominant phenotype associated with a higher feeding rate. Experimental results using dyadic fighting and the specular reflex test show, on the contrary, a reactive/subordinate character in *asip1-Tg* which directly

argues for an involvement of the melanocortin system in the regulation of fish behaviour. Improving feeding motivation without promoting aggression in fish, thus avoiding the threat to native populations in case of an escape from culture facilities, makes the inhibition of the melanocortin system, through the overexpression of asip1, a feasible target for the development of genetically modified lines, whose commercialisation is now authorised by the US governmental Food and Drug Agency (FDA).

The subordinate profile of the *asip1-Tg* animals, together with an activation of the stress axis, suggests that these animals may exhibit anxiety-like behaviour. In order to analyse this, we used several standardised behavioural tests including the "open field" (OF), "new object (NO)" and "novel tank diving test (NTDT)" tests. In all tests the results indicated that *asip1-Tg* fish show a behaviour similar to our concept of anxiety which is also closely related to a severe decrease in central serotonin (5HT) and dopamine levels as well as the elevation of their neuronal reuptake and degradation. The administration of fluoxetine, a serotonin reuptake inhibitor (SSRI), is able to recover the wild-type behavioural phenotype, mitigating anxiety behaviour in transgenic fish and restoring central 5HT levels.

This anxiety-like behaviour could have repercussions on the locomotor behaviour of the animals, so we studied potential differences in circadian rhythms of locomotor activity (LARC). The results show that *asip1-Tg* animals exhibit a complete disruption of the activity rhythm, with very high activity levels throughout the daily cycle, but especially during the night, a period in which WT animals reduce their activity to basal levels. This disruption is concomitant with a disappearance of the daily rhythm of serotonin and melatonin, a hormone secreted from the pineal gland during the nocturnal period that governs the sleep/wake periods, due to inhibition of their nocturnal secretion. In addition, the results show a loss of clock gene expression rhythms (*per1a* and *clock1a*) also showing a disruption of the molecular clock. Incubation, in

vitro, of pineal glands with Asip1 produced an inhibition of melatonin secretion replicating the results obtained in vivo and demonstrating a direct effect of Asip1, through specific receptors, on pineal physiology.

The zebrafish has become an important model organism for studies of metabolic effects on emotional and cognitive processes. In this PhD thesis, it was used as a model to investigate the effects of overfeeding-induced obesity on anxiety-like behaviour and cognitive processes (learning and memory). Obesity had no effect on anxiety, but produced a decrease in short-term memory, studied by means of aversive conditioning tests. This study also provides a reliable protocol for assessing the effect of metabolic diseases on cognitive and behavioural function, supporting zebrafish as a model for cognitive and behavioural neuroscience.

General

Introduction

The melanocortin system

The melanocortin system is essential in regulating pigmentation, stress, food intake and energy balance in vertebrates. In mammals, the system is compounded by 5 G-coupled receptors that are agonised by several peptides derived from the posttranscriptional processing of proopiomelanocortin precursor (POMC) and antagonized by agouti-related protein AGRP and agouti-signalling protein (ASIP).

POMC

Melanocortins are peptides derived from the posttranscriptional processing of proopiomelanocortin precursor (POMC). This prepropeptide has three domains that cleavage melanocyte-stimulating hormone (MSH), characterised by a core sequence HFRW that confers a high ligand-receptor affinity (Castro & Morrison, 1997). The amino-terminal region pro-γ-MSH yields γ-MSH, the adrenocorticotropin central domain: α-MSH and ACTH; and the C-terminal region ß-lipotropin encodes ß-MSH and ß-endorphin (Rocha et al., 2019; Navarro et al., 2016).

In teleost, the whole genome was duplicated 300 million years ago. As a result, two paralogue genes of POMC are found, *pomca* and *pomcb*. Partitioning of the expression has demonstrated a subfunctionalisation of these paralogue genes (de Souza et al., 2005). Expression of *pomca* has been found in a region of the hypothalamus called nucleus lateralis tuberis and in the pituitary pars intermedia (PI) and rostral pars distalis (RPD). In contrast, *pomcb* expression has been located in the preoptic area (POA) and PI. Although POA expression was also found in carp, this pattern does not match zebrafish (*Danio rerio*), where *pomcb* is found only in the PI (de Souza et al., 2005; Nasif et al., 2015). The POMC processing can result in different products. In the corticotrophs of the RPD, the prohormone convertase PC_1 cleavages POMC to

produce ACTH, whereas, in melanotrophs of the PI, $PC_1$and PC_2 generate α-MSH and ß-endorphin (Cerdá-Reverter et al., 2011; Mountjoy, 2015).

Functions of melanocortin receptors

In tetrapods, the melanocortin system functions through 5 receptors coupled to G-proteins (MC1R-MC5R). The first one, MC1R, is expressed in the skin melanocytes where the binding of α-MSH induces pigmentation by synthesising the brown pigment, eumelanin. All MCRs have an affinity for MSHs, except MC2R, which is agonised only by the adrenocorticotropin hormone (ACTH), driving the synthesis of cortisol in the adrenal cortex in mammals and the interrenal tissue in teleost (Cerdá-Reverter et al., 2011; Cone, 2006). This receptor needs the melanocortin accessory protein 1 (MRAP1) for trafficking to the plasma membrane. MRAP2 has been further reported as an inhibitor of the functional expression of MC2R by competing with MRAP1 (Chan et al., 2009). Both accessory proteins can modulate the activity of all other MCRs by increasing or reducing signalling and presence in the cellular membrane (Cerdá-Reverter et al., 2013; Chan et al., 2009). Zebrafish have two paralogues of MRAP2 regulating melanocortin receptor roles in different manners. In the case of MC4R, these genes play a crucial role in development since MRAP2a inhibits its activity, promoting growth in larvae. However, once they feed, MRAP2b increases the response to α-MSH, facilitating MC4R role in the regulation of energy homeostasis (Sebag et al., 2013).

In mammals, receptors 3 and 4 are present in the hypothalamic neurons regulating energy balance by binding γ-MSH and α-MSH with higher affinity, respectively. Teleost lack γ-MSH, and perciforms, the most modern teleost, do not express MC3R either, suggesting the coevolution of receptor/ ligand tandem. MC4R is constitutively activated and agonised by MSH to evoke satiation (Nijenhuis et al., 2001a). The

absence of MC4R expression in $MC4R^{-/-}$ mice results in a morbid obese phenotype linked to hyperphagia, hyperinsulinemia, and hyperglycemia (Huszar et al., 1997), administration of the agonist melanotan II (MTII) reduce food intake in wild-type mice but not in $MC4R^{-/-}$ mice, proving the role of MC4R in regulating energy homeostasis (Marsh et al., 1999). In humans, MC4R mutations represent the most common cause of inherited morbid obesity characterized by hyperphagia, augmented caloric intake, fat mass accumulation, elevated linear growth, and hyperinsulinemia (Farooqi et al., 2000; Hinney, 1999; Vaisse et al., 2000).

Additionally, MC4R has an affinity for ACTH after interacting with MRAP2a (Agulleiro et al., 2013; Soletto et al., 2019). In zebrafish, MC4R expression has been found in the brain, eye, intestine, and ovary (Ringholm et al., 2002). Focusing on the brain, MC4R has been found in POA (the equivalent of mammalian paraventricular nucleus, PVN) and hypothalamic lateral tuberal nucleus (the homologue of the arcuate nucleus) in goldfish *(Carassius auratus*) (Cerdá-Reverter et al., 2003a), seabass (*Dicentrarchus labrax*) (Sánchez et al., 2009) and medaka (*Oryzias latipes*) (Liu et al., 2019).

MC5R is expressed in the brain and peripheral tissues regulating secretion in exocrine glands (Chen et al., 1997) In goldfish (*Carassius auratus*), MC5R is expressed peripherally in the kidney, spleen, skin and retina, while in the brain, is present in the telencephalon, preoptic area, thalamus and hypothalamus, mesencephalon, vagal lobes, reticular formation and spinal cord (Cerdá-Reverter et al., 2003b). Two paralogues of this receptor are present in the genome of zebrafish, MC5Ra is present in the brain, ovary, intestine, and eye MC5Rb is also found in the heart (Ringholm et al., 2002).

Melanocortin receptor antagonists

The melanocortin receptor agonists compete with two endogenous antagonists, the agouti-related protein (AGRP) and the agouti-signalling protein (ASIP). Due to extra genome duplication in teleost, 4 genes encode for agouti family of peptides (Kurokawa et al., 2006; Kehoe & Volkoff, 2007 & Murashita et al., 2009). AgRP neurons release AGRP in the arcuate nucleus in mammals and the lateral tuberal nucleus in teleost (Forlano & Cone, 2007; Ollmann et al., 1997). These neurons are activated by fasting and AGRP compete with melanocortin agonists thus inducing food intake by inhibiting the activity of MC4R, which evokes hunger (Nijenhuis et al., 2001; Cerdá-Reverter & Peter, 2003; Sánchez et al., 2009)., The α-MSH binding to MC4R is not required for the receptor activity since it is constitutively activated to induce satiety. AGRP binding can modulate prevent this constitutive activity by acting as an inverse agonist (Nijenhuis et al., 2001; Sánchez et al., 2009). Furthermore, AGRP regulates homeostasis by antagonising MC3R (Koch Horvath, 2014; Cerdá-Reverter et al., 2011; Rossi et al., 1998).

In teleost, *agrp* has 2 paralogues; *agrp1* is expressed in the lateral tuberal nucleus in the head kidney and ovary (Agulleiro et al., 2014). In goldfish, fasting increases hypothalamic *agrp1* expression (Cerdá-Reverter et al., 2003a), while its overexpression in zebrafish is linked to linear growth and obesity, as well as in mice (Koch & Horvath, 2014; Song & Cone, 2007; Rossi et al., 1998). However, opposite effects have been reported in morpholino *agrp1* knockdown zebrafish (Zhang, 2012) and confirmed by the reduced food intake reported in zebrafish larvae in which *agrp1*-expressed neurons were genetically ablated (Shainer et al., 2019). These results are consistent with those obtained in mice (Gropp et al., 2005; Luquet et al., 2005). While *agrp1* regulates food intake, *agrp2* is implicated in the stress response. In the preoptic area, *agrp2*-expressing neurons project to the pars distalis of the pituitary (Herget

et al., 2014), where this antagonist is secreted to blood circulation. Interestingly, *agrp2* might act as a neurohormone in interregnal tissue where antagonising MCRs suppress cortisol synthesis and release (Shainer et al., 2019). Both paralogues of *agrp* might communicate in the hypothalamus since synaptic connections have been found, which could mean a relationship between the physiological processes they are involved in (Shainer et al., 2017).

The melanocortin system has an additional endogenous antagonist, ASIP (in vertebrates different from mouse) but Agouti mouse. It is produced in the hair follicle and inhibits the activity of MC1R, stimulating the synthesis of pheomelanin and resulting in an agouti pelage in mammals. Interestingly, when ASIP expression is ubiquitous, it antagonises MC4R in the brain, as occurs in mice with the lethal yellow mutation (Ay/Ay), resulting in a yellowish obese phenotype that develops hyperglycemia, hyperphagia and an increased linear growth (Lu et al., 1994; Michaud et al., 1993; Miller et al., 1993). As mentioned, transgenic MC4R knockdown mice are obese as well but have brown hair (Huszar et al., 1997). Also, the intracerebroventricular administration of Agouti mimetic chemicals promotes food intake, while agonist injections inhibit it (Fan et al., 1997).

In teleost, two *asip* paralogues have been described, *asip1* and *asip2*. The latter is expressed in peripheral tissues, such as the ovary and posterior kidney, and in the brain, matching with *agrp1* distribution and in the pineal gland (Agulleiro et al., 2014; Kurokawa et al., 2006b). Its function is related to the regulation of short-term fasting since *asip2* expression rises in sea bass during the first day of food privation (Agulleiro et al., 2014). Asip1 is involved in the establishment of the dorsoventral pigmentation pattern in fish, it is expressed mainly in ventral skin, promoting the synthesis of eumelanin by antagonising MC1R and its overexpression in transgenic zebrafish *asip1-Tg* results in the disruption of dorsoventral pigmentation (Cerdá-Reverter et al., 2005; Ceinos et al., 2015). Also, these transgenic fish display an

incremented food intake, linear growth, and weight, although this phenotype does not match the obese one observed in *agrp1* transgenic zebrafish fish (Song & Cone, 2007; Guillot et al., 2016; Godino-Gimeno et al., 2020).

Melanocortin system and behaviour

The melanocortin system is implicated in the regulation of feeding, aggressiveness, stress response, anxiety-like behaviour, and circadian rhythms, although underlying mechanisms are not fully described.

Feeding behaviour

Feeling hungry motivates an organism to seek food to restore energy balance. Negative energy balance is characterized by nutrient deficiency followed by low levels of leptin and insulin release by the adipose tissue and pancreas, respectively (Friedman, 2019; Woods et al., 2006). Both hormones, leptin, and insulin, in addition to glucocorticoids, regulate POMC and AgRP neurons in the arcuate nucleus thanks to specific receptors. Low leptin/insulin levels inhibit POMC expression and MSH release while promoting the opposite effect on AgRP neurons (Woods et al., 2006; Loh et al., 2017; Friedman, 2019). Moreover, fasting active ghrelin, a gastrointestinal hormone that signals to the brain through the vague nerve, also stimulates AgRP neurons through specific receptors (Date et al., 2005). As described previously, AGRP inhibits the activity of MC4R-expressing neurons, thus inducing hunger and food-seeking (Aponte et al., 2011; Padilla et al., 2016). Recent research carried out in mice indicates that MC4Rs in the dorsomedial striatum are responsible for reward food-seeking behaviour (Allen et al., 2022).

In humans, deficient MC4R function enhances emotional overeating, binge behaviour episodes and palatable high-fat food intake while attenuating satiation therefore inducing food addiction-like effects (Micioni Di Bonaventura et al., 2020). Indeed, MC4R mutations represent the most common cause of inherited morbid obesity (Farooqi et al., 2000; Hinney, 1999; Vaisse et al., 2000).

The relationship between melanocortin and serotonin systems regulates energy balance through the postprandial effects of central serotonin (Heisler et al., 2003). Satiation is evoked via 5-HT_{2C} and 5-HT_{1B} receptors expressed in POMC neurons where the increment of serotonin after eating induces α-MSH release, but also via 5-HT_{1B} 5-HT inhibits AgRP discharge, inducing anorexigenic effects either through α-MSH binding to MCRs or by decreasing the MC4R constitutive activity thanks to AgRP binding (Xu et al., 2010; Marston et al., 2011; Romanova et al., 2018). In fact, stimulation of receptors via 5-HT_{2C} and 5-HT_{1B} produces hypophagia in mammals (Compan, 2020). Under stress conditions, receptors 5-HT_4 drive a hypophagia response from the dorsal raphe nucleus (DRN) to the medial prefrontal cortex (mPFC) network (Compan, 2020). Also, decreased motivation for food has been associated with the activation of serotoninergic pathways in the nucleus acumens (NAc), involved in the regulation of motivation, locomotion, reward, addiction, and stress (Compan, 2020). Also, dopamine at the striatum is implicated in food motivation elicitation known as 'wanting'(Volkow et al., 2011).

Agonistic behaviour

Aggressiveness serves various adaptive functions, such as the establishment of dominancy and hierarchies and competition for resources such as food, shelter, mates, or territories.

The role of melanocortin in aggressiveness is linked to pigmentation and pheromone secretion. As previously described, eumelanin production in melanocytes is regulated through MC1R, and agonistic effects of α-MSH result in dark eumelanin-based colouration characteristic of more aggressive individuals within the same species (Ducrest et al., 2008). Also, a higher pheromone secretion is common in darker dominant subjects due to stimulation of MC5R in exocrine glands involved in defensive behaviour, such as Harderian, lacrimal and sebaceous glands in tetrapods (Ducrest et al., 2008) or preputial glands in mice, in which the deficiency of MC5R reduce aggressiveness (Morgan et al., 2004; Morgan & Cone, 2006).

The antagonistic effect of ASIP at the MC5R of the preputial glands of agouti mice reduces the weight of this gland but also aggression behaviour compared to non-agouti counterparts (Carola et al., 2014). In teleost, intraperitoneal administration of mammalian Asip reduces agonistic behaviour in *Astatotilapia burtoni*, a colourful cichlid from oriental Africa, while α-MSH enhances their aggressiveness (Dijkstra et al., 2017).

Stress response

The stress response is characterized by an activation of the hypothalamic–pituitary–adrenal axis (HPA axis), the tetrapod homologue of the hypothalamic-pituitary-interrenal axis (HPI) in fish. This neuroendocrine system is a feedback loop that begins with the release of the corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus to the anterior pituitary that stimulates ACTH

synthesis. The pituitary ACTH is secreted to the bloodstream and then reach the adrenal cortex where agonise MC2R in the presence of MRAP1 allowing the synthesis and secretion of cortisol. Cortisol feedback CRH neurons and corticotropic cells in the pituitary thus closing the negative loop, (Papadimitriou & Priftis, 2009). Chronic hyperstimulation of the HPA axis is associated with anxiety disorders and depression (Ceruso et al., 2020).

Studies carried out in mammals suggest an MC4R role in the regulation of the HPA axis (Markov et al., 2023; Micioni Di Bonaventura et al., 2022). Under stress conditions, the expression of POMC, α-MSH and MC4Rs rises in the hypothalamus and amygdala, both known by their role in the regulation of emotional behaviours. In mice and rats, ICV administration of α-MSH or MTII induces CRH release, correlated with increased ACTH and corticosterone synthesis, the main glococcorticoid in rodents, and promotes anxiety-like behaviours such as grooming. All these effects are reversed by MC4R antagonists such as HS014 or SHU9119 (Markov et al., 2023; Micioni Di Bonaventura et al., 2022). However, loss of MC4R function can have no effects on basal ACTH levels in rats besides HPA axis low activation under an acute stressor (Ryan et al., 2014). In humans, mutations of this receptor induce obesity and lead to enhanced emotional eating and cortisol levels (Rahati et al., 2022). Thus, the different roles of MC4R in the regulation of the HPA axis might be influenced by its localization since MC4R signalling in the medial amygdala in mice increases corticosterone levels, anxiety-like behaviour, and decreases food intake (J. Liu et al., 2013).

Glucocorticoids levels are associated to different behavioural profiles in vertebrates, especially in the establishment of fish hierarchy. Proactive fish are more aggressive in order to maintain dominance, although the HPI axis is depressed compared to reactive subordinate fish that exhibit higher HPI activity, leading to increased cortisol levels (Backström et al., 2011; Pottinger & Carrick, 1999).

Anxiety-like behaviour

The melanocortin system has been indirectly linked with mood disorders such as anxiety and depression since an unbalanced energy intake leading to either enhanced emotional eating or loss of appetite are shared symptoms (Markov et al., 2023). Common hallmarks of depression and anxiety are hyperactivation of the HPA axis (Ceruso et al., 2020), together with the alteration in central monoamine neurotransmission like low levels of serotonin, dopamine and norepinephrine (Belujon & Grace, 2017). Classically, treatment to reinstate normal levels of these neurotransmitters consists of selective reuptake inhibitors that blockade the autoreceptors (SERT in serotoninergic neurons and DAT in dopaminergic ones) that transport monoamines back into the presynaptic neurons, thus raising their concentration in the synaptic cleft and enhancing monoamines neurotransmission (Belujon & Grace, 2017; Trueta & Cercós, 2012).

The association between obesity and depression has led to the exploration of new therapeutic alternatives targeting the melanocortin system (Scott et al., 2008; Schachter et al., 2018; Markov et al., 2023). In mice, melanocortin peptides have been proven to increase dopaminergic neurotransmission (Roseberry et al., 2015). ICV injection of α-MSH into the neural network of reward and aversion, i.e. NAc, and ventral tegmental area, increases dopamine levels in NAc, while the administration of HS131, a MC4R antagonist, prevents this effect (Lindblom et al., 2001). Despite the studies on the dopaminergic system, research on central melanocortin's effects on serotonin neurotransmission is scarce. In rats, central administration of MTII increases serotoninergic activity in the dorsal raphe (Kawashima et al., 2003), while α-MSH peripheral treatment inhibits serotonin reuptake induced by experimental stressors (Racca et al., 2005). Therefore, the stimulation of the melanocortin system may induce an activation of serotoninergic transmission that can reverse anxiety-like symptoms.

Circadian rhythms

Circadian rhythms coordinate environmental and endogenous cues that occur within the 24-hour daily cycle, such as light/darkness (LD) cycle and feeding pattern. That intrinsic rhythmicity allows organisms to anticipate and prepare for predictable environmental changes or zeitgebers that are considered as inputs of the circadian system (Hastings et al., 2007; Schibler et al., 2015). Circadian rhythms are controlled by pacemaker neurons characterized by translational–transcriptional feedback loops of clock genes whose expression oscillates in a 24-hour cycle. The positive part of this molecular clock is formed by the heterodimer *clock1/bmal1,* which reaches the maximum expression at the light phase, while the negative part consists of the dimer *per/cry* genes, which peaks ending the night and inhibits *clock1/bmal1*(Hastings et al., 2007; Partch et al., 2014; Schibler et al., 2015).

The hypothalamus plays a key role in the neuronal integration of the circadian system. In mammals, the suprachiasmatic nucleus (SCN) in the anterior hypothalamus acts as a master autonomous oscillator coordinating other pacemakers (Ralph & Hurd, 1995; Schibler et al., 2015). Thus, endogenous temporal rhythmicity is transmitted centrally from SCN to other hypothalamus nuclei, pineal and pituitary glands and peripherally to the liver, intestine, adrenal gland, muscle, and adipose tissue (Schibler et al., 2015). In contrast, the teleost circadian oscillator network seems not to be coordinated by any master clock, but they have endogenous clocks in the retina, pineal gland, optic tectum, hypothalamus, and diencephalon, as well as in peripheral tissues such as the head kidney, intestine, liver and gonads (Delgado et al., 2017; Isorna et al., 2017).

In mammals, when light incises the retina, a cascade of neuronal signals travels to SCN, then PVN and continuous brainstream to the cervical ganglion until reaches the pineal gland, where the synthesis and secretion of melatonin is inhibited(Vasey

et al., 2021). Shortly after light input, a significant increase in cortisol levels characterizes the cortisol awakening response (Law & Clow, 2020) The combination of these two events, together with high levels of dopamine, results in enhanced locomotor activity as well as blood pressure and heart rate that promote wakefulness (Baghdoyan & Lydic, 2012). Contrary, darkness stimulates the synthesis and secretion of melatonin, which, together with low levels of cortisol and dopamine, results in a drastic decrease in locomotor activity, blood pressure and heart rate in order to induce the organism into sleep (Baghdoyan & Lydic, 2012). Also, scheduled feeding time represents an important input to the circadian system that generates food anticipatory activity (FAA) that interestingly does not need the coordination of SCN (Mendoza, 2007; Mistlberger, 2011). FAA is maintained independently of the action of leptin and ghrelin, and even under loss of LD rhythm due to clock gene mutation, FAA keeps its rhythmicity. Although the oscillator or molecular pacemakers underlying FAA are still unknown, the dorsomedial hypothalamus and melanocortin system have been proposed as candidates for FAA (Mistlberger, 2011; Yanik & Durhan, 2023). Concretely, FAA was lower in MC3R KO mice and, in wild type, correlated with higher expression of *bmal1*, indicating that MC3R is needed for keeping circadian rhythms patterns, although the molecular mechanism is still not described (Sutton et al., 2008). Also, lack of *bmal1* alters the rhythmicity of *agrp* expression (Clemenzi et al., 2020). Therefore, most likely, the melanocortin system is somehow regulating the circadian feeding rhythmicity.

Zebrafish as a model for neurobehavioural research

Zebrafish (*Danio rerio*) is a freshwater species originally from India that belongs to the Cyprinidae family. Due to its small size, no longer than 4 cm, and shoaling behaviour can be raised in large numbers, also breeding is easy and can performed all year round. Offspring comes in hundreds of eggs around 0.7mm and transparent, so neurodevelopmental can be observed (Kalueff & Cachat, 2011; Kawakami et al., 2016). Eggs transparency and size, together with whole genome sequencing, facilitate genetic manipulation techniques to create transgenic lines used in research of the genetic basis of neurobehavioral disorders due also to the 70% of the genome with humans (Howe et al., 2013; Kalueff et al., 2014).

Zebrafish have a nervous system that is anatomically comparable to mice except for the absence of a neocortex (McArthur et al., 2019). Early in their development, after three days after hatching, food-seeking and avoidance behaviour can already be observed (Spence et al., 2008). They reach their maturity after three months when raised at 28ºC, and complex behaviours can be observed with the aim of studying aggressiveness, stress, anxiety-like behaviour, learning and memory, (Maximino et al., 2010; Cachat et al., 2011; Schneider, 2011; Gerlai, 2011, 2016; Godwin et al., 2012; Collier et al., 2017) as will be discussed in further chapters.

Furthermore, their dopaminergic and serotoninergic networks are similar to mammals (Backström & Winberg, 2017; McArthur et al., 2019). Likewise, drugs such as benzodiazepines, serotonin and dopamine reuptake inhibitors, or other substances such as ethanol and caffeine have similar molecular and behavioural responses (Echevarria et al., 2011; Stewart et al., 2015; Srivastava & Gold, 2019).

Our model of study: zebrafish *asip1-Tg*

With the purpose of studying the role of the melanocortin system in several physiological and behavioural functions, such as pigmentation and skin cellular composition, food intake, growth, intestine permeability, puberty, behaviour and circadian rhythms, a transgenic zebrafish line overexpressing asip1, an inverse agonist of MC1R and MC4R, [Tg(Xla.Eef1a1:Cau.Asip1]iim4 or *asip1-Tg*, was generated using the Tol2 transposon system (Ceinos et al., 2015). The eGFP gene of Tol2-transposon-based vectorpT2AL200R150G was replaced with asip1 cDNA from goldfish (NCBI reference sequence: XM_001334910.3) under the control of constitutive promoter of elongation factor 1-alpha (ef1-alpha). This construct, together with a transposase mRNA, was injected in 2-cell embryos of wild-type TU strain zebrafish (Nüsslein-Volhard Lab, Tübingen, Germany) (Ceinos et al., 2015).

Previous research showed a disruption of the dorsoventral pigmentation pattern in transgenic *asip1-Tg* (Ceinos et al., 2015). Also, food intake and growth are enhanced in *asip1-Tg* compared to their counterparts (Guillot et al., 2016). Despite their bigger size, puberty is not altered because differential growth begins at 2 cm length when both genotypes have reached maturity (Navarro et al., 2021).

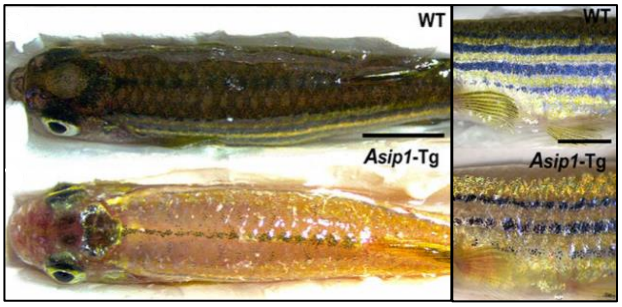

Figure 1: Disruption of the dorsoventral pigmentation pattern in transgenic *asip1-Tg*. Source: Ceinos et al., 2015.

OBJECTIVES

The main aim of this thesis was to investigate the phenotypic effects of disrupting melanocortin signalling by overexpressing the endogenous antagonist Asip1, either from an energetic/growth or behavioural point of view. Certainly, some of the objectives emerged as a consequence of previous results.

1 Our preliminary data indicated that *asip1* overexpression in transgenic zebrafish (*asip1-Tg*) was able to enhance growth when *asip1-Tg* animals were fed at the same ratio as wild-type (WT) counterparts (Guillot et al., 2016). Therefore, our first specific objective was to investigate *asip1-Tg* growth under different feeding ratios but also to assess the effects of stress on *asip1-Tg* growth. Indeed, the melanocortin system is the main hormonal system regulating the stress response in vertebrates.

2 We demonstrated that *asip1-Tg* animals were able to grow faster under the same feed ratio, suggesting that the depression of melanocortin signalling may influence either energy expenditure or feed efficiency or even both. We then explored the effect of *asip1* overexpression on intestinal function by studying transgene effects on the intestine transcriptome and gut epithelial membrane integrity, permeability and electrogenic amino acid transport.

3 *Asip1-tg* animals eat more than their WT counterparts due to a disrupted central satiety system (Guillot et al., 2016). This increased feeding suggests that transgenic animals might compete better for feed resources, showing increased aggressiveness. Therefore, our third objective focused on the study of aggressive/submissive behaviour in dyadic fights.

4 The results of the aggression studies showed that *asip1-Tg* animals exhibited submissive behaviour together with high cortisol levels, a feature of reactive behaviour. This suggested to us that the disruption of the melanocortin system might induce anxiety-like behaviour in the transgenic animals. We, therefore, set up several

behavioural tests to assess the mood of the fish. In support of this hypothesis, our previous preliminary studies showed that the overexpression of asip1 reduces the central serotoninergic tone, again indicating anxiety-like behaviour (Guillot et al., 2016). Therefore, we investigate whether the anxiety-like behaviour induced by *asip1* overexpression is mediated by central monoaminergic pathways.

5 *asip1-Tg* animals showed anxiety-like behaviour, suggesting that the activity rhythms of these animals might be disturbed. This anxiety-like behaviour could be induced by starvation, which in turn could strongly affect activity patterns. We investigated the locomotor activity patterns of *asip1-Tg* animals as an output of the circadian system but also the effects of the melanocortin system on pineal physiology.

6 In the previous experiments, we showed that changes in energy balance are associated with behavioural changes in our transgenic model. Therefore, our sixth and final objective was to study the effect of nutritional status on anxiety-like behaviour and cognitive function in zebrafish as a model organism to study neurological disorders linked to metabolic and nutritional diseases.

CHAPTER 1

Evolution of proopiomelanocortin

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Abstract

Proopiomelanocortin (*POMC*) belongs to the opioid/orphanin gene family whose peptide precursors include either opioid (YGGF) or the orphanin/nociceptin core sequences (FGGF). In addition to POMC the family includes the proenkephalin (*PENK*), prodynorphin (*PDYN*), and nociceptin/proorphanin (*PNOC*) precursors. The opioid core sequence in POMC is incorporated by the β-endorphin that occupies the C-terminal region but this propeptide also exhibits at least two "alien" melanocortin core sequences (HFRW). An ACTH/MSH fragment merged into the opioid fragment not earlier than the two tetraploidizations of the vertebrate genome. Therefore, POMC exhibit a complex "evolutionary life" since the gene has coevolved together with two different receptor systems, i.e. opioid and melanocortin following a horse trading system. In this article, we summarize the different evolutionary hypotheses proposed for POMC evolution.

Keywords: melanocortin, growth, obesity, stress, transgenic, zebrafish

1. Introduction

Proopiomelanocortin (*POMC*) gene encodes a propeptide whose posttranslational processing generates several bioactive peptides. In most vertebrates, POMC contains several domains and each integrates a core sequence (HFRW) that, in turn, distinguishes a melanocyte-stimulating hormone (MSH). The N-terminal domain integrates the pro-γ-MSH, the central domain contains the α-MSH as the N-terminal sequence of the adrenocorticotropic hormone (ACTH), and the C-terminal, β-lipotropin that includes the β-MSH (Nakanishi et al., 1979). The C terminal domain also includes β-endorphin that retains an opioid core sequence, YGGF, characteristic of the opioid/orphanin gene family (Fig.1).

The tissue-specific posttranscriptional processing rules what peptides are released from the precursor and this differential proteolytic cleavage depends on the prohormone convertase battery available in the specific tissue. In the corticotrophs of the anterior pituitary, the proprotein convertase subtilisin/kexin type 1 (Pcsk1) activity generates pro-γ-MSH, ACTH, and β-lipotropin whereas the cleavage by Pcsk1 and Pcsk1 2 produces α-MSH and β-endorphin in the melanotrophs of the pars intermedia (Castro & Morrison, 1997). *POMC* is mainly expressed in the pituitary being one of the most abundant transcripts in this gland (He, Dai, Chen, He, & Yin, 2014). However, two discrete groups of neurons in the hypothalamus and the medulla, the arcuate nucleus (NAc) and the nucleus of the solitary tract (NTS), respectively, also produce POMC (Bagnol et al., 1999). In the rat brain, POMC is mainly processed to α-MSH and β-endorphin (Castro & Morrison, 1997) but some hypothalamic ACTH-IR after RP-HPLC, elutes also at the position of ACTH, suggesting its presence in the rat brain (Smith & Funder, 1988).

The presence of opioid and melanocortin sequences in the POMC indicates that the molecular evolution of the precursor has been under constrains of either system, coevolving with different receptor systems, i.e. opioid and melanocortin, as strings pulling together in the same or different direction. In the last years, several reviews on the evolution of opioid system (Elphick, Mirabeau, & Larhammar, 2018; Larhammar, Bergqvist, & Sundstrom, 2015; Sundström, Dreborg, & Larhammar, 2010) and melanocortin system (Cerdá-Reverter et al., 2011; Cortés et al., 2014; Dores & Baron, 2011; Dores, Liang, Davis, Thomas, & Petko, 2016; Dores et al., 2014; Navarro et al., 2016; A. M. Takahashi, K.; Amano, M., 2014) have been published. We summarize here the main hypothesis on the evolutionary processes of this complex multiprecursor that amalgamates potential gene duplications, insertion, reorganization and coevolutionary process.

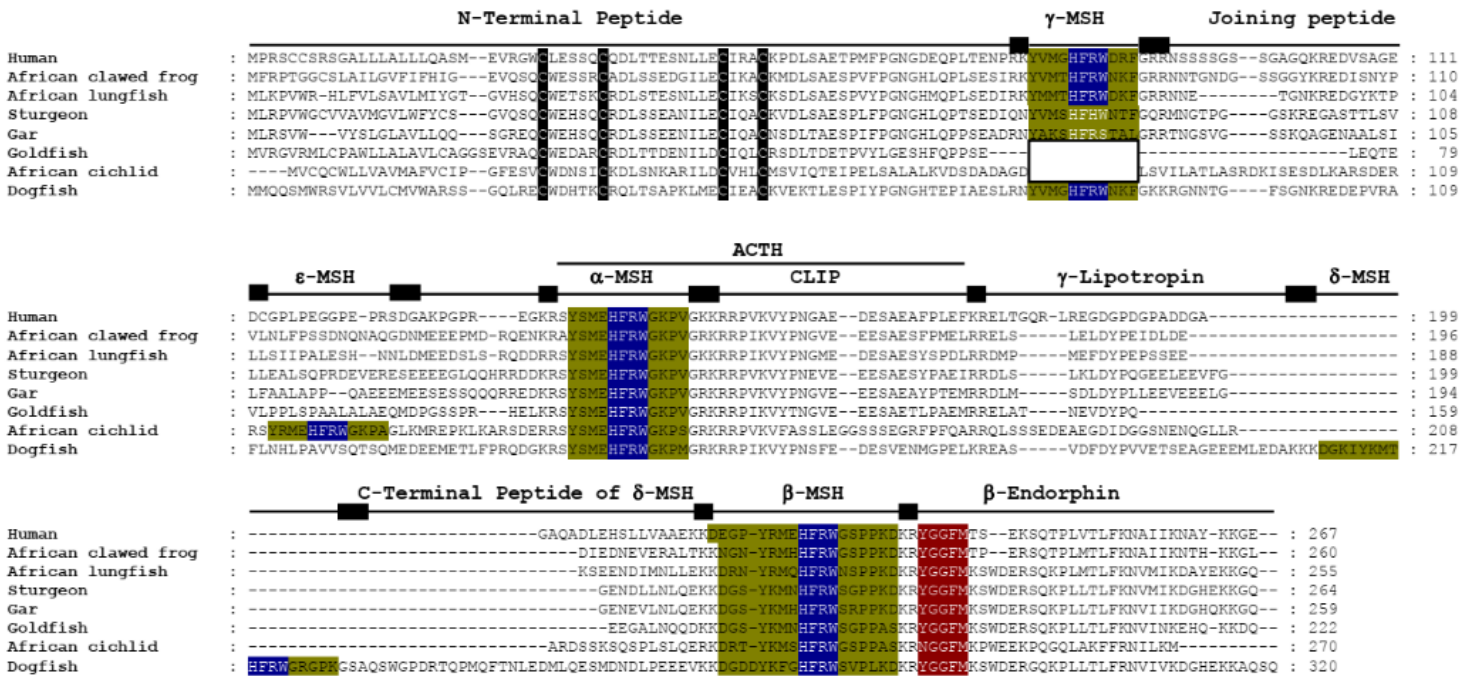

Fig. 1. Alignment of proopiomelanocortin (POMC) sequences from human (*Homo sapiens*).

White letters on black background indicate fully conserved cysteine residues in all POMC sequences. Green and red boxes show MSH peptides and endorphin core, respectively. White letters on blue background highlight melanocortin core sequences. Uncoloured boxed area demarcates γ-MSH segment in species lacking γ-MSH. Black boxes indicate endoproteolytic cleavage sites. Adapted from Navarro et al., (2016).

1. Evolution of opioid peptides and nociceptin/orphanin family

POMC gene belongs to the opioid peptides and nociception/ orphanin gene family, which also includes proenkephalin (*PENK*), prodynorphin (*PDYN*), and nociceptin/ proorphanin (*PNOC*) (Danielson & Dores, 1999). All genes from this family encode at least one opioid "core" sequence, consisting of the peptide Y/FGGF or PNOC (Dores & Lecaude, 2005; Dores, Lecaude, Bauer, & Danielson, 2002). Both PENK and PDYN contain multiple copies of the "core" opioid sequence, varying from three to seven depending on species, whereas POMC has a single core motif (as part of endorphin) and PNOC can have one or two copies depending on the class of vertebrates (Sundström et al., 2010). Outside the opioid core sequence, the members of this peptide family differ substantially among themselves and between species, but they all have retained a set of conserved cysteine residues at the N-terminal region of the molecule. Six residues are found in PENK, PDYN and PNOC, and only four in POMC. In addition, all genes from this family share the same overall structure with a single intron shortly after the region encoding the signal peptide (Larhammar et al., 2015). These unifying signatures suggest that they are all derived from a common ancestral opioid gene.

Gene families comprise several to many genes of similar nucleotide or amino acid sequences that have close functions. Several mechanisms, such as tandem duplications, segmental duplications, or even whole-genome duplications can lead to the expansion of gene families. Since many decades, gene duplication is recognized as a key mechanism of evolution. It can alter gene dosage, rescue gene function, and lead to evolution of new genetic networks, and to the re-wiring or modulation of existing ones. Whether this process is mostly neutral, or driven by natural selection,

is controversial (Lespinet, Wolf, Koonin, & Aravind, 2002; Nei, 2013). Notably, during the evolution of chordates, the ancestral deuterostome genome experienced two rounds of whole-genome duplication named 1R and 2R (Fig. 2). These events occurred before the radiation of Gnathostomes (jawed vertebrates) and there are evidences that support that both 1R and 2R happened before the divergence of Cyclostomes (lampreys and hagfishes) and Gnathostomes, approximately 500 Mya. However, an alternative scenario maintains that these two lineages might have diverged after the 1R (Smith et al., 2018) (Fig. 2). Even though most duplicated genes were secondarily lost, many evolved new functions, in support of the notion that gene and genome duplications might provide a major mechanism for generating phenotypic diversity in evolution (Ohno, 1970). The common ancestor of teleost fish has experienced an additional whole-genome duplication event approximately 320 Mya, named 3R (Amores et al., 1998; Christoffels et al., 2004; Jaillon et al., 2004; Taylor, Braasch, Frickey, Meyer, & Van de Peer, 2003) (Fig. 2). This event accounts for several divergent features in teleost genomes.

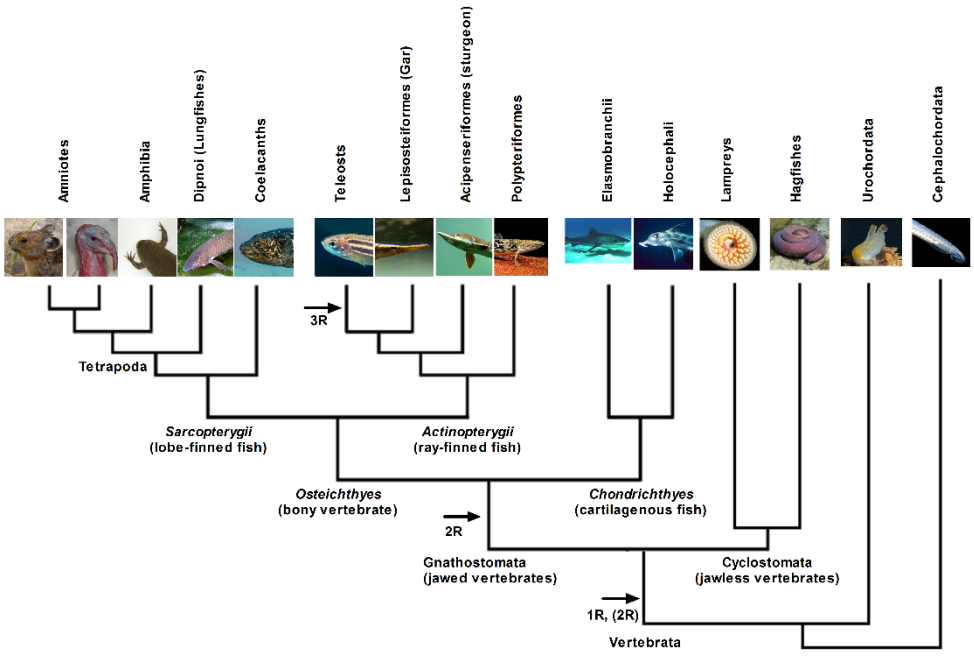

Fig. 2. Simplified phylogenetic tree of Chordate.

The phylum Chordata includes three sub-phyla: Cephalochordate, Urochordata and Vertebrata. Vertebrates are the most dominant and successful group of animals on earth, occupying both terrestrial and aquatic habitats. Vertebrates are divided into two broad groups: jawed vertebrates (Gnathostomata) and the jawless vertebrates represented by only two extant lineages, lampreys and hagfishes (Cyclostomata). Gnathostomes, conversely, comprise the vast majority of all vertebrates. There are two main groups within Gnathostomata: cartilaginous fishes (Chondrichthyes), which include sharks, rays, skates and bony vertebrates (Osteichthyes), which include ray-finned fishes, lobe-finned fishes, and tetrapods (land animals). Ray-finned fishes (Actinopterygii) comprise roughly half of all living vertebrates. Two major groups can be recognized in ray-finned fishes: teleosts and basal, nonteleost ray-finned fishes, which comprise four orders: Polypteriformes (e.g., bichir), Acipenseriformes (e.g., sturgeon and paddlefish), Lepisosteiformes (e.g., spotted gar), and Amiiformes (e.g., bowfin). Based on recent findings, it is hypothesized that two rounds of whole-genome duplication occurred, 1R and 2R, although it is still not clear when they took place. The teleost lineage went through an additional whole-genome duplication (3R).

In the human genome, *PDYN* and *POMC* are located on chromosome 20 and 2, respectively (Fig. 3). Chromosome 8 carries both *PENK* and *PNOC* genes. The chicken genome was initially thought to lack *PDYN* but recent genome assembly (Gallus_gallus-5.0) shows this gene localized on chromosome 20, pairing chicken opioid system with that of mammals. However, the chromosomal regions for the four opioid peptide precursor genes are different from those in human genome. *PENK* is found on chromosome 2, whereas both *POMC* and *PNOC* are located to chromosome 3. Therefore, *PNOC* is paired with *POMC* on chromosome 3 and not with *PENK* as occurs on human chromosome 8 (Fig. 3). Inferring the distribution pattern of the genes encoding opioid peptide precursors has demonstrate that *PENK*, *PDYN* and *PNOC* are located on three separate chromosomes in the same paralogon (set of paralogous chromosomal regions comprising syntenic genes) of the vertebrate genome (Sundström et al., 2010). The *POMC* gene in many species is locate in the same chromosomal region as the *PNOC*. They share the same chromosome in the opossum (*Monodelphis domestica*) as well as in all species of teleost fish that have been studied (Larhammar et al., 2015; Sundström et al., 2010). In fact, in the soft-shell turtle (*Pelodiscus sinensis*), *POMC* and *PNOC* are just around 1 Mb apart. The spotted gar, *Lepsisosteus oculatus*, is a basal nonteleost ray-finned fish, whose lineage diverged from the teleost lineage prior to the teleost whole-genome duplication (Fig. 2). Spotted gar possesses a typical diploid vertebrate genome similar to the human genome (Braasch et al., 2016). Comparative analyses have shown that the gar genome is organized more like chicken and human genomes than like those of teleosts (Amores, Catchen, Ferrara, Fontenot, & Postlethwait, 2011). It has fewer chromosomal rearrangements than both teleost fishes and many mammals including human, converting this ray-finned fish in an exceptional species for studies on vertebrate genome evolution. In the spotted gar *PENK* and *PDYN* are located on chromosomes LG9 and LG18, respectively. *POMC* and *PNOC* are found together on chromosome LG1

suggesting that a local gene duplication followed by fusion of an *ACTH/MSH* gene in *POMC*. The different arrangement of these two gene in the human genome could be the result of a gene translocation event, taking place in the ancestor of placental mammals. Therefore, the expansion of the opioid peptide system seems to be the result of two complete genome duplications (1R and 2R) and a single local duplication. There are three possible scenarios for the evolution of the opioid peptides. They differ in the timing of the local duplication that generated *POMC* and *PNOC* that could have taken place before 1R (scenario 1), after 1R but before 2R (scenario 2), or after 2R (scenario 3, Fig. 4) (Navarro et al., 2016). Lampreys, along with hagfishes, constitute the cyclostomes, the sole survivors of a lineage that diverged from the ancestor of the jawed vertebrates more than 500 million years ago (Docker, Hume, & Clemens, 2015). Lampreys have two *POMC* genes, proopiocortin (*POC*) and proopiomelanotropin (*POM*). *POC* encodes an ACTH sequence, a β-MSH-related sequence and a β-endorphin sequence. *POM* encodes MSH-B (an α-MSH-related peptide), MSH-A (a β-MSH-related peptide) and a β-endorphin sequence (Takahashi, Amemiya, Nozaki, et al., 1995; Takahashi, Amemiya, Sarashi, Sower, & Kawauchi, 1995). The presence of *POMC* sequences in the genome of lampreys suggests that the duplication event given rise to *PNOC* and *POMC* probably occurred before the split of cyclostomes from other chordates. If *POMC* emerged before the second genome duplication is impossible to say because both chromosomal and whole-genome duplications have played significant roles in the evolution of ancestral vertebrate lamprey genomes (Smith et al., 2018). In the whole, these considerations point to an ancient vertebrate origin of the opioid system, with all members presently found in humans already established more than 500 Mya.

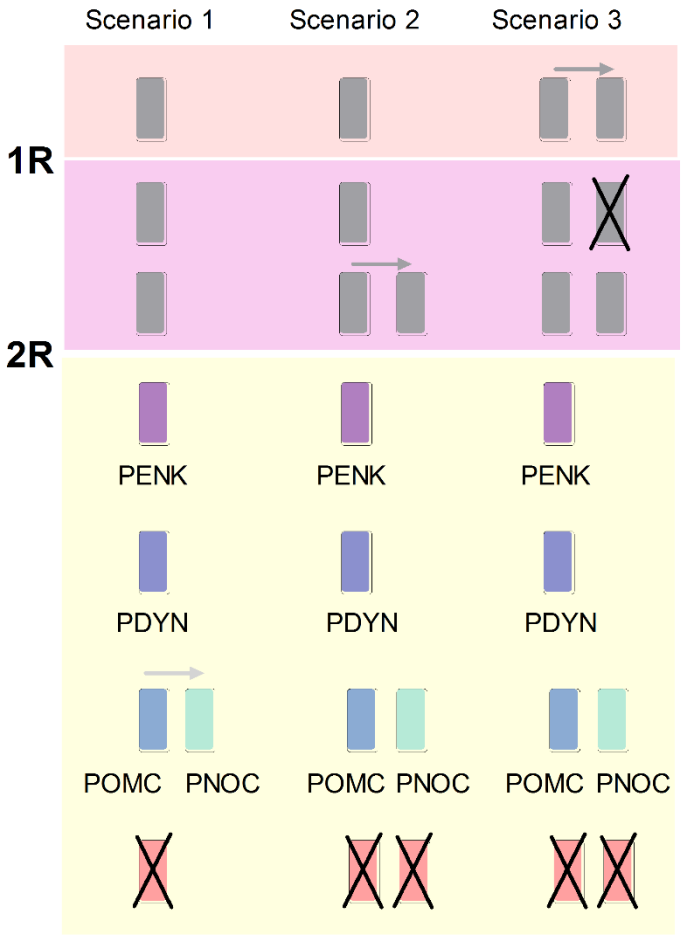

Fig. 3. Anticipated evolutionary history for the opioid peptide and nociceptin/orphanin family by a combination of whole-genome and local duplications.

Three different scenarios have been proposed to explain the timing of the duplication events that have occur in the common ancestor leading to POMC and PNOC. Local duplication, indicated by arrows, generating both POMC and PNOC could have taken place after the 2R (scenario 1), after the 1R (scenario 2) or before the 1R (scenario 3). POMC, proopiomelanocortin; PENK, proenkephalin; PDYN, prodynorphin; PNOC, nociceptin/ proorphanin. The figure is an adapted version of the figure published in Sundström et al. (2010) and Navarro et al. (2016).

Grounded in the knowledge of the existence of one opioid peptide precursor gene in the ancestor of the vertebrates prior to the 1R and 2R, efforts have been made to find related sequences also in invertebrates. However, searches on any invertebrate genome project failed to identify opioid propeptides or opioid receptor sequences, not even in deuterostomes invertebrates (reviewed in Larhammar et al., 2015). Also melanocortin receptors are still unknown outside the vertebrates (Larhammar & Bergqvist, 2013).

Mammals, and most other tetrapods, have four opioid peptide precursor genes (Dores et al., 2002). Exceptions include polyploid frogs that have additional copies. Nociceptin has not yet been reported in a shark but we have identified in the little skate (*Leucoraja erinacea*) draft genome database (LittleSkate-Transcriptome-Contigs-Build2), a sequence of a peptide that contains the opioid YGGF core motif. This peptide is found in the LittleSkate-transcriptB2-contig3929 and is 82% identical to the nociceptin peptide sequence in chicken, gar, latimera and zebrafish. Moreover,a kappa-type opioid receptor (oprk1) has been identified in the elephant shark (*Callorhinchus milii*) and therefore *PNOC* is expected to be present also in cartilaginous fish (Larhammar et al., 2015) reinforcing the idea of an ancient vertebrate origin of the opioid system. In teleost, the situation is different. The teleost-specific genome duplication is believed to be responsible for the rapid speciation and adaptive radiation of teleost. It is proposed that genome duplication events provide additional genetic raw material on which selection can act and give rise to novel phenotypes. Following genome duplication, there are three common fates for the duplicated genes. The most likely fate is the loss of one of the duplicate loci through accumulation of deleterious mutations, resulting in nonfunctionalization (Lynch & Conery, 2000). This loss of duplicated gene loci can potentially play a role in speciation via the phenomenon of divergent resolution (i.e., differential gene loss) (Semon & Wolfe, 2007; Taylor, Van de Peer, & Meyer, 2001). The other two fates of duplicated genes

are subfunctionalization (both copies are retained because of partitioning of ancestral gene functions between the duplicates) and neofunctionalization (whereby one of the copies acquires a novel function) (Force et al., 1999). The retained duplicate pairs often show a difference in overall expression patterns and expression levels (Berthelot et al., 2014). All teleost fish in which the genome has been sequences have two copies of the *pomc* genes (reviewed in Sundström et al., 2010). In the Tetraodon (*Tetraodon nigroviridis*), *pomc* paralogues have experienced subfunctionalization of both expression and peptide domains during teleost evolution (de Souza, Bumaschny, Low, & Rubinstein, 2005). *pomcα* is expressed in the nucleus lateralis tuberis of the hypothalamus, the homolog of the arcuate nucleus in fish, as well as in the rostral pars distalis and pars intermedia of the pituitary, whereas *pomcβ* is expressed in the preoptic area of the brain and weakly in the pituitary pars intermedia. *pomcβ* genes have a β-endorphin segment that lacks the consensus opioid signal and seems to be under neutral evolution in tetraodontids, whereas *pomcα* genes possess well-conserved peptide regions. Other teleost species, specifically the ones that have experienced a fourth whole-genome duplication event, e.g. rainbow trout, are found to have three copies of the *pomc* gene (Harris, Dijkstra, & Hofmann, 2014; Leder & Silverstein, 2006) but three *pomc* paralogs are also found in the barfin flounder, *Verasper moseri* (Takahashi et al., 2005), Burton's mouthbrooder (*Haplochromis burtoni*) and medaka (*Oryzias latipes*) (Harris et al., 2014). In all cases, presence of three genes should be the consequence of lineage-specific gene duplication events rather than genome duplication (Sundström et al., 2010).

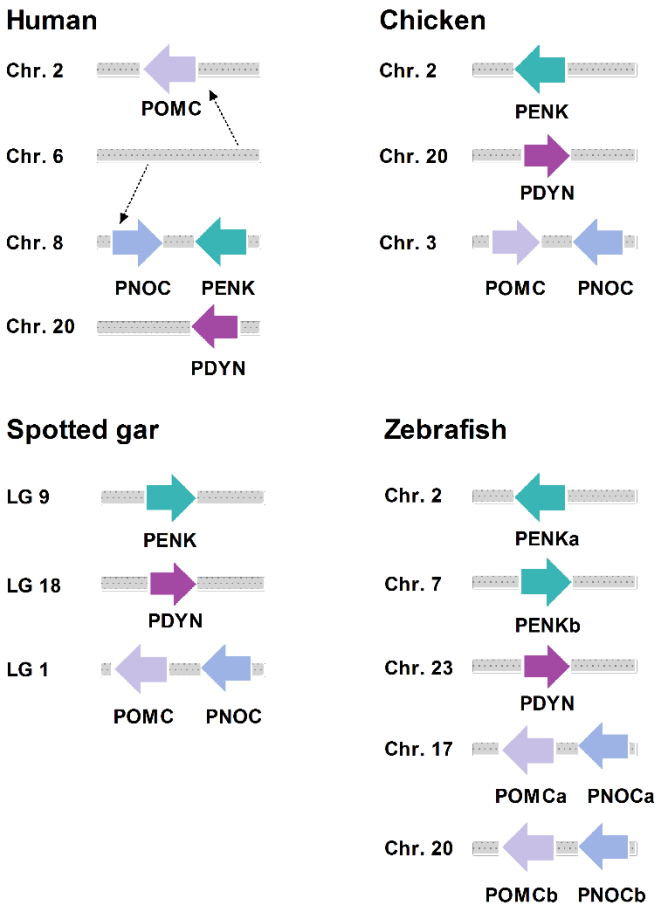

Fig. 4. Chromosomal regions for the four opioid peptide precursor genes in human (*Homo sapiens*), chicken (*Gallus gallus*), spotted gar (*Lepisosteus oculatus*) and zebrafish (*Danio rerio*).

Each arrow box represents a gene, and its orientation (forward or reverse) in the respective chromosome (horizontal lines). Dashed arrows symbolize gene translocations. Chr, chromosome; LG, linkage group. Adapted from (Larhammar et al., 2015).

2. POMC Bauplan evolution

The presence of "alien" melanocortin sequences in the primary structure of *POMC* converts this gene probably, in the most complex precursor of the opioid family. Suggestively, POMC maintains structural and sequence identities with the three other opioid precursors within the N- and C-terminal region of the precursor thus implying that this complex precursor arose when a fragment of DNA containing one or multiple MSH sequences was inserted into apreproendorphin gene. However, we cannot exclude that the ancestral melanocortin sequence resulted from accumulative mutations in the preproendorphin gene that lead to multiple melanocortin sequences after unequal crossing-over events (Dores & Baron, 2011). When the preproendorphin merged with the MSH sequence(s) is probably one of the key questions in the POMC evolution. Results suggest that a distinct proendorphin gene arose after the two tetraploidizations of the vertebrate genome (Larhammar et al., 2015; Sundström et al., 2010) and therefore POMC emerged, after the fusion of the ACTH/MSH fragment, not earlier than the second genome duplication. A gene encoding ACTH/MSH could have appeared earlier as supported by the fact that the genes encoding melanocortin receptors also arose as the result of the two duplication rounds of the vertebrate genome from a single ancestral gene present before tetraploidizations (Cortés et al., 2014).

The structural plan of the POMC precursors differs between species and these variations apply mainly for the number of MSH sequences/domains (Fig. 1 and 5). All tetrapod species exhibit three MSH core sequences (γ-MSH, α-MSH and β-MSH) suggesting that POMC evolved through intragenic duplication of an ancestral MSH gene (Nakanishi et al., 1979). Lobe-finned or sarcopterygian fish share the same organization displaying identical MSH domains (Amemiya, Takahashi, Meguro, & Kawauchi, 1999; Amemiya, Takahashi, Suzuki, Sasayama, & Kawauchi, 1999; Dores et

al., 1999) but elasmobranch fish have an additional MSH domain called δ-MSH (Amemiya, Takahashi, Suzuki, et al., 1999). Some fish like cichlid and Pomacentridae present a novel domain termed ε-MSH due to a potential duplication of the ACTH domain (Harris et al., 2014). The coincidence of the same POMC bauplan in tetrapod and lobe-finned fish suggests that the ancestral Gnathostomes exhibited the three-domain structure (γ-MSH, α-MSH and β-MSH) and this structural plan diverged concomitantly with the divergence of the ancestral Gnathostomes into cartilaginous, ray- and lobe-finned fish. Nowadays, three different structural plans coexist (Fig.5). The ancestral organization (γ-MSH, α-MSH and β-MSH) has been preserved in lobe-finned fish (lungfishes and coelacanth) and subsequently tetrapod species. The second organization plan is unique to the cartilaginous fish, which has added a fourth MSH peptide (δ-MSH). This fourth domain is placed between ACTH and β-MSH domain and probably evolved as a duplication of the C-terminal β-MSH/β-endorphin segment. The presence of this structure in elasmobranch and holocephalan species suggest that duplication occurred early after the divergence of the cartilaginous lineage.

Finally, the third organization plan is found in ray-finned fish (Actinopterygii) that presently include Polypteriformes (e.g., bichirs), Acipenseriformes (e.g., sturgeons and paddlefish), Lepisosteiformes (e.g., spotted gar), Amiiformes (bowfin) and teleost fish (Fig. 5). Most of ray-finned fish exhibit alterations of the γ-MSH domain and the zenith is reached in the teleost fish where the complete deletion of the domain was undertaken. Probably, the loss of the γ-MSH domain was progressive across the evolutionary process (Dores & Baron, 2011). In fact, basal Actinopterygii still keep the ancestral bauplan i.e. γ-MSH, α-MSH/ACTH and β-MSH/β-endorphin, but replacements at dibasic pairs flanking the MSH sequence make peptide processing and release improbable. These N- and C-terminal cleavage motifs are clearly absent in Polypteryformes and Acipensiformes precursors again making peptide processing non-

viable. Additionaly, in some species as for example the sturgeon, Pomc has mutations in the core sequence HFRW of γ-MSH, which is crucial for binding to melanocortin receptors. All this progressive degeneration of the γ-MSH domain in basal ray-finned fish finally lead to the complete deletion of this domain even affecting the spacer region between γ-MSH and α-MSH/ACTH in teleost fish (Fig.5). However, the massive deletion in the N-terminal region of the teleost precursors does not affect peptide processing in the pituitary.

Melanocortin peptides signal through a family of G-protein coupled receptors that exhibit different expression patterns and pharmacological properties (Cortés et al., 2014). The γ-MSH peptide presents major affinity biding to the melanocortin 3 receptor (MC3R) or γ-MSH receptor (Roselli-Rehfuss et al., 1993) but physiological levels of γ-MSH are commonly low and full agonist has not been characterized up to now (Hruby et al., 2007). It fills with suspicion that the precursor could be processed alternatively in the N-terminal region thus generating longer forms including the active melanocortin core much more active at the receptors binding pockets. Suggestively, the genome of most teleost fish lacks a γ-MSH receptor gene, symptomatic of a coevolutionary process of the peptide/receptor system.

Fig. 5. A) Structural plans of POMC precursor in main vertebrate groups.

Both tetrapod and lobe-finned fish share the same POMC bauplan. Cartilaginous fish exhibit and additional MSH peptide called δ-MSH whereas Actinopterygian fish lack γ-MSH peptide. Some teleost fish have an additional ε-MSH peptide as a putative tandem duplication of the α-MSH-ACTH segment.

B) Comparison of γ-MSH sequences showing the potential degenerative evolutionary process leading to the loss of γ-MSH peptide in teleost fish.

The γ-MSH sequence and the flanked proposed endoproteolytic cleavage sites for tetrapod, lobe-finned fish (lungfish), cartilaginous (spiny dogfish) and four ray-finned fish are presented. The N-terminal cleavage site (A) and the C-terminal cleavage and α-amidation site (B) are boxed. An arginine (R)-lysine (K) dibasic pair flanks tetrapod and lobe-finned fish γ-MSH peptide. An arginine (R) residue is still present in gar but lysine is replaced by an asparagine (N) residue, a common substitution in many fish species including elasmobranchs. It is uncertain whether this dibasic pair (RN) can promote γ-MSH cleavage but this motif is fully absent in Polypteriformes and Acipenseriformes precursors, which makes γ-MSH processing questionable. In addition, the C-terminal cleavage motif (RR or KK) is also absent in bichir, sturgeon and paddlefish. Moreover, sturgeon precursor carries a substitution in the MSH core sequence compromising the activity of the peptide. Adapted from Navarro et al., (2016).

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Conflict of interest

Disclosure summary: The authors have nothing to declare.

References

Amemiya, Y., Takahashi, A., Meguro, H., & Kawauchi, H. (1999). Molecular cloning of lungfish proopiomelanocortin cdna. *Gen Comp Endocrinol, 115*(3), 415-421. doi:10.1006/gcen.1999.7327

Amemiya, Y., Takahashi, A., Suzuki, N., Sasayama, Y., & Kawauchi, H. (1999). A newly characterized melanotropin in proopiomelanocortin in pituitaries of an elasmobranch, squalus acanthias. *Gen Comp Endocrinol, 114*(3), 387-395. doi:10.1006/gcen.1999.7256

Amores, A., Catchen, J., Ferrara, A., Fontenot, Q., & Postlethwait, J. H. (2011). Genome evolution and meiotic maps by massively parallel DNA sequencing: Spotted gar, an outgroup for the teleost genome duplication. *Genetics, 188*(4), 799-808. doi:10.1534/genetics.111.127324

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., Postlethwait, J. H. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science, 282*(5394), 1711-1714.

Bagnol, D., Lu, X. Y., Kaelin, C. B., Day, H. E., Ollmann, M., Gantz, I., Watson, S. J. (1999). Anatomy of an endogenous antagonist: Relationship between agouti-related protein and proopiomelanocortin in brain. *J Neurosci, 19*(18), RC26.

Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noel, B., Guiguen, Y. (2014). The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nat Commun, 5*, 3657. doi:10.1038/ncomms4657

Braasch, I., Gehrke, A. R., Smith, J. J., Kawasaki, K., Manousaki, T., Pasquier, J., Postlethwait, J. H. (2016). The spotted gar genome illuminates vertebrate evolution and facilitates human-teleost comparisons. *Nat Genet, 48*(4), 427-437. doi:10.1038/ng.3526

Castro, M. G., & Morrison, E. (1997). Post-translational processing of proopiomelanocortin in the pituitary and in the brain. *Crit Rev Neurobiol, 11*(1), 35-57.

Cerdá-Reverter, J. M., Agulleiro, M. J., R, R. G., Sanchez, E., Ceinos, R., & Rotllant, J. (2011). Fish melanocortin system. *Eur J Pharmacol, 660*(1), 53-60. doi:10.1016/j.ejphar.2010.10.108

Cortés, R., Navarro, S., Agulleiro, M. J., Guillot, R., Garcia-Herranz, V., Sanchez, E., & Cerda-Reverter, J. M. (2014). Evolution of the melanocortin system. *Gen Comp Endocrinol, 209*, 3-10. doi:10.1016/j.ygcen.2014.04.005

Christoffels, A., Koh, E. G., Chia, J. M., Brenner, S., Aparicio, S., & Venkatesh, B. (2004). Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. *Mol Biol Evol, 21*(6), 1146-1151. doi:10.1093/molbev/msh114

Danielson, P. B., & Dores, R. M. (1999). Molecular evolution of the opioid/orphanin gene family. *Gen Comp Endocrinol, 113*(2), 169-186. doi:10.1006/gcen.1998.7206

de Souza, F. S. J., Bumaschny, V. F., Low, M. J., & Rubinstein, M. (2005). Subfunctionalization of expression and peptide domains following the ancient duplication of the proopiomelanocortin gene in teleost fishes. *Molecular Biology and Evolution, 22*(12), 2417-2427. doi:10.1093/molbev/msi236

Docker, M. F., Hume, J. B., & Clemens, B. J. (2015). Introduction: A surfeit of lampreys. In M. F. Docker (Ed.), *Lampreys: Biology, conservation and control: Volume 1* (pp. 1-34). Dordrecht: Springer Netherlands.

Dores, R. M., & Baron, A. J. (2011). Evolution of pomc: Origin, phylogeny, posttranslational processing, and the melanocortins. *Ann N Y Acad Sci, 1220*, 34-48. doi:10.1111/j.1749-6632.2010.05928.x

Dores, R. M., & Lecaude, S. (2005). Trends in the evolution of the proopiomelanocortin gene. *Gen Comp Endocrinol, 142*(1-2), 81-93. doi:10.1016/j.ygcen.2005.02.003

Dores, R. M., Lecaude, S., Bauer, D., & Danielson, P. B. (2002). Analyzing the evolution of the opioid/orphanin gene family. *Mass Spectrom Rev, 21*(4), 220-243. doi:10.1002/mas.10029

Dores, R. M., Liang, L., Davis, P., Thomas, A. L., & Petko, B. (2016). 60 years of pomc: Melanocortin receptors: Evolution of ligand selectivity for melanocortin peptides. *J Mol Endocrinol, 56*(4), T119-133. doi:10.1530/JME-15-0292

Dores, R. M., Londraville, R. L., Prokop, J., Davis, P., Dewey, N., & Lesinski, N. (2014). Molecular evolution of gpcrs: Melanocortin/melanocortin receptors. *J Mol Endocrinol, 52*(3), T29-42. doi:10.1530/JME-14-0050

Dores, R. M., Sollars, C., Danielson, P., Lee, J., Alrubaian, J., & Joss, J. M. (1999). Cloning of a proopiomelanocortin cdna from the pituitary of the australian lungfish, neoceratodus forsteri: Analyzing trends in the organization of this prohormone precursor. *Gen Comp Endocrinol, 116*(3), 433-444. doi:10.1006/gcen.1999.7382

Elphick, M. R., Mirabeau, O., & Larhammar, D. (2018). Evolution of neuropeptide signalling systems. *J Exp Biol, 221*(Pt 3). doi:10.1242/jeb.151092

Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L., & Postlethwait, J. (1999). Preservation of duplicate genes by complementary, degenerative mutations. *Genetics, 151*(4), 1531-1545.

Harris, R. M., Dijkstra, P. D., & Hofmann, H. A. (2014). Complex structural and regulatory evolution of the pro-opiomelanocortin gene family. *General and Comparative Endocrinology, 195*, 107-115. doi:10.1016/j.ygcen.2013.10.007

He, W., Dai, X., Chen, X., He, J., & Yin, Z. (2014). Zebrafish pituitary gene expression before and after sexual maturation. *J Endocrinol, 221*(3), 429-440. doi:10.1530/JOE-13-0488

Hruby, V. J., Cai, M., Cain, J. P., Mayorov, A. V., Dedek, M. M., & Trivedi, D. (2007). Design, synthesis and biological evaluation of ligands selective for the melanocortin-3 receptor. *Curr Top Med Chem, 7*(11), 1107-1119.

Jaillon, O., Aury, J. M., Brunet, F., Petit, J. L., Stange-Thomann, N., Mauceli, E., Roest Crollius, H. (2004). Genome duplication in the teleost fish tetraodon nigroviridis reveals the early vertebrate proto-karyotype. *Nature, 431*(7011), 946-957. doi:10.1038/nature03025

Larhammar, D., Bergqvist, C., & Sundstrom, G. (2015). Ancestral vertebrate complexity of the opioid system. *Vitam Horm, 97*, 95-122. doi:10.1016/bs.vh.2014.11.001

Larhammar, D., & Bergqvist, C. A. (2013). Ancient grandeur of the vertebrate neuropeptide y system shown by the coelacanth latimeria chalumnae. *Front Neurosci, 7*, 27. doi:10.3389/fnins.2013.00027

Leder, E. H., & Silverstein, J. T. (2006). The pro-opiomelanocortin genes in rainbow trout (oncorhynchus mykiss): Duplications, splice variants, and differential expression. *J Endocrinol, 188*(2), 355-363. doi:10.1677/joe.1.06283

Lespinet, O., Wolf, Y. I., Koonin, E. V., & Aravind, L. (2002). The role of lineage-specific gene family expansion in the evolution of eukaryotes. *Genome Res, 12*(7), 1048-1059. doi:10.1101/gr.174302

Lynch, M., & Conery, J. S. (2000). The evolutionary fate and consequences of duplicate genes. *Science, 290*(5494), 1151-1155.

Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C., Cohen, S. N., & Numa, S. (1979). Nucleotide sequence of cloned cdna for bovine corticotropin-beta-lipotropin precursor. *Nature, 278*(5703), 423-427.

Navarro, S., Soletto, L., Puchol, S., Rotllant, J., Soengas, J. L., & Cerda-Reverter, J. M. (2016). 60 years of pomc: Pomc: An evolutionary perspective. *J Mol Endocrinol, 56*(4), T113-118. doi:10.1530/JME-15-0288

Nei, M. (2013). *Mutation-driven evolution*: Oxford University Press.

Ohno, S. (1970). *Evolution by gene duplication*. Berlin, Heidelberg: Springer.

Roselli-Rehfuss, L., Mountjoy, K. G., Robbins, L. S., Mortrud, M. T., Low, M. J., Tatro, J. B., Cone, R. D. (1993). Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci U S A, 90*(19), 8856-8860.

Semon, M., & Wolfe, K. H. (2007). Reciprocal gene loss between tetraodon and zebrafish after whole genome duplication in their ancestor. *Trends Genet, 23*(3), 108-112. doi:10.1016/j.tig.2007.01.003

Smith, A. I., & Funder, J. W. (1988). Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. *Endocr Rev, 9*(1), 159-179. doi:10.1210/edrv-9-1-159

Smith, J. J., Timoshevskaya, N., Ye, C., Holt, C., Keinath, M. C., Parker, H. J., Amemiya, C. T. (2018). The sea lamprey germline genome provides insights into programmed genome rearrangement and vertebrate evolution. *Nature Genetics, 50*(2), 270-277. doi:10.1038/s41588-017-0036-1

Sundström, G., Dreborg, S., & Larhammar, D. (2010). Concomitant duplications of opioid peptide and receptor genes before the origin of jawed vertebrates. *PLOS ONE, 5*(5), e10512. doi:10.1371/journal.pone.0010512

Takahashi, A., Amano, M., Itoh, T., Yasuda, A., Yamanome, T., Amemiya, Y., Kawauchi, H. (2005). Nucleotide sequence and expression of three subtypes of

proopiomelanocortin mrna in barfin flounder. *Gen Comp Endocrinol, 141*(3), 291-303. doi:10.1016/j.ygcen.2005.01.010

Takahashi, A., Amemiya, Y., Nozaki, M., Sower, S. A., Joss, J., Gorbman, A., & Kawauchi, H. (1995). Isolation and characterization of melanotropins from lamprey pituitary glands. *Int J Pept Protein Res, 46*(3-4), 197-204.

