BIOSÍNTESIS DE ÁCIDOS GRASOS POLIINSATURADOS DE CADENA LARGA EN GAMÁRIDOS

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Informan:

Que la presente Tesis Doctoral titulada **"Biosíntesis de ácidos grasos poliinsaturados de cadena larga en gamáridos"**, ha sido realizado por D. Alberto Ribes Navarro bajo su dirección en el grupo de Especies Auxiliares en Acuicultura, Larvicultura y Ecotoxicología. Una vez revisado y comprobado el trabajo, consideramos que reúne los requisitos necesarios para la obtención del grado de Doctor y, por consiguiente, autorizamos su presentación ante el tribunal correspondiente.

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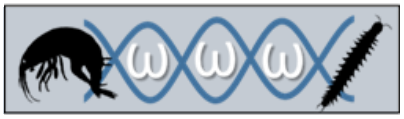
IMPROMEGA



A mis padres

A mi hermano

A ti, "Sonsi"

"¿Quién dijo que la melancolía es elegante? Quitaos esa máscara de tristeza, siempre hay motivo para cantar, para alabar al santísimo misterio, no seamos cobardes, corramos a decírselo a quien sea, siempre hay alguien que amamos y nos ama."

Gloria Fuertes

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En una región muy remota, conocida como "Iatstán", coincidió cierto grupo de personas que hacía que los días fueran muchísimo más llevaderos y agradables para sus habitantes. Gracias a todos los miembros de ese grupo de "disidentes", desde los más veteranos a los que se han ido uniendo más recientemente a la causa. Pero en Iatstán no todo era trabajar, en ocasiones, aquellos combatientes necesitaban

reponer fuerzas en los locales regentados por sus amigos "Manolo" y su "secreto con tomate" o en casa de su amiga "Tere" con su exquisito plato de conejo al ajillo. Gracias a todos de verdad, escribir todos vuestros nombres supondría llenar páginas con recuerdos con cada uno de vosotros; espero que entendáis (mediante estas "ácidas" referencias) todo lo que habéis supuesto para mí durante este proceso. No existe técnica científica que determine el grado de ayuda otorgado, es incuantificable.

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Por cierto: ¿A que la Portada es una barbaridad? Pues tiene nombre y apellido o, más bien, *nickname*: Iscariote. Dichosa casualidad la de encontrarnos de nuevo y poder enmendar ciertas actitudes del pasado por culpa de la "maldita" adolescencia. Gracias por esas partidas tan a la vez serias y absurdas al *OW2*, con nuestros memes y nuestras idioteces; de verdad que me servían de gran ayuda para desconectar de tanta ciencia. Espero que el tiempo te devuelva todo lo que te mereces, nos "vemos" pronto. Un abrazo, pichón.

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Bueno, se va acercando el final de este "textazo", el cual me ha costado más de escribir que las 200 "y pico" páginas sobre gamáridos y ácidos grasos. Se suele decir que lo mejor va al final y esta vez no podía ser menos. De verdad que estoy realmente agradecido a todas aquellas personas que he mencionado a lo largo de estos párrafos, pero existe una sin la que todo esto no hubiera sido posible, al menos no

de esta forma. Sonsi, antes de todo me gustaría pedirte perdón. Sé que no ha sido mi mejor época y que muchas veces has tenido que lidiar con una "roca con patas" pero quiero que sepas que todo ese tiempo siempre, siempre, has estado en mi corazón y, cada día que pasaba, por malo que fuera, mi amor por ti no hacía más que aumentar. Gracias por estar siempre conmigo, por quererme de esa manera tan sincera y sana. Gracias por simplemente, estar y acompañar, cuando había momentos que eso era lo único que necesitaba. Gracias por detectar mis bajones y ayudarme a recuperarme. Gracias por enseñarme de nuevo a querer, a amar. Gracias por ese inolvidable "Sí quiero" que cambió mi vida a mejor. Sigamos cruzando puentes juntos, ¿te parece? De verdad que no me imagino cómo hubieran sido todos estos años de no ser por ti y, puestos a imaginar, hagámoslo para el resto de nuestra vida. Te quiero a todos los niveles, desde las pequeñas cosas como la forma en la que mueves los pies cuando te acuestas en el sofá a hacer la siesta, hasta los grandes gestos que haces por amor, independientemente de la persona. Todo el mundo dice que les encantas por esa sinceridad que muestras al abrazar, al saludar, que se nota que abrazas y quieres de verdad. Eres maravillosa, eres real, y nos haces ser mejores a todos aquellos que te rodeamos. Muchas veces me preguntas que por qué te quiero y yo nunca sé qué responder exactamente. Quiero que veas y te des cuenta de que es imposible "dilucidar" el motivo exacto de por qué te quiero cuando son tantas las razones y virtudes que hacen de ti la persona excepcional que realmente eres. Podría seguir dedicándote miles y miles de palabras y frases en este apartado de agradecimientos, pero prefiero hacerlo de otra forma.

Te dedico mi Tesis Sonia, te amo.

Tabla de contenidos

Listado de Figuras

Listado de Tablas

Listado de Abreviaturas

aa: Aminoácido

***acc*:** Acetil-CoA carboxilasa

acot: Acil-CoA tioesterasa

***acs*:** Acil-CoA sintetasa

Acsl: Acil-CoA sintetasa de cadena larga

***actb*:** β-actina

ALA: *Alpha linoleic acid* (Ácido α-linolénico)

ANOVA: *One-Way Analysis of Variance* (Análisis de la varianza de una vía)

ARA: *Arachidonic acid* (Ácido araquidónico)

ATP: Adenosín trifosfato

BHT: *Butylated hydroxytoluene* (Butilhidroxitolueno)

BLAST: *Basic Local Alignment Search Tool*

bp: *base pair* (par de bases)

cDNA: *Complementary DNA* (ADN complementario)

***cox1*:** Citocromo c oxidasa, subunidad 1

***cpt1a*:** Carnitina palmitoiltransferasa 1A

***d9d*:** Estearil-CoA 9-desaturasa

***dgat*:** Diacilglicerol-aciltransferasa

DGE: *Differential gene expression* (Expresión diferencial de genes)

DHA: *Docosahexaenoic acid* (Ácido docosahexaenoico)

DNA: *Deoxyribonucleic acid* (Ácido desoxirribonucleico, ADN)

DPA: *Docosapentaenoic acid* (Ácido docosapentaenoico)

DTA: *Docosatetraenoic acid* (Ácido docosatetraenoico)

Eip74EF-like: *Ecdysone-induced protein 74EF-like*

Elovl: *Elongation of very long-chain fatty acid protein* (Elongasa)

EPA: Ácido eicosapentaenoico

FA: *Fatty acid* (Ácido graso)

***fabp*:** Proteína de unión de ácidos grasos

FAME: *Fatty acid methyl ester* (Éster metílico de ácido graso)

FAO: *Food and Agriculture Organization of the United Nations* (Organización de las Naciones Unidas para la Alimentación y la Agricultura)

FAS: *Fatty acid synthase* (Ácido graso sintasa)

FC: *Fold Change*

FDR: *False discovery rate* (Índice de falso positivo)

Fed: *Front-end desaturase* (desaturasa "front-end")

FM: *Fishmeal* (Harina de pescado)

FO: *Fish oil* (Aceite de pescado)

GLA: *γ-Linolenic Acid* (Ácido γ-linolénico)

GMO: *Genetically modified organism* (Organismo modificado genéticamente)

HGT: *Horizontal Gene Transfer* (Transferencia horizontal de genes)

IMTA: *Integrated Multitrophic Aquaculture* (Acuicultura Multitrófica Integrada)

IO: *Insect oil* (Aceite de insectos)

***jheh*:** *Juvenile hormone epoxide hydrolase*

LA: *Linoleic Acid* (Ácido linoleico)

LC-PUFA: *Long-chain polyunsaturated fatty acid* (Ácido graso poliinsaturado de cadena larga)

***mih*:** Hormona inhibitoria de la muda

mtDNA: DNA mitocondrial

MUFA: *Monounsaturated fatty acid* (Ácido graso monoinsaturado)

NCBI: *National Centre for Biotechnology Information*

***NFκB*:** Factor nuclear kappa b

nt: Nucleótido

OA: Oleic Acid (Ácido oleico)

ORF: *Open reading frame* (región codificante)

PA: *Palmitic acid* (Ácido palmítico)

PCA: *Principal Component Analysis* (Análisis de componentes principales)

PCR: *Polymerase Chain Reaction* (Reacción en cadena de la polimerasa)

PUFA: *Polyunsaturated fatty acid* (Ácido graso poliinsaturado)

RACE: *Rapid Amplificaction of cDNA Ends*

RAxML: *Randomized axelerated maximum likelihood*

RNA: *Ribonucleic acid* (Ácido ribonucleico, ARN)

RNASeq: Secuenciación de ARN

Scd: Estearoil-CoA desaturasa

SDA: Ácido estearidónico

SFA: *Saturated fatty acid* (Ácido graso saturado)

SGR: *Specific growth rate* (Tasa de crecimiento específico)

SRA: *Sequence Read Archive*

***srebp1*:** *Sterol regulatory element-binding protein 1*

TAG: *Triacylglyceride* (Triacilglicérido)

TSA: *Transcriptome Shotgun Assembly*

UE: Unión Europea

VLC-PUFA: *Very long-chain polyunsaturated fatty acid* (Ácido graso de cadena muy larga)

VO: *Vegetable oil* (Aceite vegetal)

WGS: *Whole Genome Shotgun*

ωx-des: *methyl-end desaturase* (desaturasa "methyl-end")

Resumen

El rápido crecimiento de la acuicultura en las últimas décadas, ha planteado una serie de problemas relacionados con la disponibilidad de ingredientes de origen marino como son las harinas y los aceites de pescado ("FM" y "FO" por sus siglas en inglés), utilizados en la elaboración de piensos. Estos ingredientes son de gran importancia en la formulación de piensos debido, entre otros factores, a su elevado contenido en ácidos grasos poliinsaturados de cadena larga (C_{20-24}, "LC-PUFAs" del inglés) de la serie omega-3, nutrientes esenciales para vertebrados como los peces. Una estrategia altamente innovadora para la obtención de nuevos ingredientes ricos en LC-PUFAs, incluidos los omega-3, consiste en el cultivo intensivo de organismos capaces de producirlos cuando son alimentados con materiales que no los contienen, especialmente si estos son subproductos de otras industrias ampliamente disponibles y de bajo coste económico. La puesta en práctica de esta estrategia exige conocer, en primer lugar, la capacidad de producción endógena (biosíntesis) de LC-PUFAs en el organismo en cuestión y, en segundo lugar, cómo esta capacidad biosintética puede modularse para favorecer la acumulación de estos compuestos esenciales en la biomasa obtenida. Esta Tesis Doctoral plantea una serie de estudios dirigidos a esclarecer el potencial de los gamáridos para aplicar esta estrategia.

La biosíntesis de LC-PUFAs en animales requiere de la acción coordinada de enzimas elongasas y desaturasas, implicadas en la conversión de ácidos grasos relativamente cortos y poco insaturados de escaso valor nutricional, en LC-PUFAs de alto valor nutricional como los omega-3. Así pues, esta Tesis Doctoral ha investigado, por un lado, la presencia y la actividad de genes involucrados en la biosíntesis de LC-PUFAs en gamáridos tanto marinos como dulceacuícolas (Capítulos 2 y 3). Por otro lado, ha estudiado cómo diferentes dietas y temperaturas afectan al metabolismo de ácidos grasos, crecimiento y supervivencia en el gamárido marino *Gammarus locusta*, tanto a nivel fisiológico y composicional (Capítulo 4), como a nivel molecular (Capítulo 5). En este último caso, se ha generado un transcriptoma de referencia a partir de muestras de *G. locusta* cultivados y alimentados en diferentes condiciones, y que ha permitido el estudio de patrones de expresión de los genes involucrados en las rutas metabólicas de interés (Capítulo 5).

Respecto a la primera parte de esta Tesis Doctoral (Capítulos 2 y 3), los resultados han demostrado que los gamáridos tienen varias elongasas (*elovl*) que participan en las rutas de biosíntesis de LC-PUFAs, pero carecen de desaturasas, necesarias para completar tales rutas metabólicas. Utilizando como modelo la especie de gamárido marino *Echinogammarus marinus*, se ha identificado un total de tres tipos de

elongasas denominadas "*elovl4*", "*elovl6*" y "*elovl1/7*-like" según la relación filogenética con sus correspondientes ortólogos de vertebrados. Estas elongasas, particularmente *elovl4* y *elovl1/7*-like, han demostrado tener la capacidad de elongación de ácidos grasos poliinsaturados necesaria para la biosíntesis de LC-PUFAs (Capítulo 2). Aunque no se han obtenido evidencias directas sobre las funciones que estas mismas enzimas tienen en gamáridos dulceacuícolas, las búsquedas de secuencias homólogas sí han permitido demostrar que la existencia de estos genes se conserva en todos los gamáridos. Sin embargo, un resultado interesante ha sido la identificación de secuencias de elongasas del tipo "Elovl2/5" en gamáridos dulceacuícolas, pero no en especies marinas. Las elongasas Elovl2/5 son enzimas muy importantes en la biosíntesis de LC-PUFAs en invertebrados como los moluscos, pero nunca antes habían sido descritas en crustáceos (Capítulo 2). Para esclarecer el origen de estas secuencias, se ha realizado una búsqueda sistemática de este tipo de elongasas en todos los transcriptomas de gamáridos disponibles en bases de datos públicas. Las búsquedas no sólo han confirmado los resultados preliminares sobre la presencia de Elovl2/5 en gamáridos de agua dulce y su ausencia en gamáridos marinos, sino que también han puesto de manifiesto el mismo patrón de distribución para las desaturasas (Capítulo 3). Concretamente, los transcriptomas de gamáridos dulceacuícolas tienen cuatro secuencias diferentes correspondientes a desaturasas "*front-end*" (*fed*) y dos correspondientes a desaturasas "*methyl-end*" (*ωx*-des), secuencias estas, ausentes en transcriptomas de gamáridos marinos, como se ha descrito anteriormente en los resultados del Capítulo 2. Análisis más profundos han permitido establecer que las secuencias de *elovl2/5*, *fed* y *ωx*-des sólo se encuentran presentes en transcriptomas de gamáridos dulceacuícolas construidos a partir de muestras consistentes en individuos completos y obtenidos del medio natural. El estudio a nivel molecular y filogenético ha permitido aclarar que las secuencias de *elovl2/5*, *fed* y *ωx*-des detectadas de forma sistemática y exclusiva en transcriptomas de gamáridos dulceacuícolas pertenecen en realidad a rotíferos bdeloideos, y no a gamáridos como cabría esperar (Capítulo 3). Este hallazgo, más allá de poner al descubierto un problema metodológico asociado a la presencia de secuencias "contaminantes" en muchas bases de datos transcriptómicas disponibles para muchos organismos, incluidos los gamáridos, ha permitido comprobar que la relación entre gamáridos de agua dulce y rotíferos bdeloideos es una constante, independientemente de la localización geográfica del ecosistema dulceacuícola considerado (Capítulo 3). Este estudio ha permitido demostrar que la dotación enzimática de los rotíferos bdeloideos, que son generalmente epibiontes de gamáridos dulceacuícolas, los capacita para biosintetizar LC-PUFAs, incluidos los omega-3, en un ambiente (ecosistemas de agua dulce) en el que estos compuestos

esenciales tienen una escasa abundancia en comparación con los ecosistemas marinos.

La segunda parte de esta Tesis Doctoral (Capítulos 4 y 5) ha investigado el efecto modulador de la dieta y de la temperatura sobre el metabolismo de ácidos grasos, y sobre procesos implicados en el crecimiento tales como la muda. Estos estudios se han realizado utilizando como modelo de estudio el gamárido marino *G. locusta*, y aplicando abordajes metodológicos diferentes pero complementarios. El Capítulo 4 recoge los estudios sobre los efectos fisiológicos y composicionales de *G. locusta* alimentados con tres dietas con un contenido variable de LC-PUFAs, y cultivados a cuatro temperaturas (5 °C, 10 °C, 15 °C y 20 °C). Los resultados han mostrado que *G. locusta* experimenta un mayor crecimiento, aunque también mayores tasas de mortalidad, cuando se cultiva a temperaturas elevadas (20 °C). Respecto a los efectos de la dieta, los análisis han mostrado que la mortalidad también aumenta cuando *G. locusta* se alimenta con la dieta denominada "Coco", consistente en la pulpa de este fruto, y caracterizada por un alto contenido en ácidos grasos saturados ("SFAs", del inglés) y ausencia de LC-PUFAs (Capítulo 4). En cuanto a los efectos a nivel composicional, los resultados han evidenciado que los perfiles de ácidos grasos de los gamáridos son muy parecidos a los de las dietas con las que han sido alimentados, independientemente de la temperatura de cultivo. Por tanto, el efecto modulador de la dieta sobre la composición corporal de *G. locusta* es tal que llega a enmascarar incluso cualquier efecto derivado de la temperatura (Capítulo 4). Este efecto se refleja también en el contenido de LC-PUFAs omega-3, pero cabe destacar que, incluso los gamáridos alimentados con dietas carentes en LC-PUFAs omega-3 conservan niveles apreciables de estos compuestos en sus lípidos corporales (Capítulo 4). Considerando que estos organismos no tienen las desaturasas necesarias para la biosíntesis de LC-PUFAs (Capítulos 2 y 3), la presencia de LC-PUFAs omega-3 en *G. locusta* alimentados con dietas carentes en estos compuestos indica una alta eficacia de retención de LC-PUFAs omega-3, aunque tampoco puede descartarse la adquisición de éstos a través de, por ejemplo, la microbiota del tracto digestivo.

Para determinar los mecanismos subyacentes a los efectos de la dieta y de la temperatura observados en el Capítulo 4, se ha llevado a cabo el análisis de expresión diferencial de genes (mediante secuenciación de ARN), en individuos de *G. locusta* de todos los tratamientos probados (Capítulo 5). Por un lado, se observa que una dieta carente de LC-PUFAs y rica en SFAs (la dieta "Coco") lleva asociada una disminución en el catabolismo de ácidos grasos, y promueve la acumulación de estos compuestos al influir en la expresión tanto de genes como de factores de transcripción involucrados en estos procesos metabólicos como pueden ser *cpt1a* y *srebp1*, entre otros. Además, una dieta carente de LC-PUFAs y rica en SFAs, afecta

negativamente al desarrollo y a la supervivencia de *G. locusta* según se desprende de los datos biométricos y de expresión de los genes involucrados en el ciclo de muda (p.ej., *mih* y *jheh*), proceso ligado al crecimiento y supervivencia (Capítulo 5). Respecto a los efectos de la temperatura, los resultados de expresión génica sugieren que, temperaturas bajas, están relacionadas con un mayor metabolismo de ácidos grasos en *G. locusta*, lo que les permite adaptarse a ambientes fríos. Esta mayor activación metabólica a temperaturas bajas está evidenciada por un incremento en la expresión tanto de genes involucrados en la biosíntesis de ácidos grasos (p. ej., *elovl3/6* y *d9d*), como de otros responsables de su catabolismo (p.ej., *cpt1a*). El aumento en la expresión de estos genes se atenúa a medida que la temperatura aumenta. Los gamáridos cultivados a 20 °C (alta temperatura) muestran unos patrones de expresión génica que indican una aceleración en el desarrollo, particularmente en el ciclo de muda debida a una baja expresión de *mih* y *jheh*, que resulta en un mayor crecimiento y tasas de mortalidad más elevadas que a temperaturas más bajas. Según los resultados obtenidos en los Capítulos 4 y 5, puede concluirse que las temperaturas elevadas y las dietas pobres en LC-PUFAs no son adecuadas para el cultivo de *G. locusta* cuando el objetivo final es la obtención de biomasa rica en estos compuestos, ya que dichas condiciones afectan negativamente al perfil nutricional del animal y a su supervivencia.

Resum

El ràpid creixement de l'aqüicultura durant les últimes dècades, ha plantejat una sèrie de problemes relacionats amb la disponibilitat d'ingredients d'origen marí com ho són les farines i els olis de peix ("FM" i "FO" per les seues sigles en anglés), emprats per a l'elaboració de pinsos. Aquests ingredients són de gran importància en la formulació de pinsos degut, entre altres factors, al seu elevat contingut en àcids grassos poliinsaturats de cadena llarga (C_{20-24}, "LC-PUFAs" de l'anglés) de la sèrie omega-3, nutrients essencials per a vertebrats com els peixos. Una estratègia altament innovadora per a l'obtenció de nous ingredients rics en LC-PUFAs, inclosos els omega-3, consisteix en el cultiu intensiu d'organismes capaços de produir-los quan són alimentats amb materials que no els contenen, especialment si aquests són subproductes d'altres indústries àmpliament disponibles i de baix cost econòmic. La posada en pràctica d'aquesta estratègia exigeix conéixer, en primer lloc, la capacitat de producció endògena (biosíntesi) de LC-PUFAs en l'organisme en qüestió i, en segon lloc, com aquesta capacitat biosintètica pot modular-se per així afavorir l'acumulació d'aquests compostos essencials en la biomassa obtinguda. Aquesta Tesi Doctoral planteja una sèrie d'estudis dirigits a esclarir el potencial dels gammàrids per a aplicar aquesta estratègia.

La biosíntesi de LC-PUFAs en animals requereix l'acció coordinada d'enzims elongases i desaturases, implicats en la conversió d'àcids grassos relativament curts i poc insaturats d'escàs valor nutricional, en LC-PUFAs d'elevat valor nutricional com els omega-3. Així doncs, aquesta Tesi Doctoral ha investigat, per una banda, la presència i activitat de gens involucrats en la biosíntesi de LC-PUFAs en gammàrids tant marins com dulciaqüícoles (Capítols 2 i 3). D'altra banda, ha estudiat com diferents dietes i temperatures afecten el metabolisme d'àcids grassos, creixement i supervivència en el gammàrid marí *Gammarus locusta*, tant des-d'un punt de vista fisiològic composicional (Capítol 4), com molecular (Capítol 5). Per a aquest últim cas, s'ha generat un transcriptoma de referència a partir de mostres de *G. locusta* cultivats i alimentats en diverses condicions experimentals, i que ha permés l'estudi de patrons d'expressió dels gens involucrats en les rutes metabòliques d'interés (Capítol 5).

Referent a la primera part d'aquesta Tesi Doctoral (Capítols 2 i 3), els resultats han demostrat que els gammàrids tenen diverses elongases (*elovl*) que participen en les rutes de biosíntesi de LC-PUFAs, però manquen de desaturases, necessàries per a completar aquestes rutes. Utilitzant com a model l'espècie de gammàrid marí *Echinogammarus marinus*, s'han identificat un total de tres tipus d'elongases

denominades "*elovl4*", "*elovl6*" i "*elovl1/7*-like" en funció de la seua relació filogenètica amb els corresponents ortòlegs de vertebrats. Aquestes elongases, particularment *elovl4* y *elovl1/7*-like, han demostrat tindre la capacitat d'elongació d'àcids grassos poliinsaturats necessària per a la biosíntesi de LC-PUFAs (Capítol 2). Tot i que no s'han obtingut evidències directes sobre les funcions que aquests mateixos enzims tenen en gammàrids dulciaqüícoles, les cerques de seqüències homòlogues sí que han permés descobrir que l'existència d'aquests gens es conserva en tots els gammàrids. Tanmateix, un resultat interessant ha sigut la identificació de seqüències elongases del tipus "Elovl2/5" en gammàrids dulciaqüícoles, però no en espècies marines. Les elongases Elovl2/5 són enzims molt importants en la biosíntesi de LC-PUFAs en invertebrats com els mol·luscos, però mai no havien sigut descrites en crustacis (Capítol 2). Per a esclarir l'origen d'aquestes seqüències, s'ha portat a terme una cerca sistemàtica d'aquest tipus d'elongases en tots els transcriptomes de gammàrids disponibles en bases de dades d'accés públic. Aquestes cerques no només han confirmat els resultats preliminars sobre la presència de Elovl2/5 únicament en gammàrids dulciaqüícoles, sinó que també han posat de manifest el mateix patró de distribució per a les desaturases (Capítol 3). Més concretament, els transcriptomes de gammàrids dulciaqüícoles tenen quatre seqüències diferents corresponents a desaturases "*front-end*" (*fed*) i dos corresponents a desaturases "*methyl-end*" (*ωx*-des), totes aquestes seqüències absents en els transcriptomes de gammàrids marins; com s'ha descrit per als resultats del Capítol 2. Anàlisis més exhaustives han permés establir que les seqüències de *elovl2/5*, *fed* i *ωx*-des només es troben presents en transcriptomes de gammàrids dulciaqüícoles elaborats a partir de mostres que pertanyen a individus complets i obtinguts directament del medi natural. L'estudi molecular i filogenètic ha permés aclarir que les seqüències de *elovl2/5*, *fed* i *ωx*-des detectades de forma sistemàtica i exclusiva en transcriptomes de gammàrids dulciaqüícoles són en realitat de rotífers bdel·loides, i no de gammàrids com caldria esperar (Capítol 3). Aquesta troballa, més enllà de posar de manifest un problema metodològic associat a la presència de seqüències "contaminants" en moltes bases de dades transcriptòmiques disponibles per a molts organismes, inclosos els gammàrids, ha permès comprovar que la relació entre gammàrids d'aigua dolça i rotífers bdel·loides és una constant, independentment de la localització geogràfica de l'ecosistema dulciaqüícola considerat (Capítol 3). Aquest estudi ha permés demostrar que la dotació enzimàtica dels rotífers bdel·loides, que generalment són epibionts de gammàrids dulciaqüícoles, els capacita per a biosintetitzar LC-PUFAs, inclosos els omega-3, en un ambient (ecosistemes d'aigua dolça) on aquests compostos tenen una escassa abundància en comparació amb els ecosistemes marins.

La segona part d'aquesta Tesi Doctoral (Capítols 4 i 5) ha investigat l'efecte modulador de la dieta i de la temperatura sobre el metabolisme d'àcids grassos, i sobre els processos implicats en el creixement, com la muda. Aquests estudis s'han realitzat utilitzant com a model d'estudi el gammàrid marí *G. locusta*, i aplicant aproximacions metodològiques diferents però complementàries. El Capítol 4 arreplega els estudis sobre els efectes fisiològics i composicionals en *G. locusta* alimentats amb tres dietes amb un contingut variable de LC-PUFAs i cultivats a quatre temperatures diferents (5 °C, 10 °C, 15 °C i 20 °C). Els resultats han mostrat que *G. locusta* experimenta un major creixement, encara que també major mortalitat, quan es cultiva a temperatures elevades (20 °C). Respecte als efectes de la dieta, les anàlisis han revelat que la mortalitat també augmenta quan *G. locusta* s'alimenta amb la dieta denominada "Coco", consistent en la polpa d'aquest fruit, i caracteritzada per un elevat contingut en àcids grassos saturats ("SFAs" de l'anglés) i absència de LC-PUFAs (Capítol 4). Referent als efectes composicionals, els resultats han evidenciat que els perfils d'àcids grassos dels gammàrids són molt pareguts als de les dietes amb què han sigut alimentats, independentment de la temperatura de cultiu. Per tant, l'efecte modulador de la dieta sobre la composició corporal de *G. locusta* és tal que pot arribar a emmascarar qualsevol possible efecte derivat de la temperatura (Capítol 4). Aquest efecte es reflecteix també en el contingut de LC-PUFAs omega-3, però cal destacar, que inclús els gammàrids alimentats amb dietes mancants de LC-PUFAs omega-3 conserven nivells apreciables d'aquests compostos en els seus lípids corporals (Capítol 4). Considerant que aquests organismes no tenen les desaturases necessàries per a la biosíntesi de LC-PUFAs (Capítols 2 i 3), la presència de LC-PUFAs omega-3 en *G. locusta* alimentats amb dietes mancants d'aquests compostos indica una alta eficàcia de retenció de LC-PUFAs omega-3, tot i que tampoc es pot descartar l'adquisició d'aquests a través de, per exemple, la microbiota del tracte digestiu.

Per a determinar els mecanismes subjacents als efectes de la dieta i la temperatura observats en el Capítol 4, s'ha portat a terme l'anàlisi d'expressió diferencial de gens (mitjançant seqüenciació de RNA), en individus de *G. locusta* de tots els tractaments experimentals (Capítol 5). Per una banda, s'observa que una dieta mancant de LC-PUFAs i rica en SFAs (la dieta "Coco") porta associada una disminució en el catabolisme d'àcids grassos, i promou l'acumulació d'aquests compostos a l'influir en l'expressió tant de gens com de factors de transcripció involucrats en aquests processos metabòlics com per exemple *cpt1a* o *srebp1*, entre d'altres. A més a més, una dieta mancant de LC-PUFAs i rica en SFAs, afecta negativament al desenvolupament i supervivència de *G. locusta* segons es desprén de les dades biomètriques i d'expressió de gens involucrats en el cicle de muda (p. ex., *mih* i *jheh*), procés lligat al creixement i supervivència (Capítol 5). Respecte als

efectes de la temperatura, els resultats d'expressió gènica suggereixen que, temperatures baixes, estan relacionades amb un increment en el metabolisme d'àcids grassos en *G. locusta* que els permet, probablement, adaptar-se millor a ambients freds. Aquesta major activació metabòlica a temperatures baixes està evidenciada per un increment en l'expressió tant de gens involucrats en la biosíntesi d'àcids grassos (p. ex., *elovl3/6* i *d9d*), com d'altres responsables en el seu catabolisme (p. ex., *cpt1a*). L'increment en l'expressió d'aquests gens disminueix a mesura que la temperatura augmenta. Els gammàrids cultivats a 20 °C (alta temperatura) mostren uns patrons d'expressió gènica que pareixen indicar una acceleració en el desenvolupament, particularment en el cicle de muda deguda una baixa expressió de *mih* i *jheh*, que resulta en un creixement major i taxes de mortalitat més elevades que a temperatures més baixes. Segons els resultats obtinguts en els Capítols 4 i 5, es pot concloure que les temperatures elevades i les dietes pobres en LC-PUFAs no són adequades per al cultiu de *G. locusta* quan l'objectiu final és l'obtenció d'una biomassa rica en aquests composts, ja que les anomenades condicions afecten negativament al perfil nutricional de l'animal i a la seua supervivència.

Summary

The fast and remarkable growth of aquaculture in the last decades, has created new challenges regarding the availability and supply of marine ingredients such as fishmeal (FM) and fish oil (FO), used for feed formulation. These ingredients are of special relevance for feed formulation due to their high content in omega-3 long-chain polyunsaturated fatty acids (C_{20-24}, LC-PUFA), which are essential nutrients for vertebrates such as fish. A novel an interesting strategy for obtaining new ingredients that are rich in LC-PUFA, including omega-3, involves culturing organisms that are capable of producing these ingredients when fed with sidestreams that do not contain them, especially if these are by-products from other industries, widely available and inexpensive. The application of this strategy requires knowing, first, the endogenous production capacity (biosynthesis) of LC-PUFA in the specific organism, and second, how this biosynthetic capacity can be modulated to favor the accumulation of these essential compounds in the resulting biomass. This Doctoral Thesis proposes a series of studies aimed at clarifying the potential of gammarids to apply this strategy.

LC-PUFA biosynthesis in animals requires the coordinated action of elongases and desaturases. These enzymes are responsible for the conversion of short-chain fatty acids (saturated or monounsaturated) into LC-PUFA with high nutritional value such as the omega-3 series. Thus, this Doctoral Thesis has aimed to elucidate, from one hand, the presence and activity of the genes responsible for LC-PUFA biosynthesis in gammarids, both marine and freshwater (Chapters 2 and 3). On the other hand, we have investigated how different diets and temperatures may modulate the fatty acid metabolism, growth and survival in the marine gammarid *Gammarus locusta* both at physiological and compositional level (Chapter 4) and at the molecular level (Chapter 5). For such molecular study, we have developed a reference transcriptome from *G. locusta* samples cultured at different conditions that has allowed us to study the differential expression of genes involved in the metabolic pathways of interest (Chapter 5).

Regarding the first part of this Doctoral Thesis (Chapters 2 and 3), results have shown that gammarids possess several elongases (*elovl*) that play a key role in LC-PUFA biosynthesis pathways. However, gammarids lack desaturases, which are required for fulfilling the LC-PUFA biosynthetic pathway. Using the marine gammarid *Echinogammarus marinus* as a model species, we have been able to identify three different types of elongases, termed as "*elovl4*, *elovl6* and *elovl1/7*-like" according to their phylogenetic relationship with their vertebrate orthologues. Functional

characterisation of these elongases revealed that *elovl4* and *elovl1/7*-like, combined, fulfill all the reactions required for LC-PUFA biosynthesis (Chapter 2). Though there are no evidences of the elongase function of these three enzymes in freshwater gammarids, further searches and phylogeny revealed that the presence of these genes is well conserved amongst all gammarids. However, an interesting finding has reported the presence of a new type of elongase (Elovl2/5) in freshwater gammarids which is absent in their marine counterparts. Elovl2/5 are enzymes with key roles in LC-PUFA biosynthesis in invertebrates like mollusks but, up to date, they have never been reported in crustaceans (Chapter 2). In order to determine the origin of this *elovl2/5*, we have performed a systematic search of this type of elongase in every gammarid transcriptome available in public databases. The abovementioned searches have confirmed the preliminary results regarding the presence of *elovl2/5* in freshwater gammarids and its absence in their marine counterparts and, additionally they have reported that this pattern is also shared with nucleotide sequences regarding desaturases (Chapter 3). More specifically, we have found four different sequences corresponding to *front-end* desaturases (*fed*) and two different sequences regarding *methyl-end* desaturases (ωx-des) to be present exclusively in transcriptomes from freshwater gammarids (Chapter 3). Further analyses have determined that *elovl2/5*, *fed* and ωx-des sequences can only be found in transcriptomes that have been generated from whole individuals of wild-caught freshwater gammarids. Additional molecular and phylogenetic analyses have reported that *elovl2/5*, *fed* and ωx-des sequences found in freshwater gammarids are not from gammarid origin and they are, in fact, typical of bdelloid rotifers (Chapter 3). This finding has uncovered a methodological problem associated with the presence of "contaminant" sequences in many transcriptomic databases available for several organisms, including gammarids. Moreover, it has allowed us to verify that the ecological relationship between freshwater gammarids and bdelloid rotifers is a constant, regardless of the geographical location of the freshwater ecosystem considered. This study has demonstrated that the enzymatic repertoire of bdelloid rotifers, which are epibionts of freshwater gammarids, enables them to biosynthesize LC-PUFA, including omega-3 series, in freshwater ecosystems; which are considered to be LC-PUFA depleted environments.