Takahashi, A., Amemiya, Y., Sarashi, M., Sower, S. A., & Kawauchi, H. (1995). Melanotropin and corticotropin are encoded on two distinct genes in the lamprey, the earliest evolved extant vertebrate. *Biochem Biophys Res Commun, 213*(2), 490-498. doi:10.1006/bbrc.1995.2158

Takahashi, A. M., K.; Amano, M. (2014). Multifunctional roles of melanocyte-stimulating hormone and melanin-concentrating hormone in fish: Evolution from classical body color change. *Aqua-BioScience Monographs, 7*(1), 46. doi:10.5047/absm.2014.00701.0001

Taylor, J. S., Braasch, I., Frickey, T., Meyer, A., & Van de Peer, Y. (2003). Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res, 13*(3), 382-390. doi:10.1101/gr.640303

Taylor, J. S., Van de Peer, Y., & Meyer, A. (2001). Genome duplication, divergent resolution and speciation. *Trends Genet, 17*(6), 299-301.

CHAPTER 2

Growth performance after agouti-signaling protein 1 (asip1) overexpression in transgenic zebrafish

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Abstract

The melanocortin system is a key structure in the regulation of energy balance. Over-expression of inverse agonists, agouti-signaling protein (ASIP) and agouti-related protein (AGRP) result in increased food intake, linear growth and body weight. ASIP regulates dorsal-ventral pigment polarity through melanocortin 1 receptor (MC1R) and overexpression induces obesity in mice by binding to central MC4R. Asip1 over-expression in transgenic zebrafish (*asip1-Tg*) enhances growth yet experiments show fish overexpressing Asip1 do not develop obesity even under severe feeding regimes. *Asip1-Tg* fish do not need to eat more to grow larger and faster thus increased food efficiency can be observed. In addition, *asip1-Tg* fish reared at high density are able to grow far more than wild type (WT) fish reared at low density, although *asip1-Tg* fish seem to be more sensitive to crowding stress than WT fish, thus making the melanocortin system a target for sustainable aquaculture especially as the US Food and Drug Association has recently approved transgenic fish trading.

Keywords: melanocortin; growth; obesity; stress; transgenic; zebrafish

1. Introduction

Melanocortin agonists are commonly encoded by a complex precursor called proopiomelanocortin (POMC). This prepropeptide displays three main domains: The N-terminal pro-γ-melanocyte-stimulating hormone (MSH), the central adrenocorti-cotropic hormone (ACTH) and the C-terminal β-lipotropin. Each domain contains one MSH peptide characterized by the presence of a core sequence HFRW, i.e. γ-MSH in pro-γ-MSH, α-MSH as N-terminal sequence of ACTH and β-MSH in the β-lipotropin domain [1,2]. POMC is mainly expressed in the pituitary and differentially processed in a tissue-specific manner according to the cellular prohormone convertase array. The proteolytic cleavage by prohormone convertase 1/3 (PC1/3) produces ACTH in

the corticotrophs of the anterior pituitary whereas cleavage by PC1 and PC2 generates α-MSH in the melanotrophs of the pars intermedia [3]. In the rodent central nervous system (CNS), two discrete groups of neurons in the hypothalamus and the medulla also produce POMC [4], which is mainly processed to α-MSH and β-endorphin [5] (Fig. 1).

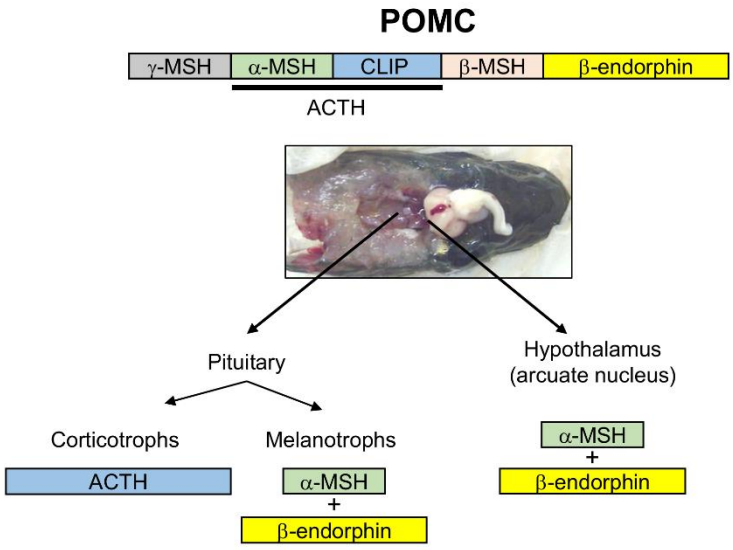


Figure 1. Scheme of the melanocortin precursor, proopiomelanocortin (POMC).

This prepropeptide shows three main domains each one containing one melanocyte-stimulating hormone (MSH). The N-terminal domain contains γ-MSH whereas the C-terminal domain carries β-MSH and the opioid peptide β-endorphin. The central domain contains α-MSH as N-terminal sequence of the adrenocorticotropic hormone. (ACTH). The C-terminal region of the ACTH is named CLIP (corticotropin-like intermediate peptide). This peptide is mainly expressed in the pituitary where it is differentially processed in a tissue-specific manner by prohormone convertases. In the corticotropes of the anterior pituitary, POMC release ACTH whereas in the melanotropes of the pars intermedia it generates α-MSH and β-endorphin. POMC is also expressed I the arcuate nucleus and nucleus of the tractus solitaries where it is processed to α-MSH and β-endorphin. See introduction for more information and references.

Melanocortins regulate a wide array of physiological functions including, melanogenesis, stress response and energy balance by binding to a family of specific G-protein-coupled receptors [6]. Five different melanocortin receptors (MC1R–MC5R) have been cloned in tetrapods. Subtype 2, or ACTH receptor, is only activated by the corticotropic hormone but requires interaction with the melanocortin receptor accessory protein subtype 1 (MRAP1) [7] whereas the four other MCRs distinctively recognize MSHs [6]. MC4R is also activated by ACTH but only after interaction with MRAP2 [8,9].

The melanocortin system also exhibits endogenous antagonists, which compete with melanocortin agonists by binding to the specific receptors [3]. Agouti-signaling protein (ASIP), designated, as agouti in mice, is a potent antagonist at MC1R and MC4R [10] whereas agouti-related protein (AGRP) competes with melanocortin agonists at MC3R and MC4R [11]. In mammals, ASIP controls the switch between the production of eumelanin (black/brown pigment) and phaeomelanin (yellow/red pigment) by antagonizing α-MSH effects on MC1R in the follicle melanocytes [12]. The atypical allele of the agouti locus, *Ay*, exhibits ubiquitous expression of the agouti gene that results in yellow hair but also in hyperphagia, increased linear growth, maturity-onset obesity and hyperinsulinemia [13,14]. This metabolic syndrome is mediated by antagonizing central MC4R signaling after ubiquitously agouti expression [10]. Intracerebroventricular (icv) administration of MC4R chemical agonists or antagonists inhibits or promotes food intake, respectively [15]. Accordingly, MC4R knockout mice exhibit a similar metabolic phenotype to that of agouti mice but with standard hair [16]. In fact, MC4R does not need α-MSH binding for signaling as it is constitutively activated [17,18], suggesting that MC4R persistent signaling is responsible for hungry constitutive inhibition. However, agouti is synthesized peripherally and only reaches CNS after ubiquitous expression. Indeed, the central function on MC4R is covered by the expression of AGRP that works as a physiological inverse

agonist at constitutively activated MC4R [17,18,19]. Central AGRP expression is confined within the arcuate nucleus [11] where its production dramatically increases with negative energy balance. Activation of AGRP neurons or icv injections lead to significant increases in food intake, but also decreased energy expenditure [20,21]. Accordingly, transgenic AGRP overexpression results in obesity with no effects on hair pigmentation [11].

Fish genome has paralogues for *agrp* and *asip* named *agrp1* and *asip1* [22,23] with controversial phylogenetic relationships [1]. Fasting increases hypothalamic *agrp1* expression in goldfish [24] whereas *agrp1* overexpression in zebrafish results in increased linear growth and weight as well as adipocyte hypertrophy [25]. *agrp1* morpholino knockdown promotes reduced growth in zebrafish larvae mediated by MC4R [26]. Our studies primarily described the involvement of ASIP1 in the regulation of melanogenesis and dorsal-ventral pigment pattern [27,28,29,30]. At metabolic levels, *asip1* overexpression resulted in increased feeding levels, linear growth and weight but different to AGRP1 overexpression [25], transgenic *asip1* zebrafish (*asip1-Tg*) display similar body fat levels to those of control fish [31]. In this experiment, both control fish and *asip1-Tg* were administered similar food quantities, suggesting an improved growth performance with no increased total fat sustained by an improved feed efficiency in *asip1-Tg* [31] despite *asip1* overexpression promoting food intake [31]. Feeding protocol for *agrp1*-Tg zebrafish was not reported [25], it is therefore plausible that feeding levels in our experiments limited obesity development in *asip1-Tg* animals [31]. In fact, diet-induced obesity has been demonstrated in zebrafish [32,33,34]. Under this framework, there was an attempt to solve two main questions: i) do *asip1-Tg* zebrafish need to eat more in order to grow further? and ii) will *asip1-Tg* zebrafish develop obesity under intense feeding protocols? Because melanocortin system is intimately linked to the stress response by regulating glucocorticoid synthesis via binding of ACTH to MC2R in the adrenal glands [35], we also

questioned if *asip1-Tg* animals could overcome the negative effects of crowding-induced stress on zebrafish growth [36].

2. Material and methods

All experiments were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals and approved by the Consejo Superior de Investigaciones Científicas (CSIC) ethics committee (project number AGL2016-74857-C3-3-R).

2.1 Effect of feeding rate on growth performance

To support the improved feeding efficiency in *asip1-Tg*, the same number of wild type (WT) and *asip1-Tg* embryos, obtained on the same day by crossing adult fish from our facilities at the Institute of Aquaculture from Torre de la Sal, were reared under the same physicochemical and volumetric conditions for four months [31]. During this period, rearing density was closely monitored to avoid density-induced growth differences. After 5 months, the initial population of each genotype was split up into four groups (n=37) following the initial sampling in which length (L) and weight (W) were registered (see below). The condition factor (CF) was calculated as follows $CF=100*W(g)/L(cm)^3$. This parameter is equivalent to body mass index (BMI) commonly used to evaluate human overweight and/or obesity. Animals were reared in 20-L glass tanks provided with autonomous filter pumps. Physicochemical conditions including pH (ranging from 7 to 7.5), photoperiod (14 light, light on at 7.00h /10 darkness, light off at 21.00h), conductivity (40-50 µS) and temperature (27 ± 1 ºC) were monitored daily and kept within the physiological range.

Two groups of each genotype (WT_LF and *asip1-Tg*_LF) were fed at 4% with commercial flakes (Vipan, Sera. 46.2% protein, 8.9% fat, 2.3% fibre, 11.9% ash, 6.7%

humidity) in two meals at 8.00 and 14.00. The remaining four groups (WT_HF and *asip1-Tg*_HF) were fed at 8% with dry feed at 8.00, 11.00 14.00 and 17.00. Food quantities and feeding times were chosen according to our previous zebrafish growth experiments [31].

Length and weight of all the animals was registered every month (see below) for three consecutive months and food rations were modified on a monthly basis according to total tank biomass. At the end of the experiment, six males and females from each treatment were used for gravimetrical quantification of total body lipids after Folch's extraction [31].

2.2 Effect of rearing density on growth performance

In order to evaluate the effect of crowding stress on growth performance of *asip1-Tg*fish, WT and *asip1-Tg* embryos, obtained on the same day by crossing adult fish from our facilities, were reared for 30 days in 15L-tanks [31].

After this initial period, animals of both genotypes were weighed in groups of five fish due to the reduced size, and distributed in eight 7.5 L-tanks as follows: WT_LD (n=12), *asip1-Tg*_LD (n=12), WT_HD (n=37) and *asip1-Tg*_HD (n=37). Fish density was chosen according to Leibold and Hammerschmidt (2015) [36].

In order to avoid differences in the water quality, potentially induced by fish density, an experiment was conducted in the re-circulatory system, provided with tandem charcoal-activated filters, where fish stocks are maintained. Physicochemical conditions were monitored and recorded as before. Animals were fed at 4% in two meals at 8.00 and 14.00 with commercial flakes. Animals were sampled for weight and length (see below) for four additional months and CF was calculated as before.

2.3 Determination of body length and whole-body weight

For determination of body length and weight, fish were not anesthetized. They were transferred to a 7.5-L sampling tank, individually captured and placed into a 100 ml plastic beaker with approximately 20 ml fresh water from the home aquaria. Subsequently, fish were pour out together with some water into a top-cut 5 ml plastic Pasteur disposable pipette. It allows fish to be immobile and positioned inside of the Pasteur pipette with enough water to reduce sampling stress. Pasteur pipet was then placed on plasticized millimeter paper (to 1 mm) and length was measured from the anterior tip of the mouth to the base of the caudal fin. Subsequently, plastic Pasteur pipette was pour out and unanesthetized fish dried on Whatman paper for approximately 5 seconds and transferred to a pre-tared plastic petri dish containing approximately 20 ml water from the home aquaria and weighted to two decimal points in grams. No mortality was registered after sampling events.

2.4 Statistical analysis

No significant differences were detected between replicate tanks after t-test student ($P<0.05$) and data were pooled for subsequent analysis. The differences between treatments were studied using two-way ANOVA (genotype vs feeding level or genotype vs rearing density) followed by Bonferroni's post-hoc test and considered significant at $P<0.05$.

3. Results

3.1 Effect of the feeding rate on growth performance

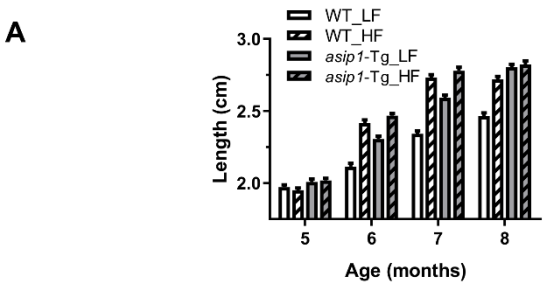
The effects of the feeding level and *asip1* overexpression on growth performance are summarized in Figure 2. In the early stage of the experiment, at five months old, animals from each genotype were statistically similar in length but *asip1-Tg* fish were already longer than WT counterparts (Figure 2A). In fact, *asip1-Tg* zebrafish were always longer than the WT animals during the whole experimental period.

The feeding level, regardless of genotype, induced significant length-growth differences on providing experimental feeding rates (6, 7 and 8 months old). Similarly, the interaction between both variables (feeding rate vs genotype) exhibited statistically significant levels during the last sampling times (Figure 2A). Such significant interaction indicates that a particular combination of both variables is responsible for a differential effect on length growth. It is particularly noticeable at the final stage of the experiment (8 months old), in which *asip1-Tg*_LF fish fed at low levels exhibited increased length when compared to WT_HF animals. Post Hoc analysis showed no differences between *asip1-Tg* fish fed with high or low levels. At this sampling point, *asip1-Tg*_LF and *asip1-Tg*_HF animals were 13.7% and 14.5% longer than WT_LF, respectively, whereas WT_HF were only 10.3% longer. On the contrary, *asip1-Tg*_HF satiated fish grew 3.8% more than the WT counterpart.

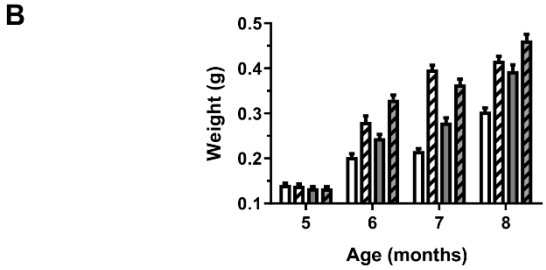
On assessing fish weight, no significant differences were displayed among the animals during the first sampling date (5 months old). Therefore, at the first stage of the experiment, *asip1-Tg* fish were longer but not heavier than WT animals (Figure 2B). Both feeding levels and genotype always enhanced fish weight but genotypic differences showed no significant relevance during the third sampling time (7 months old, Figure 2B). Once more, variable interaction reached statistical relevance

when fish were 7 and 8 months old. During the last sampling period (8 months old), *asip1-Tg* zebrafish fed at low and high levels were 30% and 52% heavier than the WT_LF, respectively whereas WT_HF were 37% heavier than fish of the same genotype fed at low levels. However, *asip1-Tg* fish were only 10% heavier than WT fish when both were fed at high levels. Statistical analysis revealed significant differences in weight within the same genotype when fed with high or low doses, respectively.

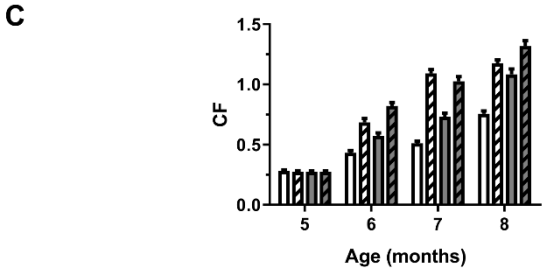
Body mass index traditionally known in fish as Condition Factor was also analysed. This parameter correlates weight and length of the animal thus providing a comparative idea of corpulence between animals. There were no differences displayed in the first stage of the experiment (Figure 2C). Subsequently, both genotype and feeding levels always induced a significant CF increase (Figure 2C). Once more, the interaction between both variables was significant at all three last sampling times. Particularly during the last sampling, CF of *asip1-Tg*_LF was similar to that of WT_HF fish whereas CF of *asip1-Tg*_HF zebrafish was statistically increased when compared to WT animals fed similarly.



	5	6	7	8
Feeding Level	p=0.7147	p<0.00001	p<0.00001	p<0.00001
Genotype	p=0.0014	p<0.00001	p<0.00001	p<0.00001
Interaction	p=0.3198	p=0.0009	p<0.00001	p<0.00001



	5	6	7	8
Feeding Level	p=0.7186	p<0.00001	p<0.00001	p<0.00001
Genotype	p=0.0829	p<0.00001	p=0.1056	p<0.00001
Interaction	p=0.7946	p=0.6916	p<0.00001	p<0.0489



	5	6	7	8
Feeding Level	0.6720	p<0.00001	p<0.00001	p<0.00001
Genotype	0.5283	p<0.00001	0.0117	p<0.00001
Interaction	0.6601	0.0236	p<0.00001	p<0.0127

Figure 2. Body Length (A), Weight (B) and Condition Factor (C) of WT and *asip1-Tg* zebrafish reared under low (LF) and high (HF) feeding regimes.

Each value is the mean ± SEM of 70 animals. Significant differences after two-way ANOVA are indicated in the table below each graph.

3.2 Feeding levels and obesity

The *agrp1* transgenic zebrafish (*agrp1*-Tg) was proposed as a model for vertebrate obesity since founder animals (+/-) overexpressing the endogenous inverse agonist exhibit adipocyte hyperplasia and higher triglyceride levels [25]. To support that *asip1* overexpression produces a similar phenotype, total lipid content in all four experimental groups was analysed. As sex can induce severe differences in the total lipid content, data were analyzed accordingly.

It was found that such data indicated that the feeding level induces increased total body lipid levels in both WT and *asip1-Tg* zebrafish (Fig 3A). Therefore, overfeeding induced an increase of 37.1% and 25.5% of total body fat in WT and *asip1-Tg* animals, respectively. The genotype also induces differences in the body fat but remarkably, *asip1-Tg* zebrafish exhibit significantly lower levels than WT fish taking into account both feeding levels (Figure 3A). WT_LF animals show 9.7% more fat content that *asip1-Tg* animals fed with similar quantities.

On feeding both genotypes at high doses, differences reached 13.9%. In contrast, sex did not induce significant differences in the lipid content of animals fed at high and low levels (Figure 3B). Remarkably, on grouping data according to sex genotype was unable to induce significant differences (P=0.057), thus suggesting that lipid content differences induced by the genotype are within the statistical limit of signification. In conclusion, on analysing data, regardless of the genotype, results demonstrate that both sex and feeding levels significantly regulate lipid content in fish (Figure 3C). Accordingly, high feeding levels induce higher amounts of total body lipids in males (13.7%) and females (12.1%), respectively but also when fed at the same level, females exhibited significantly higher lipid levels (12.2% and 10.8% for high and low feeding levels, respectively).

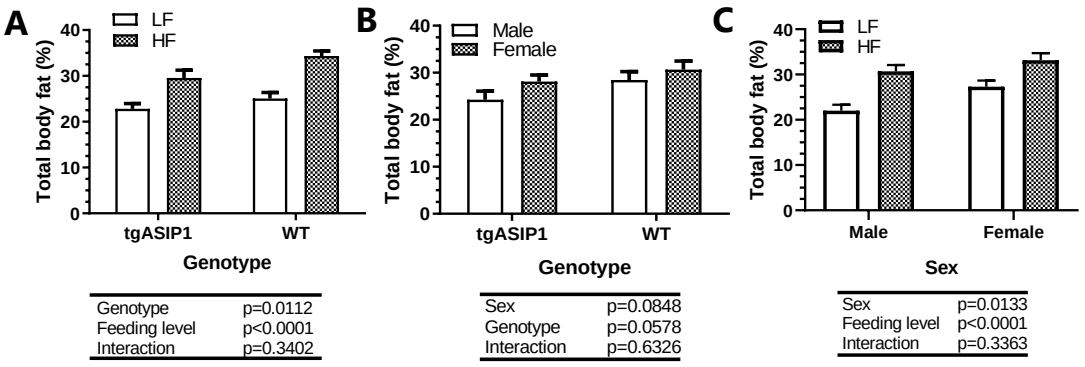


Figure 3. Total body lipids after gravimetrical determination

(A) Genotype x feeding level, (B) genotype x sex and (C) sex x feeding level. WT and *asip1-Tg* zebrafish (n=12, 6 males and 6 females each genotype) reared under low (LF) and high (HF) feeding regimens. Significant differences after two-way ANOVA are indicated in the table below each graph.

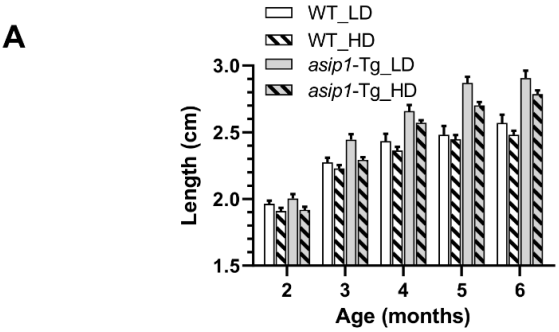
3.3 Effect of crowding stress on growth performance

The melanocortin system is a key player of the regulation stress response. It is, therefore, conceivable that stress levels can differentially affect growth in both experimental genotypes. To support this, rearing density is used as a potential stressor. Therefore, crowded tanks had a three-fold animal number (n=37) when compared to regular tanks (n=12). The initial rearing density was 0.206 and 0.128 g/l for WT_HD and *asip1-Tg*_HD and 0.07 and 0.04 g/l for WT_LD and *asip1-Tg*_LD, respectively. Concluding the experimental period, densities reached 4.84 and 4.28 g/l in *asip1-Tg*_LD and WT_LD and 13.92 and 12.40 g/l in *asip1-Tg*_HD and WT_HD, respectively. High rearing density always induced reductions in length regardless of the fish genotype (Figure 4A). In contrast, the genotype, regardless of rearing density, encouraged length growth in fish at three months of age and older, but not earlier. Interactions between both variables were never statistically significant.

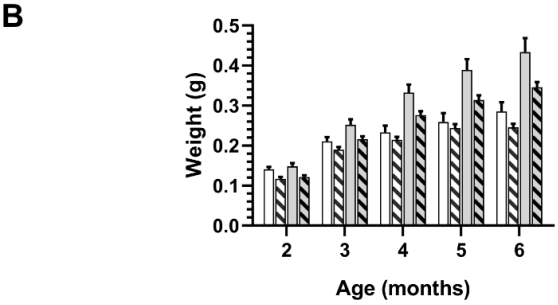
During the last sampling time, *asip1-Tg*_LD or *asip1-Tg*_HD were 13.1% and 12.1% longer than WT fish reared in similar conditions, respectively. Unexpectedly, *asip1-Tg* fish kept under crowding conditions (HD) were 8.2% longer than WT_LD fish.

At the beginning of the experiment, WT fish both in HD and LD tanks were heavier (0.21 ± 0.034 g) than *asip1-Tg* fish (0.13 ± 0.008 g) (data not shown). As early as the first experimental month, genotypic differences were abolished but density effects were already obvious (Figure 4B). Hereinafter, fish weight progressed in a parallel mode to length; therefore, density always induced significant differences in weight during the whole experimental period whereas the genotype differences were insignificant during the first sampling time. During the last sampling time, *asip1-Tg* fish reared at low or high density were 51.9% and 40% heavier than WT fish reared in similar conditions, respectively. *asip1-Tg*_HD were 21% heavier than WT_LD.

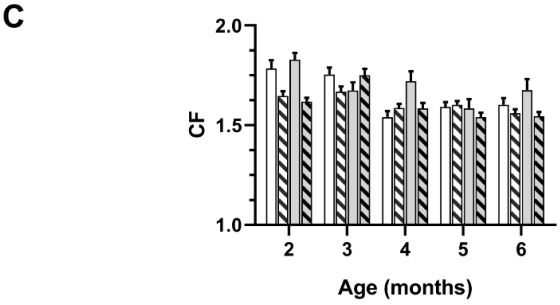
Effects of rearing density and genotype on CF are summarized in Figure 4C. At the early stage of the experiment, density had a clear effect on CF as animals reared at low density had constantly increased CF values, but these differences were reduced during the following sampling times and again reached significant relevance during the last sampling time. The genotype only induced differences in fish at four months of age. Interaction between both variables (density and genotype) was significant in fish at 4 and 5 months of age (Figure 4C).



	2	3	4	5	6
Density	p=0.0204	p=0.0019	p=0.0256	p=0.0152	p=0.0155
Genotype	p=0.3878	p=0.0002	p<0.0001	p<0.0001	p<0.0001
Interaction	p=0.6080	p=0.0918	p=0.8029	p=0.1012	p=0.6927



	2	3	4	5	6
Density	p=0.0001	p=0.0037	p=0.0039	p=0.0062	p=0.0008
Genotype	p=0.3726	p=0.0007	p<0.0001	p<0.0001	p<0.0001
Interaction	p=0.7928	p=0.4358	p=0.1446	p=0.066	p=0.2022



	2	3	4	5	6
Density	p<0.0001	p=0.8898	p=0.1821	p=0.5745	p=0.0071
Genotype	p=0.8199	p=0.9704	p=0.0084	p=0.2390	p=0.3678
Interaction	p=0.2955	p=0.0363	p=0.0072	p=0.3490	p=0.1597

Figure 4. Body (A), weight (B) and condition Factor (C) of WT and *asip1-Tg* zebrafish cultured under low and (LD) and high (HD) rearing conditions.

Each value is the mean ± SEM of 24 or 74 animals, respectively. Significant differences after two-way ANOVA are indicated in the table below each graph.

4. Discussion

The melanocortin system is intimately linked to the control of feeding behavior, obesity and growth in vertebrates [21,37], including fish [3]. In mammals, *asip* [13,14] and *agrp* [11] overexpression result in obesity as a consequence of overfeeding but also as a result of decreased energy expenditure [38]. High expression levels of both inverse agonists also result in increased linear growth and this effect is well conserved throughout the evolutionary scale [3]. In fact, overexpression of *agrp1* as well as *asip1* [31] in zebrafish enhances linear growth and body weight. Agrp-Tg zebrafish exhibit higher total triglyceride levels as well as visceral adipose hypertrophy, thus suggesting that the inhibition of melanocortin signaling promotes obesity in zebrafish as it does in mammals [25]. However, our preliminary results suggest that *asip1* overexpressing zebrafish are not obese as total body lipid levels are similar to the pair-fed WT siblings [31]. To elucidate such discrepancy, two populations of each genotype were subjected to regular (4% of body weight) and severe feeding (8% of body weight) protocols. Results determined that feeding levels enhance linear growth in zebrafish revealing the phenotypic plasticity in fish [39]. Therefore, feeding levels and no evolutionary adaptations to commercial diets explain growth differences between WT and farmed salmon [40]. Similarly, the genotype promotes length growth, regardless of feeding levels, even before starting experimental feeding protocols. However, *asip1-Tg* fish fed at a low ratio can grow as much as both WT or *asip1-Tg* fish fed at high levels showing that *asip1-Tg* fish do not need to eat more in order to enhance growth rate. In fact, growth promotion induced by severe feeding in *asip1-Tg* is reduced when compared to that induced in WT animals thus suggesting that *asip1-Tg* fish are close to the maximal phenotypic growth and demonstrate enhanced growth efficiency. Such ability requires an improved food conversion efficiency promoted by *asip1* overexpression and potentially by inhibition of

melanocortin signaling via central or peripherally expressed melanocortin receptors. Accordingly, the administration of α-MSH has been proved to stimulate energy expenditure in larval zebrafish [41], however our preliminary data shows that *asip1-Tg* fish exhibit enhanced daily locomotor activity, especially during the dark period, which suggests an enhanced energy expenditure (Godino-Gimeno A, Guillot R and Cerdá-Reverter, unpublished data). Alternatively, melanocortin signaling reduction by *asip1* overexpression in adult zebrafish could improve intestinal function. To the best of our knowledge, there is no information on melanocortin effects on gastrointestinal function but experiments carried out in zebrafish have shown a profuse expression of MC4R and MC5Ra and MC5Rb in the gastrointestinal tract [42].

Endocrine and molecular mechanisms promoting growth under decreased melanocortin signaling has been studied tentatively but yet far from being fully understood. Standard somatic growth requires AGRP signaling through MC4R in larval zebrafish [26,43]. Both POMC and AGRP hypothalamic neurons are hypophysiotropic and regulate the expression of diverse endocrine axes [26]. Therefore, *agrp1* knockdown in the morpholino zebrafish model results in decreased growth hormone (gh) expression that is concomitant with a severe increase in the expression of GH-releasing hormone and decreased somatostatin I and II expression (*sstI* and *sstII*) [26].

Recently, a proposal to involve the melanocortin system in the feeding-induced growth in larval zebrafish was made [44]. Overfeeding causes leptin resistance and reduced POMC hypothalamic levels, which lead to reduced activity of SST neurons that express *mc4r* and consequently elevated *gh* expression and somatic growth [43]. It is therefore feasible that *agrp* or *asip1* overexpression in zebrafish promotes somatic growth via MC4R in SST neurons.

Our experiments determine that feeding levels can induce obesity in zebrafish as previously proposed [35]. Total body lipid levels in both genotypes are substantial, ranging approximately from 20-30% body weight. *Asip1-Tg* animals were not found to be obese, in fact, they exhibited diminished body lipid content, regardless of feeding levels, when compared to WT fish. It contrasts sharply with the phenotype of the agouti viable yellow mice (*Ay*/+) that exhibits morbid obesity. In fact, most of the excess weight (92%) in agouti mice is due to an increase in adipose tissue mass [45]. *Asip1-Tg* fish also exhibit higher CF values than WT fish thus suggesting that *asip1-Tg* corpulence is due to an increase of muscle mass. Agrp1-Tg zebrafish were reported as obese based on percentage triglyceride levels and visceral adipocyte hypertrophy [25]. Triglyceride levels were not evaluated in *asip1-Tg* fish but total lipid levels are thought to be a sensitive maker of body fat depots as triglyceride are the main component of total lipid in fish [46,47]. It is feasible that lipid accumulation in *asip1-Tg* fish could have different targets to fat tissue [46], as membrane phospholipids. Larger animals, such as *asip1-Tg* zebrafish, could have higher amounts of structural lipid but our data is shown as a percentage of body weight. At this point, there is no explanation for such discrepancy as both AGRP and ASIP1 metabolic effects were mediated by MC4R [26,31] and further studies are required in order to evaluate potential differential effects of ASIP1 and AGRP on lipid metabolism. A plausible explanation for reduced body lipid levels could involve the enhanced locomotor activity in *asip1-Tg* fish (see above) or a potential role for MC1R in the metabolism of subcutaneous adipose tissue, one of the major fat depots together with intermuscular fat in zebrafish [46].

The melanocortin system plays a key role in the stress response in fish; it is then plausible that *asip1-Tg* fish can respond differentially to stressors and by extension exhibit differential growth response under stressful environments. To support this hypothesis, we use the crowding-induced stress as paradigm to assess *asip1*

overexpression effects on growth. Again, *asip1-Tg* fish were longer than WT counterparts regardless of the rearing density. However, crowding always reduced linear growth and body weight as previously reported [36]. *Asip1-Tg* fish reared at high densities grew even more than WT animals reared at low densities. However, *asip1-Tg* fish seem more sensitive to crowding since rearing density induces a 26% decrease in total body weight but only 16% in WT fish which applies to fish length. There are few studies regarding the effect of the melanocortin system on main behavioral aspects, different from feeding behavior, in vertebrates including fish. Our extended handling experience with *asip1-Tg* fish during samplings and husbandry suggest that these animals display higher fear levels and preliminary studies using diverse behavioral tests demonstrate that *asip1-Tg* fish exhibit higher anxiety levels as well as significant higher total cortisol levels and reduced central serotonin (5-HT). It is also conceivable that anxiety and higher food intake levels make *asip1-Tg* fish more aggressive than WT animals but again preliminary data indicates that transgenic fish are consistent losers in dyadic fights (Rocha A, Godino-Gimeno A, Cerdá-Reverter JM, unpublished results). A recent paper has shown that zebrafish *agrp2* (or *asip2b*) is expressed in the neurons of preoptic nucleus (NPO), the homologous of paraventricular nucleus (PVN) in fish [48], that project to rostral pars distalis in the pituitary to control *pomca* expression. *agrp2* knockout larvae exhibit decreased pituitary *pomca* expression and increased total cortisol levels. It seems that AGRP2 may be secreted as neurohormone acting directly on interrenal tissue, where all MCRs are expressed, to promote cortisol synthesis and release which subsequently inhibit pituitary *pomca* expression [42]. AGRP2 can bind MC1R, MC4R, MC5Ra and MC5Rb suggesting that these receptors could mediate effects on cortisol secretion [49]. Thus, suggesting that melanocortin inverse agonists are capable of controlling stress response in fish by regulating cortisol secretion. However, *asip1* overexpression displays a similar effect to the defective expression of *agrp2* on cortisol synthesis

suggesting opposite roles in stress response. ASIP1 effects were evaluated in adult fish (present study) whereas AGRP2 effects were evaluated in zebrafish larvae, it is thus feasible that melanocortin inverse agonists display opposite effects according to the developmental stage. Suggestively, AGRP1 has been shown to be a potent hipophysiotrophic peptide during the early developmental stages, [26] but not in adult fish [50].

Sustainability of fish aquaculture is compromised by the availability of raw materials for commercial diets, but also by food efficiency of reared fish [51]. FDA has recently approved the trading of GH overexpressing salmon for human consumption in the USA and Canada. It was determined that the melanocortin systems, specifically the overexpression of *asip1*, could be a potential target to develop new genetically engineered fish displaying enhanced growth (more than 50% body weight) with better food conversion ratios and no differential lipid accumulation. These animals can be reared at high density even showing higher growth rates (more than 20% in body weight) than WT fish reared at low density levels. A major issue for transgenic aquaculture involves genetically-modified organism (GMO) escapes and the displacement of natural fish population of the same and/or related species in freshwater ecosystems. Our preliminary data suggests that *asip1-Tg* fish are much less aggressive when competing with WT animals in dyadic fights but further studies are required in order to evaluate fish behavior including food competence. In addition, due to the effects of *asip1* overexpression on dorsal-ventral pigment pattern [28], these animals are easily identified in the wild after potential escapes. Commercial rejection or adverse effects on markets by morphological aspects is not expected as pigment alterations only involve the dorsal region and never the fish flanks.

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Disclosure Statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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References

1. Cortés R, Navarro S, Agulleiro MJ, Guillot R, García-Herranz V, Sánchez E et al. Evolution of the melanocortin system. Gen Comp Endocrinol 2014; 209: 3-10.
2. Navarro S, Soletto L, Puchol S, Rotllant J, Soengas JL, Cerdá-Reverter JM. 60 YEARS OF POMC: POMC: an evolutionary perspective. J Mol Endocrinol 2016; 56: 113-118.
3. Cerdá-Reverter JM, Agulleiro MJ, R R G, Sánchez E, Ceinos R, Rotllant J. Fish melanocortin system. Eur J Pharmacol 2011; 660: 53-60.
4. Bangol D, Lu X-Y, Kaelin CB, Day HEW, Ollmann M, Gantz I, et al. Anatomy of an endogenous antagonist: relationship between agouti-related protein and proopiomelanocortin in bran. J Neurosci 1999; 19: 1 –7.
5. Castro, M.G.; Morrison, E. Post-translational processing of proopiomelanocortin in the pituitary and in the brain. Crit. Rev. Neurobiol. 1997, 11, 35–57.
6. Schiöth HB, Haitina T, Ling MK, Ringholm A, Fredriksson R, Cerdá-Reverter JM et al. Evolutionary conservation of the structural, pharmacological, and genomic characteristics of the melanocortin receptor subtypes. Peptides 2005, 26, 1886-900.
7. Cerdá-Reverter JM, Agulleiro MJ, Cortés R, Sánchez E, Guillot R, Leal E, et al. Involvement of melanocortin receptor accessory proteins (MRAPs) in the function of melanocortin receptors. Gen. Comp. Endocrinol. 2013; 188: 133-136.
8. Josep Agulleiro M, Cortés R, Fernández-Durán B, Navarro S, Guillot R, Meimaridou E, et al. Melanocortin 4 receptor becomes an ACTH receptor by coexpression of melanocortin receptor accessory protein 2. Mol Endocrinol 2013; 27: 1934-45.
9. Soletto L, Hernández-Balfagó S, Rocha A, Scheerer P, Kleinau G, Cerdá-Reverter JM. Melanocortin Receptor Accessory Protein 2-Induced Adrenocorticotropic Hormone Response of Human Melanocortin 4 Receptor. J Endocr Soc 2018; 3: 314-323.
10. Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, et al. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 1994; 37: 799-802.
11. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, et al. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 1997; 278: 135-138

12. Vrieling H, Duhl DM, Millar SE, Miller KA, Barsh GS. Differences in dorsal and ventral pigmentation result from regional expression of the mouse agouti gene. Proc Natl Acad Sci USA 1994; 9: 5667-5671.
13. Michaud EJ, Bultman SJ, Stubbs LJ, Woychik RP. The embryonic lethality of homozygous lethal yellow mice (*Ay/Ay*) is associated with the disruption of a novel RNA-binding protein. Genes Dev. 1993; 7: 1203-1213.
14. Miller MW, Duhl DM, Vrieling H, Cordes SP, Ollmann MM, Winkes BM et al. Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. Genes Dev 1993; 7: 454-67.
15. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 1997; 385: 165-168.
16. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 1997; 88: 131-41.
17. Nijenhuis WA, Oosterom J, Adan RA. AgRP(83-132) acts as an inverse agonist on the human-melanocortin-4 receptor. Mol Endocrinol 2001; 15: 164-171.
18. Sánchez E, Rubio VC, Thompson D, Metz J, Flik G, Millhauser GL, et al. Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (*Dicentrarchus labrax*). Am J Physiol Regul Integr Comp Physiol 2009; 296: R1293-1306
19. Tolle V, Low MJ. In vivo evidence for inverse agonism of Agouti-related peptide in the central nervous system of proopiomelanocortin-deficient mice. Diabetes 2008; 57: 86-94.
20. Rossi M, Kim MS, Morgan DG, Small CJ, Edwards CM, Sunter D, Abusnana S, Goldstone AP, Russell SH, Stanley SA, Smith DM, Yagaloff K, Ghatei MA, Bloom SR. A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. Endocrinology. 1998; 139:4428-4431.
21. Koch M, Horvath TL. Molecular and cellular regulation of hypothalamic melanocortin neurons controlling food intake and energy metabolism. Mol Psychiatry 2014; 19: 7752-7761.
22 Klovins J, Schiöth HB. Agouti-related proteins (AGRPs) and agouti-signaling peptide (ASIP) in fish and chicken. Ann N Y Acad Sci. 2005; 1040:363-367

23. Agulleiro MJ, Cortés R, Leal E, Ríos D, Sánchez E, Cerdá-Reverter JM. Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (*Dicentrarchus labrax*). Gen Comp Endocrinol 2014; 205: 251-259.
24. Cerdá-Reverter JM, Peter RE. Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. Endocrinology 2003; 144: 4552-4561.
25. Song Y, Cone RD. Creation of a genetic model of obesity in a teleost. FASEB J 2007; 2: 2042-9.
26. Zhang C, Forlano PM, Cone RD. AgRP and POMC neurons are hypophysiotropic and coordinately regulate multiple endocrine axes in a larval teleost. Cell Metab 2012; 15: 256-64.
27. Cerdá-Reverter JM, Haitina T, Schiöth HB, Peter RE. Gene structure of the goldfish agouti-signaling protein: a putative role in the dorsal-ventral pigment pattern of fish. Endocrinology 2005; 146: 1597-1610.
28. Ceinos RM, Guillot R, Kelsh RN, Cerdá-Reverter JM, Rotllant J. Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms. Pigment Cell Melanoma Res. 2015; 28: 196-209.
29. Cal L, Suarez-Bregua P, Comesaña P, Owen J, Braasch, Kelsh R, et al. Countershading in zebrafish results from an Asip1 controlled dorsoventral gradient of pigment cell differentiation. Sci Rep 2019; 9: 3449.
30. Cal L, Suarez-Bregua P, Cerdá-Reverter JM, Braasch I, Rotllant J. Fish pigmentation and the melanocortin system. Comp Biochem Physiol A Mol Integr Physiol 2017; 211: 26-33
31. Guillot R, Cortés R, Navarro S, Mischitelli M, García-Herranz V, Sánchez E, et al. Behind melanocortin antagonist overexpression in the zebrafish brain: A behavioral and transcriptomic approach. Horm Behav 2016; 82: 87-100.
32. Oka T, Nishimura Y, Zang L, Hirano M, Shimada Y, Wang Z, et al. Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. BMC Physiol 2010; 10: 21.
33. Forn-Cuní G, Varela M, Fernández-Rodríguez CM, Figueras A, Novoa B. Liver immune responses to inflammatory stimuli in a diet induced obesity model of zebrafish J Endocrinol 2015; 224:159–170.

34. Faillaci F, Milosa F, Critelli RM, Turola E, Schepis F, Villa E. Obese zebrafish: A small fish for a major human health condition. Animal Model Exp Med 2018; 1: 255-265.
35. Delgado MJ, Cerdá-Reverter JM, Soengas JL. Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: involvement in the control of food intake. Front Neurosci 2017; 11: 354.
36. Leibold S, Hammerschmidt M. Long-term hyperphagia and caloric restriction caused by low- or high-density husbandry have differential effects on zebrafish postembryonic development, somatic growth, fat accumulation and reproduction. PLoS One 2015; 23:10 e0120776.
37. Ellacott KL, Cone RD, The role of the central melanocortin system in the regulation of food intake and energy homeostasis: lessons from mouse models. Philos Trans R Soc Lond B Biol Sci 2006; 361: 1265-1274.
38. Fekete C, Marks DL, Sarkar S, Emerson CH, Rand WM, Cone RD et al. Effect of agouti-related protein in regulation of the hypothalamic-pituitary-thyroid axis in the melanocortin 4 receptor knockout mouse. Endocrinology 2004; 145: 4816–4821
39. Johnston IA. Environment and plasticity of myogenesis in teleost fish. J. Exp. Biol. 2006; 209: 2249-2264.
40. Harvey AC, Solberg MF, Troianou E, Carvalho GR, Taylor MI, Creer S, et al. Plasticity in growth of farmed and wild Atlantic salmon: is the increased growth rate of farmed salmon caused by evolutionary adaptations to the commercial diet? BMC Evol Biol 2016; 16: 264.
41. Renquist BJ, Zhang C, Williams SY, Cone RD. Development of an assay for high-throughput energy expenditure monitoring in the zebrafish. Zebrafish 2013; 10: 343-352.
42. Ringholm A, Fredriksson R, Poliakova N, Yan YL, Postlethwait JH, Larhammar D, et a., One melanocortin 4 and two melanocortin 5 receptors from zebrafish show remarkable conservation in structure and pharmacology. J Neurochem 2002; 82: 6-18.
43. Shainer I, Michel M, Marquart GD, Bhandiwad AA, Zmora N, Ben-Moshe Livne Z, et al., Agouti-related protein 2 is a new player in the teleost stress response system. Curr Biol 2019; 29: 2009-2019.e7.

44. Löhr H, Hess S, Pereira MMA, Reinoß P, Leibold S, Schenkel C. Diet-induced growth is regulated via acquired leptin resistance and engages a pomc-somatostatin-growth hormone circuit. Cell Rep 2018; 23: 1728-1741.
45. Herberg L, Coleman DL. Laboratory animals exhibiting obesity and diabetes syndromes. Metabolism 1977; 26: 59-99.
46. Minchin JEN, Rawls JF. A classification system for zebrafish adipose tissues. Dis. Model Mech. 2017; 10: 797-809.
47. Zhang J, Tao N, Zhao Y, Wang X, Wang M. Comparison of the fatty acid and triglyceride profiles of big eye tuna (*Thunnus obesus*), atlantic salmon (*Salmo salar*) and bighead carp (*Aristichthys nobilis*) heads. Molecules 2019; 24: 3983.
48. Herget U, Wolf A, Wullimann MF, Ryu S. Molecular neuroanatomy and chemoarchitecture of the neurosecretory preoptic-hypothalamic area in zebrafish larvae. J Comp Neurol 2014; 522: 1542-1564.
49. Zhang C, Song Y, Thompson DA, Madonna MA, Millhauser GL, Toro S, et al. Pineal-specific agouti protein regulates teleost background adaptation. Proc Natl Acad Sci USA 2010; 107: 20164-20171.
50. Forlano PM, Cone RD. Conserved neurochemical pathways involved in hypothalamic control of energy homeostasis. J Comp Neurol 2007; 505: 235–248.
51. Naylor RL, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MC, Clay J, et al. Effect of aquaculture on world fish supplies. Nature 2000; 405: 1017-1024.

CHAPTER 3

Agouti overexpression in a transgenic model regulates integrity, permeability and electrogenic amino acid transport in zebrafish intestine

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Abstract

Overexpression of asip1 in transgenic zebrafish disrupts dorsoventral pigment pattern in addition to increasing food intake levels and linear growth. A higher feed intake is unnecessary in transgenic fish to enable larger and heavier growth. A plausible explanation may rely on the enhanced feeding efficiency mediated by improved nutrient absorption in transgenic animals. To test this hypothesis, wide scope transcriptomic techniques were used to elucidate the potential pathways involved in the enhanced nutrient absorption and intestinal epithelium permeability/integrity. In addition, the electrogenic capacity for amino acid transport was analysed. Transcriptomic analysis reveal that amino acid, monocarboxylates, ionic and vitamin transmembrane transporters were substantially modified. Enrichment analysis also revealed an inhibition of intestinal lipid metabolism and down-regulation of KEGG pathways related to membrane integrity suggesting augmented intestinal laxity that may enhance paracellular transport. Electrophysiological experiments carried out in Ussing chambers show that *asip1* overexpression decrease membraned tissue resistance (Rt), indicating a modification of the intestinal barrier function in ASIP1 transgenic animals. Similarly, paracellular permeability was higher in transgenic zebrafish. Both the decrease in Rt and the increase in permeability point to an ASIP1-dependent decrease in the tissue barrier function. Electrogenic amino acid transport was also enhanced in transgenic animals providing strong indication that ASIP1 fish can extract more amino acids from their diet at similar feeding levels. Both transcriptomic and electrophysiological results suggest that asip1-overexpressing zebrafish display improved nutrient absorption and by extension a higher feed efficiency which explains enhanced growth in the absence of augmented food intake. The enhanced growth of ASIP1 zebrafish potentially mediated by improved nutrient uptake and feed efficiency suggests that the melanocortin system, specifically *asip1*

overexpression, is a potential target for the development of genetically engineered fish displaying improved performance and no differential lipid accumulation.

Keywords: amino acid transport, feed efficiency, growth performance, gut, melanocortin, nutrient absorption

1. Introduction

Agouti protein, also known as agouti-signalling protein (ASIP) in species other than mice, is part of a complex hormonal system comprising the paralogue agouti-related protein (AGRP) and several melanocortin peptides encoded by the multifaceted precursor proopiomelanocortin (POMC). This prepropeptide includes different melanocyte-stimulating hormones (α-, β- and γ-MSH), as well as the adrenocorticotropic hormone (ACTH) and the C-terminal β-endorphin, an opioid peptide of the vertebrate brain (Cerdá-Reverter et al., 2011). Melanocortin signalling is mediated by a family of G-protein coupled receptors which comprises five paralogues (MC1R-MC5R) exhibiting distinct expression domains and pharmacological profiles underlying specific gene functions (Cortés et al., 2014). MC1R binds preferentially to α-MSH and is mainly expressed in the skin, where it plays a regulatory role in melanogenesis (Cal et al., 2017). MC2R is the only receptor exclusively activated by ACTH and is expressed in the adrenal gland regulating glucocorticoid synthesis and secretion. The functional expression of MC2R requires interaction with the melanocortin receptor accessory protein 1 (MRAP1) which promotes trafficking of the receptor to the plasma membrane (Cerdá-Reverter et al., 2013). MC3R and MC4R are mainly expressed in the central nervous system to regulate energy balance and feeding behaviour and energy expenditure through specific binding to the endogenous MCRs agonists γ- and β-MSH, respectively (Schiöth et al., 2005). The MC5R displays a wide

expression domain yet always at low levels. It is involved in the regulation of exocrine secretion (Schiöth et al., 2005).

ASIP and AGRP are endogenous competitive antagonists that inhibit agonists from binding the MC1R and MC4R respectively, yet ASIP also binds to MC4R. Both antagonists work as inverse agonists and inhibit the constitutive activity of their cognate receptors (Tao, 2014). The blockade of the central melanocortin signalling by suppression of MC4R expression (Huszar et al., 1997) as well as the overexpression of AGRP (Ollman et al., 1997) in mice or central AGRP administration in rats (Rossi et al., 1998) result in hyperphagia and obesity. Similarly, disruption of the melanocortin central signalling by ubiquitous constitutive expression of the agouti gene in obese yellow mice (Ay) results in hyperphagia, hyperinsulinemia, increased linear growth, maturity-onset obesity and yellow fur (Lu et al., 1994). The metabolic syndrome exhibited by agouti yellow mice is mediated by the interaction with the central MC4R, i.e., the ectopic expression of agouti acts as an endogenous antagonist in MC4R signalling in the hypothalamic neurons, thus leading to hyperphagia and obesity (Fan et al., 1997).

Due to the additional teleost specific genome duplication (TSGD or 3R), the melanocortin receptor/peptide system is greatly expanded in this lineage. Consequently, teleost fish display paralogous ASIP (ASIP1 and ASIP2) and AGRP (AGRP1 and AGRP2) genes (Agulleiro et al., 2014; Cortés et al., 2014). Central melanocortin signalling also regulates energy balance in fish as central administration of synthetic agonists results in a severe decrease in food intake levels in goldfish (Cerdá-Reverter et al., 2003 a,b). Consistently, fasting sharply up-regulates hypothalamic AGRP1 expression in the same species (Cerdá-Reverter and Peter, 2003). MC4R is constitutively activated in the brain, while AGRP1 functions as an endogenous inverse agonist (Sánchez et al., 2009). Overexpression of asip1 in transgenic zebrafish disrupts dorsoventral pigment pattern (Ceinos et al., 2015), yet also increase food intake levels and linear growth

(Guillot et al., 2016; Godino-Gimeno et al., 2020). Unlike mammalian models, an obese phenotype is absent in zebrafish overexpressing ASIP (Guillot et al., 2016), moreover, a higher intake of feed is not necessary to grow longer and heavier. This is perhaps the result of an enhanced food conversion rate (Godino-Gimeno et al., 2020). The enhanced growth may be managed at the level of muscle development through the somatotropic axis (Löhr et al., 2018) or, alternatively, ASIP1 transgenic fish could have improved intestinal function with more efficient nutrient absorption. In this study, the latter was explored by comparing the intestinal transcriptome of ASIP1 transgenic zebrafish vs wild-type animals (WT), by analysing intestinal epithelium permeability and the electrogenic capacity for amino acid transport in Ussing chambers.

2. Materials and methods

2.1 Fish and tissue sampling

WT-Tu (Tuebingen, Nüsslein-Volhard Lab) strain and transgenic [Tg(Xla.Eef1a1:Cau.Asip1)iim04] zebrafish**,** generated with the Tol2 transposon system using the elongation factor 1-alpha (*ef1-alpha*) constitutive promoter (Ceinos et al., 2015), were obtained from our facilities at the Institute of Aquaculture from Torre de la Sal (IATS), and bred under the same conditions for 6 months. Eight specimens were size-selected [bodyweight (BW)= 0.15 g ± 0.01] and kept for 24 days in 8-litre tanks equipped with a conventional re-circulatory water system to maintain the identical physicochemical and biotic conditions. Specimens were kept under 12 light (L) / 12 darkness (D) photoperiods with lights switched on at 8.00 am and fed once a day at 1.5% BW at 10.00 am (Zebrafeed, Sparos, 63% protein, 13% fat 1.8% fiber, 12% ash). Prior to tissue sampling for RNA extraction, fasting was carried out for 24h and the fish were subsequently euthanized by an overdose of anaesthesia (MS222,

tricaine methane sulfonate buffered solution; 300 mg/L). Gut samples were carefully dissected under stereomicroscope (Zeiss Stemi 305) and placed in 0.1 M phosphate saline buffer to remove any adhered non-intestinal tissue, including fat, spleen, gallbladder, gonadal tissue and free oocytes (females) with fine forceps. In addition, around 2-3 mm of the anal extreme was sectioned out with scalpel to avoid potential contamination by faeces and gonadal tissue. Experiments were carried out in accordance with the principles published in the European animal directive (86/ 609/EEC) for the protection of experimental animals and approved by the "Superior Council of Scientific Investigations" (CSIC) ethics committee (project number PID2019-103969RB-C33) and the "General Directorate of Agriculture, Livestock and Fishing of Valencia", registration number: nGVRTE/2021/217198.

2.2 RNA isolation and sequencing

Zebrafish tissue was extracted with Tri-reagent (Sigma), rapidly frozen in liquid nitrogen and stored at -70ºC. Total RNA extraction was followed by DNase (Promega) treatment and a second phenol-chloroform extraction was performed to inactivate and remove denaturalized DNase protein. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). An RIN (RNA integrity number) higher than 7 and a ratio of 28S:18S (rRNA) higher than 1.8 were required for all samples. Total RNA samples (1 µg) were used for library preparation (Illumina's TruSeq Stranded mRNA LT Sample Prep Kit with poly A selection). Sequencing was performed using an Illumina NovaSeq 6000 System (150 length, paired-end) platform (Illumina, Inc., San Diego, CA).

2.3 RNA-seq data analysis.

FASTQ files were obtained from the sequencer and subsequently the quality of the reads was evaluated using FASTQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). This was followed by a trimming step in order to remove adapters and low-quality bases using BBDuk v35.85 (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/) with the following parameters 35 bp minimum length and 25 minimum quality score. High quality reads were mapped against the *Danio rerio* genome (Ensembl GRCz11 reference genome and gene annotation) using the STAR v2.7.1a (Dobin et al., 2013). Feature Counts v 1.5.1 (Liao et al., 2014) was used to quantify gene expression as raw fragment counts. Raw count matrices were loaded in R v4.0.3 (R Core Team, 2020) and filtered using the HTSFilter package v1.30.1 (Rau et al., 2013) to remove the uninformative genes. Filtered raw counts were processed with the EdgeR package v3.32.1 (Robinson et al., 2010) to normalize the raw counts using the TMM method and perform differential gene expression analysis to identify up-regulated and/or down-regulated genes. Multiple testing correction was performed with the false discovery rate (FDR) method (Benjamini and Hochberg, 1995). The significance level was set at FDR < 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Gene Ontology (GO) term enrichment was analysed performing hypergeometric tests (R phyper function; Johnson et al., 1992) for each individual pathway and FDR correction was applied (FDR < 0.05). The pathview package v1.30.1 (Luo and Brouwer, 2013) was used to visualize gene expression in the context of functional pathways.

2.4 Expression of MCRs in the zebrafish intestine measured by qPCR.

In order to explore the expression of MCRs in the zebrafish intestine, the same total RNAs isolated for RNA-seq experiments were used. Briefly, two micrograms of DNase-treated total RNA were used for cDNA synthesis with Superscript III reverse

transcriptase (Invitrogen) primed with random hexamers and oligo(dT)12–18 (Invitrogen) in the presence of RNase inhibitors (Promega). Subsequently, one microliter of pure or diluted cDNA was added to 20µl of absolute SYBR Green fluorescein PCR master mix (ABgene, Thermo Scientific, Spain) each containing 10pmol of forward and reverse specific primers (supplementary Table 1). Reactions were carried out in triplicate in a CBX connectTM real-time system instrument (BioRad). Primer and cDNA concentrations were tested for each gene. Primer amplification efficiencies below 95% or over 110% were discarded from the analysis. The housekeeping gene β-actin was used as internal reference to normalize the cDNA template between samples. The thermal cycle protocol for all primer sets was as follows: denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 15'' and 62 °C for 30''. The melting curves (62-95 ºC) of the products were verified in order to confirm the specificity of PCR products. Normalized relative quantities of mRNA expression were calculated with the mathematical method of $\Delta\Delta$Ct (Livak and Schmittgen, 2001).

2.5 Analysis of plasma.

Blood samples were collected by ablation of the tail in heparinized capillary and plasma tubes, samples were obtained by centrifugation of the whole blood (10,000 g for 3 min) and subsequently stored at -20 ºC until further analysis. Plasma osmolality was measured in 10 µL samples with a Vapro 5520 osmometer (Wescor, South Logan, UT, USA). Sodium concentrations were measured by flame photometry (BWB-XP Performance Plus, BWB Technologies, UK). Chloride was determined by Coulombmetric titration (SAT-500, DKK-TOA, Japan). The results are expressed in mmol/L. Calcium and magnesium were measured by colorimetric tests, using commercial kits (Spinreact, Reactivos Spinreact, SA, Girona, Spain) according to the manufacturer's instructions using a Multi-Mode Microplate Reader BioTek SynergyTM 4 (BioTek▪ Instruments, Winooski, VT, USA).

2.6 Voltage clamp in Ussing chambers.

The intestine was isolated and mounted as previously described (Fuentes et al., 2010; Gregório et al., 2013) with the apical (luminal) and basolateral (blood side) sides of the tissue identified on a 0.09 cm^2 tissue holder and positioned between two half-chambers containing 2 ml of physiological saline solution.While carrying out the experiments, the tissue was gassed bilaterally with humified air, and experiments were run at a room temperature of 27-28°C. Short-circuit current (Isc, μA cm–2) was monitored by clamping the epithelia to 0 mV. Voltage clamping and current injections were performed using VCC MC6 or VCC MC8 voltage-clamp amplifiers (Physiologic Instruments, San Diego, USA). The bioelectrical parameters for each tissue were continuously recorded during the in vitro period in Labscribe3 on a Lenovo computer using IWorx188 and Lab-Trax-4 data acquisition systems, from the time of mounting and for a duration of 90 min. Epithelial resistance (Rt, $\Omega.cm^2$) was calculated manually (using Ohm's law) from the current deflections induced by a bilateral +2 mV pulse of 4 s every minute. The basolateral side of the preparation was considered as the ground. Therefore, positive currents are absorptive, while secretory currents are negative.

2.7 Permeability assay.

The ability to quantify the transepithelial transport in a barrier model is essential. Our technique is based on permeability assays and transepithelial electrical measurements. Following 20-30 minutes of tissue stabilization after mounting in vitro, the saline solution was replaced with a fresh well-gassed solution to a final volume of 2.2 ml per chamber. Fluorescein isothiocyanate–dextran (FITC, average mol wt ~4,000, Sigma) prepared as concentrated stocks of 100 mg/ml was added to final concentrations of 0.5 mg/ml to the apical chamber. A sample (0.2 ml) was collected from both the apical and the basolateral compartments after 15 min of mixing to establish

time zero. Precisely 1-hour later new samples from both the donor and receiver compartments were placed in fresh vials. Fluorescence measurements were performed using a BioTek SynergyTM 4 Multi-Mode Microplate Reader (BioTekÒ Instruments, Winooski, VT, USA) and subsequently set for excitation wavelength at 492 nm and emission wavelength at 520 nm for FITC. Concentration standards in the range of 0.2-2000 ng/ml were used to establish concentrations in the apical and basolateral chambers. The apparent permeability (Papp) was estimated using the equation (Arnold et al., 2019): Papp= (V*dC)/(A*C0*dT), where Papp is the permeability in centimetres per second, V is the volume of the receiver chamber, A is the tissue surface area in square centimetres, C0 is the initial concentration in the donor compartment (apical), and dC/dT is the rate of concentration change (ng/sec) of FITC in the recipient (basolateral) chamber.

2.8 Electrogenic amino acid transport.

The mid intestine was isolated and mounted as described above for the anterior intestine. In this subset of experiments, the apical side of the preparation is stimulated with an amino acid mixture (MEM Amino Acids Solution [50X], ThermoFisher). The amino acid pool consists of a complex mixture of essential amino acids: L-Arginine•HCl, L-Cystine•2HCl, L-Histidine•HCl•H2O, L-Isoleucine, L-Leucine L-Lysine•HCl L-Methionine L-Phenylalanine, L-Threonine, L-Tryptophan, L-Tyrosine, L- Valine. The principle of the test is to stimulate the epithelium with the presence of amino acids, thus generating a change in the current due to the cotransport of amino acids with ions (Bröer, 2008). The response is concentration-dependent, relatively fast and stabilizes within 30 min of adding amino acid. Therefore, the sequential effects of different concentrations in each epithelial preparation can be quantified. In this test, sequential concentrations of 4, 8 and 16 mM were used on the apical side. During the experimental manipulations, mannitol was added on the basolateral side at the

same time and at the same concentrations (4, 8 and 16 mM) to avoid putative effects of osmolality imbalance in the current. For calculation and data presentation, Delta Isc (μA.cm-2) was calculated as the difference between the pre-stimulation values and the steady-state current measured after each stimulation.

2.9 Statistics.

All results are shown as mean ± standard error of the mean (mean ± SEM). Furthermore, in some cases, individual values are shown to illustrate group variability. After assessing homogeneity of variance and normality, statistical analysis of the data was performed, as appropriate, by t-test or two-way analysis of variance (ANOVA) using GraphPad Prism 8. A P-value < 0.05 was considered statistically significant.

3. Results

To gain new insights into the gastrointestinal pathways involved in the melanocortin response, transcriptomic profiles of the intestine were analysed following the melanocortin pathway blockade mediated by *asip1* overexpression in transgenic zebrafish. The aim of this experiment was to identify functional classes of genes underlying susceptibility to melanocortin signalling, elucidating the functional significance of sets of differentially-expressed genes (DEG) in the intestine, with particular emphasis on epithelium permeability/integrity and amino acid transport. Complete transcriptomic results were submitted to GEO (GSE206810).

3.1 Functional classes of DEG in male zebrafish.

Principal component analysis (PCA) successfully clustered both WT and transgenic fish (data not shown). When comparing male transcriptome, 1509 genes were differentially expressed between WT and ASIP1 males with 757 and 752 up- and down-

regulated genes in transgenic animals, respectively (Supplementary Table 2a and 2b). Gene ontology (GO) enrichment analysis on up-regulated genes displayed 381 and 329 biological processes up- and down-regulated genes, respectively, the GO description "transmembrane transport" being the up-regulated biological process showing the lowest FDR and, by extension, the highest levels of significance for the enrichment analysis (Fig. 1, Supplementary Table 3a). Within this biological process, 52 genes out of 857 were up-regulated, including several components of the solute carrier family (SLC) directly involved in the transmembrane amino acid and ion transport (Supplementary Table 4). Accordingly, some biological processes related to membrane transport, including amino acid and anion transport, were significantly up-regulated by *asip1* overexpression (Supplementary Fig.1). These data are consistent with the prediction for the GO category "molecular function" in which the GO description "transmembrane transporter activity" showed the lowest FDR (Fig.1; Supplementary Table 3a). In contrast, the down-regulated biological processes showing the lowest FDR were related to lipid metabolism, including sterol synthesis as well as metabolic processes of cholesterol, isoprenoid, and steroid (Fig.2; Supplementary Table 3b), presumably crucial for membrane structure and integrity. The genes which down-regulated in the sterol and cholesterol biosynthesis processes are listed in Supplementary Table 5.

Analysis of significantly up-regulated KEGG pathways corroborated the GO results. Thirty-seven and 13 KEGG pathways were up- and down-regulated in ASIP1 animals, respectively. Several pathways related to amino acid metabolism were significantly up-regulated by *asip1* overexpression, including arginine/proline, ･-alanine, histidine metabolism and valine/leucine/isoleucine degradation (Figure 3; Supplementary Table 6a). Moreover, KEGG pathway analysis of down-regulated genes in transgenic fish revealed down-regulation of fatty acid metabolism including arachidonic acid metabolism, fatty acid elongation and steroid biosynthesis as well as functions

related to membrane integrity and permeability including focal adhesion and ECM-receptor interaction (Fig. 3; Supplementary Table 6), particularly in the integrin and laminin systems (Supplementary Table 7).

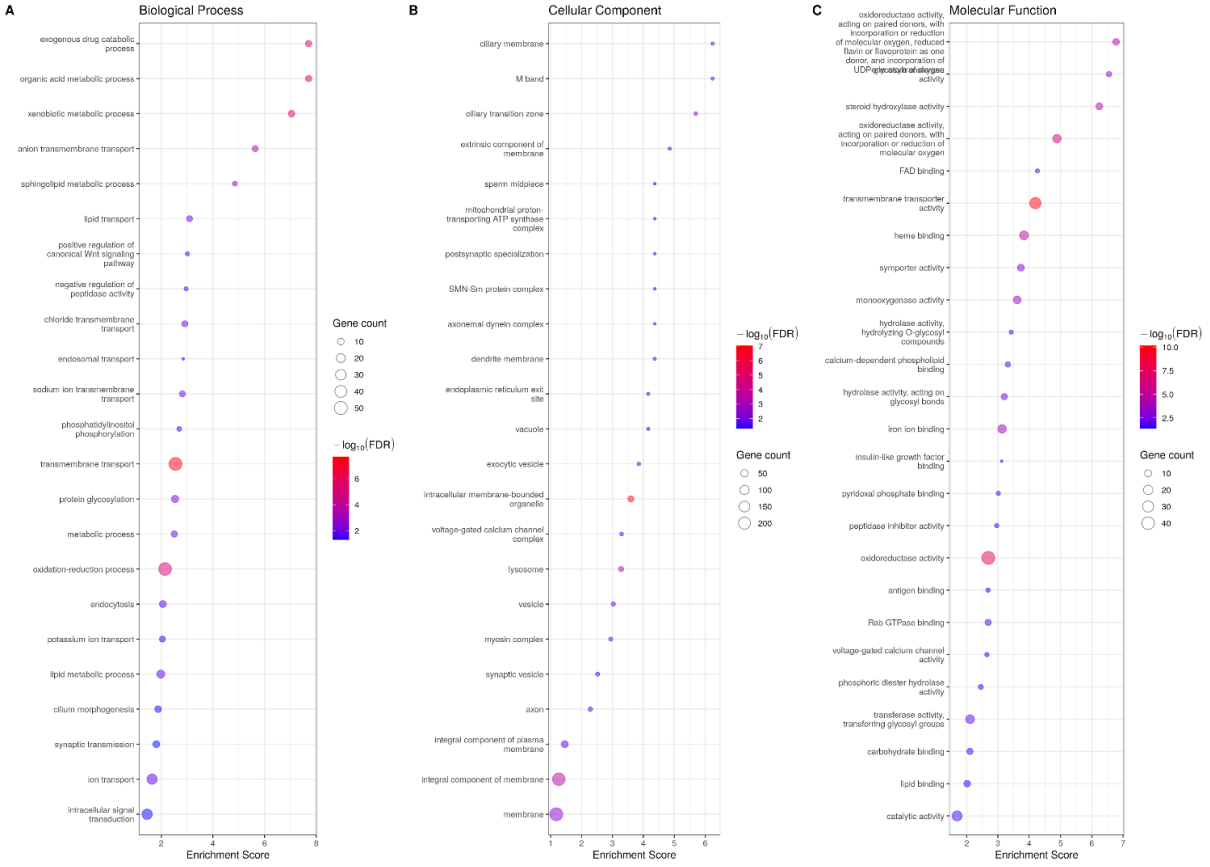

Figure 1. Bubble plot for gene ontology (GO) pathway enrichment analysis of upregulated genes in zebrafish males.

The Y-axis shows the name of Gene Ontology while the X-axis represents its enrichment. Bubble size is proportional to the number of genes assigned to Gene Ontology pathway; Colour represents enrichment significance (minus log10 transformed FDR), red indicates a greater significance level.

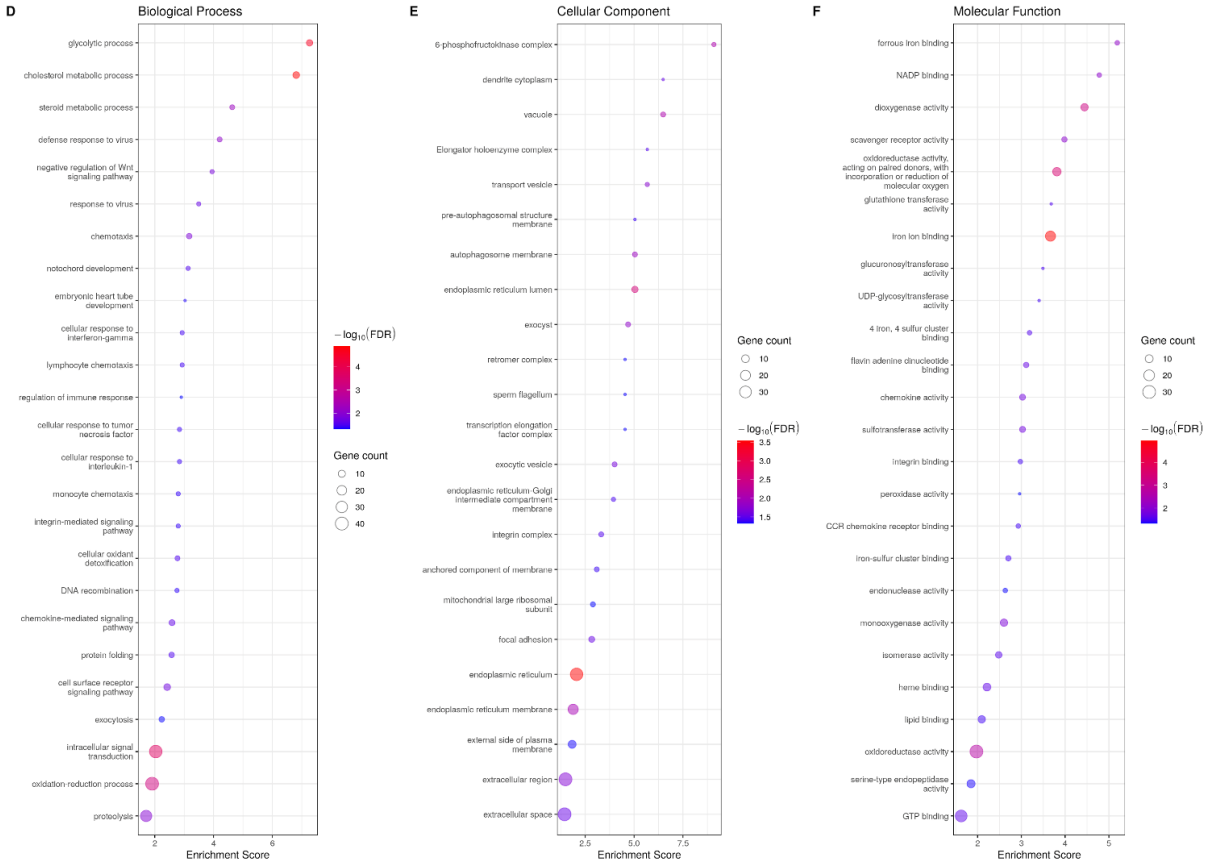

Figure 2. Bubble plot for Gene Ontology (GO) pathway enrichment analysis of down-regulated genes in zebrafish ASIP1 males.

The Y-axis shows the name of Gene Ontology while the X-axis represents its enrichment. Bubble size is proportional to the number of genes assigned to Gene Ontology pathway; Colour represents enrichment significance (minus log10 transformed FDR), red indicates a greater significance level.

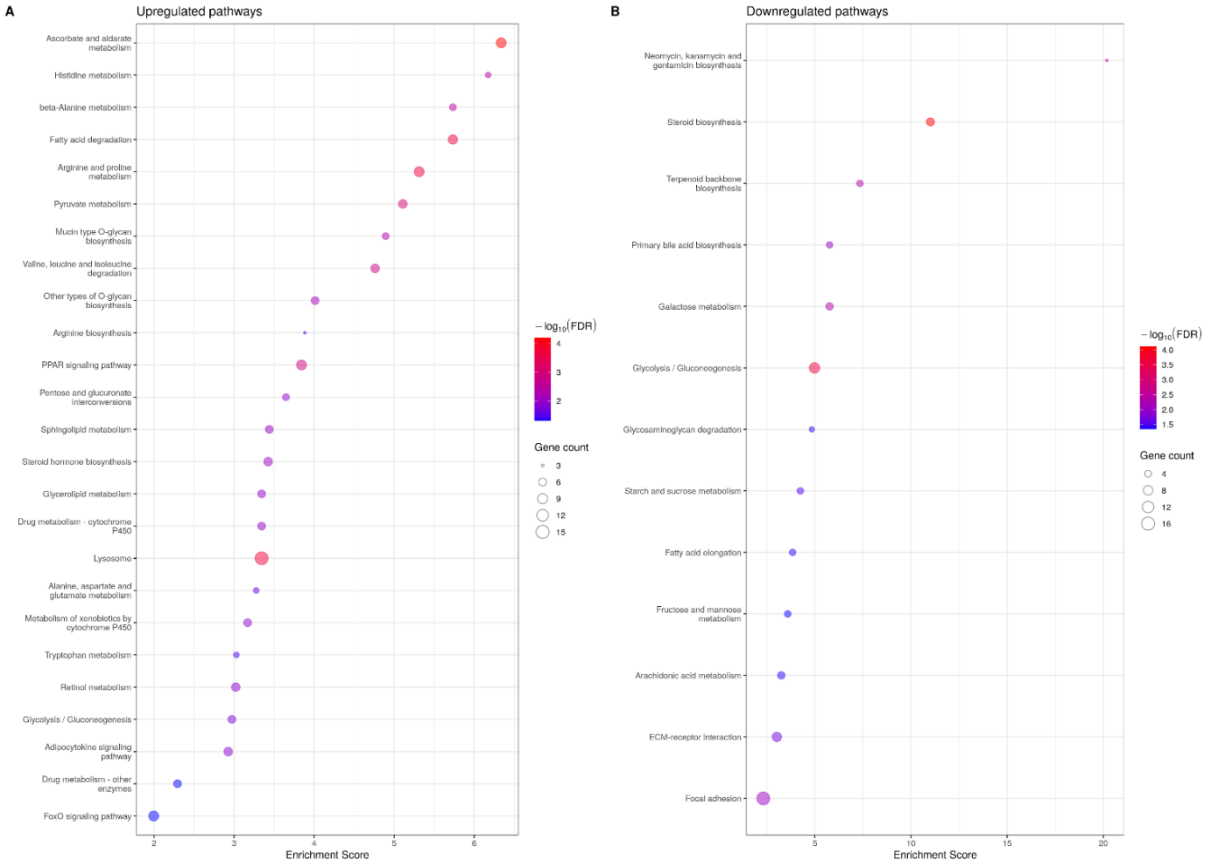

Figure 3. Bubble plot for KEGG pathway enrichment analysis of up- and down-regulated genes in zebrafish males.

The Y-axis shows the name of KEGG pathway while the X-axis represents its enrichment. Bubble size is proportional to the number of genes assigned to KEGG pathway; Colour represents enrichment significance (minus log10 transformed FDR), red indicates a greater significance level.

3.2 Functional classes of DEG in female zebrafish.

PCA successfully clustered both WT and transgenic fish (data not shown). The transcriptome of ASIP1 females showed 641 DEG with 410 and 231 up- and down-regulated genes, respectively, compared to the transcriptome of WT females maintained under identical breeding conditions (Supplementary Table 8a and 8b). GO enrichment analysis on up- and down-regulated genes revealed significant differences in 177 and 178 biological processes, respectively (Fig. 4 and 5; Supplementary Table 9a and 9b). In contrast to the male analysis, only GO-term analysis revealed differences in epithelium integrity showing down-regulation of biological processes related to skin morphogenesis, axoneme assembly, calcium-dependent cell-cell adhesion and adherent junction organization, homophilic cell adhesion via plasma membrane adhesion molecules, cell adhesion and cell-cell junction assembly among others (Fig. 5, Supplementary Table 9b). Accordingly, both the integral component of membrane and membrane were the cellular components most significantly affected (lowest FDR) by *asip1* overexpression in females (Fig. 5, Supplementary Table 9b). Interestingly, metabolism of organic acids and amino acids, including histidine, tyrosine and tryptophan were also up-regulated, as was the transport of some ions and vitamins (Fig. 4; Supplementary Table 9a).

No KEGG pathways were significantly down-regulated, yet 24 pathways were up-regulated (Fig. 6). Similar to males' transcriptome, KEGG pathways related to amino acid metabolism were up-regulated including tyrosine, tryptophan, taurine, phenylalanine, alanine/aspartate/glutamate, glycine/serine/threonine and histidine metabolism (Supplementary Fig. 2). In addition, and once again similar to the differential analysis of the transcriptome in males, fatty acid metabolism was up-regulated by *asip1* overexpression in females (Supplementary Fig. 2; Supplementary Table 10).

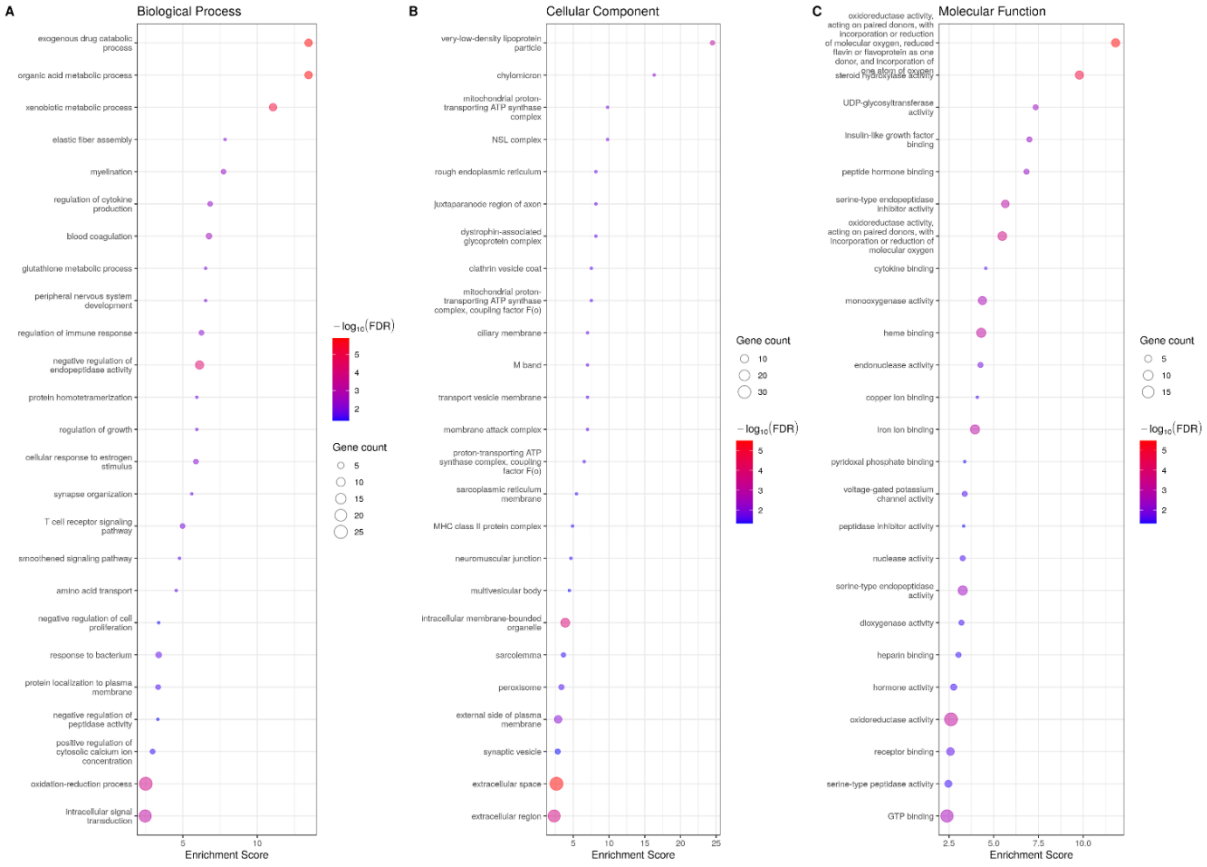

Figure 4. Bubble plot for Gene Ontology (GO) pathway enrichment analysis of up-regulated genes in zebrafish ASIP1 females.

The Y-axis shows the name of Gene Ontology while the X-axis represents its enrichment. Bubble size is proportional to the number of genes assigned to Gene Ontology pathway; Colour represents enrichment significance (minus log10 transformed FDR), red indicates a greater significance level.

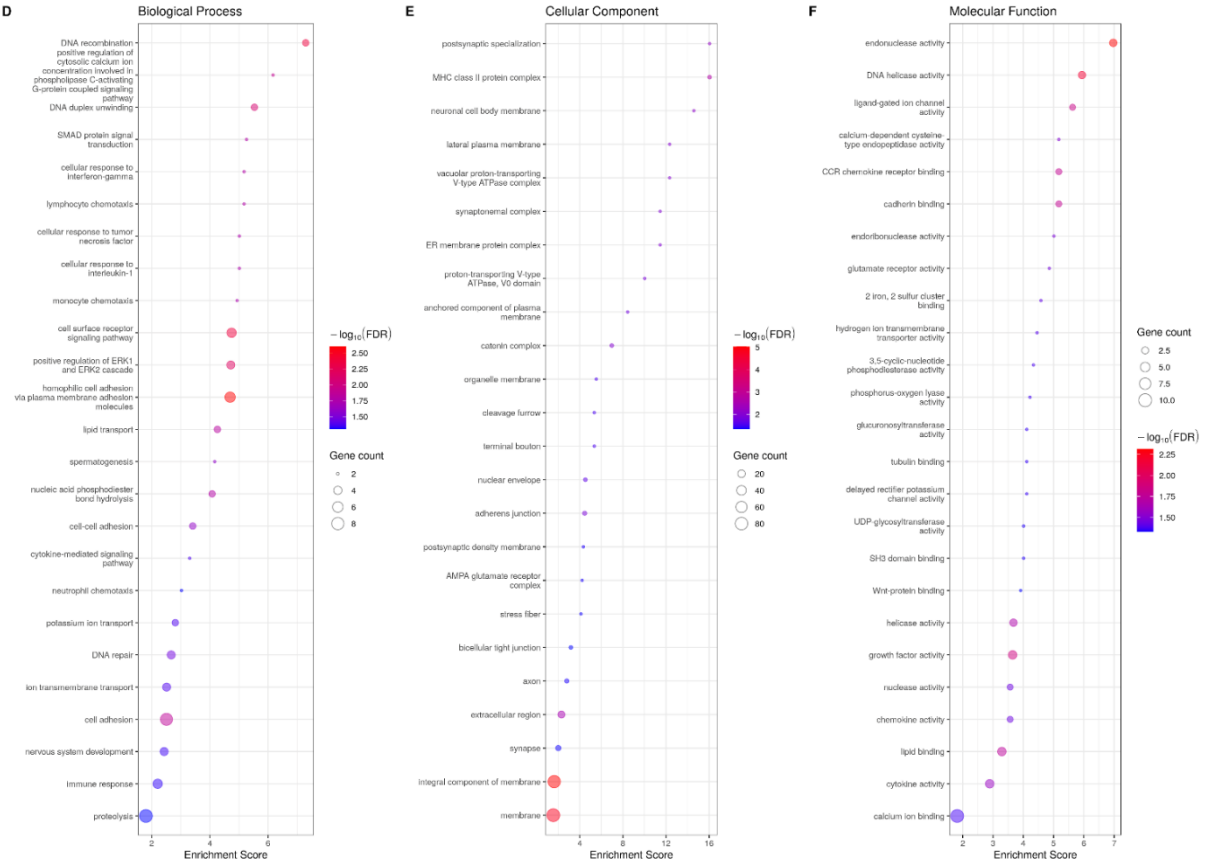

Figure 5. Bubble plot for Gene Ontology (GO) pathway enrichment analysis of down-regulated genes in zebrafish ASIP1 females.

The Y-axis shows the name of Gene Ontology while the X-axis represents its enrichment. Bubble size is proportional to the number of genes assigned to Gene Ontology pathway; Colour represents enrichment significance (minus log10 transformed FDR), red indicates a greater significance level.

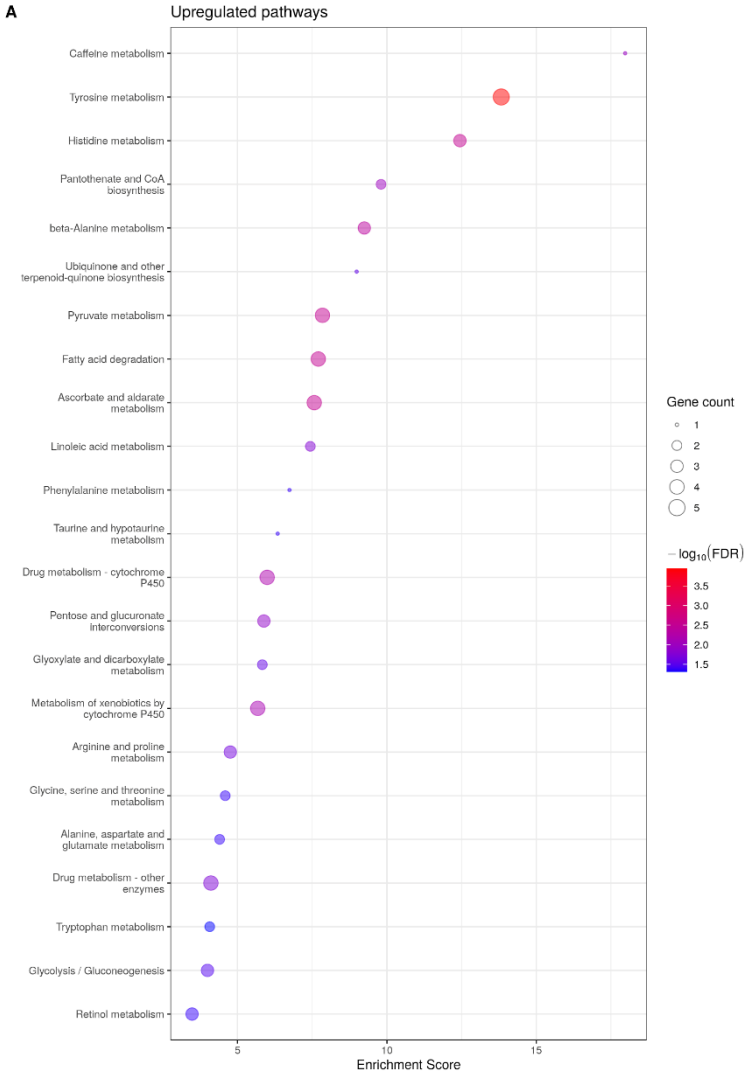

Figure 6. Bubble plot for KEGG pathway enrichment analysis of up-regulated genes in zebrafish ASIP1 females.

The Y-axis shows the name of KEGG pathway while the X-axis represents its enrichment. Bubble size is proportional to the number of genes assigned to KEGG pathway; Colour represents enrichment significance (minus log10 transformed FDR), red indicates a greater significance level.

3.3 Melanocortin receptor expression in the zebrafish intestine

Transcriptomic results failed to detect relevant expression levels of the MCRs in the zebrafish intestine as previously reported (Ringholm et al., 2002). Thus, the more sensitive real-time quantitative (Qpcr) technique was exploited to gain potential evidence for gene expression of the entire signalling repertoire of MCRs, using the same RNAs isolated from the transcriptomic experiments as templates for the Cdna synthesis. Only MC5Ra was overexpressed in ASIP1 transgenic fish after t-test ($p<0.05$) (Fig. 7). However, no effects of gender or genotype on MCR expression after two-way ANOVA analysis were found, except for MC5Ra, where overexpression of asip1 induced an increase in receptor Mrna in the ASIP1 animals, regardless of gender (Supplementary Fig 3). However, a significant interaction was found between both variables (gender x genotype) for the remaining receptors (MC1R, MC2R, MC3R, MC4R and MC5Rb). In all cases, except for MC2R and MC5Rb, ASIP1 females express higher levels of MCRs than the remaining genotypes.

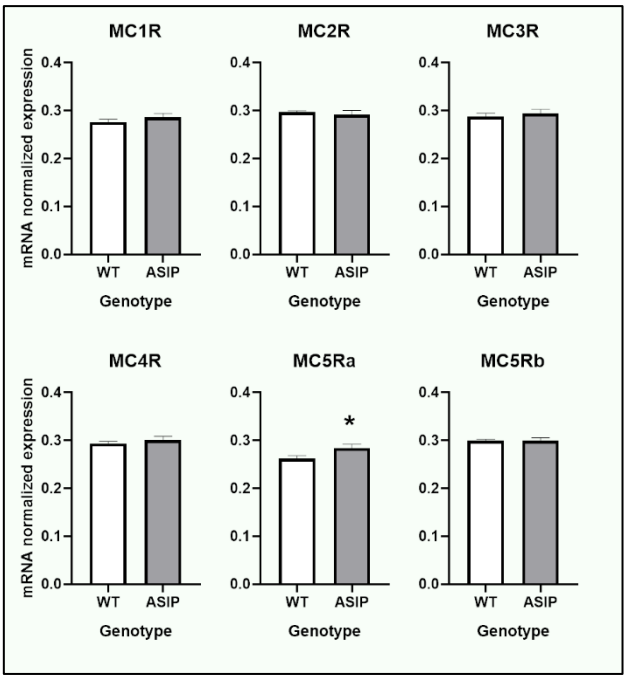

Figure 5. Intestinal melanocortin receptor as measured by RT-Qpcr.

See Material and Methods for details. Asterisk indicates significant differences after t-test ($P<0.05$).

3.4 Electrophysiological studies

3.4.1 Plasma analysis.

Ion and osmolality analysis in wild-type fish was performed to compose a Ringer solution that match the zebrafish plasma. Supplementary table 11 shows plasma measurements, while supplementary table 12 shows the resulting saline formula.

3.4.2 Intestine permeability.

Rt values for each genotype are shown in Figure 8. Overexpression of asip1 induced a reduction in epithelial resistance suggesting a decrease in tissue integrity in the transgenic genotype. In addition, short-circuit current (Isc, μAmp.cm-2) was also recorded for each epithelial preparation (Fig. 9). The intestine of asip1-overexpressing fish mounted in Ussing chambers exhibited, under voltage clamp to 0Mv and symmetric conditions, an absorptive short-circuit current (Isc, μAmp cm−2) of -9.36 ± 3.108 μAmp cm−2, whereas WT fish showed significantly lower Isc absorptive values (-0.503± 1.771 μAmp cm−2) (Fig. 8).

Paracellular transport of small macromolecules across the intestinal epithelium was assessed by 4 kDa FITC-dextran translocation. The results show that paracellular permeability of the intestinal epithelia is significantly higher in ASIP1 genotype, once more, suggesting enhanced paracellular transport and lower tissue integrity of the intestinal wall (Fig. 10).

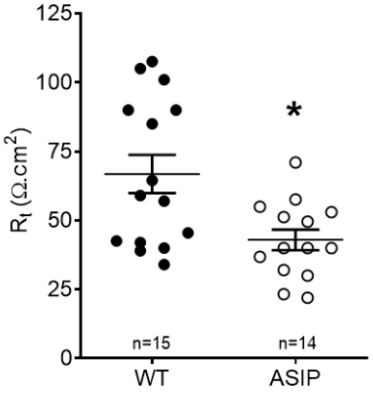

Figure 8. Trans-epithelial electrical resistance (Rt, Ω cm^2) in the intestine of zebrafish recorded in vitro during experimental testing of 90 min.

Values were averaged for whole in vitro measurements. Dot plot shows individual values for each group. Each column represents the average ± SEM of the number of measurements given in the columns. Asterisks represent significant treatments ($p < 0.05$, Sudent's t-test)

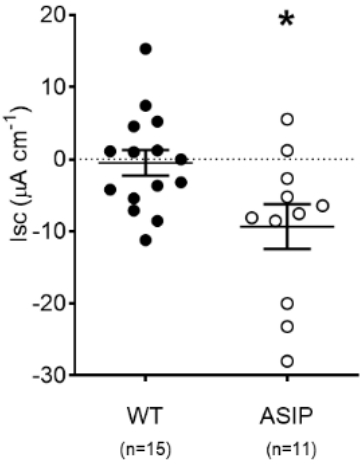

Figure 9. Short-circuit current (Isc, µAmp.cm-2) in the intestine of zebrafish recorded in vitro during experimental testing of 90 min.

Values were averaged for whole in vitro measurements. Dot plot shows individual values for each group. Each column represents the average ± SEM of the number of measurements given in the columns. Asterisks represent significant treatments (p< 0.05, Sudent's t-test).

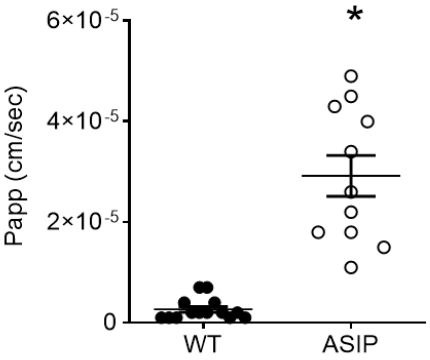

Figure 10. Permeability (Papp, cm/sec) to FITC dextran (4Kd) in the intestine of zebrafish. Dot plot shows individual values for each group.

Each column represents the average ± SEM of the number of measurements given in the columns. Asterisks represent significant treatments (p< 0.05, Sudent's t-test).

3.4.3 Intestinal transport.

Electrogenic intestinal amino acid transport was assessed by incubating intestinal epithelium in Ussing chambers with increasing concentrations of essential amino acids. Short-circuit currents showed that the intestinal epithelia of WT and ASIP1 fish exhibit a dose-dependent response in Delta Isc values at increased concentrations (Fig 11). However, these absorptive parameters in ASIP1 fish were significantly higher than those in WT fish, suggesting that ASIP1 fish can incorporate luminal amino acids more efficiently than WT animals.

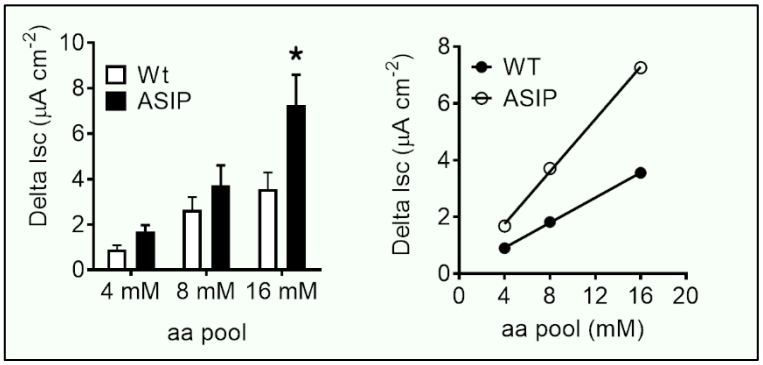

Figure 11. Electrogenic essential amino acid transport in the intestine of zebrafish recorded in vitro in Ussing chambers.

Right. Changes in short circuit current (Delta Isc, Ma.cm-2) are evoked by sequential apical addition of essential amino acid pool of 4, 8 and 16 Mm. Each column represents the average ± SEM (n=8). Two- way ANOVA table provides details of statistical analysis. Left. Using averages from the Delta Isc corresponding to stimulation with 4, 8 and 16 Mm regression analysis shows significantly different slopes p=0.0043.

4. Discussion

Previous studies demonstrated that the inhibition of melanocortin signalling mediated by *asip1* overexpression results in enhanced growth in zebrafish (Guillot et al., 2016). It was also determined that ASIP1 transgenic fish are able to eat more due to reduced sensitivity of the central satiety system (Guillot et al., 2016). However, subsequent experiments revealed that a higher intake of food was not necessary in ASIP1 zebrafish to enable larger growth, as it was observed that pair-fed ASIP1 fish grew larger than WT animals (Godino-Gimeno et al., 2020). A plausible explanation for this result may rely on the enhanced feeding efficiency mediated by improved nutrient absorption in transgenic animals. To test the hypothesis, wide scope transcriptomic techniques were used to pipe the potential pathways involved in the enhanced nutrient absorption. Transcriptomic comparisons between animals of the same sex were

considered as previous studies showed that ASIP1 can induce extensive sex-dependent differences in the brain transcriptome (Guillot et al., 2016). Gene expression divergences in male zebrafish between both genotypes showed that transmembrane transport was substantially modified in transgenic males. In particular, increased gene expression of amino acid, monocarboxylates, ionic and vitamin transporters was recorded, suggesting an improved efficiency in intestinal active transport that could lead to the enhanced anabolism and, therefore, improved growth of ASIP1 zebrafish (Li et al., 2021). Furthermore, enrichment analysis revealed an inhibition of intestinal lipid metabolism, mainly affecting sterol/cholesterol synthesis. Sterols, like cholesterol, are key components of the plasma membrane known to decrease fluidity and increase tight packaging and impermeability of the plasma membrane (Harayama and Riezman, 2018; de Mendoza and Pilon, 2019). Therefore, low expression levels of sterol-synthesis related genes in ASIP1 intestines could have impaired membrane fluidity resulting in increased membrane permeability (Subczynysk et al., 2019). Consistently, KEGG pathways related to membrane integrity, cellular cohesion and interaction with the extracellular matrix, i.e., focal adhesion and ECM-receptor interaction, were also down-regulated in ASIP1 males. This suggests that intestinal epithelium of ASIP1 males may show an augmented laxity resulting in an enhanced paracellular transport. Both the integrin and laminin systems were down-regulated in the transcriptome of ASIP1 zebrafish males. The integrins are transmembrane receptors that are essential for the formation of focal adhesions linking the actin cytoskeleton to the extracellular matrix (ECM) (Orre et al., 2019; Legerstee and Houtsmuller, 2021), thereby regulating cell adhesion and migration, ECM assembly, and mechanotransduction (Shattil et al., 2010). Integrins work as specific receptors for fibronectin and laminins, both glycoproteins of the ECM are required for the formation and function of the basement membranes (Hohenester, 2019). In summary, intestinal transcriptome enrichment analysis identifies differential gene expression

programs of key cellular pathways underlying the structure and function of the intestinal epithelium that may result in increased membrane fluidity and permeability, but also in enhanced metabolite transport and absorption.

Transcriptome differential analysis in females yielded contrasting results, as the regulated GO terms and KEGG pathways were different from those observed in males, although some biological processes were coincident. Similar to males, vitamin transport was also up-regulated, as well as some processes related to the ion transport, i.e., sodium, calcium and potassium ion homeostasis. Although amino acid transport was not up-regulated, as in males, both GO and KEGG pathways revealed an enhanced intestinal amino acid metabolism that could resemble an enriched usage of protein metabolites derived from digestive processes. Regarding membrane integrity, enrichment analysis revealed the up-regulation of genes involved in the regulation of membrane permeability, but also some processes related to cell adhesion, once more, suggesting that membrane integrity in ASIP1 females is modified. Consistently, glycerophospholipid and glycerol metabolic processes are down-regulated in ASIP1 females. Glycerophospholipid are key elements of the lipid bilayer in the plasma membrane which are also involved in the lipid emulsion (Yang et al., 2018). Although data in females are not as conclusive in males, it appears that amino acid and ion transport is enhanced in ASIP1 animals, as well as the membrane structure.

Epithelia (including the intestine) display two distinctive features: polarity and tightness. Asymmetric distribution of transporters to the apical or basolateral membrane of the intestinal cell generates epithelial polarity, whereas tight junctions regulate tissue tightness (i.e., selectivity). Several epithelial transporters and some epithelial function-related changes were identified by a transcriptomic approach. Therefore, the following step was aimed at understanding whether these changes in gene expression translate into functional changes in the intestine. Our tool of choice

was the Ussing chamber, which provides a robust experimental setup for functional analysis of the intestine (at the epithelial level). Although experimental setup is challenging in zebrafish due to the size of the tissue, when the tissue is opened flat and mounted between the two compartments of the Ussing chamber, transport, and epithelial properties can be studied using epithelial electrophysiology. No such previous studies exist in zebrafish. Therefore, in order to address this lack of knowledge, plasma measurements of the primary ion species and osmolality were carried out to formulate a zebrafish-specific Ringer's solution that mimics plasma ion levels. The experiments were run using species-specific Ringer's solution. Applying these conditions, significant changes in basal short-circuit current in ASIP1 animals were identified, reflecting changes in the transport capacity of the epithelium. Pharmacological experiments were not performed so the mechanisms responsible for this increase in the absorptive pathway remain unidentified. However, the transcriptomic approach provides a solid indication of the ample changes in transport mechanisms.

Measurement of tissue resistance (Rt) is the gold standard to show tissue integrity and selectivity changes. Unfortunately, no other studies are available in zebrafish to enable a comparison of our results. Yet, values of intestinal Rt ~75 Ω cm^2 in wild-type zebrafish are consistent with the anterior or mid intestine of other freshwater teleost such as tilapia, eel, or goldfish (Albus et al., 1979: Ruiz-Jarabo et al., 2017). However, intestinal Rt in zebrafish was much higher than those reported in medaka, a fish of similar size and equally challenging, between 3-12 Ω cm^2 (Madsen et al., 2014). In ASIP1 transgenic fish, tissue resistance showed a significant decrease to values ~40 Ω cm^2. This decrease shows a modification of the intestinal barrier function in ASIP1 transgenics, which could be explained, to a certain extent, by the molecular down-regulation of important tight junction components (Zihni et al., 2016) such as claudin (cldg, logFC=-1.59, p=0,048) or protocadherin (protocadherin 1 gamma 3, logFC=-2.74, FDR=0,0048). To further consolidate the results on functional

modification of barrier function, paracellular permeability was measured using standard methods with 4kD FITC-dextran (Balda et al., 1996) and a complementary effect was observed. Paracellular permeability was higher in ASIP1 transgenic zebrafish. Both the decrease in Rt and the increase in permeability, which measure the same aspects of tight junction functionality, are in solid agreement and point to an ASIP-dependent decrease in tissue selectivity in the intestine of ASIP1 transgenic fish.

Amino acids and small peptides (di-/ tripeptides) resulting from digestion are mainly transported into the enterocytes by several members of the group of families of solute carrier (SLC) membrane transport proteins (Broer, 2008; Broer and Fairweather, 2018). Previous studies in ASIP1 transgenic zebrafish (Godino-Gimeno et al., 2020) show improved growth in fish, which may be related to a better feed absorption. In addition, the list of up-regulated epithelial amino acid transport-related genes gleaned from transcriptomic effects provides several examples linked to proton and sodium movements. Therefore, a new set of experiments was devised to test the electrogenic amino acid transport in the zebrafish intestine. The aim of the test is to verify whether the presence of amino acids stimulates the epithelium, thus generating a change in the current due to the cotransport of amino acids with ions (Bröer, 2008). A standard mixture of essential amino acids (MEM x50 essential amino acids) was used as the stimulus for wild-type and ASIP1 transgenic zebrafish which led to the observation of concentration-dependent effects and response plateaus within 30 minutes of stimulation. Therefore, dose-response curves were constructed with the sequential addition of increased concentrations of stimulus (4, 8 and 16 mM) in each ex-vivo preparation. Responses fitted a linear regression in both cases yet with much steeper slopes in the case of ASIP1 transgenic zebrafish. Our assay shows evidence of the essential amino acid transport linked to ion movements specially sodium and protons. However, there is a strong up-regulation of several sodium and proton independent amino acid transporters such as slc7a7, slc16a10, slc43a2a, to

mention but a few, in the ASIP1 transgenic zebrafish. Such observations when translated into a functional perspective provide strong indication that ASIP1 transgenic zebrafish can extract more amino acids from their diet at similar feeding levels.

The following step was to find out whether such effects on intestinal tissue are directly mediated by melanocortin receptors. Transcriptome analysis revealed modest MCR expression in the zebrafish intestine. However, using the same RNA as those isolated for RNAseq studies, the amplifying PCR technique showed that all six receptors are expressed in the intestine, as previously demonstrated (Ringholm et al., 2002). Earlier studies demonstrated that ASIP1 functions as a competitive antagonist and inverse agonist of MCR in several fish species including zebrafish (Cerdá-Reverter et al., 2005; Sánchez et al., 2010; Guillot et al., 2016). MCR expression in the zebrafish intestine suggests that ASIP1 could block MCR signalling, even in the absence of the cognate agonist, by decreasing constitutive activity of the receptor (Sánchez et al., 2010; Guillot et al., 2016). However, a possible indirect action of the melanocortin peptides on intestinal function cannot be ruled out.

In summary, it has been demonstrated that overexpression of asip1 in zebrafish directly or indirectly regulates the intestinal barrier function by increasing the epithelium permeability and promoting enhanced electrogenic amino acid transport. Combined, such observations suggest that asip1-overexpressing zebrafish display an improved nutrient absorption and therefore, a higher feed efficiency which explains the enhanced growth in the absence of augmented food intake. Fish aquaculture sustainability is compromised not only by the availability of raw materials for commercial diets but also by food efficiency of reared fish (Naylor et al., 2000). Recently, the trading of GH overexpressing salmon for human consumption has been approved by U.S. Food and Drug Association. The enhanced growth of ASIP1 zebrafish potentially mediated by improved nutrient absorption and feed efficiency, suggests that the melanocortin system, specifically *asip1* overexpression, is a potential target

to enable the development of genetically engineered fish displaying enhanced growth with better feed conversion ratios and no differential lipid accumulation (Godino-Gimeno et al., 2020). A major issue for transgenic aquaculture involves engineered organism escapes and the displacement of the WT fish population of the same and/or related species in ecosystems. Our preliminary data suggest that ASIP1 fish are much less aggressive when competing with WT animals in dyadic fights, but further studies are required in order to evaluate fish behaviour, including feeding competition. Similar behavioural effects using exogenous Asip administration were reported before (Dijkstra et al. 2017). Furthermore, due to the effects of *asip1* overexpression on dorsal-ventral pigment pattern (Ceinos et al., 2015), these animals are easily identifiable in the wild after potential escapes. Commercial rejection or adverse effects on markets by morphological aspects are unforeseen as pigment alterations only involve the dorsal region and never the flank of the fish. However, more studies in aquaculture species are required to translate the applicability of these results.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author. Complete transcriptomic results were submitted to GEO (GSE206810).

Author contributions

Conceptualization, J.M.C.-R.; methodology, E.L., S.F.G., J.R., A.S.-V., J.F. and J.M.C.-R.; formal analysis A.S.-V., E.L., J.F. and J.M.C.-R. investigation, E.L. A.R.A., A.G., S.F.G. and J.M.C.-R.; data curation, E.L., A.S.-V; J.F. and J.M.C.-R. writing - original draft preparation, J.M.C.-R.; writing - review and editing, E.L., R.A., A.G., S.F.G., J.R., A.S.-V., J.F. and J.M.C.-R.; supervision J.M.C.-R.; project administration, J.M.C.-R.; funding acquisition,

J.M.C.-R. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest.

Authors have nothing to declare.

References

Agulleiro M.J., Cortés R., Leal E., Ríos D., Sánchez E., Cerdá-Reverter J.M. (2014). Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (*Dicentrarchus labrax*). *Gen Comp Endocrinol*. 205, 251-259. DOI: 10.1016/j.ygcen.2014.02.009.

Albus H., Groot J.A., Siegenbeek van Heukelom J. (1979). Effects of glucose and ouabain on transepithelial electrical resistance and cell volume in stripped and unstripped goldfish intestine. *Pflugers Arch*. 383, 55-66. DOI: 10.1007/BF00584475.

Balda M.S., Whitney J.A., Flores C., González S., Cereijido M., Matter K. (1996). Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol*. 134, 1031-1049. DOI: 10.1083/jcb.134.4.1031.

Benjamini Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B. 57, 289-300.
https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Broer S. and Fairweather S.J. (2018). Amino acid transport across the mammalian intestine. *Comp Physiol*. 9, 343-373. DOI: 10.1002/cphy.c170041.

Broer S. (2008). Amino acid transport across mammalian intestinal and renal epithelia. Physiol Rev. 88, 249-286. DOI: 10.1152/physrev.00018.2006.

Cal L., Braasch I., Kelsh R.N., Cerdá-Reverter J.M., Rotllant J. (2017). Fish pigmentation and melanocortin system. *Comp. Biochem. Physiol. A Mol. Integr. Physiol*. 211, 26-33. DOI: 10.1016/j.cbpa.2017.06.001.

Ceinos R.M., Guillot R., Kelsh R.N., Cerdá-Reverter J.M., Rotllant J. (2015). Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms. *Pigment Cell Melanoma Res*. 28, 196-209. DOI: 10.1111/pcmr.12335.

Cerdá-Reverter J.M. and Peter R.E. (2003). Endogenous melanocortin antagonist in fish. Structure, brain mapping and regulation by fasting of the goldfish agouti-related protein gene. *Endocrinology*. 144, 4552-4561. DOI: 10.1210/en.2003-0453.

Cerdá-Reverter J.M., Agulleiro M.J., Sánchez E., Guillot R., Ceinos R., Rotllant J. (2011). Fish Melanocortin System. *Eur J Pharmcol*. 660, 53-60. DOI: 10.1016/j.ejphar.2010.10.108.

Cerdá-Reverter J.M., Agulleiro M.J., Cortés R., Sánchez E., Guillot R., Leal E., Fernández-Durán B., Puchol S., Eley M. (2013). Involvement of MRAPs in the function of melanocortin receptors (MCRs). *Gen Comp Endocrinol*. 188, 133-136. https://doi.org/10.1016/j.ygcen.2013.01.017

Cerdá-Reverter J.M., Ling M., Schiöth H.B., Peter RE. (2003b). Molecular cloning, pharmacological characterization and brain mapping of the melanocortin 5 receptor in the goldfish. *J Neurochem*. 87, 1354-1367. DOI: 10.1046/j.1471-4159.2003.02107.x.

Cerdá-Reverter J.M., Ringholm A., Schiöth H.B., Peter R.E. (2003a). Molecular cloning, pharmacological characterization and brain mapping of the melanocortin 4 receptor in the goldfish: Involvement in the control of food intake. *Endocrinology*. 144, 2336-2349. DOI: 10.1210/en.2002-0213.

Cortés R., Navarro S., Agullerio M.J., Guillot R., García-Herranz V., Sánchez E., Cerdá-Reverter J.M. (2014). The evolution of the melanocortin system. *Gen Comp Endocrinol*. 209C, 3-10. DOI: 10.1016/j.ygcen.2014.04.005

De Mendoza D. and Pilon M. (2019). Control of membrane lipid homeostasis by lipid-bilayer associated sensors: A mechanism conserved from bacteria to humans. *Prog Lipid Res*. 76, 100996. DOI: 10.1016/j.plipres.2019.100996.

Dijkstra PD, Maguire SM, Harris RM, Rodriguez AA, DeAngelis RS, Flores SA, Hofmann HA. The melanocortin system regulates body pigmentation and social behaviour in a colour polymorphic cichlid fish. *Proc Royal Soc B: Biol Sci* 284, 20162839. DOI: 10.1098/rspb.2016.2838.

Dobin A., Davis C.A., Schlesinger F., Drenkow J., Zaleski C., Jha S., Batut P., Chaisson M., Gingeras T.R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 29, 15-21. DOI: 10.1093/bioinformatics/bts635.

Fan W., Boston B.A., Kesterson R.A., Hruby V.J., Cone R.D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*. 385, 165–168. DOI: 10.1038/385165a0.

Fuentes J., Power D.M., Canário A.V. (2010): Parathyroid hormone-related protein-stanniocalcin antagonism in regulation of bicarbonate secretion and calcium precipitation in a marine fish intestine. *Am J Physiol*. 299, R150-R158. DOI: 10.1152/ajpregu.00378.2009.

Gregório S.F., Carvalho E.S., Encarnação S., Wilson J.M., Power D.M., Canário A.V., Fuentes J. (2013). Adaptation to different salinities exposes functional

specialization in the intestine of the sea bream *(Sparus aurata* L.). *J Exp Biol*. 216, 470-479. DOI: 10.1242/jeb.073742.

Godino-Gimeno A., Sánchez E., Guillot R., Rocha A., Angotzi A.R., Leal E., Rotllant J., Cerdá-Reverter J.M. (2020). Growth performance after agouti-signaling protein 1 (asip1) overexpression in transgenic zebrafish. *Zebrafish* 17, 373-381. DOI: 10.1089/zeb.2020.1932.

Guillot R., Cortés R., Navarro S., Mischitelli M., García-Herranz V., Sánchez E., Cal L., Navarro J.C., Míguez J.M., Afanasyev S., Krasnov A., Cone R.D., Rotllant J., Cerdá-Reverter J.M. (2016). Behind melanocortin antagonist overexpression in the zebrafish brain: A transcriptomic and behavioral approach. *Horm Behav*. 82, 87-100. DOI: 10.1016/j.yhbeh.2016.04.011.

Harayama T. and Riezman H. (2018). Understanding the diversity of membrane lipid composition. *Nature Rev Mol Cell Biol.* 19, 281-296. DOI: 10.1038/nrm.2017.138.

Hohenester E. (2019). Structural biology of laminin. Essays Biochem. 63, 285-295. https://doi.org/10.1042/EBC20180075.

Huszar D., Lynch C.A., Fairchild-Huntress V., Dunmore J.H., Fang Q., Berkemeier L.R., Gu W., Kesterson R.A., Boston B.A., Cone R.D., Smith F.J., Campfield L.A., Burn P., Lee F. (1997). Targeted disruption of melanocortin-4 receptor results in obesity in mice. *Cell*. 88, 131–141. DOI: 10.1016/s0092-8674(00)81865-6.

Johnson, N. L., Kotz, S., Kemp, A. W. (1992). Univariate Discrete Distributions, Second Edition. New York: Wiley.

Legerstee K. and Houtsmuller A.B. (2021). A Layered View on Focal Adhesions. *Biology*. 10, 1189. DOI: 10.3390/biology10111189.

Li X., Zheng S., Wu G. (2021). Nutrition and functions of amino acids in fish. In Advances in Experimental Medicine and Biology.1285, 133-168.

Liao Y., Smyth G.K., Shi W. (2014). Feature counts: an efficient general-purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 30, 923-30. DOI: 10.1093/bioinformatics/btt656.

Livak K.J., Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) *Method Methods*. 25, 402-408. DOI: 10.1006/meth.2001.1262.

Löhr H., Hess S., Pereira M.M.A., Reinoß P., Leibold S., Schenkel C. (2018). Diet-induced growth is regulated via acquired leptin resistance and engages a pomc-somatostatin-growth hormone circuit. *Cell Rep*. 23, 1728–1741. DOI: 10.1016/j.celrep.2018.04.018.

Lu D., Willard D., Patel I.R., Kadwell S., Overton L., Kost T., Luhter M., Chen W., Woychik R.P., Wilkinson W.O. (1994). Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature*. 371, 799–802. DOI: 10.1038/371799a0.

Luo W. and Brouwer C. (2013). Pathview: An R/Bioconductor package for pathway-based data integration and visualization. *Bioinformatics*. 29, 1830-1831. DOI: 10.1093/bioinformatics/btt285.

Madsen S.S., Bujak J., Tipsmark C.K. (2014). Aquaporin expression in the Japanese medaka (*Oryziaslatipes*) in freshwater and seawater: challenging the paradigm of intestinal water transport? *J Exp Biol*. 217, 3108–3121. DOI: 10.1242/jeb.105098.

Naylor R.L., Goldburg R.J., Primavera J.H., Kautsky N., Beveridge M.C., Clay J., et al. (2000). Effect of aquaculture on world fish supplies. *Nature*. 405, 1017–1024. DOI: 10.1038/35016500.

Ollmann M.M., Wilson B.D., Yang Y.K., Kerns J.A., Chen I., Gantz I., Barsh G.S. (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science*. 278, 135–138. DOI: 10.1126/science.278.5335.135.

Orré T., Rossier O., Giannone G. (2019). The inner life of integrin adhesion sites: From single molecules to functional macromolecular complexes. Exp Cell Res. 379, 235-244. DOI: 10.1016/j.yexcr.2019.03.036.

R. Core Team. (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rau A., Gallopin M., Celeux G., Jaffrezic F. (2013). Data-based filtering for replicated high-throughput transcriptome sequencing experiments. *Bioinformatics*. 29, 2146-2152. DOI: 10.1093/bioinformatics/btt350.

Ringholm A., Fredriksson R., Poliakova N., Yan Y.L., Postlethwait J.H., Larhammar D., Schioth H.B. (2002). One melanocortin 4 and two melanocortin 5 receptors from zebrafish show remarkable conservation in structure and pharmacology. *J Neurochem*. 82, 6–18. DOI: 10.1046/j.1471-4159.2002.00934.x.

Robinson M.D., McCarthy D.J., Smyth G.K. Edge (2010). R: A bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 26, 139-140. DOI: 10.1093/bioinformatics/btp616.

Rossi M., Kim M.S., Morgan D.G.A., Small C.J., Edwards C.M.B., Sunter D., Abusnana S., Goldstone A.P., Russel S.H., Stanley S.A., Smith D.M., Yagaloff K., Ghatei M.A., Bloom S.R. (1998). A C-terminal fragment of Agouti-related protein increases

feeding and antagonizes the effect of alpha-melanocyte stimulating hormone *in vivo*. *Endocrinology*. 139, 4428–4431. DOI: 10.1210/endo.139.10.6332.

Ruiz-Jarabo I., Gregório S.F., Gaetano P., Trischitta F., Fuentes J. (2017). High rates of intestinal bicarbonate secretion in seawater tilapia (*Oreochromis mossambicus*). *Com Biochem Physiol A*. 207, 57-64. DOI: 10.1016/j.cbpa.2017.02.022.

Sánchez E., Rubio V.C., Thompson D., Metz J., Flik G., Millhauser G.L., Cerdá-Reverter J.M. (2009). Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein (AGRP) on melanocortin 4 receptor in sea bass (*Dicentrarchus labrax*). *A J Physiol*. 296, R1293-R1306. DOI: 10.1152/ajpregu.90948.2008.

Sánchez E., Rubio V.C., Cerdá-Reverter J.M. (2010). Molecular and Pharmacological Characterization of the Melanocortin Receptor Subtype 1 in the Sea Bass. *Gen Comp Endocrinol*. 65, 163-169. DOI: 10.1016/j.ygcen.2009.06.008.

Schiöth H.B., Haitina T., Ling M.K., Ringholm A., Fredriksson R., Cerdá-Reverter J.M., Klovins J. (2005). Evolutionary conservation of the structural, pharmacological and genomic characteristics of the melanocortin receptors subtypes. *Peptides*. 26, 1886-1900. DOI: 10.1016/j.peptides.2004.11.034.

Shattil S.J., Kim C., Ginsberg M.H. (2010). The final steps of integrin activation: the end game. *Nature Rev Mol Cell Biol*. 11, 288-300. DOI: 10.1038/nrm2871.

Subczynski W.K., Pasenkiewicz-Gierula M., Widomska J., Mainali L., Raguz M. (2017). High cholesterol/low cholesterol: Effects in biological membranes. *Cell Biochem Biophys*. 75, 369-385. DOI: 10.1007/s12013-017-0792-7.

Tao Y.X. (2014). Constitutive activity in melanocortin-4 receptor: biased signaling of inverse agonists. *Adv Pharmacol*. 70, 135-54. DOI: 10.1016/B978-0-12-417197-8.00005-5.

Yang Y., Lee M., Fairn G.D. (2018). Phospholipid subcellular localization and dynamics. *J Biol Chem* 293, 6230-6240. DOI: 10.1074/jbc.R117.000582.

Zihni C., Mills C., Matter K., Balda M.S. (2016). Tight junctions: from simple barriers to multifunctional molecular gates. *Nat Rev Mol Cell Biol*. 17, 564–580. DOI: 10.1038/nrm.2016.80.

Supplementary materials

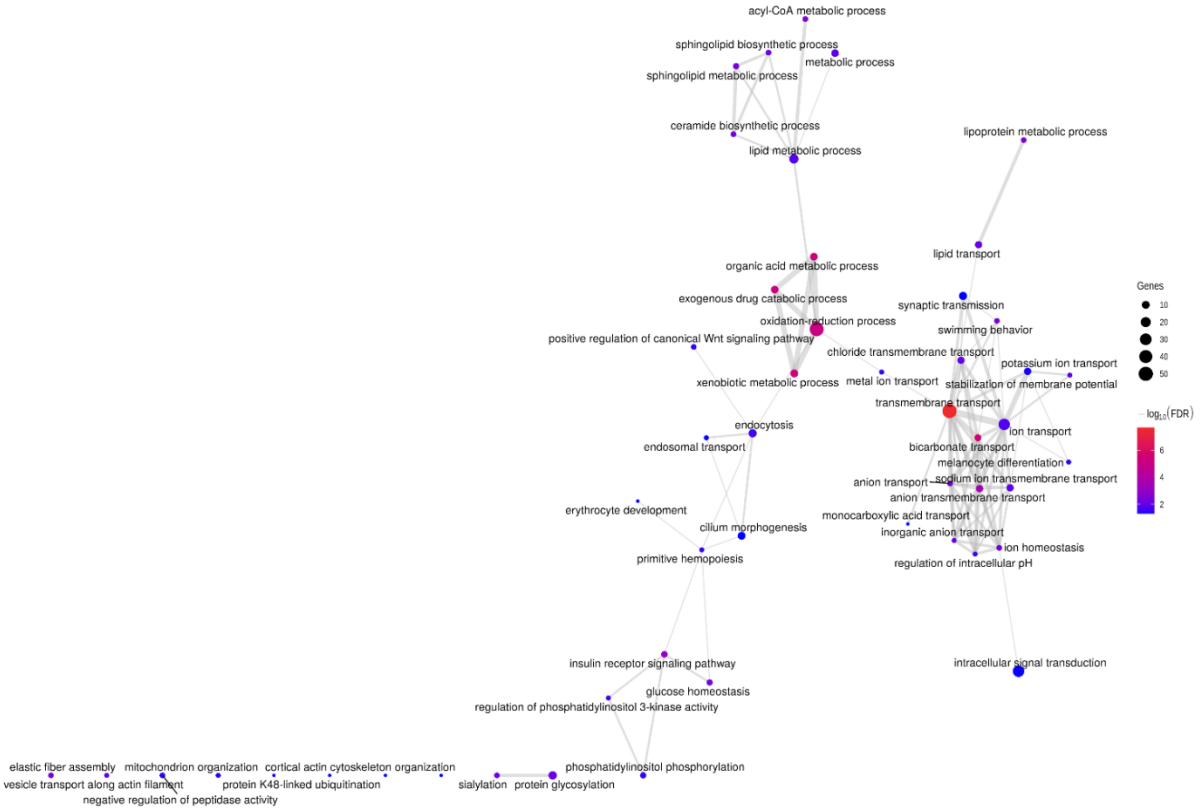

Figure S1. Network for Gene Ontology enrichment analysis of up-regulated genes in zebrafish ASIP1 males.

Nodes represent Gene Ontologies while the edge thickness is proportional to the number of common genes between the linked nodes. Node size is proportional to the number of genes assigned to Gene Ontology; Colour represents enrichment significance (minus log10 transformed FDR), red indicates a greater significance level.

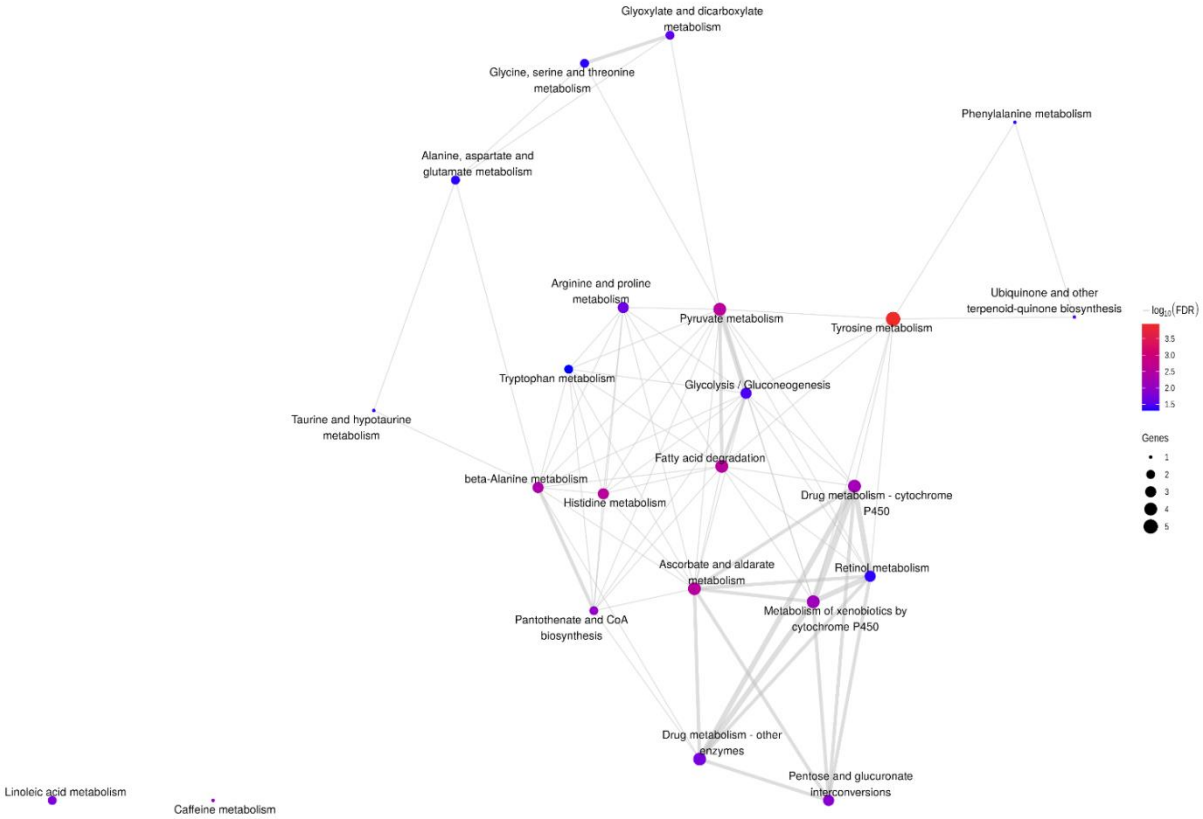

Figure S2. Network for KEGG enrichment analysis of up-regulated genes in zebrafish ASIP1 females.

Nodes represent Gene Ontologies while the edge thickness is proportional to the number of common genes between the linked nodes. Node size is proportional to the number of genes assigned to Gene Ontology; Colour represents enrichment significance (minus log10 transformed FDR), red indicates a greater significance level.

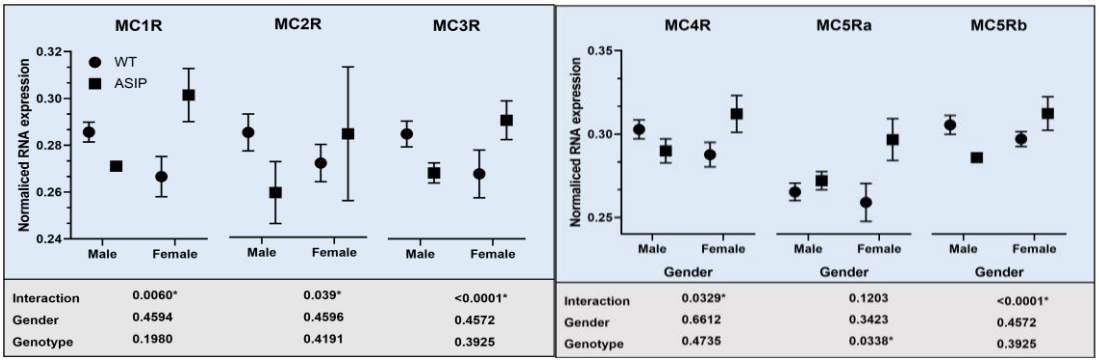

Figure S3. Sex differences in the intestinal melanocortin receptor are measured by RT-qPCR.

Significant differences after two-way ANOVA are indicated in the table below each graph.

Gene	Forward (5'-3')	Reverse (5'-3')
MC1R	TCCCACAAACCCTTACTGCAAG	TACACTGCAAAGCACCACGAAC
MC2R	CCTGTTAGCACGCCATCATG	AGGCCGCTTTTCCTGTGTT
MC3R	TGTGATTGACCCGCTCATCTATG	TCTTCCCACATCCATTCTCAGTTC
MC4R	GCCTCGCTCTACGTCCACAT	CGGCGATCCGTTTCATG
MC5aR	ATCATCTGCTGCTATAGTCTGA	ATCCACCGATCATATCCATCT
MC5bR	CGCACTCAGGAGCCAAGAGATG	AGTTCCTCCAGGCACCTTCTTC

Supplementary Table 1. Primers used for qPCR amplification of MCRs

Forward and reverse primers used to evaluate expression levels of MCRs in the intestine of zebrafish. See Material and Methods for amplification and melting curve conditions.

Additional supplementary tables are available online:

https://www.frontiersin.org/articles/10.3389/fmars.2022.1011702/full#supplementary-material

CHAPTER 4

Agouti-signalling protein overexpression reduces aggressiveness in zebrafish

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Simple Summary

Enhanced feeding can be associated with aggressive behaviour since food resources are the main responsible for agonistic behaviour (any social behaviour related to fighting). The overexpression of the gene agouti-signalling protein (asip1) in transgenic zebrafish (*asip1-Tg*) results in enhanced food intake and linear growth. Our next question was if *asip1-Tg* animals exhibit a dominant phenotype associated with the feeding-enhanced levels when compared to wild-type (WT) fish. To answer that question, we quantified the aggressive behaviour using dyadic (real oponent) fights but also confronting the animals against their specular image using mirrors. The results indicate that *asip1-Tg* are less aggressive than WT zebrafish in both dyadic fights and mirror-stimulus tests thus providing direct evidence of the role of the melanocortin system in the regulation of fish behaviour. The subordinate personality of *asip1-Tg* suggests that this transgene would be non-threatening to native populations in the event of an escape from aquaculture facilities. Such results provide a genetic modification strategy to enhance growth through high feeding motivation without promoting aggressiveness in fish, thus making the melanocortin system inhibition a feasible target for genetically engineered fish, permitted to trade by the U.S. Food and Drug Association.

Abstract

Feeding motivation is crucial for food intake and growth, it also depends on hunger and satiation, which is controlled by the melanocortin system. Overexpression of the inverse agonist agouti-signalling protein (ASIP) and agouti-related protein (AGRP) leads to enhanced food intake, linear growth, and weight. In zebrafish, Agrp overexpression induces obesity, as opposed to the phenotype observed in transgenic zebrafish overexpressing *asip1* under the control of a constitutive promoter (*asip1-*

Tg). Previous studies have demonstrated that *asip1-Tg* is a larger yet not obese fish, displaying higher feeding motivation, which in turn induces a higher feeding rate, yet higher food ration is unessential in order to grow larger than wild-type (WT) fish, this is, in all likelihood, due to their improved intestine permeability to amino acids and enhanced locomotor activity. A relationship between high feeding motivation and aggression has been previously reported in some other transgenic species showing enhanced growth. This study aims to elucidate whether the hunger observed in *asip1-Tg* is linked to aggressive behaviour. Dominance and aggressiveness were quantified using dyadic fights and mirror-stimulus tests, in addition to analysis of the basal cortisol levels. The results indicate that *asip1-Tg* are less aggressive than WT zebrafish in both dyadic fights and mirror-stimulus tests.

Keywords: ASIP1, agouti-related protein (AGRP), proopiomelanocortin (POMC), melanocyte-stimulating hormone (MSH), melanocortin, aggression, behaviour, fish

1. Introduction

The melanocortin system is essential for regulating food intake, stress, and pigmentation in vertebrates [1]. Melanocortins are peptides derived from the post-transcriptional processing of proopiomelanocortin precursor (POMC) which encodes several melanocyte-stimulating hormones (MSHs) and the adrenocorticotropic hormone (ACTH) [2]. In the tetrapod species, melanocortin signalling is mediated through five different receptors (MC1R-MC5R) which exhibit discrete functional domains and binding profiles [3]. MC2R is the only receptor exclusively activated by ACTH whereas the remaining receptors bind the different MSHs with various affinities [3]. mc1r is mainly expressed in the skin where it binds ▪-MSH to regulate melanin synthesis and skin pigmentation. *mc2r* is expressed in the adrenal cortex and mediates stress response by triggering cortisol synthesis which is released following ACTH binding.

mc3r and *mc4r* are mainly expressed in the brain where energy balance is regulated by binding MSHs. Inactivating mutations of MC4R result in enhanced linear growth and obesity in mice [4]. Finally, *mc5r* is widely expressed at low levels and appears to regulate exocrine secretion in mice [5].

Atypically, the melanocortin system is also regulated by an endogenous antagonist such as agouti-signalling protein (Asip) and agouti-related protein (Agrp). In mice, Asip regulates skin pigmentation by antagonizing ·-MSH effects on Mc1r in the follicle melanocytes. The allele *Ay* of the *asip* locus displays ubiquitous expression resulting in yellow fur but also in hyperphagia, obesity and increased linear growth thus emulating the metabolic phenotype of *mc4r (-/-)* mice [6]. The metabolic syndrome of *asip* overexpression is mediated by Mc4r given that Asip can also antagonize MSHs binding at Mc4r and depress the constitutive activity of Mc4r as an inverse agonist [7]. However, Asip is peripherally synthesized and only reaches the central nervous system (CNS) after ubiquitous expression. The central regulation of Mc4r signalling is modulated by Agrp which is profusely expressed in the arcuate nucleus under fasting conditions. Accordingly, *agrp* transgenic mice exhibit a similar metabolic phenotype to that of the allele *Ay* and *mc4r* (*-/-*) [8].

Teleost fish underwent an extra genome duplication event (TGD) that resulted in additional antagonist paralogues gene named *asip1/asip2* and *agrp1/agrp2*. Fasting increases agrp1 expression in the hypothalamus [9] and its overexpression in transgenic zebrafish (*Danio rerio*) promotes increased linear growth and weight [10]. Contrarily, *agrp1* morpholino knockdown larvae show reduced growth mediated by MC4R constitutive activity [11]. Moreover, the genetic ablation of Agrp1 neurons results in decreased food intake in zebrafish [12]. Agrp2 appears to be involved in the stress response by suppressing interrenal cortisol release [12]. *asip1* is predominantly expressed in the ventral skin of goldfish (*Carassius auratus*) and zebrafish [13,14], thus regulating both melanogenesis and chromatophore fate [14,15] through Mc1r

[16]. Recent studies have shown that *asip1* overexpression in transgenic zebrafish also results in increased food intake and linear growth yet no obese phenotype [17]. Nevertheless, *asip1* transgenic fish do not need to eat more in order to grow larger, as transgenic fish, which are fed at similar rates as wild type (WT) animals, grow larger thus suggesting an enhanced food conversion rate [18]. A link between high feeding rates, enhanced growth and dominance has been previously suggested in fish [19,20]. Growth hormone (*gh*) transgenic salmon exhibit an increased food intake and growth rate but also display pronounced dominant/aggressive behaviour [21,22]. Similarly, exogenous Gh administration promotes enhanced food motivation and increased growth, yet fish become more aggressive than WT animals [23,24]. In the present study, the potential of an *asip1-Tg* transgenic model is exploited in order to study the link between aggressiveness and enhanced feeding and growth rates as well as the involvement of the melanocortin system in zebrafish behaviour.

2. Materials and Methods

2.1 Fish and housing

Wild-type (WT) and transgenic stocks come from a background of TU (Tuebingen, Nüsslein-Volhard Lab) strain. Generation of the transgenic zebrafish line [Tg(Xla.Eef1a1:Cau.Asip1]iim4, using the Tol2 transposon system, has been previously described [14]. Zebrafish embryos were obtained from natural crosses and kept at the Institute of Aquaculture of Torre de la Sal (IATS-CSIC) facilities at 27.5- 28ºC under a 14 h/ 10 h light/dark cycle, with lights on at 7:00 A.M and off at 9:00 P.M. WT and *Asip1-Tg* experimental fish were segregated after crossing of heterozygote fish (+/*asip1* vs +/*asip1*) and inbreed for 3 generations. They were raised in mixed sex groups (~50:50 male:female ratio) of ≈ 250 (fish) in 38 L tanks. Adult fish were fed a combination of artemia and commercial flake food (Vipan, Sera. 46.2%protein,

8.9% fat, 2.3% fibre, 11.9% ash, and 6.7% humidity) three times a day until satiety was reached. The animals used in this study were experimentally naïve, and free of any signs of disease. They were tested only once, in a between subject design and returned to the stock tanks, remaining there for future experiments and breeding.

2.2 Behavioural experiments

Experiments were performed on 3- to 6-month-old male adult fish. Each test was performed on an independent cohort of 20 WT and 20 *asip1-Tg* fish (length WT = 25.2 ± 0.34; length *asip1-Tg* = 25.0 ± 0.19). Age and size were carefully paired among fish of different genotypes. At least one and a half weeks before assays, fish were transferred to a behavioural room in order to acclimatize. All behavioural assays were performed between 9:30 A.M. and 1:30 P.M, and the different genotypes were intermixed throughout the experimental period to account for possible diurnal variations in behaviour. The fish were supplied with food 30 min prior to the beginning of the trials.

Two commonly used methods were employed to quantify aggressive behaviour in zebrafish. Such methods are based on two paradigms: (1) dyadic fights test, between real opponents, WT and *asip1-Tg* and (2) mirror-stimulus tests. The protocols applied were essentially those published by [25]. The testing apparatus consisted of a 26 cm x 15 cm x 17 cm tank divided into two compartments, with one division containing a mechanical filter and a heater (set to conserve water temperature at 28ºC). The test took place in the other compartment equipped with a back perforated wall covered with white-coloured self-adhesive film to improve contrast and also fitted with a removal partition, creating two identical experimental tanks equipped with a mirror covered by a sliding opaque shield. Subjects were consistently tested in pairs, in order to provide them with conspecific odours, which would otherwise only be present in real-opponent dyads (see Figure S1 in supplementary materials).

The dyadic fights identification of individuals was carried out based on pigmentation differences among genotypes [14] thus tagging was unnecessary. Animals of each genotype were paired according to their standard length. Differences in length between opponents did not exceed 2% of the total body size. The fish remained visually isolated overnight in the experimental tank. The following day, the partitions were removed, allowing the fish to interact for 30 min, a duration that exceeded the time required to determine a clear winner of the contest. Following each interaction, the fish were once again separated by replacing the opaque partition. For the mirror-image stimulus, naïve fish (n=20) were allowed to acclimate overnight as previously described and the sliding opaque shields were removed, allowing the fish to interact with their specular image for 25 min.

2.3 Quantification of aggressive behaviour

Recording of the fish activity was performed via industrial digital cameras from IDS (UI-3240CP USB 3.0 uEye CP, IDS Imaging Development Systems GmbH) and/or Basler (Basler acA1280-60gc GigE camera, Basler AG, Germany) equipped with a high quality monofocal lens (focal length 8 mm) with a frame acquisition rate of 25 fps. Behavioural event logging was carried out using the free open-source software BORIS [26] while animal trajectory tracking was performed using EthoVision ® XT software (Noldus Inc. The Netherlands). With regard to staged fights, the latency and direction (who attacked whom) of the first territory incursion by the opponent was assessed. Behavioural interaction was analysed by an experienced observer in order to identify relevant agonistic behaviours classified as aggressive for dominant fish (bite, chase, strike) and submissive for subordinates (flee and freeze), according to the published ethogram of aggressive behaviour of male zebrafish [27]. Based on who attacks and who is submissive in the post-resolution phase of the interaction, the winner and loser of each fight was determined. For the analysis of the mirror-

image stimulus test, the arena of each individual was delimited in 3 zones: a) a safe area, consisting of the bottom part of the tank, at a distance from the mirror, b) a near area to the mirror excluding the mirror itself and c) a close area where direct contact of the fish with the mirror could take place, either by mouth or the lateral part of the body. To determine differences in risk assessment between genotypes, latency to both the first exploration and interaction and the latency to repeat such behaviour was measured. The percentage of time spent by each genotype in each of the 3 areas was also measured in order to quantify the amount of time spent interacting with their specular image. Other measurements included the number of interactions and explorations per minute at the exploration and interaction areas, respectively and the mean time employed per interaction. Furthermore, the frequency (events) or the duration (states) of typical overt and restrained aggressive behaviour such as attempted bites at the mirror reflection, lateral display, charge, and frontal and parallel swimming maintaining contact with snout to the mirror, was manually assessed for 8 pairs of fish for 10 min [25,28,29]. Ultimately, the mean distance to the mirror and the number of 360° rotations per area were measured.

2.4 Cortisol determination

Ten fish of each genotype (50:50 male-female) were placed in two tanks (10 L) for 30 days and sampled for whole-body cortisol determination [30]. The fish were euthanised by immersion in ice-cold water containing a buffered solution of tricaine methanesulfonate (MS-222, Sigma-Aldrich) (200 µg/ml) until the samples turned upside down, eyes and operculum were immobile, and no response was observed in tails upon contact. Subsequently, the fish were stored intact at -80^{0}C. Prior to hormonal determination, the fish were thawed and weighed, 0.5 ml of ice-cold 1X PBS (phosphate-buffered saline) was added prior to homogenisation using a Polytron ® PT 1200 E (Kinematica, Switzerland). Cortisol extraction was carried out by adding

5ml of diethyl ether (Fisher Scientific International, Inc., USA). After 20 minutes, the samples were centrifuged at 300 x g and the organic upper layer was transferred to new glass tubes. The extraction protocol was repeated by adding 5ml of fresh diethyl ether to ensure an optimal cortisol recovery. Subsequently, evaporation was carried out on the samples using a speed vacuum centrifuge. Cortisol levels were quantified by enzyme immunoassay (EIA, Cayman Chemical Company, USA) using a 200Pro plate reader (TECAN, Austria) and standardised according to the weight of the fish (ng cortisol/g fish).