The second part of this Doctoral Thesis (Chapters 4 and 5) has investigated the modulating effect of diet and temperature towards the fatty acid metabolism and developmental processes such as moult. These studies have been performed in the marine gammarid *G. locusta* as a model species using different but complementary approaches. Chapter 4 includes studies on the physiological and compositional effects of *G. locusta* fed with three diets with different LC-PUFA content, and grown at four temperatures (5 °C, 10 °C, 15 °C and 20 °C). The results have shown that *G.*

locusta displays greater growth, but also higher mortality rates, when grown at higher temperatures (20 °C). As for diets, the results have shown that mortality also increases when *G. locusta* is fed with the diet named "Coco", consisting of the pulp of this fruit, and characterized by a high content of saturated fatty acids (SFA) and absence of LC-PUFA (Chapter 4). Regarding the compositional effects, the results have demonstrated that the fatty acid profiles of gammarids are very similar to those of the diets they have been fed with, regardless of the culture temperature. Therefore, the modulating effect of diet on the body composition of *G. locusta* is such that it could mask any effect derived from temperature (Chapter 4). This effect is also echoed in the omega-3 LC-PUFA content, but it should also be noted that even gammarids fed with diets lacking omega-3 LC-PUFA are able to retain measurable levels of these compounds in their body lipids (Chapter 4). Considering that these organisms do not have the desaturases necessary for LC-PUFA biosynthesis (Chapters 2 and 3), the presence of omega-3 LC-PUFA in *G. locusta* fed with diets absent in these compounds indicates a high retention efficiency of omega-3 LC-PUFA, although the acquisition of these through, for example, the microbiota of the digestive tract cannot be discarded.

In order to determine the mechanisms underlying the effects of diet and temperature observed in Chapter 4, differential gene expression analysis has been carried out (by RNA sequencing) in *G. locusta* individuals from all treatments tested (Chapter 5). On one hand, diets that lack LC-PUFA and are SFA rich ("Coco" diet) are associated with a decrease in fatty acid catabolism, and promote the accumulation of these compounds by influencing the expression of both genes and transcription factors involved in these metabolic processes such as *cpta1* and *srebp1*, amongst others. Furthermore, same type of diet negatively affects the development and survival of *G. locusta* as can be presumed from the biometric data and expression of genes involved in the molting cycle (e.g., *mih* and *jheh*), a process linked to growth and survival (Chapter 5). Regarding the effects of temperature, gene expression results suggest that low temperatures are related to greater fatty acid metabolism in *G. locusta*, which allows them to adapt to cold environments. This greater metabolic activation at low temperatures is evinced by an increase in the expression of some genes involved in the biosynthesis of fatty acids (e.g., *elovl3/6* and *d9d*), and others responsible for their catabolism (e.g., *cpt1a*). The increased expression of these genes is attenuated as temperature increases. Gammarids grown at 20 °C (high temperature) show gene expression patterns that indicate an acceleration in development, particularly in the molting cycle due to a reduced expression of *mih* and *jheh*, which results into a greater growth and higher mortality rates than at lower temperatures. According to the results obtained in Chapters 4 and 5, it can be concluded that high temperatures and LC-PUFA poor diets are not suitable for the

culture of *G. locusta* when the final outcome is to obtain a LC-PUFA rich biomass, since these conditions negatively affect the nutritional profile of the animal and its survival.

1.
Introducción. Hipótesis y objetivos

1.1. Acuicultura

1.1.1. Estado actual y perspectivas futuras

La acuicultura se puede definir como «el cultivo controlado y respetuoso de organismos acuáticos destinados a cualquier fin comercial, público o recreacional» (APROMAR 2021; Mizuta *et al*., 2022). Desde sus orígenes, hace al menos 4000 años en China (Stickney y Treece, 2012), la acuicultura ha suministrado alimento para el consumo humano, principalmente plantas (algas) y animales, incluidos invertebrados como los moluscos y crustáceos entre otros, y vertebrados, principalmente peces. Entre los productos que no tienen como destino el consumo humano, la acuicultura produce especies ornamentales para acuariofilia y para la obtención de productos específicos utilizados en cosmética, farmacología y bisutería, entre muchas otras aplicaciones comerciales (Froehlich *et al*., 2017). Según el último informe de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO del inglés) sobre "El estado mundial de la pesca y la acuicultura 2022", la producción pesquera y acuícola a nivel global (excluyendo las algas) se ha incrementado significativamente en los últimos tiempos, pasando de 19 millones de toneladas en 1950 a un récord histórico de unos 179 millones de toneladas en 2018 (FAO, 2022). Tras un leve descenso en la producción en 2019, la producción alcanzó los 178 millones de toneladas en 2020 (FAO, 2022) (Figura 1.1). Según la FAO, la acuicultura

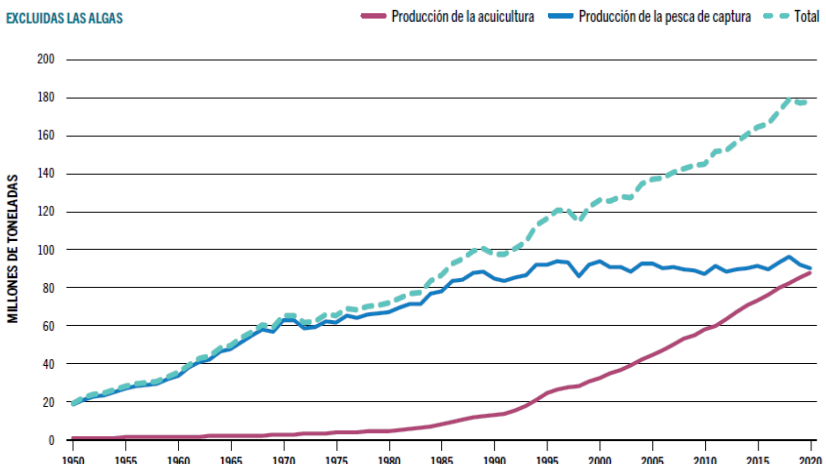

Figura 1.1. Producción mundial de la pesca de captura y de la acuicultura, excluidas las algas. Los datos se expresan en términos de equivalente en peso vivo. FAO, 2022.

ha experimentado un incremento del 609% desde 1990 hasta 2020, con un ritmo de crecimiento medio anual del 6.7%. Este crecimiento de la acuicultura ha supuesto alcanzar a la producción de pescado y marisco derivado de la pesca (Figura 1.1), y se prevé que la supere en los años venideros (FAO, 2022). En la actualidad, la pesca extractiva de especies marinas como la anchoveta (*Engraulis ringens*), el colín de Alaska (*Gadus chalcogrammus*) y el listado o "bonito" (*Katsuwonus pelanis*), entre otros, representa un 51% (91 millones de toneladas) del total de productos de origen acuático excluyendo las algas, frente al 49% (89 millones de toneladas) procedente de la acuicultura de especies de peces (57,5 millones de toneladas), moluscos (17,7 millones de toneladas) y crustáceos (11,2 millones de toneladas) (Figura 1.1) (FAO, 2022).

Cabe destacar que, a pesar del constante crecimiento que ha experimentado la acuicultura en las últimas décadas, sigue existiendo una gran diferencia en cuanto a ritmo de crecimiento según las especies cultivadas, observándose un incremento más acelerado en la producción de aquellas que precisan de la utilización de piensos

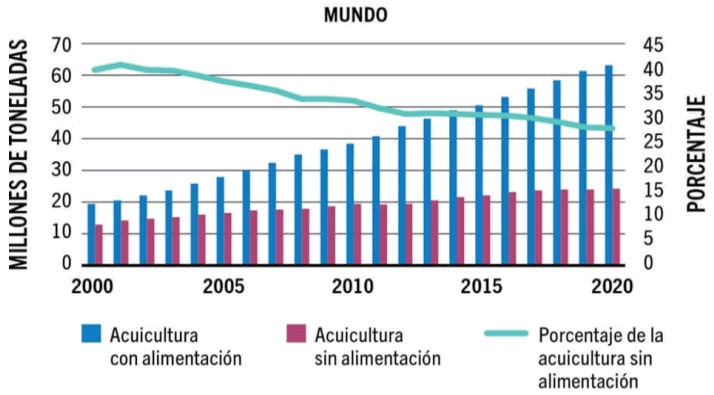

Figura 1.2 Producción mundial de la acuicultura de especies con alimentación y sin alimentación, 2000-2020. Los datos se expresan en términos de equivalente en peso vivo. FAO, 2022.

en su alimentación (peces y crustáceos decápodos, principalmente), en comparación al de otras que son cultivadas sin necesidad de la utilización de piensos (moluscos bivalvos, algas, etc.) (FAO, 2022) (Figura 1.2). En este sentido, la producción y cultivo de especies que son alimentadas con piensos formulados ha aumentado notablemente, pasando de valores cercanos al 15% (volumen de producción) antes del 2000, a unos porcentajes entre 40 y 45% en 2020 (FAO, 2022) (Figura 1.2). Esta

tendencia, cada vez más obvia hacia el aumento en la producción de especies alimentadas con pienso, repercute directamente en el incremento en la demanda de ingredientes con los que se formulan los piensos de acuicultura, situación que ha dado origen a uno de los desafíos más importantes a los que ha tenido que hacer frente el sector acuícola en los últimos tiempos y que se describe con más detalle a continuación.

1.1.2. El problema de la disponibilidad de ingredientes marinos para piensos de acuicultura

A pesar de la expansión de la acuicultura señalada anteriormente, su elevado ritmo de crecimiento a nivel global ha generado una serie de retos económicos y ecológicos que necesitan ser considerados para garantizar su sostenibilidad futura (FAO, 2022). En lo referente al cultivo y la cría de peces destinados al consumo humano, el reto más importante está relacionado con la provisión de materias primas (ingredientes) necesarias para la elaboración de los piensos. Uno de los principales objetivos marcados por la FAO para garantizar la expansión sostenible de la acuicultura propone la reducción en el uso de ingredientes de origen marino, concretamente las harinas de pescado ("FMs", del inglés *fishmeal*) y aceites de pescado ("FOs", del inglés *fish oil*) (Turchini *et al*., 2019; FAO, 2022; Tacon *et al*., 2022). Las FMs y los FOs son ingredientes de gran importancia para la alimentación en acuicultura debido a su alto valor nutricional, aportando numerosos nutrientes esenciales para animales vertebrados como los peces y que, por definición, no pueden ser sintetizados por el propio organismo a los niveles requeridos y, por ello, precisan de su provisión a través de la dieta (National Research Council, 2011).

Las FMs se caracterizan por un perfil adecuado en aminoácidos esenciales, minerales, lípidos esenciales y vitaminas (Oliva-Teles *et al*., 2015; Charles Bai *et al*., 2022). Aunque de composición variable según su origen, una FM tipo suele contener sobre 60-70% de proteína, 5-12% de lípidos, 10-12% de ceniza, y una fracción restante de agua (Oliva-Teles *et al*., 2015; Charles Bai *et al*., 2022; Hardy y Brezas, 2022). La riqueza nutricional de las FMs hace de estas un ingrediente demandado no sólo en acuicultura, sino también en otras industrias de producción animal tales como la aviar y la porcina (Jackson y Shepherd, 2004; FAO, 2022). La FM se produce a partir del procesado de peces enteros como la anchoveta (*E. ringens*) y el arenque (*Cluppea* sp.), y también a partir de subproductos de la pesca y la acuicultura obtenidos tras el fileteado del pescado (Charles Bai *et al*., 2022; FAO, 2022). Por su parte, los FOs se obtienen de las mismas fuentes señaladas anteriormente para la producción de FMs. A nivel composicional, los FOs contienen principalmente lípidos y, en niveles inferiores, otros nutrientes liposolubles como algunas vitaminas. Los FOs son la fuente principal de los denominados "ácidos grasos poliinsaturados de cadena

larga" (C_{20-24}) ("LC-PUFAs", del inglés *long-chain polyunsaturated fatty acids*) (Turchini *et al*., 2009; Tocher, 2015; Shepherd *et al*., 2017), compuestos con un protagonismo central en la presente Tesis Doctoral. A pesar de que el Apartado 1.3 ahonda en la importancia de los LC-PUFAs en nutrición animal, cabe señalar que los LC-PUFAs, particularmente los de la serie omega-3, como el ácido eicosapentaenoico (20:5n-3, EPA) y el ácido docosahexaenoico (22:6n-3, DHA), llevan consigo efectos beneficiosos en la prevención de enfermedades cardiovasculares y metabólicas, y en asegurar el correcto desarrollo neuronal y visual en vertebrados (Chow, 2007; Swanson *et al*., 2012; Calder, 2018).

La producción mundial de FM y FO para 2022 ha estado en torno a los 6,1 millones de toneladas, de los que entre 4,9 y 5,0 millones de toneladas corresponden a FM y entre 1,1 y 1,2 millones de toneladas a FO (FAO, 2022). Estas cantidades pueden oscilar de año a año, pero es importante hacer hincapié en que la producción global de FM y FO es limitada y son, por tanto, considerados recursos finitos. Como se indica anteriormente, la acuicultura es la industria con mayor demanda de ingredientes marinos, con aproximadamente un 75% de la producción mundial de FM y FO dedicada a la fabricación de piensos de acuicultura. En 2020, alrededor de 16 millones de toneladas de productos pesqueros se destinaron a la producción de FM y FO (FAO, 2022). Estos valores representan aproximadamente un 20% de la pesca de captura marina a escala global. Debido al elevado crecimiento de la acuicultura, algunas pesquerías destinadas a la producción de FM y FO están siendo explotadas por encima de los niveles que permiten la renovación de estos recursos (Naylor *et al*., 2021; FAO, 2022). La elevada demanda de FM y FO, unida a su disponibilidad no siempre predecible provocada por fenómenos climáticos globales (p. ej., El Niño) que afectan a la productividad de las pesquerías, han impulsado, entre otros factores, la búsqueda de materias primas no marinas para reemplazar estos ingredientes marinos en piensos de acuicultura usados en la fase de engorde. Como consecuencia, los piensos comerciales actuales están formulados con una proporción cada vez mayor de ingredientes de origen no marino (Ytrestøyl *et al*., 2015; Aas *et al*., 2019, 2022a, 2022b, 2022c), aunque, como se describe en el siguiente apartado, esta estrategia no está exenta de problemas vinculados con un descenso en la calidad nutricional del pescado de acuicultura.

1.1.3. Fuentes alternativas a FM y FO para los piensos de acuicultura

Desde principios de este siglo se han realizado numerosas investigaciones y financiado proyectos por parte de la Unión Europea (UE), tales como «PEPPA» (Q5RS-2000-30068; *Perspectives of Plant Protein Use in Aquaculture*, 2000-2004), «RAFOA» (Q5RS-2000-30058, *Researching alternatives to fish oil for aquaculture*, 2001-2005), «AQUAMAX» (ID: 16249, *Sustainable aquafeeds to maximise the health*

benefits of farmed fish for consumers, 2006-2010) y «ARRAINA» (ID: 28895, *Advanced Research Initiatives for Nutrition & Aquaculture*, 2012-2016), entre otros, destinados a evaluar la eficacia de fuentes alternativas a FM y FO que, como se señala anteriormente, son materias primas cuya disponibilidad es limitada, pudiendo comprometer la expansión sostenible del sector (Turchini *et al*., 2019; Naylor *et al*., 2021). Gran parte de estas investigaciones se ha centrado en el uso de materias primas derivadas de animales y plantas terrestres como sustitutivos parciales o totales de los ingredientes marinos, con resultados muy diversos respecto al grado de sustitución y el efecto sobre el producto final destinado al consumo humano (*v.g.* filete de pescado) (Gasco *et al*., 2018; Glencross *et al*., 2020; Colombo *et al*., 2023).

Los estudios sobre sustitutos de FM han evaluado la eficacia de harinas procedentes de subproductos de la industria cárnica y de harinas de origen vegetal. Las harinas de animales terrestres se elaboran a partir de materiales de desecho de las industrias de producción de aves de corral y de la ganadería, mientras que las harinas vegetales tienen su origen en los subproductos resultantes de la obtención de grano y/o aceite de plantas como la soja, el maíz, el trigo, la colza, entre otras. Tanto las harinas animales como las de plantas terrestres están consideradas como fuentes de proteínas y, por tanto, se incluyen en la formulación de piensos de acuicultura como sustitutos de FM. El contenido proteico de estas harinas es muy variable, aunque las harinas animales suelen ser más ricas en proteínas que las vegetales. En la actualidad existen los denominados "concentrados" y "aislados" de plantas como la soja, entre otras, que alcanzan niveles de proteína de hasta un 90% en el caso de los "aislados de soja" (Wang *et al*., 2004). La utilización de estos productos vegetales reduce los problemas asociados a la presencia de antinutrientes en harinas vegetales convencionales, causantes de problemas digestivos como la enteritis en peces cultivados (Aragão *et al*., 2022). Como fuentes de proteína, las harinas de animales y plantas terrestres aportan aminoácidos (aa) esenciales a la dieta. Cabe señalar que, mientras que las harinas de origen animal tienen perfiles de aa esenciales bien balanceados, las harinas vegetales suelen ser deficitarias en ciertos aa esenciales como la lisina y la metionina (Einarsson *et al*., 2019; Vélez-Calabria *et al*., 2021), problema que suele resolverse mediante la inclusión de versiones sintéticas de estos compuestos (Sánchez-Lozano *et al*., 2009; Rossi y Davis, 2012; Ceccotti *et al*., 2022).

En el contexto de esta Tesis Doctoral, una característica importante que comparten tanto las harinas de animales como las de plantas terrestres es su escaso o nulo contenido en LC-PUFAs, que contrasta con la abundancia de estos compuestos en la fracción lipídica de las FMs a las que sustituyen en las formulaciones actuales de piensos (Miller *et al*., 2008; Oliva-Teles *et al*., 2015; Turchini *et al*., 2019). Este es también el caso de las harinas de insecto, obtenidas

principalmente de la mosca soldado negra (*Hermetia illucens*) y del escarabajo de la harina (*Tenebrio molitor*), las cuales han irrumpido fuertemente en la industria acuícola en los últimos años (Henry *et al*., 2015; Gasco *et al*., 2018; Nogales-Mérida *et al*., 2019). A nivel experimental, esta carencia nutricional de las harinas de insecto se ha abordado mediante la suplementación del alimento de las fases larvarias de los insectos con algas marinas como *Ascophyllum nodosum*, las cuales contienen LC-PUFAs omega-3 que acaban acumulándose en las larvas y posteriormente en la harina producida a partir de ellas (Liland *et al*., 2017). Sin embargo, un problema asociado a esta estrategia es la acumulación de contaminantes abundantes en las propias algas como el arsénico (Biancarosa *et al*., 2018).

Respecto a la sustitución del FO en los piensos de acuicultura, los aceites vegetales ("VOs", del inglés *vegetable oils*) derivados de plantas como la soja, la colza y el girasol, entre otras, son ingredientes de uso habitual en las formulaciones actuales debido a su bajo coste y su amplia disponibilidad estacional y geográfica (Gunstone, 2011; Turchini *et al*., 2011). Su inclusión en la formulación de piensos aporta energía, pero no otros nutrientes esenciales como los LC-PUFAs que sí están presentes en los FOs. La Tabla 1.1 muestra la composición de ácidos grasos de VOs usados habitualmente en los piensos de acuicultura y, a efectos comparativos, se incluyen también los perfiles de FOs derivados de diferentes tipos de pescado y de otros aceites de origen animal incluidos los de insecto ("IOs". del inglés *insect oils*). Otras fuentes como las grasas de animales procedentes de la industria cárnica de aves, ganado porcino y ganado vacuno también han sido consideradas como sustitutos del FO en piensos de acuicultura (Bowyer *et al*., 2012; Pérez *et al*., 2014). No obstante, sus perfiles de ácidos grasos, caracterizados por un alto contenido en ácidos grasos saturados ("SFAs", del inglés *saturated fatty acids*) y muy pobres en LC-PUFAs (Tabla 1.1), los descartan como una alternativa idónea para la sustitución de FO tal y como ocurre con los VOs (Turchini *et al*., 2009; Oliva-Teles *et al*., 2015). Como se ha comentado anteriormente, la inclusión de estos aceites "alternativos" para la sustitución del FO en piensos de acuicultura conlleva una reducción en el aporte de LC-PUFAs en la dieta que trae consigo, por un lado, una pérdida de valor nutricional del producto final (filete) por un menor contenido en compuestos saludables como los LC-PUFAs omega-3 (Sprague *et al*., 2020) y, por otro, problemas de crecimiento y de salud en algunas especies de peces de cultivo que poseen una capacidad limitada para la producción endógena (biosíntesis) de LC-PUFAs desde otros precursores de cadena más corta (Tocher, 2010; Monroig *et al*., 2018, 2022). Los mecanismos que explican la biosíntesis de LC-PUFAs en animales se describen más ampliamente en el Apartado 1.4.

Tabla 1.1. Composición de ácidos grasos expresada en % de lípidos totales en diferentes FOs, VOs, grasas animales, y aceites de insectos ("IOs", del inglés), respectivamente. Datos recogidos y adaptados de diferentes publicaciones y revisiones (Turchini *et al.*, 2009; Oliva-Teles *et al.*, 2015; Li *et al.*, 2019; Pudtikajorn y Benjakul, 2020; Fawole *et al.*, 2021). SFA, ácidos grasos saturados, MUFA, ácidos grasos monoinsaturados, LA, ácido linoleico 18:2n-6, ALA ácido α linolénico 18:3n-3, ARA, ácido araquidónico 20:4n-6, EPA, ácido eicosapentaenoico 20:5n-3, DHA, ácido docosahexaenoico 22:6n-3, PUFA, ácidos grasos poliinsaturados (C_{18-20}), LC-PUFAs, ácidos grasos poliinsaturados de cadena larga (C_{20-24}). *sd, sin datos.

	SFA	MUFA	LA	ALA	ARA	EPA	DHA	n-6 PUFA	n-3 PUFA	n-3 LC-PUFA (EPA+DHA)
FO										
Anchoveta	28,8	24,9	1,2	0,8	0,1	17,0	8,8	1,3	26,6	25,8
Capelán	20	61,7	1,7	0,4	0,1	4,6	3,0	1,8	8,0	7,6
Menhaden	30,5	24,8	1,3	0,3	0,2	11,0	9,1	1,5	20,4	20,1
Arenque	20	56,4	1,1	0,6	0,3	8,4	4,9	1,4	13,9	13,3
Listado (cabeza)	54,5	32,8	1,0	0,5	1,6	1,3	6,3	2,6	9.1	7.6
Listado (ojo)	38,1	21,1	1,3	0,7	0,2	5,4	31,9	1,5	38,0	37,3
Colín de Alaska	13,9	46,5	sd	0,3	sd	7,8	4,0	sd	12,1	11,8
Hígado de bacalao	19,4	46,0	1,4	0,6	1,6	11,2	12,6	3	24,4	23,8
VO										
Palma	48,8	37,0	9,1	0,2	/	/	/	9,1	0,2	/
Soja	14,2	23,2	51,0	6,8	/	/	/	51,0	6,8	/
Colza	4,6	62,3	20,2	12,0	/	/	/	20,2	12,0	/
Girasol	1,4	19,5	65,7	/	/	/	/	65,7	/	/
Algodón	45,3	17,8	51,5	0,2	/	/	/	51,5	0,2	/
Cacahuete	11,8	46,2	32,0	/	/	/	/	32,0	/	/
Maíz	12,7	24,2	58,0	0,7	/	/	/	58,0	0,7	/
Linaza	9,4	20,2	12,7	53,3	/	/	/	12,7	53,3	/
IO										
Mosca soldado negra	66,8	13,8	0,1	/	0,1	/	/	0,2	/	/
Grasas animales										
Sebo de res	47,5	40,5	3,1	0,6	0,4	/	/	3,5	0,6	/
Manteca de cerdo	38,6	44,0	10,2	1,0	/	/	/	10,2	1,0	/
Grasa de ave	28,5	43,1	19,5	1,0	/	/	/	19,5	1,0	/

Si bien es cierto que el uso de aceites no marinos en acuicultura ha ayudado a reducir la presión sobre las pesquerías destinadas a la producción de ingredientes marinos (Oliva-Teles *et al.*, 2015; Naylor *et al.*, 2021; Davis, 2022; Colombo *et al.*,

2023), las carencias nutricionales señaladas arriba han suscitado el interés de la comunidad científica por seguir buscando y desarrollando nuevos productos ricos en LC-PUFAs omega-3 cuyo uso no conlleve los efectos negativos señalados. Entre estos destacan los aceites de plantas transgénicas cuyos aceites, al contrario que VOs convencionales, tienen altos niveles de LC-PUFAs omega-3 tales como EPA y DHA (Napier y Sayanova, 2005; Sayanova y Napier, 2011; Napier *et al*., 2015; West *et al*., 2021). En la actualidad existen aceites de variantes transgénicas de colza (*Brassica napus*) comercializados por *Nuseed* como *Aquaterra®*, aunque es cierto que la mayoría de estudios sobre los efectos de estos productos en peces como el salmón, la dorada y la trucha arcoíris, se han realizado con aceites de variantes transgénicas del sésamo bastardo (*Camelina sativa*) (Betancor *et al*., 2015, 2016, 2017; Osmond *et al*., 2021). No obstante, pese a que la actual legislación de la UE permite el uso de ciertos organismos modificados genéticamente ("GMOs", del inglés *genetic modified organisms*) para alimentación (Oficina de Publicaciones de la Unión Europea, 2014), existe todavía un cierto grado de reticencia por parte del consumidor sobre la incorporación de GMOs en piensos para animales, y sobre el consumo directo de los mismos GMOs (Singh *et al*., 2006; Tocher, 2009; Peter *et al*., 2019). Otros aceites que pueden ser usados para la sustitución de FO son aquellos obtenidos de microalgas fotosintéticas y heterótrofas, las cuales producen aceites con niveles particularmente altos en EPA y DHA, respectivamente (Khozin-Goldberg *et al*., 2011; Sprague *et al*., 2017; Remize *et al*., 2021). Actualmente existen varios aceites disponibles en el mercado derivados de microalgas como son *AlgaPrime™* DHA-liquid y *Veramaris® Oil*. Sin embargo, aunque hay diversos estudios que demuestran la efectividad de estos productos como sustitutos de FO en piensos de acuicultura (Santigosa *et al*., 2020, 2021; Carvalho *et al*., 2022; Zatti *et al*., 2023), su elevado coste limita todavía su uso como ingrediente en piensos de acuicultura (Tocher *et al*., 2019; Liu *et al*., 2022; Santin *et al*., 2022).

Frente a las ventajas que ofrecen los ingredientes "alternativos" considerados anteriormente, y cuyo uso ha permitido racionalizar el de materias primas finitas y altamente demandadas como son las FMs y los FOs, resulta también obvio observar que la utilización de estos ingredientes conlleva algunas desventajas que limitan su eficacia como ingredientes de piensos, por motivos que van desde la disminución en la calidad de producto hasta una menor rentabilidad económica, pasando por la posible reticencia de los consumidores. Por tanto, es necesario seguir trabajando en la búsqueda de candidatos para la sustitución de FM y FO que 1) tengan niveles elevados de nutrientes esenciales tales como los LC-PUFAs, y que 2) permitan su producción en sistemas sencillos y de fácil escalado. Con estas premisas, el cultivo de ciertas especies de invertebrados de bajo nivel trófico se vislumbra como una estrategia novedosa para la producción de ingredientes de origen marino tan

demandados por la industria acuícola. Los gamáridos han sido el modelo de estudio elegido en este trabajo de investigación.

1.2. Gamáridos

Los gamáridos (Figura 1.3) son crustáceos anfípodos de bajo nivel trófico que habitan normalmente en las zonas béntica e intermareal de cualquier medio acuático, desde ríos y lagunas, hasta salinas, mares y océanos (Costa y Costa, 2000; Väinölä *et al*., 2008; Baeza-Rojano *et al*., 2014; Guerra-García *et al*., 2014). Esta familia de crustáceos

Figura 1.3 Gamáridos liofilizados de la especie marina *Gammarus locusta*. Se muestra una ampliación de uno de los ejemplares de mayor tamaño (~1 cm).

cuenta con más de 200 especies descritas, la mayoría de las cuales son de agua dulce (Macneil *et al*., 1999; Väinölä *et al*., 2008; Costa *et al*., 2009). Los gamáridos suelen habitar bajo las rocas y la grava, y también sobre la vegetación tanto viva como en descomposición, donde encuentran refugio contra depredadores y consiguen alimento (Kunz *et al*., 2010). La alimentación de los gamáridos se basa principalmente en detritos y restos de cualquier material orgánico, aunque también pueden alimentarse de animales de menor tamaño, huevos, larvas y algas (Kelly *et al*., 2002; Christie y Kraufvelin, 2004). Los gamáridos sirven, a su vez, como fuente de alimento para una gran variedad de animales como peces y cefalópodos (Costa y Costa, 2000; Baeza-Rojano *et al*., 2010, 2013; Ashour *et al*., 2021), de forma que juegan un papel muy importante en la ecología trófica de los ecosistemas acuáticos. Además, los gamáridos se caracterizan por su rápido crecimiento, con una elevada tasa de fertilidad y un particular desarrollo sin fases larvarias, ya que del huevo eclosiona directamente un juvenil. La gran capacidad de adaptación de los gamáridos les permite habitar en zonas geográficas de muy distinta latitud y climatología

(Neuparth *et al*., 2002; Costa *et al*., 2009; Whiteley *et al*., 2011). Los mecanismos de adaptación a estas variaciones de condiciones ambientales afectan al ciclo de vida y reproductivo de estos crustáceos como ilustra el caso de *Gammarus duebeni*, especie para la cual la temperatura influye directamente en la determinación del sexo y de la proporción entre machos y hembras en un proceso conocido como "determinación ambiental del sexo" (Dunn *et al.*, 2005). Como se señala anteriormente, esta alta capacidad de adaptación al entorno ha permitido a estos animales ocupar con relativa facilidad una gran variedad de ecosistemas acuáticos (Gerhardt *et al*., 2011). Por tales motivos, los gamáridos, especialmente las especies dulceacuícolas *Gammarus pulex* y *Gammarus fossarum*, se han utilizado a lo largo de los años como modelos para estudios de ecotoxicología y sobre la calidad ambiental de agua (Kunz *et al*., 2010; Gerhardt *et al*., 2011; Chaumot *et al*., 2015; Harlıoğlu y Farhadi, 2018).

La composición de los gamáridos se caracteriza por un alto contenido en proteínas (40-50%) y, en menor medida, lípidos (5-15%) relativamente ricos en LC-PUFAs omega-3 (Tabla 1.2) (Kolanowski *et al*., 2007; Baeza-Rojano *et al*., 2014; Harlıoğlu y Farhadi, 2018; Jiménez-Prada *et al*., 2018). Durante la última década han sido varios los estudios que han mostrado que los gamáridos pueden ser alimentados con materiales de desecho o subproductos de industrias como la agricultura y la propia acuicultura, sin que esta práctica repercuta negativamente en términos de crecimiento o de composición nutricional (contenido de LC-PUFAs omega-3), al compararse con gamáridos alimentados con algas marinas (Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020). Estos resultados sugieren que los gamáridos tienen mecanismo de biosíntesis y/o de acumulación selectiva de LC-PUFAs, lo que abre la posibilidad a producir biomasas ricas en estos compuestos aprovechando productos de desecho de bajo coste como alimento. Esta estrategia es la que se ha explorado en los proyectos *BIOCYCLES* (2019-2022), financiado por el Research Council of Norway, y *SIDESTREAM* (2020-2023), financiado a través del programa EraNet BlueBio Cofund, en los que se enmarca esta Tesis Doctoral, y que han abordado el estudio sobre la eficacia de varios materiales de desecho o subproductos de industrias agro-alimentarias, entre otras, como alimento para gamáridos, para determinar la calidad nutricional de la biomasa resultante y poder identificar las condiciones de cultivo óptimas que maximicen dicha calidad. Los lípidos en general y los LC-PUFAs omega-3 en particular determinan en gran medida la calidad nutricional de biomasas que pueden ser en última instancia convertidas en harinas para piensos. En el siguiente Apartado (1.3), se realiza una breve revisión sobre lípidos de elevada importancia en nutrición de animales acuáticos, con especial énfasis en los LC-PUFAs omega-3 y los mecanismos que permiten su biosíntesis.

Tabla 1.2. Selección de ácidos grasos de gamáridos marinos y dulceacuícolas. Datos recogidos y adaptados de diferentes publicaciones (Biandolino y Prato, 2006; Kolanowski *et al*., 2007; Makhutova *et al*., 2011; Baeza-Rojano *et al*., 2014; Jiménez-Prada *et al*., 2018, 2020; Alberts-Hubatsch *et al*., 2019). *sd, sin datos.