2.5 Statistical Analysis

Statistical analyses were carried out using the GraphPad Prism 8 software (GraphPad Software Inc, San Diego, CA). For the dyadic fights, an Unpaired t-test was used to compare the latency for the first territory incursion between genotypes. Fisher's exact test was used to compare differences in performing the first bite by individual fish between genotypes. The number of dominant, subordinate or undetermined fish was reported per genotype and results were analysed by the Chi-square test.

In the mirror-image stimulus test, differences in the latency to both the first visit and latency to revisit the close and near zones to the mirror those behaviours, 360° rotations per zone were analysed by repeated measures mixed-effects model followed by the Sidak's multiple comparison test. The time each fish spent in each area and the visit velocities, expressed as the number of entries per minute spent close or near to the mirror, were studied using a two-way ANOVA repeated measures and followed by the Sidak's multiple comparison test An Unpaired t-test was employed to investigate differences in the meantime per interaction, mean distance to the mirror and the number of lateral displays, charges, frontal swimming and bites. This method was also applied to compare the frequency of charges, bites and lateral

display, and the duration of frontal swimming. An Unpaired t-test was also used so as to compare the cortisol levels between *asip1-Tg* and WT fish. All data are presented as mean ± SEM, the significance level was set at $P<0.05$.

3. Results

3.1 Agonistic behaviour in zebrafish

Aggressiveness serves various adaptive functions, such as the establishment of dominance relationships and hierarchies and competition for key resources such as food, shelter, mates or territories. Zebrafish are a gregarious species which exhibit shoaling behaviour in captivity. However, several studies have previously demonstrated that both male and female fish exhibit aggressive behaviour [27,31]. In the present study, dyadic fights between size-paired males were used in order to examine aggressiveness in transgenic zebrafish which overexpress an antagonist of the melanocortin system. Male zebrafish dyads follow stereotyped behavioural patterns, with a well-structured temporal pattern which has been previously and thoroughly characterized thus allowing for standardization [27].

During the experimental period of this study, two phases were easily differentiated. The initial phase consisted of mutual assessment behaviours, with fish circling, and biting each other in order to determine the relative fighting ability of the opponent. Such phase started with the first interaction of the trial and ended when the first chase/retreat was observed, thus marking the resolution point of the fight. Since bite is the most frequent behaviour, representing over 50% of all behaviour types exhibited by zebrafish in the first phase, it was decided to measure which type of fish bite first, it was found that WT significantly bit first ($p = 0.0019$) (Fig. 1A) although

there were no significant differences in latency to the first territory intrusion ($p = 0.2291$) (Fig. 1B). During the second phase, which is characterized by chasing and retreating until the subordinate fish typically falls into a freeze type behaviour, *asip1-Tg* fish fled when chased by WT animals consequently remaining immobile in one corner at the bottom of the tank (freezing behaviour). Approximately 74% (14 out of 20) of *asip1-Tg* fish exhibited the aforementioned behaviour during dyadic fights. Transgenic fish only exhibited dominant behaviour in one of the experimental conflicts and 4 dyadic pairs showed no apparent agonistic behaviour (Fig. 1c). The results show an evident subordinate personality in *asip1-Tg* ($p<0.0001$) (Fig 1d).

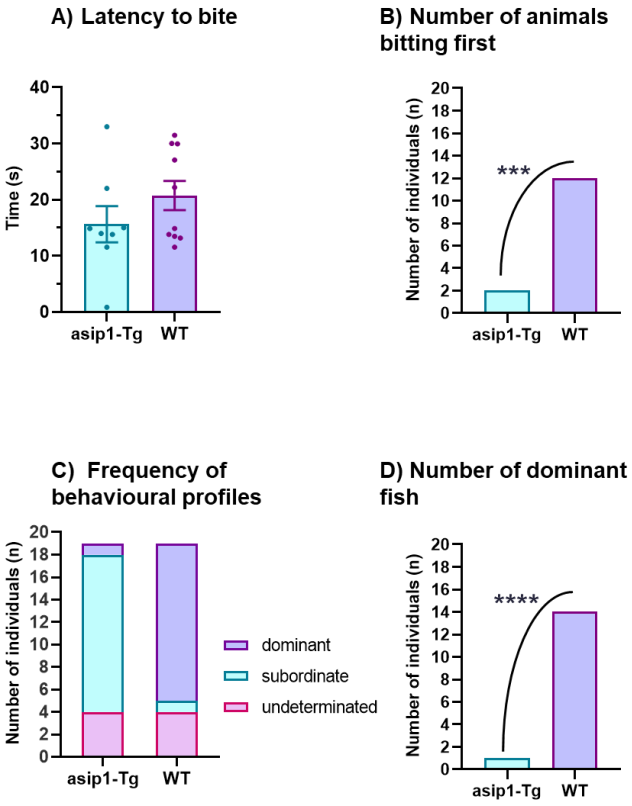
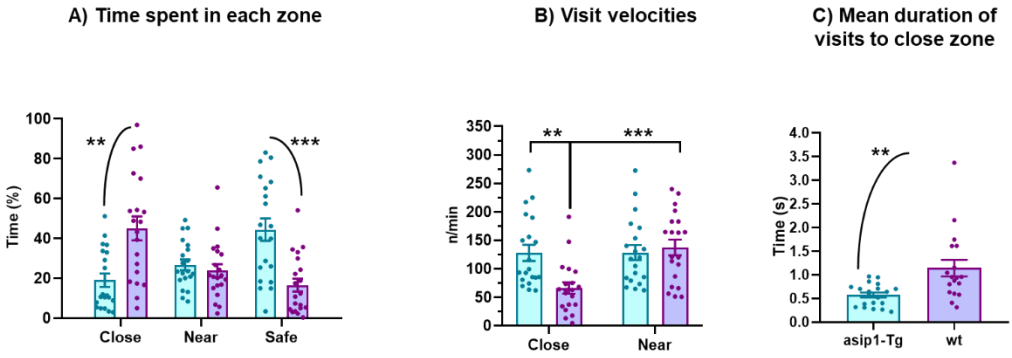


Figure 1. Behavioural differences between transgenic (*asip1-Tg*) and wild-type (WT) zebrafish in dyadic fights.

(A) Latency to first bite bite, (B) Number of animals bitting first, (C Frequency of behavioural profiles and (D) Number of dominant fish. Experiments were performed on 20 WT and 20 *asip1-Tg* fish paired by age and size. Data are represented as mean ± SEM and analysed by Unpaired t-test (latency to first territory intrusion) or as the number of fish and analysed by Chi-square test (dominant fish) or Fisher's exact test (first to bite). Asterisks indicate statistical differences between genotypes (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001).

Agonistic behaviour was also quantified by using the mirror-image stimulus. Fish fail the self-recognition test, thus attack the image reflected in the mirror as a conspecific or rival [32,33]. Significant differences were found in the latency to the first visit, specifically *asip1-Tg* entered the zone near the mirror first than WT, but they did not differ in getting the close zone to the mirror area in terms of time (Fig 2A), Nevertheless, no differences between genotypes were found in the latency to revisit either of the zones (Fig. 2B).

Transgenic fish spent significantly less time close to the mirror and more time in the safe area (Fig. 3A). Surprisingly, the visits' velocity, e.g., entries per minute spent in an area, was significantly higher close to the mirror in *asip1-Tg* than in WT fish (Fig. 3B). Despite of this, WT animals spent the double of time close to the mirror in each visit compared to transgenic fish (*asip1-Tg* mean = 0.571s, WT mean =1.143s; p = 0.0022) (Fig. 3C). Significant differences in visits' velocity between zones close and near the mirror were only found in WT group (p = 0.0016) (Fig. 3B). Taken together, these results suggest a significant difference in fighting behaviour between genotypes. While *asip1-Tg* preferred the safe area, WT fish confronted their specular image for longer periods of time close to the mirror, contrary to transgenic animals which approach the mirror with higher frequency but for a shorter period of time.



Two-way ANOVA RM	P value	Sidak's multiple comparisons	
Interaction	<0.0001	*asip1*-Tg - WT	P value
Zone	0.361	Close	0.0021
Genotype	0.3328	Near	0.8673
Subject	>0.9999	Safe	0.0005

Two-way ANOVA	P value	Sidak's multiple comparisons	
Interaction	0.0145	*asip1*-Tg - WT	P value
Zone	0.0132	Close	0.0022
Genotype	0.032	Near	0.8657
Subject	0.829	Close - Near	
		asip1-Tg	0.9995
		WT	0.0016

Figure 3. Behavioural differences between *asip1-Tg* and WT in the mirror-image stimulus test.

(A) Time spent in three previously defined areas i) a safe area, consisting of the bottom part of the tank, ii) an area near to the mirror and iii) an area close to the mirror where direct contact of the fish with the mirror may take place, (B) Visits' velocity computed as the number of entries per minute spent near or close to the mirror, (c) Mean duration of visits to the close area. Experiments were performed on 20 WT and 20 *asip1-Tg* fish. Data are represented as mean ± SEM and analysed by two-way ANOVA repeated measures (RM) followed by the Sidak's multiple comparison test for (A) and (B) or Unpaired t-test for (C). Asterisks indicate statistical differences between genotypes (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001). Blue and purple colours represent *asip1-Tg* and WT fish, respectively.

Such behaviour is reflected in the heatmaps analysis, where the colour patterns show that *asip1-Tg* fish appear to flee from their own reflection in the mirror (Fig. 4). It is also supported by the higher number of rotations per minute displayed by *asip1-Tg* in the safe area and the almost double mean distance from the mirror kept by *asip1-Tg* compared to WT (see also Figure S2 and S3 in supplementary materials).

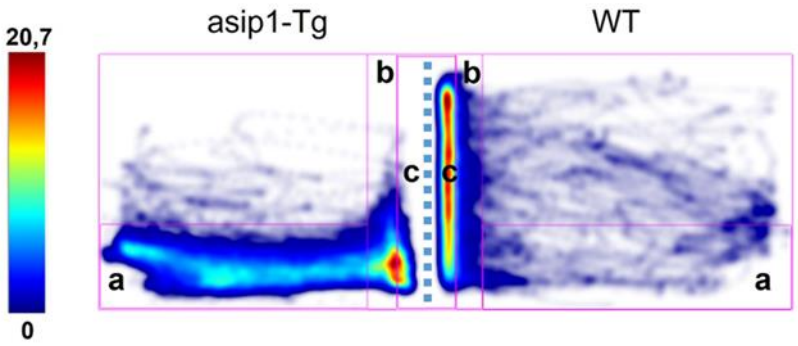


Figure 4. Representative heatmaps of *asip1-Tg* (right) and WT (left).

The colour scale represents the cumulative time spent in each of the previously defined arena zones a) a safe area, consisting of the bottom part of the tank, b) near to the mirror zone, an exploration area where fish can approach but not touch the mirror and c) a close to the mirror area where direct contact of the fish with the mirror can take place. The dotted line represents the position of the mirror.

The frequency of WT overt aggressive behaviour was higher than that observed in *asip1-Tg*, with WT fish attempting to bite their own reflection more frequently than *asip1-Tg*, although not statistically different ($p = 0.0908$) (Fig. 5B). WT also perform frontal and parallel swimming, maintaining contact with snout to the mirror significantly longer than *asip1-Tg* ($p < 0.0001$) (Fig. 5C and 5D). On the contrary, the frequency of restrained aggressive behaviour events, charges and lateral display, behaviours with no physical contact, were significantly higher in *asip1-Tg* fish than WT ($p = 0.0321$ and $p = 0.0015$, respectively) (Fig. 5). Such findings provide evidence, which once again, reinforces the subordinate personality of *asip1-Tg* zebrafish

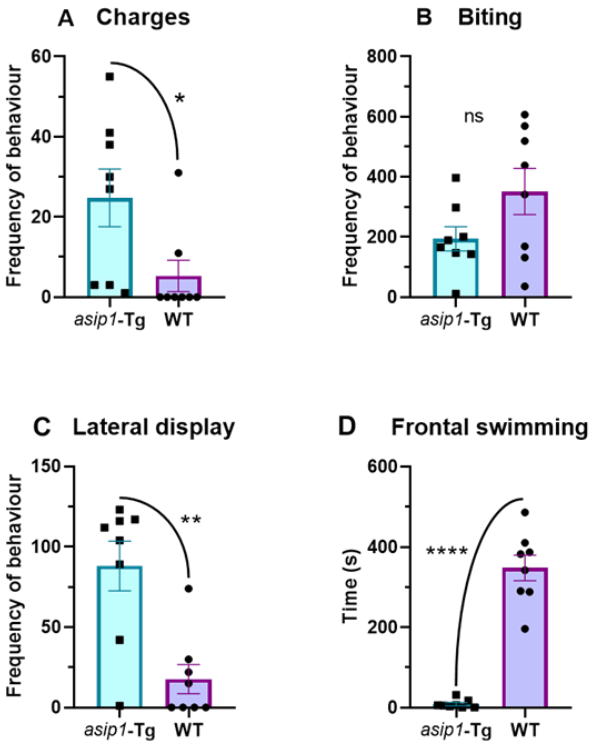


Figure 5. Frequency and duration of aggressive-related behaviours of *asip1-Tg* and WT in the mirror-stimulus test.

(A) Charges, (B) Biting, (C) Lateral display, (D) Frontal swimming. Data relate to 8 WT and 8 *asip1-Tg* fish and are represented as mean ± SEM. Data was analysed by an Unpaired t-test. Asterisks indicate statistical differences between genotypes (*p ≤ 0.05, **p ≤ 0.01, **** p ≤ 0.0001).

3.2 Whole-body cortisol levels

Whole-body cortisol basal levels of both genotypes were determined by EIA assays, it was observed that *asip1-Tg* zebrafish exhibited significantly higher total cortisol levels than WT animals (p=0.0146) (Fig. 6).

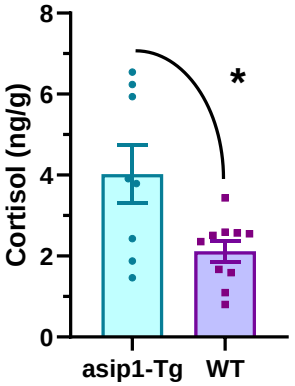


Figure 6. Whole-body basal cortisol levels in *asip1-Tg* and WT zebrafish.

Ten fish of each genotype were homogenized, cortisol was extracted and quantified by EIA. Asterisk indicates statistical differences between genotypes (*p ≤ 0.05).

4. Discussion

Previous studies have demonstrated that *asip1* overexpression in a transgenic model promotes a profuse change in the dorsoventral pigment pattern in zebrafish [14,15]. In addition, *asip1-Tg* fish exhibit higher food intake levels which result in a greater growth rate, yet animals are found not to be obese. Such increased food intake appears to be the result of a desensitized satiety system which promotes enhanced feeding motivation [17,18]. Although *asip1-Tg* zebrafish grow faster, puberty is not reached in advance, as the effects of the transgene on growth are only noticeable after a threshold length when puberty has already been attained [34]. It is therefore assumed that being larger, *asip1-Tg* fish could compete more efficiently for food,

while exhibiting aggressive behaviour. Indeed, aggression serves several adaptive functions including the competition for key resources such as food, territories or mates [27]. Live conspecific and mirror-image stimuli were used to enable characterization of agonistic behaviour in transgenic animals. As zebrafish are territorial, this simple behavioural paradigm, in which fish are pre-exposed to 16h of social isolation, promotes fighting behaviour, even in the presence of abundant, not-limititing resources [27]. Results demonstrate that although the opposing fish invade the territory of others regardless of their genotype, showing no significant differences in risk assessment, *asip1-Tg* fish have a subordinate behaviour in dyadic interactions with WT animals, as only one transgenic fish, out of 20, became a dominant winner. The outcome of the behaviour observed during the conspecific fights was similar to the aggressive behaviour expressed towards a mirror in the mirror-image stimulus test. Subjects of the *asip1-Tg* genotype spent significantly less time interacting with their specular images and more time than WT at the safe area, thus avoiding their own reflection in the mirror. Such observations reflect a cautious and elusive behaviour under a given potential risk. Even though *asip1-Tg* reacted against their specular image, they carried out significantly more restrained aggressive displays, charges and lateral displays, behaviours which display no physical attacks. On the other hand, WT fish maintained contact with snout to the mirror for most of the experimental period and attempted to bite their own reflection more frequently than *asip1-Tg* although no significant differences were observed. On the whole, results from the mirror-image stimulus corroborate the submissive phenotype of *asip1-Tg* fish. While the mirror-image test is a standardized assay for quantification of aggressive behaviour, the physiological underpinnings are not completely replicated, particularly at the level of the central transcriptome and/or endocrine system, and behavioural responses [28,35,36]. In fact, a true subordinate phenotype, observed in real-opponent fights, is never expressed, as fight has no outcome and the expression of aggressiveness is

uncoupled from the experience of the contest result [35]. Nevertheless, the total level of aggressiveness and the stereotyped components of the aggression between the males exposed to real and mirror-image opponents were similar in *Astatotilapia burtoni* [28] and zebrafish [37]. A recent report showed that intraperitoneal administration of α-MSH and mammalian Asip increased and reduced, respectively, the rate of aggressiveness in the cichlid fish *Astatotilapia burtoni* [38], thus corroborating our results and further supporting the role of the melanocortin system in the modulation of agonistic behaviour in fish.

Although *asip1-Tg* zebrafish grow faster and longer than WT siblings [17,18], the differences observed cannot be attributed to the size of the animals, as opponents were paired by body length. The age of both genotypes was also paired therefore, the reproductive status was similar for both genotypes. The diminished amount of time of interaction with the mirror could also arise from a reduced locomotor activity of the transgenic animal stemming from genetic manipulation. However, no differences were observed in the total distance travelled and mean velocity between phenotypes (data not shown). Recent studies have suggested that subordinate animals are capable of adapting their behavioural output to novel social contests in order to reduce energetic expenditure and lower the risk of injuries and exhaustion, by exhibiting submissive behaviour, such as behavioural inhibition, particularly under increased dominance threat [39,40]. In the present experiment, encounters between *asip1-Tg* and a WT conspecific showed there was predominantly unidirectional aggression from WT fish towards *asip1-Tg*. Twelve WT fish out of twenty bit the opponent first while only two *asip1-Tg* attacked first. Thus *asip1-Tg* fish were found not to challenge the WT fish for dominance and instead decide to flee or remain motionless in the bottom corner. It can be argued that the social competence of *asip1-Tg*, defined as an individual's ability to use social information in order to optimise its social behaviour [39,40] allows such fish to conserve energy (by avoiding the fight for

dominance) and evade aggression. In such context, it is noteworthy that being subordinate may be costly for the individual, as it is associated with low activity levels, reduced growth, suppressed feeding and reduced reproduction [41-43]. Nevertheless, such negative outcomes are unobserved in the *asip1-Tg* as overexpression of Asip1 results in increased food intake and linear growth. Furthermore, our unpublished results indicate that *asip1-Tg* fish exhibit an increased locomotor activity during the circadian period, particularly during the night periods (Godino-Gimeno A, Puchol S, Rocha A and Cerdá-Reverter JM), in all likelihood, this is a result of increased foraging behaviour due to the desensitized satiety system [17].

asip1 transgene overexpression yields a paler colour of the dorsal pigmentation in zebrafish [14]. It cannot entirely be disregarded that our behavioural results are, to some extent, due to differences in the dorsoventral pigmentation pattern. In fact, fish coloration is an important visual signal linked to aggressive behaviour and/or social rank used by territorial animals [44]. Therefore, the WT animals interpret the paler dorsal pigmentation of *asip1-Tg* as a marginal phenotype associated to a suboptimal physiological condition thus providing a chance for the opponent to reach social dominance. Under this hypothesis, *asip1-Tg* animals should exhibit similar aggressive levels to those of the WT animals in the mirror-stimulus test, as the image stimulus provided corresponds to a paler animal, *asip1-Tg* animals also observe a paler animal considering that fish cannot self-recognize [36 see also 45-47 for controversial discussion]. However, even so, transgenic fish display a submissive-like behaviour in front of the mirror which is suggested by the reduced interaction with their own image. It can therefore be argued that the dorsoventral pigment pattern had little effect on behaviour outcome.

Endocrine and neuroendocrine systems play a crucial role in the regulation of social behaviour, including aggressiveness and the acquisition of a social status (dominance vs subordination) in fish [44, 48-50]. However, the involvement of the

melanocortin system in the regulation of social behaviour has been scarcely studied. The neuronal mechanisms responsible for the behavioural phenotype behind *asip1* overexpression are unknown. It is acknowledged that Mc4r agonists improve the social deficits shown by NFK1-/- mice through the oxytocin pathway [51] yet ‥Msh central administration has been shown also to decrease social rewards in an oxytocin-dependent manner in Syrian hamsters (*Mesocricetus auratus*) [52]. Previous experiments in goldfish [53], sea bass [54] and zebrafish [55] have demonstrated that MC4R is profusely expressed in the parvocellular and magnocellular preoptic area as well as within the whole extension of the tuberal hypothalamus. Such diencephalic region of fish produces two nonapeptides, i.e., isotocin (It, analogue to mammalian oxytocin) and arginine-vasoticin (Avt, analogue to the mammalian arginine-vasopressin) which play critical roles in the social behaviour of fish, including aggressiveness [48,49,56]. Dominant zebrafish exhibit more Avt magno- and giganto-cellular neurons of the preoptic area (POA) after dyadic tests, while subordinate or loser animals display an abundant presence of Avt neurons in the parvocellular POA [60]. In other species, such as the *Oreochromis mossambicus*, the aggressive behaviour correlates to the number of Avt cells in the parvocellular region whereas the social subordination is associated to changes in the Avt cell populations in the magno- and giganto-cellular preoptic neurons [59] [61]. Therefore, teleost brains have multiple nonapeptidergic pathways that modulate social responses associated to parvo and magnocellular neurons of the POA.

It can be assumed that melanocortins modulate Avp availability in the preoptic region, via Mc4r, thus promoting a submissive phenotype. The regulation of the diencephalic nonapeptidergic system by the melanocortin system has already been reported in rats. Central administration of MC3/4R agonist melanotan II (MTII) increases c-Fos expression in AVP neurons of the paraventricular hypothalamus (PVH), a mammalian homologue of the non-tetrapod magnocellular preoptic nucleus [57],

to acutely inhibit food intake. In addition, MTII fails to fully suppress feeding in mice with virally-mediated PVH-AVP neuron ablation [62]. Therefore, it can be assumed that preoptic Mc4r could induce Avt expression in the parvocellular nucleus and/or reduce the number of Avt neurons in the magnocellular region to promote submissive behaviour and modulate food intake levels. Melanocortin-induced modulation of social behaviour would be modulated by the parvocellular Avt neurons whereas food intake levels would be modulated by magnocellular Avt neurons. In fact, intracerebroventricular injections of Avt inhibit food intake and induce anxiety-like behaviour in goldfish and rainbow trout [63,64]. Suggestively, our unpublished results demonstrate that *asip1-Tg* animals display anxiety-like behaviour after several behavioural assays including novel tank test, although Asip1 effects appear to be mediated via the serotoninergic system (unpublished results).

The submissive behaviour of *asip1-Tg* animals could be part of a behavioural adjustment in order to reduce their stress load as transgenic zebrafish also showed increased whole-body cortisol levels compared to WT fish. Subordinate individuals in groups with stable hierarchies, in which dominance is attained by aggression and intimidation, show the greatest physiological indices of stress [65] and it is therefore essential for such individuals to adjust their behaviour, such as adopting submissive behaviour, in order to reduce their stress load.

Proactive and reactive animals respond differently to stressful events with proactive animals being more aggressive and mobile yet also by lower activity of the hypothalamic-pituitary-interrenal axis (HPI, the fish homologue of the tetrapod hypothalamic-pituitary-adrenal axis, HPA) [66,67]. On the contrary, reactive/submissive animals exhibit higher HPI activity which leads to increased cortisol levels [66,67]. The associated genotypes can be genetically segregated into different lines as high/proactive (HR) and low/reactive (LR) strains [66,67]. Numerous studies have shown that individuals which respond to stress with high HPA/HPI axis reactivity are less

aggressive than those which respond with lower HPA axis reactivity [68]. Accordingly, HR rainbow trout displayed more aggressiveness than LR strain in resident-intruder tests [67]. Our previous experiments in zebrafish have shown that whole body cortisol levels can be a good indicator of HPI axis activity [69] providing more support to the submissive behavior of the *asip1-Tg* genotype.

5. Conclusions

In conclusion, results show that the reduced melanocortin signalling imposed by the overexpression of endogenous antagonists leads to less aggressiveness in animals thus suggesting a submissive phenotype. Behavioural phenotype is in accordance with physiological data, as *asip1-Tg* fish also display higher cortisol levels, which is distinctive of reactive/submissive phenotypes. These results imply direct evidence of the role of the melanocortin system in the regulation of fish behaviour and provide new central mechanisms for future behavioural studies.

Author Contributions

A.R.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing-original draft, review and editing, Visualization, Supervision. A.G.-G.: Validation, Formal analysis, Data Curation, Visualization, Writing-original draft. J.R.: Methodology, Resources, Review and editing. J.M.C.-R.: Conceptualization, Formal analysis, Funding acquisition, Investigation, Supervision, Writing-original draft, review and editing.

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Institutional Review Board Statement:

All experiments were performed in accordance with the Spanish (Royal Decree 53/2013) and European (2010/63/EU) legislation for the protection of animals used for experimentation. The protocols applied were approved by the IATS Ethics Committee (Register Number 09-0201) under the supervision of the Secretary of State for Research, Development and Innovation of the Spanish Government.

Data Availability Statement:

Data are available at:

https://data.mendeley.com/datasets/999v2k53fv/draft?a=ba0ce768-20be-4782-acc3-ba32b2dfe2db.

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Conflicts of Interest:

The authors declare no competing interests.

References

1. Cerdá-Reverter, J.M.; Agulleiro, M.J.; R, R.G.; Sánchez, E.; Ceinos, R.; Rotllant, J. Fish melanocortin system. *Eur. J. Pharmacol*. **2011**, *660*, 53-60.
2. Rocha, A.; Godino-Gimeno, A.; Cerdá-Reverter, J.M. Evolution of proopiomelanocortin. Vitam. Horm. **2019**, *111*, 1-16.
3. Schiöth, H.B.; Haitina, T.; Ling, M.K.; Ringholm, A.; Fredriksson, R.; Cerdá-Reverter, J.M.; Klovins, J. Evolutionary conservation of the structural, pharmacological, and genomic characteristics of the melanocortin receptor subtypes. *Peptides*. **2005**, *26*, 1886-1900.
4. Huszar, D., Lynch, C.A.; Fairchild-Huntress, V.; Dunmore, J.H.; Fang, Q.; Berkemeier, L.R.; Gu, W.; Kesterson, R.A.; Boston, B.A.; Cone, R.D.; Smith, F.J.; Campfield, L.A.; Burn, P.; Lee, F. Targeted disruption of melanocortin-4 receptor results in obesity in mice. *Cell* **1997**, *88*, 131–141
5. Chen, W.; Kelly, M.A.; Opitz-Araya, X.; Thomas, R.E.; Low, M.J.; Cone, R.D. Exocrine glands dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell*. **1997**, *91*, 89-798.
6. Miller, M.W.; Duhl, D.M.J.; Vrieling, H.; Cordes, S.P.; Ollmann, M.M.; Winkes, B.M.; Barsh, G.S. Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying a lethal yellow mutation. *Genes Dev*. **1993**, *7*, 454–467
7. Tolle, V.; Low, M.J. In vivo evidence for inverse agonism of Agouti-related peptide in the central nervous system of proopiomelanocortin-deficient mice. *Diabetes* **2008**, *57*, 86–94.
8. Ollmann, M.M.; Wilson, B.D.; Yang, Y.K.; Kerns, J.A.; Chen, I.; Gantz, I.; Barsh, G.S. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* **1997**, *278,* 135–138.

9. Cerdá-Reverter, J.M.; Peter, R.E. Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. *Endocrinology*. **2003**, *144*, 4552-4561.

10. Song, Y.; Cone, R.D. Creation of a genetic model of obesity in a teleost. *FASEB J*. **2007**, *21*, 2042–2049

11. Zhang, C.; Forlano, P.M.; Cone, R.D. AgRP and POMC neurons are hypophysiotropic and coordinately regulate multiple endocrine axes in a larval teleost. *Cell Metab*. **2012**, *15*, 256-264.

12. Shainer, I.; Michel, M.; Marquart, G.D.; Bhandiwad, A.A.; Zmora, N.; Ben-Moshe Livne, Z.; Zohar, Y.; Hazak, A.; Mazon, Y.; Förster, D.; Hollander-Cohen, L.; Cone, R.D.; Burgess, H.A.; Gothilf, Y. Agouti-Related Protein 2 Is a New Player in the Teleost Stress Response System. *Curr. Biol.* **2019**, *29*, 2009-2019.e7.

13. Cerdá-Reverter, J.M.; Haitina, T.; Schiöth, H.B.; Peter, R.E. Gene structure of the goldfish agouti-signaling protein: a putative role in the dorsal-ventral pigment pattern of fish. *Endocrinology*. **2005**, *146*, 1597-1610.

14. Ceinos, R.M.; Guillot, R.; Kelsh, R.N.; Cerdá-Reverter, J.M.; Rotllant, J. Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms *Pigment Cell Melanoma Res*. **2015**, *28*,196-209.

15. Cal, L.; Suarez-Bregua, P.; Comesaña, P.; Owen, J.; Braasch, I.; Kelsh, R.; Cerdá-Reverter, J.M.; Rotllant, J. Countershading in zebrafish results from an Asip1 controlled dorsoventral gradient of pigment cell differentiation. *Sci. Rep*. **2019**, *9*, 3449.

16. Cal, L.; Suarez-Bregua, P.; Braasch, I.; Irion, U.; Kelsh, R.; Cerdá-Reverter, J.M.; Rotllant, J. Loss-of-function mutations in the melanocortin 1 receptor cause disruption of dorso-ventral countershading in teleost fish. *Pigment Cell Melanoma Res*. **2019**, *32*, 817-828.

17. Guillot, R.; Cortés, R.; Navarro, S.; Mischitelli, M.; García-Herranz, V.; Sánchez, E.; Cal, L.; Navarro, J.C.; Míguez, J.M.; Afanasyev, S.; Krasnov, A.; Cone, R.D.; Rotllant, J.; Cerdá-Reverter, J.M. Behind melanocortin antagonist overexpression in the zebrafish brain: A behavioral and transcriptomic approach. *Horm. Behav.* **2016**, *82*, 87-100.

18. Godino-Gimeno, A.; Sánchez, E.; Guillot, R.; Rocha, A.; Angotzi, A.R.; Leal, E.; Rotllant, J.; Cerdá-Reverter, J.M. Growth performance after agouti-signaling protein 1 (asip1) overexpression in transgenic zebrafish. *Zebrafish*. **2020**, *17*, 373-381.

19. Metcalfe, N.B.; Huntingford, F.A.; Graham, W.D.; Thorpe, J.E. Early social status and the development of life-history strategies in Atlantic salmon. *Proc. Royal Soc. B*. **1989**, *236*, 7-19.

20. Lahti, K.; Laurila, A.; Enberg, K.; Piironen, J. Variation in aggressive behaviour and growth rate between populations and migratory forms in the brown trout, *Salmo trutta*. *Anim. Behav*. **2001**, 62, 935–944.

21. Devlin, R.H.; D'Andrade, M.; Uh, M.; Biagi, C.A. Population effects of growth hormone transgenic coho salmon depend on food availability and genotype by environment interactions. *Proc. Natl. Acad. Sci*. **2004**, *101*, 9303.

22. Hallerman, E.M.; McLean, E.; Fleming, I.A. Effects of growth hormone transgenes on the behavior and welfare of aquacultured fishes: A review identifying research needs. In *Applied Animal Behaviour Science* Vol. 104, **2007**. pp. 265–294.

23. Johnsson, J.I.; Bjornsson, B.T. Growth-enhanced fish can be competitive in the wild. *Funct. Ecol.* **2001**, *15*: 654-659

24. Jonsson E, Johnsson JI, Bjornsson BT 1989 Growth hormone increases aggressive behavior in juvenile rainbow trout. *Horm. Behav.* **1989**, *33*, 9-15

25. Teles, M.C.; Oliveira, R.F. Quantifying Aggressive Behavior in Zebrafish. In: *Zebrafish: methods and protocols*, 2nd ed.; Kawakami, K., Patton, E.E., Orger, M. Eds.; Springer NY, **2016;** pp.293-305.

26. Friard, O.; Gamba, M. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Meth. Ecol. Evol*. **2016**, *7*, 1325–1330.

27. Oliveira, R.F.; Silva, J.F.; Simoes, J.M. Fighting zebrafish: characterization of aggressive behavior and winner-loser effects. *Zebrafish* **2011**, *8*, 73-81

28. Desjardins, J.K.; Fernald, R.D. What do fish make of mirror images? *Biol. Lett*. **2010,** 23, 744-747.

29. Way GP, Rulh n, Sneker JL, Kiesel AL, McRobert SP A Comparison of Methodologies to Test Aggression in Zebrafish. *Zebrafish* **2016**, *2*, 144-151

35. Oliveira, R.F.; Simoes, J.M.; Teles, M.C.; Oliveira, C.R.; Becker, J.D.; Lopes, J.S. Assessment of fight outcome is needed to activate socially driven transcriptional changes in the zebrafish brain**.** *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E654-E661

31. Spence, R.; Gerlach, G.; Lawrence, C.; Smith, C. The behaviour and ecology of the zebrafish, Danio rerio. *Biol. Rev.* 2008, *83*, 13–34

30. Cortés, R.; Teles, M.; Oliveira, M.; Fierro-Castro, C.; Tort, L.; Cerdá-Reverter, J.M. Effects of acute handling stress on short-term central expression of orexigenic/anorexigenic genes in zebrafish. *Fish Physiol. Biochem*. **2018**, *44*, 257-272

32. Gallup, Jr. G.G. Mirror-image stimulation. *Psychol. Bull*. **1968**, *70*, 782–793.

33. Balzarini, V.; Taborsky, M.; Wanner, S.; Koch, F.; Frommen, J.G. Mirror, mirror on the wall: the predictive value of mirror tests for measuring aggression in fish. *Behav. Ecol. Sociobiol.* **2014**, *68,* 871-878.

34. Navarro, S.; Crespo, D.; Schultz, R.W.; Ge, W.; Rotllant, J.; Cerda-Reverter, J.M.; Rocha, A. Role of melanocortin system in gonadal steroidogenesis of zebrafish. *Animals*, **2022**, *12*, 2737.

36. Oliveira, R.F.; Carneiro, L.A.; Canario, A.V.M. No hormonal response in tied fights. *Nature*. **2005**, *437*, 207-208

37. Teles, M.C.; Dahlbom, S.J.; Winberg, S.; Oliveira, R.F. Social modulation of brain monoamine levels in zebrafish. *Behav. Brain Res*. **2013**, *253*, 17-24.

38. Dijkstra, P.D.; Maguire, S.M.; Harris, R.M.; Rodriguez, A.A.; DeAngelis, R.S.; Flores, S.A.; Hofmann, H.A. The melanocortin system regulates body pigmentation and social behaviour in a colour polymorphic cichlid fish. *Proc. Biol. Sci*. 2017 284, 20162838.

39. Taborsky, B. Oliveira, R. Social competence: an evolutionary approach. Trends Ecol. Evol. **2012**, *27*, 679-688.

40. Taborsky, B.; Arnold, C.; Junker, J.; Tschopp, A. The early social environment affects social competence in a cooperative breeder. *Anim. Behav.* **2012**, *83*, 1067-1074.

41. Amorim, M.C.P.; Almada, V.C. The outcome of male–male encounters affects subsequent sound production during courtship in the cichlid fish Oreochromis mossambicus. *Animal Behav.* **2005,** *69*, 595-601.

42. Oliveira, R.F.; Almada, V.C. Androgenization of Dominant Males in a Cichlid Fish: Androgens Mediate the Social Modulation of Sexually Dimorphic Traits. *Ethology*. **2010**, *104*, 841-858.

43. Sørensen, C.; Johansen, I. B.; Øverli, Ø. Chapter 9. In: *The physiology of fishes*. 2nd ed. Evans, D. H., Clairborne, J. B., Currie, S. Eds.; Editorial Taylor and Francis Group: Washington USA, **2013**: pp. 289

44. Maruska KP, Anselmo CM, King T, Mobley RB, Ray EJ, Wayne R 2022 Endocrine and neuroendocrine regulation of social status in cichlid fishes. *Horm. Behav*. **2022**, *139*, 105110.

45. Kohda, M.; Hotta, T.; Takeyama, T.; Awata, S.; Tanaka, H.; Asai, J.Y.; Jordan, A.L. If a fish can pass the mark test, what are the implications for consciousness and self-awareness testing in animals? *PloS Biol*. **2019**, *17*, e3000021.

46. Vonk, J. A fish eye view of the mirror test. *Learn. Behav*. **2020**, *48*, 193-194.

47. Kohda, M.; Sogawa, S.; Jordan, A.L.; Kubo, N.; Awata, S.; Satoh, S.; Kobayashi, T.; Fujita, A.; Bshary, R. Further evidence for the capacity of mirror self-recognition in cleaner fish and the significance of ecologically relevant marks. *PloS Biol*. **2022**, *20*, e3001529

48. Goodson, J.L. Nonapeptides and the evolutionary patterning of sociality. In Progress in Brain Research, Neumann, I.D., Landgraf, R. Eds., Elsevier **2008**; Volume 170, pp. 3-15.

49. Godwin, J. Thompson, R. Nonapeptides and Social Behavior in Fishes *Horm. Behav*. **2012**, *61*, 230–238.

50. Quintana, L.; Jalabert, C.; Fokidis, H.B.; Soma, K.K.; Zubizarreta, L. Neuroendocrine mechanisms underlying non-breeding aggression: common strategies between birds and fish. *Front. Neural Circuits*. **2021**, 15, 716605.

51. Mastinu, A.; Premoli, M.; Maccarinelli, G.; Grilli, M.; Memo, M.; Bonini S.A. Melanocortin 4 receptor stimulation improves social deficits in mice through oxytocin pathway. *Neuropharmacology*. **2018**, *133*, 366-374.

52. Grieb, Z.A., Cross, E.A., Albers, H.E. Alpha-melanocyte-stimulating hormone (αMSH) modulates the rewarding properties of social interactions in an oxytocin receptor-dependent manner in Syrian hamsters (*Mesocricetus auratus*). *Physiol. Behav*. **2022**, *252*, 113828.

53. Cerdá-Reverter, J.M.; Ringholm, A.; Schiöth, H.B.; Peter, R.E. Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: involvement in the control of food intake. *Endocrinology*. **2003**, *144*, 2336-2349.

54. Sánchez, E.; Rubio, V.C.; Thompson, D.; Metz, J.; Flik, G.; Millhauser, G.L.; Cerdá-Reverter, J.M. Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (*Dicentrarchus labrax*). *Am. J. Physiol*. **2009**, *296*, R1293-306.

55. Josep Agulleiro, M.; Cortés, R.; Fernández-Durán, B.; Navarro, S.; Guillot, R.; Meimaridou, E.; Clark, A.J.; Cerdá-Reverter, J.M. Melanocortin 4 receptor becomes an ACTH receptor by coexpression of melanocortin receptor accessory protein 2. *Mol. Endocrinol*. **2013**, *27*, 1934-1945.

56. Filby, A.L.; Paull, G.C.; Bartlett, E.J.; Van Look, K.J.; Tyler, C.R. Physiological and health consequences of social status in zebrafish (*Danio rerio*). *Physiol. Behav*. **2010,** *101,* 576-87.

57. Cerdá-Reverter, J.M.; Canosa, L.F. 2009 Neuroendocrine Systems of the Fish Brain. In: *Fish Neuroendocrinology*. *Fish Physiology*, Bernier, N.J., Van Der Kraak, G., Farrell, A.P., Brauner, C.J., Eds., Academic Press, London, UK, **2009**; Volume 28, pp. 3-74.

58. Greenwood, A.K.; Wark, A.R.; Fernald, R.D.; Hofmann, H.A. Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. *Proc. R. Soc. B*. **2008**, *275*, 2393–2402.

59. Backström, T.; Winberg, S. 2009 Arginine-vasotocin influence on aggressive behavior and dominance in rainbow trout. *Physiol. Behav*. **2009**, *96*, 470-475.

60. Larson, E.T.; O'Malley, D.M.; Melloni, R.H. Jr. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. *Behav. Brain Res.* **2006**, *167*, 94-102.

61. Almeida, O.; Oliveira, R.F. Social Status and Arginine Vasotocin Neuronal Phenotypes in a Cichlid Fish. *Brain Behav. Evol.* **2015**, *85,* 203-213.

62. Pei, H.; Sutton, A.K.; Burnett, K.H.; Fuller, P.M.; Olson, D.P. AVP neurons in the paraventricular nucleus of the hypothalamus regulate feeding. *Mol. Metab*. **2014**, *3*, 209–215.

63. Araishi, K.; Watanabe, K.; Yamazaki, T.; Nakamachim T.; Matsuda, K. Intracerebroventricular administration of arginine vasotocin (AVT) induces anorexigenesis and anxiety-like behavior in goldfish. *Peptides* **2019**, *119*, 170118.

64. Gesto, M.; Soengas, J.L.; Rodriguez-Illamola, A.; Miguez, J.M. Arginine Vasotocin Treatment Induces a Stress Response and Exerts a Potent Anorexigenic Effect in Rainbow Trout, Oncorhynchus mykiss. *J. Endocrinol*. **2014**, *26,* 89-99.

65. Sapolsky, R.M. The influence of social hierarchy on primate health. *Science* **2005**, *308*, 5722.

66. Pottinger, T.G.; Carrick, T.R. A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Aquaculture*. **1999**, *175*, 351-363. 67. Backström, T.; Schjolden, J.; Overli, O.; Thornqvist, P.O.; Winberg, S. Stress effects on AVT and CRF systems in two strains of rainbow trout (*Oncorhynchus mykiss*) divergent in stress responsiveness *Horm. Behav*. **2011**, *59*, 180-186.

67. Schjolden, J.; Backström, T.; Pulman, K.G.T.; Pottinger, T.G.; Winberg, S. Divergence in behavioural responses to stress in two strains of rainbow trout (Oncorhynchus mykiss) with contrasting stress responsiveness *Horm. Behav.* **2005**, *48*, 537–544.

68. Summers, C.H.; Winberg, S. Interactions between the neural regulation of stress and aggression. *J. Exp. Biol*. **2006**, *209*, 4581-4589.

69. Cortés, R., Teles, M., Oliveira, M., Fierro-Castro, C., Tort, L., Cerdá-Reverter, J.M. Whole body cortisol and mRNA levels of orexigenic/anorexigenic genes in *Danio rerio* after an acute handling stress. *Fish Physiol. Biochem*. **2018**, *44*, 257-272.

Supplementary materials

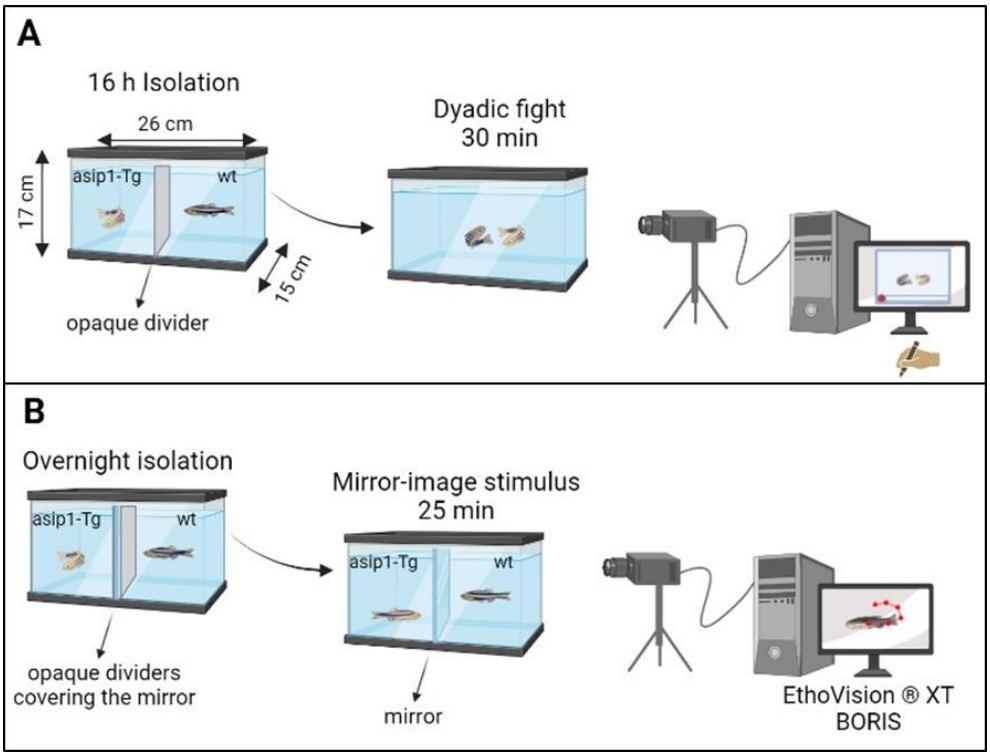


Figure S1. Behavioural tests setups, a) dyadic fights setup, an *asip1-Tg* and a WT males were placed in a tank and kept separated for 16 hours Subsequently, the opaque divider was removed to allow fish to interact for 30 minutes, the estereotipated behaviours was recorded and analysed manually. b) Mirror-image setup, a mirror was placed in between two divisors and an *asip1-Tg* and a WT males were separated overnight. Subsequently, the opaque dividers were removed and fish exposed to their specular image, video recording, trajectory tracking, and analysis were carried out using EthoVision ® XT software (Noldus Inc. The Netherlands) and behavioural event loggings with BORIS software. See material and methods for more information.

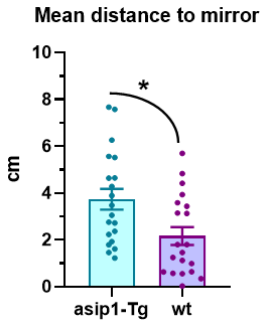
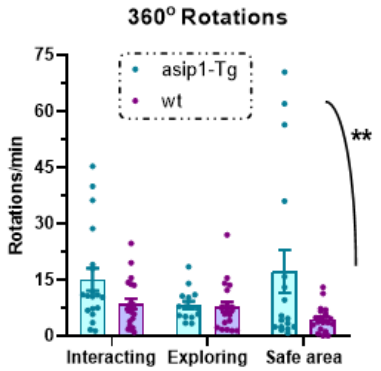


Figure S2: Mean distance to mirror of *asip1-Tg* (n = 20) and WT (n = 20) in the mirror-image stimulus test.

Data were represented as mean ± SEM and analysed by Unpaired t-test. Asterisks indicate statistical differences between genotypes (*p ≤ 0.05).



Two-way ANOVA	P value	Sidak's multiple comparisons test	
Interaction	0,0868	asip1-Tg - wt	P value
Behaviour	0,3456	Interacting	0,2224
Genotype	0,0035	Exploring	0,9991
		Safe area	0,0034

Figure S3. 360o Rotations per minute in the mirror-image stimulus test.

Three arena zones were previously defined i) a safe area, consisting of the bottom part of the tank, ii) an exploration zone close to the mirror and iii) an interaction zone where direct contact of the fish with the mirror can occur. Experiments were performed on 20 WT and 20 *asip1-Tg* fish. Data are represented as mean ± SEM and analysed by two-way ANOVA followed by Sidak's multiple comparison test (latency to first). Asterisks indicate statistical differences between genotypes (*p ≤ 0.05, **p ≤ 0.01).

CHAPTER 5

Agouti-signalling protein 1 (Asip1) overexpression induces anxiety-like behaviour in zebrafish

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Abstract

Hunger and satiation are vital to maintaining energy balance and are controlled by the melanocortin system. The overexpression of two inverse agonists, agouti-related protein (AGRP) and agouti-signalling protein (ASIP), promotes an enhanced food intake and body size. Contrary to zebrafish overexpressing Agrp obesogenic phenotype, overexpressing asip1 under the control of a constitutive promoter (*asip1-Tg*) enhances linear growth and weight but not obesity. Our previous research relates greater food take in *asip1-Tg* zebrafish to enhanced feeding motivation, albeit no higher feeding rate is required for their increased growth. This is explained by significant feeding efficiency due to a differential intestine permeability that facilitates nutrient absorption in *asip1-Tg*. Our previous behavioural studies showed that the bigger size of *asip1-Tg* is not enhancing aggressiveness. In fact, we observed that these transgenic fish often choose to flee to safer zones than confront a possible threat. That is what inspired us to study the anxiety-like behaviour of *asip1-Tg* zebrafish. To investigate locomotion and exploration, we performed the open-field test, where transgenic fish displayed a higher mean velocity and visit velocity in the central zone and marked freezing behaviour compared to their counterparts. To continue, we placed an object in the arena, which most *asip1-Tg* still did not explore. Anxiety-like behaviour was approached using the novel tank diving test (NTDT), anxiety indicators such as mean velocity, acceleration, angular velocity, freezing and latency to visit the top zone were significantly more elevated in *asip1-Tg* than in WT fish. We confirmed the transgenics anxiogenic phenotype by analysing serotoninergic and dopaminergic pathways metabolites, where we found significantly lower serotonin and dopamine levels in *asip1-Tg*, together with high turnover rates. Finally, we reverse the anxiogenic phenotype with a chronic fluoxetine treatment, a selective serotonin reuptake inhibitor (SSRI). Anxiety-like behaviour of transgenics observed previously

in the NTDT was mitigated and confirmed by no longer differences in serotonin between *asip1-Tg* and WT. Our results showed behavioural aspects of the relationship between monoaminergic neurotransmission and the melanocortin system. Furthermore, these findings indicate that inhibiting the melanocortin system has an anxiogenic effect that, together with our previous works, suggests that constant feeding motivation enhances anxiety in fish. We believe that these anxiogenic effects may promote continuous food searching, impairing circadian rhythms by enhancing locomotor activity in *asip1-Tg* fish.

Key words: Melanocortins · MC4R · Asip1 · Anxiety · Serotonin · Dopamine · Food intake · Behaviour

1. Introduction

The melanocortin system regulates crucial physiological functions such as melanogenesis, stress response, and energy balance (Cerdá-Reverter et al., 2011). This complex system is composed of peptides derived from the posttranscriptional processing of proopiomelanocortin precursor (POMC) that yields γ-MSH, α-MSH, ACTH, and ß-MSH and ß-endorphin (Rocha et al., 2019; Navarro et al., 2016). In corticotrophs of the anterior pituitary, the prohormone convertase PC_1 cleavages POMC to yield ACTH, whereas, in melanotrophs of the pars intermedia, PC_1 and PC_2 generate α-MSH and ß-endorphin (Cerdá-Reverter et al., 2011; Mountjoy, 2015).

Melanocortin functions are regulated through five receptors coupled to G-proteins (MC1R-MC5R) with different affinities for their agonists, i.e. MSHs and ACTH (Schiöth et al., 2005) and endogenous antagonists agouti-signalling protein (ASIP) and agouti-related protein (AGRP) (Cerdá-Reverter et al., 2005; Cerdá-Reverter & Peter, 2003). MC2R is the only receptor that exclusively binds ACTH, whereas the remaining receptors bind the MSHs with different affinities. Binding of ACTH to MC2R

requires interaction with the melanocortin receptor accessory protein (MRAP1), whereas the paralog MRAP2 dimerises with the remaining MCRs and modulates their signalling. Furthermore, the interaction of MRAP2/MC4R is able to modify the binding profile of the receptor, conferring ACTH sensitivity (Agulleiro et al., 2013; Soletto et al., 2019).

ACTH binding to the MC2R/MRAP1 complex in the adrenal or interrenal gland (fish homologue) modulates the stress response by promoting cortisol release (Cerdá-Reverter et al., 2011). MC1R is mainly expressed in the skin and α-MSH binding regulates eumelanin (dark pigment) synthesis in melanocytes. Antagonism of ASIP1 reduces MC1R signalling and promotes the switch to pheomelanin (yellow pigment) synthesis in melanocytes, thus controlling pigmentation patterns (Vrieling et al., 1994). MC5R is present at low levels in many tissues and has been shown to regulate exocrine secretion (Chen et al., 1997). Finally, MC3R and MC4R are key encephalic receptors for the regulation of energy balance, i.e. feed intake and energy expenditure (Cone, 2006). MC4R is constitutively active (Nijenhuis et al., 2001; Sánchez et al., 2009; Tolle & Low, 2008) and thus imposes an inhibitory tone on feed intake that is overcome by the binding of AGRP to induce hunger (Cone, 2006). In the encephalon, AGRP is expressed exclusively in the arcuate nucleus and its overexpression induces increased feed intake levels and obesity without pigmentation changes (Ollmann et al., 1997). AGRP overexpression mimics MC4R (-/-) phenotype supporting MC4R-mediation of AGRP effects (Huszar et al., 1997). Although ASIP expression is restricted to peripheral tissues, in the rare allele of the agouti locus, Ay, the gene is under the control of the Raly promoter, an RNA binding protein that is ubiquitously expressed (Michaud et al., 1993; Miller et al., 1993). The *Ay* phenotype is characterised by a pheomelanised yellow coat and hyperphagia leading to obesity. The metabolic effects in *Ay* mice are due to the ectopic encephalic expression of ASIP and its binding to MC4R, which mimics AGRP overexpression (Fan et al., 1997; Lu et

al., 1994). In teleosts, an additional genome duplication resulted in two paralogues for both Asip and Agrp, whose phylogenetic relationships remain controversial (Cortés et al., 2014). In goldfish, fasting increases agrp1 expression (Cerdá-Reverter et al., 2003a). Similar to mice, agrp1 overexpression in zebrafish promotes linear growth and obesity (Song & Cone, 2007), whereas opposite effects were reported in agrp1 morpholino knockdown zebrafish (Zhang, 2012) and confirmed by genetic ablation of AgRP1-expressing neurons (Shainer et al., 2019). Previous results have shown that Asip1 is expressed in the ventral skin and regulates melanogenesis (Cerdá-Reverter et al., 2005). Interestingly, the phenotype observed in asip1-overexpressing transgenic zebrafish overexpressing asip1 under the control of a constitutive promoter (*asip1-Tg*) shows not only a disruption of the dorsoventral pigmentation pattern (Ceinos et al., 2015), but also an increase in linear growth, body weight and food intake, although not an obese phenotype (Song & Cone, 2007; Guillot et al., 2016; Godino-Gimeno et al., 2020). Our recent studies suggest that asip1 Tg fish have enhanced nutrient absorption compared to wild type (WT), resulting in higher feed efficiency, which may explain the observed enhanced growth without increased food intake (Godino-Gimeno et al., 2020; Guillot et al., 2016; Leal et al., 2022). Surprisingly, this enhanced growth and higher feeding rates are not associated with accelerated puberty (Navarro et al., 2021), as in other fish species (Lampert et al., 2010; Liu et al., 2020).

From a behavioural point of view, our previous results showed that asip1 Tg fish display a submissive personality, which is perfectly in line with increased plasma cortisol levels (Rocha et al., 2023). Asip1 administration studies have also confirmed our phenotypic results (Øverli et al., 2002). Furthermore, a decrease in serotonin (5-HT) levels is common in anxiety syndromes, and low central 5HT (Winberg & Thörnqvist, 2016; Mondanelli & Volpi, 2021;) and dopamine (Cabib & Puglisi-Allegra, 2012; Thörnqvist et al., 2019) and high cortisol levels (Alsop & Vijayan, 2008; Ramsay et al.,

2009) have also been described in subordinate/reactive fish. As asip1 Tg fish exhibit a subordinate phenotype and our preliminary studies have shown reduced central 5HT levels (Guillot et al., 2016), it is plausible that our transgenic fish exhibit anxiety-like behaviour.

In this study, we used different behavioural test to demonstrate that Asip Tg fish show an anxious phenotype linked to depressed central 5HT levels. The administration fluoxetine, a selective serotonin reuptake inhibitor (SSRI) that acts at presynaptic neuron blocking receptor 5HT1A (Sohel et al., 2022) was enough to rescue the WT phenotype suggesting that central 5HT mediates the melanocortin antagonist-induced anxiety in zebrafish.

2. Materials and methods

2.1 Animals and housing conditions

WT and *Asip1-Tg* zebrafish [Tg(Xla.Eef1a1:Cau.Asip1]iim4 (Ceinos et al., 2015) belonging to the TU strain (Nüsslein-Volhard Lab, Tuebingen, Germany) were bred and housed into a recirculating system at the Institute of Aquaculture of Torre de la Sal (IATS-CSIC) facilities. Water temperature was kept at 27.5 – 28ºC and pH at 7.5-8. The photoperiod was 14L:10D, from 7 am until 9 pm. Fish were raised at a density of approximately 6 fish/L and 50:50 sex ratio and fed thrice daily with artemia and commercial flakes (Vipan, Sera, Heinsberg, Germany). The animals used in this study were experimentally naïve and free of any signs of disease. Experiments were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals and approved by the "Superior Council of Scientific Investigations" (CSIC) ethics committee (project number

PID2019-103969RB-C33) and the "General Directorate of Agriculture, Livestock and Fishing of Valencia", registration number: nGVRTE/2021/217198.

2.2 Experiment design

Six-months-old fish of both genotypes, with a sex ratio 50:50, were separated from the stock and kept in two 20-litre tanks in the isolated behavioural test room. Water conditions, photoperiod and feeding protocol were maintained as in the zebrafish facility. Fish were acclimated for two weeks prior to testing. First, two tests were conducted to investigate differences in exploratory behaviour: the open field test and the novel object test. Both were conducted in 22 cm diameter cylindrical filled with 2 litres of unchlorinated water at the same temperature and pH as the water in the home tanks. To investigate anxiety-like behaviour, we used the novel tank diving test (NTDT). Subsequently, the brains of both genotypes were dissected to measure metabolites from serotoninergic and dopaminergic pathways by high-performance liquid chromatography (HLPC). Finally, to rescue the behavioural phenotype of *asip1-Tg*, we treated both genotypes with 0.25µg/g of fluoxetine, an SSRI, administered in the diet for two weeks. After the dietary treatment, control and treated animals from both genotypes were behaviourally tested in the NTDT, and the levels of metabolites belonging to the serotoninergic and dopaminergic pathways were measured by HPLC.

2.3 Behavioural data acquisition

In all the tests, videos were recorded using with Basler (Basler acA1280-60gc GigE camera, Basler AG, Ahrensburg, Germany) equipped with a high-quality monofocal lens (focal length 8 mm) at 25 frames per second (fps). Fish tracking data were analysed with Ethovision XT 16 (Noldus Inc., Wageningen, the Netherlands). All experiments were conducted between 9:30 and 13:30 hrs.

2.3.1 Open field and novel object tests

Zebrafish of both genotypes (WT = 20, *asip1-Tg* = 20) were initially tested in the open field for 10 minutes, after 30 seconds of habituation. To determine thigmotaxis behaviour, time spent in the peripheral and central zones, distance travelled, mean central speed and central entry speed were measured. To investigate possible anxiety-like behaviour in this novel environment, we measured freezing behaviour, periods of immobility during which the fish only moved the operculum, either in terms of time or in terms of the percentage of the population of WT and *asip1-Tg* that experienced one or more freezing bout(s). After acclimation to the open field, we performed the novel object test by placing a 1.5 cm dark blue marble in the centre of the circular tank and observing for 10 min whether the subject explored the novel object.

2.3.2 Novel tank diving test

For the novel tank diving test (NTDT), fish of each genotype (WT n=24; *asip1-Tg* n=24) were removed from their home tank and placed in a trapezoidal tank (L:27cm x H:15cm x W:5cm, Aquaneering ZT180T) filled with 1.8 litres of chlorine-free water at the same temperature and pH as their home tank. We placed 2 tanks on a bench, one for WT and one for *asip1-Tg*, so that 2 fish were tested simultaneously, but the tanks were shielded to avoid visual contact with each other. The sides of the tanks were covered with duct tape to avoid eye contact between subjects, and an infrared light board was placed behind the bench to improve contrast in the video. The NTDT lasted 10 minutes after a 30 seconds period of accommodation. At the end of the trial, the fish were returned to the home system in 38L tanks, and the test trapezoidal tanks were rinsed with 96% ethanol. To analyse the natural bottom-dwelling response to novelty, known as geotaxis, followed by a gradual exploration of the new environment, each arena was divided into 2 equal zones: top and bottom. Parameters

such as mean velocity, angular velocity, acceleration, distance moved, and time spent moving were evaluated for each zone. Additional anxiety indicators were measured, such as latency to the first visit to the top zone and habituation expressed as time spent in freezing along the trial, as well as the percentage of each genotype that was always moving *vs* those that were immobile since the start of the test, after 30 seconds or after 60 seconds.

2.3.3 Novel tank diving test after chronic fluoxetine treatment

Two groups of each genotype (n=16) were initially separated (WT, WT + fluoxetine, *asip1-Tg*, *asip1-Tg* + fluoxetine). The fish were kept in 5-litre tanks equipped with mechanical filters and filled with chlorine-free water at the same temperature and pH as the recirculation system. Approximately 30% of the total volume of water was changed daily. Fluoxetine (F132, Sigma-Aldrich, Germany) was included in the diet and administered daily at a dose of 0,25µg/g for 15 days. After chronic treatment with fluoxetine, the geotaxis of the 4 groups was tested in the NTDT according to the methodology described above. In order to investigate the effects of the treatment on both genotypes, the following parameters were analysed in each zone: mean velocity, distance travelled, acceleration, time spent moving, latency to the first visit to the top and time spent in freezing.

2.4 Monoamine Neurotransmitters analysis

Brains of both genotypes, either fluoxetine-treated or control (n=10), were dissected and stored at -80 ºC until analysed. Samples were then thawed in ice-cold NaH_2PO_4 buffer (10.2g/L) and homogenised using an ultrasonic disruptor. The levels of 5-hydroxytryptophan (5HTP), 5HT and 5-hydroxy indoleacetic acid (5HIAA) were determined by HPLC with electrochemical detection (Godino-Gimeno et al., 2023). In addition, metabolites of the dopaminergic pathway were analysed: L-

dihydroxyphenylalanine (L-dopa), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and noradrenaline (NA) were assayed. The mobile phase (pH = 2.95) consisted of 63.9 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.80 mM sodium 1-octanesulfonate and 15.3% methanol (v/v). The HPLC equipment consisted of a Jasco pump (PU-2080); an analytical 5µm column (Phenomenex, Nucleosil C18, 150 mm x 4.6 mm); an M5011 double analytical cell (first electrode: +40mV; second electrode +340mV) for oxide analytes and an ESA Coulochem II detector. Results were obtained using ChromNAV 1.12 software (Jasco Corporation). The levels of each metabolite *per* total brain protein (measured by BCA) and the 5HIAA/5HT, as well as the DOPAC/DA ratios, were calculated.

2.5 Gene expression determination

Brains of ten animals of each genotype were dissected and rapidly placed in Trireagent (Invitrogen), homogenized and stored a -80ºC for RNA extraction following the manufacturer's instructions. Subsequently, total RNA was re-suspended in RNase-free water and quantified using a Nanodrop 2000 spectrophotometer. Potential genomic DNA was removed with Turbo DNAse-RNAse-free (Thermo Scientific) according to the manufacturer's instructions. One microgram of DNA-free total RNA was reverse transcribed using the SuperScript III reverse transcriptase (Thermo Scientific) and oligo (dT)12-18 (0.5 µg/ml), as indicated by the manufacturer. cDNA was diluted in nuclease-free water and stored at −20 °C until use. Real-time PCR was performed in a CBX ConnectTM Real-Time System instrument (BioRad) using Abgene's SYBR® Green QPCR Master Mix (Thermo Scientific, Spain) in a total reaction volume of 15 µL (Thermo Scientific) and specific primers (Forward: 5' AAGCCTACATCCGCAAGCAT 3' and Reverse: 5' AGGTGGCAAGGAACCAGAGT 3'), previously optimized, to evaluate the expression of aromatic L-amino acid decarboxylase (*aadc*). Thermal cycling conditions were 95ºC for 15 min, followed by 40 cycles of 95ºC for

15 s, annealing temperature (55ºC) for 30 s and 72ºC for 30 s; followed by 95ºC for 30 s, 55ºC for 30 s. The expression of target-specific genes was normalized to the relative expression of zebrafish β-*actin,* (Forward: 5′ GATGAGGAATCGCTGCCCT 3′ and Reverse: 5′ GTCCTTCTGTCCCATGCCAA 3′) and fold change gene expression was performed using the mean normalized expression (MNE) method of the Q-Gene application. Negative controls with no template were included. A melting curve for each PCR was determined by reading fluorescence every degree between 55°C and 95°C to ensure the specific amplification.

2.6 Statistics

All statistical analyses were performed using GraphPad Prism 8. In the open field test, statistical differences between genotypes in travel time and distance travelled *per* zone were analysed by two-way ANOVAs, and no multiple comparison tests were performed as interactions were not significant. Significant differences in mean central velocity, entry velocity, and time spent in freezing between WT and *asip1-Tg* were examined using unpaired t-tests. Differences in the novel object test were assessed using the chi-squared test. The results of the NTDT were analysed to investigate significant differences between the WT and *asip1-Tg* groups using an unpaired t-test, except for time spent in freezing per minute, where we used the two-way ANOVA for repeated measures followed by Sidak's multiple comparisons test. An unpaired t-test was also used to genotype examine differences in monoamine levels. In the NTDT, following chronic fluoxetine treatment, significant differences between groups were assessed by two-way ANOVA followed by Sidak's multiple comparisons test when interactions were significant. Only significant differences in time spent in freezing between control and fluoxetine-treated transgenic fish were assessed by unpaired t-test. Finally, two-way ANOVA was used to study differences in monoamine

levels between control and fluoxetine-treated groups. All data are represented as mean ± SEM.

3. Results

3.1 Thigmotaxis did not differ between genotypes, but *asip1-Tg* showed footprints of anxiety and low exploratory behaviour.

We use the open field test to assess differential thigmotaxis between both genotypes by analysing locomotor parameters in the peripheral and central zones of the test tank. asip1 Tg fish spent more time moving in the peripheral zone than WT animals, but no significant differences in distance travelled were recorded (Fig. 1A, 1B). We also analysed parameters related to anxiety-like behaviour, including mean central velocity, entry velocity and time spent freezing. *asip1-Tg* fish consistently showed higher levels for all variables (Fig. 1C, 1D). In addition, 80% of the WT fish were constantly moving, but 50% of the *asip1-Tg* animals experienced freezing episodes during the test (Supplementary Fig. S1). Accordingly, the time spent freezing was significantly higher in *asip1-Tg* fish (Fig. 1E). Different tracking patterns in the central and peripheral areas can be observed in Figure 1F. Subsequently, after the animals had become acclimated to the novel environment of the open field test tank, a novel object was placed in the centre of the test tank to compare the exploratory abilities of both genotypes. The majority of *asip1-Tg* animals did not approach the novel object, showing an opposite behaviour to that observed in WT animals (Figure 1G).

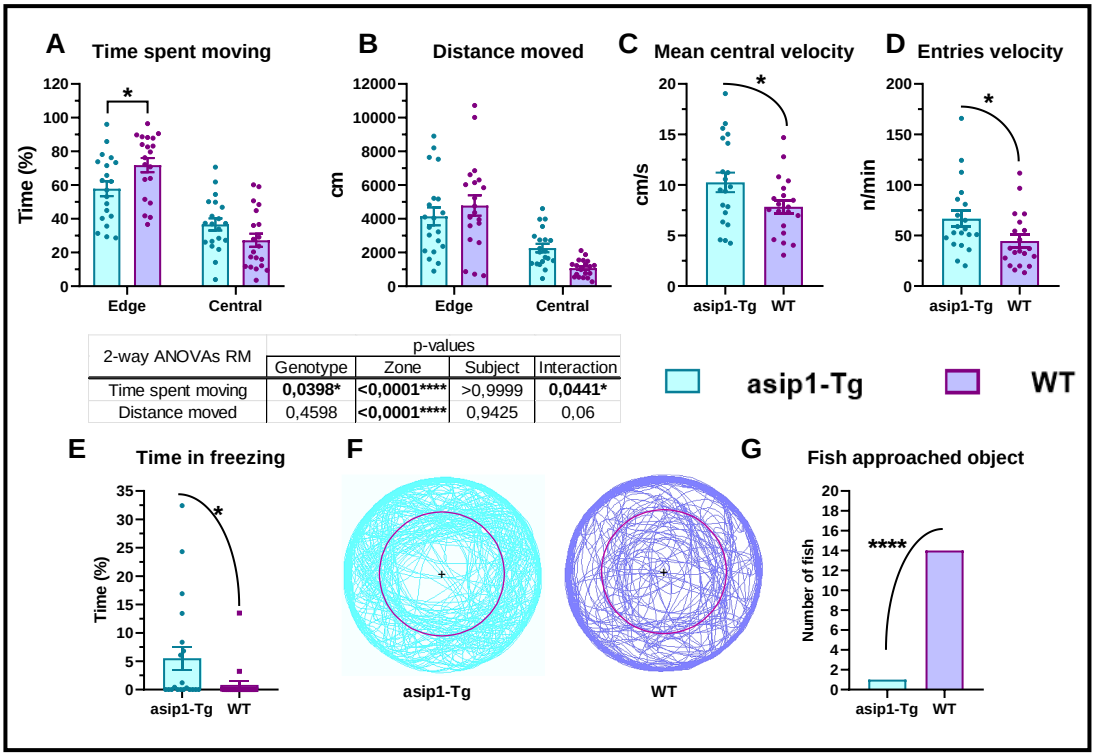
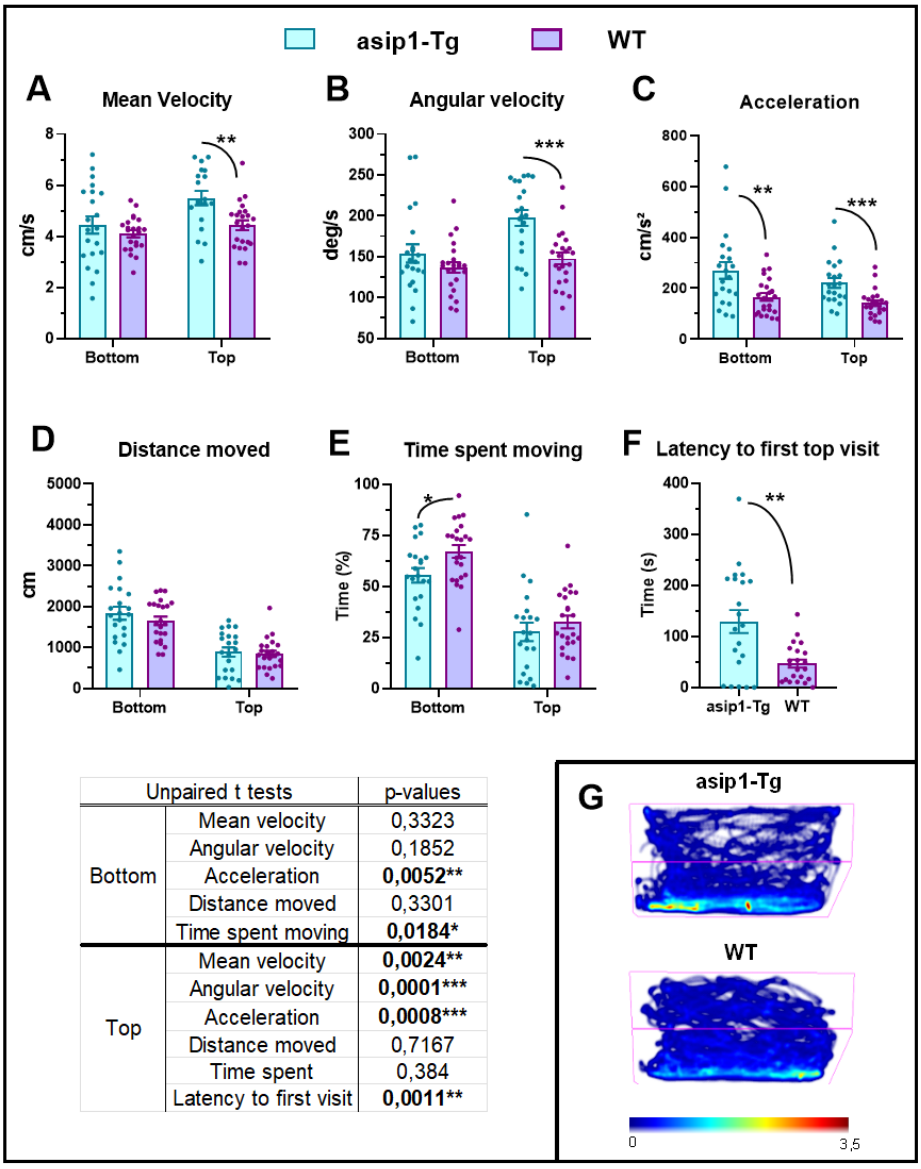


Figure 1: Open field (A-F) and novel object test results (G).