Especies	**% Ácidos grasos (peso seco)**								
Marinas	**14:0**	**16:0**	**18:0**	**18:1n-9**	**18:2n-6**	**18:3n-3**	**20:4n-6**	**20:5n-3**	**22:6n-3**
Gammarus insensibilis	1,2	5,9	1,7	4,9	4,8	0,6	1,4	5,1	3,3
Echinogammarus marinus	0,8 ± 0,1	16,7 ± 0,5	2,6 ± 0,1	22,1 ± 0,3	4,4 ± 0,4	2,8 ± 0,3	9,3 ± 0,8	19,7 ± 0,6	5,0 ± 0,7
Gammarus aequicauda	1,9	33,7	5,8	16	3,5	sd	6,6	7,5	2,5
Dulceacuícolas	**14:0**	**16:0**	**18:0**	**18:1n-9**	**18:2n-6**	**18:3n-3**	**20:4n-6**	**20:5n-3**	**22:6n-3**
Gammarus fossarum	4,1 ± 0,4	17,2 ± 0,4	2,9 ± 0,1	23,2 ± 0,8	18,2 ± 0,2	6,4 ± 0,6	2,9 ± 0,2	9,3 ± 0,8	2,0 ± 0,2
Gammarus pulex	2,2 ± 0,6	24,5 ± 1,1	5,5 ± 0,2	28,5 ± 1,1	13,0 ± 0,5	4,5 ± 0,2	2,2 ± 0,6	5,0 ± 1,1	1,5 ± 0,7
Eulimnogammarus viridis	3,8 ± 0,6	19,0 ± 0,8	2,0 ± 0,2	17,8 ± 1,0	2,5 ± 0,3	2,8 ± 0,4	15,0 ± 0,3	16,5 ± 1,0	4,9 ± 1,1
Echinogammarus sp.	2,8	17,0	4,5	24,2	9,8	4,7	2,4	8,5	0,9

1.3. Lípidos y ácidos grasos

El término "lípido" se utiliza de un modo general para describir aquellas moléculas orgánicas que tienen como característica común su insolubilidad en agua y su alta solubilidad en solventes orgánicos tales como cloroformo o alcoholes (Gurr *et al*., 2002; Berg *et al*., 2015; Nelson y Cox, 2021). Los lípidos son macronutrientes que se encuentran presentes en todos los seres vivos, en los que juegan un papel fundamental en numerosas funciones esenciales tales como el almacenamiento de energía y el mantenimiento de la integridad estructural de las células, además de actuar como precursores de oxilipinas y eicosanoides, y como cofactores para reacciones enzimáticas y transportadores de proteínas liposolubles (Turchini *et al*., 2022; Berg *et al*., 2015; Nelson y Cox, 2021). Debido a la complejidad y diversidad de los lípidos, estos pueden clasificarse de dos formas distintas. La primera está basada en la complejidad de su estructura, diferenciando los lípidos simples cuando están formados por uno o dos tipos de componentes básicos (ácidos grasos y triacilglicéridos son ejemplos de lípidos simples), de los lípidos complejos cuando están formados por tres o más componentes, como ocurre con los fosfolípidos (Gurr *et al*., 2002; Berg *et al*., 2015; Nelson y Cox, 2021). Una segunda forma de clasificación

de los lípidos se basa en su polaridad, diferenciando entre lípidos polares y lípidos neutros. De esta forma, los lípidos polares incluyen principalmente a los fosfolípidos y los esfingolípidos, mientras que los lípidos neutros engloban a los acilglicéridos (mono-, di- y triacilglicéridos), esteroles y sus derivados éster, ácidos grasos libres y ceras (Gurr *et al*., 2002; Berg *et al*., 2015; Nelson y Cox, 2021). A continuación, se describen las características de los lípidos polares y neutros más comunes.

1.3.1. Lípidos polares

Los lípidos polares tienen funciones directamente relacionadas con el mantenimiento y la estructura de las membranas celulares, y se pueden distinguir dos grandes clases: los fosfolípidos y los esfingolípidos. La estructura básica tanto de los fosfolípidos como de los esfingolípidos consta de dos partes principales: una región polar (cabeza) con un grupo fosfato; y una parte apolar (cola) formada por las cadenas de ácidos grasos. Así pues, los lípidos polares son moléculas anfipáticas, característica que les confiere la capacidad de asociarse para formar membranas biológicas, gracias a la unión entre sus partes hidrófobas (ácidos grasos) y sus cabezas polares. De esta forma, los lípidos polares forman la bicapa lipídica de las células y de ciertos orgánulos, en la que los ácidos grasos se disponen en la parte interna, interaccionando entre ellos, y las cabezas polares en la zona acuosa, tanto de la célula (citoplasma) como del medio (Figura 1.4). La bicapa lipídica forma membranas semipermeables que permiten el tráfico de nutrientes e iones a través

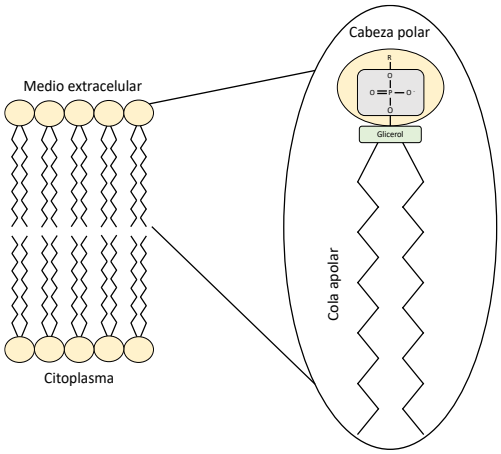

Figura 1.4 Estructura general de la membrana lipídica y de un fosfolípido.

de la misma de forma selectiva, además de la unión de diferentes proteínas de membrana implicadas en numerosas funciones biológicas (Gurr *et al*., 2002; Berg *et al*., 2015; Nelson y Cox, 2021).

1.3.2. Lípidos neutros

Los acilglicéridos constituyen el grupo más abundante de lípidos neutros. Son, en esencia, ésteres derivados de ácidos grasos unidos a una molécula de glicerol, y cuya función principal es la del almacenamiento de energía. Según el número de ácidos grasos que se encuentran esterificados al glicerol, podemos distinguir entre monoacilglicéridos ("MAGs", del inglés) con un solo ácido graso, diacilglicéridos ("DAGs", del inglés) con dos ácidos grasos, y triacilglicéridos ("TAGs", del inglés) con tres ácidos grasos (Figura 1.5). Los TAGs son la clase lipídica predominante en grasas

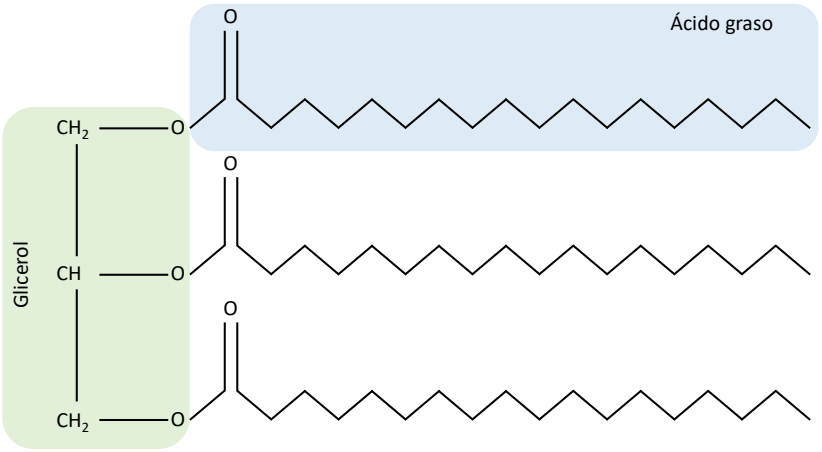

Figura 1.5 Estructura general de un triacilglicérido (TAG). Se observan tres moléculas de ácido graso (azul) unidas a una de glicerol (verde).

sólidas y líquidas (aceites) (Gurr *et al*., 2002; Berg *et al*., 2015; Nelson y Cox, 2021). Tanto los animales como las plantas utilizan los TAGs como reservorio de energía en tejidos especializados, mayormente en el tejido adiposo en los animales, y en las semillas en el caso de las plantas. En animales, cuando la cantidad de energía obtenida mediante la dieta excede la cantidad requerida por el organismo, esta suele almacenarse en forma de TAGs en el tejido adiposo de forma anhidra, debido a la naturaleza hidrófoba de los TAGs. Este tipo de almacenamiento permite liberar más energía que el metabolismo convencional de glucógeno en el hígado y en el

músculo, por lo que normalmente los TAGs almacenan energía para consumir a largo plazo mientras que el glucógeno, forma de almacenamiento de energía de los carbohidratos, libera energía de forma más inmediata y rápida (Gurr *et al*., 2002; Berg *et al*., 2015; Nelson y Cox, 2021).

1.3.3. Ácidos grasos

Los ácidos grasos pueden definirse como moléculas orgánicas compuestas por cadenas hidrocarbonadas de longitud variable, con un grupo carboxilo (-COOH) y otro metilo (-CH_3) en sus dos extremos (Figura 1.6). La nomenclatura de los ácidos

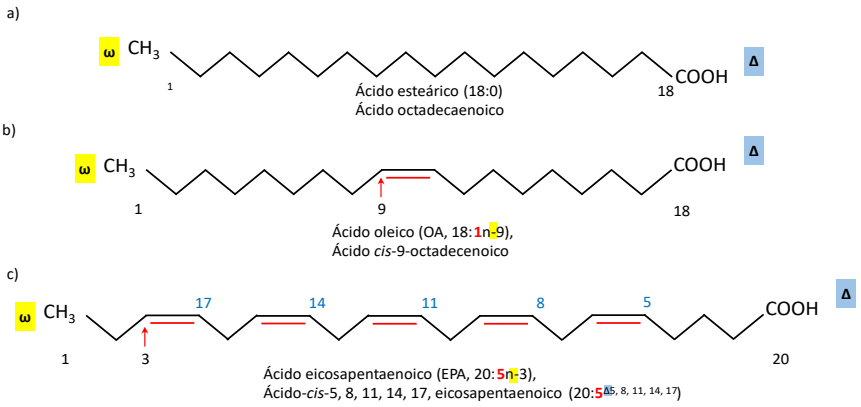

Figura 1.6 Tipos de ácidos grasos y sus diferentes nomenclaturas. a) ácido graso saturado de 18 carbonos, **b)** ácido graso monoinsaturado de 18 carbonos, y **c)** ácido graso poliinsaturado de 20 carbonos con 5 dobles enlaces.

grasos consiste principalmente en dos numeraciones separadas por dos puntos (A:Bn-x o A:Bωx). El conjunto numérico que precede a los dos puntos (A) indica los átomos de carbono de la cadena hidrocarbonada, mientras que el número después de los dos puntos (B) indica los dobles enlaces (insaturaciones) de la molécula. En la notación n-x (o ωx), la "x" hace referencia a la posición donde se encuentra el primer doble enlace desde el grupo metilo (Figura 1.6). Por tanto, un ácido graso de 18 carbonos sin dobles enlaces (saturado) se nombra como "18:0", mientras que un ácido graso de 20 carbonos con 5 dobles enlaces, el primero de estos en la posición 3 desde el carbono del grupo metilo, se nombra como "20:5n-3" (Figura 1.6). Como se ha indicado anteriormente, esta nomenclatura permite conocer la localización del primer doble enlace en la cadena hidrocarbonada (indicada con "x"). La posición de

este primer doble enlace desde el grupo metilo es el que da nombre a la "serie" del ácido graso (p. ej., n-3 u ω3, n-6 u ω6) y marca las propiedades fisiológicas y nutricionales de este. Cada ácido graso, además de su fórmula química, tiene un nombre común, algunos de los cuales se inspiran en sus fuentes de origen. Por ejemplo, el ácido hexadecanoico (16:0) y el ácido *cis*-9-octadecenoico (18:1n-9) pueden llamarse también ácido palmítico ("PA", del inglés) y ácido oleico ("OA", del inglés), respectivamente, ya que el primero es abundante en el aceite de palma y el segundo en el de oliva. Tanto PA como OA son ejemplos de ácidos grasos conocidos por su nombre común. Sin embargo, la forma más habitual para nombrar a los ácidos grasos es la que se basa en su estructura química. Por ejemplo, es mucho más reconocible por la comunidad científica el nombre "ácido eicosapentaenoico (EPA)" para referirse al "20:5n-3" que su nombre común ("ácido timnodónico"). Además, para definir la acción de las enzimas desaturasas involucradas en la biosíntesis de los ácidos grasos (ver Apartado 1.4), los ácidos grasos se pueden nombrar con la llamada "nomenclatura Δ", en la que se indican cada una de las posiciones de los carbonos en los que hay una insaturación, tomando como referencia en este caso el carbono del grupo carboxilo (Figura 1.6). Así pues, el "20:5n-3" puede nombrarse como "20:5 $^{\Delta 5,8,11,14,17}$" (Figura 1.6). A pesar de ser de gran utilidad en la definición de los denominados en inglés "*non-methylene interrupted fatty acids"*, un tipo particular de ácidos grasos abundantes en invertebrados acuáticos (Barnathan, 2009), la nomenclatura Δ no será utilizada en esta Tesis Doctoral. La Tabla 1.3 muestra una lista de los nombres y abreviaturas de los ácidos grasos más comunes en el contexto de la presente Tesis Doctoral.

Tabla 1.3. Lista de los ácidos grasos más comunes en este estudio, su nombre, abreviatura usada y clasificación según las definiciones hechas en el Apartado 1.3.3.

Nombre	Nomenclatura	Abreviatura	Tipo
Ácido mirístico	14:0	MA	SFA
Ácido palmítico	16:0	PA	SFA
Ácido esteárico	18:0	SA	SFA
Ácido oleico	18:1n-9	OA	MUFA
Ácido linoleico	18:2n-6	LA	PUFA n-6
Ácido α-linolénico	18:3n-3	ALA	PUFA n-3
Ácido araquidónico	20:4n-6	ARA	LC-PUFA n-6
Ácido eicosapentaenoico	20:5n-3	EPA	LC-PUFA n-3
Ácido docosahexaenoico	22:6n-3	DHA	LC-PUFA n-3

Los ácidos grasos se pueden clasificar según diferentes características moleculares como son el número de dobles enlaces o insaturaciones, y la longitud de la cadena hidrocarbonada (número de carbonos). De esta forma, los ácidos grasos saturados ("SFAs" del inglés) son aquellos que no tienen dobles enlaces en la cadena hidrocarbonada, los ácidos grasos monoinsaturados ("MUFAs" del inglés) tienen un único doble enlace, y los ácidos grasos poliinsaturados ("PUFAs" del inglés) tienen dos o más dobles enlaces. La longitud de la cadena hidrocarbonada determina en gran medida las funciones biológicas de los PUFAs, hecho que conlleva a la distinción del término "LC-PUFAs" para referirnos a PUFAs cuya longitud de cadena está entre 20 y 24 carbonos (C_{20-24}) y "VLC-PUFAs" para referirse a PUFAs de cadena "muy larga" con una longitud de más de 24 carbonos ($>C_{24}$) (Poulos, 1995). Esta Tesis Doctoral sigue la clasificación de los distintos tipos de ácidos grasos según las definiciones aquí detalladas.

Las características moleculares de los ácidos grasos descritas arriba determinan las propiedades físico-químicas y sus funciones biológicas. Por ejemplo, el grado de polaridad de los ácidos grasos y su temperatura de fusión aumentan con el número de carbonos y, por otro lado, el número de insaturaciones también contribuye a la disminución de la temperatura de fusión (Gurr *et al*., 2002; Berg *et al*., 2015; Nelson y Cox, 2021). Los ácidos grasos juegan un papel primordial en el mantenimiento y estructura de la membrana lipídica y, por tanto, la longitud y el número de insaturaciones de los ácidos grasos que componen los fosfolípidos de membrana afecta directamente a la fluidez de la misma, así como también lo hace la proporción entre SFAs, MUFAs y PUFAs (Feller *et al*., 2002; Huber *et al*., 2002; Feller, 2008). Esta proporción de ácidos grasos es de vital importancia en la denominada "adaptación homeoviscosa", mediante la cual ciertos organismos modifican la composición de ácidos grasos de sus membranas para mantener la homeostasis (Moyes y Ballantyne, 2011; Ernst *et al*., 2016). Además, este proceso es especialmente importante en animales poiquilotermos como los crustáceos aquí estudiados, por ser organismos cuya temperatura corporal depende de la del medio en el que habitan (Macdonald, 1981; Pruitt, 1990).

1.4. Biosíntesis de ácidos grasos poliinsaturados de cadena larga

La presencia de LC-PUFAs (C_{20-24}) en los lípidos de animales depende principalmente de su entrada a través de la dieta, así como de la capacidad de producción endógena (biosíntesis), considerándose la dieta el factor más determinante. Los ecosistemas marinos se caracterizan por ser la principal fuente de LC-PUFAs n-3 (omega-3) a escala global, debido a la gran abundancia de organismos con la capacidad para biosintetizarlos, los cuales ocupan la base de la cadena trófica marina (Arts *et al*.,

2009). Históricamente, los microorganismos como las microalgas, bacterias y protistas, se han considerado como los principales y prácticamente únicos productores primarios de LC-PUFAs n-3 en los mares y océanos (Nichols, 2003; Arts *et al*., 2009). Otros organismos situados en eslabones superiores como, por ejemplo, los peces presentan niveles altos de LC-PUFAs n-3 por el consumo de presas que los contienen. Por tal motivo, es habitual encontrar en la bibliografía especializada menciones sobre la incapacidad de los peces marinos para la síntesis de LC-PUFAs n-3, aludiendo a la pérdida de enzimas desaturasas y/o elongasas implicadas en el proceso de biosíntesis (Apartado 1.4.1) durante su evolución y adaptación en medios marinos, con alto aporte por la dieta (Castro *et al*., 2016). Por el contrario, a los peces dulceacuícolas y a los salmónidos se les atribuye una alta capacidad de biosíntesis de LC-PUFAs n-3 que han conservado por no tener el aporte suficiente de estos nutrientes esenciales a través de la dieta, ya que los ecosistemas de agua dulce tienen poca producción primaria de LC-PUFAs n-3 (Twining *et al*., 2021). Hoy en día es bien conocido que la distinción entre las capacidades de biosíntesis de LC-PUFAs n-3 de peces marinos y peces dulceacuícolas no es tan obvia como se creía y existen numerosas excepciones que se han puesto de manifiesto al ampliarse el número de especies estudiadas (Monroig *et al*., 2018, 2022). En cualquier caso, e independientemente del hábitat (marino o dulceacuícola) o de otros factores implicados, parece claro que lo que verdaderamente determina la capacidad de biosíntesis de LC-PUFAs n-3 en cualquier especie animal incluidos los peces es la dotación y funciones de las desaturasas y elongasas de dicha especie. En general, las especies con una dotación completa de estos enzimas pueden biosintetizar LC-PUFAs n-3, mientras que las que carecen de ciertas actividades enzimáticas tienen una capacidad de biosíntesis limitada. En peces cultivados, esta cuestión tiene implicaciones prácticas en la formulación de piensos. Por un lado, aquellas especies de peces con baja capacidad biosintética de LC-PUFAs n-3 dependen de un mayor aporte de estos nutrientes esenciales en su dieta y, por tanto, requieren piensos con altos niveles de inclusión de FM y FO. Por otro lado, las especies con alta capacidad biosintética requieren niveles más bajos de LC-PUFAs n-3 en los piensos (baja inclusión de FM y FO) ya que son capaces de producirlos a partir de precursores de cadena corta (C_{18}) presentes, por ejemplo, en los VOs con los que se formulan hoy en día los piensos de acuicultura (Apartado 1.1.3) (Bell y Koppe, 2010; Colombo *et al*., 2020; Turchini *et al*., 2022). Sin embargo, otros organismos de eslabones superiores de la cadena trófica como los humanos, dependen fundamentalmente de la incorporación en la dieta de fuentes ricas en LC-PUFAs para un correcto funcionamiento del organismo, y para la prevención de varias enfermedades, como por ejemplo las cardiovasculares, debido a las limitaciones enzimáticas que presentan (Guillou *et al*., 2010; Swanson *et al*., 2012; Akbar *et al*., 2021).

1.4.1. Rutas de biosíntesis de LC-PUFAs

La biosíntesis de LC-PUFAs varía en función del tipo de organismo considerado (Qiu *et al.*, 2020; Monroig *et al.*, 2022). Algunos microorganismos como la bacteria *Shewanella* sp. pueden sintetizar LC-PUFA como DHA mediante el complejo enzimático policétido sintasa ("PKS" del inglés) (Metz *et al.*, 2001). Este complejo enzimático produce LC-PUFAs a través de la denominada "ruta anaeróbica", denominada así porque no requiere de oxígeno para la introducción de nuevos dobles enlaces (desaturaciones) como en las rutas aeróbicas (Hopwood y Sherman, 1990; Qiu, 2003). En animales, así como en la gran mayoría de organismos eucariotas, la biosíntesis de LC-PUFAs transcurre a través de rutas aeróbicas, las cuales dependen de la acción de tres enzimas principales: las desaturasas *methyl-end* (ωx-des), las desaturasas *front-end* ("Fed", del inglés), y las elongasas ("Elovl", de *elongation of very long-chain fatty acid protein* en inglés) (Monroig *et al.*, 2022). La acción de estas enzimas tiene lugar sobre el MUFA ácido oleico (OA, 18:1n-9) que es sintetizado a su vez por la estearil-CoA desaturasa (Δ9 desaturasa) a partir del ácido esteárico (18:0) (Figura 1.7). De esta forma, se sintetizan, en primer lugar, PUFAs de 18 carbonos como el ácido linoleico (LA, 18:2n-6) y el ácido α-linolénico (ALA, 18:3n-3), y tras la acción de desaturasas y elongasas, se producen otros PUFAs más insaturados y más largos entre los que se encuentran los LC-PUFAs (Figura 1.7).

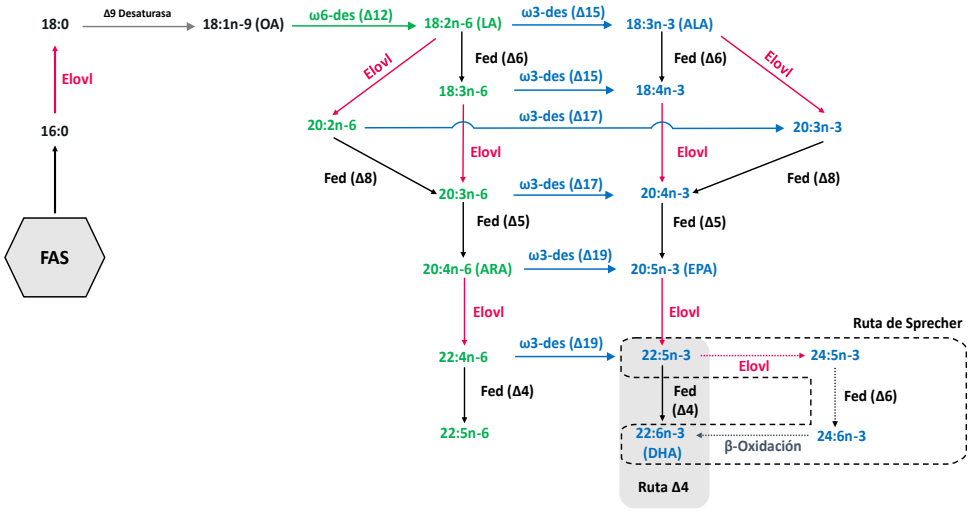

Figura 1.7 Rutas biosintéticas de LC-PUFAs en invertebrados. Adaptada de Monroig y Kabeya (2018).

1.4.1.1. Desaturasas "methyl-end" (ωx-des)

Como se ha introducido anteriormente, las rutas de biosíntesis de LC-PUFAs parten desde LA y ALA, precursores de las rutas las rutas de biosíntesis de omega-6 y omega-3, respectivamente. Las ωx-des son las enzimas responsables de la transformación del OA (18:1n-9) a LA (18:2n-6), y de este último a ALA (18:3n-3) (Figura 1.7). Las ωx-des encargadas de introducir el doble enlace en el OA para convertirlo en LA se conocen como "ω6 desaturasas", mientras que las responsables de la conversión de LA en ALA se denominan "ω3 desaturasas". La nomenclatura de las ωx-des viene dada en función de la posición donde estas enzimas introducen el doble enlace tomando como referencia el grupo metilo (Figura 1.6). La estructura molecular de las ωx-des consta de tres dominios característicos de cualquier tipo de desaturasa de ácidos grasos y que denominan "cajas de histidina" ("H-box" en inglés) (Hashimoto *et al.*, 2008; Kabeya *et al.*, 2018). La secuencia de aa de la tres H-box y su ubicación relativa en la estructura proteica son características de cada tipo de desaturasa (Hashimoto *et al.*, 2008). En el caso de las ωx-des, las tres H-box constan de 5 aa, concretamente HXXXH en el caso de la H-box1 y HXXHH para las H-box2 y H-box3 (Hashimoto *et al.*, 2008; Kabeya *et al.*, 2018). Por otro lado, otra particularidad de las ωx-des es la ausencia del denominado en inglés como "*cytochrome b5 heme binding domain*" (secuencia "HPGG"), motivo implicado en la transferencia de electrones durante las reacciones redox (Gostinčar *et al.*, 2010) y el cual sí está presente en las desaturaras Fed, también implicadas en las rutas de biosíntesis de LC-PUFAs (Apartado 1.4.1.2) (Gostinčar *et al.*, 2010; Kabeya *et al.*, 2018; Boyen *et al.*, 2023). A diferencia de las desaturasas Fed, la acción de las ωx-des sobre un ácido graso sustrato implica un cambio en la serie del mismo, pasando de n-9 a n-6 en el caso de las ω6 desaturasas y de n-6 a n-3 en el caso de las ω3 desaturasas (Figura 1.7). Es importante señalar que los vertebrados no tienen ωx-des ni, por tanto, capacidad de interconversión entre series diferentes de ácidos grasos.

La especificidad de sustratos de las ωx-des puede también expresarse en función del carbono en el que se introduce el nuevo doble enlace tomando como referencia en este caso, el grupo carboxilo, como se explica posteriormente en el Apartado 1.4.1.2 para las Fed. Así, la ω6 desaturasa tiene actividad Δ12 desaturasa (ocurre entre el carbono 12 y 13 contando desde el grupo carboxilo) cuando convierte OA en LA (Figura 1.7). La desaturación de LA a ALA llevada a cabo por una ω3 desaturasa es una desaturación Δ15 (ocurre entre el carbono 15 y 16 contando desde el grupo carboxilo), pero es muy común que las ω3 desaturasas tengan también actividad Δ15 sobre otros C_{18} PUFAs n-6, además de actuar como Δ17 desaturasas cuando usan como sustratos C_{20} PUFAs, y como Δ19 desaturasas cuando lo hacen sobre sustratos C_{22} PUFAs (Figura 1.7) (Monroig *et al.*, 2022).

La creencia generalizada de que los animales carecen de ωx-des sentó las bases para considerar a LA y ALA como "ácidos grasos esenciales" para todos los animales. Sin embargo, los resultados del estudio de Kabeya *et al*. (2018) y otros publicados posteriormente (Kabeya *et al*., 2020, 2021; Boyen *et al*., 2023), han permitido demostrar que algunos invertebrados entre los que se encuentran ciertos crustáceos, moluscos y poliquetos, entre otros, son capaces de biosintetizar LA y ALA a partir de OA al poseer ωx-des con capacidad de desaturación Δ12 y Δ15, respectivamente (Figura 1.7) (Kabeya *et al*., 2018, 2020, 2021; Monroig *et al*., 2022). Por este motivo, LA y ALA no deben considerarse como nutrientes esenciales en estos animales invertebrados. Más allá de las implicaciones que la presencia de ωx-des en invertebrados tiene sobre el carácter esencial de ciertos ácidos grasos, la existencia de enzimas ω3 desaturasas en invertebrados permite la bioconversión de un amplio espectro de sustratos ω6 (n-6) en sus correspondientes productos de desaturación ω3 (n-3) (Figura 1.7), ampliándose notablemente su capacidad de producción endógena de LC-PUFAs n-3 en comparación con los vertebrados, en los que no se da este tipo enzimático.

1.4.1.2. Desaturasas "front-end" (Fed)

Tanto el LA como el ALA actúan como precursores a partir de los cuales se pueden sintetizar los LC-PUFAs n-6 y n-3, respectivamente, mediante la acción orquestada de dos tipos de enzimas, las desaturasas *front-end* (Fed) y las elongasas (Apartado 1.4.1.3). Las Fed son enzimas encargadas de introducir dobles enlaces en la cadena hidrocarbonada entre uno ya existente y el grupo carboxilo (Sperling *et al*., 2003; Castro *et al*., 2016). Como se ha mencionado anteriormente, la estructura molecular de las Fed se caracteriza por contener un "*cytochrome b5 heme binding domain*" con la secuencia "HPGG" situado en el extremo N terminal de la secuencia aminoacídica. Además, las Fed tienen también tres H-box como las ωx-des, aunque con estructuras y composición diferentes a estas últimas (Apartado 1.4.1.1). Mientras que la primera H-box de las Fed coincide con las de las ωx-des (HXXXH), la segunda H-box de las Fed tiene un total de 6 aa (HXXXHH) en vez de 5 aa como en las ωx-des (Hashimoto *et al*., 2008). Por otro lado, la tercera H-box de las Fed está caracterizada por tener una glutamina (Q) en la primera posición (QXXHH) en lugar de una histidina (H) como ocurre en las ωx-des (Hashimoto *et al*., 2008). La presencia de esta Q en la primera posición de la tercera H-box ha demostrado ser crucial para una correcta función de las Fed, así como para su especificidad de sustratos (Sayanova *et al*., 2001).

Los diferentes tipos de Fed se establecen en función de la posición en la que introducen el nuevo doble, tomando como referencia el grupo carboxilo ya que este representa el "*front-end*" en la estructura molecular de los ácidos grasos (Figura 1.6). De esta forma, una Fed con actividad Δ6 desaturasa es la que introduce un doble

enlace entre los carbonos 6 y 7 contando desde el carbono del grupo carboxilo. Las actividades Δ4, Δ5, Δ6 y Δ8 desaturasa son las más comunes en Fed de animales (Monroig *et al*., 2022). Tales actividades desaturasa pueden existir de forma exclusiva en las denominadas Fed "monofuncionales" o bien como combinaciones de varias de dos de ellas en las Fed "bifuncionales" como las Δ6/Δ8 o Δ6/Δ5 (Castro *et al*., 2016; Monroig *et al*., 2022).

Tras el estudio pionero de la Δ5 Fed del pulpo común (*Octopus vulgaris*) (Monroig *et al*., 2012a), la presencia y la actividad de enzimas Fed ha sido objeto de estudio en numerosos invertebrados acuáticos (Monroig y Kabeya, 2018; Monroig *et al.,* 2022). En el caso de los moluscos, por ejemplo, se ha demostrado la presencia de dos tipos de Fed, nombradas como "FadsA" y "FadsB" para señalar su cercana relación filogenética (Surm *et al*., 2015, 2018). La caracterización funcional de enzimas pertenecientes al grupo de las FadsA de varias especies de moluscos y de equinodermos ha demostrado que son Δ5 desaturasas (Surm *et al*., 2015, 2018). Por otro, las FadsB pueden ser Δ6 (Ran *et al*., 2018), Δ8 (Liu *et al*., 2014) o Δ6/Δ8 desaturasas (Ramos-Llorens *et al*., 2023a). La existencia en una misma especie animal de Fed con actividades Δ5 y Δ6 o con actividades Δ5 y Δ8, bien como dos enzimas monofuncionales independientes o como uno solo bifuncional, permite cubrir todas las reacciones de desaturación requeridas para la biosíntesis LC-PUFAs tales como ARA y EPA a partir de LA y ALA, respectivamente (Figura 1.7).

En el caso de los crustáceos, la presencia de Fed con funciones en la biosíntesis de LC-PUFAs se ha demostrado únicamente en copépodos harpacticoides tales como *Tigriopus californicus* (Kabeya *et al*., 2021) y *Platychelipus littoralis* (Boyen *et al*., 2023). Si bien es cierto que se ha sugerido la presencia de Fed en ciertos crustáceos decápodos (Yang *et al*., 2013; Chen *et al*., 2017; Lin *et al*., 2017; Wu *et al*., 2018; Chen *et al*., 2024), ninguna de estas enzimas ha sido caracterizada funcionalmente y, por ende, no queda claro si juegan un papel relevante en la biosíntesis de LC-PUFAs en estos animales. Además, la estructura molecular de estas desaturasas de decápodos presenta variaciones en la secuencia correspondiente a la tercera H-box (Monroig *et al*., 2022) que, como se ha mencionado previamente, es un dominio clave para su función como Fed. De hecho, esta variación en la secuencia aminoacídica de la tercera H-box, sugiere que estas desaturasas son de un tipo diferente a las Fed, conocidas como "*sphingolipid desaturases"* (Hashimoto *et al*., 2008), cuya función en la biosíntesis de LC-PUFAs no ha sido demostrada.