A: Time spent moving in the edge and central zones; significant differences were found between genotypes and zones; *asip1-tg* (blue) spent significantly less time moving at the edge than WT (purple) (see table below). B: Distance moved per zone; only significant differences were found between zones but not due to genotype (*asip1-Tg* blue, WT: purple) (see table below). C: Mean central velocity, *asip1-Tg*, showed higher velocity when moving in the central zone than WT (*p ≤ 0.05). D: Entries velocity, *asip1-Tg* entries per minute, was significantly higher than WT (*p ≤ 0.05). E: Time spent in freezing, *asip1-Tg* spent significantly more time in freezing than WT (*p ≤ 0.05). F: Representative tracks of *asip1-Tg* (blue) and WT (purple) moving in the open field test, circles delimit edge and central zones and cross the arena centre where marble was placed in the posterior novel object test. G: Number of fish from both genotypes approached the new object, and significantly more WT fish explored the novel object. Data are expressed by mean ±SEM *asip1-Tg* n = 20; WT = 20. A and B were analysed by two-way ANOVA repeated measures and Sidak's multiple comparations test. C-E data were analysed with an Unpaired t-test and F by Chi-square test. Asterisks indicate significant differences between *asip1-Tg* and WT (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001).

3.1 *Asip1-Tg* displayed an enhanced anxiety-like behaviour in the NTDT.

Next, we used the NTDT to further investigate anxiety-like behaviour in *asip1-Tg* fish. We evaluated several parameters, including mean velocity, angular velocity, acceleration, distance travelled, and time spent travelling in the top and bottom areas of the tank. Mean, and angular velocities were always higher in the top area of the tank in *asip1-Tg* animals, whereas acceleration was higher in all areas of the tank (Fig 2.A, 2B, 2C). No differences were observed in the distance travelled between the two phenotypes (Fig. 2D), but the time spent travelling at the bottom was increased in WT animals (Fig. 2E). We also measured two variables that are well related to anxiety, i.e. the latency to first visit the upper region of the tank (Fig. 2F) and habituation to the novel environment, assessed as the number of freezing bouts per minute (Fig. 3). *asip1-Tg* animals took significantly more time to first visit the upper region of the tank, suggesting a fearful/anxious behaviour (Fig. 2F). Furthermore, *asip1-Tg* exhibited significantly more freezing bouts than WT during the first 3 minutes (Fig. 3). Only 19% of *asip1-Tg* were constantly moving during the NTDT compared to almost 87% of WT. Of those transgenics that freezed, 52.4% showed freezing behaviour from the start of the test, 14% after 30 seconds and the same percentage after 60 seconds (Supplementary Fig. S2).

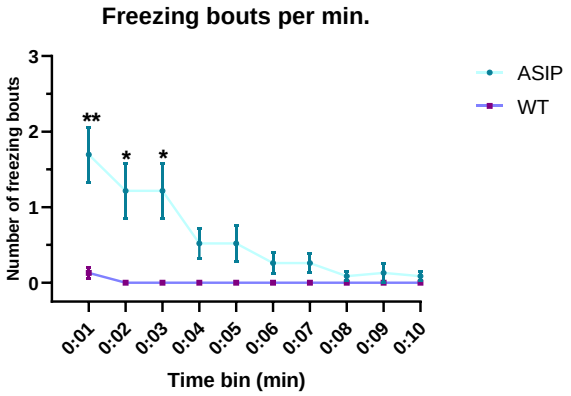


Unpaired t tests		p-values
Bottom	Mean velocity	0,3323
	Angular velocity	0,1852
	Acceleration	**0,0052****
	Distance moved	0,3301
	Time spent moving	**0,0184***
Top	Mean velocity	**0,0024****
	Angular velocity	**0,0001*****
	Acceleration	**0,0008*****
	Distance moved	0,7167
	Time spent	0,384
	Latency to first visit	**0,0011****

Figure 2: Novel tank diving test (NTDT) parameters of anxiety-like behaviours, measured in two zones: bottom and top.

A) Mean velocity of *asip1-Tg* fish was significantly higher than WT in the top zone. B) Angular velocity of *asip1-Tg* fish was significantly more elevated than WT in the top zone. C) Acceleration in both zones was significantly higher in the transgenic group. D) Distance moved was similar in both genotypes. E) Time spent moving was significantly less in the *asip1-Tg* group.

F) Latency to visit the top zone was significantly more reduced in WT than in transgenic fish. G) Representative heatmaps of *asip1-Tg* and WT fish; the colour scale represents the cumulative time spent in each of the previously defined arena zones: bottom and top. Data are represented by mean ±SEM and analysed by Unpaired t-test (see table below). Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01***p ≤ 0.001, **** p ≤ 0.0001).



2-way ANOVA RM: Freezing bouts			
p-values			
Genotipe	Time	Subject	Interaction
<0,0001****	**<0,0001******	**<0,0001******	**<0,0001******
Sidak's multiple comparations test: asip1-Tg vs WT			
0:01	**0,003****	0:06	0,5793
0:02	**0,0271***	0:07	0,436
0:03	**0,0271***	0:08	0,8289
0:04	0,1394	0:09	0,9813
0:05	0,3531	0:10	0,8289

Figure 3: Habituation in the NTDT measured in freezing bouts per minute.

Significant differences were found between genotypes, being *asip1-Tg*, the group displaying significantly more freezing bouts in the first 3 minutes, and the behaviour decreased slower in transgenic fish, indicating worse habituation to the novel environment. Data are represented by mean ±SEM and analysed by Two-way ANOVA Repeated Measures and, Sidak's multiple comparations test (see table below). Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01***p ≤ 0.001, **** p ≤ 0.0001).

3.3 *Asip1-Tg* showed lower levels of serotonin, dopamine, and noradrenaline in the encephalon.

To investigate whether differences in monoamine biosynthetic pathways could explain the anxiogenic profile observed in *asip1-Tg*, we analysed the metabolites of serotoninergic and dopaminergic neurotransmission pathways by HPLC. We found no genotypic differences in the serotonin precursor 5HTP (Fig. 4A), but serotonin levels were significantly lower in *asip1-Tg*, while the metabolite 5HIAA was slightly higher in *asip1-Tg*, but without reaching statistical differences (Fig. 4B, 4C). The metabolisation ratio 5HIAA/5HT was significantly higher in *asip1-Tg* than in their counterparts, as shown in Figure 4D. Analyses of the dopaminergic pathway showed no genotypic differences in the dopamine precursor L-dopa (Fig. 5A), but dopamine levels were significantly lower in *asip1-Tg* (Fig. 5B). Again, the metabolite of dopamine, DOPAC, was slightly more elevated in *asip1-Tg*, but no statistical differences were reached (Fig. 5C), yet the DOPAC/DA ratio was significantly higher in *asip1-Tg*, as shown in Fig. 4D. Dopamine is the precursor of norepinephrine, the levels of which were also significantly reduced in the transgenic fish (Fig. 5E). The concurrent reduction in central levels of both 5HT and dopamine suggested that the enzyme Aadc, which is common to both serotoninergic and dopaminergic biosynthetic pathways, may have reduced activity. To explore this hypothesis, we evaluated the genotypic expression levels of aadc in the brain by qPCR, which showed no significant differences.

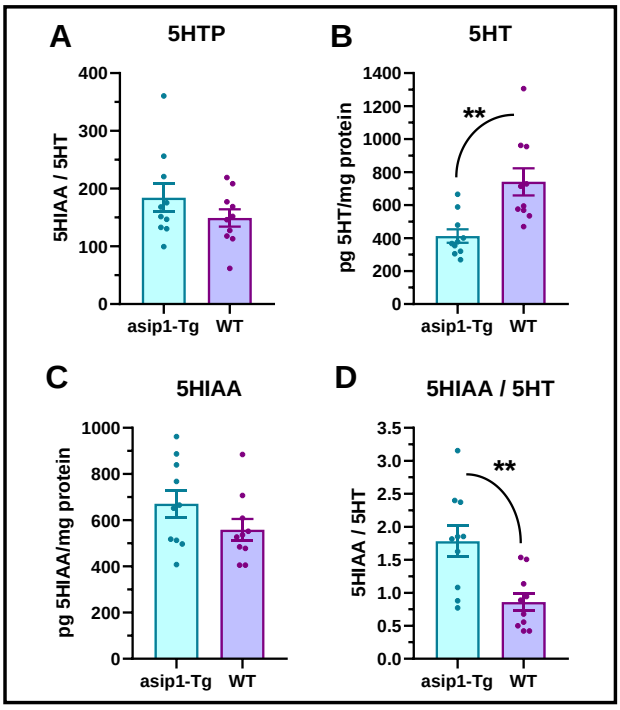


Figure 4: Brain serotoninergic biosynthesis pathways metabolites and their degradation ratio, comparison between *asip1-Tg* and WT fish.

A) Levels of serotonin precursor 5-hydroxytryptophan (5HTP), were similar in both genotypes. B) Serotonin (5HT) levels were significantly reduced in *asip1-Tg*. C) Levels of 5-hydroxy indoleacetic acid (5HIAA) did not differ statistically between genotypes. D) The ratio of 5HIAA/5HT was significantly higher in transgenic fish compared to WT. Dissected brains (*asip1-Tg* n=10, WT n=8, sex ratio 50:50) were analysed by HPLC. Results are expressed in pg of metabolite per mg of total protein; data are represented by mean ±SEM analysed by Unpaired t-test. Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01)

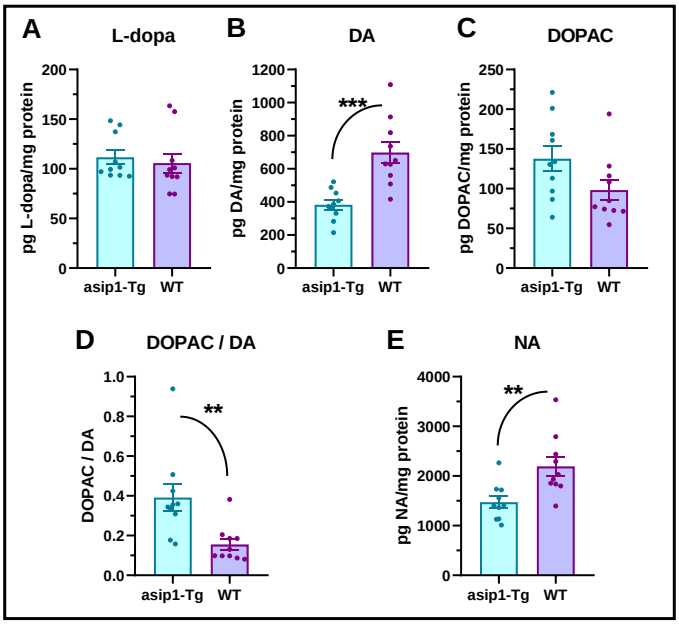


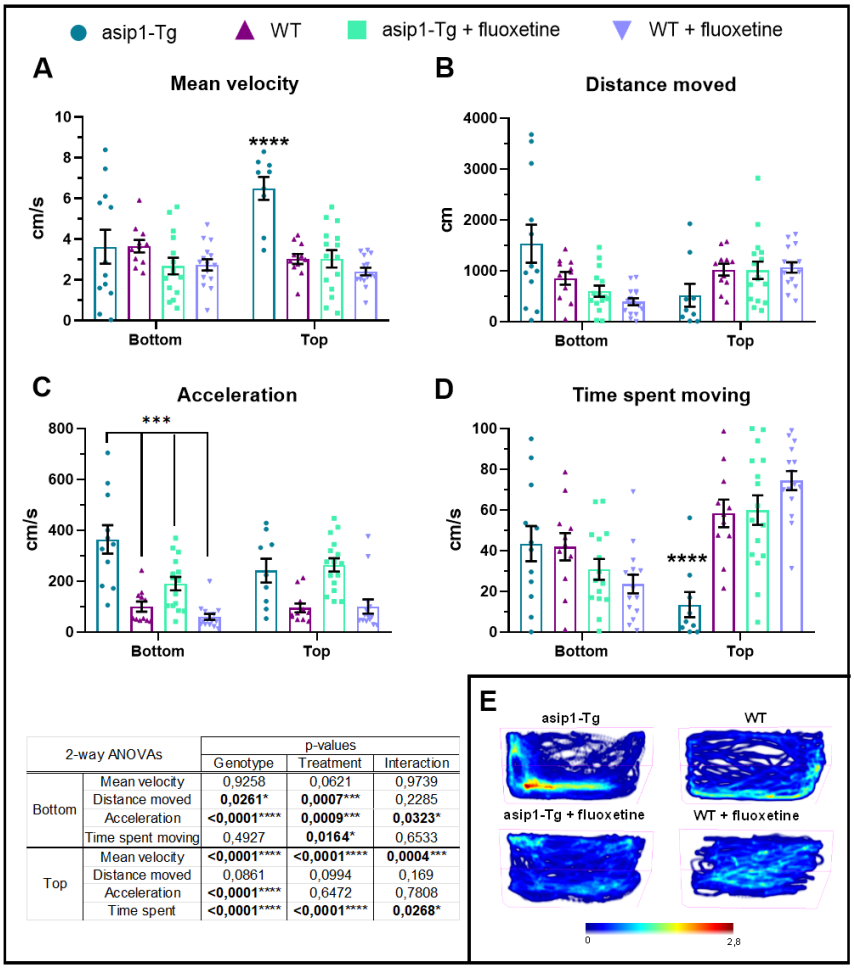
Figure 5: Brain dopaminergic biosynthesis pathways metabolites and their degradation ratio, comparison between *asip1-Tg* and WT fish.

A) Levels of dopamine precursor L-dihydroxyphenylalanine (L-dopa) were similar in both genotypes. B) Dopamine (DA) levels were significantly reduced in *asip1-Tg*. C) Levels of dihydroxyphenylacetic acid (DOPAC) did not differ statistically between genotypes. D) The ratio of DOPAC/DA was significantly higher in transgenic fish compared to WT. E) Norepinephrine (NE) levels, synthesized from DA, were significantly lower in *asip1-Tg*. Dissected brains (*asip1-Tg* n=10, WT n=10, sex ratio 50:50) were analysed by HPLC. Results are expressed in pg of metabolite per mg of total protein; data are represented by mean ±SEM analysed by Unpaired t-test. Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01)

3.4 Chronic fluoxetine treatment reverses anxiety-like behaviour in *asip1-Tg*

The results suggested that the anxiety-like behaviour induced by *asip1* overexpression might be mediated via central serotoninergic pathways. To test this hypothesis, we treated both genotypes with oral fluoxetine for two weeks and used NTDT to assess whether the reuptake inhibitor was able to rescue the phenotype of the transgenic animals. We found no differences in mean velocity, distance travelled and time spent travelling in the lower region of the tank (Fig. 6A, 6B, 6D). The distance travelled in the upper region was also similar between the two genotypes, treated or not (Fig. 6B). However, *asip1*-Tg showed an increased mean velocity in the upper region, which was reversed by oral fluoxetine to that observed in treated WT (Fig. 6A). Fluoxetine treatment also induced a decrease in mean velocity in WT animals, but the values did not reach statistical significance (Fig. 6A). Also in the upper region of the tank, the time spent moving was reduced in *asip1-Tg* when compared to all three remain experimental groups. Oral fluoxetine reversed transgene effects increasing the levels of the variable to those of the WT-treated animals (Fig. 6D). Again, treatment increased the time spent moving in WT animals, when compared to WT untreated animals, but levels did not reach significant relevance (Fig. 6D). The acceleration of *asip1-Tg* animals was higher in the bottom region of the tank and the fluoxetine treatment reversed the phenotype, however acceleration levels still remained slightly high when compared to WT and/or WT-treated animals (Fig. 6C). This time, fluoxetine significantly reduces acceleration levels in WT-treated animals when compared to WT untreated animals (Fig. 6C). Representative heat maps for all experimental groups are shown in Fig. 6E. Finally, we also evaluated the latency to the first visit of the top area corroborating that *asip1-Tg* animals take more time to overcome fear condition, however SSRI treatment reversed values to those exhibited WT and

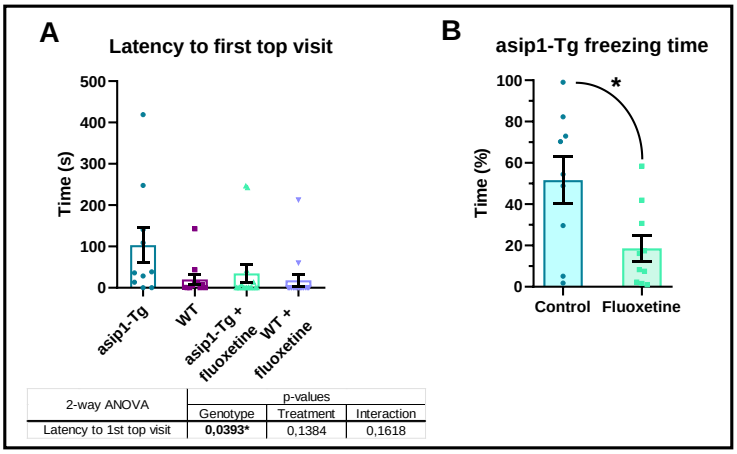
WT-treated fish but not significant relevance was reached (Fig. 7A). Freezing time was also reduced in *asip1-Tg* treated fish (Fig. 7B).



2-way ANOVAs		p-values		
		Genotype	Treatment	Interaction
Bottom	Mean velocity	0,9258	0,0621	0,9739
	Distance moved	**0,0261***	**0,0007*****	0,2285
	Acceleration	**<0,0001********	**0,0009*****	**0,0323***
	Time spent moving	0,4927	**0,0164***	0,6533
Top	Mean velocity	**<0,0001********	**<0,0001********	**0,0004*****
	Distance moved	0,0861	0,0994	0,169
	Acceleration	**<0,0001********	0,6472	0,7808
	Time spent	**<0,0001********	**<0,0001********	**0,0268***

Figure 6: Novel tank diving test (NTDT) results per zone, comparison between genotypes after chronic treatment with fluoxetine (*asip1-Tg* + fluoxetine, WT + fluoxetine) and control non-treated fish (*asip1-Tg*, WT).

A) Mean velocity was only higher in *asip1-Tg*, while *asip1-Tg* showed similar results than WT and WT+fluoxetine. B) Distance moved differences at the bottom zone were due to genotype and treatment (see table below). C) Acceleration differences in both zones were found between genotypes; at the bottom zone, acceleration was significantly reduced by treatment in both genotypes, albeit differences between *asip1-Tg* + fluoxetine and WT + fluoxetine remain significant in this area. D) Time spent moving at the bottom was reduced by treatment, while at the top zone was significantly augmented; the *asip1-Tg* tendency to significantly spent less time on top was reversed by fluoxetine treatment. E) Representative heatmaps of controls, *asip1-Tg* and WT, and treated fish *asip1-Tg* and fluoxetine, WT + fluoxetine; the colour scale represents the cumulative time spent in each of the previously defined arena zones: bottom and top. Data are represented by mean ±SEM and analysed by Two-way ANOVA (see table below), and when interaction was significant, Sidak's multiple comparations test Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01***p ≤ 0.001, **** p ≤ 0.0001).



2-way ANOVA	p-values		
	Genotype	Treatment	Interaction
Latency to 1st top visit	**0,0393***	0,1384	0,1618

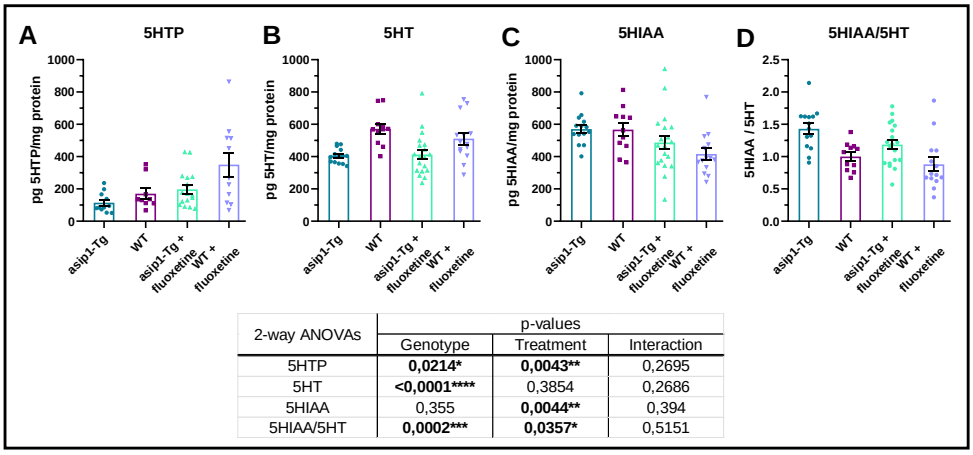
Figure 7: Novel tank diving test (NTDT) additional anxiety-like behaviour parameters.

A) Latency to first top visit, comparison between genotypes after chronic treatment with fluoxetine (*asip1-Tg* + fluoxetine, WT + fluoxetine) and control non-treated fish (*asip1-Tg*, WT), significant differences due to genotype disappeared with fluoxetine treatment. B) Time spent in freezing observed mainly in *asip1-Tg* was significantly reduced in the treated transgenic group. Data are represented by mean ±SEM and analysed by Two-way ANOVA (A) and Unpaired t-test (B). Asterisks indicate significant differences (*p ≤ 0.05).

3.5 High serotonin reuptake induces low 5HT levels and, consequently, anxiety in *asip1-Tg*.

Brains from either control or fluoxetine-treated animals (see above) were prepared for HPLC monoamine determination as before, to confirm the effects of SSRI treatment on central neurotransmitter levels. Again, 5HT levels were lower in *asip1-Tg* animals regardless of SSRI treatment as oral fluoxetine was unable to restore whole brain 5HT levels to those observed in WT animals (Figure 8B). Fluoxetine treatment increased 5HT precursor (5HTP) levels in both genotypes (Fig. 8A) as well as 5HIIA levels (Fig. 8C). Therefore, the 5HIIA/5HT ratio was higher in *asip1-Tg* animals, but was reduced by SSRI treatment in both genotypes (Fig. 8D). Ratio levels in treated *asip1-Tg* animals reached similar levels to those observed in untreated WT fish.

Following the dopaminergic pathways, *asip1-Tg* always showed lower dopamine and norepinephrine levels and DOPAC/dopamine ratios, irrespective of SSRI treatment (Supplementary Fig. S4). Thus, all observed differences were induced by genotype, but never by fluoxetine treatment.



2-way ANOVAs	p-values		
	Genotype	Treatment	Interaction
5HTP	**0,0214***	**0,0043****	0,2695
5HT	**<0,0001**** **	0,3854	0,2686
5HIAA	0,355	**0,0044****	0,394
5HIAA/5HT	**0,0002*****	**0,0357***	0,5151

Figure 8: Brain serotoninergic biosynthesis pathways metabolites and their degradation ratio, comparison between genotypes after chronic treatment with fluoxetine (*asip1-Tg* + fluoxetine, WT + fluoxetine) and control non-treated fish (*asip1-Tg*, WT).

A) Levels of 5HTP were significantly different according to genotype and treatment. B) Serotonin (5HT) levels were significantly reduced due to transgenic genotype; such difference disappeared with fluoxetine treatment. C) Levels of 5-hydroxy indoleacetic acid (5HIAA) did not differ statistically between genotypes but were significantly reduced in treated groups. D) The ratio of 5HIAA/5HT was significantly higher in the transgenic genotype; although such difference was reduced in treated fish, significant differences were still found after treatment. Brains were dissected after NTDT and were analysed by HPLC. Results are expressed in pg of metabolite per mg of total protein; data are represented by mean ±SEM analysed by Two-way ANOVA. Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01***p ≤ 0.001, **** p ≤ 0.0001).

4. Discussion

Our previous results have shown that asip1 Tg animals exhibit a subordinate/reactive phenotype, as demonstrated in dyadic fights (Rocha et al., 2023). This phenotype correlates well with the high cortisol levels (Rocha et al., 2023) that are commonly exhibited by subordinate fish (Pottinger & Carrick, 1999; Øverli et al., 2002). In addition, our preliminary studies have also suggested that *asip1-Tg* have low central 5HT levels (Guillot et al., 2016), which is a hallmark of subordinate behaviour in (Winberg & Thörnqvist, 2016). All these data suggest that *asip1-Tg* animals might exhibit anxiety-like behaviour induced by melanocortin antagonists and mediated by the central serotoninergic system. To further investigate this hypothesis, we developed several standard behavioural tests focused on revealing anxiety-like behaviour in fish. We first examine the exploratory capacity of *asip1-Tg* animals by examining locomotor patterns in the open field and new object tests (Stewart et al., 2012), as it has been shown to be impaired in the reactive/subordinate animals (Godwin et al., 2012). The open field and novel object tests have been suggested as good indicators of anxiety-like behaviour in fish (Godwin et al., 2012) and their efficacy in measuring benzodiazepine- and 5HT 1A receptor agonist-induced anxiety has been well established (Prut & Belzung, 2003). The increased freezing behaviour during exposure to a novel environment in *asip1-Tg* fish suggests that these animals exhibit increased anxiety-like behaviour. It is also significant that freezing behaviour increases in response to the alarm pheromone in studies of anxiety-related behaviour in zebrafish (Speedie & Gerlai, 2008) . Increased freezing time is also associated with faster swimming in anxious animals, but the total distance travelled was similar (Godwin et al., 2012). Accordingly, we found that *asip1-Tg* moved faster and had a higher entry speed in the central zone of the test tank than WT animals. Since the distance travelled is similar in both genotypes, we believe that the thigmotaxis response is not affected

by *asip1* overexpression, yet it induces a strong anxiogenic effect, as evidenced by the weight on more related anxiety variables, i.e. freezing time and swimming speed. The low edge movement time could be the result of the extensive immobilisation/freezing time of *asip1-Tg* in the peripheral part of the test tank. Similar results were obtained in goldfish after ICV administration of the MC4R chemical antagonist HS024 (Watanabe et al., 2021). This result has been interpreted as a reversal effect of the antagonist on the anxiogenic effect of centrally administered α-MSH (Watanabe et al., 2021), but assessing only time spent in the peripheral area does not seem to fully predict anxiety-like behaviour in fish (Prut & Belzung, 2003; Godwin et al., 2012; Johnson et al., 2023). When a new object was introduced into the central area of the test tank after the animals had already adapted to the new environment, most of the asip1 Tg animals failed to approach the object again. This cautious/fearful behaviour also indicates and reinforces anxiety-like responses in transgenic animals (Johnson et al., 2023).

The NTDT has been shown to be the most sensitive test for assessing anxiety-like behaviour in fish (Cachat et al., 2010; Cachat et al., 2011; Egan et al., 2009; Kysil et al., 2017; Stewart et al., 2012), and the results correlate well with those of the open field test (Godwin et al., 2012; Stewart et al., 2012; Johnson et al., 2023). Our findings in the NTDT confirmed the increased anxiety-like behaviour in the fish. The higher mean and angular velocity in the upper area, as well as the increased acceleration all around, are indicative of erratic movements that are fully compatible with anxiety-like behaviour (Quadros et al., 2016; Volgin et al., 2019). These results, together with the increased latency to visit the potentially dangerous upper area of the tank, highlight the anxiogenic profile of *asip1-Tg* (Egan et al., 2009; Cachat et al., 2010; Cachat et al., 2011). The anxiogenic effect of the transgene is confirmed by the increase in freezing episodes, which is associated with less travelling spent time in the bottom region, but also with a longer period of adaptation to the new conditions of the

assip1-Tg animals. The results are in good agreement with those obtained in other genetic backgrounds showing high levels of anxiety, such as albino, TL or leopard zebrafish (Egan et al., 2009; Maximino et al., 2013a). Similar to *asip1-Tg*, leptin receptor knockout zebrafish exhibits enhanced food intake levels but opposite behavioural response since *lepa* KO zebrafish display hyperactivity and depressed freezing behaviour (Audira et al., 2018). Interestingly, these mutants had low serotonin levels, albeit high dopamine and cortisol levels (Audira et al., 2018).

Our next question addressed the potential mechanism by which melacortinergic pathways modulate behavioural response. Preliminary data suggest that *asip1* overexpression regulates central monoamine metabolism (Guillot et al., 2016), which is implicated in anxiety disorders (Albert & Benkelfat, 2013; Blier & El-Mansari, 2013; Winberg & Thörnqvist, 2016; Soares et al., 2018; Strekalova et al., 2021). Both central 5HT and dopamine levels were depressed in *asip1-Tg* animals, confirming our previous findings (Guillot et al., 2016). Intriguingly, we did not find any differences in the monoamine precursors, i.e. L-Dopa and 5HTP. This suggested to us that the common enzyme in the monoamine synthetic pathways, i.e. AADC (Verbeek et al., 2007), might be dysregulated, however the expression levels of *aadc* were similar in both genotypes. On the contrary, the levels of 5HT (5HIAA) and dopamine (DOPAC) metabolites and turnover rates (5HIAA/5HT and DOPAC/dopamine) were increased in the zebrafish brain, suggesting that the reduced monoamine levels may be the result of increased monoamine reuptake by their respective transporters, i.e. SERT and DAT, and concomitant neurotransmitter degradation (Lillesaar, 2011; Kacprzak et al., 2017). In leopard zebrafish, low 5HT levels and high neurotransmitter turnover ratio were correlated to increased bottom-dwelling in NTDT, demonstrating the relationship between anxiety-like behaviour and serotonin turnover. (Maximino et al., 2013b;c). Studies in hierarchical populations of salmonids have further shown that once dominance is established, 5HIAA/5HT ratios remain elevated in subordinate

fish, whereas they return to basal levels in dominant fish. Indeed, in groups of juvenile salmonids, there is a correlation between social status and brain 5HT turnover ratio, with the highest levels found in subordinate individuals (Winberg & Thörnqvist, 2016). Furthermore, 5HT brain levels have been shown to decline in subordinate salmonids after long-term intense social interactions (Cubitt et al., 2008). Not only 5HT metabolism has been shown to influence anxiety-like behaviour, as central dopamine levels are critical in coping with stress (Cabib & Puglisi-Allegra, 2012). In fish, subordinate salmonids have also been shown to have an increased dopamine turnover rate and high cortisol levels (Øverli et al., 1999). However, *dat* knockout zebrafish exhibited increased bottom-dwelling and thigmotaxis, suggesting enhanced anxiety-like behaviour, as observed with ageing and after cocaine administration (Kacprzak et al., 2017).

Next, we treated our transgenic animals with the SSRI (fluoxetine) to increase serotoninergic synaptic transmission by inhibiting 5HT reuptake and rescue the behavioural phenotype. Fluoxetine has been widely shown to be an anxiolytic in vertebrates (Belujon & Grace, 2017; Blier & El-Mansari, 2013; Sohel et al., 2022). In zebrafish, fluoxetine treatment reduces erratic movements and latency to visit the upper zone of the test tank in NTDT and increases the time spent in the latter area. It is also able to reverse the anxiety-like phenotype in leopard zebrafish (Egan et al., 2009; Maximino et al., 2013a) and prevent the stress response in adult zebrafish (de Abreu et al., 2014; Giacomini et al., 2016). We examined anxiety-like behaviour in treated fish using NTDT and showed that fluoxetine was able to reverse several anxiety-like behaviour parameters in *asip1-Tg* to levels similar to those found in WT animals. These results suggest that the effects of *asip1* overexpression on anxiety-like behaviour may be mediated through serotoninergic pathways. Indeed, fluoxetine treatment reduced the 5HIAA/5HT ratio, yet no effect on total 5HT levels was observed, indicating that fluoxetine reduces serotonin reuptake, thereby increasing

serotonergic neurotransmission, which in turn induces the observed behavioural changes (Sinyakova et al., 2018). No changes were observed in the dopaminergic neurotransmission further supporting a specific key role of the serotoninergic pathway.

How melanocortinergic signalling reaches 5HT-dependent behavioural pathways is unknown. The encephalic distribution of tryptophan hydroxylases (Tph1 and Tph2) has been studied in several vertebrate species, including zebrafish (Panula et al., 2010; Gaspar & Lillesaar, 2012). In mammalian species, peripheral tissues use Tph1 for 5HT synthesis, whereas Tph2 is essentially central and its expression is restricted to raphe neurons in the brainstem. Therefore, all behavioural effects of 5HT are modulated in the raphe. In fish, both *tphs* are expressed in the encephalon, but only *tph2* is expressed in the raphe (Chivite et al., 2021). *tph2* is further expressed in the pretectal area and the ventral thalamus. In contrast, *tph1* expression is restricted to diencephalic areas, including the caudal hypothalamus and the posterior tuberculum, both of which are densely innervated by Agrp1 and α-MSH fibres in zebrafish (Forlano & Cone, 2007), but have also been shown to express both *mc4r* (Cerdá-Reverter et al., 2003c) and *mc5r* (Cerdá-Reverter et al., 2003b) in goldfish. The presence of melanocortinergic fibres, either Agrp1 and/or α-MSH, in the zebrafish raphe appears to be very reduced but still present (Forlano & Cone, 2007) but no expression of *mc4r* and *mc5r* was reported (Cerdá-Reverter et al., 2003b; c). Unfortunately, the serotoninergic pathways involved in the regulation of fish behaviour, and in particular anxiety-like behaviour, are poorly understood and our future studies will focus the morphofunctional dissection of the serotonin-dependent behavioural pathways of the zebrafish brain and the interaction with the melanocortinergic system.

In conclusion, we show that the reduction of melanocortinergic signalling by ubiquitous overexpression of the endogenous antagonist asip1 has a potent anxiogenic effect that seems to be dependent of the modulation of serotoninergic pathways as

indicated by a severe increase of serotonin turnover (5HIAA/5HT ratio). The behavioural phenotype can be rescued by the oral treatment with SSRIs which induce a decrease of 5HIAA/5HT ratio and by extension of the serotoninergic function.

Author Contributions

A.G.-G.: conceptualization, methodology, validation, formal and statistical analysis, investigation, data curation, visualization, writing—original draft, reviewing and editing. A.R: supervision, reviewing and editing. MC: methodology, reviewing and editing. JR: resources, reviewing and editing. JMM: supervision, reviewing and editing. J.M.C.-R.: conceptualization, methodology, validation, funding acquisition, investigation, supervision, writing—original draft, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

All experiments were performed following guidelines of the Spanish (Royal Decree 53/2013) and the European Union Directive on the Protection of Animals Used for Scientific Purposes (Directive 2010/63/EU). The protocols applied were approved by IATS Ethics Committee (Register Number 09-0201) under the supervision of the

Secretary of State for Research, Development and Innovation of the Spanish Government.

Conflicts of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Agulleiro, M. J., Sánchez, E., Leal, E., Corté, R., Fernández-Durán, B., Guillot, R., Davis, P., Dores, R. M., Gallo-Payet, N., Cerdá -Reverter, J. M., & Fuentes, J. (2013). Molecular Characterization and Functional Regulation of Melanocortin 2 Receptor (MC2R) in the Sea Bass. A Putative Role in the Adaptation to Stress. *Molecular Endocrinology* 27:1934-1945. https://doi.org/10.1371/journal.pone.0065450

Albert, P. R., & Benkelfat, C. (2013). The neurobiology of depression-revisiting the serotonin hypothesis. II. Genetic, epigenetic and clinical studies. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1615). https://doi.org/10.1098/rstb.2012.0535

Alsop, D., & Vijayan, M. M. (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *294*(3). https://doi.org/10.1152/AJPREGU.00671.2007

Audira, G., Sarasamma, S., Chen, J. R., Juniardi, S., Sampurna, B. P., Liang, S. T., Lai, Y. H., Lin, G. M., Hsieh, M. C., & Hsiao, C. Der. (2018). Zebrafish Mutants Carrying Leptin a (lepa) Gene Deficiency Display Obesity, Anxiety, Less Aggression and Fear, and Circadian Rhythm and Color Preference Dysregulation. *International Journal of Molecular Sciences*, *19*(12), 4038. https://doi.org/10.3390/IJMS19124038

Belujon, P., & Grace, A. A. (2017). Dopamine system dysregulation in major depressive disorders. *International Journal of Neuropsychopharmacology*, *20*(12), 1036–1046. https://doi.org/10.1093/IJNP/PYX056

Blier, P., & El-Mansari, M. (2013). Serotonin and beyond: Therapeutics for major depression. In *Philosophical Transactions of the Royal Society B: Biological Sciences* (Vol. 368, Issue 1615). Royal Society. https://doi.org/10.1098/rstb.2012.0536

Cabib, S., & Puglisi-Allegra, S. (2012). The mesoaccumbens dopamine in coping with stress. *Neuroscience and Biobehavioral Reviews*, *36*(1), 79–89. https://doi.org/10.1016/J.NEUBIOREV.2011.04.012

Cachat, J. M., Canavello, P. R., Elegante, M. F., Bartels, B. K., Elkhayat, S. I., Hart, P. C., Tien, A. K., Tien, D. H., Beeson, E., Mohnot, S., Laffoon, A. L., Stewart, A. M., Gaikwad, S., Wong, K., Haymore, W., & Kalueff, A. V. (2011). Modeling stress and anxiety in zebrafish. *Neuromethods*, *52*, 73–88. https://doi.org/10.1007/978-1-60761-922-2_3

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., … Kalueff, A. V. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols*, *5*(11), 1786–1799. https://doi.org/10.1038/nprot.2010.140

Ceinos, R. M., Guillot, R., Kelsh, R. N., Cerdá-Reverter, J. M., & Rotllant, J. (2015). Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms. *Pigment Cell & Melanoma Research*, *28*(2), 196–209. https://doi.org/10.1111/pcmr.12335

Cerdá-Reverter, J. M., Agulleiro, M. J., Guillot R, R., Sánchez, E., Ceinos, R., & Rotllant, J. (2011). Fish melanocortin system. *European Journal of Pharmacology*, *660*(1), 53–60. https://doi.org/10.1016/j.ejphar.2010.10.108

Cerdá-Reverter, J. M., Haitina, T., Schiöth, H. B., & Peter, R. E. (2005). Gene structure of the goldfish agouti-signaling protein: A putative role in the dorsal-ventral pigment pattern of fish. *Endocrinology*, *146*(3), 1597–1610. https://doi.org/10.1210/EN.2004-1346

Cerdá-Reverter, J. M., Ling, M. K., Schiöth, H. B., & Peter, R. E. (2003). Molecular cloning, characterization and brain mapping of the melanocortin 5 receptor in the goldfish. *Journal of Neurochemistry*, *87*(6), 1354–1367. https://doi.org/10.1046/J.1471-4159.2003.02107.X

Cerdá-Reverter, J. M., & Peter, R. E. (2003). Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-

related protein gene. *Endocrinology*, *144*(10), 4552–4561. https://doi.org/10.1210/EN.2003-0453

Cerdá-Reverter, J. M., Ringholm, A., Schiöth, H. B., & Peter, R. E. (2003). Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: Involvement in the control of food intake. *Endocrinology*, *144*(6), 2336–2349. https://doi.org/10.1210/en.2002-0213

Cerdá-Reverter, J. M., Schiöth, H. B., & Peter, R. E. (2003). The central melanocortin system regulates food intake in goldfish. *Regulatory Peptides*, *115*(2), 101–113. https://doi.org/10.1016/S0167-0115(03)00144-7

Chen, W., Kelly, M. A., Opitz-Araya, X., Thomas, R. E., Low, M. J., & Cone, R. D. (1997). Exocrine Gland Dysfunction in MC5-R-Deficient Mice: Evidence for Coordinated Regulation of Exocrine Gland Function by Melanocortin Peptides. In *Cell* (Vol. 91).

Chivite, M., Leal, · Esther, Míguez, J. M., Jose, ·, & Cerdá-Reverter, M. (2021). Distribution of two isoforms of tryptophan hydroxylase in the brain of rainbow trout (Oncorhynchus mykiss). An in situ hybridization study. *Brain Structure and Function*, *226*, 2265–2278. https://doi.org/10.1007/s00429-021-02322-8

Chivite, M., Naderi, F., Conde-Sieira, M., Soengas, J. L., Lopez-Patiño, M. A., & Míguez, J. M. (2021). Central serotonin participates in the anorexigenic effect of GLP-1 in rainbow trout (Oncorhynchus mykiss). *General and Comparative Endocrinology*, *304*. https://doi.org/10.1016/j.ygcen.2021.113716

Cone, R. D. (2006). Studies on the physiological functions of the melanocortin system. *Endocrine Reviews*, *27*(7), 736–749. https://doi.org/10.1210/ER.2006-0034

Cortés, R., Agulleiro, M. J., Navarro, S., Guillot, R., Sánchez, E., & Cerdá-Reverter, J. M. (2014). Melanocortin receptor accessory protein 2 (MRAP2) interplays with the zebrafish melanocortin 1 receptor (MC1R) but has no effect on its pharmacological profile. *General and Comparative Endocrinology*, *201*, 30–36. https://doi.org/10.1016/J.YGCEN.2014.03.009

Cubitt, K. F., Winberg, S., Huntingford, F. A., Kadri, S., Crampton, V. O., & Øverli, Ø. (2008). Social hierarchies, growth and brain serotonin metabolism in Atlantic salmon (Salmo salar) kept under commercial rearing conditions. *Physiology & Behavior*, *94*(4), 529–535. https://doi.org/10.1016/J.PHYSBEH.2008.03.009

de Abreu, M. S., Koakoski, G., Ferreira, D., Oliveira, T. A., da Rosa, J. G., Abreu, M. S. de, Koakoski, G., Ferreira, D., Oliveira, T. A., Rosa, J. G. S. da, Gusso, D., Giacomini, A. C. V., Piato, A. L., & Barcellos, L. J. G. (2014). Diazepam and Fluoxetine Decrease the Stress Response in Zebrafish. *PLoS ONE*, *9*(7), e103232. https://doi.org/10.1371/journal.pone.0103232

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., Elkhayat, S. I., Bartels, B. K., Tien, A. K., Tien, D. H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., & Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research*, *205*(1), 38–44. https://doi.org/10.1016/j.bbr.2009.06.022

Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., & Cone, R. D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*, *385*(6612), 165–168. https://doi.org/10.1038/385165A0

Forlano, P. M., & Cone, R. D. (2007). Conserved Neurochemical Pathways Involved in Hypothalamic Control of Energy Homeostasis. *J. Comp. Neurol*, *505*, 235–248. https://doi.org/10.1002/cne.21447

Gaspar P., & Lillesaar C. (2012) Probing the diversity of serotonin neurons. Philos Trans R Soc Lond B Biol Sci 367:2382-94. https://doi.org/10.1098/rstb.2011.0378

Giacomini, A. C. V. V., Abreu, M. S., Giacomini, L. V., Siebel, A. M., Zimerman, F. F., Rambo, C. L., Mocelin, R., Bonan, C. D., Piato, A. L., & Barcellos, L. J. G. (2016). Fluoxetine and diazepam acutely modulate stress induced-behavior. *Behavioural Brain Research*, *296*, 301–310. https://doi.org/10.1016/j.bbr.2015.09.027

Godino-Gimeno, A., Sánchez, E., Guillot, R., Rocha, A., Angotzi, A. R., Leal, E., Rotllant, J., & Cerdá-Reverter, J. M. (2020). Growth Performance after Agouti-Signaling Protein 1 (Asip1) Overexpression in Transgenic Zebrafish. *Zebrafish*, *17*(6), 373–381. https://doi.org/10.1089/zeb.2020.1932

Godwin, J., Sawyer, S., Perrin, F., Oxendine, S. E., & Kezios, Z. D. (2012). *Adapting the Open Field Test to Assess Anxiety-Related Behavior in Zebrafish* (pp. 181–189). https://doi.org/10.1007/978-1-61779-597-8_13

Guillot, R., Cortés, R., Navarro, S., Mischitelli, M., García-Herranz, V., Sánchez, E., Cal, L., Navarro, J. C., Míguez, J. M., Afanasyev, S., Krasnov, A., Cone, R. D., Rotllant, J., & Cerdá-Reverter, J. M. (2016). Behind melanocortin antagonist overexpression

in the zebrafish brain: A behavioral and transcriptomic approach. *Hormones and Behavior*, *82*, 87–100. https://doi.org/10.1016/j.yhbeh.2016.04.011

Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P., & Frank, L. (1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell*, *88*(1), 131–141. https://doi.org/10.1016/S0092-8674(00)81865-6

Johnson, A., Loh, E., Verbitsky, R., Slessor, J., Franczak, B. C., Schalomon, M., & Hamilton, T. J. (2023). Examining behavioural test sensitivity and locomotor proxies of anxiety-like behaviour in zebrafish. *Scientific Reports*, *13*(1). https://doi.org/10.1038/S41598-023-29668-9

Kacprzak, V., Patel, N. A., Riley, E., Yu, L., Yeh, J.-R. J., & Zhdanova, I. V. (2017). *Dopaminergic control of anxiety in young and aged zebrafish*. https://doi.org/10.1016/j.pbb.2017.01.005

Kysil, E. V., Meshalkina, D. A., Frick, E. E., Echevarria, D. J., Rosemberg, D. B., Maximino, C., Lima, M. G., Abreu, M. S., Giacomini, A. C., Barcellos, L. J. G., Song, C., & Kalueff, A. V. (2017). Comparative Analyses of Zebrafish Anxiety-Like Behavior Using Conflict-Based Novelty Tests. *Zebrafish*, *14*(3), 197–208. https://doi.org/10.1089/zeb.2016.1415

Lampert, K. P., Schmidt, C., Fischer, P., Volff, J. N., Hoffmann, C., Muck, J., Lohse, M. J., Ryan, M. J., & Schartl, M. (2010). Determination of onset of sexual maturation and mating behavior by melanocortin receptor 4 polymorphisms. *Current Biology : CB*, *20*(19), 1729–1734. https://doi.org/10.1016/J.CUB.2010.08.029

Leal, E., Angotzi, A. R., Godino-Gimeno, A., Gregório, S. F., Rotllant, J., Saera-Vila, A., Fuentes, J., & Cerdá-Reverter, J. M. (2022). Agouti overexpression in a transgenic model regulates integrity, permeability and electrogenic amino acid transport in zebrafish intestine. *Frontiers in Marine Science*, *9*. https://doi.org/10.3389/fmars.2022.1011702

Lillesaar, C. (2011). The serotonergic system in fish. *Journal of Chemical Neuroanatomy*, *41*, 294–308. https://doi.org/10.1016/j.jchemneu.2011.05.009

Liu, R., Du, K., Ormanns, J., Adolfi, M. C., & Schartl, M. (2020). Melanocortin 4 receptor signaling and puberty onset regulation in Xiphophorus swordtails. *General*

and Comparative Endocrinology, *295*. https://doi.org/10.1016/J.YGCEN.2020.113521

Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., Wilkison, W. O., & Cone, R. D. (1994). Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature 1994 371:6500*, *371*(6500), 799–802. https://doi.org/10.1038/371799a0

Maximino, C., Araujo, J., Matos Oliveira, K. R., & Manoel Herculano, A. (2013c). *The Serotonergic System of Zebrafish: Genomics, Neuroanatomy and Neuropharmacology*. https://www.researchgate.net/publication/257931575

Maximino, C., Puty, B., Benzecry, R., Araújo, J., Lima, M. G., De Jesus Oliveira Batista, E., Renata De Matos Oliveira, K., Crespo-Lopez, M. E., & Herculano, A. M. (2013b). Role of serotonin in zebrafish (Danio rerio) anxiety: Relationship with serotonin levels and effect of buspirone, WAY 100635, SB 224289, fluoxetine and para-chlorophenylalanine (pCPA) in two behavioral models. *Neuropharmacology*, *71*, 83–97. https://doi.org/10.1016/j.neuropharm.2013.03.006

Maximino, C., Puty, B., Matos Oliveira, K. R., & Herculano, A. M. (2013a). Behavioral and neurochemical changes in the zebrafish leopard strain. *Genes, Brain, and Behavior*, *12*(5), 576–582. https://doi.org/10.1111/GBB.12047

Michaud, E. J., Bultman, S. J., Stubbs, L. J., & Woychik, R. P. (1993). The embryonic lethality of homozygous lethal yellow mice (A(y)/A(y)) is associated with the disruption of a novel RNA-binding protein. *Genes and Development*, *7*(7 A), 1203–1213. https://doi.org/10.1101/gad.7.7a.1203

Miller, M. W., Duhl, D. M. J., Vrieling, H., Cordes, S. P., Ollmann, M. M., Winkes, B. M., & Barsh, G. S. (1993). Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. *Genes & Development*, *7*(3), 454–467. https://doi.org/10.1101/GAD.7.3.454

Mondanelli, G., & Volpi, C. (2021). The double life of serotonin metabolites: in the mood for joining neuronal and immune systems. *Current Opinion in Immunology*, *70*, 1–6. https://doi.org/10.1016/J.COI.2020.11.008

Mountjoy, K. G. (2015). Pro-Opiomelanocortin (POMC) Neurones, POMC-Derived Peptides, Melanocortin Receptors and Obesity: How Understanding of this System has Changed Over the Last Decade. *Journal of Neuroendocrinology*, *27*(6), 406–418. https://doi.org/10.1111/JNE.12285

Navarro, S., Guillot, R., Crespo, D., Schulz, R. W., Ge, W., Rotllant, J., Cerdá-Reverter, J. M., & Rocha, A. (2021). Enhanced growth without accelerated puberty in fish: A role for the melanocortin system. *Aquaculture*, *540*, 736721. https://doi.org/10.1016/J.AQUACULTURE.2021.736721

Navarro, S., Soletto, L., Puchol, S., Rotllant, J., Soengas, J. L., & Cerdá-Reverter, J. M. (2016). 60 YEARS OF POMC: POMC: an evolutionary perspective. *Journal of Molecular Endocrinology*, *56*(4), T113–T118. https://doi.org/10.1530/JME-15-0288

Nijenhuis, W. A. J., Oosterom, J., & Adan, R. A. H. (2001). AgRP(83-132) acts as an inverse agonist on the human-melanocortin-4 receptor. *Molecular Endocrinology (Baltimore, Md.)*, *15*(1), 164–171. https://doi.org/10.1210/MEND.15.1.0578

Ollmann, M. M., Wilson, B. D., Yang, Y. K., Kerns, J. A., Chen, Y., Gantz, I., & Barsh, G. S. (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science (New York, N.Y.)*, *278*(5335), 135–138. https://doi.org/10.1126/SCIENCE.278.5335.135

Øverli, Ø., Harris, C. A., & Winberg, S. (1999). Short-Term Effects of Fights for Social Dominance and the Establishment of Dominant-Subordinate Relationships on Brain Monoamines and Cortisol in Rainbow Trout. *Brain Behavior and Evolution*, *54*(5), 263–275. https://doi.org/10.1159/000006627

Øverli, Ø., Pottinger, T. G., Carrick, T. R., Øverli, E., & Winberg, S. (2002). Differences in behaviour between rainbow trout selected for high- and low-stress responsiveness. *Journal of Experimental Biology*, *205*(3), 391–395. https://doi.org/10.1242/jeb.205.3.391

Pottinger, T. G., & Carrick, T. R. (1999). A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Aquaculture*, *175*(3–4), 351–363. https://doi.org/10.1016/S0044-8486(99)00107-6

Prut, L., & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. In *European Journal of Pharmacology* (Vol. 463, Issues 1–3, pp. 3–33). Elsevier. https://doi.org/10.1016/S0014-2999(03)01272-X

Quadros, V. A., Silveira, A., Giuliani, G. S., Didonet, F., Silveira, A. S., Nunes, M. E., Silva, T. O., Loro, V. L., & Rosemberg, D. B. (2016). Strain- and context-dependent

behavioural responses of acute alarm substance exposure in zebrafish. *Behavioural Processes*, *122*, 1–11. https://doi.org/10.1016/j.beproc.2015.10.014

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L., & Schreck, C. B. (2009). Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture (Amsterdam, Netherlands)*, *297*(1–4), 157–162. http://www.ncbi.nlm.nih.gov/pubmed/25587201

Rocha, A., Godino-Gimeno, A., & Cerdá-Reverter, J. M. (2019). Evolution of proopiomelanocortin. *Vitamins and Hormones*, *111*, 1–16. https://doi.org/10.1016/BS.VH.2019.05.008

Rocha, A., Godino-Gimeno, A., Rotllant, J., & Cerdá-Reverter, J. M. (2023). Agouti-Signalling Protein Overexpression Reduces Aggressiveness in Zebrafish. *Biology*, *12*(5), 712. https://doi.org/10.3390/biology12050712

Sánchez, E., Rubio, V. C., Thompson, D., Metz, J., Flik, G., Millhauser, G. L., & Cerdá-Reverter, J. M. (2009). Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (Dicentrarchus labrax). *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *296*(5). https://doi.org/10.1152/AJPREGU.90948.2008

Schiöth, H. B., Haitina, T., Ling, M. K., Ringholm, A., Fredriksson, R., Cerdá-Reverter, J. M., & Klovins, J. (2005). Evolutionary conservation of the structural, pharmacological, and genomic characteristics of the melanocortin receptor subtypes. *Peptides*, *26*(10), 1886–1900. https://doi.org/10.1016/J.PEPTIDES.2004.11.034

Shainer, I., Michel, M., Marquart, G. D., Bhandiwad, A. A., Zmora, N., Ben-Moshe Livne, Z., Zohar, Y., Hazak, A., Mazon, Y., Förster, D., Hollander-Cohen, L., Cone, R. D., Burgess, H. A., & Gothilf, Y. (2019). Agouti-Related Protein 2 Is a New Player in the Teleost Stress Response System. *Current Biology : CB*, *29*(12), 2009-2019.e7. https://doi.org/10.1016/J.CUB.2019.05.021

Sinyakova, N. A., Kulikova, E. A., Englevskii, N. A., & Kulikov, A. V. (2018). Effects of Fluoxetine and Potential Antidepressant 8-Trifluoromethyl 1,2,3,4,5-Benzopentathiepin-6-Amine Hydrochloride (TC-2153) on Behavior of Danio rerio Fish in the Novel Tank Test and Brain Content of Biogenic Amines and Their Metabolites. *Bulletin of Experimental Biology and Medicine*, *164*(5), 620–623. https://doi.org/10.1007/S10517-018-4045-6

Soares, M. C., Gerlai, R., & Maximino, C. (2018). The integration of sociality, monoamines and stress neuroendocrinology in fish models: applications in the neurosciences. *Journal of Fish Biology*, *93*(2), 170–191. https://doi.org/10.1111/jfb.13757

Sohel, A. J., Shutter, M. C., & Molla, M. (2022). Fluoxetine. *XPharm: The Comprehensive Pharmacology Reference*, 1–9. https://doi.org/10.1016/B978-008055232-3.61768-4

Soletto, L., Hernández-Balfagó, S., Rocha, A., Scheerer, P., Kleinau, G., & Cerdá-Reverter, J. M. (2019). *Melanocortin Receptor Accessory Protein 2-Induced Adrenocorticotropic Hormone Response of Human Melanocortin 4 Receptor*. https://doi.org/10.1210/js.2018-00370

Song, Y., & Cone, R. D. (2007). Creation of a genetic model of obesity in a teleost. *The FASEB Journal*, *21*(9), 2042–2049. https://doi.org/10.1096/FJ.06-7503COM

Speedie, N., & Gerlai, R. (2008). Alarm substance induced behavioral responses in zebrafish (Danio rerio). *Behavioural Brain Research*, *188*(1), 168–177. https://doi.org/10.1016/j.bbr.2007.10.031

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., & Kalueff, A. V. (2012). Modeling anxiety using adult zebrafish: A conceptual review. *Neuropharmacology*, *62*(1), 135. https://doi.org/10.1016/J.NEUROPHARM.2011.07.037

Stewart, A. M., Gaikwad, S., Kyzar, E., & Kalueff, A. V. (2012). *Understanding spatiotemporal strategies of adult zebrafish exploration in the open field test*. https://doi.org/10.1016/j.brainres.2012.02.064

Strekalova, T., Svirin, E., Waider, J., Gorlova, A., Cespuglio, R., Kalueff, A., Pomytkin, I., Schmitt-Boehrer, A. G., Lesch, K. P., & Anthony, D. C. (2021). Altered behaviour, dopamine and norepinephrine regulation in stressed mice heterozygous in TPH2 gene. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *108*. https://doi.org/10.1016/J.PNPBP.2020.110155

Thörnqvist, P.-O., Mccarrick, S., Ericsson, M., Roman, E., & Winberg, S. (2019). *Bold zebrafish (Danio rerio) express higher levels of delta opioid and dopamine D2 receptors in the brain compared to shy fish*. https://doi.org/10.1016/j.bbr.2018.06.017

Tolle, V., & Low, M. J. (2008). In vivo evidence for inverse agonism of Agouti-related peptide in the central nervous system of proopiomelanocortin-deficient mice. *Diabetes*, *57*(1), 86–94. https://doi.org/10.2337/DB07-0733

Verbeek, M. M., Geurtz, P. B. H., Willemsen, M. A. A. P., & Wevers, R. A. (2007). Aromatic l-amino acid decarboxylase enzyme activity in deficient patients and heterozygotes. *Molecular Genetics and Metabolism*, *90*(4), 363–369. https://doi.org/10.1016/J.YMGME.2006.12.001

Volgin, A. D., Yakovlev, O. A., Demin, K. A., Alekseeva, P. A., & Kalueff, A. V. (2019). Acute behavioral effects of deliriant hallucinogens atropine and scopolamine in adult zebrafish. *Behavioural Brain Research*, *359*, 274–280. https://doi.org/10.1016/j.bbr.2018.10.033

Vrieling, H., Duhl, D. M. J., Millar, S. E., Miller, K. A., & Barsht, G. S. (1994). Differences in dorsal and ventral pigmentation result from regional expression of the mouse agouti gene. In *Proc. Natd. Acad. Sci. USA* (Vol. 91).

Watanabe, K., Konno, N., Nakamachi, T., & Matsuda, K. (2021). Intracerebroventricular administration of α-melanocyte-stimulating hormone (α-MSH) enhances thigmotaxis and induces anxiety-like behavior in the goldfish Carassius auratus. *Peptides*, *145*. https://doi.org/10.1016/J.PEPTIDES.2021.170623

Winberg, S., & Thörnqvist, P.-O. (2016). Role of brain serotonin in modulating fish behavior. *Current Zoology*, *62*(3), 317–323. https://doi.org/10.1093/CZ/ZOW037

Zhang, C. (2012). Elucidation of the roles of agouti related proteins in zebrafish. *Scholar Archive*, *755*. https://digitalcommons.ohsu.edu/etd/755

Supplementary materials

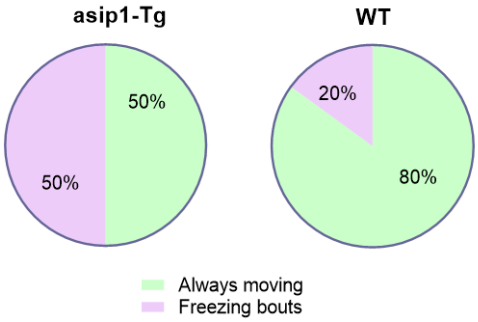


Figure S1: Open field test results.

The percentage of the *asip1-Tg* population that displayed freezing bouts was larger than WT fish.

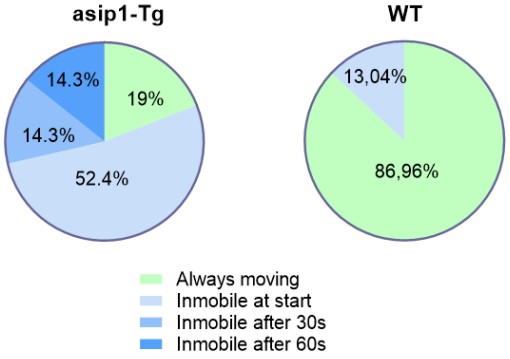
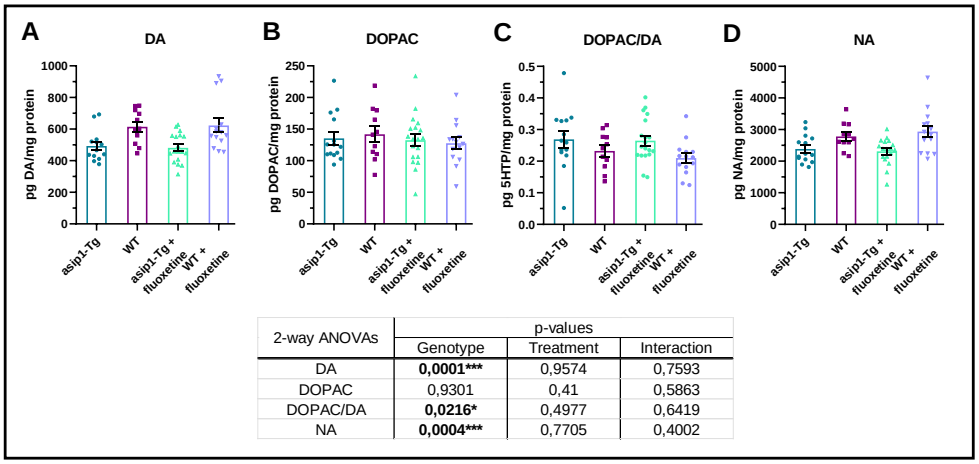


Figure S2: NTDT results.

The percentage of the *asip1-Tg* population that displayed freezing bouts was larger than WT fish, being more those transgenic fish that were in freezing since the beginning of the test.



2-way ANOVAs	p-values		
	Genotype	Treatment	Interaction
DA	**0,0001*** **	0,9574	0,7593
DOPAC	0,9301	0,41	0,5863
DOPAC/DA	**0,0216***	0,4977	0,6419
NA	**0,0004*** **	0,7705	0,4002

Figure S3: Brain dopaminergic biosynthesis pathways metabolites and their degradation ratio, comparison between genotypes after chronic treatment with fluoxetine (*asip1-Tg* + fluoxetine, WT + fluoxetine) and control non-treated fish (*asip1-Tg*, WT).

A) Levels of DA were significantly different only according to genotype. B) DOPAC levels did not differ statistically between genotypes or due to treatment. C) The ratio of DOPAC/DA was significantly higher due to genotype; such differences were not found after treatment. D) NA levels were significantly different only according to genotype. Brains were dissected after NTDT and were analyzed by HPLC. Results are expressed in pg of metabolite per mg of total protein; data are represented by mean ±SEM analyzed by Two-way ANOVA. Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01***p ≤ 0.001, **** p ≤ 0.0001).

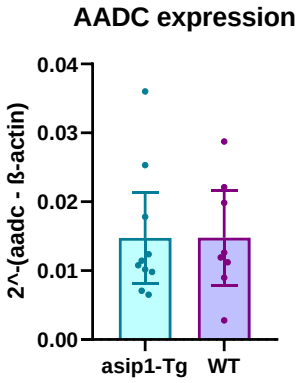


Figure S4: Comparison of aadc gene expression, an enzyme called aromatic l-amino acid decarboxylase necessary to transform 5HTP and L-dopa into serotonin and dopamine.

No differences were found between *asip1-Tg* and WT fish. Dissected brains (*asip1-Tg* n=10, WT n=8, sex ratio 50:50) were analyzed by qPCR. Results are normalized according to β-actin gene expression; data are represented by mean ±SEM analyzed by Unpaired t-test

CHAPTER 6

Role of the melanocortin system in the locomotor activity rhythms and melatonin secretion as revealed by agouti-signalling protein (asip1) overexpression in zebrafish.

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Abstract

Temporal traits such as light/temperature cycles modulate animal physiology and behaviour. The organism should be able to predict these cycle changes by reading environmental features to get better environmental fitness. In fish, the pineal gland, through the secretion of melatonin, appears to play a critical role in the circadian system, probably acting as the master clock. An important output of this circadian clock is the locomotor activity circadian rhythm LACR, which is adapted to the photoperiod and thus determines whether animals are diurnal or nocturnal. Using a transgenic zebrafish strain [Tg(Xla.Eef1a1:Cau.Asip1)iim04] overexpressing the agouti signalling protein 1 (asip1), an endogenous antagonist of the melanocortin system, we observed a complete disruption of the LACR, which correlates with the ablation of the melatonin daily rhythm. Consistent with this, in vitro experiments also showed that Asip1 inhibits melatonin secretion from the zebrafish pineal gland, most likely through the melanocortin receptors expressed in the gland. Asip1 overexpression also disrupted the central expression of clock genes, including per1a and clock1a, thereby masking circadian oscillation. Collectively, these results suggest that the melanocortin system plays an important role in modulating pineal physiology and, by extension, circadian organisation in zebrafish.

1. Introduction

Melanocortin peptides are processed from the complex precursor proopiomelanocortin (POMC), which is mainly synthesised in the pituitary gland. In mammals, two small neuronal populations in the arcuate nucleus of the hypothalamus and the nucleus of the solitary tract (NTS) of the medulla also produce POMC, which is processed to yield melanocyte-stimulating hormone (α-MSH) and β-endorphin (Cerdá-Reverter et al., 2011). Melanocortin peptides bind to five different G-coupled receptors (MC1R-MC5R). Subtype 2 only binds adrenocorticotropic hormone (ACTH), while the other four distinctively recognise the different MSH peptides (α-, β- and γ-MSH) (Cortés et al., 2014). MCRs mediate a wide range of functions, including skin/fur pigmentation (MC1R), energy balance (MC3R and MC4R) and stress response (MC2R) (Cerdá-Reverter et al., 2011). Atypically, melanocortin receptors are also regulated by endogenous antagonists called agouti-signalling protein (ASIP) and agouti-related protein (AGRP). Asip is mainly produced in the hair follicle, where it regulates the pigment synthesis in melanocytes by antagonising the effects of α-MSH on MC1R. Agrp is synthesised in the arcuate nucleus, inhibiting melanocortin signalling at MC3R and MC4R to promote food intake (Cone, 2006).

The teleost-specific genome duplication (TSGD) promoted the emergence of paralogous genes for *asip* (*asip1* and *asip*2) and *agrp* (*agrp1* and *agrp*2), although the evolutionary history of the agouti family is controversial (Cortés et al., 2014; Guillot et al., 2016). *asip1* is mainly expressed in the ventral skin (Cerdá-Reverter et al., 2005) to regulate the dorsoventral pigment pattern (Ceinos et al., 2015; Cal et al., 2019) whereas *agrp1* is expressed in the lateral tuberal nucleus of the hypothalamus (Cerdá-Reverter & Peter, 2003) a homologue of the mammalian arcuate nucleus (Cerdá-Reverter & Canosa, 2009), and regulates food intake (Cerdá-Reverter & Peter, 2003; Shainer et al., 2019; Lin et al., 2023). *agrp*2 is expressed in the retinal pigment

epithelium-like (RPE) cells of the zebrafish pineal complex (Zhang et al., 2010; Shainer et al., 2019) and in a small neuronal subpopulation of the preoptic area, the homologous region of the mammalian paraventricular hypothalamic nucleus (PVN) (Herget et al., 2014). The pineal expression does not appear to be not involved in the regulation of circadian rhythms of locomotor activity (LA), yet preoptic expression appears to play a role in the stress response (Shainer et al., 2019). The function of *asip*2 remains unexplored, yet it is overexpressed in the skin of pseudo-albino flatfish (Pinto et al., 2019). Our recent studies have shown that zebrafish overexpressing *asip1* (*asip1-Tg*) exhibit hyperphagia and reduced satiety signalling (Guillot et al., 2016; Godino-Gimeno et al., 2020). *asip1-Tg* also exhibits behavioural disturbances, with transgenic animals exhibiting less aggressiveness than their wild-type counterparts (Rocha et al., 2023). Intriguingly, *asip1-Tg* shows reduced melatonin levels during scotophase (Guillot et al., 2016). This suggests that they may exhibit disrupted LA circadian rhythms, as blockade of the circadian oscillator in the melatonin-producing pineal photoreceptors disrupts LA rhythms (Ben-Moshe Livne et al., 2016), and ablation of melatonin synthesis in the pineal gland disrupts the circadian adjustment of the sleep-wake cycles (Gandhi et al., 2015). Our research shows that nocturnal melatonin secretion in *asip1-Tg* is severely disrupted, consistent with nocturnal hyperactivity locomotor patterns. Accordingly, daily rhythms of central serotonin (5-HT) and clock gene expression are also disrupted. Finally, we demonstrated that Asip1 dose-dependently inhibits *in vitro* melatonin secretion, thus supporting a role for the melanocortin system in pineal physiology.

2. Material and methods

2.1. Animals and housing conditions

Zebrafish WT and transgenic (*asip1-Tg*) belong to the TU strain (Nüsslein-Volhard Lab, Tuebingen, Germany). Transgenic line, [Tg(Xla.Eef1a1:Cau.Asip1]iim4 was developed previously by the Tol2 transposon system (Ceinos et al., 2015). Both WT and transgenic were bred and housed in a recirculating system designed for zebrafish at the Institute of Aquaculture of Torre de la Sal (IATS-CSIC) facilities. Water temperature was kept at 27.5 – 28ºC and pH at 7.5-8. At the facility, the photoperiod was 14L:10D, from 7 AM until 9 PM Fish were raised at a density of approximately 6 fish/L and 50:50 sex ratio and fed thrice daily with artemia and commercial flake food (Vipan, Sera, Heinsberg, Germany), which formulation met the nutritional requirements of zebrafish: 6.2% protein, 8.9% fat, 2.3% fibre, 11.9% ash, and 6.7% humidity.

Experiments were performed with adult zebrafish from each genotype 50:50 sex ratio (WT n=150, 0.24g±0.06; *asip1-Tg* n = 150 0.30g±0.11) kept at 1,6 fish/L density in a light- and sound-isolated room at 28ºC and 12h light (L)/12h darkness (D) (lights on at 9 AM and off at 9 PM). In all experiments, animals were acclimated to the experimental room for 30 days, where no access was allowed, except for feeding (1.5% of biomass) once a day at 11 AM.

The animals used in all experiments were naïve and free of any signs of disease. Experiments were carried out following the principles published in the European animal directive (86/ 609/EEC) for the protection of experimental animals and approved by the "Superior Council of Scientific Investigations" (CSIC) ethics committee (project number PID2019-103969RB-C33) and the "General Directorate of Agriculture, Livestock and Fishing of Valencia", registration number: nGVRTE/2021/217198.

2.2. Activity experiments

Locomotor activity (LA) was tested in triplicate, each replica consisted of two 6-litre tanks per genotype (WT n=10 fish, *asip1-Tg* n=10; 50:50 sex ratio) provided with two infrared sensors (E3ZD822, Omron) placed in the superior and inferior parts (Supplementary Fig. S1) connected to a computer system (AUTOMATA) that recorded sensor activations. No access to the experimental area was allowed, except for feeding (1.5% of biomass) once a day at 11 AM. After accommodation, LA was monitored for 28 consecutive days at the LD cycle, and data was integrated every 10 min. Subsequently, the LD cycle was inverted, and LA was screened for another 12 days, again, data was integrated every 10 min. LA was further monitored for 6 days under constant darkness conditions (DD) following the same experimental protocol. AUTOMATA software was used for data acquisition and integration.

2.3. Gene expression study

Fish of each genotype were distributed in twelve 6-litre tanks (6 tanks per genotype n = 10 fish per tank; 50:50 sex ratio) and acclimated to the experimental room at conditions previously described. After 30 days at LD cycle, one tank from each genotype (WT n=10; *asip1-Tg* n=10) was euthanised by an overdose of anaesthesia (MS222, tricaine methane sulfonate; 300 mg/L) for each sampling point every 4 hours starting at 9 AM and following 1 PM, 5 PM, 9 AM, 1 AM and 5 AM. Nocturnal samplings were done under dim red light. Animals were fasted for 24 hours prior to the sampling. Brains were dissected and rapidly placed in Tri-reagent (Invitrogen), homogenised and stored at -80ºC for RNA extraction following the manufacturer's instructions. Subsequently, total RNA was re-suspended in RNase-free water, quantified using a Nanodrop 2000 spectrophotometer, and stored at −80°C. Potential genomic DNA was removed with Turbo DNAse-RNAse-free (Thermo Scientific) according to the manufacturer's instructions. One microgram of DNA-free total RNA

was reverse transcribed using the SuperScript III reverse transcriptase (Thermo Scientific) and oligo (dT)12-18 (0.5 µg/ml), as indicated by the manufacturer. cDNA was diluted in nuclease-free water and stored at −20°C until use. Real-time PCR (qPCR) was performed in a CBX ConnectTM Real-Time System instrument (BioRad) using Abgene's SYBR® Green QPCR Master Mix (Thermo Scientific, Spain) in a total reaction volume of 20 µL (Thermo Scientific) and specific primers, previously optimised (Table 1), to evaluate the expression of period circadian clock 1a (*per1a*), clock circadian regulator 1a *clock1a*, cryptochrome circadian regulator 1a (*cry1a*), basic helix-loop-helix aryl hydrocarbon receptor nuclear translocator (ARNT) like 1a (*bmal1a*), nuclear receptor subfamily 1, group d, member 1 (*nr1d1*) and proopiomelanocortin a and b (*pomca* and *pomcb*). Thermal cycling conditions were 95ºC for 15 min, followed by 40 cycles of 95ºC for 15 s, annealing temperature (55ºC) for 30 s and 72ºC for 30 s; followed by 95ºC for 30 s, 55ºC for 30 s. The expression of target-specific genes was normalised to the relative expression of zebrafish *β-actin*, and fold change gene expression was performed using the Q-Gene application's mean normalised expression (MNE) method. Negative controls with no template were included in all the experiments. A melting curve for each PCR was determined by reading fluorescence every degree between 55°C and 95°C to ensure the specific amplification.

Gene	Forward primer sequence 5′ → 3′	Reverse primer sequence 5′ → 3′
β-actin	GATGAGGAATCGCTGCCCT	GTCCTTCTGTCCCATGCCAA
clock1a	CTGAGATCTCCGCAGACAAGT	TCGCTCTAGGGCTCCTT
bmal1	GACCCGTGGACTTCAGTGAC	TAGAGCGCTGTTTGCTGATG
cry1a	GGCTCCACGACAATCCTTCA	TGGGGAAGACATCGGTAGGT
per1a	GAATCTGGGGGAGATTCACA	GACAGAGATGCCCTGAAAGC
nr1d1	TGCAACAAGACAACAGCCTC	AGCCAGCAGAATCTCCTTGT
pomca	AAATGACCCATTTCCGCTGGAG	CCCACCTTCGTTTCTATGCATG
pomcb	AAACAACGGGAAGTATCGCATG	TCTGTGAACTGCTGTCCATTGC

Table 1: Oligonucleotide sequence 5′ → 3′ of forward and reverse primers designed for qPCR assay.

The reference gene is *β-actin*, while circadian clock genes are *clock1a, bmal1, cry1a, per1a and nr1d1*, and genes related to food intake control: *pomca* and *pomb*. Both forward and reverse primers were used at 10μM.

2.4. Pineal gland perfusion and treatments

Adult WT zebrafish (n=16) were acclimated to the experimental room at conditions previously described. After acclimation at the LD cycle, fish were euthanised) in the middle of the dark period by an overdose of anaesthesia (MS222, tricaine methane sulfonate; 300 mg/L and placed in an ice water bath and pineal gland dissection was carried out under a microscope and immediately transferred to oxygenated and sterile Hank's culture medium (140mM NaCl, 5mM KCl, 1mM $CaCl_2$, 4mM $MgSO_4$, 3mM NaH_2PO_4, 4mM KH2PO4, 4mM NaHCO3 and 0.5μM tryptophan, 6mM glucose, pH=7.4). The perfusion system was inside a thermostatic oven (25˚C) and consisted of a peristaltic pump (KD Scientific 780100V Syringe Infusion Pump) connected to a reduced volume (100 μL) chamber made from a plastic tube with silicone plugs at its ends, which were perforated in its central part to allow connection to the inlet and outlet tubing. A 0.45μM pore cellulose filter was included at the outlet end to prevent the pineal tissue from exiting the chamber. The chamber was filled with culture

medium and 4 pineal organs, perfused with a constant flow rate of 5µL/min. Fractions were collected every 30 min in refrigerated glass microtubes (Bioanalytical Systems HoneyComb fraction collector). Samples were stored at -80˚C until the melatonin quantification, usually performed on the same day. For assays, human Asip1 was dissolved in the same culture medium at different concentrations: 5nM, 50nM and 1µM and perfused for 30-minute intervals.

2.5. Melatonin and monoamine determination

2.5.1 Melatonin quantification

Melatonin concentration was quantified by high-performance liquid chromatography (HPLC) with fluorescence detection as previously described (López-Patiño et al., 2014), with slight modifications. Brains were kept from light in a water/ice bath and homogenised by ultrasonic disruption in 100µL of cold HPLC mobile phase, then centrifuged (16000g, 15 min). While for pineal melatonin quantification, the fractions were collected from pineal superfusion. Briefly, 50µl of each sample was injected into a system consisting of a JASCO autosampler (AS-4150) connected to a quaternary pump (PU-4180) and a fluorescence detector (FP-4025) set at 285/345 excitation/emission wavelengths. Separation was performed on a Kinetex C-18 column (2.6 µm particles, 150 mm × 4.6 mm, Phenomenex, USA) maintained at 30°C in a JASCO column oven (CO-4060). The mobile phase consisting of 50mM sodium acetate and 22% (v/v) acetonitrile, pH adjusted to 4.5 with acetic acid, was pumped isocratically at a flow rate of 1.0 mL/min. ChromNAV 2.0 HPLC software was used for chromatogram acquisition and integration. The detection limit of the technique was set at 0.1pg, with a noise/signal ratio of 1:3. The brain protein content measured by the bicinchoninic acid method (Smith et al., 1985) was used as a reference for the concentration calculation.

2.5.2 Brain monoamines quantification

The contents of 5-hydroxytryptamine (5HT), 5-hydroxyindolacetic acid (5HIAA), dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by HPLC with electrochemical detection, according to Chivite et al. (2021). Brains were kept in a water/ice bath and homogenised by ultrasonic disruption in 250µL of cold HPLC mobile phase, then centrifuged (16000g, 15 min). The supernatants were again diluted (1:3) in the mobile phase, and 20µL of each sample was injected into the HPLC system. The system was a Jasco HPLC equipped with an AS-2055 autosampler, a PU-2080 pump (Jasco, Japan) and a CO-4060 column oven. Chromatographic separation was achieved with a 5 µm analytical column (Phenomenex, Nucleosil C18, 150 mm length × 4.6 mm diameter) and 1 mL/min isocratic flow rate of mobile phase (63.9mM NaH_2PO_4, 0.1mM Na_2EDTA, 0.80mM sodium 1-octanesulfonate and 13% (v/v) methanol, pH adjusted to 2.95 with orthophosphoric acid). The analytes were detected with an ESA Coulochem II detector equipped with a dual analytical cell (M5011) set at + 40 mV (first electrode) and + 340 mV (second electrode). ChromNAV version 1.12 software (Jasco Corp.) was used for chromatogram acquisition and integration. The brain protein content measured by the bicinchoninic acid method (Smith et al., 1985) was used as a reference for the concentration calculation.

2.6. Statistical analyses

Differences between genotypes in brain gene expression, melatonin and monoaminergic synthesis and transmission among the different 24-hour sampling points analysed by two-way ANOVA using GraphPad Prism 8. Differences in melatonin levels of perfusion fractions or average levels were assayed by one-way ANOVA. LA actograms and mean waves were plotted with "El Temps" software v 1.313 (http://el-temps.com). The rhythmicity in brain gene expression, melatonin and monoamines was checked by cosinor analysis run in the previous software and "Cosinor Online"

software (https://cosinor.online/app/cosinor.php). Differences between average levels of LA during L and D periods in both genotypes were assayed by two-way ANOVA using GraphPad Prism 8, whereas differences between average levels of brain melatonin and monoamines in both genotypes were assayed by t-test. Differences were considered significant when $p<0.05$, data represented mean with ± SEM.

3. Results

3.1 Activity patterns

WT exposed to 12L/12D photoperiod exhibited marked circadian rhythms of LA. When the light cycle was inverted to 12D/12L WT, animals rapidly fit their activity pattern to new light conditions, as shown in figures 1 and 2. *asip1-Tg* also exhibited circadian LA patterns as revealed by the cosinor fitting (Fig. 2), yet activity levels were much higher than that recorded for WT animals as shown in figures 2 and 3 (see also Fig. S2a,b, S3, S4). There were no differences in the average levels of LA between light and dark phases in *asip1-Tg* under LD, but LA was higher during the dark phase under DL photoperiod (Fig. 3). Diurnal LA of WT was higher than nocturnal activity even when photoperiod was inverted, although diurnal LA was slightly lower in DL as shown in figure 3. *Asip1*-Tg animals also exhibit enhanced activity under constant DD (constant darkness) conditions (Fig. S5 and S6).

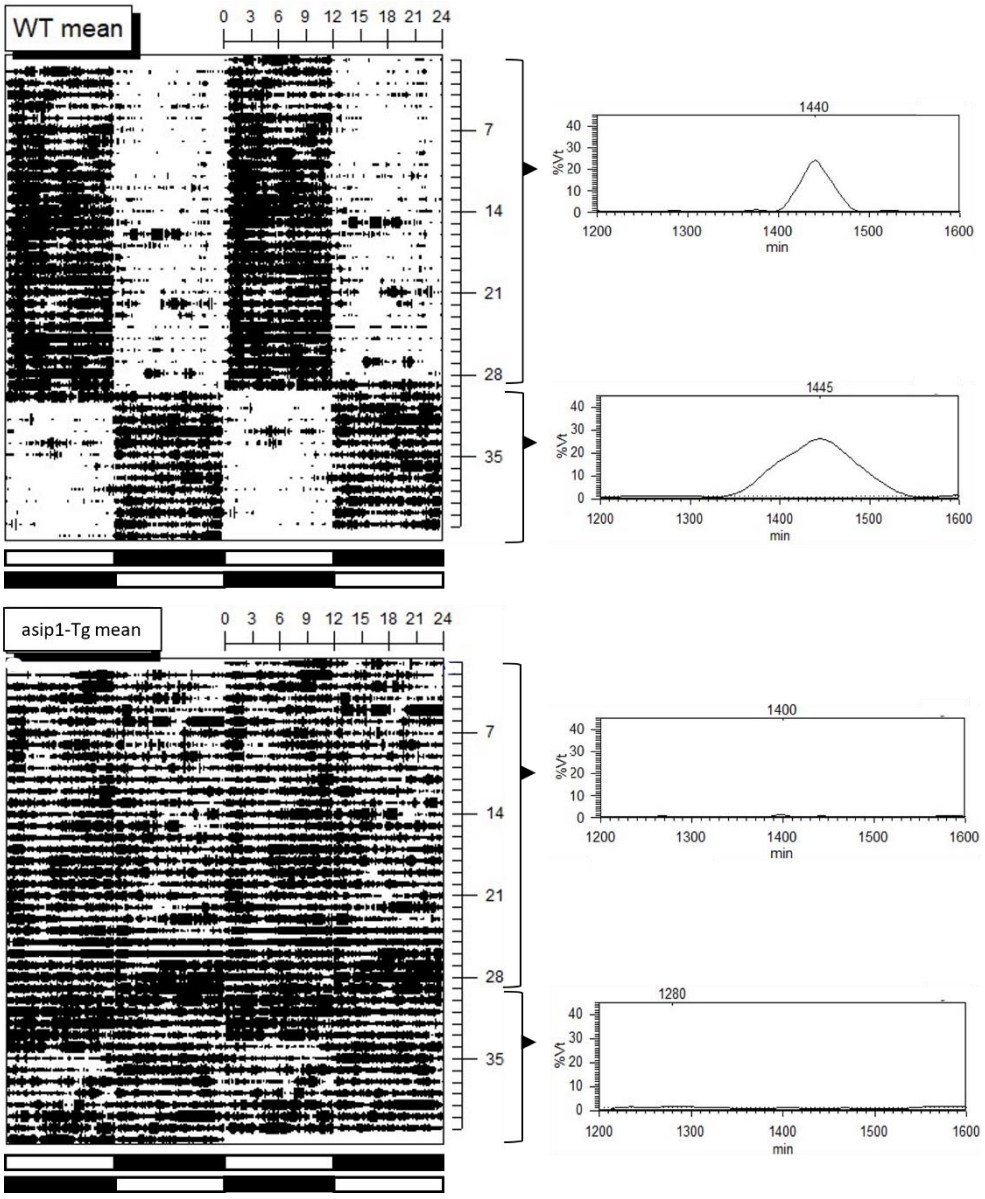

Figure 1: Actograms and periodograms. The left panels show the actograms of locomotor activity of WT and *asip1-Tg* animals maintained under constant LD photoperiod for 28 days. Subsequently, the LD cycle was inverted, and LA screened for 12 days. Actograms are presented in a double plot format (48 h time scale) to improve visualization. The horizontal bar at the bottom represents day (in white) and night (in black) hours of two consecutive days. The right panels show periodogram analysis providing the periodicity of the data with tau values (L/D and D/L) close to 1440 m (24h) for WT animals.

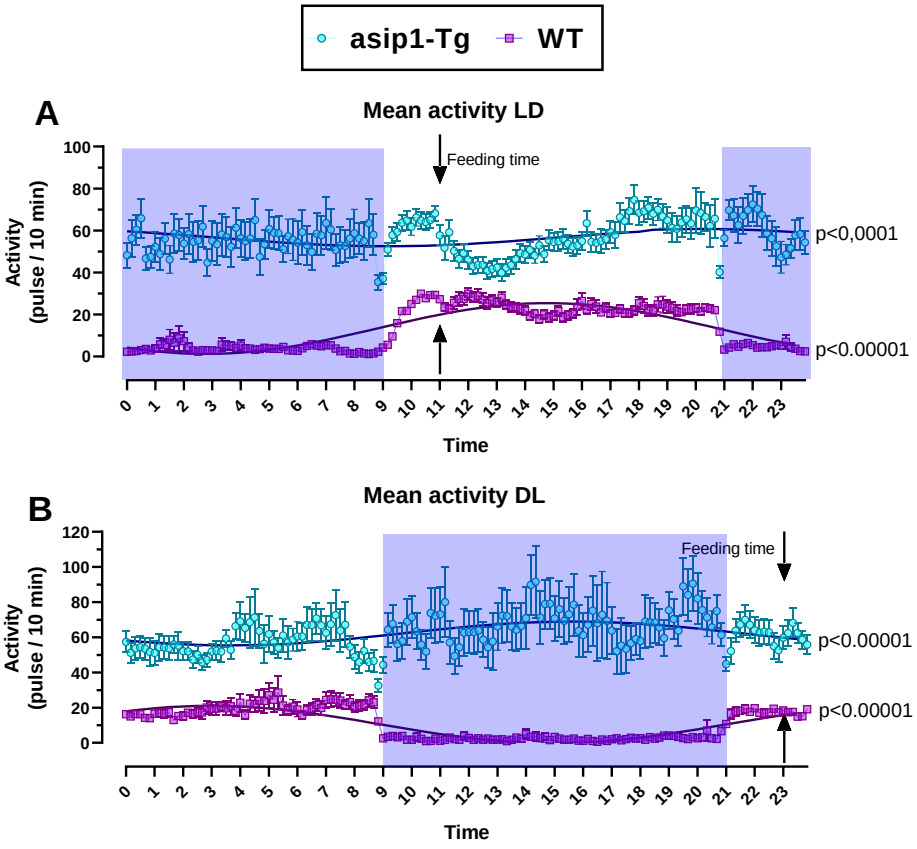

Figure 2: The average locomotor activity (LA) of adult *asip1-Tg* and WT zebrafish under constant LD photoperiod (12L/12D, lights on/off at 9 and 21 h, respectively).

Infrared sensors recorded LA for 28 consecutive days, and pulses were integrated every 10 min. Each point represents the mean ± SEM of three 6L tanks with 10 animals for each genotype. Rhythmicity was checked by cosinor fit run in El Temps v 1.313 (http://el-temps.com) and Cosinor Online (https://cosinor.online/app/cosinor.php) software. The significance level of the cosinor fit is indicated on the right-hand side of the figure. Black arrows indicate the feeding time, and dark purple panels indicate the scotophase of the photoperiod, respectively.

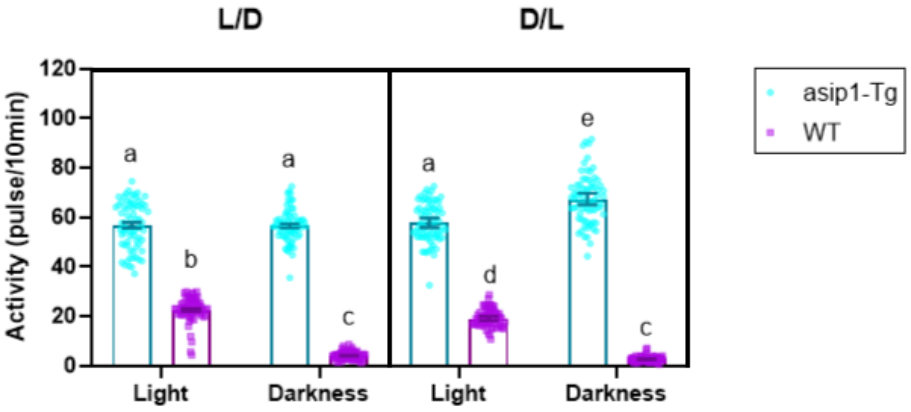

Figure 3: Total average locomotor activity (LA) during the light and dark period of *asip1-Tg* and WT animals.

The left panel (L/D) shows values recorded for the first experimental phase, which extended for 28 days. The right panel (D/L) displays LA values when the photoperiod was inverted subsequently for 12 additional days. The different letter indicates significant differences in the two-way ANOVA test ($p<0.05$). Data are represented by mean ± SEM. See figure 2 for further details.

3.2 Melatonin and monoamine brain levels

We then studied whole-brain melatonin because of the small size of the zebrafish pineal gland and monoamine levels in animals of both genotypes subjected to a 12L:12D photoperiod. WT animals exhibited significant daily variations in melatonin and 5-HT levels but only melatonin fitted to circadian conditions. However, *asip1-Tg* lost melatonin circadian rhythms and 5-HT daily variations (Fig. 4). Both central dopamine and noradrenaline did not exhibit significant daily variations. Dopaminergic and serotoninergic activity, as revealed by DOPAC/dopamine and 5-HIIA/5-HT ratios, displayed significantly inverted fluctuations through the LD phases still only dopaminergic waves were fitted to circadian periods in both WT and *asip1-Tg* genotypes (Fig. 4). WT animals exhibited higher melatonin and 5-HT average levels whereas both average dopaminergic and serotoninergic activity were increased in *asip1-Tg* (Fig.5).

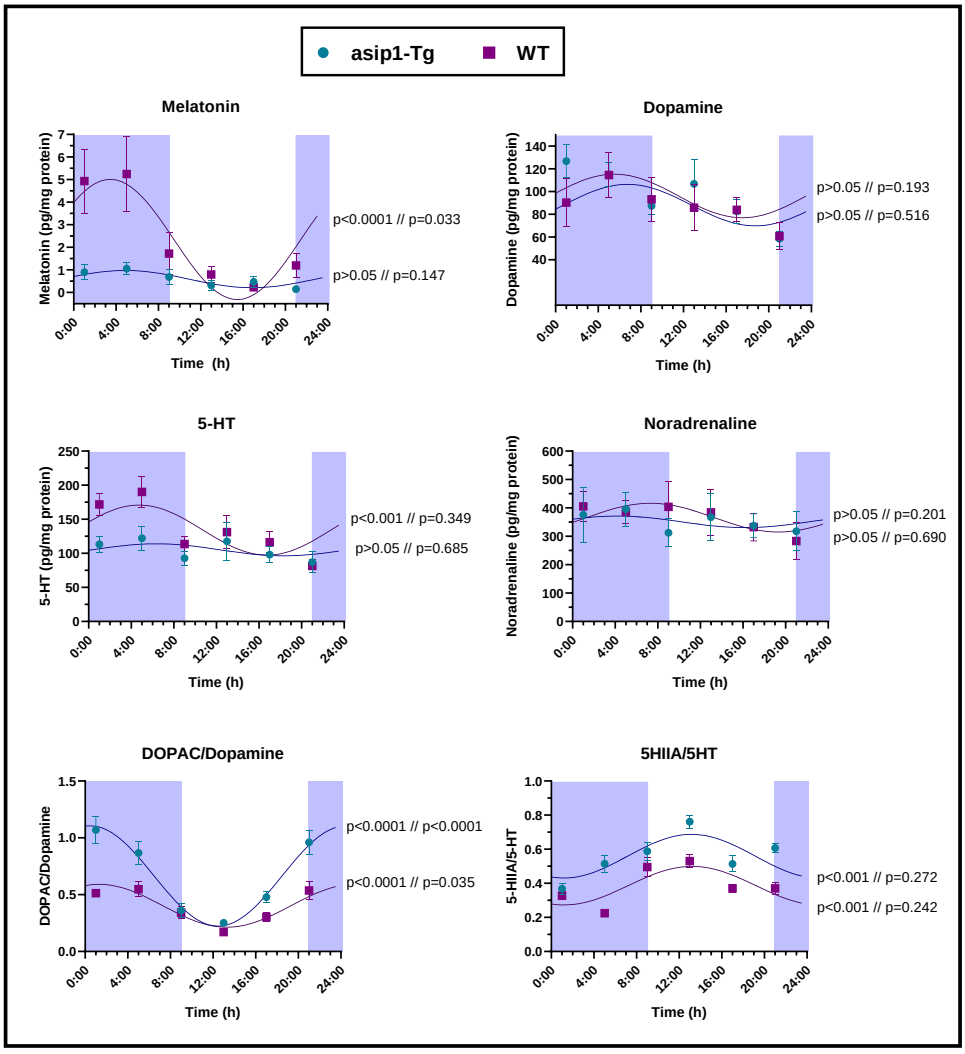

Figure 4: Neurotransmitters and melatonin daily rhythms of *asip1-Tg* and WT animals (n=10 per genotype and sampling time) kept under constant LD photoperiod for 30 days. Dark purple bars indicate the dark phase of the photoperiod.

Results are expressed in pg of metabolite per mg of total protein. Data are represented by mean ±SEM. Differences among sampling times of the same genotype were assessed by one-way ANOVA and average values fitted to Cosinor curves. Significance values for each analysis are given close to the cosinor curves (ANOVA // Cosinor).

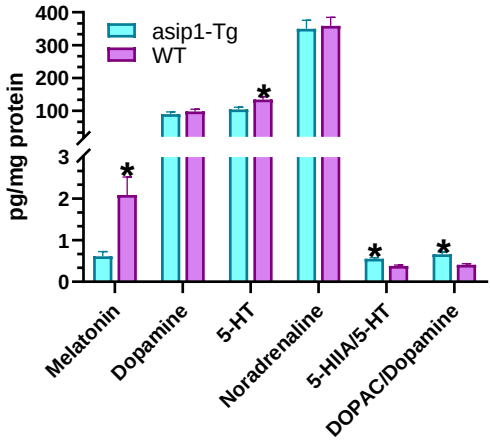

Figure 5: Brain melatonin, neurotransmitters serotonin (5HT), and dopamine and turnover their ratios (5HIAA/5HT and DOPAC/DA) *asip1-Tg* and WT animals (n=60 per genotype) kept under constant LD for 30 days.

Results are expressed in pg of metabolite per mg of total protein. Data are represented by mean ±SEM analysed by an unpaired t-test (* $p<0.05$).

3.3. *In vitro* effects of melanocortin antagonists on pineal melatonin release

In vivo experiments suggested that *asip1* overexpression can reduce melatonin synthesis and/or secretion. We prefunded zebrafish pineal glands with graded mammalian Asip concentrations to further test this hypothesis. Higher doses of asip induced a significant time-course decrease in melatonin secretion. The effect was only detected after the peptide perfusion in the lower doses (Fig. 6). Accordingly, average melatonin secreted levels were significantly lower in higher asip-perfused doses.

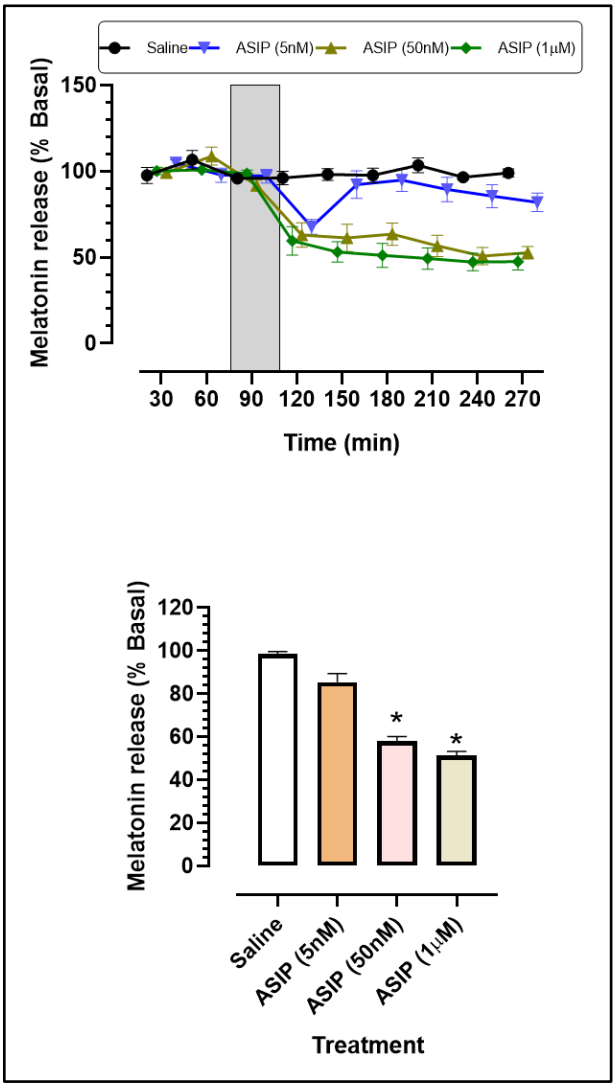

Figure 6: Upper panel shows melatonin levels secreted from pineal glands superperfused with a constant flow rate of 5μL/min with saline or melatonin. Fractions were collected every 30 min.

Each point represents the mean ± SEM (saline and 1mM n=5, 5 and 50 nM n=6). The grey bar indicates the perfusion period. Experiments were done in triplicate. The bottom panel displays average levels of melatonin released from pineal glands after perfusion. Each bar represents the mean ± SEM (n=10) from all collected fractions (saline and 1mM n=45, 5 and 50 nM n=54)

3.4. Clock genes expression

Subsequently, we hypothesised that the rhythmic expression of central clock genes could be masked in *asip1-Tg*. Transgenic animals lost *per1a* and *clock1a* gene expression rhythmicity that was fitted to circadian patterns in WT fish. *cry1a*, *bmal1a,* and *nr1dr1* gene expression exhibited similar significant daily expression patterns in both genotypes. Still, nocturnal expression levels of nr1d1, but no diurnal levels, were significantly higher in WT animals (Fig 7).

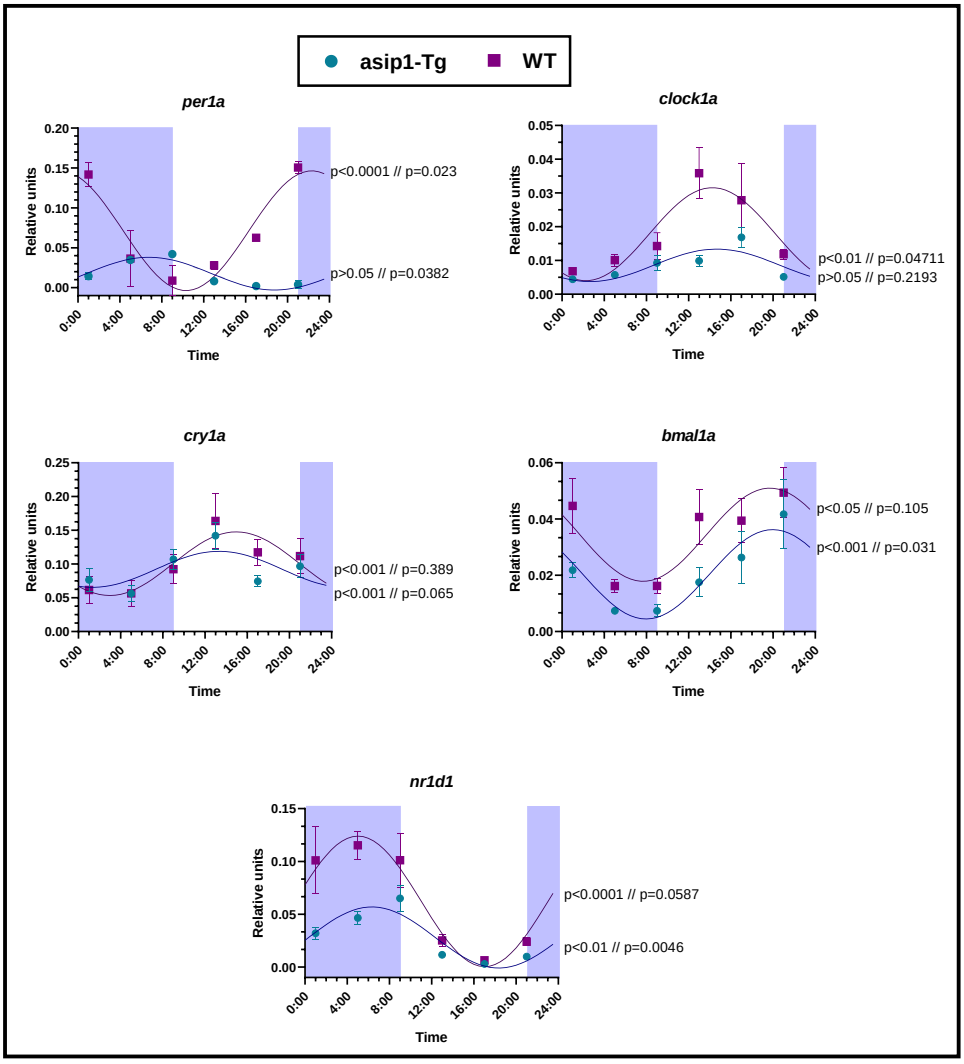

Figure 7. Daily rhythms of clock gene expression of *asip1* Tg and WT animals (n=10) kept under constant LD photoperiod (12L/12D, lights on/off at 9 and 21 h, respectively) for 30 days.

Grey bars indicate the dark phase of the photoperiod. Each point represents the mean ± SEM (n=10). Difference among sampling times of the same genotype were assessed by one-way ANOVA and average values fitted to Cosinor curves. Significance values for each analysis are given close to the Cosinor curves (ANOVA // Cosinor).

3.5 Hypothalamic POMC expression

We further postulated that the increased LA in *asip1-Tg* could be associated with feed anxiety, as suggested by increased food intake levels in *asip1-Tg* (Guillot et al., 2016; Godino-Gimeno et al., 2020) and the reduced 5-HT levels in *asip1-Tg*. Expression studies displayed depressed *pomc a* and *b* expression levels during the dark phase of the photoperiod in transgenic animals. In fact, daily significant variations in pomc expression levels were masked in *asip1-Tg* (Fig. 8).

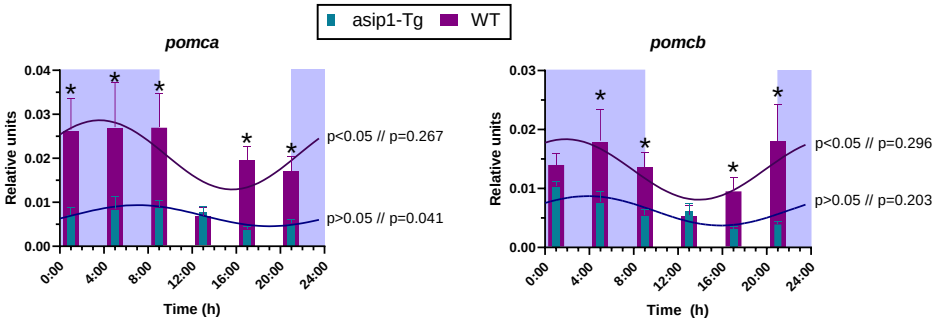

Figure 8: Daily rhythms of *pomca* (left panel) and *pomcb* (right panel) brain expression of *asip1-Tg* and WT animals (n=10 per genotype and sampling time) kept under constant LD photoperiod for 30 days.

Dark purple bars indicate the scotophase of the photoperiod. Data are represented by mean ±SEM. Differences among sampling times of the same genotype were assessed by one-way ANOVA and average values fitted to Cosinor curves. Significance values for each analysis are given close to the Cosinor curves (ANOVA // Cosinor).

4. Discussion

Central melanocortin agonists and antagonists are essential peptides in the control of feed intake and energy expenditure in vertebrates (Cone, 2006), including fish (Cerdá-Reverter & Peter, 2003; Cerdá-Reverter et al., 2003; Shainer et al., 2019). Accordingly, *asip1* overexpression results in increased food intake, linear growth and food conversion, yet not an obese phenotype (Guillot et al., 2016; Godino-Gimeno et al., 2020; Leal et al., 2022) *agrp1* overexpression results in a similar phenotype (Song and Cone, 2007; Shainer et al., 2019; Lin et al., 2023). Food intake is the final step of a complex behavioural process, including food seeking, sensory evaluation, learning, and final consumption. Thus, the feeding acts should be associated with daily LA patterns during the active phase of the sleep-wake cycle, suggesting a coordinated behavioural regulation. Our previous studies showed that *asip1-Tg* has a reduced nocturnal melatonin surge (Guillot et al., 2016), suggesting a potential role of the melanocortin system in circadian behaviour. We have now examined LA patterns in *asip1-Tg* as an output of the circadian clock under constant LD conditions, showing severe LA rhythms disruption due to very high activity during the scotophase. In fact, *asip1-Tg* always showed an increased LA during both the photophase and the scotophase, but unlike the WT genotype, the activity level did not decrease during the nocturnal period. The persistence under constant conditions (DD) suggests an effect on endogenous rhythmicity. These results coincided with a severe disruption of melatonin secretion that completely inhibited the nocturnal pulse, masking its circadian rhythm in *asip1-Tg*. Similarly, *asip1* overexpression disrupted 5-HT circadian cycles in the brain by reducing nocturnal secretion/synthesis, suggesting that reduced nocturnal melatonin levels could be due to reduced 5-HT availability.

The melatonin rhythm is an output of the vertebrate circadian clock (Elbaz et al., 2013). The molecular architecture of this clock is highly conserved, but there are considerable differences in the organisation of circadian systems. Mammalian species have a master circadian clock in the suprachiasmatic nucleus (SCN) that regulates the pineal gland through a multisynaptic pathway. In birds, the pineal gland can also function independently of SCN innervation using an intrinsic clock (Ben-Moshe Livne et al., 2016). The fish pineal is also photoreceptive and can function independently thanks to an intrinsic circadian oscillator (Elbaz et al., 2013). As no functional equivalent of the SCN has been found in fish, the pineal gland is considered a master clock organ. However, the presence of photoreceptive peripheral oscillators that can be entrained by light (Whitmore et al., 2000), with similar molecular structure (Vallone et al., 2004, 2005), suggests a diffuse circadian system that challenges the idea of a master pineal clock. To obtain further evidence for disrupting the circadian system in *asip1-Tg*, we examined the expression of key clock genes in the whole zebrafish brain, including the pineal gland. Again, *asip1* overexpression disrupted the circadian expression of clock genes, including *per1a* and *clock1a*, and reduced the amplitude of *nr1d1* gene expression during the scotophase. In vertebrates, the transcription factors Clock and Bmal bind as heterodimers to E-box enhancers of the transcriptional repressors Per and Cry, which in turn repress the transcriptional activity of *clock/bmal*, reducing the expression of their own genes (*per* and *cry*) and thus closing the feedback loop (Vallone et al., 2004). Our results suggest that *asip1* overexpression disrupts the central molecular clock, subsequently promoting the abolishment of the melatonin circadian rhythm and ultimately disrupting the LA circadian rhythm. The pineal gland has been proposed as a behaviour-regulating clock centre in zebrafish as a blockade of *clock* gene expression masks circadian rhythms of vertical positioning in the water column under constant darkness conditions (DD) (Ben-Moshe Livne et al., 2016). However, the LA pattern was not eliminated under DD in these animals

(ΔClock), yet the amplitude was significantly reduced, suggesting that additional central clock centres may play a role in controlling circadian behaviours. Suggestively, ΔClock animals exhibited normal melatonin rhythms under constant LD conditions but were disrupted under DD conditions. In *asip1-Tg*, melatonin and the LA pattern were completely disrupted under constant LD conditions, suggesting that the melanocortin system could use additional central clocks, not just the pineal molecular clock, to mask melatonin and behavioural outputs.

It is challenging to think that the alterations in the dorsoventral pigment pattern of *asip1-Tg* could alter the sensibility of the pineal gland to light exposure. *asip1* overexpression inhibits dorsal melanogenesis and manages the chromophore fate control, drastically reducing the number of melanophores (Cerdá-Reverter et al., 2005; Ceinos et al., 2015; Cal et al., 2019). The re-evaluation of the dorsal morphology of *asip1-Tg* shows that the reduction in the number of melanocytes exclusively affects the dorsal trunk but not the dorsal region of the head, including the pineal window (Cal et al., 2019). Therefore, a morphological explanation of the results is unlikely. This brings us to the next question, which concerns how melanocortin encoding information reaches the pineal complex to inhibit the nocturnal melatonin surge and disrupt the circadian LA patterns. The pineal gland is known to express some melanocortin receptors, suggesting a direct role of the melanocortinergic peptides in pineal physiology (Shainer et al., 2017). To address this hypothesis, we dissected zebrafish pineal glands and incubated them with graded concentrations of mammalian Asip1. The results show that Asip1 can reduce melatonin secretion dose-dependently, suggesting that inhibiting endogenous melanocortinergic activity through specific receptors leads to inhibition of melatonin secretion. Therefore, the *in vitro* results mimic those observed in *asip1-Tg*, further supporting the role of Asip1 in pineal physiology.

The zebrafish pineal gland is reactive to Agrp antibodies but not to α-MSH (Forlano and Cone, 2007), probably due to cross-reactivity with Agrp2, which is highly expressed in the zebrafish pineal complex (Zhang et al., 2010). However, larval *agrp2* knockouts and ablated retinal pigment epithelium (RPE)-like cells, which express *agpr2* in the pineal, exhibit robust circadian LA rhythms under constant dim light conditions, suggesting that Agrp2 and/or RPE cells are not required for the maintenance of locomotor behaviour in zebrafish larvae (Shainer et al., 2019). Although our experimental conditions and the age of the animals are significantly different (larvae *vs.* adult fish), the agrp2 knockout results suggest that the potential Asip1/Agrp2 structural mimicry cannot explain the circadian disruption in *asip1-Tg* animals. However, we cannot exclude the possibility that Agrp2 may play an alternative role in regulating the pineal circadian clock in adult animals.

Asip1 can antagonise all MCRs with different efficiency (Guillot et al., 2016), but it is not expressed in the fish pineal gland (data obtained in sea bass *Dicentrarchus labrax* by RT-PCR (Agulleiro et al., 2014). We re-evaluated *asip1* expression in our *Drer.asip1-iTol2-eGFP*-BAC transgenic line (Cal et al., 2017) and no expression signal was obtained in the pineal complex. However, animals exhibit sound expression levels in the retina (Supplementary Fig. 5). Furthermore, minimal levels of *asip1* expression have been demonstrated in the zebrafish brain, as detected by RT-PCR (Cal et al., 2017). Therefore, it is plausible that the ubiquitous *asip1* expression in our transgenic model may mimic the role of hypothalamic *agrp1*, potentially projecting to the pineal gland. Tracking studies have shown that no pinealopetal cells are labelled in the zebrafish brain after tract-tracing with carbocyanine dyes (DiI) (Yáñez et al., 2009). However, the presence of *mc1r*, *mc3r*, and *mc4r* mRNAs in the zebrafish pineal has been demonstrated by RT-PCR (Shainer et al., 2017). The absence of pinealopetal neurons has also been reported in several fish species, but other species, including sea bass (*Dicentrarchus labrax*) and sturgeon, have an extensive pinealopetal system

(Servili et al., 2011). Such a discrepancy could be due to species-specific differences but also to the plasticity of the pinealopetal system in response to different physiological and/or environmental conditions (Servili et al., 2011). Again, the available information does not support the presence of interneurons connecting the tuberal hypothalamus, where both *agrp1* and *pomcs* are expressed, and the pineal complex in zebrafish, although the potential plasticity of the pinealopetal system cannot completely rule out this hypothesis. Alternatively, melanocortin agonists and/or antagonists could reach the pineal gland via systemic circulation. The pineal complex has an extensive choroid plexus, the *saccus dorsalis*, which is highly vascularized and can be accessed by peripheral hormones for regulatory purposes. Indeed, the fish pineal gland seems modulated by systemic hormones in zebrafish, as, for example, the absence of leptin promotes a decrease in brain melatonin levels (Audira et al., 2018).

Signalling of the MCRs could be considered as another additional option. Our previous studies have shown that fish MC1R and MC4R can signal in the absence of agonists with constitutive activity and that both Asip1 and Agrp1 can act as inverse agonists (Sánchez et al., 2009, 2010; Guillot et al., 2016). It is then plausible that the pineal MCRs impose a melanocortinergic tone required for the regular functioning of the molecular clock, which is disrupted by the ectopic *asip1* overexpression. Further research is needed to explore the physiological significance of the melanocortin-induced inhibition of melatonin secretion and explain how melanocortins reach the pineal complex.

asip1-Tg exhibit hyperphagia (Guillot et al., 2016; Godino-Gimeno et al., 2020) due to a depressed satiety system (Guillot et al., 2016). Our results further showed that transgenic fish exhibit subordinate personality, elevated cortisol levels (Rocha et al., 2023) and high anxiety levels mediated by depressed central 5-HT levels (Guillot et al., 2016; Rocha et al., 2023). We now further show that 5-HT circadian rhythmicity is masked in *asip1-Tg*. 5-HT is an anorexigenic neurotransmitter in vertebrates,

including zebrafish (Wee et al., 2019), which acts in part through the regulation of hypothalamic POMC neurons (Heisler et al., 2002). In addition, activation of arcuate Agrp neurons inhibits POMC neurons throughout the GABAergic projections (Cowley et al., 2001). Our transgenic animals exhibit reduced levels of 5-HT and *pomc a/b* expression during the scotophase, suggesting they may be hungry during the nocturnal period. Unfortunately, we could not replicate the zebrafish self-feeding systems (Del Pozo et al., 2011) to estimate nocturnal food intake. It is, therefore, plausible that the high activity level of *asip1-Tg* is related to the increased anxiety promoted by appetite, which ultimately results in increased foraging behaviour and locomotor activity. Food intake is regulated multifacetedly, including homeostatic and hedonic aspects and temporary traits (Delgado et al., 2017). How all this information is centrally integrated into a single output, feeding behaviour, is unknown. However, our results suggest that the melanocortin system may be involved, and the *asip1-Tg* model can help to elucidate how all three regulatory aspects are integrated.

Melatonin is also required for the control of sleep, which is regulated by a homeostatic process that responds to internal cues, as evidenced by a compensatory increase in intensity and duration after deprivation, and a circadian process that responds to external cues and sets the timing of sleep (Elbaz et al., 2013; Gandhi et al., 2015). In mammals, sleep, like other circadian rhythms, is controlled by the SCN, which regulates melatonin secretion in the pineal gland and the rhythmic secretion of neuropeptides and monoamines (Elbaz et al., 2013). Melatonin is a potent sleep-promoting hormone in fish (Zhdanova, 2006). Since nocturnal melatonin levels in *asip1-Tg* are comparable to those of WT during the light period, the obvious question is whether transgenic animals sleep during the nocturnal period. Adult zebrafish show reduced locomotor activity and an increased arousal threshold after short-term forced wakefulness. However, after long-term sleep deprivation, many animals become hyperactive even shortly after the deprivation has ended without sleep

rebound (Zhdanova, 2011). It is, therefore, plausible that the hyperactivity observed in *asip1-Tg* is forced by the potential sleep deprivation imposed by low nocturnal melatonin levels. The hyperactivity observed in *asip1-Tg*, even during the daytime, could reflect the absence of homeostatic sleep regulation in adult fish, as previously suggested (Cirelli & Tononi, 2008; Zhdanova, 2011).

In conclusion, we have shown that overexpression of endogenous melanocortin antagonists disrupts the central circadian clock in zebrafish, coinciding with the masking of central melatonin circadian rhythms. Furthermore, *in vitro* experiments mimic *in vivo* Asip1-induced inhibition of melatonin secretion. The absence of nocturnal melatonin correlates well with the ablation of LA rhythms, suggesting that Asip1-induced disruption of the central clock promotes the masking of melatonin rhythms and the subsequent ablation of LA circadian rhythms. How all this downstream sequence of the molecular clock relates to the sleep-wake and feeding cycles is unknown. However, the *asip1-Tg* model could help to elucidate the integration between feeding and locomotor behaviour during the circadian period.

References

Agulleiro, M. J., Cortés, R., Leal, E., Ríos, D., Sánchez, E., and Cerdá-Reverter, J. M. (2014). Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (Dicentrarchus labrax). *Gen Comp Endocrinol* 205, 251–259. doi: 10.1016/j.ygcen.2014.02.009.

Audira, G., Sarasamma, S., Chen, J. R., Juniardi, S., Sampurna, B. P., Liang, S. T., et al. (2018). Zebrafish Mutants Carrying Leptin a (lepa) Gene Deficiency Display Obesity, Anxiety, Less Aggression and Fear, and Circadian Rhythm and Color Preference Dysregulation. *Int J Mol Sci* 19, 4038. doi: 10.3390/IJMS19124038.

Ben-Moshe Livne, Z., Alon, S., Vallone, D., Bayleyen, Y., Tovin, A., Shainer, I., et al. (2016). Genetically Blocking the Zebrafish Pineal Clock Affects Circadian Behavior. *PLoS Genet* 12, 1006445. doi: 10.1371/journal.pgen.1006445.

Cal, L., Megías, M., Cerdá-Reverter, J. M., Postlethwait, J. H., Braasch, I., and Rotllant, J. (2017). BAC recombineering of the agouti loci from spotted gar and zebrafish reveals the evolutionary ancestry of dorsal-ventral pigment asymmetry in fish HHS Public Access. *J Exp Zool B Mol Dev Evol* 328, 697–708. doi: 10.1002/jez.b.22748.

Cal, L., Suarez-Bregua, P., Comesaña, P., Owen, J., Braasch, I., Kelsh, R., et al. (2019). Countershading in zebrafish results from an Asip1 controlled dorsoventral gradient of pigment cell differentiation. *Scientific Reports 2019 9:1* 9, 1–13. doi: 10.1038/s41598-019-40251-z.

Ceinos, R. M., Guillot, R., Kelsh, R. N., Cerdá-Reverter, J. M., and Rotllant, J. (2015). Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms. *Pigment Cell Melanoma Res* 28, 196–209. doi: 10.1111/pcmr.12335.

Cerdá-Reverter, J., and Canosa, F. (2009). "Neuroendocrine Systems of the Fish Brain," in *Fish Neuroendocrinology*, 3–74.

Cerdá-Reverter, J. M., Agulleiro, M. J., Guillot R, R., Sánchez, E., Ceinos, R., and Rotllant, J. (2011). Fish melanocortin system. *Eur J Pharmacol* 660, 53–60. doi: 10.1016/j.ejphar.2010.10.108.

Cerdá-Reverter, J. M., Haitina, T., Schiöth, H. B., and Peter, R. E. (2005). Gene structure of the goldfish agouti-signaling protein: A putative role in the dorsal-ventral pigment pattern of fish. *Endocrinology* 146, 1597–1610. doi: 10.1210/EN.2004-1346.

Cerdá-Reverter, J. M., and Peter, R. E. (2003). Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. *Endocrinology* 144, 4552–4561. doi: 10.1210/EN.2003-0453.

Cerdá-Reverter, J. M., Schiöth, H. B., and Peter, R. E. (2003). The central melanocortin system regulates food intake in goldfish. *Regul Pept* 115, 101–113. doi: 10.1016/S0167-0115(03)00144-7.

Chivite, M., Leal, · Esther, Míguez, J. M., Jose, ·, and Cerdá-Reverter, M. (2021). Distribution of two isoforms of tryptophan hydroxylase in the brain of rainbow trout (Oncorhynchus mykiss). An in situ hybridization study. *Brain Struct Funct* 226, 2265–2278. doi: 10.1007/s00429-021-02322-8.

Cirelli, C., and Tononi, G. (2008). Is Sleep Essential? *PLoS Biol* 6, e216. doi: 10.1371/JOURNAL.PBIO.0060216.

Cone, R. D. (2006). Studies on the physiological functions of the melanocortin system. *Endocr Rev* 27, 736–749. doi: 10.1210/ER.2006-0034.

Cortés, R., Agulleiro, M. J., Navarro, S., Guillot, R., Sánchez, E., and Cerdá-Reverter, J. M. (2014). Melanocortin receptor accessory protein 2 (MRAP2) interplays with the zebrafish melanocortin 1 receptor (MC1R) but has no effect on its pharmacological profile. *Gen Comp Endocrinol* 201, 30–36. doi: 10.1016/J.YGCEN.2014.03.009.

Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdán, M. G., Diano, S., Horvath, T. L., et al. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411, 480–484.

Del Pozo, A., Antonio Sánchez-Férez, J., Javier Sánchez-Vázquez, F., and Sánchez-Vázquez, F. J. (2011). Circadian Rhythms of Self-feeding and Locomotor Activity in Zebrafish (Danio Rerio). *Chronobiol Int* 28, 39–47. doi: 10.3109/07420528.2010.530728.

Delgado, M. J., Cerdá-Reverter, J. M., and Soengas, J. L. (2017). Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: Involvement in the control of food intake. *Front Neurosci* 11. doi: 10.3389/fnins.2017.00354.

Elbaz, I., Foulkes, N. S., Gothilf, Y., and Appelbaum, L. (2013). Circadian clocks, rhythmic synaptic plasticity and the sleep-wake cycle in zebrafish. *Front Neural Circuits* 7, 41303. doi: 10.3389/FNCIR.2013.00009/BIBTEX.

Forlano, P. M., and Cone, R. D. (2007). Conserved Neurochemical Pathways Involved in Hypothalamic Control of Energy Homeostasis. *J. Comp. Neurol* 505, 235–248. doi: 10.1002/cne.21447.

Gandhi, A. V, Mosser, E. A., Oikonomou, G., and Correspondence, D. A. P. (2015). Melatonin Is Required for the Circadian Regulation of Sleep. *Neuron* 85. doi: 10.1016/j.neuron.2015.02.016.

Godino-Gimeno, A., Sánchez, E., Guillot, R., Rocha, A., Angotzi, A. R., Leal, E., et al. (2020). Growth Performance after Agouti-Signaling Protein 1 (Asip1) Overexpression in Transgenic Zebrafish. *Zebrafish* 17, 373–381. doi: 10.1089/zeb.2020.1932.

Guillot, R., Cortés, R., Navarro, S., Mischitelli, M., García-Herranz, V., Sánchez, E., et al. (2016). Behind melanocortin antagonist overexpression in the zebrafish brain: A behavioral and transcriptomic approach. *Horm Behav* 82, 87–100. doi: 10.1016/j.yhbeh.2016.04.011.

Herget, U., Wolf, A., Wullimann, M. F., and Ryu, S. (2014). Molecular neuroanatomy and chemoarchitecture of the neurosecretory preoptic-hypothalamic area in zebrafish larvae. *Journal of Comparative Neurology* 522, 1542–1564. doi: 10.1002/CNE.23480.

Leal, E., Angotzi, A. R., Godino-Gimeno, A., Gregório, S. F., Rotllant, J., Saera-Vila, A., et al. (2022). Agouti overexpression in a transgenic model regulates integrity, permeability and electrogenic amino acid transport in zebrafish intestine. *Front Mar Sci* 9. doi: 10.3389/fmars.2022.1011702.

Lin, C.-Y., Yeh, K.-Y., Lai, H.-H., and Her, G. M. (2023). AgRP Neuron-Specific Ablation Represses Appetite, Energy Intake, and Somatic Growth in Larval Zebrafish. *Biomedicines* 11, 499. doi: 10.3390/BIOMEDICINES11020499.

López-Patiño, M. A., Gesto, M., Conde-Sieira, M., Soengas, J. L., and Míguez, J. M. (2014). Stress inhibition of melatonin synthesis in the pineal organ of rainbow trout (Oncorhynchus mykiss) is mediated by cortisol. *Journal of Experimental Biology* 217, 1407–1416. doi: 10.1242/JEB.087916/257641/AM/STRESS-INHIBITION-OF-MELATONIN-SYNTHESIS-IN-THE.

Pinto, P. I. S., Guerreiro, C. C., Costa, R. A., Martinez-Blanch, J. F., Carballo, C., Codoñer, F. M., et al. (2019). Understanding pseudo-albinism in sole (Solea senegalensis): a transcriptomics and metagenomics approach. *Sci Rep* 9. doi: 10.1038/S41598-019-49501-6.

Rocha, A., Godino-Gimeno, A., Rotllant, J., and Cerdá-Reverter, J. M. (2023). Agouti-Signalling Protein Overexpression Reduces Aggressiveness in Zebrafish. *Biology (Basel)* 12, 712. doi: 10.3390/biology12050712.

Sánchez, E., Rubio, V. C., and Cerdá-Reverter, J. M. (2010). Molecular and pharmacological characterization of the melanocortin type 1 receptor in the sea bass. *Gen Comp Endocrinol* 165, 163–169. doi: 10.1016/J.YGCEN.2009.06.008.

Sánchez, E., Rubio, V. C., Thompson, D., Metz, J., Flik, G., Millhauser, G. L., et al. (2009). Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (Dicentrarchus labrax). *Am J Physiol Regul Integr Comp Physiol* 296. doi: 10.1152/AJPREGU.90948.2008.

Servili, A., Herrera-Pérez, P., Yáñez, J., and Muñoz-Cueto, J. A. (2011). Afferent and efferent connections of the pineal organ in the European sea bass Dicentrarchus labrax: a carbocyanine dye tract-tracing study. *Brain Behav Evol* 78, 272–285. doi: 10.1159/000330824.

Shainer, I., Buchshtab, A., Hawkins, T. A., Wilson, S. W., Cone, R. D., and Gothilf, Y. (2017). Novel hypophysiotropic AgRP2 neurons and pineal cells revealed by BAC transgenesis in zebrafish. *Sci Rep* 7. doi: 10.1038/srep44777.

Shainer, I., Michel, M., Marquart, G. D., Bhandiwad, A. A., Zmora, N., Ben-Moshe Livne, Z., et al. (2019). Agouti-Related Protein 2 Is a New Player in the Teleost Stress Response System. *Curr Biol* 29, 2009-2019.e7. doi: 10.1016/J.CUB.2019.05.021.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* 150, 76–85. doi: 10.1016/0003-2697(85)90442-7.

Song, Y., and Cone, R. D. (2007). Creation of a genetic model of obesity in a teleost. *The FASEB Journal* 21, 2042–2049. doi: 10.1096/FJ.06-7503COM.

Vallone, D., Gondi, S. B., Whitmore, D., and Foulkes, N. S. (2004). E-box function in a period gene repressed by light. Available at: www.pnas.orgcgi-doi10.1073pnas.0305436101 [Accessed September 22, 2023].

Vallone, D., Lahiri, K., Dickmeis, T., and Foulkes, N. S. (2005). Zebrafish Cell Clocks Feel the Heat and See the Light! *https://home.liebertpub.com/zeb* 2, 171–187. doi: 10.1089/ZEB.2005.2.171.

Wee, C. L., Song, E. Y., Johnson, R. E., Ailani, D., Randlett, O., Kim, J. Y., et al. (2019). A bidirectional network for appetite control in larval zebrafish. *Elife* 8. doi: 10.7554/ELIFE.43775.

Whitmore, D., Foulkes, N. S., and Sassone-Corsi, P. (2000). Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature* 404, 87–91. doi: 10.1038/35003589.

Yáñez, J., Busch, J., Anadón, R., and Meissl, H. (2009). Pineal projections in the zebrafish (Danio rerio): overlap with retinal and cerebellar projections. *Neuroscience* 164, 1712–1720. doi: 10.1016/J.NEUROSCIENCE.2009.09.043.

Zhang, C., Song, Y., Thompson, D. A., Madonna, M. A., Millhauser, G. L., Toro, S., et al. (2010). Pineal-specific agouti protein regulates teleost background adaptation. *Proc Natl Acad Sci U S A* 107, 20164–20171. doi: 10.1073/PNAS.1014941107.

Zhdanova, I. V. (2006). Sleep in zebrafish. *Zebrafish* 3, 215–226. doi: 10.1089/ZEB.2006.3.215.

Zhdanova, I. V. (2011). Sleep and its regulation in zebrafish. *Rev Neurosci* 22, 27–36. doi: 10.1515/RNS.2011.005.

Supplementary materials

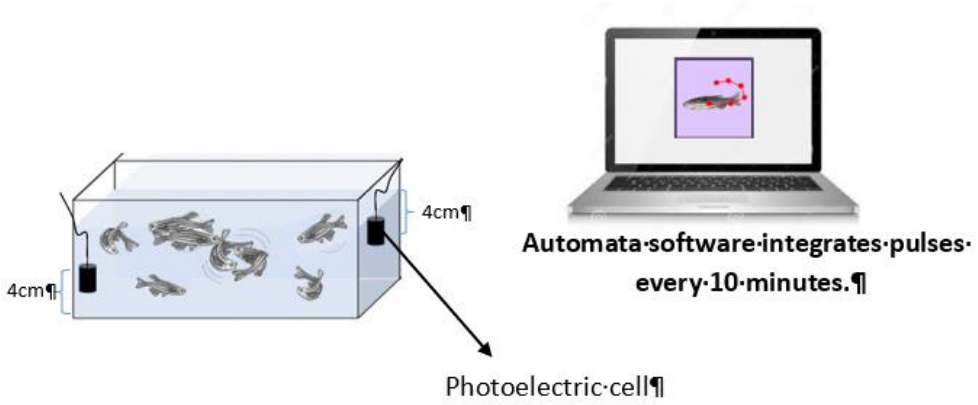

Figure S1: Activity monitoring system used in locomotor activity studies located in light- and sound-isolated room at 28ºC and 12h light (L)/12h darkness (D) (lights on at 9 AM and off at 9 PM).

In all experiments, animals were acclimated to the experimental room for 30 days, where no access was allowed, except for feeding (1.5% of biomass) once a day at 11 AM. Fish were placed in 6-litre tanks provided with two infrared photoelectric cell sensors (E3ZD822, Omron) placed in the superior and inferior parts connected to a computer system (AUTOMATA) that recorded sensor activations (pulses) and integrated them every 10 minutes.

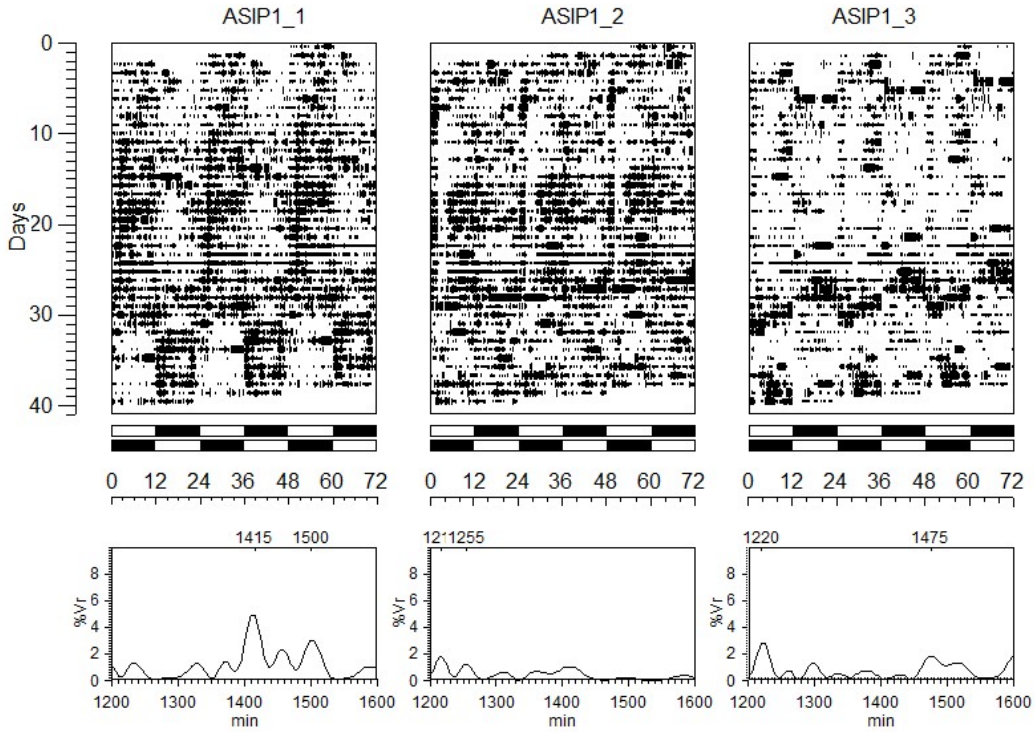

Figure S2a. Actograms of locomotor activity of *asip1-Tg* animals maintained under constant LD photoperiod for 28 days.

Subsequently, the LD cycle was inverted, and LA screened for 12 days. Actograms are presented in a triple plot format (72 h time scale) to improve visualization. The horizontal bar at the bottom represents day (in white) and night (in black) hours of three consecutive days. Down panels show periodogram analysis providing the periodicity of the data with tau values (L/D and D/L).

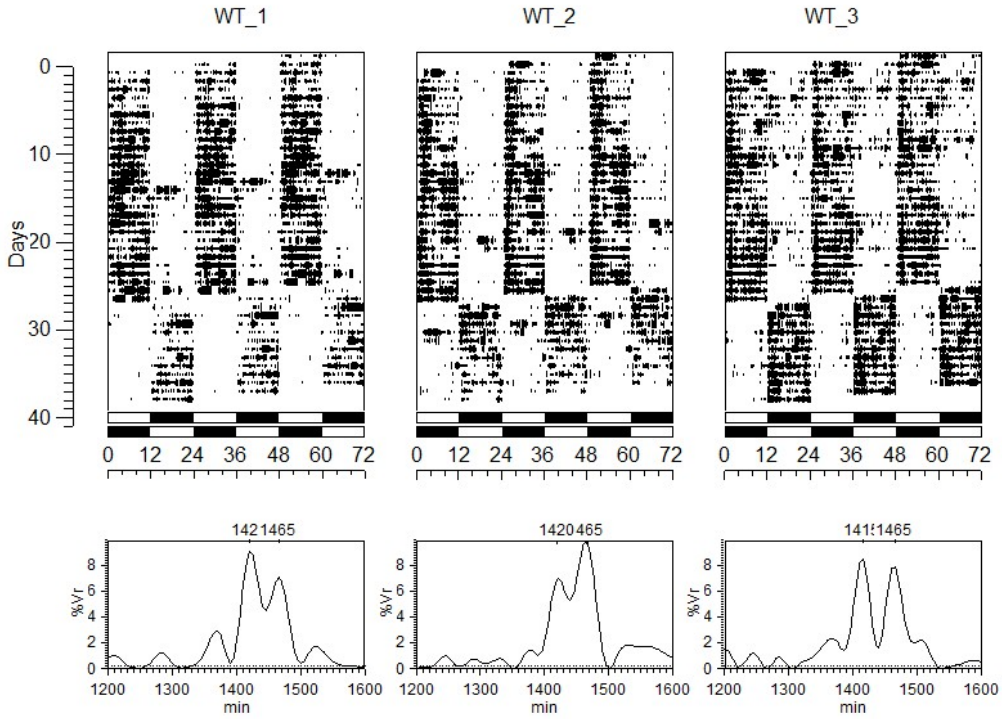

Figure S2b. Actograms of locomotor activity of WT animals maintained under constant LD photoperiod for 28 days.

Subsequently, the LD cycle was inverted, and LA screened for 12 days. Actograms are presented in a triple plot format (72 h time scale) to improve visualization. The horizontal bar at the bottom represents day (in white) and night (in black) hours of three consecutive days. Down panels show periodogram analysis providing the periodicity of the data with tau values (L/D and D/L) close 1440 m (24h) for WT animals.

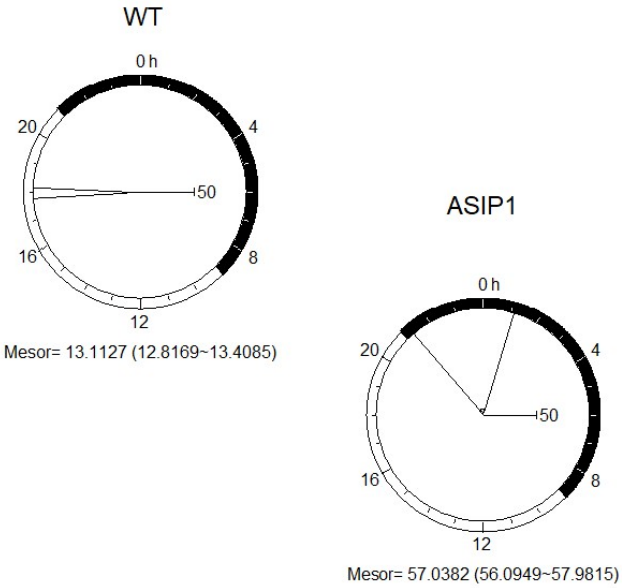

Figure S3. Cosinor fitness parameters of WT (upper left panel) and *asip1-Tg* fish (down-right panel) locomotor activity under 12L/12D photoperiod shown using circular graphs.

Data form all three replicas (n=3) were integrated for cosinor fitness (see material and methods for additional information). The black section of the cycle indicates the dark phase of the photoperiod. Mesor indicates the mean value of the adjusted rhythm and numbers between parenthesis indicates 95% confidence limits. Region inside the triangle indicates position of the rhythm acrophase (time for maximal amplitude of the rhythm) and 95% confidence limits.

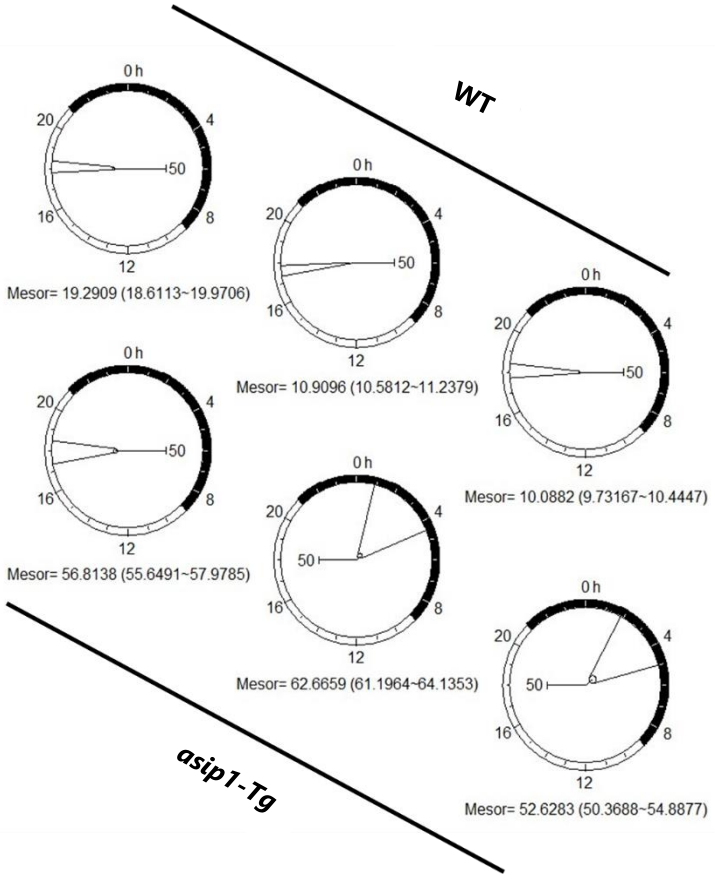

Figure S4: Cosinor fitness parameters of WT (down panels) and *asip1-Tg* fish (upper panels) of locomotor activity rhythms under 12L/12D photoperiod shown using circular graphs. Fitness for individual experimental tanks. See material and methods and supplementary Fig. 3 for additional information). The black section of the cycle indicates the dark phase of the photoperiod. Mesor indicates the mean value of the adjusted rhythm and numbers between parenthesis indicates 95% confidence limits. Region inside the triangle indicates position of the rhythm acrophase (time for maximal amplitude of the rhythm) and 95% confidence limits.

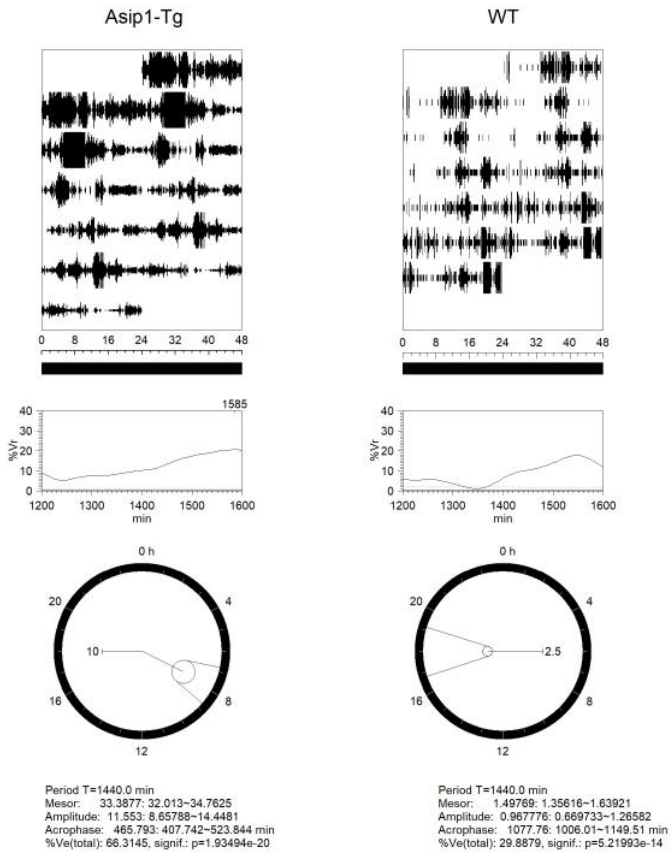

Figure S5: Charts (up) show the actograms of locomotor activity of WT (right) and *asip1*-Tg (left) animals maintained under a constant DD photoperiod for 6 days. Actograms are presented in a dual-plot format (48 h time scale) to facilitate visualization. Horizontal bars in black represent night for two consecutive days. The X axes indicate time of day (h) and Y axes indicate time (days). Periodogram analysis providing the periodicity of the data with tau values close to 1440 m (24h) for WT animals are shown just below periodograms. No periodicity was detected in both WT and asip1-Tg animals. Cosinor fitness parameters are shown at the bottom of the figure (see Fig S3 for more details).