1.4.1.3. Elongasas de ácidos grasos (Elovl)

Las elongasas de ácidos grasos (Elovl) son, al igual que las desaturasas (ωx-des y Fed), enzimas de membrana localizadas en el retículo endoplasmático. Las Elovl son

las responsables de la reacción de condensación por la que un ácido graso sustrato activado (acil-CoA) se une a malonil-CoA para formar 3-cetoacil-CoA (Figura 1.8) (Castro *et al.*, 2016; Gao *et al.*, 2021). Posteriormente, el 3-cetoacil-CoA pasa un

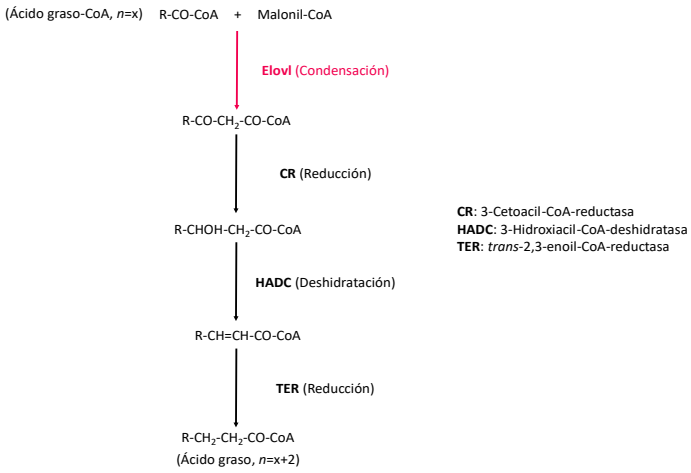

Figura 1.8. Ciclo de elongación de ácidos grasos. A partir de un ácido graso activado (unido a acetil-CoA) de longitud *n*=x, se consigue alargar su longitud en dos carbonos mediante las reacciones que se muestran en la figura. Adaptada de Castro *et al* (2016).

proceso de reducción-deshidratación-reducción para acabar resultando en un ácido graso producto con dos carbonos adicionales a los que tenía el sustrato al comienzo de este ciclo (Figura 1.8) (Castro *et al.*, 2016; Gao *et al.*, 2021). El ácido graso resultante puede pasar varias veces por el mismo proceso cíclico para seguir alargando su cadena hidrocarbonada. La estructura molecular de las Elovl está caracterizada por la presencia de los dominios conservados KXXEXXDT, HXXMYXYY y TXXQXXQ, y una única H-box (HXXHH) (Jakobsson *et al.*, 2006). Los aa que preceden a la H-box de las Elovl determinan en parte su afinidad por el tipo de ácidos grasos a los que reconocen como sustratos (Hashimoto *et al.*, 2008). De esta forma, si en la posición -5 con respecto a la primera histidina (H) de la H-box se encuentra una prolina (P), la Elovl suele tener más afinidad por SFAs y MUFAs; por otro lado, si hay una glutamina (Q) en esa misma posición, la Elovl suele presentar más afinidad por sustratos PUFAs (Hashimoto *et al.*, 2008). En animales vertebrados se han identificado ocho tipos diferentes de Elovl ("Elovl1-8"), entre los que Elovl2, Elovl4, Elovl5 y Elovl8 son consideradas elongasas con afinidad por PUFAs y, por tanto, con roles importantes en la biosíntesis de LC-PUFAs (Monroig *et al.*, 2022). Además del

tipo de ácido graso que reconoce como sustrato de elongación, la capacidad elongasa de las Elovl queda también definida por la afinidad que muestran en función de la longitud (número de carbonos) de los sustratos (Castro *et al*., 2016; Monroig *et al*., 2022).

Los invertebrados tienen Elovl con capacidad elongasa sobre PUFAs, aunque el número de tipos no está tan claro como ocurre en los vertebrados y es habitual encontrar, por ejemplo, Elovl nombradas como "Novel Elovl" (Surm *et al*., 2015, 2018). Un caso que merece especial atención es de la elongasa "Elovl2/5", la cual corresponde a una proteína ancestral de la que originaron posteriormente las Elovl2 y Elovl5 de vertebrados (Monroig *et al*., 2016). Como ocurre con Elovl2 y Elovl5 de vertebrados, la Elovl2/5 tiene alta afinidad por sustratos PUFAs y es de gran importancia en la biosíntesis de LC-PUFAs en invertebrados como los moluscos (Monroig *et al*., 2012b; Castro *et al*., 2016; Ran *et al*., 2019). Además, otra Elovl con afinidad por PUFAs descrita en moluscos es la denominada "Elovl4" por su proximidad filogenética a su ortólogo en vertebrados, la cual se ha caracterizado funcionalmente en el pulpo común *O. vulgaris* (Monroig *et al*., 2017) y en la almeja navaja china *Sinonovacula constricta* (Ran *et al*., 2019). Hasta la fecha, ninguna Elovl8 ha sido descrita en moluscos en particular ni en invertebrados en general, a excepción de la caracterizada para el crustáceo branquiópodo *Artemia fransciscana* (Ramos-Llorens *et al*., 2023b), que se explica más adelante.

Respecto a los crustáceos, varios estudios han demostrado la presencia de tres tipos de elongasas diferentes en la clase *Malacostraca* con actividades relevantes en la biosíntesis de LC-PUFAs (Lin *et al*., 2018; Mah *et al*., 2019; Sun *et al*., 2020; Ting *et al*., 2020). Estas secuencias corresponden a las elongasas Elovl4, Elovl6 y Elovl1/7-like, y son propias de crustáceos de la clase *Malacostraca* tales como los cangrejos de tierra *Scylla olivacea* (Mah *et al*., 2019; Ting *et al*., 2020) y *Scylla paramamosain* (Lin *et al*., 2018), y el cangrejo caballo (*Portunus trituberculatus*) (Sun et al., 2020b). La Elovl1/7-like descrita en *S. olivacea* parece ser un ancestro común de las Elovl1 y Elovl7 de vertebrados aunque, a diferencia de las Elovl1 y Elovl7 de vertebrados caracterizadas por su afinidad por sustratos SFAs (Guillou *et al*., 2010), la Elovl1/7-like de crustáceos sí que posee actividad elongasa sobre PUFAs (Mah *et al.*, 2019). Otros crustáceos de diferentes clases como los copépodos y los branquiópodos también tienen los tres tipos de elongasas descritos en *S. olivacea*, pero resulta interesante observar la existencia de otras Elovl, algunas de las cuales con demostrada capacidad elongasa sobre sustratos PUFAs (Kabeya *et al*., 2021; Ramos-Llorens *et al*., 2023b). En este sentido, un estudio reciente ha demostrado que el branquiópodo *A. franciscana* tiene una elongasa Elovl8 con una marcada capacidad elongasa sobre PUFAs, resultados que sugieren que el rol de esta elongasa en la biosíntesis de LC-PUFAs está conservado en los animales (Ramos-Llorens *et al*.,

2023b). Cabe destacar que ninguno de los estudios realizados sobre caracterización de Elovl de crustáceos han confirmado la presencia de Elovl2/5 en este grupo animal, resultados que evidencian la ausencia en crustáceos de este gen con un papel muy relevante en la biosíntesis de LC-PUFAs de algunos invertebrados como se señala anteriormente para los moluscos (Monroig *et al*., 2022).

1.5. Economía circular y acuicultura

La Comisión Europea define la economía circular como la estrategia para reducir la dependencia de los recursos naturales como materias primas, promover el uso sostenible de los recursos naturales obtenidos de la agricultura, pesquerías y acuicultura, y su transformación en productos que puedan ser aprovechados por las industrias, ya sea en forma de energía o de nuevos materiales, con un valor añadido, a la vez que se generan nuevos puestos de trabajo e industrias (Comisión Europea, 2018). La economía circular tiene como propósito final la conversión de un modelo de producción lineal, en el que la mayor parte de los desechos que se generan no se aprovechan, hacia otro circular en el que una industria obtiene un producto con valor añadido usando para ello materiales de desecho y subproductos de otras industrias o de la suya propia. De esta forma, se pasa de un paradigma donde se prioriza una mayor producción a costa de una menor sostenibilidad, a otro en el que se busca un mejor aprovechamiento de los recursos y residuos, que lleven como consecuencia una industria mucho más eficiente y sostenible.

Los ejemplos de aplicación de los principios en los que se basa la economía circular en el ámbito de la acuicultura son diversos, pero la mayoría se centran en estrategias dirigidas a optimizar la alimentación (Colombo *et al*., 2023; Verreth *et al*., 2023). Un ejemplo claro de circularidad en acuicultura es el que se comenta anteriormente (Apartado 1.1.2) sobre el reciclado de subproductos derivados de la industria acuícola y pesquera para la elaboración de FMs y FOs que son usados como ingredientes para piensos (Naylor *et al*., 2002; Turchini *et al*., 2019; Boyd *et al*., 2020; FAO, 2022; Colombo *et al*., 2023). Otra estrategia de aprovechamiento de nutrientes es la que propone la "Acuicultura Multitrófica Integrada" ("IMTA", del inglés), que consiste en la producción simultánea de diversas especies acuáticas de diferentes niveles tróficos (Troell *et al*., 2009; Chopin, 2010, 2013; FAO, 2022). Así pues, en un sistema IMTA, el alimento que no es consumido por las especies de mayor nivel trófico (peces, cefalópodos, etc.) es aprovechado por otras de nivel trófico inferior como organismos filtradores y detritívoros (moluscos bivalvos, anfípodos, etc.) que, además, pueden recuperar parte de los nutrientes contenidos en las heces de los primeros (Guerra-García *et al*., 2016; Fernandez-Gonzalez, 2017; Fernandez-Gonzalez *et al*., 2018; Jiménez-Prada *et al.*, 2018, 2020; Fraga-Corral *et al*., 2022).

Otra clara demostración de implementación de principios de economía circular en acuicultura se refleja en la utilización cada vez mayor de ingredientes de insectos como la mosca soldado negra (*H. illucens*), la mosca común (*Musca domestica*) y el gusano de la harina (*T. molitor*), que se producen usando subproductos de la industria agroalimentaria como fuente de alimento (Henry *et al*., 2015; Gasco *et al.*, 2018; Nogales-Mérida *et al*., 2019; Sangiorgio *et al*., 2022). Ejemplos de empresas productoras de insectos en España son InsectEAT (https://www.insecteat.es/) y Tebrio (https://tebrio.com/). El procesado de la biomasa obtenida genera como productos harinas y aceites de insecto que, como se ha descrito en el Apartado 1.1.2, han demostrado ser ingredientes con cualidades adecuadas para la alimentación de peces, aunque desprovistos de LC-PUFAs n-3, lo que compromete su valor nutricional como sustitutos de ingredientes marinos. La presente Tesis Doctoral ha explorado una estrategia similar con gamáridos que, además de permitir aprovechar subproductos de otras industrias, resulte también en una biomasa con alto valor nutricional (alto contenido en LC-PUFAs n-3).

A lo largo de esta introducción se han detallado los principales retos a los que se enfrenta actualmente la acuicultura en relación a la provisión de materias primas (FMs y FOs) para la elaboración de piensos, y qué estrategias se están adoptando para garantizar una expansión sostenible de la acuicultura en el futuro (Apartado 1.1). Parece evidente que el modelo productivo lineal (Figura 1.9) que sigue imperando en acuicultura intensiva de peces y crustáceos, y que implica a menudo la sobreexplotación de pesquerías para la producción de materias primas para los piensos, plantea serios problemas de sostenibilidad ambiental y económica que pueden comprometer su expansión en el futuro cercano. Como se ilustra en Figura 1.9, el modelo más sostenible basado en principios de economía circular en el que se basa este trabajo de investigación propone aprovechar la capacidad detritívora de los gamáridos, junto con su facilidad para adaptarse a una gran variedad de condiciones ambientales (Apartado 1.2), para la generación de una biomasa de estos animales criados con subproductos de industrias agroalimentarias, que pueda ser procesada posteriormente y utilizada en última instancia como ingrediente rico en LC-PUFAs n-3 en piensos de acuicultura.

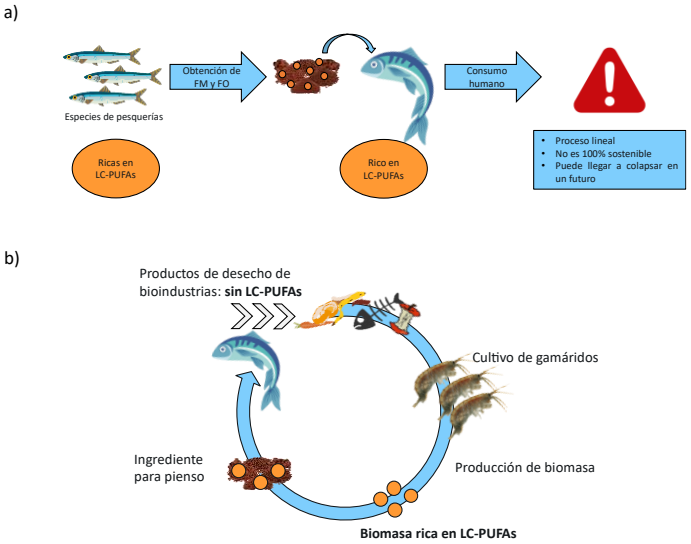

Figura 1.9. Modelos de producción lineal y circular en acuicultura intensiva. a) Sistema actual de producción de peces de acuicultura basado en un modelo lineal. **b)** Modelo de bioeconomía circular propuesto utilizando gamáridos alimentados con subproductos de otras industrias.

1.6. Hipótesis y objetivos

1.6.1. Hipótesis

Los gamáridos tienen la capacidad de producir y/o retener LC-PUFAs incluidos los denominados omega-3 a partir de precursores metabólicos presentes en subproductos de industrias agroalimentarias. Además, tal capacidad metabólica de los gamáridos puede modularse a través de la dieta y de factores ambientales como la temperatura, para asegurar niveles altos de LC-PUFAs en la biomasa resultante, de tal forma que se garantice su calidad nutricional como ingrediente para piensos de acuicultura.

1.6.2. Objetivo general

El objetivo general de esta Tesis Doctoral es dilucidar la capacidad biosintética de LC-PUFAs en gamáridos y cómo esta puede modularse a través de la dieta y la temperatura. Para ello, se ha investigado la dotación completa de genes que

codifican para elongasas y desaturasas implicadas en la biosíntesis de LC-PUFAs en gamáridos tanto marinos como dulceacuícolas, y se ha determinado la función de los enzimas correspondientes (Objetivo 1). Además, se ha estudiado la influencia de factores externos como la dieta y la temperatura en la biosíntesis de LC-PUFAs en el gamárido marino *Gammarus locusta*, así como en el crecimiento y la supervivencia, analizando su composición química (Objetivo 2) y la respuesta metabólica a nivel molecular mediante una aproximación transcriptómica (Objetivo 3).

1.6.3. Objetivos específicos

1. Caracterización molecular y funcional de elongasas y desaturasas en gamáridos marinos (Capítulo 2) y dulceacuícolas (Capítulo 3).
2. Determinación del perfil lipídico en gamáridos marinos alimentados con diferentes dietas y cultivados a diferentes temperaturas (Capítulo 4).
3. Construcción de un transcriptoma *de novo* de *G. locusta* y su utilización para estudiar la respuesta metabólica de estos gamáridos frente a dietas de composición variada y cultivados a diferentes temperaturas (Capítulo 5).

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2.

Biosynthesis of long-chain polyunsaturated fatty acids in marine gammarids: Molecular cloning and functional characterisation of three fatty acyl elongases

Abstract

Long-chain ($C_{20–24}$) polyunsaturated fatty acids (LC-PUFAs) are essential nutrients that are mostly produced in marine ecosystems. Previous studies suggested that gammarids have some capacity to endogenously produce LC-PUFAs. This study aimed to investigate the repertoire and functions of elongation of very long-chain fatty acid (Elovl) proteins in gammarids. Our results show that gammarids have, at least, three distinct elovl genes with putative roles in LC-PUFA biosynthesis. Phylogenetics allowed us to classify two elongases as Elovl4 and Elovl6, as they were bona fide orthologues of vertebrate Elovl4 and Elovl6. Moreover, a third elongase was named as "Elovl1/7like" since it grouped closely to the Elovl1 and Elovl7 found in vertebrates. Molecular analysis of the deduced protein sequences indicated that the gammarid Elovl4 and Elovl1/7-like were indeed polyunsaturated fatty acid (PUFA) elongases, whereas Elovl6 had molecular features typically found in non-PUFA elongases. This was partly confirmed in the functional assays performed on the marine gammarid *Echinogammarus marinus* Elovl, which showed that both Elovl4 and Elovl1/7-like elongated PUFA substrates ranging from C_{18} to C_{22}. *E. marinus* Elovl6 was only able to elongate C_{18} PUFA substrates, suggesting that this enzyme does not play major roles in the LC-PUFA biosynthesis of gammarids.

2.1. Introduction

Long-chain ($C_{20–24}$) polyunsaturated fatty acids (LC-PUFAs), including arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are physiologically essential compounds required for normal growth and development of vertebrates (Guillou *et al*., 2010). Moreover, the n-3 (or omega-3) LC-PUFAs EPA and DHA have beneficial roles in human health (Swanson *et al*., 2012). Since n-3 LC-PUFAs are nearly exclusively produced in marine ecosystems by low trophic marine organisms, such as microbes (Nichols, 2003; Pereira *et al*., 2003; Khozin-Goldberg *et al*., 2011) and certain invertebrates (Kabeya *et al*., 2018; Monroig and Kabeya, 2018), marine products are regarded as unique sources of EPA and DHA in the human diet (Gladyshev *et al*., 2013). Products derived from marine aquaculture have traditionally guaranteed the supply of health-promoting n-3 LC-PUFAs due to the fact that aquafeeds were formulated with high inclusion levels of the so-called "marine ingredients" fishmeal (FM) and fish oil (FO), naturally rich in n-3 LC-PUFAs (Tocher, 2015; Shepherd *et al*., 2017). FM and FO are mostly produced from feed-grade fish species and, with the rapid expansion of aquaculture worldwide, pressure on fisheries exploited for FM and FO production has remarkably increased, reaching and, on occasion, exceeding their ecological sustainability limit. This prompted interest in exploring alternative ingredients for aquaculture and, currently, raw materials derived from land animals or plants are commonly used to partly or totally replace FM and/or FO in aquafeeds (Turchini *et al*., 2009; Jannathulla *et al*., 2019; Galkanda-Arachchige *et al*., 2020). With respect to FO alternatives, oils derived from microalgae and transgenic oilseed crops have become available in recent years, representing promising sources of the n-3 LC-PUFAs (Sprague *et al*., 2017; Tocher *et al*., 2019; Napier *et al*., 2020). Currently, the use of vegetal oils (VOs) as substitutes for FO in fish feed has become a widely extended practice (Turchini *et al*., 2011a; Tocher, 2015; Aas *et al*., 2019). However, the use of non-marine ingredients is often associated with a loss of nutritional value of farmed fish products mainly due to decreased contents of LC-PUFAs, especially EPA and DHA, which are absent or at low levels in non-marine ingredients. Biomasses derived from low trophic marine crustaceans, such as krill and copepods, as well as the ingredients derived from their processing, have been demonstrated to be excellent sources of essential nutrients including n-3 LC-PUFAs and thus arise as promising alternative raw materials for aquafeed formulation (McKinnon *et al*., 2003; Naylor *et al*., 2009; Dhont *et al*., 2013). However, the exploitation of wild stocks for the production of crustacean-derived ingredients poses the same ecological sustainability issues alluded to above for reduction fisheries. Alternatively, mass production of marine crustaceans appears to

be a reasonable strategy to address such a sustainability hurdle, particularly with species of low trophic level with high nutritional value (i.e., high n-3 LC-PUFAs) and culture performance output.

Gammarids, crustacean amphipods, are aquatic invertebrates that are abundant in benthic communities in virtually all aquatic environments (Odum and Heald, 1972; Dauby *et al*., 2001). Previous studies have shown that gammarids' nutritional profiles are characterised by a high protein content, low levels of carbohydrates, and relatively high contents of n-3 LC-PUFAs (Köprücü and Özdemir, 2005; Kolanowski *et al*., 2007; Baeza-Rojano *et al*., 2014; Jiménez-Prada *et al*., 2018, 2020; Makhutova *et al*., 2018). Such characteristics, along with the possibility to establish high density cultures (Evjemo, 2011, 2016; Jiménez-Prada *et al*., 2018), have prompted interest for using gammarids in aquaculture (Harlıoğlu and Farhadi, 2018). Importantly, some investigations reported on the ability of gammarids to be used in a wide range of sidestreams, in bioindustries such as aquaculture, forestry and agriculture (Evjemo, 2011, 2016; Jiménez-Prada *et al*., 2018; Alberts-Hubatsch *et al*., 2019). Consequently, gammarids arise as promising candidates to apply circular economy principles by which sidestreams can be utilised for the production of biomass with high nutritional value. Moreover, in the scenario of a limited availability of marine ingredients alluded to above, it is important to elucidate whether marine gammarids are able to bioconvert fatty acids (FAs) present in sidestreams into the high-value physiologically essential LC-PUFAs.

The ability of animals for endogenous production (biosynthesis) of LC-PUFAs depends upon the gene repertoire and function of two types of enzymes (Monroig *et al*., 2013b; Monroig and Kabeya, 2018). On one hand, frontend desaturases (Fad) introduce double bonds (unsaturations) into polyunsaturated fatty acid (PUFA) substrates; on the other hand, elongation of very long-chain fatty acid (Elovl) proteins (or commonly known as "elongases") catalyse the usually rate-limiting reaction (condensation) in the FA elongation pathway and results in the extension of the pre-existing FA in two carbons (Castro *et al*., 2016). To the best of our knowledge, no studies have reported on the molecular and functional characterisation of *fad* and *elovl* genes from gammarids, and the LC-PUFA biosynthetic capacity of gammarids has been only inferred from feeding experiments assessing the impact of dietary FA on body FA composition (Kolanowski *et al*., 2007; Baeza-Rojano *et al*., 2014; Jiménez-Prada *et al*., 2018, 2020; Alberts-Hubatsch *et al*., 2019). While the presence of Fad-like enzymes in crustaceans remains to be clarified (Monroig and Kabeya, 2018), *elovl* genes with roles in LC-PUFA biosynthesis have been recently reported in crabs such as the mud crab *Scylla paramamosain* (Lin et al., 2018b), the orange mud crab *Scylla olivacea* (Mah *et al*., 2019; Ting *et al*., 2020) and the swimming crab *Portunus trituberculatus* (Sun et al., 2020b). To date, three different elongases named Elovl4,

Elovl6 and Elovl7 have been reported in crustaceans (Lin et al., 2017; Mah et al., 2019; Sun et al., 2020b; Ting et al., 2020). While the vertebrate Elovl4 has been demonstrated to elongate PUFA substrates, Elovl6 and Elovl7 have not been reported to play a role in LC-PUFA biosynthesis of vertebrates (Guillou *et al*., 2010; Castro *et al*., 2016), suggesting functional diversification of animal Elovl (Monroig and Kabeya, 2018). Our overall aim is to elucidate the LC-PUFA biosynthetic pathways in gammarids in order to identify species with a high capacity to convert short-chain FA compounds available in various sidestreams potentially used as feed into high nutritional value LC-PUFAs. Specifically, the present study aimed to characterise molecularly and functionally the set of *elovl* genes identified in *Echinogammarus marinus*, a gammarid species in which capacity for LC-PUFA endogenous production was previously reported (Alberts-Hubatsch *et al*., 2019). We here provide compelling evidence showing that *E. marinus* possess three *elovl* genes, named Elovl4, Elovl6 and Elovl1/7-like, with roles in LC-PUFA biosynthesis.

2.2. Results

2.2.1. Phylogeny of the *E. marinus* Elovl

Our search strategy enabled us to identify three *elovl* sequences from *E. marinus*, which were homologous to the three Elovls found in the amphipod reference genome from *Hyalella azteca* (Poynton *et al*., 2018). The newly cloned *E. marinus elovl4* cDNA (deposited in GenBank database with accession number MW660836) has an open reading frame (ORF) of 960 base pairs (bp), encoding a putative protein of 319 amino acids (aa). The *E. marinus elovl6* cDNA has an ORF of 1029 bp (MW659697) that translates to a putative protein of 342 aa residues, whereas *elovl1/7*-like contains an ORF of 1077 bp (MW659696) that encodes for a putative protein of 358 aa. The putative aa sequences deduced from the *E. marinus elovl* have some of the distinctive characteristics of fatty acyl elongases, such as the conserved domains KXXEXXDT, NXXXHXXMYXYY and TXXQXXQ, a histidine box (HXXHH) and a specific number of transmembrane-spanning regions (Figure 2.1). More specifically, prediction of transmembrane-spanning domains showed that the deduced aa sequences of the three *E. marinus* Elovls contained seven transmembrane-spanning regions (Figure S2.1 a–c). The histidine box (HXXHH), a common feature shared with desaturase and hydrolase enzymes, and crucial for the coordination of electron exchange during FA elongation (Jakobsson *et al*., 2006; Hashimoto *et al*., 2008), was found in all three *E. marinus* Elovls (Figure 2.1). However, the sequence of the N-terminal side of the histidine box of the three *E. marinus* Elovls presented some noteworthy differences. On one hand, Elovl4 and Elovl1/7-like from *E. marinus* had,

respectively, a glutamine (Q) and a histidine (H) at position -5 preceding their histidine boxes; on the other hand, the *E. marinus* Elovl6 had a proline (P) at position -5 and a lysine (L) at position -4 (Figure 2.1). Subcellular location analysis revealed that these three elongases are located in the endoplasmic reticulum (ER) membrane (Table .2.1 a, b).

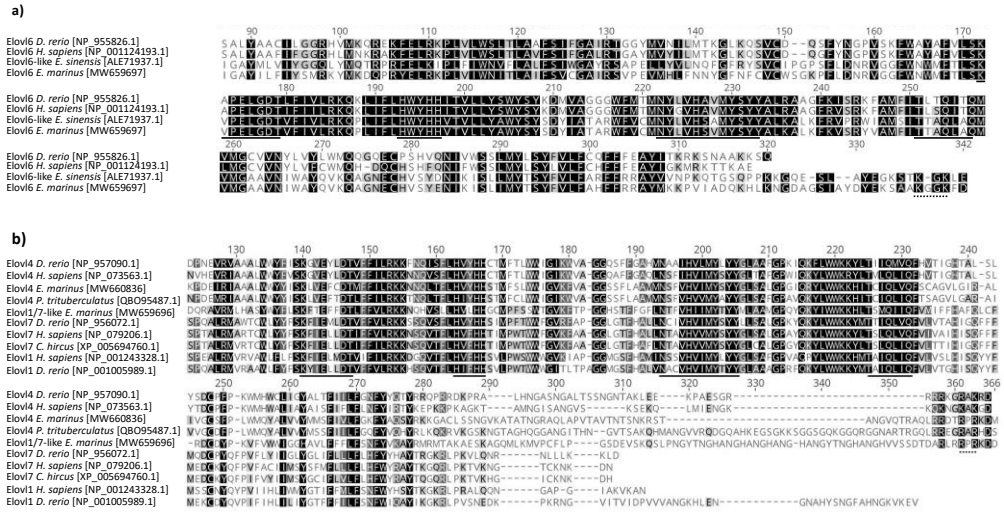

Figure 2.1. Alignments of the partial deduced amino acid (aa) sequences from *Echinogammarus marinus* (a) SFA/MUFA like elongase *elovl6* (MW659697) and **(b)** PUFA-like elongases *elovl4* (MW660836) and *elovl1/7*-like (MW659696), with several elongases already characterised from vertebrates and crustaceans including the swimming crab *P. trituberculatus*. Conserved domains KXXEXXDT, NXXXHXXMYXYY and TXXQXXQ, and the histidine box (HXXHH) are underlined (solid line). Additionally, aa residues corresponding to putative endoplasmic reticulum retention signal have a dotted underline.

In order to elucidate the orthology of the newly cloned *elovl* from *E. marinus*, a phylogenetic tree was constructed to compare them with Elovl proteins from a variety of animal groups, including both invertebrates and vertebrates (Figure 2.2). Phylogenetic analysis of the aa sequences showed that the putative *E. marinus* Elovl4 grouped together with Elovl4 from the crustaceans *P. trituberculatus* and *S. olivacea*, as well as vertebrate Elovl4. The *E. marinus* Elovl6 protein grouped closely to Elovl6 sequences from other crustaceans such as the Chinese mitten crab *E. sinensis* and more distantly from their vertebrate orthologues. With respect to the phylogeny of the Elovl1/7-like retrieved from *E. marinus*, the putative protein clustered together

with a group of as yet unnamed putative Elovl proteins from other crustaceans like *P. trituberculatus* and *S. paramamosain*, themselves being closely related to *H. azteca* (XP_018024127.1) and *S. olivacea* (AWM30548.1) that were originally annotated as "Elovl7-like". Three *elovl2/5*-like sequences were identified from freshwater gammarids including *Echinogammarus berilloni*, *Gammarus wautieri* and *Gammarus pulex*, but no homologous sequences of this *elovl* type could be found in transcriptomic databases from either *E. marinus* or *G. locusta*.

All putative Elovl proteins found in *E. marinus* were further confirmed to exist in other gammarids including both freshwater and marine species from the *Gammaridae* family (Figure 2.2). Given the close taxonomic relationship between *Echinogammarus* and *Gammarus* species, the Elovl proteins from the different gammarid species shared a high homology and therefore clustered together in the tree (Figure 2.2).

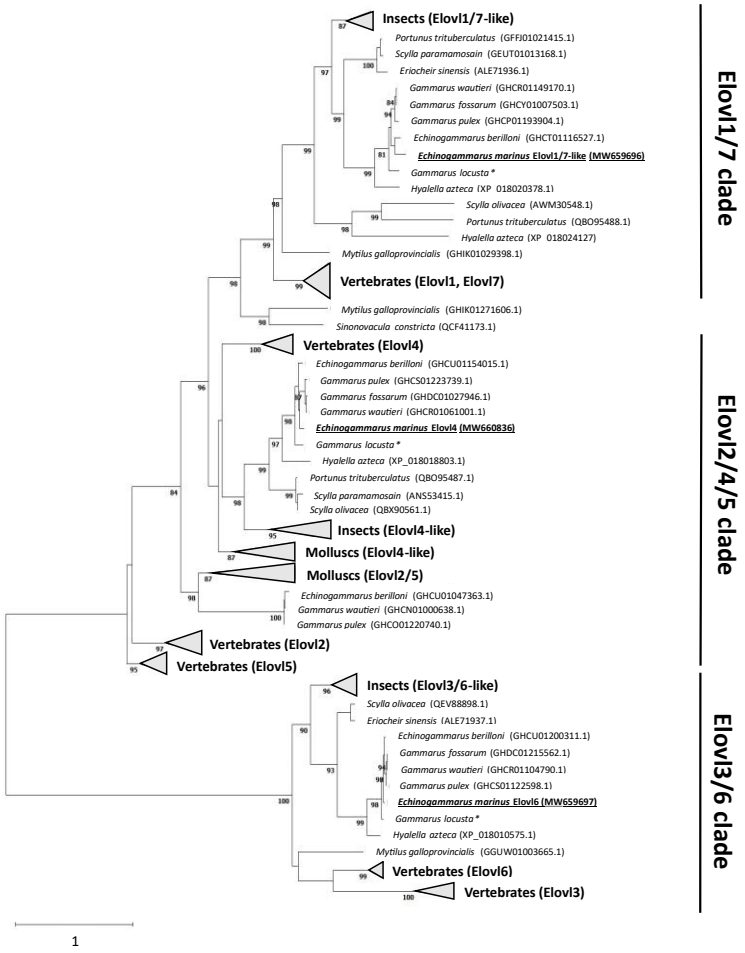

Figure 2.2. Phylogenetic tree comparing gammarid Elovl with several elongases retrieved from other crustaceans, as well as molluscs and vertebrates. There are three different clades that grouped the majority of the sequences analysed, namely Elovl1/7 clade, Elovl2/4/5 clade and Elovl3/6 clade. Two sequences that do not correspond to any of the previously defined clusters are PUFA elongases found in *Mytilus galloprovincialis* and *Sinonovacula constricta* (Ran *et al*., 2019). The protein evolutionary model used was LG4X (Le *et al*., 2012). The tree was constructed using the maximum likelihood method by randomized axelerated maximum likelihood (RAxML) within the CIPRES external server. Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations. The transfer distance bootstrap support value (%) is given in each node. Values lower than 80% are not shown. Accession numbers according to the NCBI database are given for each sequence. * Elovls from *Gammarus locusta* were retrieved from blasting the transcriptome assembled by Neuparth *et al*., (2020).

2.2.2. Roles of the *E. marinus* Elovl4, Elovl6 and Elovl1/7-like in LC-PUFA biosynthesis

The role of the *E. marinus* Elovl proteins in LC-PUFA biosynthesis was investigated by expressing their ORFs in yeast. Transgenic yeast expressing each of the *E. marinus elovl* sequences were grown in the presence of exogenously supplied PUFA substrates including linoleic acid (LA, 18:2n-6), α-linolenic acid (ALA, 18:3n-3), γ-linolenic acid (GLA, 18:3n-6), stearidonic acid (SDA, 18:4n-3), ARA (20:4n-6), EPA (20:5n-3), docosatetraenoic acid (DTA, 22:4n-6), docosapentaenoic acid (DPA, 22:5n-3) and DHA (22:6n-3). The elongase conversions are shown in Table 2.1. For the *E. marinus* Elovl4, the results showed that this elongase has the ability to elongate all PUFA substrates to the corresponding 2-carbon longer products (Table 2.1). Conversions of C_{18} and C_{20} substrates were all above 1%, whereas conversions towards C_{22} were below 1% (Table 2.1). A second elongation product was detected when 18:4n-3, 20:5n-3, 20:4n-6 and 22:5n-3 were used as substrates, resulting in the production of 22:4n-3, 24:5n-3, 24:4n-6 and 26:5n-3 (Table 2.1). The *E. marinus* Elovl6 was able to elongate only C_{18} PUFA, with conversions to the corresponding C_{20} products being below 0.4 % in all cases (Table 2.1). Functional characterisation of the *E. marinus* Elovl1/7-like revealed that, like Elovl4, this enzyme was able to elongate all substrates assayed (Table 2.1). It is worth mentioning that conversions towards the C_{20} substrates EPA and ARA were relatively high (13.17 and 5.75 %, respectively), and also a second elongation product was detected in both cases (Figure 2.3). However, unlike Elovl4, the *E. marinus* Elovl1/7-like did not produce 26:5n-3.

Table 2.1. Functional characterisation of the *Echinogammarus marinus* Elovl4, Elovl6 and Elovl1/7-like. Conversions of exogenously supplied fatty acid (FA) substrates were calculated according to the formula [areas of all products with longer chain than substrate/(areas of all products with longer chain than substrate + substrate area)] × 100. nd, not detected.

FA substrate	FA product	Elovl4	Elovl6	Elovl1/7-like	Activity
18:3n-3	20:3n-3	2.67	0.22	0.29	C18→C20
18:2n-6	20:2n-6	1.32	0.11	0.19	C18→C20
18:4n-3	20:4n-3	1.82	0.33	1.46	C18→C20
	22:4n-3	0.02	nd	0.02	C20→C22
18:3n-6	20:3n-6	1.21	0.35	0.87	C18→C20
20:5n-3	22:5n-3	2.52	nd	13.17	C20→C22
	24:5n-3	0.05	nd	0.17	C22→C24
20:4n-6	22:4n-6	1.61	nd	5.75	C20→C22
	24:4n-6	0.02	nd	0.09	C22→C24
22:5n-3	24:5n-3	0.92	nd	2.16	C22→C24
	26:5n-3	0.09	nd	nd	C24→C26
22:4n-6	24:4n-6	0.46	nd	0.72	C22→C24
22:6n-3	24:6n-3	0.38	nd	0.54	C22→C24

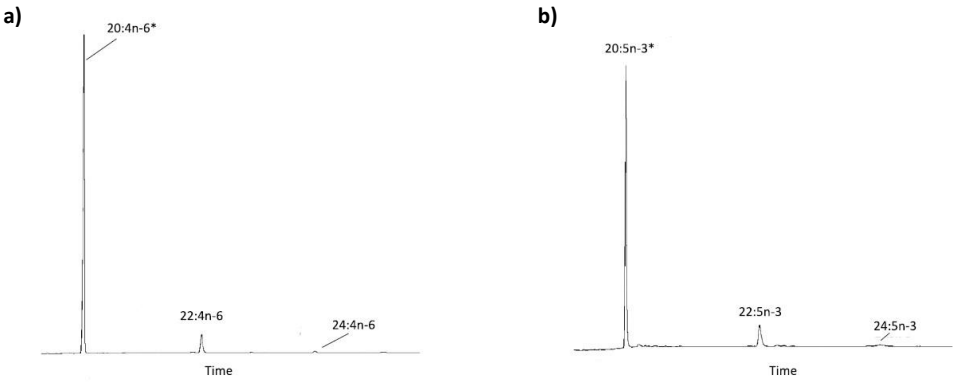

Figure 2.3. Functional characterisation of the *E. marinus* Elovl1/7-like activity towards C_{20} PUFA substrates. Yeast (*S. cerevisiae*) expressing the *E. marinus elovl1/7*-like gene was grown in the presence of (a) arachidonic acid (ARA) (20:4n-6) and (b) eicosapentaenoic acid (EPA) (20:5n-3). Substrates (*) and their corresponding elongation products are indicated in each panel.

2.3. Discussion

There exists a growing interest in using gammarids in aquaculture associated with their nutritional profiles, characterised by relatively high contents of n-3 LC-PUFA(Baeza-Rojano *et al*., 2014; Jiménez-Prada *et al*., 2018). Moreover, gammarids have shown an ability to be grown on a varied range of sidestreams from bio-based industries (Evjemo, 2011, 2016; Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020). Recently, Alberts-Hubatsch *et al*. (2019) reported that the gammarids *E. marinus* and *G. locusta* had some capacity for "trophic upgrading", implying that these crustaceans can bioconvert FA present in sidestreams from agriculture into high nutritional value LC-PUFA (Alberts-Hubatsch *et al*., 2019). However, the actual enzymes accounting for such FA conversions remained to be elucidated and, therefore, this study contributes to investigating the role that endogenous Elovls play in gammarids' trophic upgrading capacity. We herein report on three elongases with putative functions in the LC-PUFA biosynthesis of gammarids, and demonstrate that the relatively high LC-PUFA contents of gammarids grown on agriculture sidestreams can account for the endogenous enzymatic capacity existing in gammarids themselves.

Our phylogenetic analysis revealed a well-conserved pattern among gammarids, consisting of three Elovl proteins that have been here referred to as Elovl4, Elovl6 and Elovl1/7-like. The phylogenetic analysis clearly established that both *elovl4* and *elovl6* found in multiple gammarids are closely related to the Elovl4 and Elovl6 present in higher metazoans (Guillou *et al*., 2010; Castro *et al*., 2016), and previously characterised in other non-gammarid crustaceans like the mud crab *S. paramamosain* (Lin et al., 2018b), the swimming crab *P. trituberculatus* (Sun et al., 2020b) and the orange mud crab *S. olivacea* (Ting *et al*., 2020). Interestingly, we named a further *elovl* as "*elovl1/7*-like" since the phylogenetic analyses illustrated that this elongase grouped closely with the vertebrate Elovl1 and Elovl7 subfamilies. Moreover, our results showed that this elongase is closely related to an Elovl named as "Elovl7-like" for *S. olivacea* (Mah *et al*., 2019). It is important to note that some invertebrates such as molluscs possess an elongase named Elovl2/5 (Monroig *et al*., 2012, 2016a; Liu *et al*., 2014; Ran *et al*., 2019), which represents an ancestor elongase that gave rise to the Elovl2 and Elovl5 protein families present in vertebrates (Monroig *et al*., 2016b). Like Elovl2 and Elovl5 (Castro *et al*., 2016), the mollusc Elovl2/5 has well established roles in the elongation of PUFAs (Monroig *et al*., 2012, 2016a; Liu *et al*., 2014; Ran *et al*., 2019). Our *elovl*-like sequence search strategy identified three putative *elovl2/5* sequences from freshwater gammarids, namely *Echinogammarus berilloni*, *Gammarus wautieri* and *Gammarus pulex*. However, multiple attempts to isolate a partial sequence of this elongase from marine

gammarids including *E. marinus* and *G. locusta* did not result in any conclusive result, suggesting that these elongases could be absent from genomes of these species or, alternatively, are expressed at relatively low levels compared to those of the elongases characterised in this study.

We further characterised the sequences of the *elovl* genes present in the marine gammarid *E. marinus*, a species in which capacity for trophic upgrading of dietary FA had been previously reported (Alberts-Hubatsch *et al.*, 2019), and is now also confirmed to be a valid representative of marine gammarids as it possesses at least one copy of the *elovl4*, *elovl6* and *elovl1/7*-like genes. The newly cloned *elovl4*, *elovl6* and *elovl1/7*-like from *E. marinus* have all the distinctive characteristics shared by Elovls (Jakobsson *et al.*, 2006) and were further confirmed to exist in orthologues from *S. paramamosain, S. olivacea* and *P. trituberculatus* (Lin et al., 2018b; Sun et al., 2020b; Ting et al., 2020). Such Elovl characteristic traits include the conserved domains KXXEXXDT, NXXXHXXMYXYY, TXXQXXQ, a histidine box (HXXHH) and a specific number of transmembrane-spanning regions. However, the above-described differences in the residues located in the N-terminal side of the histidine box between the *E. marinus* Elovl4 and Elovl1/7-like and Elovl6 suggest different putative functions according to the categorisation of PUFA vs. non-PUFA elongases proposed by Hashimoto *et al.* (2008). Clearly, our results established that both Elovl4 and Elovl1/7-like from *E. marinus* and other gammarids, such as *G. locusta*, have the diagnostic residues in the surrounding region of the histidine box that are a common feature of PUFA elongases (Hashimoto *et al.*, 2008). On the contrary, the gammarid Elovl6 contains the diagnostic residues that are typically found in Elovls involved in elongation of saturated and monounsaturated fatty acids (Hashimoto *et al.*, 2008). The predicted subcellular localisation results showed that all three Elovls are located in the ER, the cellular site where LC-PUFA biosynthesis takes place (Jakobsson *et al.*, 2006; Guillou *et al.*, 2010; Castro *et al.*, 2016). Retention in the ER is crucial for Elovl to exert its function and, consistently, the *E. marinus* Elovl4 and Elovl1/7like, but not Elovl6, have the RXR motif that is also associated with ER retention (Zerangue *et al.*, 1999) and predicted in the *P. trituberculatus* Elovl4 (Sun et al., 2020b). Collectively, the molecular characterisation results showed that the *E. marinus* Elovl4 and Elovl1/7-like have distinctive features with respect to Elovl6, which can partly account for the substrate specificities revealed in our functional assays in yeast.

The functional characterisation of the herein studied *E. marinus* Elovls revealed that these enzymes, particularly Elovl4 and Elovl1/7-like, play roles in the LC-PUFA biosynthesis of gammarids, which likely account for the trophic upgrading capacity of these crustaceans (Alberts-Hubatsch *et al.*, 2019). Indeed, the gammarid Elovl4 and Elovl1/7-like were able to utilise a variety of C_{18-22} PUFAs as substrates and convert them into longer products, including multiple LC-PUFAs. On the contrary,

the chain length of PUFA substrates recognised by the *E. marinus* Elovl6 was limited to C_{18} and with relatively low conversions throughout. While our results do not allow us to completely rule out a putative role of the *E. marinus* Elovl6 in PUFA elongation, the restricted elongation capacity towards C_{18} PUFA, along with the abovementioned sequence characteristics of non-PUFA Elovl (Hashimoto *et al*., 2008), suggests that Elovl6 does not play a relevant role in LC-PUFA biosynthesis in gammarids. Importantly, the elongation abilities contained within the gammarid Elovl4 and Elovl1/7-like enable these crustaceans to catalyse all elongation reactions involved in the LC-PUFA biosynthetic pathway, even in the absence or low expression of the PUFA elongase *elovl2/5* in marine species such as *E. marinus* and *G. locusta*, as pointed out above. The ability of the gammarid Elovl4 and Elovl1/7-like to produce 24:5n-3 from 22:5n-3, but also from 20:5n-3, suggests that these elongases can contribute to the biosynthesis of DHA via the Sprecher pathway, requiring 24:5n-3 as an intermediate for a Δ6 desaturase to produce 24:6n-3, which is subsequently β-oxidised to DHA (22:6n-3) (Sprecher, 2000). This key metabolic pathway is found in some mammals (Sprecher, 2000), fish (Deák *et al*., 2019) and molluscs (Ran *et al*., 2019), where Δ6 desaturases that are able to convert 24:5n-3 into 24:6n-3 have been reported. However, to the best of our knowledge, no front-end desaturases from gammarids having such capacity have been yet reported in the literature. While further studies aiming to characterise the gene complement and function of front-end desaturases from gammarids are needed, the current evidence suggest that it is unlikely that DHA can be produced via the Sprecher pathway in gammarids. Likewise, biosynthesis of other physiologically important LC-PUFA such as EPA and ARA appear to not be possible either, unless the existence of front-end desaturases, particularly Δ5 desaturases, is demonstrated to exist in gammarids. Rather than biosynthetic products, however, EPA and ARA appear to be metabolic precursors in gammarids, according to the results of the yeast assays showing that these compounds can be efficiently elongated to the corresponding C_{22} elongation products. Conversion of EPA to 22:5n-3 (DPA) was particularly high (13.17%) for Elovl1/7-like, and considering the low concentration of DPA in detritus that gammarids naturally feed on, it is tempting to speculate that part of the DPA found in wild caught *E. marinus* (Alberts-Hubatsch *et al*., 2019) derives from biosynthesis.

Beyond its role in LC-PUFA biosynthesis, the gammarid Elovl4 can also participate in the biosynthesis of the so-called very long-chain (>C_{24}) PUFAs (VLC-PUFAs). Our functional characterisation assays showed that transgenic yeast expressing the *E. marinus elovl4* were able to produce 26:5n-3 when 22:5n-3 was supplied as a substrate. Such elongation capacity was found to be unique among the three gammarid elongases characterised in the present study, since neither the *E. marinus* Elovl6 nor Elovl1/7-like were able to synthesise VLC-PUFAs. These results are

consistent with those reported previously for the crab *P. trituberculatus* Elovl4 (Sun et al., 2020b) and illustrate putative roles of crustacean Elovl4 in the biosynthesis of VLC-PUFAs as occurs for vertebrates (Jakobsson *et al*., 2006; Guillou *et al*., 2010; Monroig *et al*., 2018; Deák *et al*., 2019), as well as other invertebrates such as molluscs (Liu *et al*., 2014; Monroig *et al*., 2017; Ran *et al*., 2019). Vertebrate Elovl4 is widely expressed in the central nervous system and photoreceptors of the retina (Deák *et al*., 2019) and, in fish, pineal gland (Monroig *et al*., 2010), and hence it is believed to play pivotal roles in brain functioning and photoreception by providing VLC-PUFAs that guarantee normal function. Analysis of VLC-PUFAs from natural samples is technically challenging (Agbaga *et al*., 2010; Serrano *et al*., 2020) and it can be only accurately performed on lipid samples prepared from specific polar lipid fractions collected from key tissues such as the central nervous system or retina. For that reason, it was not possible to clarify whether the ability of the gammarid Elovl4 for the biosynthesis of 26:5n-3 shown here occurs in vivo.

In conclusion, the research described in this work demonstrates that gammarids possess at least three distinct *elovl* genes, namely *elovl4*, *elovl6* and *elovl1/7*-like, with putative roles in the biosynthesis of LC-PUFAs. Molecular and functional characterisation of the set of *elovl* sequences from the marine gammarid *E. marinus* revealed that gammarids' Elovl4 and Elovl1/7-like are PUFA elongases with affinity towards PUFA substrates ranging from C_{18} to C_{22}, and account, by themselves, for all the elongation reactions required for LC-PUFA biosynthesis from C_{18} biosynthetic precursors. On the contrary, the gammarid Elovl6 sequence contained characteristics typically found in non-PUFA elongases which, along with its elongase capacity being restricted to C_{18} PUFA substrates, indicated that this enzyme does not play major roles in LC-PUFA biosynthesis in gammarids. Elovl4 was the sole Elovl found in gammarids with the ability to produce PUFAs of up to 26 carbons. Overall, the present study provides insight into the endogenous machinery enabling gammarids to bioconvert dietary fatty acids into high nutritional value LC-PUFAs.

2.4. Materials and methods

2.4.1. Molecular cloning of full-length cDNAs of three Elovls from *E. marinus*

Several *E. marinus* individuals collected from the wild in the intertidal zone in Trondheim, Norway, were preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and sent to the facilities of the Instituto de Acuicultura Torre de la Sal, Spain, for further analysis. Total RNA was extracted from one single whole individual using the Maxwell® instrument and the Maxwell® 16 LEV simplyRNA Tissue Kit

(Promega, Madison, WI, USA) following the manufacturer's instructions. First strand complementary DNA (cDNA) was synthesised from 2 µg of total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega) following the manufacturer's instructions.

The strategy to retrieve the *elovl* sequences from *E. marinus* was established by considering that this species possesses the same *elovl* gene complement as *Hyalella azteca*, a closely related amphipod species with a reliable genome annotation (www.hgsc.bcm. edu/arthropods/hyalella-azteca-genome-project (accessed on 10 September 2019); www. ncbi.nlm.nih.gov/bioproject/243935 (accessed on 10 September 2019)). Searches for *elovl* homologues within the *H. azteca* genome resulted in the identification of four sequences: 1) gb|XM_018163314.1|, *H. azteca* elongation of very long-chain fatty acids protein 4-like; 2) gb|XM_018155086.1|, *H. azteca* elongation of very long-chain fatty acids protein 6-like; 3) gb|XM_018168638.1|, *H. azteca* elongation of very long-chain fatty acids protein 7-like; and 4) gb|XM_018164888.1|, *H. azteca* elongation of very long-chain fatty acids protein AAEL008004-like. Preliminary analysis of the sequences suggested that sequence 3) did not translate to any potentially functional putative elongases in *E. marinus*, nor in any other gammarid. Therefore, only sequences 1) "*elovl4"*; 2) "*elovl6"*; and 4), which we hereafter refer to as "*elovl1/7*-like", were used for further analyses. The *H. azteca elovl*like sequences were used as queries to run BLAST searches for homologous sequences within the Transcriptomic Shotgun Assembly (TSA) database of the *Gammarus* BioProject (PRJNA497972) (accessed on 10 September 2019) as follows. First, *H. azteca elovl4* (gb|XM_018163314.1|) was used to search homologous sequences from gammarid species available at NCBI (www.ncbi.nlm.nih.gov (accessed on 10 September 2019)), which included several gammarid species such as *Echinogammarus berilloni* (GHCU01154015.1), *Gammarus fossarum* (GHDC01027946.1) and *Gammarus pulex* (GHCS01223739.1). Alignment of these sequences (Geneious Prime Software, Version 2019.0.3, www.geneious.com (accessed on 10 September 2019)) (Kearse *et al*., 2012) enabled the design of the degenerate primers EM_ELO4_F1 and EM_ELO4_R2 on conserved regions (Table 2.2). A polymerase chain reaction (PCR) using a high-fidelity polymerase (Phusion Green HighFidelity DNA Polymerase, Thermo Fisher Scientific) was performed in order to amplify the first DNA fragment of the *E. marinus* putative *elovl4*. Primer sequences and PCR conditions are given in Table 2.2. A PCR product of the expected size (~350 bp) was obtained and then purified on a 1% (*w*/*v*) agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega). The identity of the peak was confirmed by DNA sequencing (DNA Sequencing Service, IBMCP-UPV, Valencia, Spain). Subsequently, gene-specific primers based on the first fragment sequence of the *E. marinus* putative *elovl4* were designed in order to obtain the full-length open

reading frame (ORF) sequence by rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE Kit, Thermo Fisher Scientific). A two-round (nested) PCR using the *Taq* Polymerase GoTaq®G2 Green Master Mix (Promega) was performed. Briefly, the first round of the 3′ RACE PCR was run with the gene-specific primer EM_ELOVL4_F3 (Table 2.2) and the primer 3′ RACE Outer (provided by the kit), and *E. marinus* 3′RACE cDNA as a template. The second-round PCR was performed using the gene-specific primer EM_ELOVL4_F5 (Table 2.2) and the primer 3′RACE Inner (provided by the kit), and the first-round PCR product as a template. A 3′ RACE PCR product was obtained, which was subsequently purified and sequenced as above.

In order to obtain the 5′ end of the *E. marinus elovl4* ORF, a BLASTn (Megablast) was performed using the partial *elovl4* cDNA generated previously as a query against the TSA database of the *Gammarus* BioProject. We identified a high identity sequence from *Marinogammarus marinus* (GHCW01145927.1), a former species name for *E. marinus* (Horton *et al*., 2021). This sequence (GHCW01145927.1), containing part of the 5′ untranslated region (5′UTR) and the 5′ end of the ORF, aligned with the 3′ end of the partial first fragment *elovl4* sequence obtained in previous steps. Gene-specific primers designed in the 5′UTR (EM_ELOVL4_ORF_U5F) and the 3′UTR (EM_ELOVL4_ORF_U3R) were used to obtain a 1246 bp product by running a PCR (Phusion Green High-Fidelity DNA Polymerase), which encompassed the ORF of the putative *E. marinus elovl4*. The PCR product was purified and sequenced as described above. The ORF sequence was predicted using the NCBI ORF Finder tool (www.ncbi.nlm.nih.gov/orffinder, accessed on 17 September 2019).

Table 2.2. Primer sets and corresponding PCR conditions used in the cloning of the *E. marinus elovl4*, *elovl6* and *elovl1/7*-like genes.

Gene	Aim	Primer name	Primer sequence	Cycles	Tm	Extension
ELOVL4	1st fragment generation	EM_ELOVL4_ F1	TCTACAACCTTGCTGTCATG	35	55 ° C	72 ° C (1 min)
		EM_ELOVL4_ R2	TGCACAAAGCTGTTCATCAT			
	3'RACE PCR	3'Outer_Primer	GCGAGCACAGAATTAATACGACTCACTATAGGT 12	35	55 ° C	72 ° C (75 s)
		3'Inner_Primer	CGCGGATCCGAATTAATACGACTCACTATAGGT 12			
		EM_ELOVL4_F3	CACGTGTATCACCACTCGAC			
		EM_ELOVL4_F4	GGATTGGAGTCAAGTTTGTGG			
		EM_ELOVL4_F5	CCTGGCGGCAATGATGAACA			
	Full ORF	EM_ELOVL4_ORF_U5F	ATGAATTTAGTGAACAACAA	35	62° C (10 cycles) 58° C (25 cycles)	72 ° C (1 min)
		EM_ELOVL4_ORF_U3R	CAAGATGCCTGAACTCCCGGT			
	Functional characterisation	EM_FW_ELOVL4_HindIII	CCCAAGCTTACAATGGCTGCCTCTGTT	35	62 ° C (10 cycles) 58 ° C (25 cycles)	72 ° C (45 s)
		EM_RV_ELOVL4_XbaI	CCGTCTAGACTACATGTCCTTTCGAGG			
ELOVL6	1st fragment generation	EM_ELOVL6_ F1	GGCTTCTGGAACTGGATGTT	35	55° C	72 ° C (1 min)
		EM_ELOVL6_ R2	TTCATGTAGGCACGACGGAA			
	Full ORF	EM_ELOVL6_ORF_U5F	CTTTACCACGTTTTACTGGG	35	62 ° C (10 cycles) 58 ° C (25 cycles)	72 ° C (1 min)
		EM_ELOVL6_ORF_U3R	ACTGGTAGTTTTGTATGCAT			
	Functional characterisation	EM_FW_ELOVL_HindIII	CCCAAGCTTACGATGGCCCTCTCGGAC	35	62 ° C (10 cycles) 58 ° C (25 cycles)	72 ° C (45 s)
		EM_RV_ELOVL_XbaI	CCGTCTAGATTAATCGAACTTCCCTCCCT			
ELOVL1/7-LIKE	1st fragment generation	EM_ELOVL_ F1	GTCATCCACCACGGATGCATG	35	55 ° C	72 ° C (1 min)
		EM_ELOVL_ R1	GCCTTCACGTAGAAGTTGGAG			
	Full ORF	EM_ELOVL_ORF_U5F	AAAAACGTGTTCTCGGCCAG	35	62 ° C (10 cycles) 58 ° C (25 cycles)	72 ° C (1 min)
		EM_ELOVL_ORF_U3R	GAGGCTTAACTAAAACGAAC			
	Functional characterisation	EM_FW_ELOVL_HindIII	CCCAAGCTTAAGATGGCGGGTACAGCA	35	62 ° C (10 cycles) 58 ° C (25 cycles)	72 ° C (45 s)
		EM_RV_ELOVL_XbaI	CCGTCTAGATCAGTCGTCCTTCCGAGG			

For the cloning of the *E. marinus elovl6* and *elovl1/7*-like ORF, we identified contig assemblies containing full-length sequences of the ORF by searching within the *M. marinus* (or *E. marinus*) BioSample (SAMN10259948). Briefly, the *H. azteca* XM_018155086.1 (*elovl6*) and XM_018164888.1 (*elovl1/7*-like) sequences were translated to their corresponding protein sequences and used as queries for TBLASTn searches against the *E. marinus* BioSample (SAMN10259948). These searches allowed the identification of the highly homologous sequences GHCW01079894.1 and GHCW01079198.1, containing, respectively, the putative *elovl6* and *elovl1/7*-like from *E. marinus*. Due to the large size of these sequences (~5.7 to 6.5 Kb), a preliminary analysis was performed in order to predict the ORF sequences for the putative elongases (NCBI ORF Finder). The predicted proteins were subsequently analysed using the Pfam online tool (https://pfam.xfam.org/, accessed on 15 October 2019) and those containing the complete "ELO" domain (pfam 01151) were selected as putative Elovls and considered for further analysis. Primer pairs for each target gene (*elovl6* or *elovl1/7*-like) were designed in the putative 5' and 3'UTR (Table 2.2), and subsequently used for PCR amplification as described previously to obtain PCR products of 1405 bp for *elovl6*, and 1233 bp for the *elovl1/7*-like.

2.4.2. Sequence and phylogenetic analysis of the *E. marinus* elongases

The putative aa sequences of the three newly cloned *elovl* from *E. marinus* were predicted using the ORF Finder tool available from NCBI. The transmembrane regions and protein domains of the *E. marinus* Elovl were predicted using the

TMHMM online tool (www.cbs.dtu.dk/services/TMHMM/, accessed on 18 October 2019). Moreover, the subcellular location of the newly cloned elongases was predicted using the DeepLoc 1.0 online tool (www.cbs.dtu.dk/services/DeepLoc/, accessed on 29 March 2021). A phylogenetic analysis comparing the Elovl aa sequences from model vertebrates such as *Danio rerio* and *Mus musculus*, as well as Elovl protein sequences from crustacean species such as Elovl4 from the swimming crab *P. trituberculatus* (QBO95487.1), and Elovl4 (QBX90561.1), Elovl6 (QEV88898.1) and Elovl7 (AWM30548.1) from the orange mud crab *S. olivacea*, was performed. In addition, several putative Elovl protein sequences from other crustacean species, including gammarids from the genus *Gammarus* and other amphipods, were retrieved and also included in the phylogenetic analysis. Briefly, 1) orthologues for the *E. marinus* Elovl sequences were retrieved by tblastn (NCBI) within the *Gammarus* TSA database (PRJNA497972), 2) putative Elovl sequences were analysed using the Pfam online tool and the ones containing the "ELO" domain (pfam 01151) were selected as candidates to be included in the phylogenetic analysis. The phylogenetic tree and all the corresponding analyses were performed using the CIPRES platform (Miller *et al*., 2010) (https://www. phylo.org/, accessed on 29 March 2021). Briefly, first, a MAFFT alignment and subsequent trimming (TrimAl) were performed. Then, a model test was run in order to select the best evolutionary model for aa substitution. Finally, randomized axelerated maximum likelihood (RAxML) was performed in order to build the phylogenetic tree and the LG4X was used as an evolutionary model (Le *et al*., 2012). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations, following the transfer distance bootstrap approach (Lemoine *et al*., 2018; Lutteropp *et al*., 2020).

2.4.3. Functional characterisation of the *E. marinus* Elovl

The ORF sequences of the three *E. marinus elovl* sequences were cloned into the yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA). Briefly, primers containing *Hin*d III (forward) and *Xba*I (reverse) restriction sites were designed for amplification of the whole ORF of the three *E. marinus elovl* genes (Table 2.2). The PCRs were performed using the high-fidelity polymerase Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific), with conditions used for each gene detailed in Table 2.2. Then, PCR products were purified as above and subsequently digested with the corresponding restriction enzymes before being ligated into similarly restricted pYES2. Ligation reactions were transformed into the *E. coli* competent TOP10™ strain (Invitrogen) and positive colonies were grown overnight in Luria–Bertani (LB) broth containing ampicillin (100 µg/mL). Next, plasmids were purified using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, St

Louis, MO, USA) following the manufacturer's instructions prior to DNA sequencing (IBMCP-UPV) in order to confirm the sequence correctness of the inserts.

The plasmid constructs pYES2-Elovl4, pYES2-Elovl6 and pYES2-Elovl (Elovl1/7-like) were transformed into *Saccharomyces cerevisiae* competent cells (strain INVSc1) (Invitrogen). The recombinant yeast were selected on *S. cerevisiae* minimal medium minus uracil (SCMM^{-ura}) agar plates for 3 d at 30 °C. The culture of recombinant yeast was carried out as described in detail by Jin *et al*., (2017). Briefly, one single recombinant colony from each transformation (*E. marinus elovl* pYES2 constructs) was grown in SCMM^{-ura} broth for 2 d at 30 °C to produce a bulk culture with an OD600 of 8–10. Subsequently, an appropriate volume of the yeast bulk cultures was inoculated in 5 mL of SCMM^{-ura} broth contained in individual 150 mL Erlenmeyer flasks to provide an OD600 of 0.4. The recombinant yeast in each Erlenmeyer flasks was grown for 4 h at 30 °C and under constant shaking (250 rpm) until they reached an OD600 of ~1, at which point the transgene expression was induced by supplementing the culture media with galactose at 2% (w/v). Moreover, in order to test the ability of the *E. marinus* Elovl to elongate PUFA substrates, recombinant yeast expressing the *E. marinus elovl4*, *elovl6* and *elovl1/7*-like were supplemented with one of the following PUFA substrates in the growth media: α-linolenic acid (18:3n-3), linoleic acid (18:2n-6), stearidonic acid (18:4n-3), γ-linolenic acid (18:3n-6), eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6), docosapentaenoic acid (22:5n-3), docosatetraenoic acid (22:4n-6) and docosahexaenoic acid (22:6n-3). Final concentrations of the exogenously supplied PUFA substrates were 0.5 mM (C_{18}), 0.75 mM (C_{20}), 1.0 mM (C_{22}) as uptake efficiency decreases with increasing chain length (Lopes-Marques *et al*., 2017). A control consisting of yeast transformed with the empty pYES2 was run exactly as described above for the three *E. marinus* elongases. Except stearidonic acid (18:4n-3), all FA substrates (>98–99% pure) used for the functional characterisation assays were obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA). Yeast culture reagents including galactose, nitrogen base, raffinose, tergitol NP-40, stearidonic acid (>99%) and uracil dropout medium were obtained from Sigma-Aldrich (St. Louis, MO, USA). The yeast cultures were maintained at 30 °C and under constant shaking (250 rpm) for 2 d until yeast was harvested by centrifugation at 1500 g for 2 min. Yeast pellets were washed twice with 5 mL of ddH_2O, homogenised in 6 mL of 2:1 (*v/v*) chloroform:methanol containing 0.01% (*w/v*) butylated hydroxytoluene (BHT, Sigma-Aldrich) as antioxidant and stored at −20 °C for a minimum of 24 h in an oxygen-free atmosphere until further analysis.

2.4.4. Fatty acid analysis

The FA composition of yeast containing either the ORF of the *E. marinus* elongases (i.e., transformed with either pYES2-Elovl4, pYES2-Elovl6 or pYES2-Elovl) or empty

pYES2 (control) was determined using the method described by Monroig *et al*., (2013a). Briefly, total lipids were extracted from the homogenised yeast samples using the Folch method (Folch *et al*., 1957). Subsequently, total lipids were used to prepare fatty acid methyl esters (FAME), which were analysed using gas chromatography coupled with mass spectrometry (GC–MS) (Monroig *et al*., 2010). The elongation of exogenously supplemented PUFA substrates by the *E. marinus* Elovl4, Elovl6 and Elovl1/7-like was calculated by the proportion of substrate FA converted to elongated FA product(s) as [areas of all products with longer chain than substrate/(areas of all products with longer chain than substrate + substrate area)] × 100.

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Biosynthesis of long-chain polyunsaturated fatty acids in marine gammarids: Molecular cloning and functional characterisation of three fatty acyl elongases

3.

Examination of gammarid transcriptomes reveals a widespread occurrence of key metabolic genes from epibiont bdelloid rotifers in freshwater species

Abstract

Previous data revealed the unexpected presence of genes encoding for long-chain polyunsaturated fatty acid (LC-PUFA) biosynthetic enzymes in transcriptomes from freshwater gammarids but not in marine species, even though closely related species were compared. This study aimed to clarify the origin and occurrence of selected LC-PUFA biosynthesis gene markers across all published gammarid transcriptomes. Through systematic searches, we confirmed the widespread occurrence of sequences from seven elongases and desaturases involved in LC-PUFA biosynthesis, in transcriptomes from freshwater gammarids but not marine species, and clarified such occurrence is independent from the gammarid species and geographical origin. The phylogenetic analysis established that the retrieved elongase and desaturase sequences were closely related to bdelloid rotifers, confirming that multiple transcriptomes from freshwater gammarids contain contaminating rotifers' genetic material. Using the *Adineta steineri* genome, we investigated the genomic location and exon-intron organisation of the elongase and desaturase genes, establishing they are all genome-anchored and importantly, identifying instances of horizontal gene transfer. Finally, we provide compelling evidence demonstrating Bdelloidea desaturases and elongases enable these organisms to perform all the reactions for *de novo* biosynthesis of PUFA and, from them, LC-PUFA, an advantageous trait when considering the low abundance of these essential nutrients in freshwater environments.

3.1. Introduction

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), and more specifically, the omega-3 (ω3 or n-3) LC-PUFA eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are of high relevance for animal health and development (Innis, 2007; Swanson *et al*., 2012; Bazinet and Layé, 2014; Tocher, 2015; Závorka *et al*., 2022). Aquatic ecosystems, and particularly marine habitats, are main sources of n-3 LC-PUFA due to the abundance of low trophic organisms with the ability for their endogenous production (biosynthesis) (Twining *et al*., 2021). Microorganisms such as photosynthetic microalgae, heterotrophic protists and bacteria have been historically regarded as primary producers of n-3 LC-PUFA in the ocean (Nichols, 2003; Pereira *et al*., 2003; Khozin-Goldberg *et al*., 2011). However, more recently, certain invertebrates have been also shown to contribute to n-3 LC-PUFA production in both aquatic and terrestrial ecosystems (Kabeya *et al*., 2018; Monroig and Kabeya, 2018; Menzel *et al*., 2019; Monroig *et al*., 2022). Flux of n-3 LC-PUFA from primary producers to upper trophic levels is crucial for marine ecosystems since fish, as all vertebrates, depend upon provision of pre-formed health beneficial n-3 LC-PUFA in their diet (Twining *et al*., 2021). Interest to understand the mechanisms by which living organisms biosynthesise n-3 LC-PUFA has prompted in recent years due, among other reasons, to the decrease in global availability of these essential nutrients predicted to occur because of climate change (Hixson and Arts, 2016; Twining *et al*., 2021; Holm *et al*., 2022).