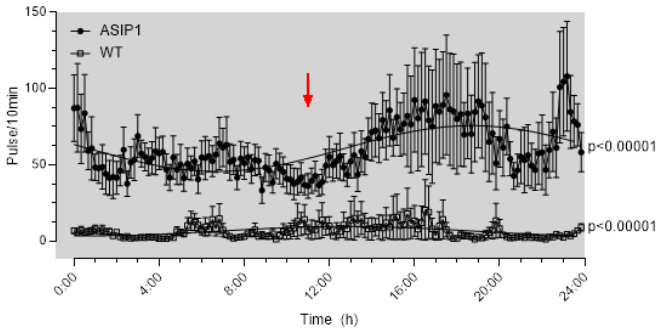

Figure S6: The average waveform of locomotor activity (LA) for adult asip1-Tg and WT zebrafish kept under constant DD photoperiod. Infrared sensors recorded LA for 6 consecutive days, and pulses were integrated every 10 min. Each point represents the mean ± SEM of three 6L tanks with 10 animals for each genotype. Rhythmicity was checked by cosinor fit run in El Temps v 1.313 (http://el-temps.com) and Cosinor Online (https://cosinor.online/app/cosinor.php) software. The significance level of the cosinor fit is indicated on the right-hand side of the figure. Red arrow indicate the feeding time.

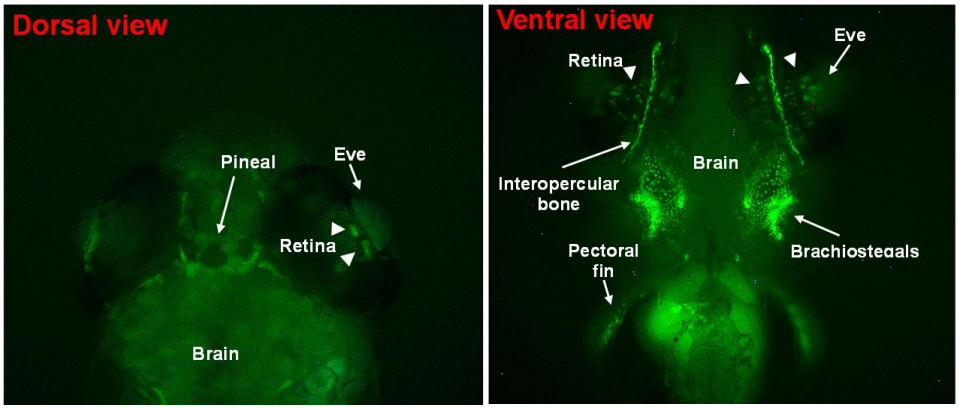

Figure S7: Green fluorescent expression under the control of *asip1* promoter in transgenic zebrafish (Drer.asip1-iTol2-eGFP-BAC) showing the absence of *asip1* expression in the pineal gland.

Right and left panel are dorsal and ventral views, respectively of a 30-days adult zebrafish. Note the significant asip1 expression in the retina.

CHAPTER 7

Obesity impairs cognitive function with no effects on anxiety-like behaviour in zebrafish

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Abstract

Over the last decade, the zebrafish has emerged as an important model organism for behavioural studies and neurological disorders, as well as for the study of metabolic diseases. This makes zebrafish an alternative model for studying the effects of energy disruption and nutritional quality on a wide range of behavioural aspects. Here, we used the zebrafish model to study how obesity induced by overfeeding regulates emotional and cognitive processes. Two groups of fish (n=16 per group) were fed at 2% (CTRL) and 8% (overfeeding-induced obesity, OIO) for 8 weeks and tested for anxiety-like behaviour using the novel tank diving test (NTDT). Fish were first tested in short-term memory test (STM) and then trained for four days for long-term memory tests (LTM). At the end of the experiment, fish were euthanised for biometric sampling, total lipid content and triglyceride analysis. In addition, brains (8 per treatment) were dissected for HPLC determination of monoamines. Overfeeding induced faster growth and obesity, as indicated by increased total lipid content. OIO had no effect on anxiety-like behaviour. Animals were then tested for cognitive function (learning and memory) using the aversive learning test in Zantiks AD units. Results show that both OIO and CTRL animals were able to associate the aversive stimulus with the conditioned stimulus (conditioned learning), but OIO impaired STM regardless of fish sex, revealing the effects of obesity on cognitive processes in zebrafish. Obese fish did not show a deficiency in monoaminergic transmission, as revealed by quantification of total brain levels of dopamine and serotonin and their metabolites. This provides a reliable protocol for assessing the effect of metabolic disease on cognitive and behavioural function, supporting zebrafish as a model for behavioural and cognitive neuroscience.

Keywords: Obesity; Overfeeding; Fat; BMI; Memory; Anxiety; Monoamines; Zebrafish

1. Introduction

There is currently an increasing volume of research focusing on the effects of metabolic disorders on cognitive and neurodegenerative processes [1]. Significantly, Alzheimer's disease has been proposed as "type III diabetes" not only because of the high risk of dementia associated with type II diabetes, but also due to the large amount of evidence on the existence of resistance to insulin in the brain during Alzheimer's disease [2]. Diet and nutrition are also tightly associated with mood disorders, including anxiety and depression, as well as other neuropsychiatric conditions [3]. Obesity, which is a global concern, challenges the health systems in Western societies and is also a major factor in metabolic syndrome, which has profound negative effects on brain structure [4] by impairing cognitive function and emotional states [1,5] thus exacerbating the development of learning/memory dysfunction and promoting anxiety-related responses [3,5].

In the last decade, zebrafish has emerged as a key model organism for behavioural studies [6] and neurological disorders [7,8], as well as for the study of metabolic diseases [9–12]. This makes zebrafish an interesting model for exploring the effects of energy disruption on behavioural aspects. Nutri-behavioural experiments mirroring those in mammalian species have also demonstrated the effect of diet-induced obesity (DIO), particularly high fat and/or carbohydrate diets, on cognitive function [13] and anxiety-like behaviour [14]. There is some controversy on the behavioural effects of obesity according to the method involved in promoting obesity. Overfeeding with regular diets not only promotes obesity but also metabolically healthy fish, in contrast to high-fat induced DIO, which generates hyperglycemia, ectopic lipid accumulation, and adipocyte hypertrophy, thus increasing visceral adipose tissue depots and inducing the expression of genes related to inflammation, fibrosis and lipid metabolism [15]. However, overfeeding-induced obesity (OIO) with

commercial food does induce deleterious effects on brain homeostasis and neuronal plasticity, thus promoting anxiety-like responses in zebrafish [16]. Although similar experiments following an analogous approach to generate OIO in zebrafish were not able to replicate anxiety-like responses or find differences in aversive learning assays [17,18]. In the face of such controversy, the effects of OIO on anxiety-like behaviour and short and long-term memory using aversive learning tests in zebrafish were explored. Moreover, the potential involvement of the central monoaminergic systems in obesity-induced behavioural and cognitive impairment was studied.

2. Materials and Methods

2.1. Animals and housing conditions

Zebrafish (*Danio rerio*) AB strains, purchased from the Zebrafish core facility at Karolinska Institutet, Solna, Sweden, were bred and housed in a recirculating Aquaneering system for zebrafish (Aquaneering, San Diego, CA 92126, USA) at Uppsala University Biomedical Centre. The system used copper-free non-chlorinated water from Uppsala municipality, 10% of which was exchanged daily. The temperature was set at 27ºC ±1.5 SD, pH at 7.5-8 and the photoperiod was 14-hours light and 10-hours darkness with lights on at 7 am. Fish were fed twice a day with rotifers and granulated feed (ZebrafishFeed, 63% protein, 13% fat, 1.8% fibre, and 1.2% ash, Sparos I&D, Olhao, Portugal) measured for each developmental stage. Subsequently, six-month-old zebrafish were distributed in 9L tanks (~50:50 sex ratio). Body weight (W) and length (L) were measured, and body mass index was calculated [$BMI=W(g)/L(cm)^2$] to ensure the absence of initial significant differences. Uppsala Animal Ethical Committee (permit 5.8.18-10125/2018) approved the use of animals in this study following the guidelines of the Swedish Legislation on Animal Experimentation (Animal

Welfare Act SFS1998:56) and the European Union Directive on the Protection of Animals Used for Scientific Purposes (Directive 2010/63/EU).

2.2. Experimental design

Two groups of 28 weight-graded fish were fed as previously mentioned at 2% (control, W=0.504g ± 0.975; L=2.882 ± 0.178, and BMI=0.060 ± 0.006) and 8% (overfeeding-induced obesity, OIO, W=0.510g ± 0.075, L=2.854 ± 0.129 and BMI=0.626 ± 0.007 SD) (Supplementary Fig. 1). No significant differences were detected at the beginning of the experiment as revealed by unpaired t-test ($p<0.05$). Biometrical samplings were carried out every two weeks for eight weeks (Fig.6A, 1A), such time was reported to be sufficient for inducing overfeeding obesity in zebrafish [59]. The animals were tested for anxiety-like behaviour using novel tank diving test (NTDT) (see below), and subsequently sampled for morphological parameters and individualised in tanks for acclimation for 24 hours prior to memory tests (Fig. 6A). Fish were first tested in short-term memory test (STM) and subsequently trained for four days for long-term memory tests (LTM) (Fig. 6A). At the end of the experiment, fish were euthanised by immersion in benzocaine (5ml/L) and biometrically sampled. Whole-body samples (n=16; eight per treatment, sex ratio 50:50) were taken for total lipids content and triglycerides analysis. In addition, brains (n=16; eight per treatment, sex ratio 50:50) were dissected for monoamine HPLC determination.

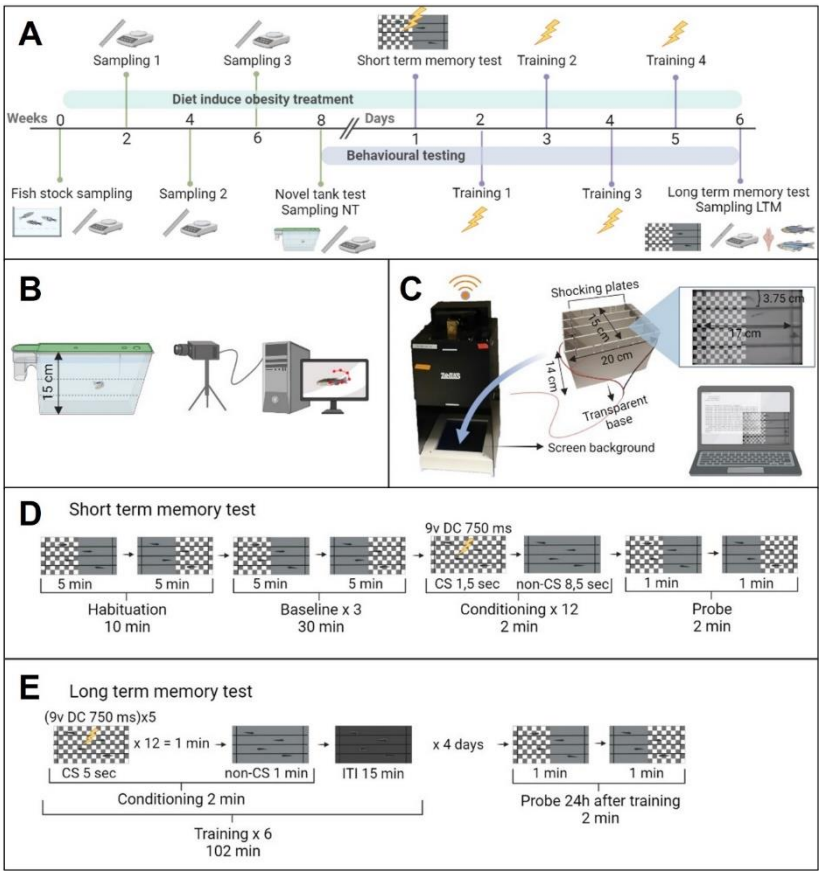


Figure 6. Experimental design diagram.

(A) Fish from the stock were weight graded and divided into control and overfeeding-induced obesity (OIO) groups. Animals were fed at 2 and 8%, respectively for 8 weeks and sampled for morphological parameters every two weeks. Following the feeding protocol, the behavioural tests began with novel tank diving test (NTDT). Animals were subsequently isolated in individual tanks for 24 hours (n=16 each treatment). Fish were then tested for short-term memory (STM), and subsequently trained for 4 days for LTM tests. Finally, fish were euthanised and sampled for morphological parameters. Eight fish were used for lipid quantification and the brain from the remaining eight fish dissected for monoamine determination by HPLC. (B) Novel tank diving test setup, fish were placed in trapezoidal tanks, and their trajectories were acquired for 10 minutes using Ethovision 16 XT. (C) Zantiks AD unit representation, used for aversive learning tests. (D) Short-term memory test protocol. Fish were habituated for 10 minutes to the experimental conditions in which the screen was half-divided into 'check' and 'grey' switching positions every 5 minutes. The potential preference for any background was recorded for 30 minutes (baseline). The same schedule used for the habituation period was then repeated 3 times. Throughout the conditioning phase, conditioned stimulus (CS) was

displayed for 1.5s and paired to the unconditioned stimulus (US, 9V DC shock for 750 ms) followed by 8.5s of non-conditioned stimulus ('check' or 'grey'). This protocol was repeated 12 times and learning and STM tested for 2 minutes. (E) Long-term memory test protocol. Fish were initially trained for 4 consecutive days. The conditioning phase was followed by an inter-trial interval (ITI) of 15 min to allow memory consolidation and the probe phase was performed 24 hours after the last training session. CS was displayed for 5 seconds and linked to the US (5 shocks of 9V DC, 750ms each). This protocol was repeated 12 times and subsequently non-CS was shown for 1 minute followed by the ITI (absence of stimulus). Conditioning + ITI were repeated 6 times for each training session. Finally, 24 hours after the last training session, the probe phase took place. In both STM and LTM tests, the preference for conditioned stimulus (PCS) was calculated as total time in CS / (time in CS + time in non-CS). Significant differences between baseline and probe PCS's were analysed. The significantly lower probe PCS, the memory enhancer was achieved.

2.3. Novel tank diving test (NTDT)

For NTDT, fish from each experimental group (Control, n=24 and OIO, n=24) were scooped from the home tank and placed in a trapezoidal tank (L:27cm x H:15cm x W:5cm, Aquaneering ZT180T) filled with 1.8 L of tap water as described in Fig. 6B. Four fish were tested simultaneously on a shelve, but tank sides were light-shielded to avoid visual contact between subjects. An infrared light was placed behind the shelf to improve contrast during recordings. The NTDT took 10 minutes after 30 seconds of habituation. Video recording and tracking data acquisition were carried out with Basler acA 1300 camera and Ethovision XT 16 (Noldus Inc., Wageningen, the Netherlands) (Fig. 6B). All trials were performed on the same day (9 am – 12 am). After NTDT, each fish was individualised in 2.8L tanks (Aquaneering ZT280T) for subsequent memory tests (see 2.4). In order to analyse the bottom-dwelling natural response to novelty, known as geotaxis [19] (Cachat et al., 2010), followed by a gradual exploration of the new environment, each arena was divided into 3 equal zones: top, middle and bottom (Fig. 6B); distance travelled, mean velocity, angular velocity, mean turn angle and time spent moving were measured for each zone. Latency to the first top zone visit, frequency of visits and the number of freezing bouts were also recorded.

2.4. Aversive learning methodology

To test the effect of obesity on cognitive traits, conditioned aversive learning was adopted. This behavioural paradigm involves associative learning to induce fish to link a visual cue to an aversive stimulus. Individual fish previously tested in NTDT were randomly used for aversive learning experiments. Tests were carried out using four Zantiks AD units (Zantiks Ltd., Cambridge, UK), equipped with fully automated systems which require minimal intervention in order to avoid disturbances during trials. Each unit consists of a camera, computer and floor screen to display visual cues and a tank (H:15cm x W:14cm x D:20cm) equipped with two stainless steel plates connected to a power supply to deliver electric shocks. Zantiks AD technology allows the testing of 4 fish simultaneously, two control and two OIO (Fig. 6C) [20] (Brock et al., 2017). Zantiks AD tanks were filled with 3 L of tap water at the same temperature as their home tank, and fish were then randomly placed individually in each compartment. In both short and long-term memory tests, the visual stimulus consisted of two different patterns selected and named 'check' (black or white alternated squares) and 'grey' (solid light grey) (Fig. 6C) [21–23] Conditioned stimulus (CS) was 'check' while non-conditioned stimulus (non-CS) was grey. The unconditioned stimulus (US) was a 9 V DC electric shock in both memory tests.

2.4.1. Short-term memory test (STM)

The STM testing protocol was an adaptation of the method employed by previous authors [20,22–24] and was tested in preliminary experiments (data not shown). The script developed for Zantiks web application to carry out this test is available on GitHub (https://github.com/Godino-Gimeno-A/aversive-learning/tree/Short-term-memory-test). As described in Fig. 6D, fish were acclimated for 10 min, displaying the visual stimuli in a half-divided background screen. The patterns were side switched after 5 minutes. This habituation phase was followed by a baseline period, which is

essential in order to ensure that fish avoid any place preferences. The baseline period lasted 30 minutes, during which, the same switching protocol used in habituation was repeated 3 times. Conditioning was then conducted to induce the fish to associate visual stimuli with a negative reward. CS ('check' or 'grey') was presented in the full screen for 1.5 seconds, paired with the US (9V DC shock for 750 ms) and followed by 8.5 seconds of non-CS displaying ('check' or 'grey'), this procedure was repeated 12 times. Finally, subjects were tested to determine whether short-term memory was achieved, by observing whether the animals learned and remembered to avoid CS. The test phase lasted 2 minutes, throughout which, the fish were exposed to both CS and non-CS, which switched position after 1 minute in order to detect any place preference disturbances. The preference for conditioned stimulus (PCS) in baseline and test phases were calculated as Total time in CS / (Time in CS + Time in non-CS).

2.4.2. Long-term memory test (LTM)

The LTM in zebrafish using the Zantiks AD system was adapted for aversive learning on the previous work in Drosophila [25]. The script for this test was developed after several preliminary trials (data not shown) and is available on GitHub (https://github.com/Godino-Gimeno-A/aversive-learning/tree/Long-term-memory-test). This protocol lasted 5 days (Fig. 6E) and consisted of 4 training sessions followed by the test. The first training session was carried out the day after the STM test, and the previous CS were maintained for each fish. As shown in figure 6E, "conditioning", the CS was displayed for 5 seconds, matched with the US (5 shocks of 9V DC for 750ms each) and repeated 12 times. Non-CS was then shown in the whole arena for a minute. After conditioning, an inter-trial interval (ITI), during which no stimulus was applied, was performed for 15 minutes to allow memory consolidation. Each training session comprised 6 consecutive trials (conditioning + ITI). Training trials were carried out at 9 am for 4 consecutive days. 24 hours after the last training

session, the test was performed for 2 minutes following the same protocol as that used in STM tests, subsequently, PCS were calculated.

2.5. Total lipids and triglycerides

Whole-body samples from both groups (8 of each genotype, sex ratio 50:50) were lyophilised, weighed, and homogenised into fine particles. Lipid extraction was carried out following Folch's method [26] and was quantified gravimetrically. Subsequently, the percentage of total lipids was calculated for each sample. A fraction of each sample (~50 mg) was used in the Triglyceride colourimetric assay kit from Cayman Chemical (Cayman Chemical Company, Ann Arbor, USA) and the percentage of triglycerides was subsequently calculated.

2.6. Monoamine analysis

At the end of the study, OIO and control brains were dissected (8 of each genotype, sex ratio 50:50), and stored at -80^{0}C. The samples were thawed in ice-cold phosphate buffer (pH= 7.4, 1M) and homogenised using an ultrasonic disruptor. Levels of 5-hydroxytryptophan (5HTP), serotonin (5-hydroxytryptamine, 5-HT) and 5-hydroxy indoleacetic acid (5HIAA) were determined using HPLC with electrochemical detection [27]. Additionally, metabolites of the dopaminergic pathway were measured: L-dihydroxyphenylalanine (L-dopa), dopamine (DA) and dihydroxyphenylacetic acid (DOPAC). The mobile phase (pH = 2.95) content was 63.9mM NaH_2PO_4, 0.1mM Na_2EDTA, 0.80mM sodium 1-octanesulfonate and methanol 15.3% (v/v). HPLC equipment consisted of a Jasco pump (PU-2080); a reverse-phase analytical column (Phenomenex, Kinetex C18, 5 µm, 100 Å, 150 mm x 4.6 mm); an M5011 double analytical cell (first electrode: +40mV; second electrode +340mV) to oxide analytes and an ESA Coulochem II detector. Results were obtained using ChromNAV 1.12 software (Jasco

Corporation). Levels of each metabolite per total protein content in the brain (measured with BCA), 5HIAA/5HT ratio, and DOPAC/DA ratio were calculated.

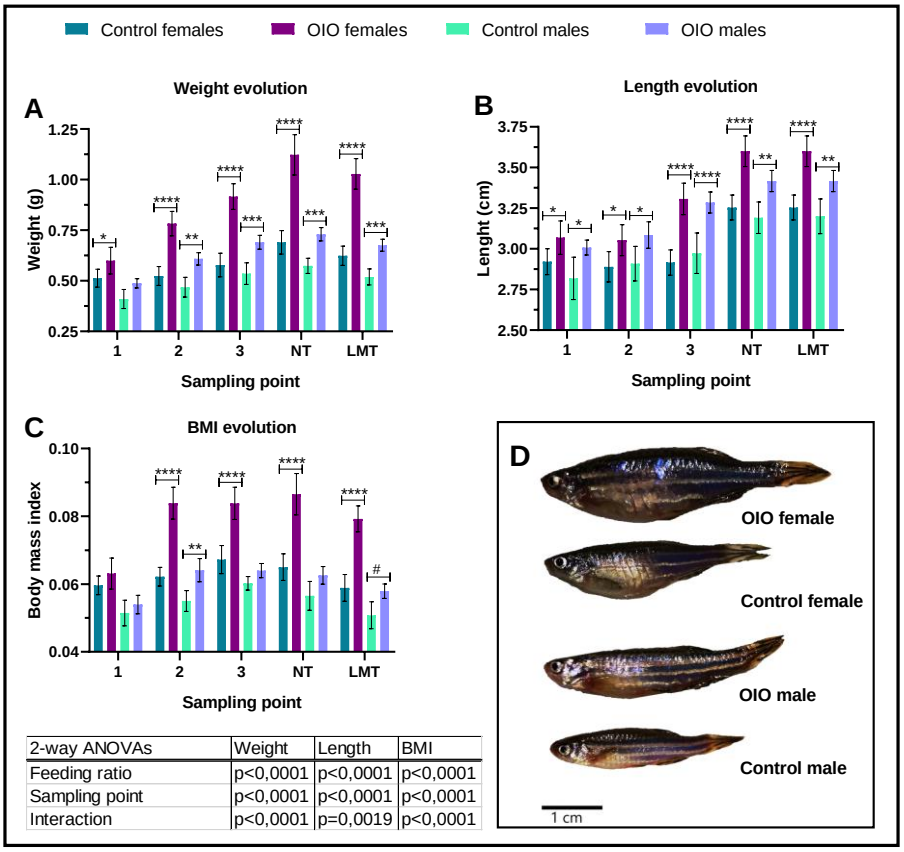
2.7. Statistics

Statistical analyses were performed with GraphPad Prism 8. A two-way ANOVA and Tukey's multiple comparison test were performed for morphological parameters. Regarding NTDT, significant differences between the control and OIO groups and between sexes with Two-way ANOVA were researched. No multiple comparison tests were run since interactions were not significant. In STM and LTM tests, significant differences between the PCS baseline and the tests within both experimental groups and between control and OIO were assessed by mixed-effects two-way ANOVA and Sidak's multiple comparisons test. An unpaired t-test was employed in studying differences between groups in the percentage of total lipids content and triglycerides and in monoamines levels. All data were represented as mean with ± SEM.

3. Results

3.1. Overfeeding induces obesity in zebrafish.

Overfeeding induced an increase in weight and length of OIO animals of both sexes (Fig. 1). Significant differences in weight appeared earlier in females (2 weeks) than males (4 weeks), yet differences in length began as early as 2 weeks in both sexes (Fig. 1A, B) and were maintained until the end of the experiment. After 8 weeks, at the beginning of behavioural studies, OIO females and males were 62% and 27% heavier than control counterparts, respectively (Fig. 1A). Significant differences in BMI started after a month of overfeeding in females and males yet were only maintained at significant levels until the end of the experiment in OIO females (Fig. 1C). Accordingly, OIO fish exhibited a higher lipid percentage (~ 200%) than control animals (Fig. 2A) yet triglyceride levels were similar (Fig. 2B). Together, biochemical and morphological data support overfeeding induced growth and obesity in zebrafish (Fig. 1D).



2-way ANOVAs	Weight	Length	BMI
Feeding ratio	p<0,0001	p<0,0001	p<0,0001
Sampling point	p<0,0001	p<0,0001	p<0,0001
Interaction	p<0,0001	p=0,0019	p<0,0001

Figure 1. Experimental dynamics of morphological parameters.

Fish were sampled every two weeks in 1, 2, 3 and NT (NTDT testing day) and one week between NT and LTM (long-term memory test probe phase). (A) Weight (B) Length and (C) Biomass index (BMI). (D) Representative images of control and OIO, females and males at the end of the study. Data are expressed by mean ±SEM analysed by two-way ANOVA and Tukey's multiple comparison test. Asterisks indicate significant differences between OIO and control within the same gender (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001), and # p = 0.051.

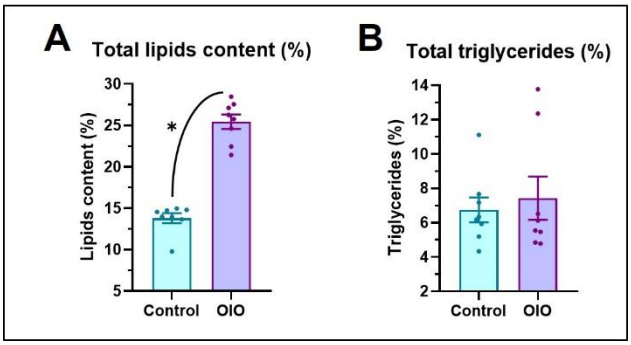
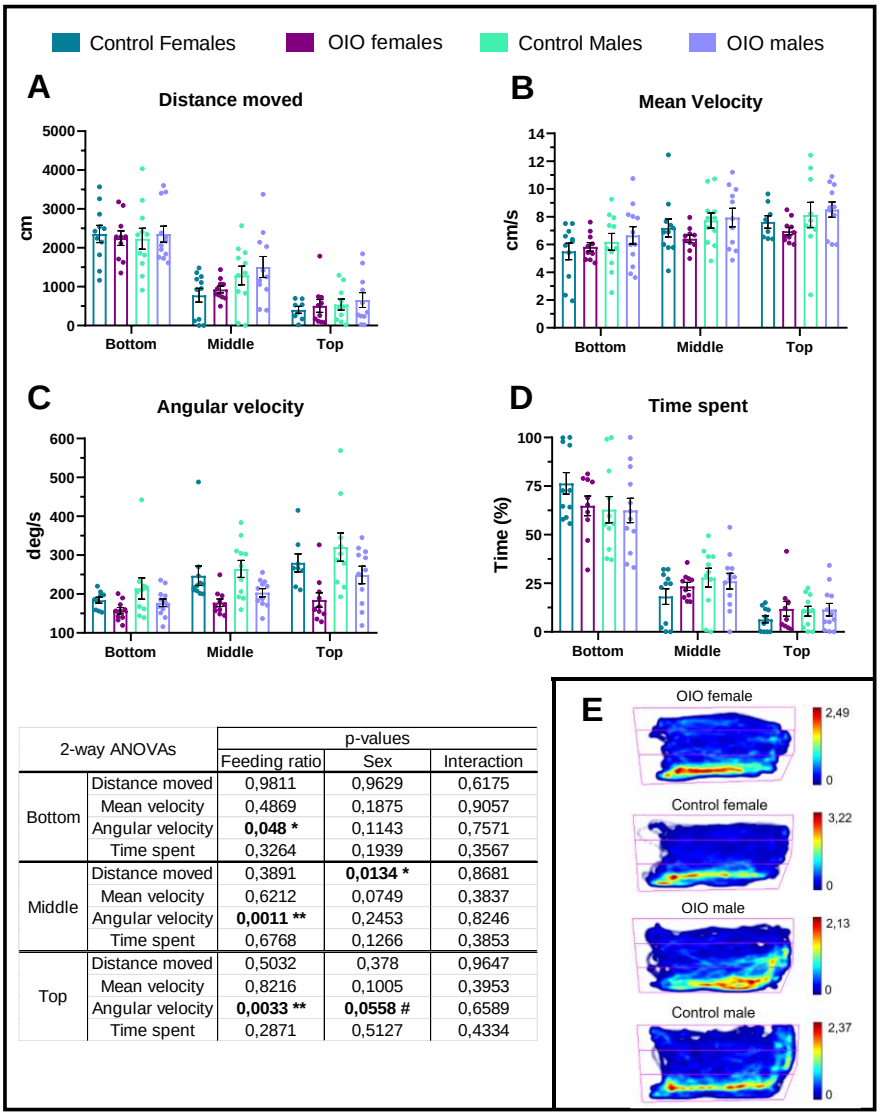


Figure 2. (A) The percentage of total lipids. (B) Triglycerides content

(n=8 per treatment, sex ratio 50:50). Data are expressed by mean ± SEM and analysed by unpaired t-test (*p ≤ 0.05).

3.2. Obesity does not promote anxiety-like behaviour in zebrafish.

In order to study the anxiety-like behaviour in obese zebrafish, the geotaxis of OIO and control animals using NTDT was assessed. Distance travelled was similar regardless of the feeding level in all tank areas (Fig. 3A) yet the females covered less distance in the middle region of the tank (Fig. 3A). The mean velocity was similar in every animal regardless of the feeding ration and sex (Fig. 3B) yet remarkably, angular velocity was always depressed in OIO animals in all tank areas (Fig. 3C). Time spent in each tank area was similar in every animal and arena (Fig. 3D, 3E). Another two potential indicators of anxiety-like behaviour are the latency to the top zone and frequency of visits to this zone, but no difference was observed between feeding levels (Supplementary Fig. 3). Remarkably, the sex had a significant effect on latency, since males, regardless of the feeding level, took much less time to visit the upper region of the tank (Supplementary Fig. 3). Finally, effects on freezing bouts were not studied due to the limited number of fish displaying a brief single freezing episode (2 control females and 1 male, 2 =IO males) (data not shown).



2-way ANOVAs		p-values		
		Feeding ratio	Sex	Interaction
Bottom	Distance moved	0,9811	0,9629	0,6175
	Mean velocity	0,4869	0,1875	0,9057
	Angular velocity	**0,048 ***	0,1143	0,7571
	Time spent	0,3264	0,1939	0,3567
Middle	Distance moved	0,3891	**0,0134 ***	0,8681
	Mean velocity	0,6212	0,0749	0,3837
	Angular velocity	**0,0011 ****	0,2453	0,8246
	Time spent	0,6768	0,1266	0,3853
Top	Distance moved	0,5032	0,378	0,9647
	Mean velocity	0,8216	0,1005	0,3953
	Angular velocity	**0,0033 ****	**0,0558** #	0,6589
	Time spent	0,2871	0,5127	0,4334

Figure 3. Novel tank diving test (NTDT).

Anxiety-like behaviours were measured in three zones: bottom, middle, and top. (A) distance travelled, (B) mean velocity per zone, (C) angular velocity and (D) time spent in each zone. (E) Representative heatmaps of fish movements. The colour scale represents the cumulative time spent in each of the previously defined arena zones: bottom, middle and top. Data are represented by mean ±SEM and analysed by two-way ANOVA. Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01), and hashtag indicates an apparent significant difference (# p = 0.051-0.06).

3.3. Obesity does not alter central monoamine levels in zebrafish.

To determine whether diet-induced obesity has any effect on brain serotoninergic and dopaminergic pathways, the central levels of the metabolites by HPLC were analysed. No significant differences in either monoamines and/or degradation metabolites induced by obesity were found (Fig. 4).

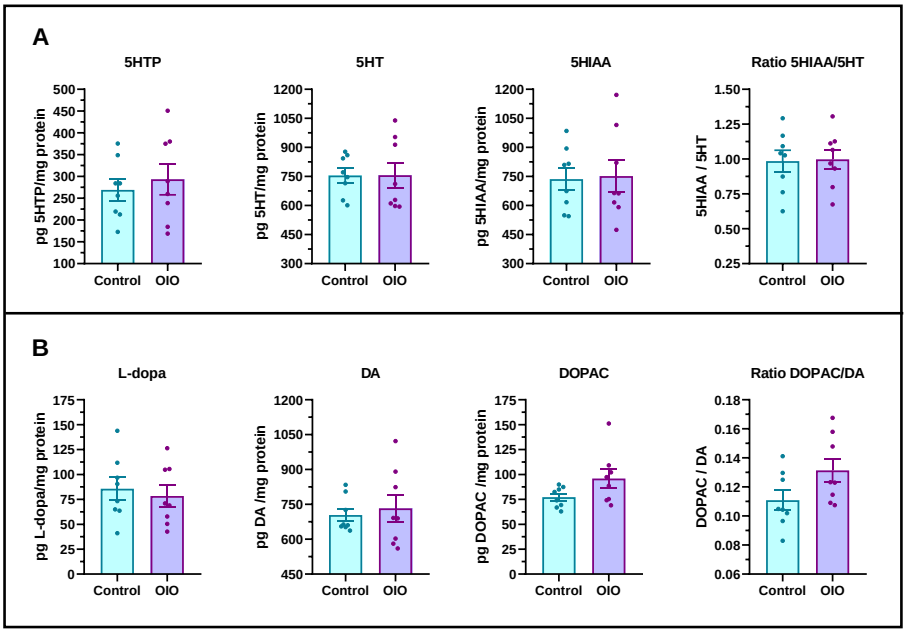
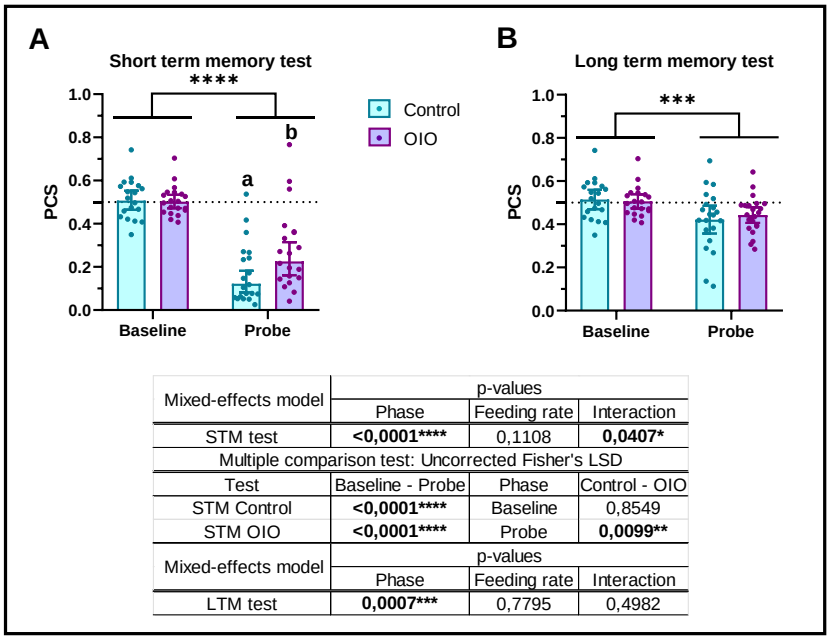


Figure 4. Brain serotoninergic and dopaminergic metabolites.

(A) 5-hydroxytryptophan (5HTP), serotonin (5HT) and 5HIAA/5HT ratio. (B) Levels of L-dihydroxyphenylalanine (L-dopa), dopamine (DA), and dihydroxyphenylacetic acid (DOPAC) and DOPAC/DA ratio (n=8 each per treatment sex ratio 50:50). Results are expressed in pg of metabolite per mg of total protein. Data are represented by mean ±SEM analysed by unpaired t-test.

3.4. Obesity impairs short-term memory in zebrafish with no effects on LTM.

In order to investigate the effects of obesity on memory, two different tests for STM and LTM were carried out. Initial analysis indicated that both sexes responded similarly regardless of the feeding ratio (Supplementary Fig. 4), so both sexes were coupled in posterior analysis to increase the sample size. Regarding the STM, both groups exhibited similar PCS during the baseline phase (PCs = 0.5). Significant differences between baseline and test phase demonstrated that both groups learned and memorized to avoid CS (Fig. 5A). However, OIO fish exhibited significantly higher PCS level than control fish, supporting less efficiency in short-term memory (Fig. 5A). LTM showed that zebrafish consolidated the information to avoid CS after 24h, however, no significant differences were found between both feeding rates during the test phase (Fig 5B).



Mixed-effects model	p-values		
	Phase	Feeding rate	Interaction
STM test	**<0,0001******	0,1108	**0,0407***
Multiple comparison test: Uncorrected Fisher's LSD			
Test	Baseline - Probe	Phase	Control - OIO
STM Control	**<0,0001******	Baseline	0,8549
STM OIO	**<0,0001******	Probe	**0,0099****
Mixed-effects model	p-values		
	Phase	Feeding rate	Interaction
LTM test	**0,0007*****	0,7795	0,4982

Figure 5. Effects of obesity on short and long-term memory.

(A) Short-term memory (STM), (B) long-term memory (LTM). PCS, preference for conditioned stimulus. Data are represented by mean ± SEM. The dotted line indicates no stimulus preference (PCS = 0.5), low and high PCS mean less or more time spent in conditioned stimulus (CS), respectively. Results were analysed by repeated measures mixed-effect model and, when interaction was significant, uncorrected Fisher's LSD test for multiple comparisons. Asterisks indicate significant differences between phases, baselines and probes (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001), and letters indicate significant differences between control and OIO probe.

4. Discussion

Obesity is a major health concern for Western societies and to a great degree responsible for the metabolic syndrome which promotes an elevated risk of cardiovascular diseases and type II diabetes, in addition to neuropsychiatric and neurodegenerative disorders such as Parkinson's and Alzheimer's diseases [5]. Zebrafish can be used as a model species to study such central disorders since obesity can be easily induced by overfeeding on regular or high-fat diets [15] and, thereby, an excellent model for comparative approaches in behavioural neurosciences [6]. In this study, the effect of OIO on anxiety-like behaviour and cognitive processes in zebrafish is explored. To induce obesity, a previously successful overfeeding protocol for zebrafish Tu strain [28] was used, which is based exclusively on the increase of daily dry-food ratio (2% vs 8%). In the present studies, the AB strain was used as it is commonly accepted to study obesity and obesity-related processes [15]. The increase of the feeding rate in OIO fish resulted in increased W, L and BMI, in addition to elevated total fat percentage compared to control animals (~60%), supporting the obese condition of OIO fish as previously reported [15–17,28]. Females consistently exhibited an increased BMI from the second week of the overfeeding protocol, yet no differences were detected in males from 3 weeks to the end of the experimental period. Therefore, the growth dynamic in the sexes appears to be different, with the result that females are more adept at increasing energy density when overfed than males, likely, due to the increased oocyte production. Similar results were obtained in our previous experiments [28,29] and by other authors [16]. In any case, BMI obtained in overfed fish (>0.08 and 0.06 in females and males, respectively) were higher than those reported in previous experiments using similar overfeeding protocols [15,16]. Overall, growth performance parameters, high BMIs and much higher fat percentage levels support OIO in AB zebrafish.

The manner in which obesity is induced in zebrafish seems to have effects on behavioural responses and particularly in anxiety-like responses. Zebrafish, either larvae or adult specimens, fed with obesogenic diets containing high-fat levels exhibit anxiety-like behaviours [14,30], however, obese fish having regular diets at high ratios failed to exhibit such behaviour [17,18], yet only the total distance travelled and time spent in the bottom were evaluated in NTDT. On the contrary, OIO protocols severely decreased locomotor activity in zebrafish, which was restored when animals switched to a standard dietary ratio [16]. Due to the wide spectrum of behavioural responses displayed in NTDT by zebrafish [31–33], it was decided to enlarge the behavioural variables to corroborate previous results and study the activation of the central monoaminergic circuitry. Our results support the absence of anxiety-like behaviours in obese animals. Differences linked only to feeding levels in the angular velocity and mean turn angle in all three areas of the experimental arena were found. It is thought that these differences alone are not indicative of anxiety-like behaviours since they could be explained by less agile movements of obese fish due to their rounded-body shape in contrast to the lean control animals. However, such results reveal the sensitivity of the geotaxis assays to decipher potential behavioural differences. In addition, sex-induced differences were also found in the total distance travelled and latency to the top zone of the arena by females which was inferior to that observed in males. Previous studies have demonstrated that females might exhibit lower locomotor activity regardless of body weight and length [34]. Once more, it is thought that the differences observed are not indicative of a sex-linked anxiety-like response. It is unknown why the way in which obesity is induced promotes different effects on anxiety-like behaviour. Previous studies compared both DIO and OIO effects on zebrafish physiology [15]. DIO animals exhibited metabolic alterations in contrast to OIO zebrafish, including hyperglycaemia, adipocyte hypertrophy, ectopic hepatic lipid accumulation as well as key differences for gene markers in

inflammation, lipid metabolism and fibrosis [15]. Similar studies using OIO protocols in adult males did report hyperglycaemia, increased oxidative stress and reduced neuronal plasticity, which was revealed by a decrease in the cell proliferation in the telencephalon and diencephalon of zebrafish [16]. In addition, the integrity of the brain blood barrier (BBB) in the hypothalamic parenchyma is slightly challenged in OIO zebrafish fed with dry food in the absence of neuro-inflammatory markers [16]. Contrary, the combination of dry food and artemia promotes severe BBB impairment and neuro-inflammation [35], suggesting once more that the diet composition, most likely, the lipid levels and quality, may induce different effects on brain homeostasis [12]. Overall, present reported data does not allow a proposal of any mechanism as DIO and OIO differentially regulate anxiety-like behaviour in zebrafish and a standardization of DIO/OIO protocols is needed for a correct comparison of experimental results. Anxiety-like behaviour in zebrafish is mediated partially by serotoninergic neurons in the posterior raphe of the caudal brain [36], as well as sensory responsiveness during arousal [37]. The potential effect of OIO on the activation of central monoaminergic circuits was measured by quantifying total 5-HT and dopamine levels and their primary metabolites in the whole zebrafish brain. The absence of significant differences supports previous results showing no effects of OIO on anxiety-like behaviour.

Obesity has been previously reported to impair cognitive function in vertebrates, including zebrafish [1,5,12]. Learning can lead to both STM and LTM [38], presumably managed and stored by different, yet interconnected, neuronal systems [39]. STM last from seconds to minutes and its formation depends on biochemical changes, whereas LTM lasts from hours to years and its formation relies on new protein synthesis [38,40]. In adult zebrafish, experiments focused on the study of social memory have estimated the period of 24h as a long-lasting memory [41], although a similar social reward induced 36h long-lasting memory in larval zebrafish [40]. Using the

active avoidance test, the effect of OIO on learning capacity, STM and LTM was studied. Results, expressed as the relation between the time expended in the CS versus total time (PCS), demonstrate that the animals were able to identify and avoid the CS. This implies that fish learn and memorize the association between the negative reward with the CS, as shown by a severe decrease in PCS after short-term periods. Animals were further able to retain this association for a 24h period (LTM), although PCS values were higher than those in STM, showing a higher efficiency of STM when compared to LTM. OIO animals exhibited higher short-term PCS levels than control animals suggesting that STM performance in obese animals is impaired. Although animals retained the stimulus/reward association for 24h corroborating the LTM, there were no differences in PCS values between both metabolic conditions, thus suggesting OIO had no effects on LTM. The existence of STM and LTM has been demonstrated in zebrafish [41], and the effects of DIO on STM [13] and LTM have previously been studied [30]. DIO induced an impairment of both STM and LTM, but OIO protocols had no effect on STM formation [17], yet intergenerational effects of obesity were reported [18]. Unfortunately, OIO effects on LTM response were not studied. The present study focused on both STM and LTM, showing that OIO had significant effects on STM but no effects on LTM. Once more, the procedure involved in inducing obesity (DIO vs OIO) in zebrafish appears to severely affect the cognitive process. Therefore, DIO exhibits consistent effects on both STM and LTM, whereas OIO had no effect on LTM and controversial effects on STM ([17] vs the present study). Since diet composition appears to be critical for obesity-induced cognitive and behavioural impairment, the aforementioned controversy may stem from the dietary components involved in generating obesity by overfeeding. Previous studies involved solely freshly hatched artemia [42] or combined with commercially available fish food [17]. However, in this study, rotifer was used in combination with a dry diet which was specifically formulated for zebrafish husbandry, firmly showing a different

nutritional composition to that used in previous studies [17,18,42]. Therefore, standardization of dietary protocols, diet composition and ulterior studies on the effect of specific nutrients in obesity-induced cognitive and behavioural impairment appears to be critical for future investigation.

The underlying mechanisms responsible for the obesity-induced effects on cognitive performance are unknown, however, obesity is associated with a decreased hippocampal neurogenesis [43], a function which has been shown to be enhanced during active learning [44]. Such obesity-induced effect on neurogenesis is probably mediated by neuro-inflammatory processes deriving from high-fat diets [43]. Interestingly, studies in DIO zebrafish have shown enhanced oxidative stress and decreased cell proliferation in neurogenic niches of the diencephalon and ventral and dorsomedial telencephalon [14,16], a fish homologue of the mammalian amygdala [45]. This complex pallial/subpallial structure plays a key role in Pavlovian fear conditioning [46]. The glutamatergic nuclei of the pallial amygdala are essential for the association of CS and US, which sends projections to the subpallial amygdala that serves as a primary output region projecting to different brain nuclei for the control of fear response [47–49]. The surgical or genetic ablation of the dorsomedial telencephalon promotes deficits in performing the avoidance response, thus indicating a role in the conditioned avoidance response [47,48]. LTM formation in zebrafish depends on protein synthesis and glutamatergic transmission since the incubation of larval zebrafish with protein synthesis inhibitors or NMDA receptor antagonists during training periods prevented or impaired memory formation, respectively [40]. Suggestively, the dorsomedial telencephalon in zebrafish has been shown to be rich in glutamatergic and GABAergic neurons, which mainly project to the subpallial and hypothalamic areas [47]. Since high-fat diets have been shown to induce changes in hippocampal glutamate metabolism and glutamatergic transmission [50], it is,

therefore, conceivable that obesity-induced injuries in the glutamatergic population of dorsomedial pallium may promote deficits in the aversive learning assays.

5. Conclusions

In summary, it can be observed that OIO has no effect on anxiety-like behaviour and LTM acquisition but impairs STM regardless of the fish sex, revealing the obesity effects on cognitive processes in zebrafish. Results further suggest that these effects are entirely dependent on food protocols and/or diet composition. Obese fish exhibited no deficiency of monoaminergic transmission, revealed by the quantification of total brain levels of dopamine and 5-HT and their metabolites. Finally, a reliable protocol is provided to assess the effect of metabolic diseases on cognitive and behavioural function, thus supporting zebrafish as a model in behavioural and cognitive neuroscience.

Author Contributions

A.G.-G.: conceptualization, methodology, validation, formal and statistical analysis, investigation, data curation, visualization, writing—original draft, revising and editing. P.-O.T.: conceptualization, methodology, validation, investigation, supervision, reviewing and editing. MC: HLPC analysis, reviewing and editing. JMM: supervision, reviewing and editing. SW: conceptualization, methodology, validation, funding acquisition, investigation, supervision, reviewing and editing. J.M.C.-R.: conceptualization, methodology, validation, funding acquisition, investigation, supervision, writing—original draft, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

All experiments were performed following guidelines of the Spanish (Royal Decree 53/2013), the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Union Directive on the Protection of Animals Used for Scientific Purposes (Directive 2010/63/EU). The protocols applied were approved by the Uppsala Animal Ethical Committee and IATS Ethics Committee (Register Number 09-0201) under the supervision of the Secretary of State for Research, Development and Innovation of the Spanish Government.

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Conflicts of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Sarangi, M.; Dus, M. Crème de La Créature: Dietary Influences on Behavior in Animal Models. Front Behav Neurosci 2021, 15, 746299, doi:10.3389/FNBEH.2021.746299/BIBTEX.
2. Hamzé, R.; Delangre, E.; Tolu, S.; Moreau, M.; Janel, N.; Bailbé, D.; Movassat, J. Type 2 Diabetes Mellitus and Alzheimer's Disease: Shared Molecular Mechanisms and Potential Common Therapeutic Targets. Int J Mol Sci 2022, 23, doi:10.3390/IJMS232315287.
3. Adan, R.A.H.; Cirulli, F.; Dye, L.; Higgs, S.; Aarts, K.; van der Beek, E.M.; Buitelaar, J.K.; Destrebecq, F.; De Witte, E.; Hart-mann, T.; et al. Towards New Nutritional Policies for Brain Health: A Research Perspective on Future Actions. Brain Behav Immun 2022, 105, 201–203, doi:10.1016/J.BBI.2022.07.012.
4. Medawar, E.; Witte, A.V. Impact of Obesity and Diet on Brain Structure and Function: A Gut-Brain-Body Crosstalk. Proc Nutr Soc 2022, 81, doi:10.1017/S0029665122002786.
5. Zhang, Q.; Jin, K.; Chen, B.; Liu, R.; Cheng, S.; Zhang, Y.; Lu, J. Overnutrition Induced Cognitive Impairment: Insulin Re-sistance, Gut-Brain Axis, and Neuroinflammation. Front Neurosci 2022, 16, 884579, doi:10.3389/FNINS.2022.884579/BIBTEX.
6. Gerlai, R. Zebrafish (Danio Rerio): A Newcomer with Great Promise in Behavioral Neuroscience. Neurosci Biobehav Rev 2023, 144, doi:10.1016/J.NEUBIOREV.2022.104978.
7. Chia, K.; Klingseisen, A.; Sieger, D.; Priller, J. Zebrafish as a Model Organism for Neurodegenerative Disease. Front Mol Neurosci 2022, 15, 940484, doi:10.3389/FNMOL.2022.940484/BIBTEX.
8. Liu, Y. Zebrafish as a Model Organism for Studying Pathologic Mechanisms of Neurodegenerative Diseases and Other Neural Disorders. Cellular and Molecular Neurobiology 2023 2023, 1–18, doi:10.1007/S10571-023-01340-W.
9. Zang, L.; Maddison, L.A.; Chen, W. Zebrafish as a Model for Obesity and Diabetes. Front Cell Dev Biol 2018, 6.
10. Williams, M.B.; Watts, S.A. Current Basis and Future Directions of Zebrafish Nutrigenomics. Genes Nutr 2019, 14, 1–10, doi:10.1186/S12263-019-0658-2/METRICS.
11. Chowanadisai, W.; Hart, M.D.; Strong, M.D.; Graham, D.M.; Rucker, R.B.; Smith, B.J.; Keen, C.L.; Messerli, M.A. Genetic and Genomic Advances in Developmental Models: Applications for Nutrition Research. Adv Nutr 2020, 11, 971–978, doi:10.1093/ADVANCES/NMAA022.

12. Ghaddar, B.; Diotel, N. Zebrafish: A New Promise to Study the Impact of Metabolic Disorders on the Brain. Int J Mol Sci 2022, 23, doi:10.3390/IJMS23105372.
13. Meguro, S.; Hosoi, S.; Hasumura, T. High-Fat Diet Impairs Cognitive Function of Zebrafish. Sci Rep 2019, 9, doi:10.1038/s41598-019-53634-z.
14. Türkoğlu, M.; Baran, A.; Sulukan, E.; Ghosigharehagaji, A.; Yildirim, S.; Akgül Ceyhun, H.; Bolat, İ.; Arslan, M.; Buğrahan Ceyhun, S. The Potential Effect Mechanism of High-Fat and High-Carbohydrate Diet-Induced Obesity on Anxiety and Off-spring of Zebrafish. Eating and Weight Disorders - Studies on Anorexia, Bulimia and Obesity 2022, 27, 163–177, doi:10.1007/s40519-021-01140-5.
15. Landgraf, K.; Schuster, S.; Meusel, A.; Garten, A.; Riemer, T.; Schleinitz, D.; Kiess, W.; Körner, A. Short-Term Overfeeding of Zebrafish with Normal or High-Fat Diet as a Model for the Development of Metabolically Healthy versus Unhealthy Obesity. BMC Physiol 2017, 17, 1–10, doi:10.1186/s12899-017-0031-x.
16. Ghaddar, B.; Bringart, M.; Lefebvre d'Hellencourt, C.; Meilhac, O.; Diotel, N. Deleterious Effects of Overfeeding on Brain Homeostasis and Plasticity in Adult Zebrafish. Zebrafish 2021, zeb.2020.1962, doi:10.1089/zeb.2020.1962.
17. Anwer, H.; O'Dea, R.E.; Mason, D.; Zajitschek, S.; Klinke, A.; Reid, M.; Hesselson, D.; Noble, D.W.A.; Morris, M.J.; Lagisz, M.; et al. The Effects of an Obesogenic Diet on Behavior and Cognition in Zebrafish (Danio Rerio): Trait Average, Variability, Repeatability, and Behavioral Syndromes. Ecol Evol 2022, 12, doi:10.1002/ece3.9511.
18. Anwer, H.; Mason, D.; Zajitschek, S.; Hesselson, D.; Noble, D.W.A.; Morris, M.J.; Lagisz, M.; Nakagawa, S. Intergenerational Effects of Overfeeding on Aversive Learning in Zebrafish (Danio Rerio). Ecol Evol 2022, 12, doi:10.1002/ece3.9423.
19. Cachat, J.; Stewart, A.; Grossman, L.; Gaikwad, S.; Kadri, F.; Chung, K.M.; Wu, N.; Wong, K.; Roy, S.; Suciu, C.; et al. Meas-uring Behavioral and Endocrine Responses to Novelty Stress in Adult Zebrafish. Nat Protoc 2010, 5, 1786–1799, doi:10.1038/nprot.2010.140.
20. Brock, A.J.; Sudwarts, A.; Daggett, J.; Parker, M.O.; Brennan, C.H. A Fully Automated Computer-Based 'Skinner Box' for Testing Learning and Memory in Zebrafish. bioRxiv 2017, 110478, doi.org/10.1101/110478
21. Valente, A.; Huang, K.-H.; Portugues, R.; Engert, F. Ontogeny of Classical and Operant Learning Behaviors in Zebrafish. Learning & Memory 2012, 19, 170–177, doi:10.1101/lm.025668.112.
22. Cleal, M.; Parker, M.O. Moderate Developmental Alcohol Exposure Reduces Repetitive Alternation in a Zebrafish Model of Fetal Alcohol Spectrum Disorders. Neurotoxicol Teratol 2018, 70, 1–9, doi:10.1016/j.ntt.2018.09.001.

23. Fontana, B.D.; Cleal, M.; Parker, M.O. Female Adult Zebrafish (Danio Rerio) Show Higher Levels of Anxiety-like Behavior than Males, but Do Not Differ in Learning and Memory Capacity. European Journal of Neuroscience 2020, 52, 2604–2613, doi:10.1111/EJN.14588.
24. Vossen, L.E.; Cerveny, D.; Österkrans, M.; Thörnqvist, P.O.; Jutfelt, F.; Fick, J.; Brodin, T.; Winberg, S. Chronic Exposure to Oxazepam Pollution Produces Tolerance to Anxiolytic Effects in Zebrafish (Danio Rerio). Environ Sci Technol 2020, 54, 1760–1769, doi:10.1021/acs.est.9b06052.
25. Jacob, P.F.; Waddell, S. Spaced Training Forms Complementary Long-Term Memories of Opposite Valence in Drosophila. Neuron 2020, 106, 977-991.e4, doi:10.1016/j.neuron.2020.03.013.
26. Folch, J.; Lees, M.; Sloane, G.H. A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES*. 1957, doi:10.1016/S0021-9258(18)64849-5.
27. Chivite, M.; Naderi, F.; Conde-Sieira, M.; Soengas, J.L.; Lopez-Patiño, M.A.; Míguez, J.M. Central Serotonin Participates in the Anorexigenic Effect of GLP-1 in Rainbow Trout (Oncorhynchus Mykiss). Gen Comp Endocrinol 2021, 304, doi:10.1016/j.ygcen.2021.113716.
28. Godino-Gimeno, A.; Sánchez, E.; Guillot, R.; Rocha, A.; Angotzi, A.R.; Leal, E.; Rotllant, J.; Cerdá-Reverter, J.M. Growth Performance after Agouti-Signaling Protein 1 (Asip1) Overexpression in Transgenic Zebrafish. Zebrafish 2020, 17, 373–381, doi:10.1089/zeb.2020.1932.
29. Guillot, R.; Cortés, R.; Navarro, S.; Mischitelli, M.; García-Herranz, V.; Sánchez, E.; Cal, L.; Navarro, J.C.; Míguez, J.M.; Afanasyev, S.; et al. Behind Melanocortin Antagonist Overexpression in the Zebrafish Brain: A Behavioral and Transcriptomic Approach. Horm Behav 2016, 82, 87–100, doi:10.1016/j.yhbeh.2016.04.011.
30. Picolo, V.L.; Quadros, V.A.; Canzian, J.; Grisolia, C.K.; Goulart, J.T.; Pantoja, C.; de Bem, A.F.; Rosemberg, D.B. Short-Term High-Fat Diet Induces Cognitive Decline, Aggression, and Anxiety-like Behavior in Adult Zebrafish. Prog Neuropsycho-pharmacol Biol Psychiatry 2021, 110, doi:10.1016/j.pnpbp.2021.110288.
31. Egan, R.J.; Bergner, C.L.; Hart, P.C.; Cachat, J.M.; Canavello, P.R.; Elegante, M.F.; Elkhayat, S.I.; Bartels, B.K.; Tien, A.K.; Tien, D.H.; et al. Understanding Behavioral and Physiological Phenotypes of Stress and Anxiety in Zebrafish. Behavioural Brain Research 2009, 205, 38–44, doi:10.1016/j.bbr.2009.06.022.

32. Kalueff, A. V.; Gebhardt, M.; Stewart, A.M.; Cachat, J.M.; Brimmer, M.; Chawla, J.S.; Craddock, C.; Kyzar, E.J.; Roth, A.; Landsman, S.; et al. Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. Zebrafish 2013, 10, 70–86, doi:10.1089/zeb.2012.0861.
33. Collier, A.D.; Kalueff, A. V.; Echevarria, D.J. Zebrafish Models of Anxiety-like Behaviors. In The Rights and Wrongs of Zebrafish: Behavioral Phenotyping of Zebrafish; 2017; pp. 45–72 ISBN 9783319337746, doi: 10.1007/978-3-319-33774-6_3
34. Tran, S.; Gerlai, R. Individual Differences in Activity Levels in Zebrafish (Danio Rerio). Behavioural brain research 2013, 257, 224, doi:10.1016/J.BBR.2013.09.040.
35. Ghaddar, B.; Veeren, B.; Rondeau, P.; Bringart, M.; Lefebvre d'Hellencourt, C.; Meilhac, O.; Bascands, J.L.; Diotel, N. Impaired Brain Homeostasis and Neurogenesis in Diet-Induced Overweight Zebrafish: A Preventive Role from A. Borbonica Extract. Sci Rep 2020, 10, doi:10.1038/s41598-020-71402-2.
36. Herculano, A.M.; Maximino, C. Serotonergic Modulation of Zebrafish Behavior: Towards a Paradox. Prog Neuropsycho-pharmacol Biol Psychiatry 2014, 55, 50–66, doi:10.1016/j.pnpbp.2014.03.008.
37. Yokogawa, T.; Hannan, M.C.; Burgess, H.A. The Dorsal Raphe Modulates Sensory Responsiveness during Arousal in Zebrafish. J Neurosci 2012, 32, 15205–15215, doi:10.1523/JNEUROSCI.1019-12.2012.
38. Reemst, K.; Shahin, H.; Shahar, O.D. Learning and Memory Formation in Zebrafish: Protein Dynamics and Molecular Tools. Front Cell Dev Biol 2023, 11, doi:10.3389/fcell.2023.1120984.
39. Norris, D. Short-Term Memory and Long-Term Memory Are Still Different. Psychol Bull 2017, 143, 992–1009, doi:10.1037/BUL0000108.
40. Hinz, F.I.; Aizenberg, M.; Tushev, G.; Schuman, E.M. Protein Synthesis-Dependent Associative Long-Term Memory in Larval Zebrafish. J Neurosci 2013, 33, 15382–15387, doi:10.1523/JNEUROSCI.0560-13.2013.
41. Madeira, N.; Oliveira, R.F. Long-Term Social Recognition Memory in Zebrafish. Zebrafish 2017, 14, 305–310, doi:10.1089/zeb.2017.1430.
42. Oka, T.; Nishimura, Y.; Zang, L.; Hirano, M.; Shimada, Y.; Wang, Z.; Umemoto, N.; Kuroyanagi, J.; Nishimura, N.; Tanaka, T. Diet-Induced Obesity in Zebrafish Shares Common Pathophysiological Pathways with Mammalian Obesity. BMC Physiol 2010, 10, 21, doi:10.1186/1472-6793-10-21.

43. Cai, D. Neuroinflammation and Neurodegeneration in Overnutrition-Induced Diseases. Trends Endocrinol Metab 2013, 24, 40–47, doi:10.1016/J.TEM.2012.11.003.
44. Gould, E.; Beylin, A.; Tanapat, P.; Reeves, A.; Shors, T.J. Learning Enhances Adult Neurogenesis in the Hippocampal For-mation. Nat Neurosci 1999, 2, 260–265, doi:10.1038/6365.
45. Salas, C.; Broglio, C.; Rodríguez, F. Evolution of Forebrain and Spatial Cognition in Vertebrates: Conservation across Diversity. Brain Behav Evol 2003, 62, 72–82, doi:10.1159/000072438.
46. Bouton, M.E.; Maren, S.; McNally, G.P. Behavioral and neurobiological mechanism of pavlovian and instrumental extinction learning. Physiol Rev 2021, 101, 611–681, doi:10.1152/PHYSREV.00016.2020.
47. Lal, P.; Tanabe, H.; Suster, M.L.; Ailani, D.; Kotani, Y.; Muto, A.; Itoh, M.; Iwasaki, M.; Wada, H.; Yaksi, E.; et al. Identification of a Neuronal Population in the Telencephalon Essential for Fear Conditioning in Zebrafish. BMC Biol 2018, 16, doi:10.1186/S12915-018-0502-Y.
48. Portavella, M.; Torres, B.; Salas, C. Avoidance Response in Goldfish: Emotional and Temporal Involvement of Medial and Lateral Telencephalic Pallium. Journal of Neuroscience 2004, 24, 2335–2342, doi:10.1523/JNEUROSCI.4930-03.2004.
49. Northcutt, R.G. Connections of the Lateral and Medial Divisions of the Goldfish Telencephalic Pallium. J Comp Neurol 2006, 494, 903–943, doi:10.1002/CNE.20853.
50. Valladolid-Acebes, I.; Merino, B.; Principato, A.; Fole, A.; Barbas, C.; Lorenzo, M.P.; García, A.; del Olmo, N.; Ruiz-Gayo, M.; Cano, V. High-Fat Diets Induce Changes in Hippocampal Glutamate Metabolism and Neurotransmission. Am J Physiol En-docrinol Metab 2012, 302,396–402, doi: 10.1152/ajpendo.00343.2011.

Supplementary material

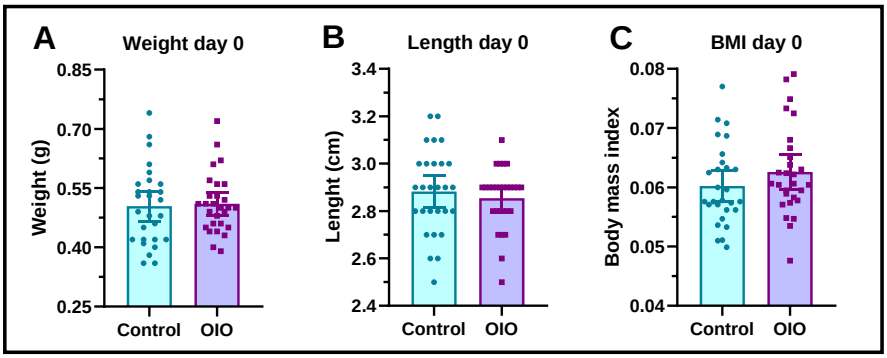
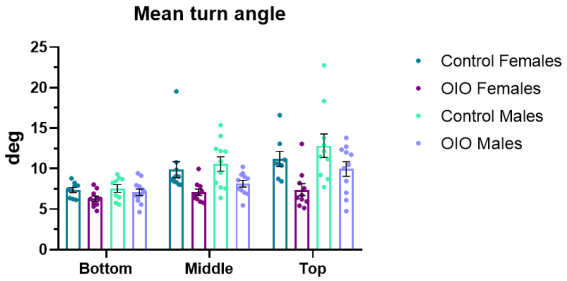


Figure S1. Morphological parameters of control and OIO groups at the beginning of the experiment.

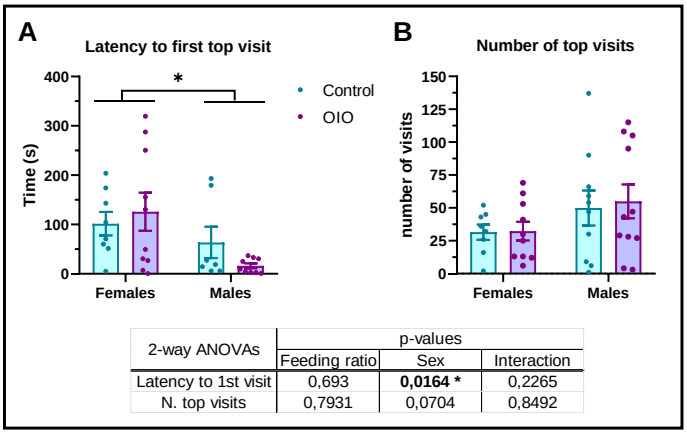
(A) Weight (B) Length and (C) Body mass index (BMI). Data are expressed by mean ±SEM analysed by unpaired t-test.



2-way ANOVAs		p-values		
		Feeding ratio	Sex	Interaction
Mean Turn angle	Bottom	**0,0553 #**	0,2041	0,4262
	Middle	**0,0011 ****	0,2453	0,8246
	Top	**0,0033 ****	**0,0558 #**	0,6589

Figure S2. Mean turn angle in the bottom, middle and top area during NDTD.

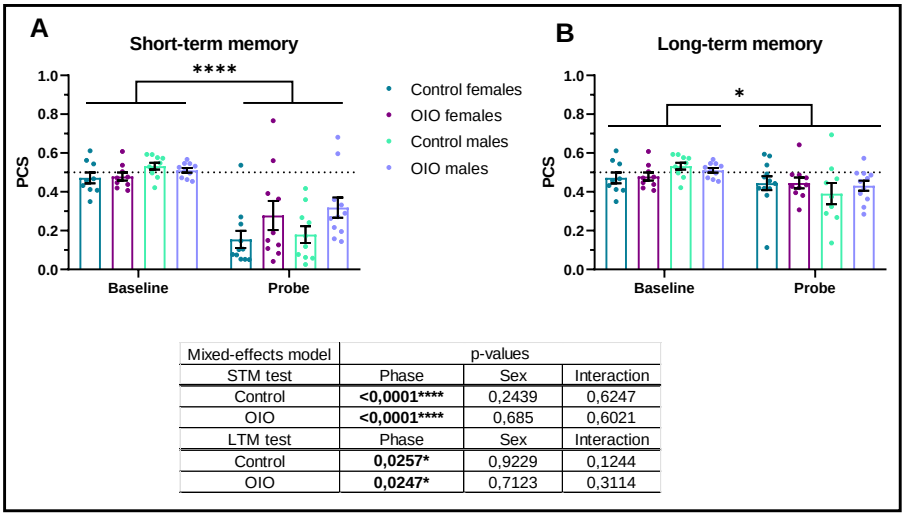
Significant differences induced by OIO are found in the middle and top zones, but no influence of sex. Data are represented by mean ±SEM and analysed by two-way ANOVA. Asterisks indicate significant differences (*p ≤ 0.05, **p ≤ 0.01), and hashtag indicates an apparent significant difference (# p = 0.051-0.06).



2-way ANOVAs	p-values		
	Feeding ratio	Sex	Interaction
Latency to 1st visit	0,693	**0,0164 ***	0,2265
N. top visits	0,7931	0,0704	0,8492

Figure S3. Anxiety-like parameters in the top area after NTDT.

(A) Latency to the zone. (B) Number of visits to the top area. Data are represented by mean ±SEM and analysed by two-way ANOVA, asterisks indicate significant differences (*p ≤ 0.05).



Mixed-effects model	p-values		
STM test	Phase	Sex	Interaction
Control	**<0,0001****	0,2439	0,6247
OIO	**<0,0001****	0,685	0,6021
LTM test	Phase	Sex	Interaction
Control	**0,0257***	0,9229	0,1244
OIO	**0,0247***	0,7123	0,3114

Figure S4. Effects of gender on STM and LTM in control and OIO fish.

(A) Short-term memory test and (B) long-term memory test. Data are represented by mean ± SEM. The dotted line indicates no stimulus preference (PCS = 0.5), low and high PCS mean less or more time spent in conditioned stimulus (CS), respectively. Results were analysed by Repeated Measures Mixed-effect model; asterisks indicate significant differences between phases, baselines and probes (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, **** p ≤ 0.0001).

General Discussion

1. Melanocortin system inhibition, growth and intestinal permeability

The melanocortin system has been implicated in the control of feeding behaviour, obesity and growth in vertebrates (Ellacott & Cone, 2006; Koch & Horvath, 2014), including fish (Cerdá-Reverter et al., 2011). In mammals, overexpression of *asip* (Michaud et al., 1993; Miller et al., 1993) and *agrp* (Ollmann et al., 1997) results in linear growth and obesity caused by overfeeding and reduced energy expenditure (Fekete et al., 2004). In zebrafish, *agrp1* overexpression increases linear growth, body weight, triglyceride levels and adipose hypertrophy (Song & Cone, 2007), suggesting that inhibition of melanocortin signalling promotes obesity in zebrafish, as in mammals. Despite the increased linear growth and weight in *asip1*-overexpressing zebrafish, which correlates with increased food intake (Guillot et al., 2016), these transgenic fish do not develop obesity, as whole-body lipid levels are similar to pair-fed WT siblings (Godino-Gimeno et al., 2020). In addition, *asip1-Tg* fish have better growth efficiency, gaining more than 50% more weight than pair-fed WT fish without differential lipid accumulation, suggesting a better food conversion ratio, even under high-density culture conditions (Godino-Gimeno et al., 2020). The possible reason for the improved feed efficiency in *asip1-Tg*, which allows to reach the maximum phenotypic growth, is probably a better efficiency in intestinal absorption driven by a differential expression of genes related to the transport of amino acids, monocarboxylates, ions and vitamins (Leal et al., 2022), which could lead to an improved anabolism and thus to an enhanced growth in transgenic fish (Li et al., 2021). This potential improved absorption also correlates well with low cellular adhesion and increased membrane laxity and permeability due to decreased expression of genes involved in sterol/cholesterol (de Mendoza & Pilon, 2019; Harayama & Riezman,

2018) and glycerophospholipid and glycerol synthesis (Yang et al., 2018) in *asip1-Tg* males and females, respectively (Leal et al., 2022). This is further supported by the study of intestinal electrogenic amino acid transportation, which showed improved essential amino acid transport of *asip1-Tg* compared to WT fish, suggesting that transgenic fish can take up more amino acids from their diet than pair-fed WT, which also supports their improved food conversion rate, linear growth and body weight in the absence of obesity (Godino-Gimeno et al., 2020; Leal et al., 2022). Therefore, inhibition of the melanocortin system through *asip1* overexpression represents a potential target to enable the development of genetically modified fish or genetic selection programmes for aquaculture. This strategy is of particular interest since the US Food and Drug Administration (FDA) has approved the marketing of GH-overexpressing salmon for human consumption.