Microorganisms can biosynthesise n-3 LC-PUFA using either anaerobic or aerobic metabolic pathways, each of them involving different enzymatic components (Jovanovic *et al*., 2021). The anaerobic pathways occur mostly in prokaryotes and some eukaryotic organisms, and are mainly driven by the polyketide synthase (PKS) complex (Metz *et al*., 2001). However, most eukaryotic organisms including animals operate aerobic pathways enabling LC-PUFA biosynthesis through the orchestrated action of three key enzymes, namely methyl-end desaturases (ωx), front-end desaturases (Fed), and elongation of very long-chain fatty acid (Elovl) proteins (Monroig and Kabeya, 2018; Monroig *et al*., 2022). Briefly, the monoene oleic acid (OA, 18:1n-9), biosynthesised from stearic acid (18:0) by a Δ9 desaturase virtually occurring in all living organisms (Castro *et al*., 2011), can be converted into linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), which represent the simplest cases of n-6 and n-3 polyunsaturated fatty acids (PUFA), respectively (Figure 3.1). With some exceptions (Zhou *et al*., 2008; Haritos *et al*., 2012, 2014; Semmelmann *et al*., 2019), the *de novo* biosynthesis of n-6 and n-3 PUFA (i.e., LA and ALA) is carried out by ωx enzymes (Figure 3.1). The ωx responsible for the biosynthesis of LA from

OA are commonly known as Δ12 or ω6 desaturases, whereas those enabling the conversion of LA into ALA are known as Δ15 or ω3 desaturases (Figure 3.1). Interestingly, ω3 desaturases typically recognise a variety of n-6 PUFA as substrates, converting them into n-3 products (Figure 3.1). LA and ALA have no major biological functions themselves but serve as precursors of the physiologically important LC-PUFA by the action of further enzymes including Fed and Elovl (Figure 3.1). Like ωx, Fed introduce double bonds (unsaturations) into the pre-existing fatty acyl chains, but such insertion takes place in the "front end" of the fatty acid acting as substrate, more specifically between an existing double bond and the carboxyl terminus (-COOH) (Sperling *et al*., 2003; Castro *et al*., 2016). Typical activities reported in Fed include Δ4, Δ5, Δ6 and Δ8 (Figure 3.1) (Monroig *et al*., 2022). Besides, Elovl catalyse the condensation reaction in the fatty acid elongation pathway that results in the extension of an existing fatty acid in two carbons (Castro *et al*., 2016; Monroig *et al*., 2022) (Figure 3.1). Both invertebrates and vertebrates possess Fed and Elovl involved in the biosynthesis of LC-PUFA from C_{18} PUFA precursors (Figure 3.1). However, a distinctive trait among animals' LC-PUFA biosynthetic capacity is that invertebrates, but not vertebrates, can have ωx enabling them the *de novo* biosynthesis of PUFA (i.e., LA and ALA) described above (Figure 3.1).

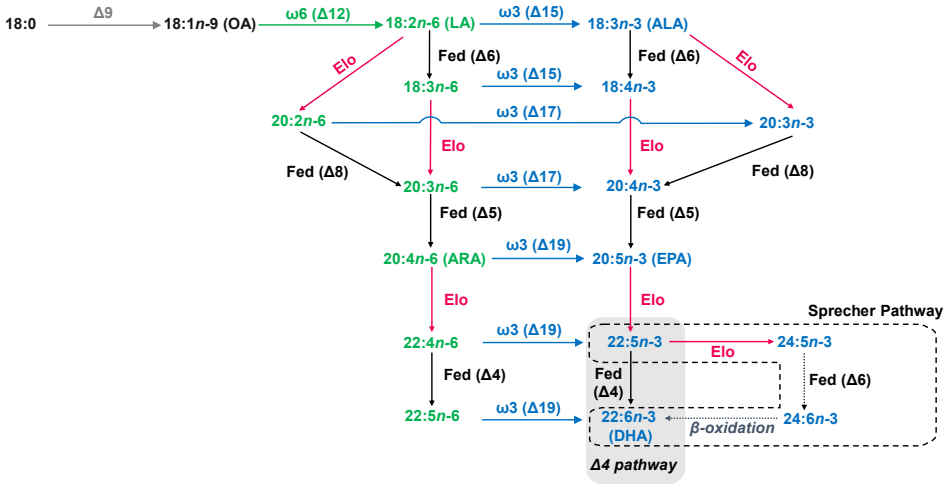


Figure 3.1. General overview of the LC-PUFA biosynthesis pathways in animals. Reactions catalysed by front-end desaturases are marked as "Fed" along with their specific Δy activity (y indicates position of double bond from the -COOH terminus of the fatty acid). Reactions catalysed by methyl-end desaturases are denoted as "ωx" (x indicates position of double bond from the -CH_3 terminus of the fatty acid), and their specific Δy activity. Reactions marked with "Elo" are catalysed by elongation of very long-chain fatty acid (Elovl) proteins. Two distinct pathways enabling DHA biosynthesis are possible, namely the "Δ4 pathway" (grey background) and the "Sprecher pathway" (dashed line).

Evidence supporting the capacity of invertebrates for LC-PUFA biosynthesis had been mostly collected from compositional studies and biochemical assays aiming to determine *in vivo* bioconversions of labelled fatty acid substrates (Schauer and Simpson, 1985; Bell *et al.*, 2007; De Troch *et al.*, 2012; Pairohakul *et al.*, 2013; Helenius *et al.*, 2020; Pérez *et al.*, 2022; Titocci and Fink, 2022). However, the ever-increasing availability of genomic data from multiple invertebrates now offers the opportunity to identify the specific genes encoding LC-PUFA biosynthetic enzymes, thus often enabling to distinguish the invertebrate's enzymatic capacity from that of (micro)organisms that may inhabit their hosts (Jøstensen and Landfald, 1997). Studies aiming to characterise LC-PUFA biosynthetic genes from invertebrates have helped to predict the gene complement and function in certain groups (Kabeya *et al.*, 2018; Monroig *et al.*, 2022; Tan and Zheng, 2022). Crustaceans have been the focus of particular attention due to their central roles in trophic ecology of essential fatty acids in aquatic ecosystems, as well as their commercial interest in aquaculture (Mah et al., 2019; Sun et al., 2020b; Ting et al., 2020, 2022; Kabeya et al., 2021; Ramos-Llorens et al., 2023b). Current evidence suggests that both ωx and Fed enzymes occur

in certain copepods (Kabeya *et al*., 2018, 2021; Boyen *et al*., 2023), but are absent in other crustaceans (Monroig *et al*., 2022). Comparatively to desaturases, the repertoire of Elovl with functions along the LC-PUFA biosynthetic pathways in crustaceans is far more varied, with occurrence of multiple *elovl*-like genes, not only in copepods (Nielsen *et al*., 2019; Kabeya *et al*., 2021; Boyen *et al*., 2023) but also decapods (Lin et al., 2017, 2018a; Sun et al., 2020b; Ting et al., 2020), branchiopods (Ramos-Llorens et al., 2023b) and gammarids (Ribes-Navarro *et al*., 2021). Such an Elovl diversity has been hypothesised to compensate for the apparent absence of Elovl2/5 in crustaceans, a pivotal PUFA Elovl reported in molluscs and amphioxus (Monroig *et al*., 2012; Liu *et al*., 2014; Ran *et al*., 2019). Surprisingly, Ribes-Navarro *et al*. (2021) found putative *elovl2/5* sequences in freshwater gammarids, but not in marine species, an unexpected finding considering they are very closely related species, often from the same genus (e.g., *Gammarus*).

The present study aimed to clarify the origin and occurrence of sequences of LC-PUFA biosynthesising genes, such as *elovl*2/5 and further biosynthetic gene components including Fed and ωx in freshwater gammarids. To do so, we conducted a systematic search of diagnostic motifs characteristic of each three LC-PUFA biosynthetic enzyme families within publicly available transcriptomes from both freshwater and marine gammarids (Gismondi and Thomé, 2016; Naumenko *et al*., 2017; Cogne *et al*., 2019; Drozdova *et al*., 2019; Caputo *et al*., 2020; Neuparth *et al*., 2020a; Collins *et al*., 2021; Lipaeva *et al*., 2021). Our results confirmed that sequences encoding putative Elovl2/5, Fed and ωx are widespread in transcriptomic databases from freshwater gammarids but absent in those from marine species. We further analysed the phylogenetic relationship of the retrieved desaturases and elongases. Strikingly, we unequivocally established that they were closely related to bdelloid rotifers. Using the genome of the freshwater bdelloid rotifer *Adineta steineri* as a proxy-model, we investigated the genomic location and exon-intron organisation of all the desaturase and elongase genes used as diagnostic markers. We establish they are all genome-anchored and clarify some instances of horizontal gene transfer (HGT). Finally, we provide compelling evidence that the Bdelloidea desaturase and elongase sequences encode enzymes enabling all the reaction for *de novo* biosynthesis of PUFA and, from them, LC-PUFA.

3.2. Materials and methods

3.2.1. Retrieval of LC-PUFA biosynthesis genes in gammarid transcriptomes

Sequences from functionally characterised LC-PUFA biosynthetic desaturases and elongases from aquatic invertebrates including annelids, molluscs and arthropods

were used as queries for BLAST searches (*tblastn*) within transcriptomes from a variety of gammarids, including freshwater and marine species (Gismondi and Thomé, 2016; Naumenko *et al*., 2017; Cogne *et al*., 2019; Drozdova *et al*., 2019; Caputo *et al*., 2020; Neuparth *et al*., 2020a; Collins *et al*., 2021; Lipaeva *et al*., 2021). Hits were only selected for further analysis when they contained the full-length open reading frame (ORF) and their predicted protein sequences (https://www.ncbi.nlm.nih.gov/orffinder) contained specific features according to Hashimoto *et al*. (2008). Briefly, Fed had to contain three diagnostic histidine boxes (H-box) "HXXXH", "HXXXHH" (or "HXXHH") and "QXXHH", and a heme binding motif (HPGG) in the cytochrome b_5 domain. Putative desaturase sequences with the third H-box "HXXHH" instead of "QXXHH" were not regarded as Fed based on evidence collected from other crustaceans suggesting these enzymes lack fatty acyl desaturation capacity (Monroig and Kabeya, 2018; Monroig *et al*., 2022). Selection of ωx was made based on the presence of the three H-boxes as "HXXXH", "HXXHH" and "HXXHH", and absence of cytochrome b_5 domain (Hashimoto *et al*., 2008). As for elongases, the deduced amino acid (aa) sequences had to contain the conserved domains "KXXEXXDT", "NXXXHXXMYXYY" and "TXXQXXQ", and a H-box as "HXXHH".

3.2.2. Phylogeny of LC-PUFA biosynthesis genes retrieved from freshwater gammarid transcriptomes

The deduced aa sequences of the *elovl2/5*, *fed* and *ωx* retrieved from freshwater gammarid transcriptomes were used for phylogenetic analysis, along with a wide range of previously characterised genes from vertebrates and invertebrates (Kabeya *et al*., 2018, 2021; Ribes-Navarro *et al*., 2021). Putative desaturase and elongase sequences from Rotifera transcriptomes (PRJNA295486-295489) were also included in the analysis (Eyres *et al*., 2015). Besides, for the Fed tree, several sphingolipid desaturases from crustaceans were used as outgroups. The resulting dataset was aligned with a MAFFT E-INS-i (*--maxiterate 1000 --genafpair*) (Katoh *et al*., 2019). Subsequently, trimming with a gap threshold of 95% was performed for each alignment (trimAl) (Capella-Gutiérrez *et al*., 2009). Finally, next generation version of randomised axelerated maximum likelihood (RAxML-NG) (Kozlov *et al*., 2019) was performed in order to build the phylogenetic tree with LG+I+G4 as the best aa substitution model calculated by Modeltest-NG (Flouri *et al*., 2015; Darriba *et al*., 2020). Confidence in the resulting phylogenetic tree branch topology was measured with all-in-one mode (*--all*). Auto bootstrapping (autoMRE, cutoff: 0.03) was converged after 350 replicates for front-end desaturase tree, 650 replicates for methyl-end desaturase tree and 450 replicates for elongase tree.

3.2.3. Screening gammarid transcriptomes for "contaminant" gene sequences from Rotifera symbionts

Since freshwater gammarids have often been reported to have bdelloid rotifers as epibionts (De Smet and Verolet, 2016; Bojko *et al*., 2017; Bojko and Ovcharenko, 2019), we analysed the occurrence of Rotifera in published transcriptomes from freshwater (*Gammarus pulex*, *Gammarus fossarum*, *Gammarus wautieri* and *Echinogammarus berilloni*) and marine (*Echinogammarus marinus*) wild caught gammarids (Cogne *et al*., 2019). We chose the dataset from Cogne *et al*. (2019), not only because of its species coverage including both freshwater and marine species, but also because it is deposited as sequence read archive (SRA) enabling analysis and reproducibility of the raw sequencing data. Sequences retrieved from the above searches were introduced into a newly developed pipeline for the selection of the mitochondrial gene cytochrome c oxidase subunit 1 (*cox1*) to identify genetic contamination from the phylum Rotifera in the gammarid transcriptomes. Briefly, all the Metazoan complete mitochondrial (mtDNA) genomes available of NCBI (February of 2021) were retrieved and used as a reference to extract mitochondrial reads from the gammarids SRA, using the tool bbmap.sh and reformat.sh from BBMap v.37.77 (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbmap-guide/). The filtered mtDNA reads for each sample were assembled using rnaSPAdes v3.11.1 with default parameters (Bushmanova *et al*., 2019). Assembled contigs were blast searched against the nucleotide database of NCBI (NCBI-nt, Download; 24/08/2021), using the blastn from BLAST+ v. 2.11.0 (Camacho *et al*., 2009), and all contigs with hits with the phylum Rotifera retrieved. The mtDNA gene *cox1* is used as a standardised molecular identification system in most metazoan (Hebert *et al*., 2003), thus one of the most sequenced genes available on NCBI. Therefore, to identify the putative Rotifera species present within each sample, all *cox1* sequences from Rotifera were retrieved from NCBI and used as a reference database for a blast search (blastn) to identify Rotifera *cox1* containing contigs. All hits that resulted in DNA sequences with identity scores higher than 75% were considered as positive for genetic contamination.

3.2.4. Detection of LC-PUFA biosynthesis genes in gammarids collected from the wild and laboratory cultures

In order to validate the occurrence of rotifer-origin genetic material in gammarid transcriptomes, we developed a polymerase chain reaction (PCR) assay to determine the presence of LC-PUFA biosynthetic gene sequences in *G. pulex* adults collected from the wild and laboratory cultures. The herein referred to as "wild-captured" *G. pulex* sample consisted of one sole whole individual collected from a small freshwater

stream in Northern Germany (53°29'52"N, 8°46'08"E), which was preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) until further analysis. The "laboratory cultured" *G. pulex* sample consisted of one sole adult of an apparent same size as the wild-captured *G. pulex* sample, and derived from the second generation resulting from maintaining wild-captured *G. pulex* from the above location. The laboratory cultured *G. pulex* were maintained in fresh water at 18 °C with coarse gravel at substrate and fed a mixed diet containing pellets made of dried carrot leaves, sugar beet and brewer grains. Furthermore, a second adult specimen of laboratory cultured *G. pulex* was dissected for collecting a hepatopancreas sample. Total RNA was extracted the three *G. pulex* samples (wild-captured, laboratory cultured and hepatopancreas) using the Illustra™ RNAspin Mini kit (Cytiva, Marlborough, MA, USA) following the manufacturer's instructions. The extracted RNA was treated with DNase I to avoid potential genomic DNA contamination. Subsequently, first strand complementary DNA (cDNA) was synthesised from 2 µg of total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega, Madison, WI, USA) and stored at -20 °C until further analysis. Using the three cDNA samples (wild-captured whole individual, laboratory cultured whole individual, laboratory culture hepatopancreas) as templates, PCR (GoTaq® G2 Green Master Mix, Promega) were run using gene-specific primers targeting the sequences of *elovl2/5*, four front-end desaturases (*fed1-4*) and two methyl-end desaturases (*ωx1-2*) retrieved from *G. pulex* transcriptomes (Table S3.1). Moreover, a PCR with degenerate primers targeting a conserved region of the housekeeping gene *β-actin* (*actb*) for *Rotaria* species was also run to check for possible rotifer genetic material within the above three template cDNA samples (Table S3.1). Specific primers targeting the *G. pulex actb* were used for PCR as positive controls. Primer sequences and PCR conditions are indicated in Tabla S5.1. The resulting PCR products were purified on a 1% (w/v) agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega) and their identity confirmed by DNA sequencing (DNA Sequencing Service, IBMCP-UPV, Valencia, Spain) (Figure S3.1).

3.2.5. Sequence identity of the LC-PUFA biosynthesis genes among gammarid transcriptomes

The intra- and inter-specific diversity of the LC-PUFA biosynthesis genes within gammarid transcriptomes was analysed by estimating the nucleotide (nt) identity scores (https://www.ebi.ac.uk/Tools/msa/clustalo/) as follows. First, we compared the elongase and desaturase nt sequences retrieved from transcriptomes of *Gammarus* species sampled at two geographical sites (intra-specific identity). The publicly available transcriptomes (Table 3.1) enabled us to estimate the intra-specific identity

for 1) *G. pulex*, comparing sequences occurring in transcriptomes built from samples collected in Germany (53°29'52"N 8°46'08"E) and France (45°57'21"N, 5°15'44"E) (BioProject ID: PRJNA497972), and 2) *G. fossarum*, comparing sequences occurring in transcriptomes built from samples collected at two distinct French sites (47°53'31"N, 6°58'53"E, and 45°57'21"N, 5°15'44"E) (BioProject ID: PRJNA497972). Second, we determined the inter-specific identity of the elongase and desaturase nt sequences retrieved from transcriptomes of two *Gammarus* species (*G. pulex* and *G. fossarum*) sampled at the same site, more specifically the Pollon River, Saint-Maurice de Rémens, France (45°57'21"N, 5°15'44"E) (BioProject ID: PRJNA497972). Finally, we analysed the sequence nt identity between two different gammarid species (*G. pulex* vs *G. lacustris*) sampled at very geographically distant locations, namely Germany (53°29'52"N, 8°46'08"E) and Russia (55°55'14.39"N, 105°4'19.48"E), respectively. Further details of the location and sample type can be found in Table 3.1.

Examination of gammarid transcriptomes reveals a widespread occurrence of key metabolic genes from epibiont bdelloid rotifers in freshwater species

Table 3.1. Transcriptomic databases from gammarids mined for occurrence of key metabolic LC-PUFA genes (*elovl2/5*, *fed* and *ωx*). Details on species, geographical location of the sampling site and genes found (number in brackets) are indicated. All databases were built from analysis of specimens collected from the wild except for *G. locusta* (PRJNA600472; laboratory culture), and correspond to whole individuals except for *G. pulex* hepatopancreas (PRJEB13055) and *G. fossarum* "internal tissues" (PRJNA556212).

SPECIES NAME	BIOPROJECT ID	LOCATION	GENES FOUND
Gammarus pulex	PRJNA497972 (Cogne et al., 2019)	Pollon River, Saint-Maurice de Rémens, France (45° 57′21″N, 5° 15′44″E)	*elovl*2/5, *fed* (4), *ωx* (2)
Gammarus pulex	PRJEB13055 (Gismondi and Thomé, 2016)	Blanc-Gravier River, Liege, Belgium (50° 34′60″N, 5° 34′60″E)	None
Gammarus pulex	PRJEB13055 (Gismondi and Thomé, 2016)	Vesdre River, Vaux-sous-Chevremont, Belgium (50° 36′00″N, 5° 37′58″E)	None
Gammarus fossarum	PRJNA497972 (Cogne et al., 2019)	Pollon River, Saint-Maurice de Rémens, France (45° 57′21″N, 5° 15′44″E)	*elovl*2/5, *fed* (4), *ωx* (2)
Gammarus fossarum	PRJNA497972 (Cogne et al., 2019)	Seebach River, Fellering, France (47° 53′31″N, 6° 58′53″E)	*elovl*2/5, *fed* (4), *ωx* (2)
Gammarus fossarum	PRJNA556212 (Caputo et al., 2020)	Elgg, Switzerland (47° 30′04.23″N, 8° 51′09.40″ E)	*elovl*2/5, *fed* (2), *ωx* (1)
Gammarus wautieri	PRJNA497972 (Cogne et al., 2019)	Galaveyson River, Le Grand Serre, France (45° 16′27″N, 5° 07′08″E)	*elovl*2/5, *fed* (4), *ωx* (2)
Echinogammarus berilloni	PRJNA497972 (Cogne et al., 2019)	Saucats River, Saucats, France (44° 39′34″N, 0° 34′25″W)	*elovl*2/5, *fed* (4), *ωx* (2)
Gammarus lacustris	PRJNA660769 (Lipaeva et al., 2021)	Lake nº14, Baikal Lake, Irkutsk, Russia (51° 55′14.39″N, 105° 4′19.48″E)	*elovl*2/5, *fed* (2), *ωx* (2)
Gammarus lacustris	PRJNA505233 (Drozdova et al., 2019)	Lake nº14, Baikal Lake, Irkutsk, Russia (51° 55′14.39″N, 105° 4′19.48″E)	*elovl*2/5, *fed* (2), *ωx* (2)
Hyalellopsis costata	PRJNA32160 (Naumenko et al., 2017)	Baikal, Bolshie Koty, across from ISU Biostation (51.90 N, 105.07 E)	*elovl*2/5, *fed* (2), *ωx* (2)
Echinogammarus marinus	PRJNA497972 (Cogne et al., 2019)	Sea Coast, Portsmouth, UK (50° 47′41″N, 1° 01′50″W)	None
Echinogammarus marinus	PRJEB34316 (Collins et al., 2021)	Intertidal mudflat, Saltash, UK (50° 47′41″N, 1° 01′50″W)	None
Gammarus locusta	PRJNA600472 (Neuparth et al., 2020)	South margin of the Sado estuary, Portugal (38 ° 27 ´ N, 08 ° 43 ´ W)	None

3.2.6. Genome location and exon-intron structure analysis of LC-PUFA biosynthesis genes in bdelloid rotifers

Expanding on the phylogeny results suggesting that sequences for LC-PUFA biosynthesis genes found in freshwater gammarid transcriptomes belong to Bdelloid rotifers, we identified the corresponding LC-PUFA biosynthesis gene sequences from *A. steineri* and studied their genome location (WGS Project: CAJNOJ01). In order to gain insight into the evolutionary origin of the LC-PUFA biosynthesis genes, we determined the exon-intron structure using the Exon-Intron Graphic Maker webpage (http://wormweb.org/exonintron, accessed 29th March, 2023). Due to the exon-intron organisations of the *fed3* and *fed4* genes, as well as their unexpected location in the phylogenetic tree, we examined their genomic neighbourhood of along the *A. steineri* genome in order to clarify whether these genes were not a spurious artefact of the genome assembly. We further addressed the evolutionary origin of the flanking genes with respect to the taxonomic distribution with blast (animal *vs* non-animal presence).

3.2.7. Functional characterisation of LC-PUFA biosynthetic elongases and desaturases in yeast

Elongase and desaturase genes isolated from wild-captured *G. pulex* were functionally characterised by expressing their ORF in brewer's yeast *Saccharomyces cerevisiae*. Primers containing restriction sites for further cloning into the yeast expression vector pYES2 (Thermo Fisher Scientific) were used to amplify the ORF sequences using the high-fidelity polymerase Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific) (Table S3.1). The PCR template consisted of cDNA prepared from total RNA of wild-captured *G. pulex* as described above. Each of the PCR products were digested with the corresponding restriction enzymes (Table S3.1) and further ligated into a similarly restricted pYES2 plasmid using T4 DNA ligase (Promega). The pYES2 constructs containing the ORF of the genes of interest (pYES2-Elovl25, pYES2-Fed1, pYES2-Fed2, pYES2Fed3, pYES2-Fed4, pYES2-ωx1 and pYES2-ωx2) were individually transformed into INV*Sc*1 *S. cerevisiae* competent cells (Thermo Fisher Scientific) using the *S.c.* EasyComp™ yeast transformation kit (Thermo Fisher Scientific). Selection and growth of recombinant yeast were carried out as described by Ribes-Navarro *et al*. (2021). To test the capacity of the elongase and desaturase enzymes, recombinant yeast expressing one sole LC-PUFA biosynthesis gene were supplemented with one of the following potential PUFA substrates selected according to LC-PUFA biosynthetic pathways (Figure 3.1). For Elovl2/5, the assayed PUFA substrates included 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3, 22:4n-6 and 22:6n-3, whereas the exogenously PUFA supplied for transgenic yeast

expressing front-end desaturases were 18:3n-3 and 18:2n-6 (Δ6 desaturation), 20:3n-3 and 20:2n-6 (Δ8 desaturation), 20:4n-3 and 20:3n-6 (Δ5 desaturation), and 22:5n-3 and 22:4n-6 (Δ4 desaturation). For methyl-end desaturases, the assayed PUFA included 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. Each PUFA substrate was supplemented as sodium salt at a final concentration of 0.5 mM (C_{18}), 0.75 mM (C_{20}), 1.0 mM (C_{22}) in order to compensate for the decreased uptake efficiency as chain length increases. All fatty acid substrates (>98-99% pure) used for the functional characterisation assays were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Immediately after addition of the PUFA substrates, further supplementation of galactose (2 %, w/v) induced gene expression. Along their activity towards exogenously supplied PUFA substrates, we tested the ability of the two ωx to desaturate yeast endogenous fatty acid as previously reported (Kabeya *et al*., 2020, 2021). For that purpose, transgenic yeast transformed with pYES2-ωx1 or pYES2-ωx2, as well as yeast transformed with the empty pYES2 vector (control), were grown in the absence of exogenously added PUFA substrates. Gene expression was induced with galactose as described above. After induction, yeast cultures were maintained at 30 °C and under constant shaking (250 rpm) for 2 d until yeast were harvested by centrifugation at 1500 g for 2 min. Yeast pellets were washed twice with 5 mL of ddH_2O, homogenised in 6 mL of 2:1 (v/v) chloroform:methanol containing 0.01% (w/v) butylated hydroxytoluene (BHT, Sigma-Aldrich) as antioxidant and stored at -20 °C for a minimum of 24 h in an oxygen-free atmosphere until further analysis. Yeast culture reagents including galactose, yeast nitrogen base without aa, raffinose, Tergitol™ NP-40, and uracil dropout medium were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2.8. Fatty acid analysis

The fatty acid composition of yeast collected from functional assays was determined using the method described by Monroig *et al*. (2013). Briefly, extracts of total lipids from yeast (Folch *et al*., 1957) were used to prepare fatty acid methyl esters (FAME) that were analysed using gas chromatography coupled with a flame ionisation detector (FID) (GC-FID) (Ribes-Navarro *et al*., 2022). The GC-FID was equipped with a fused silica 30 m × 0.25 mm open tubular column (TRACE™ TR-WAX, film thickness: 0.25 µm, Thermo Fisher Scientific) fitted with an on-column injection system and using helium as a carrier gas. Conversions of the LC-PUFA biosynthesising enzymes towards the exogenously supplied PUFA substrates were calculated according to the formula [all product areas / (all product areas + substrate area)] x 100. Where necessary, further confirmation of FA products was carried out using an Agilent 6850 GC equipped with a mass spectrometry (MS) detector (5975 Series) and a 30 m ×

0.25 mm open tubular column (DB5-MS, film thickness 0.25 µm, Agilent, Santa Clara, CA, USA) and comparing the spectra against those from the NIST library (MS Search v.2.0).

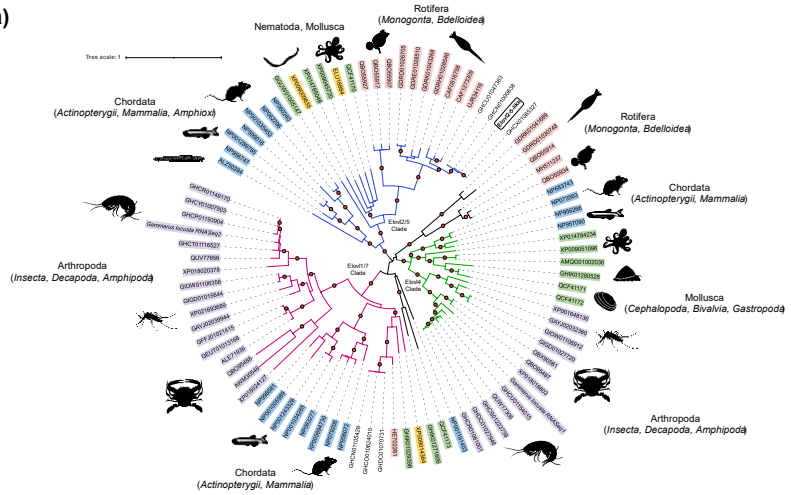
3.3. Results

3.3.1. Phylogeny of the LC-PUFA biosynthesis gene sequences retrieved from gammarid transcriptomes

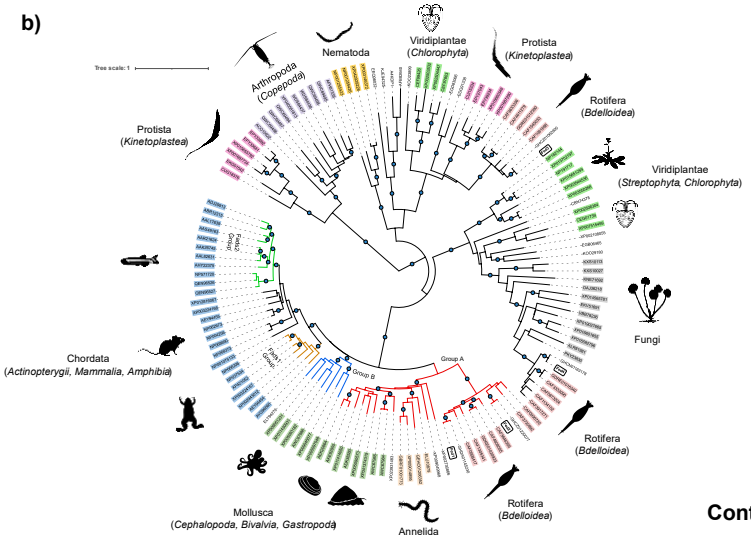
Full-length ORF sequences of putative PUFA elongases (*elovl2/5*), front-end desaturases, and methyl-end desaturases could be only found in transcriptomes from freshwater gammarids but not marine species (Table 3.1). Interestingly, no sequences encoding the above diagnostic LC-PUFA biosynthetic genes were found in the freshwater gammarid *G. pulex* transcriptome built using RNA extracted from hepatopancreas instead of whole individuals (PRJEB13055, Table 3.1). Irrespectively of the enzyme type, the phylogenetic analyses showed that the aa sequences retrieved from freshwater gammarid transcriptomes clustered closely (bootstrap > 90%) with their orthologues from rotifers, specifically from the Bdelloidea class (Figure 3.2a; Figure S3.1). First, *elovl2/5* from the freshwater gammarids *G. pulex*, *G. fossarum*, *G. wautieri* and *E. berilloni* grouped together with putative *elovl2/5* from several *Rotaria* species, and more distantly with orthologues from other invertebrate groups including the functionally characterised Elovl2/5 from molluscs and amphioxus (Figure 3.2a; Figure S3.1). Regarding front-end desaturases, the phylogenetic tree showed three well-differentiated clusters grouping all the gammarid sequences in two of these three clusters (Figure 3.2b, Figure S3.2). The first cluster included two distinct sequences (Fed1 and Fed2) that could be consistently identified in transcriptomes from the freshwater gammarids analysed herein (Figure 3.2b, Figure S3.2). These sequences clustered closely with front-end desaturase sequences belonging to the invertebrate Group A desaturases (Surm *et al*., 2015, 2018) and, more distantly, vertebrate sequences (Figure 3.2b, Figure S3.2). Moreover, the first cluster included gammarid's transcriptomic sequences termed as "Fed4", which grouped along fatty acyl desaturases from several Fungi (Figure 3.2b, Figure S3.2). The second cluster of front-end desaturases retrieved from gammarid transcriptomes (termed herein as "Fed3") located closely to non-metazoan sequences from e.g., *Kinetoplastea* (Figure 3.2b, Figure S3.2). Noteworthy, all Fed sequences retrieved from gammarid transcriptomes clustered closely to their rotifer orthologues. For methyl-end desaturases, all putative sequences retrieved from freshwater gammarids transcriptomes clustered within the so-called "Clade 3" (Kabeya *et al*., 2018), along with methyl-end desaturases from Mollusca, Annelida,

Arthropoda and Rotifera (Figure 3.2c; Figure S3.3). Two distinct sequences termed herein as "*ωx1*" and "*ωx2*" were identified in each gammarid transcriptome from which methyl-end desaturases were retrieved (Table 3.1). Importantly, the *ωx* sequences retrieved from freshwater gammarid transcriptomes grouped more closely to Rotifera sequences rather than other invertebrates including Arthropoda (Figure 3.2c; Figure S3.3).

a)



b)



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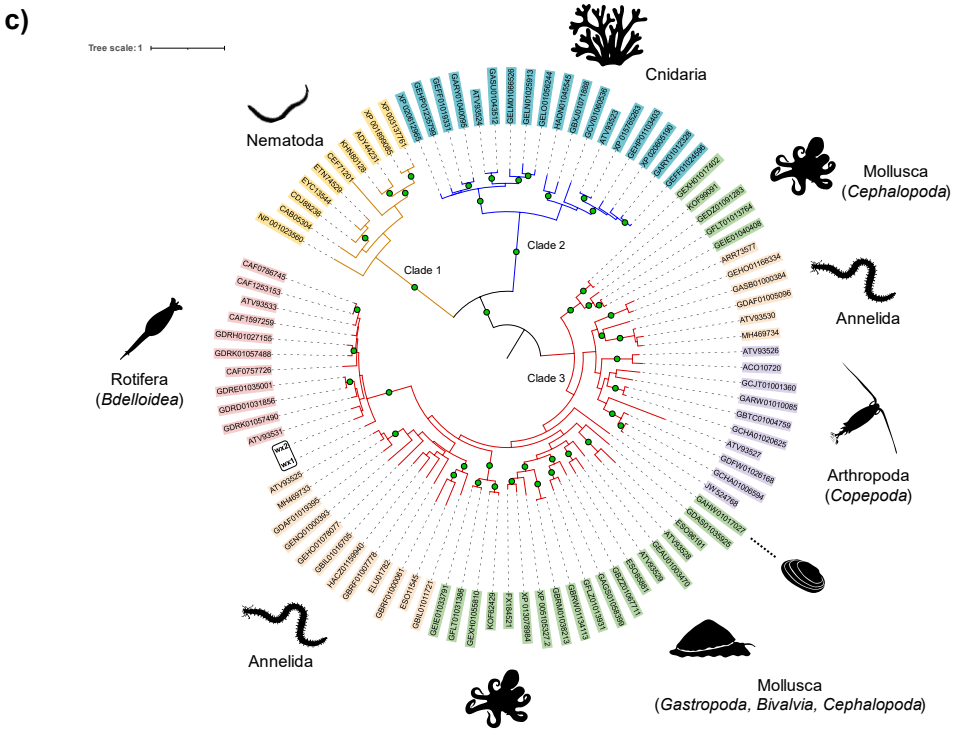

Figure 3.2. Condensed maximum likelihood phylogenetic trees of (a) elongases, (b) front-end desaturases, and (c) methyl-end desaturases. The protein evolutionary model was LG+I+G4. The tree was constructed using the maximum likelihood method by next generation version of randomised axelerated maximum likelihood (RAxML-NG). Confidence in the resulting phylogenetic tree branch topology was measured by auto-bootstrapping mode. Coloured dots on the nodes represent supporting values of 80% or above for RAxML. For each sequence, the accession numbers are given according to the information contained in the corresponding NCBI databases. Sequences subject to functional characterisation in this study are rounded and highlighted in bold. Raw trees are available in the Supplementary Data.

3.3.2. Occurrence of Rotifera mitochondrial genes in gammarid transcriptomes

Sequence homology searches of the *cox1* gene showed a very high identity with bdelloid rotifer *cox1* sequences (99.61% *Rotaria magnacalcarata*; 98.78% *Rotaria socialis*) within *G. pulex* RNASeq data (SRR8089720) (Table S3.2). Additionally, several

other hits with a lower percentage of identity (79.4%-86.6%) with other bdelloid rotifers were also identified. On the other hand, sequence homology searches of *cox1* within RNASeq from marine gammarids did not show any relevant identity with rotifer *cox1* sequences (Table S3.2).

3.3.3. Sequence identity of *elovl2/5*, *fed* and *ωx* retrieved from gammarid transcriptomes

The identity scores of the nt sequences from the LC-PUFA biosynthesis genes retrieved from transcriptomes of *Gammarus* species sampled at two geographical sites was determined to estimate the intra-specific diversity. The results showed that the identity scores of each homologous gene between *G. pulex* from Germany and France were all ≥ 96.9% (Table 3.2). Similarly, comparison of sequences of each gene between *G. fossarum* from two distinct sites in France showed identity scores ranged between 96.9 to 99.5 % (Table 3.2). Subsequently, we analysed the inter-specific identity of sequences encoding LC-PUFA biosynthetic enzymes from *G. fossarum* and *G. pulex* sampled at the same site in France (Pollon River, Saint-Maurice-de-Rémens). The results showed identity scores ranging between 98.7 to 99.9 % (Table 3.2). Finally, the results of the sequence comparison between *G. pulex* and *G. lacustris* sampled at very geographically distant locations (Germany and Russia, respectively) showed identity scores >98% (Table 3.2).

Table 3.2. Intra- and inter-specific identity of the nucleotide sequences of selected LC-PUFA biosynthetic genes retrieved from transcriptomes of *Gammarus* spp. a) Intra-specific comparisons made between sequences retrieved from *G. pulex* sampled in France (45°57′21″N, 5°15′44″E) and Germany (53°29'52"N, 8°46'08"E), and *G. fossarum* sampled at two French sites (47°53′31″N, 6°58′53″E and 45°57′21″N, 5°15′44″E). **b)** Inter-specific comparisons made between *G. pulex* and *G. fossarum* sampled at the same geographical location (Pollon River, Saint-Maurice de Rémens, France; 45°57′21″N, 5°15′44″E). **c)** Inter-specific comparisons made between *G. pulex* sampled in Germany (53°29'52"N, 8°46'08"E) and *G. lacustris* sampled in Russia (Lake nº14, Baikal Lake, Irkutsk, 51°55′14.39N, 105°4′19.48"E). * No gene found in the *G. lacustris* transcriptome (PRJNA660769), as indicated in Table 3.1.

	% Identity						
	***elovl*2/5**	***fed*1**	***fed*2**	***fed*3**	***fed*4**	***ωx*1**	***ωx*2**
a) Intra-specific							
Gammarus pulex	99.6	99.9	98.4	99.8	97.3	99.6	99.7
Gammarus fossarum	97.9	96.9	98.5	99.5	97.5	98.2	99.5
b) Inter-specific							
G. pulex vs *G. fossarum*	99.6	99.5	98.7	99.8	99.9	99.7	99.8
b) Inter-specific							
G. pulex vs *G. lacustris*	98.1	99.7	99.4	*	*	99.5	99.5

3.3.4. Occurrence of LC-PUFA biosynthesis genes in gammarids collected from the wild and laboratory cultures

We next confirmed the occurrence of rotifer genetic material in freshwater gammarids by detecting the *in silico* retrieved LC-PUFA biosynthesis genes by RT-PCR on cDNA prepared from laboratory cultured whole individual adult *G. pulex* (L), hepatopancreas from adult *G. pulex* (H), wild-captured adult *G. pulex* (W) (Figure 3.3). RT-PCR targeting each of the candidate LC-PUFA biosynthetic genes were positive for W, but not L and H (Figure 3.3.). Similarly, only the W sample, but not L and H, showed a positive band when targeting the *Rotaria actb* chosen as diagnostic gene to detect the presence of epibiotic rotifers (Figure 3.3, Figure S3.4). Moreover, sequencing results for the *Rotaria actb* band confirmed its identity as a rotifer gene. A positive control targeting the *G. pulex actb* was positive in all three cDNA samples (L, H and W) (Figure 3.3).

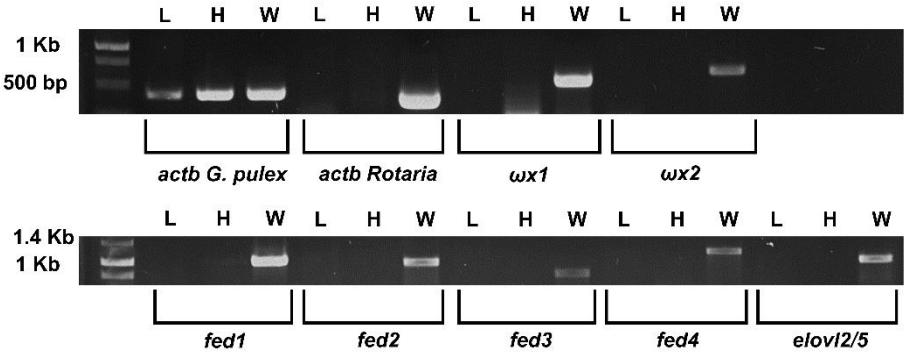

Figure 3.3. RT-PCR assays for detection of candidate genes (*ωx1*, *ωx2*, *fed1*, *fed2*, *fed3*, *fed4*, and *elovl2/5*) and housekeeping genes (*actb* from *G. pulex* and *Rotaria*) in laboratory cultured whole individual *G. pulex* (L), hepatopancreas (H) and wild caught whole individual *G. pulex* (W).

3.3.5. Exon-intron organisation of LC-PUFA biosynthesis genes from Bdelloidea rotifers

To confirm the results from the phylogenetic analysis suggesting that the LC-PUFA biosynthesis genes retrieved from gammarid transcriptomes belong to epibiotic bdelloid rotifers, we mapped them into the genome of *A. steineri* (WGS project: CAJNOM01; Assembly: GCA_905250015.1) and determined their exon-intron organisation (Figure 3.4). Our results revealed that the seven LC-PUFA biosynthesis genes retrieved from gammarid transcriptomes, namely one elongase (*elovl2/5*), four

fed, and two *ωx*, are genome-anchored in *A. steineri*. The analysis of their exon-intron organisation showed that the front-end desaturase herein termed "*fed3*" is an intronless gene (Figure 3.4). Besides, the *fed4* sequence contained characteristic features of "introner-like" elements (Van Der Burgt *et al*., 2012), including a relatively long intron (third, 940 bp), conserved motifs with pyrimidine rich regions, and acceptor and donor sites for recognition by the spliceosome (Figure 3.5). The particular exon-intron organisation of *fed3* and *fed4*, along with their close phylogenetic relationship with sequences from kinetoplastids and fungi, respectively, suggested HGT as the evolutionary origin of these genes in bdelloid rotifers. To exclude the possibility that such kinetoplastid and fungi sequences might be a contamination in bdelloids, we further examined the genomic neighbourhood of both *fed3* and *fed4* along the *A. steineri* genome (Figure 3.4a). The *A. steineri fed3* and *fed4* are both flanked by genes that have orthologues in other metazoan species, confirming that both *fed3* and *fed4* are real components of the *A. steineri* genome and not a putative contamination of kinetoplastids and fungi (Figure 3.4b).

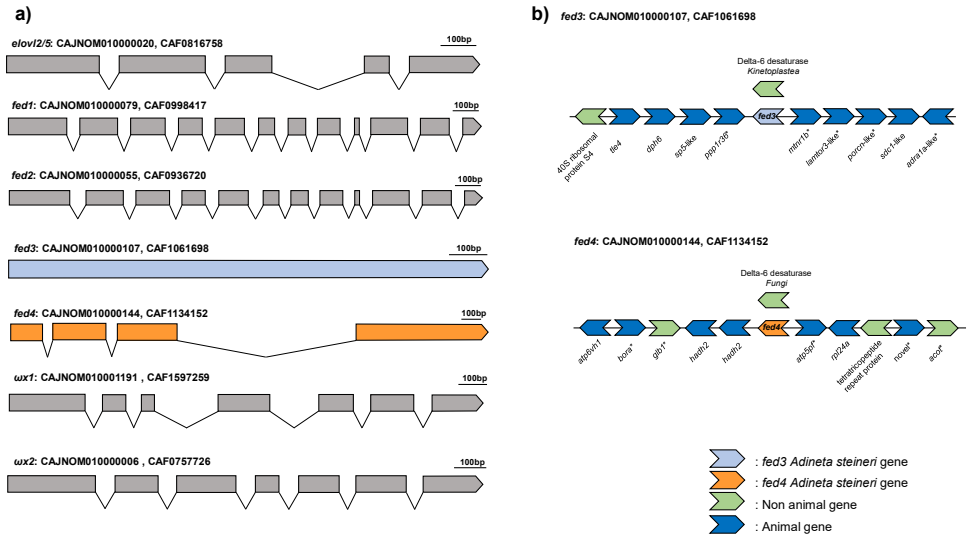

Figure 3.4. Exon-intron organisation and genomic neighbourhood of LC-PUFA biosynthetic genes from *Adineta steineri*. a) Exon-intron structure of candidate LC-PUFA biosynthetic genes (*elovl2/5*, *fed1*, *fed2*, *fed3*, *fed4*, *ωx1*, and *ωx2*) in the *A. steineri* genome. Introns are indicated by solid lines between exons (coloured boxes). All genes analysed have introns except for *fed3*, which is intronless. Besides, *fed4* has an exon-intron organisation consistent with its HGT origin. **b)** Distribution of the *fed3* and *fed4* genes across the *A. steineri* genome. Both genes are flanked by animal genes in the genome according to identity analyses results (BLAST). Moreover, BLAST results also revealed that *fed3* has a protist origin whereas *fed4* has fungi origin. * Identity scores less than 45%.

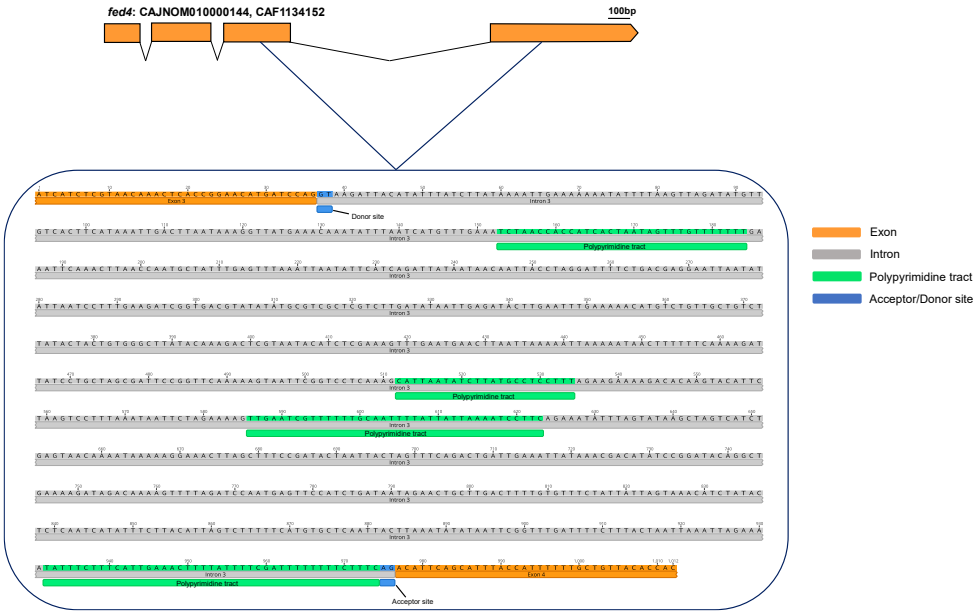

Figure 3.5. Nucleotide sequence of the *Adineta steineri fed4* genomic sequence. The *A. steineri fed4* genomic sequence (CAJNOM010000144, CAF1134152) has characteristic motifs of an introner-like sequence (ILE). Intron 3 (grey) is flanked by exons 3 and 4 (orange). The particular nucleotide sequences typical from regular spliceseosomal introns (RSI) are polypyrimidine tracts (green) and acceptor/donor sites (blue) for the spliceosome recognition and removal (Van Der Burgt *et al.*, 2012; Gozashti *et al.*, 2022).

3.3.6. Functional characterisation of LC-PUFA biosynthesis enzymes from bdelloid rotifers

The activities of the LC-PUFA biosynthetic enzymes encoded by the diagnostic genes from bdelloid rotifers was investigated by expressing their ORF in yeast. Transgenic yeast expressing the *elovl2/5* were able to elongate both C_{18} and C_{20} PUFA substrates, but no C_{22}, to their corresponding C_{20} and C_{22} elongation products, respectively (Table S3.3). Regarding front-end desaturases, Fed1 showed Δ5 desaturase activity as denoted by the conversions of 20:4n-3 and 20:3n-6 into 20:5n-3 (EPA) and 20:4n-6 (ARA), respectively (Table S3.4). Functional characterisation of Fed3 showed this enzyme is a Δ6 desaturase as it was able to convert 18:3n-3 and 18:2n-6 into 18:4n-3 and 18:3n-6, respectively (Table S3.4). Fed2 and Fed4 did not show activity towards any of the assayed substrates (data not shown). For methyl-end desaturases, both characterised enzymes showed dual Δ12 and Δ15 activities as they were able to

desaturate 18:1n-9 to 18:2n-6 (Δ12 desaturation), as well as 18:2n-6 and 18:3n-6 to 18:3n-3 and 18:4n-3, respectively (Δ15 desaturations) (Table S3.5).

3.4. Discussion

Our sequence retrieval strategy has revealed a markedly different repertoire of LC-PUFA biosynthetic genes in transcriptomes from freshwater gammarids compared to marine species, with the former having a generally conserved number of *elovl2/5* (1 gene), *fed* (4 genes) and *ωx* (2 genes), and none in the latter. Instances of animal lineages with enhanced LC-PUFA biosynthetic capacity in species inhabiting freshwater ecosystems compared to that of marine counterparts, have been previously reported in sticklebacks and American soles (Ishikawa *et al*., 2019; Matsushita *et al*., 2020). However, such an evolutionary innovation enabling colonisation of freshwater environments by these teleosts occurred in one specific front-end desaturase gene, *fads2* (Ishikawa *et al*., 2019; Matsushita *et al*., 2020), rather than an acquisition of multiple genes as suggested by the gammarid's transcriptomic database searches. The present study provides compelling evidence demonstrating that the unexpected occurrence of key desaturase and elongase genes in freshwater gammarid transcriptomes, rather than gammarids' themselves, is accounted for the presence of bdelloid rotifers in the analysed samples.

Our phylogenetic analysis revealed that, with the exception of the herein termed *fed3* and *fed4*, the LC-PUFA biosynthetic genes used as diagnostic markers clustered together with orthologues from bdelloid rotifers, particularly *Rotaria* and *Adineta* species. These results strongly suggested that multiple transcriptomes from freshwater gammarids contain rotifers' genetic material contaminating those publicly available databases. Due to the fact that bdelloid rotifers are typical epibionts of freshwater gammarids associated to their branchial plates and appendages (e.g., 22 different species reported in *G. pulex*), their high prevalence (up to 80%), and their relatively small size (ranging from 150 to 700 µm approx.) (Ricci and Melone, 2000; De Smet and Verolet, 2016; Bojko *et al*., 2017; Bojko and Ovcharenko, 2019), it is reasonable to speculate that the gammarid specimens used for RNA sequencing contained rotifers that were not removed prior analysis. As opposed to whole individuals (Cogne *et al*., 2019), the transcriptome built from *G. pulex* hepatopancreas (Gismondi and Thomé, 2016) did not result in any positive hit against any of the LC-PUFA biosynthesis gene markers assessed, confirming that internal body parts of the gammarid are free from epibiotic rotifers. Unlike freshwater species, marine gammarids such as *E. marinus* (Cogne *et al*., 2019; Collins *et al*., 2021) and *G. locusta* (Neuparth *et al*., 2020) did not have any of the diagnostic LC-PUFA biosynthesis genes, consistent with the fact that the above described epibiotic

relationship between gammarids (host) – bdelloid rotifers (epibiont) is restricted to freshwater environments (De Smet and Verolet, 2016). Our data further support that the abundance of rotifers in the gammarid samples used for RNA sequencing was not negligible according to the presence of a remarkable amount of rotifer-derived mitochondrial genes (*cox*) in some of the available RNA sequencing data (Cogne *et al*., 2019). It is also worth noting that, while pipelines for *de novo* transcriptome assembly typically imply filtering strategies (Grabherr *et al*., 2011; Haas *et al*., 2013; Davidson and Oshlack, 2014; Caputo *et al*., 2020), these appear not have been effective in removing rotifers' contamination from the analysis. This probably explains why, despite not being the targeted species (gammarid), full-length sequences for all diagnostic LC-PUFA biosynthesis gene markers were found in most freshwater gammarid transcriptomes when such genomic platforms were built with freshly sampled wild-captured whole individuals. Laboratory cultured gammarids that have progressively lost epibionts after successive generations and/or moulting events, as well as internal tissues such as the abovementioned hepatopancreas (Gismondi and Thomé, 2016), arise as safe, clean alternative samples, as supported by the PCR-based determinations of gene markers in *G. pulex*. As noted above, our phylogenetic analyses could only identify *Rotaria* and less probably *Adineta* as the most likely bdelloid genera contributing to the occurrence of LC-PUFA biosynthetic gene sequences in gammarid transcriptomes. Interestingly, only slight variations along the nucleotide sequences of the analysed genes were found, even when comparing sequences from samples collected from very distantly located sites like Central Europe (France, Belgium, Germany and Switzerland) and Baikal Lake (Russia). These results show that all epibiotic rotifers inhabiting the exoskeleton of freshwater gammarids are very closely related irrespectively of their geographical location and biotope.

Phylogenetics suggested that Fed3 and Fed4 are from a heterologous origin unlike other LC-PUFA biosynthesis genes investigated herein, since they clustered together with front-end desaturases from kinetoplastids (protists) and fungi, respectively. To clarify this point, we examined the genomic location of *fed3* and *fed4* within the genome of the bdelloid rotifer *A. steineri* (Nowell *et al*., 2021), demonstrating both genes are genome-anchored. Similarly, other LC-PUFA biosynthetic genes, namely *elovl2/5*, *fed1*, *fed2*, *ωx1* and *ωx2*, were also confirmed to be genome-anchored, demonstrating that the complement of LC-PUFA biosynthetic genes in bdelloid rotifers goes well beyond *ωx1* and *ωx2* previously reported in *Adineta vaga* (Kabeya *et al*., 2018). Importantly, in agreement with their unexpected location in the phylogenetic trees, we established that *fed3* and *fed4* likely originate via HGT, a well-documented evolutionary mechanism by which

bdelloid rotifers have gained bacterial, protist and fungal genes (Gladyshev *et al*., 2008; Boschetti *et al*., 2012; Flot *et al*., 2013; Eyres *et al*., 2015; Hespeels *et al*., 2015; Debortoli *et al*., 2016). Moreover, analysis of the exon-intron organisation in *fed3* and *fed4* further supported these were likely instances of HGT. The *A. steineri fed3* is intronless, characteristic of horizontally transferred genes by RNA retrotransposition (Syvanen, 1994; Pontarotti, 2014; Villa and Viñas, 2019) likely from kinetoplastids that are often prevalent in the freshwater habitats occupied by bdelloid rotifers (Arndt, 1993; Von der Heyden and Cavalier-Smith, 2005; Mukherjee *et al*., 2019; José de Paggi *et al*., 2022). Unlike *fed3*, *fed4* contains introns suggesting a relatively more complex mechanism by which *fed4* has been transferred into the *A. steineri* genome, combining RNA retrotransposition as described for *fed3* and/or further intron gain likely occurring *via* the intron-generating transposons called "Introners" (Schwartz *et al*., 2008; Worden *et al*., 2009). Consistently, the structure of the third intron of the *A. steineri fed4*, includes typical features of "introner-like" elements (Van Der Burgt *et al*., 2012), particularly those belonging to the "novel RNA-propagated elements" family, which have been described in fungi (Gozashti *et al*., 2022). Introners had been originally described in the picoeukaryote *Micromonas* (Worden *et al*., 2009), the pelagic tunicate *Oikopleura dioica* (Denoeud *et al*., 2010), and the dothideomycete fungus *Meloidogyne graminicola* (Torriani *et al*., 2011). However, it has been recently discovered that they are more widespread in eukaryotes than originally anticipated and, interestingly, disproportionately common in aquatic lineages (Gozashti *et al*., 2022). While fungi, identified as potential donors justifying the origin of *fed4*, have been previously reported to explain HGT to bdelloid rotifers (Boschetti *et al*., 2012; Debortoli *et al*., 2016), no previous studies have reported HGT to bdelloid rotifers from kinetoplastids as suggested herein for *fed3*. However, abundance of kinetoplastids in aquatic ecosystems, particularly freshwater environments, makes them likely donors in a HGT of *fed3* to rotifers (Bouck, 1993; Arndt *et al*., 2000; Von der Heyden and Cavalier-Smith, 2005; Mukherjee *et al*., 2019). In agreement, the substrate specificity of kinetoplastid front-end desaturases shared features with that of the bdelloid rotifer Fed3-like desaturase characterised in our study, further supporing a common origin.

The bdelloid rotifer Fed3 was functionally characterised as a Δ6 desaturase and showed no activity as Δ8 desaturase. These results are in agreement with functions reported in one of the three front-end desaturases from the kinetoplastid *Leishmania major*, which was shown to have Δ6 activity but no Δ8 (Tripodi *et al*., 2006). Such regioselectivity is markedly different to multiple animal front-end desaturases characterised from mammals (Park *et al*., 2009), teleosts (e.g., Monroig *et al*., 2011) and copepods (Kabeya *et al*., 2021) that, in addition to Δ6, have also Δ8 desaturase activity. Exceptions to the above pattern can be found in e.g. the bivalve mollusc

Sinonovacula constricta Δ6 Fed (Ran *et al*., 2018), although this protein is phylogenetically unrelated to those from the cluster formed by kinetoplastid and bdelloid rotifer Fed3 (Monroig *et al*., 2022). A more widespread regioselectivity among invertebrates' Fed is Δ5 desaturase (Monroig *et al*., 2022), herein demonstrated to occur in the bdelloid rotifer Fed1 but not Fed2 despite both genes being duplicates as our phylogenetics and exon-intron organisation analyses in *A. steineri* clearly demonstrated. Consequently, since our yeast system did not show any detectable activity ruling out neofunctionalisation beyond Δ5 activity, it can be hypothesised that functional redundancy has driven loss of function in Fed2 (Wagner, 1998; Birchler and Yang, 2022). Reasons accounting for the loss function in Fed4 are less clear, although they could be related to their HGT origin as reported elsewhere in the literature (Vogan and Higgs, 2011; Husnik and McCutcheon, 2018). Along the functions of front-end desaturases, characterisation of the two methyl-end desaturases (ωx1 and ωx2) and Elovl2/5 demonstrate that bdelloid rotifers have complete enzymatic capacity that enable them to biosynthesise *de novo* LC-PUFA up to ARA and EPA (Figure 3.1). Indeed, both ωx1 and ωx2 from bdelloid rotifers have Δ12 and Δ15 desaturase activities, enabling the conversion of OA to LA and, subsequently ALA (Figure 3.1). While missing the Δ17 activity also contained in the *A. vaga* methyl-end desaturases (Kabeya *et al*., 2018), the combined Δ12/Δ15 regioselectivities within the same enzyme demonstrated herein for both ωx desaturase enzymes is consistent with their bdelloid rotifer origin and contrasts that from non-rotifer methyl-end desaturases in which the gene complement typically includes also an ω3 desaturase (Monroig *et al*., 2022). Elovl2/5 exhibited activity towards C_{18} and C_{20} PUFA substrates, converting them into the corresponding C_{20} and C_{22} products, respectively. Such elongation abilities by Elovl2/5 account for all the elongations required to biosynthesise ARA and EPA (Figure 3.1). However, DHA biosynthesis capacity in bdelloid rotifers appears to be limited in the apparent absence of a Δ4 front-end desaturase as our results suggest (Figure 3.1). Potential DHA synthesis in bdelloid rotifers, if existing, would necessarily proceed via the so-called Sprecher pathway but, additionally to Elovl2/5, participation of further PUFA elongases with the ability to elongate 22:5n-3 to 24:5n-3 would be mandatory (Figure 3.1). Importantly, the above results raise the question of whether bdelloid rotifers can supply their hosts (gammarids) with physiologically essential LC-PUFA originated via biosynthesis, which might be highly advantageous for the gammarids according to their limited biosynthetic capacity (Ribes-Navarro *et al*., 2021) and the relatively low availability of preformed LC-PUFA in freshwater environments (Colombo *et al*., 2016; Twining *et al*., 2021).

In conclusion, the present study demonstrates multiple transcriptomes from freshwater gammarids contain sequences that can be unequivocally assigned to bdelloid rotifers, well-established epibionts of gammarids. More specifically, we determined the systematic occurrence of key metabolic enzymes of LC-PUFA biosynthesis, and clarified that such occurrence is independent from the species and their geographical origin when transcriptomes are built from freshly sampled wild-captured whole individuals. Genomic location and exon-intron organisation further demonstrated that the diagnostic elongase and desaturase genes are *bona fide* components of the *A. steineri* (and presumably other bdelloid rotifers) genome. Moreover, we provide compelling evidence demonstrating that two of the front-end desaturase genes, namely *fed3* and *fed4*, have been acquired via HGT. Our findings offer a critical example of the value of functional genomics and critical assessment of sequence data to infer ecological impacts in the age of *Omics*.

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Effects of diet and temperature on the fatty acid composition of the gammarid *Gammarus locusta* fed alternative terrestrial feeds

Abstract

The fast and remarkable growth of global aquaculture in recent years has created new challenges, such as guaranteeing a sustainable supply of raw materials used for aquafeed formulation. Gammarids are low-trophic crustaceans with an increasing interest in aquaculture due to their high nutritional profiles and their capacity to grow under high-density conditions. Moreover, gammarids have the ability to thrive on a wide range of sidestreams while accumulating relatively high levels of long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA). In the present study, juveniles of the marine gammarid *Gammarus locusta* were cultured at four different temperatures (5 °C, 10 °C, 15 °C, and 20 °C) for 21 days and fed three diets, including the seaweed *Fucus* spp. as control, and carrot leaves and coconut flesh representing two agri-food industry sidestreams. Our results indicate that both the survival and biomass of *G. locusta* were highly affected by diet, with coconut showing the lowest growth performance. The temperature had no effect on biomass, although high temperature (20 °C) resulted in a decrease in survival. The effects of temperature on the gammarid fatty acids were not evident, with diet being the main modulator of the profiles. Furthermore, the results also reveal that the *Fucus* spp. diet was associated with relatively high percentages of n-3 and n-6 LC-PUFA. Interestingly, essential LC-PUFA such as eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids were detected in gammarids fed on either Fucus sp. or any of the sidestreams irrespectively of their presence in the diets. These results suggest an ability of *G. locusta* for LC-PUFA biosynthesis (trophic upgrading) and/or retention, making this species a promising candidate for the production of high-value ingredients for aquafeeds.

4.1. Introduction

Rapidly expanding aquaculture worldwide has created new economic and ecological challenges (FAO, 2020). With regard to fish farming, such challenges have been mostly linked to modifying the supply of raw materials used for feed formulation in order to reduce the current usage of finite resources such as the so-called marine ingredients fishmeal (FM) and fish oil (FO). FM and FO are regarded as major sources of essential nutrients for aquafeeds, including long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) (Tocher, 2015; Shepherd *et al*., 2017). The current production of FM and FO largely relies on feed-grade species fisheries and, with the expansion of aquaculture worldwide, pressure on these fisheries has grown to an extent that they may not sustainably fulfill the increasing demand (Naylor *et al*., 2009; FAO, 2020).

Several efforts have been made to find alternative feeding sources to alleviate the abovementioned pressure on fisheries and reduce the dependence upon FM and FO in finfish aquaculture (Naylor *et al*., 2009; Turchini *et al*., 2011b; Jannathulla *et al*., 2019). Up to the present, the use of raw materials derived from animals or plants has become a widely extended practice (Turchini *et al*., 2009; Jannathulla *et al*., 2019; Galkanda-Arachchige *et al*., 2020). However, the replacement of FM and FO with non-marine ingredients has often been associated with decreased nutritional value of fish farming products, including reduced levels of the health-promoting n-3 LC-PUFA eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), as well as suboptimal growth (Bell *et al*., 2001; Mourente and Bell, 2006; Turchini *et al*., 2011a; Yıldız *et al*., 2018; Romano *et al*., 2020). Therefore, high- quality alternative ingredients are needed in order to successfully replace traditional sources of FM and FO in aquafeed while maintaining growth performance and nutritional value (i.e., levels of n-3 LC-PUFA) of farmed fish (Alberts-Hubatsch *et al*., 2019). Indeed, the search for novel aquatic ingredients for aquafeed is a priority within the EU, as is their production through Integrative Multi-Trophic Aquaculture (IMTA) strategies (Guerra-García *et al*., 2016).

Ingredients derived from biomasses of low-trophic marine crustaceans such as gammarids, krill, and copepods have been regarded as promising candidates for aquafeed formulations due to their balanced profiles of essential nutrients, including n-3 LC-PUFA (McKinnon *et al*., 2003; Suontama *et al*., 2007; Naylor *et al*., 2009; Dhont *et al*., 2013; Harlıoğlu and Farhadi, 2018). While the exploitation of wild populations of marine crustaceans poses negative ecological impacts similar to those of wild-capture fisheries alluded to above, biomass production using intensive aquaculture systems arises as an interesting strategy. Gammarids are abundant in benthic

communities and inhabit practically all aquatic environments, which is reflected in their high diversity of feeding habits (Costa and Costa, 2000; Harlıoğlu and Farhadi, 2018). Moreover, gammarids are fish's natural prey, and their use as an alternative ingredient for fish feeding has been explored (Ashour *et al*., 2021). Gammarids can be grown and maintained in laboratory cultures and adapt well to different culture conditions and diets (Sexton, 1928; Costa and Costa, 2000; Neuparth *et al*., 2002; Ahyong and Hughes, 2016; Beermann *et al*., 2018; Alberts-Hubatsch *et al*., 2019). Interestingly, recent investigations have also demonstrated that gammarids can be fed on a wide range of sidestreams from bioindustries such as agriculture and aquaculture itself (Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020). Such sidestreams are characterized by being deprived or having low contents of LC-PUFA, but, intriguingly, gammarids fed on these sidestreams have shown relatively high levels of LC-PUFA, suggesting that gammarids have some capacity for trophic upgrading *via* endogenous lipid metabolism (Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020). Thus, applying circular bioeconomy strategies by which sidestreams derived from bioindustries are used for the production of high nutritional value gammarid biomasses as potential fish feed ingredients has been proposed (Jiménez-Prada *et al*., 2020). However, up to date, little is known about the optimal culture conditions for reliable large-scale cultures of marine gammarids (Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020). Several factors, including diet, temperature, and salinity can modulate LC-PUFA metabolism, growth, and survival of aquatic invertebrates (Neuparth *et al*., 2002; Monroig and Kabeya, 2018; Alberts-Hubatsch *et al*., 2019). Hence, it is necessary to fine tune the culture conditions for the production of LC-PUFA–rich gammarid biomass.