2. Melanocortin system inhibition and agonistic behaviour.

One of the concerns about the development of genetically modified fish aquaculture is precisely the risk of displacement of native fish populations by dominant fish in the event of escape from facilities. The association of high feeding rates and enhanced growth with the dominant phenotype has already been suggested in fish (Metcalfe et al., 1989; Lahti et al., 2001). Growth hormone (GH)-transgenic salmon exhibit increased food intake and growth rate together with pronounced aggressive behaviour promoting a dominant profile (Devlin et al., 2004; Hallerman et al., 2007), similar to the phenotype of fish treated with exogenous GH (Jönsson et al., 1998; Jöhnsson & Björnsson, 2001). Therefore, one of the aims of this work was to investigate whether the high feeding motivation promoted by *asip1* overexpression in *asip1-Tg* fish is associated with increased aggressive behaviour. After confronting WT and *asip1-Tg* males, a clear submissive behaviour was found in *asip1-Tg*, which were unable to win most of the dyadic fights against WT fish (Rocha et al., 2023).

Furthermore, when faced with their own reflection, *asip1-Tg* displayed a submissive profile, despite approaching the mirror, they avoided physical contact with the mirror and preferred to flee to a safe zone, contrary to the behaviour observed in WT fish (Rocha et al., 2023). The high basal cortisol levels found in *asip1-Tg*, resulting from HPI activation, also confirmed the subordinate profile of the transgenic animals (Backström et al., 2011; Pottinger & Carrick, 1999). Therefore, inhibition of the melanocortin system by overexpression of asip1 promotes submissive behaviour in fish, which reduces their chances of survival under natural conditions and is unlikely to be able to overcome competition from native fish species.

3. Melanocortin system inhibition and anxiety-like behaviour

After describing the reactive/subordinate profile of *asip1-Tg* fish linked to the activaton of stress axis (Øverli et al., 2002; Rocha et al., 2023), we investigate whether the exploration and locomotion patterns of *asip1-Tg* fish might differ from their proactive WT counterparts, as found in other reactive zebrafish lines (Godwin et al., 2012). The reactive profile of *asip1-Tg* was well-correlated with a lack of exploration as previously reported (Wright et al., 2003). Although thigmotaxis was not affected by the overexpression of *asip1*, these transgenic fish showed a marked anxiety pattern in OFT characterised by enhanced freezing behaviour and faster swimming than WT. This phenotypical pattern has been also described in other reactive zebrafish lines (Godwin et al., 2012). Moreover, *asip1-Tg* showed a pronounced bottom-dwelling together with frequent immobile periods and poor habituation to a new environment in NTDT, all indicators associated with anxiety-like behaviour (Egan et al., 2009; Cachat et al., 2010; Wong et al., 2010). Also, transgenic *asip1-Tg* exhibited additional anxiety parameters as prolonged latency to visit the top zone (Egan et al., 2009; Cachat et al., 2010; Cachat et al., 2011) and erratic movements (Quadros et al., 2016; Volgin et al., 2019). These findings correlate with the anxiety-like behaviour of *asip1-*

Tg observed in OFT, NOT and NTDT and highlight their subordinate/reactive profile (Lawther et al., 2020). At central levels, *asip1-Tg* exhibited low dopamine levels and enhanced neurotransmitter reuptake, as previously reported in reactive fish (Cabib & Puglisi-Allegra, 2012), as well as in subordinate rainbow trout (*Oncorhynchus mykiss*) that showed elevated dopamine turnover rate and high cortisol levels (Øverli et al., 1999). The anxiety-like behaviour of *asip1-Tg* was also tightly linked to low central serotonin levels due to a high neurotransmitter reuptake, as reported in subordinate salmonid species (Winberg & Thörnqvist, 2016) and zebrafish (Dahlbom et al., 2012). Serotonin reuptake blockade using the SSRI agents (fluoxetine) in *asip1-Tg* animals enhanced serotoninergic neurotransmission thus rescuing the behavioural phenotype (Maximino et al., 2013b; c).

We have established that overexpressing Asip1 promotes anxiety-like behaviour due mainly to reduced central serotoninergic neurotransmission. Central 5HT has anorexic effects, we therefore postulate that the enhanced motivational feeding in *asip1-Tg* (Godino-Gimeno et al., 2020; Guillot et al., 2016), could be interpreted as a feeding-related anxiety, similar to those experienced during dieting, which would be driven by the interaction between melanocortinergic and serotoninergic pathways. Consequently, *asip1-Tg* could represent a suitable model organism for biomedical research regarding anxiety and eating disorders (Compan, 2020; Micioni Di Bonaventura et al., 2020; Rahati et al., 2022).

4. Role of the melanocortin system in the regulation of circadian rhythms

Feeding behaviour is associated with daily patterns of locomotor activity (LA) during the active phase of the sleep-wake cycle, suggesting coordinated behavioural regulation. Our previous preliminary results showed that *asip1-Tg* has a reduced nocturnal melatonin surge (Guillot et al., 2016), suggesting a potential role of the melanocortin system in the regulation of circadian behaviour. Our experiments on LA showed an increased activity in *asip1-Tg* during the photophase, which did not decrease during the scotophase. These results are consistent with a disruption of melatonin secretion in *asip1-Tg* together with a disrupted serotonin daily cycle in the encephalon, suggesting that low nocturnal melatonin levels could be due to reduced serotonin availability (Appelbaum et al., 2009; Elbaz et al., 2013; Tricklebank, 2019; Trueta & Cercós, 2012).

Analysis of the circadian molecular machinery confirmed the disruption of the circadian system in the transgenic animals, as the daily expression of the clock genes, *per1a* and *clock1a* was masked during this nocturnal phase, together with a severe reduction in the amplitude of *nr1d1* gene expression. Therefore, *asip1* overexpression disrupts the central molecular clock, subsequently promoting the abolition of the melatonin circadian rhythm and ultimately disrupting the LA circadian rhythm. As no functional equivalent of the SCN has been found in fish, the pineal gland is considered as the master clock organ, although the presence of photoreceptive peripheral oscillators may challenge this hypothesis (Whitmore et al., 2000; Vallone et al., 2004, 2005; Ben-Moshe Livne et al., 2016). The zebrafish pineal gland is known to express *mc1r*, *mc3r*, and *mc4r* mRNAs (Shainer et al., 2017), suggesting a direct role of the melanocortinergic peptides in pineal physiology. Accordingly, the incubation of zebrafish pineal glands with Asip1 strongly reduced melatonin secretion in

absence of any agonist and in a dose-dependent manner thus mimicking *in vivo* observations and further supporting a role for Asip1 in the pineal physiology. Indeed, both Asip1 and Agrp1 can work as inverse agonist of the constitutively activated MC1R and MC4R (Guillot et al., 2016; Sánchez et al., 2009, 2010). Therefore, a plausible explanation of our results involves that the constitutive signalling of the melanocortin receptors in the pineal is critical for the good performance of the molecular clock and by overexpressing *asip1* ubiquitously, we have disrupted the circadian output masking melatonin rhythms and by extension the locomotor activity circadian rhythms. Obviously, more studies are required to corroborate this hypothesis and to explore the endogenous mechanism behind the melanocortin effects on circadian system. Additionally, *pomca* and *pomcb* reduced expression during the full light-cycle again suggest that *asip1-Tg* animals are constantly hungry and the increased locomotor activity may represent an exacerbated foraging behaviour driven by appetite.

In summary a new role of the melanocortin system in the regulation of circadian behaviour has been uncovered making *asip1-Tg* an interesting model for biomedical research on physiological mechanisms controlling sleep-wakefulness and feeding behaviour.

5. Influence of obesity on memory and anxiety-like behaviour

Obesity challenges health systems in Western societies and is also a major contributor to metabolic syndrome, which has profound negative effects on brain function (Medawar & Witte, 2022) by impairing cognitive function and emotional states (Sarangi & Dus, 2021; Zhang et al., 2022), thereby exacerbating learning and memory dysfunction and promoting anxiety-related responses (Sarangi & Dus, 2021; Q. Zhang et al., 2022). Zebrafish have high genetic similarities and shared pathophysiological pathways with mammalian diet-induced obesity models (Oka et al., 2010;

Howe et al., 2013; Zang et al., 2018) and have been consolidated as a model organism in the study of brain disorders such as anxiety and cognitive function (Linker et al., 2011; Kalueff et al., 2014; Meshalkina et al., 2017).

Overfeeding the AB zebrafish strain at 8% feeding rate for 2 months with a zebrafish formulated diet induced obesity. After treatment, both obese males and females were heavier and longer than controls. Accordingly, BMIs in OIO were the highest than those reported in previous experiments using similar overfeeding protocols (Landgraf et al., 2017; Ghaddar et al., 2021). In terms of lipid content, OIO fish reached 25% of total body weight, while controls remained around 12.5%. Despite the apparent obesity reported in OIO fish, anxiety-like behaviour was not affected, in contrast to that observed in high-fat obesity-induced zebrafish (Landgraf et al., 2017). However, in the NTDT, each of the anxiety indicators, such as bottom dwelling, immobility, prolonged latency to visit the top area or erratic movements, were more pronounced in the obese fish. Similarly, serotonin and dopamine synthesis and neurotransmission did not differ between obese and lean animals. The reason for the different effects on anxiety between high-fat-induced obese fish (Meguro et al., 2019; Picolo et al., 2021; Türkoğlu et al., 2022) and overfed-induced obese fish (Ghaddar et al., 2021; Godino-Gimeno et al., 2023) are unknow but may be due to the different effects of the diets on BBB integrity and neuroinflammation (Landgraf et al., 2017; Blüher, 2020;)

Learning can lead to both short-term memory (STM) and long-term memory (LTM) (Reemst et al., 2023), which are presumably managed and stored by distinct but interconnected neural systems (Norris, 2017). STM lasts from seconds to minutes and its formation depends on biochemical changes, whereas LTM lasts from hours to years and its formation depends on new protein synthesis (Hinz et al., 2013; Reemst et al., 2023). OIO animals had poorer retention of STM compared to controls, suggesting that obesity impairs STM. Obesity has been associated with reduced

hippocampal neurogenesis, which is associated with memory impairment (Bracke et al., 2019; Park et al., 2010; Purkayastha & Cai, 2013; Ramos-Rodriguez et al., 2014). Interestingly, Ghaddar et al, (2021) also reported increased central oxidative stress and decreased cell proliferation in neurogenic niches of the diencephalon and ventral and dorsomedial telencephalon (Dm), a homologous structure of the amygdala that is also rich in glutamatergic neurons and involved in aversive learning and memory (Northcutt, 2006; Meshalkina et al., 2017; Lal et al., 2018). Therefore, we believe that similar neurogenic damage may underlie the STM impairment found in obese OIO fish in our study, in line with findings in high-fat diet-induced obese fish (Meguro et al., 2019; Picolo et al., 2021). Surprisingly, a related experiment showed that artemia-overfed obese fish showed no effects on cognitive function (Anwer et al., 2022), indicating that cognitive impairment in obese zebrafish is highly dependent on the type of diet administered and the protocol for the aversive learning test.

Our experiments confirmed that LTM acquisition can be maintained for 24 h in zebrafish, but the nutritional state induced by OIO had no effect. In zebrafish, LTM has been achieved in social memory experiments (Madeira & Oliveira, 2017) and inhibitory avoidance learning (Blank et al., 2009; Nam et al., 2004), challenging zebrafish's natural preference for dark places with electrical socks. This methodology is controversial, as proactive or reactive fish may have different latencies to explore the dark zone, along with human manipulation and electric shock intensity (Manuel et al., 2014). To our knowledge, the aversive learning protocol used in this study (Godino-Gimeno et al., 2023) is the first used to assess LTM in zebrafish. As a fully automated system, it prevents human manipulation bias and allows fine control of electric shock intensity, duration and frequency. Furthermore, aversive learning has been shown to be effective in LTM research in *Drosophila melanogaster* (Jacob & Waddell, 2020; Xia et al., 2005), *Caenorhabditis elegans* (Rose et al., 2002; Amano &

Maruyama, 2011; Shibutani et al., 2022) and mice (Reolon et al., 2006; Acuña et al., 2023).

In conclusion, obesity induced by overfeeding has no effect on anxiety-like behaviour, monoaminergic transmission and LTM acquisition, but impairs STM. Furthermore, these effects are entirely dependent on the feeding protocols and/or diet composition. Finally, a reliable protocol is provided to assess the effect of metabolic disease on cognitive and behavioural function, supporting zebrafish as a model in behavioural and cognitive neuroscience.

Conclusions

1. Depressing melanocortin signalling by ubiquitous overexpression of *asip1* in a transgenic zebrafish model enhances structural growth at equal feeding rates suggesting an improved food efficiency in the genetically modified fish.
2. *asip1* overexpression regulates intestinal function by increasing epithelial permeability and amino acid absorption, providing a physiological basis for improved feed efficiency that supports enhanced growth at similar feeding rates.
3. Overexpression of *asip1* promotes activation of the stress axis as well as a reactive/subordinate behavioural phenotype, which would significantly reduce the potential risk of displacement of native fish populations in the event of escape. Together with the easy identification of transgenic animals by dorsoventral pigment pattern disruption, asip1 represents a potential target to enable the development of genetically engineered fish or genetic selection programmes for aquaculture.
4. *Asip1-Tg* zebrafish exhibit an anxiety-like phenotype caused by a disruption of central serotonergic activity, making the transgenic model suitable for studying mood disorders.
5. Overexpression of asip1 masks daily locomotor activity patterns and greatly increases total locomotor activity, particularly at night, by disrupting the daily melatonin rhythm and the circadian molecular machinery. In vitro pineal organotypic experiments further demonstrate that Asip1 can directly regulate melatonin secretion through pineal receptors, suggesting that the constitutive activity of the melanocortin receptors is critical for the proper functioning of the circadian system.
6. Overfeeding-induced obesity affects cognitive processes by impairing short-term memory without affecting long-term memory or anxiety-like behaviour. These findings make zebrafish a viable model for studying the effects of metabolic disease on cognitive and behavioural functions.

General references

Acuña, L. R., Back, F., Barp, C. G., Guilherme Tassoni Bortoloci, J., Assreuy, J., & Carobrez, A. P. (2023). Role of nitric oxide on defensive behavior and long-term aversive learning induced by chemical stimulation of the dorsolateral periaqueductal gray matter. *Neurobiology of Learning and Memory*, *200*, 107735. https://doi.org/10.1016/J.NLM.2023.107735

Agulleiro, M. J., Cortés, R., Fernández-Durán, B., Navarro, S., Guillot, R., Meimaridou, E., Clark, A. J. L., & Cerdá-Reverter, J. M. (2013). Melanocortin 4 receptor becomes an ACTH receptor by coexpression of melanocortin receptor accessory protein 2. *Molecular Endocrinology*, *27*(11), 1934–1945. https://doi.org/10.1210/me.2013-1099

Agulleiro, M. J., Cortés, R., Leal, E., Ríos, D., Sánchez, E., & Cerdá-Reverter, J. M. (2014). Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (Dicentrarchus labrax). *General and Comparative Endocrinology*, *205*, 251–259. https://doi.org/10.1016/j.ygcen.2014.02.009

Allen, A. T., Heaton, E. C., Shapiro, L. P., Butkovich, L. M., Yount, S. T., Davies, R. A., Li, D. C., Swanson, A. M., & Gourley, S. L. (2022). Inter-individual variability amplified through breeding reveals control of reward-related action strategies by Melanocortin-4 Receptor in the dorsomedial striatum. *Communications Biology*, *5*(1). https://doi.org/10.1038/S42003-022-03043-2

Amano, H., & Maruyama, I. N. (2011). Aversive olfactory learning and associative long-term memory in Caenorhabditis elegans. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *18*(10), 654–665. https://doi.org/10.1101/LM.2224411

Anwer, H., O'Dea, R. E., Mason, D., Zajitschek, S., Klinke, A., Reid, M., Hesselson, D., Noble, D. W. A., Morris, M. J., Lagisz, M., & Nakagawa, S. (2022). The effects of an obesogenic diet on behavior and cognition in zebrafish (Danio rerio): Trait average, variability, repeatability, and behavioral syndromes. *Ecology and Evolution*, *12*(11). https://doi.org/10.1002/ece3.9511

Aponte, Y., Atasoy, D., & Sternson, S. M. (2011). AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nature Neuroscience*, *14*(3), 351–355. https://doi.org/10.1038/NN.2739

Appelbaum, L., Wang, G. X., Maro, G. S., Mori, R., Tovin, A., Marin, W., Yokogawa, T., Kawakami, K., Smith, S. J., Gothilf, Y., Mignot, E., & Mourrain, P. (2009). Sleep-wake regulation and hypocretin-melatonin interaction in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(51), 21942–21947. https://doi.org/10.1073/pnas.906637106

Backström, T., Schjolden, J., Øverli, Ø., Thörnqvist, P. O., & Winberg, S. (2011). Stress effects on AVT and CRF systems in two strains of rainbow trout (Oncorhynchus mykiss) divergent in stress responsiveness. *Hormones and Behavior*, *59*(1), 180–186. https://doi.org/10.1016/J.YHBEH.2010.11.008

Backström, T., & Winberg, S. (2017). Serotonin Coordinates Responses to Social Stress—What We Can Learn from Fish. *Frontiers in Neuroscience*, *11*(OCT), 595. https://doi.org/10.3389/FNINS.2017.00595

Baghdoyan, H. A., & Lydic, R. (2012). *The Neurochemistry of Sleep and Wakefulness* (8th ed.).

Belujon, P., & Grace, A. A. (2017). Dopamine system dysregulation in major depressive disorders. *International Journal of Neuropsychopharmacology*, *20*(12), 1036–1046. https://doi.org/10.1093/IJNP/PYX056

Ben-Moshe Livne, Z., Alon, S., Vallone, D., Bayleyen, Y., Tovin, A., Shainer, I., Nisembaum, L. G., Aviram, I., Smadja-Storz, S., Fuentes, M., Falcó, J., Eisenberg, E., Klein, D. C., Burgess, H. A., Foulkes, N. S., Gothilf, Y., & Livne, B.-M. Z. (2016). Genetically Blocking the Zebrafish Pineal Clock Affects Circadian Behavior. *PLoS Genet*, *12*(11), 1006445. https://doi.org/10.1371/journal.pgen.1006445

Blank, M., Guerim, L. D., Cordeiro, R. F., & Vianna, M. R. M. (2009). A one-trial inhibitory avoidance task to zebrafish: Rapid acquisition of an NMDA-dependent long-term memory. *Neurobiology of Learning and Memory*, *92*(4), 529–534. https://doi.org/10.1016/J.NLM.2009.07.001

Blüher, M. (2020). Metabolically Healthy Obesity. *Endocrine Reviews*, *41*(3), 405–420. https://doi.org/10.1210/ENDREV/BNAA004

Bracke, A., Domanska, G., Bracke, K., Harzsch, S., van den Brandt, J., Bröker, B., & von Bohlen und Halbach, O. (2019). Obesity Impairs Mobility and Adult Hippocampal Neurogenesis. *Journal of Experimental Neuroscience*, *13*. https://doi.org/10.1177/1179069519883580

Cabib, S., & Puglisi-Allegra, S. (2012). The mesoaccumbens dopamine in coping with stress. *Neuroscience and Biobehavioral Reviews*, *36*(1), 79–89. https://doi.org/10.1016/J.NEUBIOREV.2011.04.012

Cachat, J. M., Canavello, P. R., Elegante, M. F., Bartels, B. K., Elkhayat, S. I., Hart, P. C., Tien, A. K., Tien, D. H., Beeson, E., Mohnot, S., Laffoon, A. L., Stewart, A. M., Gaikwad, S., Wong, K., Haymore, W., & Kalueff, A. V. (2011). Modeling stress and anxiety in zebrafish. *Neuromethods*, *52*, 73–88. https://doi.org/10.1007/978-1-60761-922-2_3

Cachat, J. M., Canavello, P. R., Elkhayat, S. I., Bartels, B. K., Hart, P. C., Elegante, M. F., Beeson, E. C., Laffoon, A. L., Haymore, W. A. M., Tien, D. H., Tien, A. K., Mohnot, S., & Kalueff, A. V. (2011). Video-aided analysis of zebrafish locomotion and anxiety-related behavioral responses. In *Zebrafish Neurobehavioural Protocols* (Vol. 51, pp. 1–14). https://doi.org/10.1007/978-1-60761-953-6_1

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., … Kalueff, A. V. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols*, *5*(11), 1786–1799. https://doi.org/10.1038/nprot.2010.140

Carola, V., Perlas, E., Zonfrillo, F., Soini, H. A., Novotny, M. V., & Gross, C. T. (2014). Modulation of social behavior by the agouti pigmentation gene. *Frontiers in Behavioral Neuroscience*, *8*(AUG). https://doi.org/10.3389/FNBEH.2014.00259/ABSTRACT

Castro, M. G., & Morrison, E. (1997). Post-translational processing of proopiomelanocortin in the pituitary and in the brain. *Critical Reviews in Neurobiology*, *11*(1), 35–57. https://doi.org/10.1615/CRITREVNEUROBIOL.V11.I1.30

Ceinos, R. M., Guillot, R., Kelsh, R. N., Cerdá-Reverter, J. M., & Rotllant, J. (2015). Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms. *Pigment Cell & Melanoma Research*, *28*(2), 196–209. https://doi.org/10.1111/pcmr.12335

Cerdá-Reverter, J. M., Agulleiro, M. J., Cortés, R., Sánchez, E., Guillot, R., Leal, E., Fernández-Durán, B., Puchol, S., & Eley, M. (2013). Involvement of melanocortin receptor accessory proteins (MRAPs) in the function of melanocortin receptors.

General and Comparative Endocrinology, *188*(1), 133–136. https://doi.org/10.1016/j.ygcen.2013.01.017

Cerdá-Reverter, J. M., Agulleiro, M. J., Guillot R, R., Sánchez, E., Ceinos, R., & Rotllant, J. (2011). Fish melanocortin system. *European Journal of Pharmacology*, *660*(1), 53–60. https://doi.org/10.1016/j.ejphar.2010.10.108

Cerdá-Reverter, J. M., Haitina, T., Schiöth, H. B., & Peter, R. E. (2005). Gene structure of the goldfish agouti-signaling protein: A putative role in the dorsal-ventral pigment pattern of fish. *Endocrinology*, *146*(3), 1597–1610. https://doi.org/10.1210/EN.2004-1346

Cerdá-Reverter, J. M., & Peter, R. E. (2003). Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. *Endocrinology*, *144*(10), 4552–4561. https://doi.org/10.1210/EN.2003-0453

Cerdá-Reverter, J. M., Ringholm, A., Schiöth, H. B., & Peter, R. E. (2003). Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: Involvement in the control of food intake. *Endocrinology*, *144*(6), 2336–2349. https://doi.org/10.1210/en.2002-0213

Cerdá-Reverter, J. M., Schiöth, H. B., & Peter, R. E. (2003). The central melanocortin system regulates food intake in goldfish. *Regulatory Peptides*, *115*(2), 101–113. https://doi.org/10.1016/S0167-0115(03)00144-7

Ceruso, A., Martínez-Cengotitabengoa, M., Peters-Corbett, A., Diaz-Gutierrez, M. J., & Martínez-Cengotitabengoa, M. (2020). Alterations of the HPA Axis Observed in Patients with Major Depressive Disorder and Their Relation to Early Life Stress: A Systematic Review. *Neuropsychobiology*, *79*(6), 417–427. https://doi.org/10.1159/000506484

Chan, L. F., Webb, T. R., Chung, T. T., Meimaridou, E., Cooray, S. N., Guasti, L., Chapple, J. P., Egertová, M., Elphick, M. R., Cheetham, M. E., Metherell, L. A., & Clark, A. J. L. (2009). MRAP and MRAP2 are bidirectional regulators of the melanocortin receptor family. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(15), 6146–6151. https://doi.org/10.1073/PNAS.0809918106/SUPPL_FILE/0809918106SI.PDF

Chen, W., Kelly, M. A., Opitz-Araya, X., Thomas, R. E., Low, M. J., & Cone, R. D. (1997). Exocrine Gland Dysfunction in MC5-R-Deficient Mice: Evidence for

Coordinated Regulation of Exocrine Gland Function by Melanocortin Peptides. In *Cell* (Vol. 91).

Clemenzi, M. N., Martchenko, A., Loganathan, N., Tse, E. K., Brubaker, P. L., & Belsham, D. D. (2020). Analysis of Western diet, palmitate and BMAL1 regulation of neuropeptide Y expression in the murine hypothalamus and BMAL1 knockout cell models. *Molecular and Cellular Endocrinology*, *507*, 110773. https://doi.org/10.1016/J.MCE.2020.110773

Collier, A. D., Kalueff, A. V., & Echevarria, D. J. (2017). Zebrafish models of anxiety-like behaviors. In *The Rights and Wrongs of Zebrafish: Behavioral Phenotyping of Zebrafish* (pp. 45–72). https://doi.org/10.1007/978-3-319-33774-6_3

Compan, V. (2020). Serotonin in eating behavior. In *Handbook of Behavioral Neuroscience* (Vol. 31, pp. 489–503). Elsevier B.V. https://doi.org/10.1016/B978-0-444-64125-0.00028-1

Cone, R. D. (2006). Studies on the physiological functions of the melanocortin system. *Endocrine Reviews*, *27*(7), 736–749. https://doi.org/10.1210/ER.2006-0034

Dahlbom, S. J., Backström, T., Lundstedt-Enkel, K., & Winberg, S. (2012). Aggression and monoamines: Effects of sex and social rank in zebrafish (Danio rerio). *Behavioural Brain Research*, *228*, 333–338. https://doi.org/10.1016/j.bbr.2011.12.011

Date, Y., Toshinai, K., Koda, S., Miyazato, M., Shimbara, T., Tsuruta, T., Niijima, A., Kangawa, K., & Nakazato, M. (2005). Peripheral Interaction of Ghrelin with Cholecystokinin on Feeding Regulation. *Endocrinology*, *146*(8), 3518–3525. https://doi.org/10.1210/EN.2004-1240

de Mendoza, D., & Pilon, M. (2019). Control of membrane lipid homeostasis by lipid-bilayer associated sensors: A mechanism conserved from bacteria to humans. *Progress in Lipid Research*, *76*. https://doi.org/10.1016/J.PLIPRES.2019.100996

de Souza, F. S. J., Bumaschny, V. F., Low, M. J., & Rubinstein, M. (2005). Subfunctionalization of expression and peptide domains following the ancient duplication of the proopiomelanocortin gene in teleost fishes. *Molecular Biology and Evolution*, *22*(12), 2417–2427. https://doi.org/10.1093/MOLBEV/MSI236

Delgado, M. J., Cerdá-Reverter, J. M., & Soengas, J. L. (2017). Hypothalamic integration of metabolic, endocrine, and circadian signals in fish: Involvement in the

control of food intake. *Frontiers in Neuroscience*, *11*(JUN). https://doi.org/10.3389/fnins.2017.00354

Devlin, R. H., D'Andrade, M., Uh, M., & Biagi, C. A. (2004). Population effects of growth hormone transgenic coho salmon depend on food availability and genotype by environment interactions. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(25), 9303. https://doi.org/10.1073/PNAS.0400023101

Dijkstra, P. D., Maguire, S. M., Harris, R. M., Rodriguez, A. A., DeAngelis, R. S., Flores, S. A., & Hofmann, H. A. (2017). The melanocortin system regulates body pigmentation and social behaviour in a colour polymorphic cichlid fish†. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1851). https://doi.org/10.1098/RSPB.2016.2838

Ducrest, A. L., Keller, L., & Roulin, A. (2008). Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends in Ecology and Evolution*, *23*(9), 502–510. https://doi.org/10.1016/j.tree.2008.06.001

Echevarria, D. J., Buske, C., Toms, C. N., & Jouandot, D. J. (2011). A novel test battery to assess drug-induced changes in zebrafish social behavior. *Neuromethods*, *51*, 109–124. https://doi.org/10.1007/978-1-60761-953-6_9

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., Elkhayat, S. I., Bartels, B. K., Tien, A. K., Tien, D. H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., & Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research*, *205*(1), 38–44. https://doi.org/10.1016/j.bbr.2009.06.022

Elbaz, I., Foulkes, N. S., Gothilf, Y., & Appelbaum, L. (2013). Circadian clocks, rhythmic synaptic plasticity and the sleep-wake cycle in zebrafish. *Frontiers in Neural Circuits*, *7*(JAN), 41303. https://doi.org/10.3389/FNCIR.2013.00009/BIBTEX

Ellacott, K. L. J., & Cone, R. D. (2006). The role of the central melanocortin system in the regulation of food intake and energy homeostasis: lessons from mouse models. *Philosophical Transactions of The Royal Society*, *361*, 1265–1274. https://doi.org/10.1098/rstb.2006.1861

Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., & Cone, R. D. (1997). Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*, *385*(6612), 165–168. https://doi.org/10.1038/385165A0

Farooqi, I. S., Yeo, G. S. H., Keogh, J. M., Aminian, S., Jebb, S. A., Butler, G., Cheetham, T., & O'Rahilly, S. (2000). Dominant and recessive inheritance of morbid obesity associate with melanocortin 4 receptor deficiency. *Journal of Clinical Investigation*, *106*(2), 271–279. https://doi.org/10.1172/JCI9397

Fekete, C., Marks, D. L., Sarkar, S., Emerson, C. H., Rand, W. M., Cone, R. D., & Lechan, R. M. (2004). *Effect of Agouti-Related Protein in Regulation of the Hypothalamic-Pituitary-Thyroid Axis in the Melanocortin 4 Receptor Knockout Mouse*. https://doi.org/10.1210/en.2004-0476

Forlano, P. M., & Cone, R. D. (2007). Conserved Neurochemical Pathways Involved in Hypothalamic Control of Energy Homeostasis. *J. Comp. Neurol*, *505*, 235–248. https://doi.org/10.1002/cne.21447

Friedman, J. M. (2019). Leptin and the endocrine control of energy balance. *Nature Metabolism 2019 1:8*, *1*(8), 754–764. https://doi.org/10.1038/s42255-019-0095-y

Gerlai, R. (2011). Associative Learning in Zebrafish (Danio rerio). In *Methods in Cell Biology* (Vol. 101). https://doi.org/10.1016/B978-0-12-387036-0.00012-8

Gerlai, R. (2016). Learning and memory in zebrafish (Danio rerio). In *Methods in Cell Biology* (Vol. 134). Elsevier Ltd. https://doi.org/10.1016/bs.mcb.2016.02.005

Ghaddar, B., Bringart, M., Lefebvre d'Hellencourt, C., Meilhac, O., & Diotel, N. (2021). Deleterious Effects of Overfeeding on Brain Homeostasis and Plasticity in Adult Zebrafish. *Zebrafish*, zeb.2020.1962. https://doi.org/10.1089/zeb.2020.1962

Godino-Gimeno, A., Sánchez, E., Guillot, R., Rocha, A., Angotzi, A. R., Leal, E., Rotllant, J., & Cerdá-Reverter, J. M. (2020). Growth Performance after Agouti-Signaling Protein 1 (Asip1) Overexpression in Transgenic Zebrafish. *Zebrafish*, *17*(6), 373–381. https://doi.org/10.1089/zeb.2020.1932

Godino-Gimeno, A., Thörnqvist, P. O., Chivite, M., Míguez, J. M., Winberg, S., & Cerdá-Reverter, J. M. (2023). Obesity Impairs Cognitive Function with No Effects on Anxiety-like Behaviour in Zebrafish. *International Journal of Molecular Sciences*, *24*(15). https://doi.org/10.3390/ijms241512316

Godwin, J., Sawyer, S., Perrin, F., Oxendine, S. E., & Kezios, Z. D. (2012). *Adapting the Open Field Test to Assess Anxiety-Related Behavior in Zebrafish* (pp. 181–189). https://doi.org/10.1007/978-1-61779-597-8_13

Gropp, E., Shanabrough, M., Borok, E., Xu, A. W., Janoschek, R., Buch, T., Plum, L., Balthasar, N., Hampel, B., Waisman, A., Barsh, G. S., Horvath, T. L., & Brüning, J. C.

(2005). Agouti-related peptide-expressing neurons are mandatory for feeding. *Nature Neuroscience*, *8*(10), 1289–1291. https://doi.org/10.1038/nn1548

Guillot, R., Cortés, R., Navarro, S., Mischitelli, M., García-Herranz, V., Sánchez, E., Cal, L., Navarro, J. C., Míguez, J. M., Afanasyev, S., Krasnov, A., Cone, R. D., Rotllant, J., & Cerdá-Reverter, J. M. (2016). Behind melanocortin antagonist overexpression in the zebrafish brain: A behavioral and transcriptomic approach. *Hormones and Behavior*, *82*, 87–100. https://doi.org/10.1016/j.yhbeh.2016.04.011

Hallerman, E. M., McLean, E., & Fleming, I. A. (2007). Effects of growth hormone transgenes on the behavior and welfare of aquacultured fishes: A review identifying research needs. In *Applied Animal Behaviour Science* (Vol. 104, Issues 3–4, pp. 265–294). https://doi.org/10.1016/j.applanim.2006.09.008

Harayama, T., & Riezman, H. (2018). Understanding the diversity of membrane lipid composition. *Nature Reviews. Molecular Cell Biology*, *19*(5), 281–296. https://doi.org/10.1038/NRM.2017.138

Hastings, M., O'Neill, J. S., & Maywood, E. S. (2007). Circadian clocks: regulators of endocrine and metabolic rhythms. *The Journal of Endocrinology*, *195*(2), 187–198. https://doi.org/10.1677/JOE-07-0378

Heisler, L. K., Cowley, M. A., Kishi, T., Tecott, L. H., Fan, W., Low, M. J., Smart, J. L., Rubinstein, M., Tatro, J. B., Zigman, J. M., Roger D Cone, & Joel K Elmquist. (2003). Central Serotonin and Melanocortin Pathways Regulating Energy Homeostasis. *Annals New York Academy of Sciences*, *994*, 169–174.

Herget, U., Wolf, A., Wullimann, M. F., & Ryu, S. (2014). Molecular neuroanatomy and chemoarchitecture of the neurosecretory preoptic-hypothalamic area in zebrafish larvae. *Journal of Comparative Neurology*, *522*(7), 1542–1564. https://doi.org/10.1002/CNE.23480

Hinney, A. (1999). Several Mutations in the Melanocortin-4 Receptor Gene Including a Nonsense and a Frameshift Mutation Associated with Dominantly Inherited Obesity in Humans. *Journal of Clinical Endocrinology & Metabolism*, *84*(4), 1483–1486. https://doi.org/10.1210/JC.84.4.1483

Hinz, F. I., Aizenberg, M., Tushev, G., & Schuman, E. M. (2013). Protein synthesis-dependent associative long-term memory in larval zebrafish. *Journal of Neuroscience*, *33*(39), 15382–15387. https://doi.org/10.1523/JNEUROSCI.0560-13.2013

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., Mclaren, K., Matthews, L., Mclaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G.-J., White, S., … Stemple, D. L. (2013). *The zebrafish reference genome sequence and its relationship to the human genome*. https://doi.org/10.1038/nature12111

Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P., & Frank, L. (1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell*, *88*(1), 131–141. https://doi.org/10.1016/S0092-8674(00)81865-6

Isorna, E., de Pedro, N., Valenciano, A. I., Alonso-Gómez, Á. L., & Delgado, M. J. (2017). Interplay between the endocrine and circadian systems in fishes. *Journal of Endocrinology*, *232*(3), R141–R159. https://doi.org/10.1530/JOE-16-0330

Jacob, P. F., & Waddell, S. (2020). Spaced Training Forms Complementary Long-Term Memories of Opposite Valence in Drosophila. *Neuron*, *106*(6), 977-991.e4. https://doi.org/10.1016/j.neuron.2020.03.013

Johnsson, J. I., & Björnsson, B. T. (2001). Growth-enhanced fish can be competitive in the wild. *Functional Ecology*, *15*(5), 654–659. https://doi.org/10.1046/J.0269-8463.2001.00566.X

Jönsson, E., Johnsson, J. I., & Björnsson, B. T. (1998). Growth hormone increases aggressive behavior in juvenile rainbow trout. *Hormones and Behavior*, *33*(1), 9–15. https://doi.org/10.1006/HBEH.1997.1426

Kalueff, A. V., & Cachat, J. M. (2011). Zebrafish Neurobehavioral Protocols. In *Zebrafish Neurobehavioral Protocols* (51st ed., Vol. 51). Neuromethods. https://doi.org/10.1007/978-1-60761-953-6

Kalueff, A. V, Stewart, A. M., & Gerlai, R. (2014). Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol Sci*, *35*(2), 63–75. https://doi.org/10.1016/j.tips.2013.12.002

Kawakami, K., Patton, E. E., & Orger, M. (2016). *Zebrafish Methods and Protocols* (2nd ed., Vol. 1451). http://www.springer.com/series/7651

Kawashima, N., Chaki, S., & Okuyama, S. (2003). Electrophysiological effects of melanocortin receptor ligands on neuronal activities of monoaminergic neurons in

rats. *Neuroscience Letters*, *353*(2), 119–122. https://doi.org/10.1016/j.neulet.2003.09.024

Kehoe, A. S., & Volkoff, H. (2007). Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (Gadus morhua). *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *146*(3), 451–461. https://doi.org/10.1016/j.cbpa.2006.12.026

Koch, M., & Horvath, T. L. (2014). Molecular and cellular regulation of hypothalamic melanocortin neurons controlling food intake and energy metabolism. *Molecular Psychiatry*, *19*(7), 752–761. https://doi.org/10.1038/MP.2014.30

Kurokawa, T., Murashita, K., & Uji, S. (2006a). Characterization and tissue distribution of multiple agouti-family genes in pufferfish, Takifugu rubripes. *Peptides*, *27*(12), 3165–3175. https://doi.org/10.1016/J.PEPTIDES.2006.09.013

Kurokawa, T., Murashita, K., & Uji, S. (2006b). Characterization and tissue distribution of multiple agouti-family genes in pufferfish, Takifugu rubripes. *Peptides*, *27*(12), 3165–3175. https://doi.org/10.1016/j.peptides.2006.09.013

Lahti, K., Laurila, A., Enberg, K., & Piironen, J. (2001). Variation in aggressive behaviour and growth rate between populations and migratory forms in the brown trout, Salmo trutta. *Animal Behaviour*, *62*(5), 935–944. https://doi.org/10.1006/anbe.2001.1821

Lal, P., Tanabe, H., Suster, M. L., Ailani, D., Kotani, Y., Muto, A., Itoh, M., Iwasaki, M., Wada, H., Yaksi, E., & Kawakami, K. (2018). Identification of a neuronal population in the telencephalon essential for fear conditioning in zebrafish. *BMC Biology*, *16*(1). https://doi.org/10.1186/S12915-018-0502-Y

Landgraf, K., Schuster, S., Meusel, A., Garten, A., Riemer, T., Schleinitz, D., Kiess, W., & Körner, A. (2017). Short-term overfeeding of zebrafish with normal or high-fat diet as a model for the development of metabolically healthy versus unhealthy obesity. *BMC Physiology*, *17*(1), 1–10. https://doi.org/10.1186/s12899-017-0031-x

Law, R., & Clow, A. (2020). Stress, the cortisol awakening response and cognitive function. *International Review of Neurobiology*, *150*, 187–217. https://doi.org/10.1016/BS.IRN.2020.01.001

Lawther, A. J., Hale, M. W., & Lowry, C. A. (2020). Serotonin and the neurobiology of anxious states. In *Handbook of Behavioral Neuroscience* (Vol. 31, pp. 505–520). Elsevier B.V. https://doi.org/10.1016/B978-0-444-64125-0.00029-3

Leal, E., Angotzi, A. R., Godino-Gimeno, A., Gregório, S. F., Rotllant, J., Saera-Vila, A., Fuentes, J., & Cerdá-Reverter, J. M. (2022). Agouti overexpression in a transgenic model regulates integrity, permeability and electrogenic amino acid transport in zebrafish intestine. *Frontiers in Marine Science*, *9*. https://doi.org/10.3389/fmars.2022.1011702

Li, X., Zheng, S., & Wu, G. (2021). Nutrition and Functions of Amino Acids in Fish. *Advances in Experimental Medicine and Biology*, *1285*, 133–168. https://doi.org/10.1007/978-3-030-54462-1_8

Lindblom, J., Opmane, B., Mutulis, F., Mutule, I., Petrovska, R., Klusa, V., Bergström, L., & Wikberg, J. E. S. (2001). The MC4 receptor mediates alpha-MSH induced release of nucleus accumbens dopamine. *Neuroreport*, *12*(10), 2155–2158. https://doi.org/10.1097/00001756-200107200-00022

Linker, A., Stewart, A., Gaikwad, S., Cachat, J. M., Elegante, M. F., Kalueff, A. V., & Warnick, J. E. (2011). Assessing the maximum predictive validity for neuropharmacological anxiety screening assays using zebrafish. *Neuromethods*, *51*, 181–190. https://doi.org/10.1007/978-1-60761-953-6_15

Liu, J., Garza, J. C., Li, W., & Lu, X. Y. (2013). Melanocortin-4 receptor in the medial amygdala regulates emotional stress-induced anxiety-like behaviour, anorexia and corticosterone secretion. *International Journal of Neuropsychopharmacology*, *16*(1), 105–120. https://doi.org/10.1017/S146114571100174X

Liu, R., Kinoshita, M., Adolfi, M. C., & Schartl, M. (2019). Analysis of the role of the MC4R system in development, growth, and puberty of medaka. *Frontiers in Endocrinology*, *10*(APR), 213. https://doi.org/10.3389/FENDO.2019.00213/FULL

Loh, K., Zhang, L., Brandon, A., Wang, Q., Begg, D., Qi, Y., Fu, M., Kulkarni, R., Teo, J., Baldock, P., Bruning, J. C., Cooney, G., Neely, G. G., & Herzog, H. (2017). Corrigendum to "Insulin controls food intake and energy balance via NPY neurons" [Mol Metabol 6 (2017) 574–584]. *Molecular Metabolism*, *6*(11), 1562. https://doi.org/10.1016/J.MOLMET.2017.06.017

Lu, D., Willard, D., Patel, I. R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R. P., Wilkison, W. O., & Cone, R. D. (1994). Agouti protein is an

antagonist of the melanocyte-stimulating-hormone receptor. *Nature 1994 371:6500*, *371*(6500), 799–802. https://doi.org/10.1038/371799a0

Luquet, S., Perez, F. A., Hnasko, T. S., & Palmiter, R. D. (2005). NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science (New York, N.Y.)*, *310*(5748), 683–685. https://doi.org/10.1126/SCIENCE.1115524

Madeira, N., & Oliveira, R. F. (2017). Long-Term Social Recognition Memory in Zebrafish. *Zebrafish*, *14*(4), 305–310. https://doi.org/10.1089/zeb.2017.1430

Manuel, R., Gorissen, M., Piza Roca, C., Zethof, J., Van De Vis, H., Flik, G., & Van Den Bos, R. (2014). Inhibitory Avoidance Learning in Zebrafish (Danio Rerio): Effects of Shock Intensity and Unraveling Differences in Task Performance. *Zebrafish*, *11*(4), 341. https://doi.org/10.1089/ZEB.2013.0970

Markov, D. D., Dolotov, O. V., & Grivennikov, I. A. (2023). The Melanocortin System: A Promising Target for the Development of New Antidepressant Drugs. *International Journal of Molecular Sciences 2023, Vol. 24, Page 6664*, *24*(7), 6664. https://doi.org/10.3390/IJMS24076664

Marsh, D. J., Hollopeter, G., Huszar, D., Laufer, R., Yagaloff, K. A., Fisher, S. L., Burn, P., & Palmiter, R. D. (1999). *Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides*. http://genetics.nature.com

Marston, O. J., Garfield, A. S., & Heisler, L. K. (2011). Role of central serotonin and melanocortin systems in the control of energy balance. In *European Journal of Pharmacology* (Vol. 660, Issue 1, pp. 70–79). https://doi.org/10.1016/j.ejphar.2010.12.024

Maximino, C., Araujo, J., H Matos Oliveira, K. R., & Manoel Herculano, A. (2013). *The Serotonergic System of Zebrafish: Genomics, Neuroanatomy and Neuropharmacology*. https://www.researchgate.net/publication/257931575

Maximino, C., Marques De Brito, T., De Mattos Dias, C. A. G., Gouveia, A., & Morato, S. (2010). Scototaxis as anxiety-like behavior in fish. *Nature Protocols*, *5*(2), 221–228. https://doi.org/10.1038/NPROT.2009.225

Maximino, C., Puty, B., Benzecry, R., Araújo, J., Lima, M. G., De Jesus Oliveira Batista, E., Renata De Matos Oliveira, K., Crespo-Lopez, M. E., & Herculano, A. M. (2013). Role of serotonin in zebrafish (Danio rerio) anxiety: Relationship with serotonin levels and effect of buspirone, WAY 100635, SB 224289, fluoxetine and para-

chlorophenylalanine (pCPA) in two behavioral models. *Neuropharmacology*, *71*, 83–97. https://doi.org/10.1016/j.neuropharm.2013.03.006

McArthur, K. L., Chow, D. M., & Fetcho, J. R. (2019). Zebrafish as a model for revealing the neuronal basis of behavior. In *The Zebrafish in Biomedical Research: Biology, Husbandry, Diseases, and Research Applications* (pp. 593–617). Elsevier. https://doi.org/10.1016/B978-0-12-812431-4.00046-4

Medawar, E., & Witte, A. V. (2022). Impact of obesity and diet on brain structure and function: a gut-brain-body crosstalk. *The Proceedings of the Nutrition Society*, *81*(4). https://doi.org/10.1017/S0029665122002786

Meguro, S., Hosoi, S., & Hasumura, T. (2019). High-fat diet impairs cognitive function of zebrafish. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-53634-z

Mendoza, J. (2007). Circadian clocks: setting time by food. *Journal of Neuroendocrinology*, *19*(2), 127–137. https://doi.org/10.1111/J.1365-2826.2006.01510.X

Meshalkina, D. A., Kizlyk, M. N., Kysil, E. V., Collier, A. D., Echevarria, D. J., Abreu, M. S., Barcellos, L. J. G., Song, C., & Kalueff, A. V. (2017). Understanding zebrafish cognition. *Behavioural Processes*, *141*(Pt 2), 229–241. https://doi.org/10.1016/J.BEPROC.2016.11.020

Metcalfe, N. B., Huntingford, F. A., Graham, W. D., & Thorpe, J. E. (1989). Early social status and the development of life-history strategies in Atlantic salmon. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, *236*(1282), 7–19. https://doi.org/10.1098/RSPB.1989.0009

Michaud, E. J., Bultman, S. J., Stubbs, L. J., & Woychik, R. P. (1993). The embryonic lethality of homozygous lethal yellow mice (A(y)/A(y)) is associated with the disruption of a novel RNA-binding protein. *Genes and Development*, *7*(7 A), 1203–1213. https://doi.org/10.1101/gad.7.7a.1203

Micioni Di Bonaventura, E., Botticelli, L., Del Bello, F., Giorgioni, G., Piergentili, A., Quaglia, W., Romano, A., Gaetani, S., Micioni Di Bonaventura, M. V., & Cifani, C. (2022). Investigating the role of the central melanocortin system in stress and stress-related disorders. *Pharmacological Research*, *185*, 106521. https://doi.org/10.1016/J.PHRS.2022.106521

Micioni Di Bonaventura, E., Botticelli, L., Tomassoni, D., Tayebati, S. K., Micioni Di Bonaventura, M. V., & Cifani, C. (2020). The Melanocortin System behind the

Dysfunctional Eating Behaviors. *Nutrients 2020, Vol. 12, Page 3502*, *12*(11), 3502. https://doi.org/10.3390/NU12113502

Miller, M. W., Duhl, D. M. J., Vrieling, H., Cordes, S. P., Ollmann, M. M., Winkes, B. M., & Barsh, G. S. (1993). Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. *Genes & Development*, *7*(3), 454–467. https://doi.org/10.1101/GAD.7.3.454

Mistlberger, R. E. (2011). Neurobiology of food anticipatory circadian rhythms. *Physiology & Behavior*, *104*(4), 535–545. https://doi.org/10.1016/J.PHYSBEH.2011.04.015

Morgan, C., & Cone, R. D. (2006). Melanocortin-5 receptor deficiency in mice blocks a novel pathway influencing pheromone-induced aggression. *Behavior Genetics*, *36*(2), 291–300. https://doi.org/10.1007/S10519-005-9024-9/FIGURES/2

Morgan, C., Thomas, R. E., Ma, W., Novotny, M. V., & Cone, R. D. (2004). Melanocortin-5 Receptor Deficiency Reduces a Pheromonal Signal for Aggression in Male Mice. *Chemical Senses*, *29*(2), 111–115. https://doi.org/10.1093/CHEMSE/BJH011

Mountjoy, K. G. (2015). Pro-Opiomelanocortin (POMC) Neurones, POMC-Derived Peptides, Melanocortin Receptors and Obesity: How Understanding of this System has Changed Over the Last Decade. *Journal of Neuroendocrinology*, *27*(6), 406–418. https://doi.org/10.1111/JNE.12285

Murashita, K., Kurokawa, T., Ebbesson, L. O. E., Stefansson, S. O., & Rønnestad, I. (2009). Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (Salmo salar). *General and Comparative Endocrinology*, *162*(2), 160–171. https://doi.org/10.1016/J.YGCEN.2009.03.015

Nam, R. H., Kim, W., & Lee, C. J. (2004). NMDA receptor-dependent long-term potentiation in the telencephalon of the zebrafish. *Neuroscience Letters*, *370*(2–3), 248–251. https://doi.org/10.1016/J.NEULET.2004.08.037

Nasif, S., de Souza, F. S. J., González, L. E., Yamashita, M., Orquera, D. P., Low, M. J., & Rubinstein, M. (2015). Islet 1 specifies the identity of hypothalamic melanocortin neurons and is critical for normal food intake and adiposity in adulthood. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(15), E1861–E1870. https://doi.org/10.1073/pnas.1500672112

Navarro, S., Guillot, R., Crespo, D., Schulz, R. W., Ge, W., Rotllant, J., Cerdá-Reverter, J. M., & Rocha, A. (2021). Enhanced growth without accelerated puberty in fish: A role for the melanocortin system. *Aquaculture*, *540*, 736721. https://doi.org/10.1016/J.AQUACULTURE.2021.736721

Navarro, S., Soletto, L., Puchol, S., Rotllant, J., Soengas, J. L., & Cerdá-Reverter, J. M. (2016). 60 YEARS OF POMC: POMC: an evolutionary perspective. *Journal of Molecular Endocrinology*, *56*(4), T113–T118. https://doi.org/10.1530/JME-15-0288

Nijenhuis, W. A. J., Oosterom, J., & Adan, R. A. H. (2001a). AgRP(83-132) acts as an inverse agonist on the human-melanocortin-4 receptor. *Molecular Endocrinology (Baltimore, Md.)*, *15*(1), 164–171. https://doi.org/10.1210/MEND.15.1.0578

Nijenhuis, W. A. J., Oosterom, J., & Adan, R. A. H. (2001b). AgRP(83-132) acts as an inverse agonist on the human-melanocortin-4 receptor. *Molecular Endocrinology (Baltimore, Md.)*, *15*(1), 164–171. https://doi.org/10.1210/MEND.15.1.0578

Norris, D. (2017). Short-term memory and long-term memory are still different. *Psychological Bulletin*, *143*(9), 992–1009. https://doi.org/10.1037/BUL0000108

Northcutt, R. G. (2006). Connections of the lateral and medial divisions of the goldfish telencephalic pallium. *The Journal of Comparative Neurology*, *494*(6), 903–943. https://doi.org/10.1002/CNE.20853

Oka, T., Nishimura, Y., Zang, L., Hirano, M., Shimada, Y., Wang, Z., Umemoto, N., Kuroyanagi, J., Nishimura, N., & Tanaka, T. (2010). Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. *BMC Physiology*, *10*(1), 21. https://doi.org/10.1186/1472-6793-10-21

Ollmann, M. M., Wilson, B. D., Yang, Y. K., Kerns, J. A., Chen, Y., Gantz, I., & Barsh, G. S. (1997). Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science (New York, N.Y.)*, *278*(5335), 135–138. https://doi.org/10.1126/SCIENCE.278.5335.135

Øverli, Ø., Harris, C. A., & Winberg, S. (1999). Short-Term Effects of Fights for Social Dominance and the Establishment of Dominant-Subordinate Relationships on Brain Monoamines and Cortisol in Rainbow Trout. *Brain Behavior and Evolution*, *54*(5), 263–275. https://doi.org/10.1159/000006627

Øverli, Ø., Pottinger, T. G., Carrick, T. R., Øverli, E., & Winberg, S. (2002). Differences in behaviour between rainbow trout selected for high- and low-stress

responsiveness. *Journal of Experimental Biology*, *205*(3), 391–395. https://doi.org/10.1242/jeb.205.3.391

Padilla, S. L., Qiu, J., Soden, M. E., Sanz, E., Nestor, C. C., Barker, F. D., Quintana, A., Zweifel, L. S., Rønnekleiv, O. K., Kelly, M. J., & Palmiter, R. D. (2016). AgRP Neural Circuits Mediate Adaptive Behaviors in the Starved State. *Nature Neuroscience*, *19*(5), 734. https://doi.org/10.1038/NN.4274

Papadimitriou, A., & Priftis, K. N. (2009). Regulation of the hypothalamic-pituitary-adrenal axis. *Neuroimmunomodulation*, *16*(5), 265–271. https://doi.org/10.1159/000216184

Park, H. R., Park, M., Choi, J., Park, K. Y., Chung, H. Y., & Lee, J. (2010). A high-fat diet impairs neurogenesis: involvement of lipid peroxidation and brain-derived neurotrophic factor. *Neuroscience Letters*, *482*(3), 235–239. https://doi.org/10.1016/J.NEULET.2010.07.046

Partch, C. L., Green, C. B., & Takahashi, J. S. (2014). Molecular Architecture of the Mammalian Circadian Clock. *Trends in Cell Biology*, *24*(2), 90. https://doi.org/10.1016/J.TCB.2013.07.002

Picolo, V. L., Quadros, V. A., Canzian, J., Grisolia, C. K., Goulart, J. T., Pantoja, C., de Bem, A. F., & Rosemberg, D. B. (2021). Short-term high-fat diet induces cognitive decline, aggression, and anxiety-like behavior in adult zebrafish. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *110*. https://doi.org/10.1016/j.pnpbp.2021.110288

Pottinger, T. G., & Carrick, T. R. (1999). A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Aquaculture*, *175*(3–4), 351–363. https://doi.org/10.1016/S0044-8486(99)00107-6

Purkayastha, S., & Cai, D. (2013). Disruption of Neurogenesis by Hypothalamic Inflammation in Obesity or Aging. *Reviews in Endocrine & Metabolic Disorders*, *14*(4), 351–356. https://doi.org/10.1007/S11154-013-9279-Z

Quadros, V. A., Silveira, A., Giuliani, G. S., Didonet, F., Silveira, A. S., Nunes, M. E., Silva, T. O., Loro, V. L., & Rosemberg, D. B. (2016). Strain- and context-dependent behavioural responses of acute alarm substance exposure in zebrafish. *Behavioural Processes*, *122*, 1–11. https://doi.org/10.1016/j.beproc.2015.10.014

Racca, S., Spaccamiglio, A., Esculapio, P., Abbadessa, G., Cangemi, L., DiCarlo, F., & Portaleone, P. (2005). Effects of swim stress and α-MSH acute pre-treatment on brain 5-HT transporter and corticosterone receptor. *Pharmacology Biochemistry and Behavior*, *81*(4), 894–900. https://doi.org/10.1016/J.PBB.2005.06.014

Rahati, S., Qorbani, M., Naghavi, A., & Pishva, H. (2022). Association and interaction of the MC4R rs17782313 polymorphism with plasma ghrelin, GLP-1, cortisol, food intake and eating behaviors in overweight/obese Iranian adults. *BMC Endocrine Disorders*, *22*(1). https://doi.org/10.1186/S12902-022-01129-W

Ralph, M. R., & Hurd, M. W. (1995). Circadian pacemakers in vertebrates. *Ciba Foundation Symposium*, *183*. https://doi.org/10.1002/9780470514597.CH5

Ramos-Rodriguez, J. J., Molina-Gil, S., Ortiz-Barajas, O., Jimenez-Palomares, M., Perdomo, G., Cozar-Castellano, I., Lechuga-Sancho, A. M., & Garcia-Alloza, M. (2014). Central proliferation and neurogenesis is impaired in type 2 diabetes and prediabetes animal models. *PloS One*, *9*(2). https://doi.org/10.1371/JOURNAL.PONE.0089229

Reemst, K., Shahin, H., & Shahar, O. D. (2023). Learning and memory formation in zebrafish: Protein dynamics and molecular tools. *Frontiers in Cell and Developmental Biology*, *11*. https://doi.org/10.3389/fcell.2023.1120984

Reolon, G. K., Braga, L. M. E., Camassola, M., Luft, T., Henriques, J. A. P., Nardi, N. B., & Roesler, R. (2006). Long-term memory for aversive training is impaired in Idua(-/-) mice, a genetic model of mucopolysaccharidosis type I. *Brain Research*, *1076*(1), 225–230. https://doi.org/10.1016/J.BRAINRES.2006.01.008

Ringholm, A., Fredriksson, R., Poliakova, N., Yan, Y. L., Postlethwait, J. H., Larhammar, D., & Schiöth, H. B. (2002). One melanocortin 4 and two melanocortin 5 receptors from zebrafish show remarkable conservation in structure and pharmacology. *Journal of Neurochemistry*, *82*(1), 6–18. https://doi.org/10.1046/J.1471-4159.2002.00934.X

Rocha, A., Godino-Gimeno, A., & Cerdá-Reverter, J. M. (2019). Evolution of proopiomelanocortin. *Vitamins and Hormones*, *111*, 1–16. https://doi.org/10.1016/BS.VH.2019.05.008

Rocha, A., Godino-Gimeno, A., Rotllant, J., & Cerdá-Reverter, J. M. (2023). Agouti-Signalling Protein Overexpression Reduces Aggressiveness in Zebrafish. *Biology*, *12*(5), 712. https://doi.org/10.3390/biology12050712

Romanova, I. V., Derkach, K. V., Mikhrina, A. L., Sukhov, I. B., Mikhailova, E. V., & Shpakov, A. O. (2018). The Leptin, Dopamine and Serotonin Receptors in Hypothalamic POMC-Neurons of Normal and Obese Rodents. *Neurochemical Research*, *43*(4), 821–837. https://doi.org/10.1007/s11064-018-2485-z

Rose, J. K., Kaun, K. R., & Rankin, C. H. (2002). A new group-training procedure for habituation demonstrates that presynaptic glutamate release contributes to long-term memory in Caenorhabditis elegans. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *9*(3), 130–137. https://doi.org/10.1101/LM.46802

Roseberry, A. G., Stuhrman, K., & Dunigan, A. I. (2015). Regulation of the mesocorticolimbic and mesostriatal dopamine systems by α-melanocyte stimulating hormone and agouti-related protein. *Neuroscience and Biobehavioral Reviews*, *56*, 15–25. https://doi.org/10.1016/J.NEUBIOREV.2015.06.020

Rossi, M., Kim, M. S., Morgan, D. G. A., Small, C. J., Edwards, C. M. B., Sunter, D., Abusnana, S., Goldstone, A. P., Russell, S. H., Stanley, S. A., Smith, D. M., Yagaloff, K., Ghatei, M. A., & Bloom, S. R. (1998). A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. *Endocrinology*, *139*(10), 4428–4431. https://doi.org/10.1210/ENDO.139.10.6332

Ryan, K. K., Mul, J. D., Clemmensen, C., Egan, A. E., Begg, D. P., Halcomb, K., Seeley, R. J., Herman, J. P., & Ulrich-Lai, Y. M. (2014). Loss of melanocortin-4 receptor function attenuates HPA responses to psychological stress. *Psychoneuroendocrinology*, *42*, 98–105. https://doi.org/10.1016/J.PSYNEUEN.2014.01.010

Sánchez, E., Rubio, V. C., & Cerdá-Reverter, J. M. (2010). Molecular and pharmacological characterization of the melanocortin type 1 receptor in the sea bass. *General and Comparative Endocrinology*, *165*(1), 163–169. https://doi.org/10.1016/J.YGCEN.2009.06.008

Sánchez, E., Rubio, V. C., Thompson, D., Metz, J., Flik, G., Millhauser, G. L., & Cerdá-Reverter, J. M. (2009). Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (Dicentrarchus labrax). *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *296*(5). https://doi.org/10.1152/AJPREGU.90948.2008

Sarangi, M., & Dus, M. (2021). Crème de la Créature: Dietary Influences on Behavior in Animal Models. *Frontiers in Behavioral Neuroscience*, *15*, 746299. https://doi.org/10.3389/FNBEH.2021.746299/BIBTEX

Schachter, J., Martel, J., Lin, C. S., Chang, C. J., Wu, T. R., Lu, C. C., Ko, Y. F., Lai, H. C., Ojcius, D. M., & Young, J. D. (2018). Effects of obesity on depression: A role for inflammation and the gut microbiota. *Brain, Behavior, and Immunity*, *69*, 1–8. https://doi.org/10.1016/J.BBI.2017.08.026

Schibler, U., Gotic, I., Saini, C., Gos, P., Curie, T., Emmenegger, Y., Sinturel, F., Gosselin, P., Gerber, A., Fleury-Olela, F., Rando, G., Demarque, M., & Franken, P. (2015). Clock-Talk: Interactions between Central and Peripheral Circadian Oscillators in Mammals. *Cold Spring Harbor Symposia on Quantitative Biology*, *80*, 223–232. https://doi.org/10.1101/SQB.2015.80.027490

Schneider, H. (2011). Measuring agonistic behavior in zebrafish. *Neuromethods*, *51*, 125–134. https://doi.org/10.1007/978-1-60761-953-6_10

Scott, K. M., McGee, M. A., Wells, J. E., & Oakley Browne, M. A. (2008). Obesity and mental disorders in the adult general population. *Journal of Psychosomatic Research*, *64*(1), 97–105. https://doi.org/10.1016/j.jpsychores.2007.09.006

Sebag, J. A., Zhang, C., Hinkle, P. M., Bradshaw, A. M., & Cone, R. D. (2013). Developmental Control of the Melanocortin-4 Receptor by MRAP2 Proteins in Zebrafish. *Science (New York, N.Y.)*, *341*(6143), 278. https://doi.org/10.1126/SCIENCE.1232995

Shainer, I., Buchshtab, A., Hawkins, T. A., Wilson, S. W., Cone, R. D., & Gothilf, Y. (2017). Novel hypophysiotropic AgRP2 neurons and pineal cells revealed by BAC transgenesis in zebrafish. *Scientific Reports*, *7*. https://doi.org/10.1038/srep44777

Shainer, I., Michel, M., Marquart, G. D., Bhandiwad, A. A., Zmora, N., Ben-Moshe Livne, Z., Zohar, Y., Hazak, A., Mazon, Y., Förster, D., Hollander-Cohen, L., Cone, R. D., Burgess, H. A., & Gothilf, Y. (2019). Agouti-Related Protein 2 Is a New Player in the Teleost Stress Response System. *Current Biology : CB*, *29*(12), 2009-2019.e7. https://doi.org/10.1016/J.CUB.2019.05.021

Shibutani, M., Vibulyaseck, S., & Maruyama, I. N. (2022). Aversive Associative Learning and Memory Formation by Pairing Two Chemicals in Caenorhabditis elegans. *Journal of Visualized Experiments*, *2022*(184). https://doi.org/10.3791/64137

Song, Y., & Cone, R. D. (2007). Creation of a genetic model of obesity in a teleost. *The FASEB Journal*, *21*(9), 2042–2049. https://doi.org/10.1096/FJ.06-7503COM

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biological Reviews of the Cambridge Philosophical Society*, *83*(1), 13–34. https://doi.org/10.1111/J.1469-185X.2007.00030.X

Srivastava, A. B., & Gold, M. S. (2019). Translating Neurobiology into Practice in Tobacco, Alcohol, Drug, and Behavioral Addictions. In *Handbook of Behavioral Neuroscience* (Vol. 29, pp. 389–401). Elsevier B.V. https://doi.org/10.1016/B978-0-12-803161-2.00028-X

Stewart, A. M., Grieco, F., Tegelenbosch, R. A. J., Kyzar, E. J., Nguyen, M., Kaluyeva, A., Song, C., Noldus, L. P. J. J., & Kalueff, A. V. (2015). A novel 3D method of locomotor analysis in adult zebrafish: Implications for automated detection of CNS drug-evoked phenotypes. *Journal of Neuroscience Methods*, *255*, 66–74. https://doi.org/10.1016/j.jneumeth.2015.07.023

Sutton, G. M., Perez-Tilve, D., Nogueiras, R., Fang, J., Kim, J. K., Cone, R. D., Gimble, J. M., Tschöp, M. H., & Butler, A. A. (2008). The Melanocortin-3 Receptor Is Required for Entrainment to Meal Intake. *The Journal of Neuroscience*, *28*(48), 12946. https://doi.org/10.1523/JNEUROSCI.3615-08.2008

Tricklebank, M. D. (2019). Serotonin and sleep. In *The Serotonin System: History, Neuropharmacology, and Pathology* (pp. 181–192). Elsevier. https://doi.org/10.1016/B978-0-12-813323-1.00010-4

Trueta, C., & Cercós, M. G. (2012). Liberacion sináptica y extrasináptica de serotonina. In *Salud Mental* (Vol. 35, Issue 5).

Türkoğlu, M., Baran, A., Sulukan, E., Ghosigharehagaji, A., Yildirim, S., Akgül Ceyhun, H., Bolat, İ., Arslan, M., & Buğrahan Ceyhun, S. (2022). The potential effect mechanism of high-fat and high-carbohydrate diet-induced obesity on anxiety and offspring of zebrafish. *Eating and Weight Disorders - Studies on Anorexia, Bulimia and Obesity*, *27*, 163–177. https://doi.org/10.1007/s40519-021-01140-5

Vaisse, C., Clement, K., Durand, E., Hercberg, S., Guy-Grand, B., & Froguel, P. (2000). Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *Journal of Clinical Investigation*, *106*(2), 253–262. https://doi.org/10.1172/JCI9238

Vallone, D., Gondi, S. B., Whitmore, D., & Foulkes, N. S. (2004). *E-box function in a period gene repressed by light*. www.pnas.orgcgidoi10.1073pnas.0305436101

Vallone, D., Lahiri, K., Dickmeis, T., & Foulkes, N. S. (2005). Zebrafish Cell Clocks Feel the Heat and See the Light! *Https://Home.Liebertpub.Com/Zeb*, *2*(3), 171–187. https://doi.org/10.1089/ZEB.2005.2.171

Vasey, C., McBride, J., & Penta, K. (2021). Circadian Rhythm Dysregulation and Restoration: The Role of Melatonin. *Nutrients*, *13*(10). https://doi.org/10.3390/NU13103480

Volgin, A. D., Yakovlev, O. A., Demin, K. A., Alekseeva, P. A., & Kalueff, A. V. (2019). Acute behavioral effects of deliriant hallucinogens atropine and scopolamine in adult zebrafish. *Behavioural Brain Research*, *359*, 274–280. https://doi.org/10.1016/j.bbr.2018.10.033

Volkow, N. D., Wang, G. J., & Baler, R. D. (2011). Reward, dopamine and the control of food intake: implications for obesity. *Trends in Cognitive Sciences*, *15*(1), 37. https://doi.org/10.1016/J.TICS.2010.11.001

Whitmore, D., Foulkes, N. S., & Sassone-Corsi, P. (2000). Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature*, *404*(6773), 87–91. https://doi.org/10.1038/35003589

Winberg, S., & Thörnqvist, P.-O. (2016). Role of brain serotonin in modulating fish behavior. *Current Zoology*, *62*(3), 317–323. https://doi.org/10.1093/CZ/ZOW037

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K. M., Stewart, A., Gilder, T., … Kalueff, A. V. (2010). Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research*, *208*(2), 450–457. https://doi.org/10.1016/j.bbr.2009.12.023

Woods, S. C., Lutz, T. A., Geary, N., & Langhans, W. (2006). Pancreatic signals controlling food intake; insulin, glucagon and amylin. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *361*(1471), 1219. https://doi.org/10.1098/RSTB.2006.1858

Wright, D., Rimmer, L. B., Pritchard, V. L., Krause, J., & Butlin, R. K. (2003). Inter and intra-population variation in shoaling and boldness in the zebrafish (Danio rerio). *Die Naturwissenschaften*, *90*(8), 374–377. https://doi.org/10.1007/S00114-003-0443-2

Xia, S., Miyashita, T., Fu, T. F., Lin, W. Y., Wu, C. L., Pyzocha, L., Lin, I. R., Saitoe, M., Tully, T., & Chiang, A. S. (2005). NMDA Receptors Mediate Olfactory Learning and Memory in Drosophila. *Current Biology : CB*, *15*(7), 603. https://doi.org/10.1016/J.CUB.2005.02.059

Xu, Y., Jones, J. E., Lauzon, D. A., Anderson, J. G., Balthasar, N., Heisler, L. K., Zinn, A. R., Lowell, B. B., & Elmquist, J. K. (2010). A serotonin and melanocortin circuit mediates D-fenfluramine anorexia. *Journal of Neuroscience*, *30*(44), 14630–14634. https://doi.org/10.1523/JNEUROSCI.5412-09.2010

Yang, Y., Lee, M., & Fairn, G. D. (2018). Phospholipid subcellular localization and dynamics. *The Journal of Biological Chemistry*, *293*(17), 6230–6240. https://doi.org/10.1074/JBC.R117.000582

Yanik, T., & Durhan, S. T. (2023). Specific Functions of Melanocortin 3 Receptor (MC3R). *Journal of Clinical Research in Pediatric Endocrinology*, *15*(1), 5–21. https://doi.org/10.4274/JCRPE.GALENOS.2022.2022-5-21

Zang, L., Maddison, L. A., & Chen, W. (2018). Zebrafish as a model for obesity and diabetes. In *Frontiers in Cell and Developmental Biology* (Vol. 6, Issue AUG). Frontiers Media S.A. https://doi.org/10.3389/fcell.2018.00091

Zhang, C. (2012). Elucidation of the roles of agouti related proteins in zebrafish. *Scholar Archive*, *755*. https://digitalcommons.ohsu.edu/etd/755

Zhang, C., Song, Y., Thompson, D. A., Madonna, M. A., Millhauser, G. L., Toro, S., Varga, Z., Westerfield, M., Gamse, J., Chen, W., & Cone, R. D. (2010). Pineal-specific agouti protein regulates teleost background adaptation. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(47), 20164–20171. https://doi.org/10.1073/PNAS.1014941107

Zhang, Q., Jin, K., Chen, B., Liu, R., Cheng, S., Zhang, Y., & Lu, J. (2022). Overnutrition Induced Cognitive Impairment: Insulin Resistance, Gut-Brain Axis, and Neuroinflammation. *Frontiers in Neuroscience*, *16*, 884579. https://doi.org/10.3389/FNINS.2022.884579/BIBTEX

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