A previous study comparing the effects of several diets on two gammarid species, namely *Gammarus locusta* and *Echinogammarus marinus*, suggested that *G. locusta* is the best candidate regarding LC-PUFA composition, growth, and survival when fed sidestreams (Alberts-Hubatsch *et al*., 2019). However, to the best of our knowledge, information on the combined effects of diet and environmental factors, on survival, growth, and LC-PUFA content of marine gammarids is lacking. The main goal of this study was to elucidate the effects of three diets and four temperatures on the FA profile and growth performance of the marine gammarid *G. locusta*. For this purpose, cultures were carried out at 5 °C, 10 °C, 15 °C, and 20 ° C, representing ambient temperatures in the wild, with *G. locusta* occurring in coastal areas of the North Atlantic (Costa and Costa, 2000). *Fucus* spp. was used as a natural marine diet, and carrot leaves and coconut flesh as two different non-marine diets, mimicking agriculture sidestreams of different nutritional values.

4.2. Materials and methods

4.2.1. Animal collection and culture

All *G. locusta* specimens used in the experiments were obtained from a laboratory culture at the Alfred Wegener Institute, with stock cultures originating from the German Bight, North Sea. Specimens were reared in the laboratory prior to the experiment at temperatures of 18 °C, a salinity of 33–34 ppt, and pH 8. Broodstock cultures and juvenile *G. locusta* were raised on a mixture of dried *Fucus* spp. and vegetable greens (mainly carrot greens and kale leaves). Juvenile *G. locusta* (28 days post-hatch) were collected from the same batch culture, maintained at 10 °C for 5 days, and starved for 24 h prior to the experiment.

4.2.2. Experimental setup

The juvenile gammarids (5.327 ± 1.54 mm SD, n = 960) were randomly transferred into white 1-L buckets, filled with 500 ml of freshly filtered seawater (5µm tube filter) at 33–34 ppt and equipped with a mesh (70 × 70 mm, 5 mm mesh size) and an oyster shell as substrate. Each container was stocked with 20 specimens at 10 °C and randomly allocated to the respective temperatures (5 °C, 10 °C, 15 °C, and 20 °C) and diet in quadruplicates. The temperature was slowly adjusted by transferring the containers into water baths at the desired temperature. Three different diets were prepared: one natural marine food source, thalli of *Fucus* spp. containing LC- PUFA (hereafter referred to as "Fucus"), and two non-marine diets: carrot leaves (hereafter referred to as "Carrot"), an agricultural sidestream, (high shorter-chain ($<C_{20}$) PUFA content), and a diet that mimics a potential sidestream rich in saturated fatty acids (SFA) consisting on coconut flesh (hereafter referred to as "Coco"). All diets were rinsed in fresh water and dried at 55 °C for 24 h. The temperature in the experimental containers was recorded twice a day, and water exchange with fresh seawater adjusted to the respective temperature was done every second day. Feeding was done *ad libitum* with remaining food items removed and replaced during water exchange. Dead individuals were removed on a daily basis. Gammarids were cultured at the corresponding diet vs. temperature combination for 21 days, until sexual maturity was reached in the higher temperature treatments.

4.2.3. Growth and survival

Initially, a subsample of 100 juvenile gammarids (average 5 mm) was analysed. Total body lengths of gammarids were measured at the beginning and end of the experiment by analysing pictures of each replicate taken on scale paper using Fiji ImageJ (vers. 1.53q, Schneider *et al*., 2012). Total lengths were measured from the

basal point of the antennae to the third urosome segment. Specific growth rate (SGR, in % day^{-1}) was calculated as length gain per experimental duration for each pool as follows: SGR = 100 × [ln (final length) − ln (initial length)/time interval]. Initial individual weights could not be taken at the beginning of the experiment due to the vulnerability of the early life stages. Therefore, at the end of the experiment, all remaining specimens were counted and wet biomass per replicate was recorded using an analytical scale (Sartorius Practum 213-S1, *d* = 0.001 g). The gammarids were rinsed in Milli-Q water twice, placed into Eppendorf tubes, and immediately frozen at −80 °C. The frozen samples were then freeze-dried at −52 °C within 2 weeks after the experiment and thereafter stored at −80 °C until further analyses.

4.2.4. Fatty acid analysis

Total lipids and FA were analysed from gammarid samples as well as from experimental diets. Briefly, total lipids were extracted from the homogenized samples using the Folch method (Folch *et al*., 1957). Subsequently, total lipids were used to prepare fatty acid methyl esters (FAME), which were analysed using a Thermo Trace GC Ultra Gas Chromatograph (Thermo Electron Corporation, Waltham, MA, USA), equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 mm, Teknokroma, Sant-Cugat del Vallés, Spain), fitted with an on-column injection system, using helium as a carrier gas, and a flame ionization detector (FID). The analytical temperature was programmed from 50 °C to 220 °C. Chromatograms were integrated and analysed with Azur Datlys (St Martin d'Heres, France) software. FAs were identified by comparison of retention times of each peak with those of well-characterized standards.

4.2.5. Statistical analysis

All data were subjected to statistical analyses using PAST (vers. 4.09) (Hammer *et al*., 2001). Prior to the analyses, data were tested for normality (Shapiro–Wilk) and homogeneity (Levene's test). After assuring that normality and homogeneity criteria were met, data were analysed using analysis of variance (fixed effects two-way ANOVA) with Tukey's *post-hoc* test for multiple comparisons. Principal component analysis (PCA) was used to analyse and visualize the relationship between diet and FA profiles of gammarids. Differences in FA levels among treatments were tested by using a two-way permutational multivariate analysis of variance (PERMANOVA) with factors: "Temperature," four different conditions (5 °C, 10 °C, 15 °C, and 20 °C), and "Diet," three different treatments (Fucus, Carrot, Coco). One-way PERMANOVA was further used to compare the scores of the dietary groups. Ellipses were fitted to the scores at 95% confidence. Additionally, the percentage of similarity analysis (SIMPER) was used to determine the FA responsible for dissimilarities between conditions

within the dietary groups. Unless otherwise stated, statistical significance was tested at 95% confidence level ($p \leq 0.05$). The Unsaturation Index (UI) was used to establish a relationship between the FA levels of diets used and the FA levels of gammarids fed on the corresponding diets in order to determine how diet can affect the gammarids' FA profiles and to ascertain previous retention. The UI was calculated according to the following formula: ∑ [area of fatty acid * number of unsaturations].

4.3. Results

4.3.1. Growth and survival

The two-way ANOVA revealed that both temperature ($F(3, 36) = 12.43$, $p < 0.0001$) and diet ($F(2, 36) = 18.08$, $p < 0.0001$) had a significant effect on the survival of G. locusta, although no interacting effects of temperature and diet on survival were observed ($F(6, 36) = 0.38$, $p = 0.89$). Pairwise comparisons of the survival of gammarids at different temperatures revealed that the highest temperature (20 °C) resulted in significantly lower survival rates, whereas no differences were observed between the temperatures (Table 4.1). Regarding the diets, Coco resulted in significantly lower survival of gammarids, whereas no differences between the effects of Carrot and Fucus were found. The highest mortality was obtained with the Coco diet at 20 °C (Table 4.1).

Regarding total biomass, there was a significant interacting effect of temperature and diet as revealed by the two-way ANOVA ($F(6, 36) = 3.17$, $p = 0.01$). Here, our analysis shows no significance of temperature ($F(3, 36) = 1.63$, $p = 0.20$) but a highly significant effect of diet ($F(2, 36) = 29.07$, $p < 0.001$). Pairwise comparisons of the biomass output revealed highly significant differences ($p < 0.005$) between all diet groups, with the Fucus diet resulting in the highest (Table 4.1). No significant differences were found in the gammarid biomasses when comparing temperature groups.

The final length was not affected by the interaction of temperature and diet ($F(6,35) = 1.47$, $p = 0.22$). In this case, the diet had no effect on final length ($F(2, 35) = 3.03$, $p = 0.06$), in contrast to temperature, which strongly affected final length ($F(3, 35) = 20, 73$, $p < 0.001$). When looking at pairwise comparisons, it becomes evident that this effect was caused by the 20 °C treatment, which resulted in significantly higher final lengths compared to all other temperature groups ($p < 0.001$), whereas no differences were found between the other groups (Table 4.1).

The SGR followed a similar pattern as the final length data, with temperature having a highly significant effect on SGR ($F(3, 35) = 31.83$, $p < 0.001$). This was also explained by the higher SGR in the 20 °C treatment, which was significantly different

from all other temperature treatments ($p < 0.001$) while the other temperatures were not different (Table 4.1). Diet had a significant effect on SGR ($F(2, 35) = 5.26$, $p = 0.01$), which was caused by the low SGR when regarding pairwise comparisons, in which Coco was significantly different from Fucus but not from Carrot (Table 4.1). This was biased by the very high mortality in this treatment group (see above). Similarly, a significant interaction effect of diet and temperature on SGR ($F(6, 35) = 3.44$, $p = 0.008$) was observed.

Table 4.1. Survival, total biomass, and total length of *G. locusta* in response to different diets and temperatures after the 21-day feeding trial. Fucus, *Fucus* spp.; Carrot, carrot leaves; Coco, coconut flesh. Data are expressed as mean of the different replicates per condition ± standard deviation (SD). Statistical differences (Tukey's pairwise comparisons, $p \leq 0.05$) are indicated by different letters: lowercase letters (a–d) indicate differences in temperature and uppercase letters (A–D) indicate differences in diet.

% Survival (± SD)	**5°C**	**10°C**	**15°C**	**20°C**
Fucus	73.75 ± 10.31 $^{a, A}$	65.00 ± 10.80 $^{a, A}$	71.25 ± 14.93 $^{a, A}$	45.00 ± 28.28 $^{b, A}$
Carrot	71.25 ± 6.29 $^{a, A}$	60.00 ± 10.80 $^{a, A}$	66.25 ± 27.80 $^{a, A}$	32.50 ± 10.41 $^{b, A}$
Coco	47.50 ± 8.66 $^{a, B}$	23.75 ± 23.94 $^{a, B}$	47.50 ± 8.66 $^{a, B}$	8.75 ± 4.79 $^{b, B}$
Biomass (mg ± SD)				
Fucus	76.00 ± 11.86 $^{a, A}$	122.50 ± 31.03 $^{ab, A}$	121.25 ± 38.35 $^{b, A}$	159.00 ± 36.39 $^{ab, A}$
Carrot	67.50 ± 20.04 $^{a, B}$	78.00 ± 26.89 $^{ab, B}$	95.75 ± 41.10 $^{b, B}$	86.50 ± 27.74 $^{ab, B}$
Coco	64.00 ± 43.14 $^{a, C}$	27.25 ± 9.00 $^{ab, C}$	51.25 ± 16.66 $^{b, C}$	27.25 ± 12.47 $^{ab, C}$
Lenght (mm ± SD)				
Fucus	6.92 ± 0.85 ^{a}	8.28 ± 1.44 ^{a}	8.24 ± 0.1 ^{a}	11.59 ± 2.45 ^{b}
Carrot	6.85 ± 0.73 ^{a}	7.10 ± 0.32 ^{a}	7.62 ± 1.05 ^{a}	9.81 ± 1.73 ^{b}
Coco	7.26 ± 2.63 ^{a}	5.31 ± 1.57 ^{a}	6.75 ± 0.3 ^{a}	10.89 ± 8.0 ^{b}
Specific growth rate (SGR ± SD)				
Fucus	0.37 ± 0.12 $^{a, A}$	0.89 ± 0.11 $^{a, A}$	0.89 ± 0.13 $^{a, A}$	1.68 ± 0.32 $^{b, A}$
Carrot	0.53 ± 0.08 $^{a, AB}$	0.71 ± 0.04 $^{a, AB}$	0.74 ± 0.04 $^{a, AB}$	1.29 ± 0.44 $^{b, AB}$
Coco	0.43 ± 0.36 $^{a, B}$	-0.10 ± 0.58 $^{a, B}$	0.66 ± 0.22 $^{a, B}$	1.48 ± 0.39 $^{b, B}$

4.3.2. Fatty acid profiles of gammarids

The FA analysis of the diets revealed that Fucus was rich in oleic acid (18:1n-9) (29.8%), whereas a-linolenic acid (ALA, 18:3n-3) was the most abundant FA in carrot leaves (26.5%). On the other hand, the Coco diet showed high levels of SFA (90.4%), such as lauric acid (12:0) (48.7%) and myristic acid (14:0) (19.9%) (Table 4.2). Low levels of DHA (22:6n-3) were detected only in the Fucus diet (1.1%), whereas EPA (20:5n-3) was also present in Fucus (4.8%) and only in trace amounts (0.2%) in Carrot. Moreover, the Coco diet had the lowest UI (6.2) whereas the Fucus and Carrot diets

showed similar levels (150.4 and 138.3, respectively), reflecting that the former is very poor in PUFA and MUFA and rich in SFA, as compared to the other treatments.

Table 4.2. Relative fatty acid (FA) composition (% of total FA) of diets used in the feeding trial. Fucus = *Fucus* spp., Carrot = carrot leaves, Coco = coconut flesh. OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; UI, unsaturation index; nd, not detected.

	Fucus	Carrot	Coco
12:0	1.1	1.1	48.7
14:0	11.4	1.4	19.9
16:0	13.6	17.5	9.3
16:1n-7	1.5	0.2	0.1
18:0	0.7	1.3	3.5
18:1n-9 (OA)	29.8	2.1	4.7
18:2n-6 (LA)	8.8	14.3	0.7
18:3n-3 (ALA)	3.3	26.5	0.1
20:4n-6 (ARA)	12.0	0.4	nd
20:5n-3 (EPA)	4.8	0.2	nd
22:5n-3 (DPA)	nd	nd	nd
22:6n-3 (DHA)	1.1	nd	nd
SFA	27.7	23.2	90.4
MUFA	32.4	2.8	4.7
n-3 PUFA	11.6	26.7	0.1
n-6 PUFA	21.6	14.9	0.7
n-3 LC-PUFA	6.0	0.3	nd
n-6 LC-PUFA	12.8	0.6	nd
UI	150.4	138.3	6.2

FA analysis of cultured *G. locusta* showed similar profiles when different temperature treatments were compared (Table 4.3). However, the two-way ANOVA of the FA analyses only showed significant differences when comparing the different dietary treatments (Table 4.4). One-way ANOVA of gammarids fed on the different diets (Table 4.5) did not show significant differences in EPA and DHA levels. The two-way PERMANOVA results did not show differences in FA among different temperatures ($F(3, 28) = 1.20$, $p = 0.29$), or the interaction temperature and diet ($F(6, 28) = 0.73$, $p = 0.74$). Moreover, the two-way PERMANOVA revealed diet as the main modulator of FA profiles ($F(2, 28) = 8.97$, $p < 0.001$). PCA revealed that the first component (PC1) accounted for the 60.81% of variance of this dataset, whereas the PC2 accounted for the 19.19% of variance (Figure 4.1). The PCA loading plot showed saturated fatty acids such as lauric acid (12:0) and myristic acid (16:0) are separated

on the negative side of PC1 from unsaturated and polyunsaturated fatty acids including LC-PUFA such as arachidonic acid (20:4 n-6, ARA), EPA (20:5n-3), docosapentaenoic acid (22:5n-3, DPA), and DHA (22:6n-3), which load on the positive side (Figure 4.1). On the other hand, 16:1n-7, LA (18:2n-6), and ALA (18:3n-3) load on the negative side of PC2. This variable distribution drives the scores segregation (Figure 4.1). The FA profiles of *G. locusta* fed on Coco are correlated with SFA (Figure 4.1). Those fed carrots are associated with LA and ALA, whereas those fed the Fucus diet are associated with LC- PUFA, among others. It is also interesting to note that the scores of the FA profiles of gammarids fed on the Fucus diet showed less dispersion than those fed the carrot leaves and coconut diets (95% ellipses, Figure 4.1). One-way PERMANOVA (F = 9.38, $p < 0.0001$), however, showed that the three dietary groups were significantly different (Supplementary Table S4.1). No significant differences were found for PC2. Additional SIMPER analysis (Supplementary Tables S4.2-S4.4) revealed that oleic acid (18:1n-9, OA) was the FA with more dissimilarity between the Fucus diet and the other diet groups (Carrot and Coco). On the other hand, the SIMPER analysis comparing Carrot vs. Coco showed that the SFA were the FA characteristic of the Coco diet.

Table 4.3. Relative fatty acid (FA) composition (% of total FA) of samples of *G. locusta* fed different diets at four temperatures. OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; UI, unsaturation index; nd, not detected.

	5°C Fucus	5°C Carrot	5°C Coco	10°C Fucus	10°C Carrot	10°C Coco	15°C Fucus	15°C Carrot	15°C Coco	20°C Fucus	20°C Carrot	20°C Coco
12:0	0.2 ± 0.1	0.1 ± 0.08	6.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	11.6	0.7 ± 0.2	0.2 ± 0.1	8.2	0.2 ± 0.2	0.3 ± 0.1	8.7 ± 1.7
14:0	3.0 ± 0.4	0.7 ± 0.1	4.9 ± 0.1	3.4 ± 0.7	1.0 ± 0.2	12.9	2.8 ± 0.2	1.0 ± 0.3	9.1	2.4 ± 1.0	1.1 ± 0.5	7.3 ± 2.0
16:0	15.0 ± 0.3	17.2 ± 1.3	15.7 ± 0.8	16.2 ± 0.6	15.3 ± 1.5	31.8	16.6 ± 0.2	16.4 ± 0.6	37.0	18.9 ± 1.4	18.3 ± 2.6	19.7 ± 2.9
16:1n-7	2 ± 0.5	1.5 ± 0.5	1.7 ± 0.9	2.2 ± 0.5	2.5 ± 1.4	0.8	2.0 ± 0.6	3.4 ± 0.4	0.9	1.5 ± 0.2	2.4 ± 0.6	1.8 ± 0.7
18:0	2.5 ± 1.4	3.4 ± 0.6	3.9 ±0.69	2.1 ± 0.2	2.8 ± 0.4	9.1	3.6 ± 0.9	3.3 ± 0.5	12.5	3.3 ± 1.0	5.6 ± 1.6	5.2 ± 0.7
18:1n-9 (OA)	29.7 ± 4.0	17.5 ± 2.5	26.2 ± 2.4	27.2 ± 3.4	17.6 ± 6.1	18.3	27.7 ± 3.0	14.0 ± 2.7	8.7	24.9 ± 2.6	16.1 ± 2.7	14.2 ± 4.9
18:2n-6 (LA)	7.3 ± 0.7	8.6 ± 1.7	4.5 ± 0.2	6.7 ± 1.1	10.0 ± 0.0	2.2	6.0 ± 0.6	13.9 ± 1.4	0.7	5.5 ± 0.8	7.8 ± 3.3	2.4 ± 0.1
18:3n-3 (ALA)	2.3 ± 0.7	5.4 ± 1.9	0.4 ± 0.0	2.6 ± 0.3	8.5 ± 2.4	nd	2.1 ± 0.6	12.0 ± 1.5	1.1	1.8 ± 0.4	5.9 ± 0.8	0.3
20:4n-6 (ARA)	10.1 ± 3.6	10.9 ± 2.6	13.2 ± 0.3	12.1 ± 0.8	8.3 ± 1.3	3.5	12.3 ± 1.3	4.8 ± 1.0	0.8	13.5 ± 1.9	4.1 ± 1.6	4.6 ± 0.1
20:5n-3 (EPA)	7.1 ± 3.5	7.5 ± 2.3	8.9 ± 0.3	9.9 ± 0.8	6.3 ± 1.1	1.7	10.8 ± 1.4	4.1 ± 0.7	1.4	12.6 ± 3.7	5.9 ± 0.8	9.7 ± 2.1
22:5n-3 (DPA)	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.5 ± 0.1	0.1 ± 0.2	nd	0.6 ± 0.1	0.2 ± 0.1	nd	0.7 ± 0.3	0.2 ± 0.1	0.3 ± 0.1
22:6n-3 (DHA)	2.3 ± 0.6	3.3 ± 0.9	2.3 ± 1.7	2.4 ± 0.7	3.0 ± 0.4	0.6	2.6 ± 0.9	1.7 ± 0.4	0.4	3.0 ± 0.8	3.0 ± 1.1	3.0 ± 0.6
SFA	20.9 ± 0.7	22.1 ± 1.6	32.0 ± 1.6	22.2 ± 0.9	22.3 ± 0.9	66.8	24.1 ± 0.2	22.3 ± 0.7	69.2	25.8 ± 3.7	24.6 ± 4.9	37.1 ± 11.4
MUFA	36.0 ±4.0	23.7 ± 3.0	31.9 ± 0.2	35.7 ± 1.8	25.3 ± 8.8	22.4	34.2 ± 2.0	24.0 ± 3.8	12.5	30.9 ± 2.3	23.9 ± 1.9	23.7 ± 2.7
n-3 PUFA	16.6 ± 1.3	20.0 ± 1.2	12.8 ± 0.3	18.3 ± 1.6	20.2 ± 1.1	2.3	17.6 ± 1.5	21.8 ± 3.1	2.9	19.7 ± 4.1	21.1 ± 6.5	15.2 ± 1.9
n-6 PUFA	21.2 ± 0.3	23.0 ± 2.3	19.0 ± 0.1	20.8 ± 0.9	20.1 ± 1.0	6.0	19.6 ± 1.3	20.4 ± 2.0	1.6	20.5 ± 1.4	19.0 ± 1.9	10.8 ± 5.5
n-3 LC-PUFA	12.2 ± 0.4	13.8 ± 0.6	12.4 ± 0.3	13.6 ± 1.4	11.6 ± 1.1	2.3	14.3 ± 2.3	9.7 ± 4.0	1.8	16.5 ± 4.8	12.7 ± 6.2	13.2 ± 2.4
n-6 LC-PUFA	13.5 ± 0.3	12.6 ± 2.6	14.5 ± 0.1	13.6 ± 0.8	10.1 ± 1.0	3.8	13.8 ± 1.3	6.5 ± 1.4	0.8	15.0 ± 2.1	9.4 ± 3.3	5.3 ± 0.2
UI	184.0 ± 2.2	193.7 ± 9.1	165.0 ± 2.1	189.5 ± 9.9	165.3 ± 2.2	53.8	184.5 ± 9.6	162.1 ± 11.8	30.0	196.7 ± 26.6	184.4 ± 40.6	136.9 ± 19.3

Table 4.4. Two-way ANOVA results of the fatty acid composition of samples of *G. locusta* fed different diets at four temperatures. OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; UI, unsaturation index; nd, not detected.

	Temperature		Diet		Interaction	
	F test (2, 38)	*p* value	F test (3, 37)	*p* value	F test (6, 40)	*p* value
12:0	0.56	0.65	19.78	< 0.001	0.32	0.92
14:0	0.27	0.84	14.09	< 0.001	0.25	0.96
16:0	0.32	0.81	1.15	0.329	0.34	0.91
16:1n-7	0.63	0.60	1.34	0.275	1.38	0.26
18:0	0.75	0.53	9.24	< 0.001	1.02	0.43
18:1n-9 (OA)	1.23	0.31	32.13	< 0.001	0.89	0.51
18:2n-6 (LA)	1.59	0.21	8.79	< 0.001	1.84	0.12
18:3n-3 (ALA)	1.87	0.15	11.71	< 0.001	1.60	0.18
20:4n-6 (ARA)	1.19	0.33	8.96	< 0.001	1.79	0.14
20:5n-3 (EPA)	1.56	0.22	4.28	0.021	0.74	0.62
22:5n-3 (DPA)	1.07	0.37	8.82	< 0.001	0.45	0.84
22:6n-3 (DHA)	2.06	0.12	0.85	0.437	0.75	0.61
SFA	0.19	0.90	7.09	0.002	0.30	0.93
MUFA	0.80	0.50	25.03	< 0.001	0.54	0.78
n-3 PUFA	0.13	0.94	4.36	0.019	0.49	0.81
n-6 PUFA	0.84	0.48	7.38	0.002	0.76	0.60
n-3 LC-PUFA	1.19	0.33	2.93	0.065	0.61	0.72
n-6 LC-PUFA	1.14	0.35	8.72	< 0.001	0.99	0.44

Table 4.5. Tukey's *post-hoc* test after ANOVA of the fatty acid composition of *G. locusta* fed different diets. OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; UI, unsaturation index; nd, not detected.

	p value		
	Fucus *vs* Carrot	Fucus *vs* Coco	Carrot *vs* Coco
12:0	0.95	<0.001	<0.001
14:0	0.25	<0.001	<0.001
16:0	0.52	0.34	0.90
16:1n-7	0.63	0.69	0.25
18:0	0.04	<0.001	0.13
18:1n-9 (OA)	<0.001	<0.001	0.45
18:2n-6 (LA)	0.08	0.08	<0.001
18:3n-3 (ALA)	<0.001	0.77	<0.001
20:4n-6 (ARA)	0.004	0.002	0.002
20:5n-3 (EPA)	0.033	0.07	1.00
22:5n-3 (DPA)	0.010	<0.001	<0.001
22:6n-3 (DHA)	0.94	0.41	0.61
SFA	0.58	0.002	0.023
MUFA	<0.001	<0.001	<0.001
n-3 PUFA	0.99	0.03	0.04
n-6 PUFA	0.49	<0.001	0.03
n-3 LC-PUFA	0.21	0.07	0.76
n-6 LC-PUFA	0.01	<0.001	0.62

Generally, *G. locusta* fed on Fucus showed FA profiles richer in LC-PUFA (C_{20-24}) than those fed on Carrot or Coco, whereas *G. locusta* fed on Carrot showed the highest levels of PUFA ($<C_{20}$) such as LA and ALA (Table 4.3). Notably, levels of EPA and DHA were detected in *G. locusta* fed on either Carrot or Coco regardless of these FA not being present in those diets. Regarding SFA, *G. locusta* fed on Coco showed higher percentages as compared to *G. locusta* fed on either Fucus or Carrot (Table 4.3). It is important to note as well that lower percentages of SFA were detected in cultured *G. locusta* fed on Coco in comparison with the Coco diet itself, while maintaining moderate LC-PUFA levels.

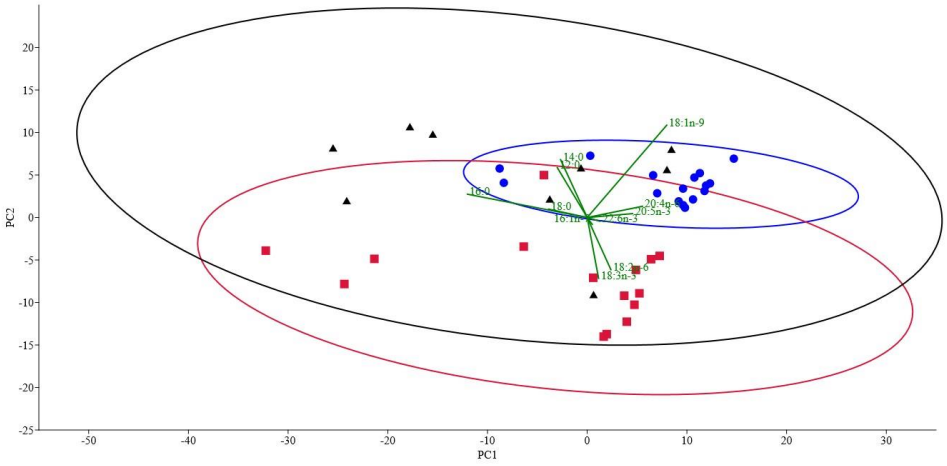

Figure 4.1. PCA of the fatty acid composition of *G. locusta* fed different diets and reared at different temperatures. Dots (blue), squares (red), and triangles (black) are the scores of *G. locusta* fed on *Fucus* spp., carrot leaves, and coconut, respectively. Fatty acids responsible for the grouping pattern are displayed in the biplot (green vectors); 95% confidence ellipses are shown. For the sake of clarity, and due to the lack of statistical significance, temperature labels are not shown.

4.4. Discussion

The nutritional profiles of gammarids have been studied as alternative sustainable ingredients for aquafeeds (Kolanowski *et al.*, 2007; Baeza-Rojano *et al.*, 2010, 2013, 2014; Jiménez-Prada *et al.*, 2018) due to the potential of these organisms to feed on a wide range of sidestreams (Harlıoğlu and Farhadi, 2018). However, only a few

investigations have focused on culture strategies based on the use of aquaculture and/or agriculture waste as primary food (Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020), and up to date, there are no investigations reporting on the effect of food and different environmental conditions on the fatty acid profile, growth, and survival of these organisms. In the present study, juveniles of *G. locusta* survived and reached sexual maturity in all the conditions tested for both temperature and diet groups. Interestingly, the survival of *G. locusta* was significantly lower at 20 °C than at the rest of the temperatures. This result supports the hypothesis that at 20 °C there is an acceleration of growth and reduction of the life cycle of *G. locusta*, resulting in a faster length growth and therefore metabolism, which results in an increased mortality (Neuparth *et al*., 2002). Regarding the effect of different diets, survival was significantly lower in *G. locusta* fed on Coco, whereas no differences were found between those of gammarids fed on the Carrot and Fucus diets. The absence of physiologically essential compounds such as LC-PUFA in the coconut diet may explain this difference (Jump, 2002; Monroig *et al*., 2022). Indeed, it is tempting to hypothesize that the high content of SFA in the diet may be related to an impairment of the membrane composition and properties, ultimately resulting in an increase in mortality under the most stressful temperature conditions (Hazel and Eugene Williams, 1990; Hazel, 1995; Ernst *et al*., 2016; Laila, 2021; Zinoöcker *et al*., 2021). Noticeably, cannibalistic behaviour was observed in gammarids fed on a coconut diet, suggesting a potential corrective mechanism towards the nutritional impairment. Although LC-PUFA was low in carrot leaves, the survival of gammarids fed on this diet was similar to that obtained with Fucus. As previously reported by Alberts- Hubatsch *et al*. (2019), this higher survival may be partially explained by the presence of other health-promoting nutrients like carotenoids that can thus compensate for suboptimal levels of LC-PUFA (Saini *et al*., 2015; González-Peña *et al*., 2021). Indeed, carotenoids are regarded as biomolecules that protect against unspecific and oxidant stress conditions such as suboptimal temperatures, which can ultimately cause increased mortality in gammarids (Agnew and Taylor, 1986; Neuparth *et al*., 2002; Wijnhoven *et al*., 2003). Significant differences in total gammarid biomass outputs were found when comparing different diets, and, regarding temperature, only significant differences were found between 5 °C and 15 °C, the latter having been reported as the optimal growth temperature for *G. locusta* (Neuparth *et al*., 2002). Interestingly, culturing gammarids at 20 °C and fed on Fucus resulted in the highest output of biomass as well as final length and SGR. This may be due to the combined effects of the high nutritional value of the Fucus diet (Alberts-Hubatsch *et al*., 2019) as well as the increased growth performance of *G. locusta* when grown at 20 °C (Neuparth *et al*., 2002) that compensates for the

biomass loss associated with the abovementioned high mortality rates detected at 20 °C.

Under the present experimental conditions, *G. locusta* showed similar FA profiles regardless of the rearing temperature. Only slight differences among temperatures within the same diet were evident, indicating either an apparent lack of temperature effect or a much more predominant dietary phenotypic impact. The FA dietary fingerprint was noticeable in the Fucus treatment in the LC- PUFA of the gammarid's profiles, whereas Carrot treatment resulted in higher levels of LA and ALA, and Coco induced higher amounts of SFA such as lauric and myristic acids in the final biomass. Interestingly, *G. locusta* fed on either Carrot or Coco still showed EPA and DHA percentages similar to those of *G. locusta* fed on Fucus, regardless of the absence of these LC- PUFA in the diets. This finding is in agreement with the potential capacity of gammarids for trophic upgrading, as has been suggested by several authors (Baeza-Rojano *et al*., 2013; Baeza-Rojano *et al*., 2014; Jiménez-Prada *et al*., 2018; Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020), indicating that these animals could bioconvert the short-chain FA present in sidestreams into high nutritional value LC-PUFA *via* their biosynthetic pathway.

LC-PUFA biosynthesis relies especially upon the gene repertoire and in the coordinated action of two types of enzymes (Monroig and Kabeya, 2018; Monroig *et al*., 2022). These two enzymes, elongation of very long-chain-fatty acid proteins (commonly known as "elongases"), and front-end desaturases (also named "desaturases"), need to be present and active in the organism in order to efficiently biosynthesize LC-PUFA such as EPA and DHA from dietary FA (Castro *et al*., 2016; Monroig and Kabeya, 2018; Monroig *et al*., 2022). A recent report on the three different elongases in the marine gammarid *E. marinus* (Ribes-Navarro *et al*., 2021) indicates the potential capacity for endogenous production of LC-PUFA in marine gammarids, but the presence and activity of desaturases remain to be elucidated. The presence of EPA and DHA can be due to reasons other than endogenous biosynthesis, such as the accumulation and retention of these FA from sources other than the diet provided, like the occasional and practically unavoidable presence of prey organisms such as copepods and rotifers in the culture tanks during the experiment. These organisms, particularly copepods, are characterized by having high LC-PUFA contents (McKinnon *et al*., 2003; Miller *et al*., 2008; Naylor *et al*., 2009; Nielsen *et al*., 2019; Boyen *et al*., 2020; Kabeya *et al*., 2021). It should be noted, however, that during the experiment, it was evident that gammarids fed actively on the vegetal substrates, and although impossible to rule out, the possible contribution of the above-mentioned accompanying fauna entering the system as well as that of the rapid development of biofilm seems at most testimonial. Other factors, like the fungal contribution described in the digestive process for the freshwater *G. pulex*

(Chamier and Willoughby, 1986), can also be invoked to explain the presence of unexpected components in the final profiles, but this can only be clarified in experiments run in controlled conditions, far beyond the scope of the present experimental design.

In summary, the present study demonstrates that gammarids can be fed on agricultural sidestreams (carrot leaves) without drastically affecting their growth performance and survival when reared at temperatures ranging from 5 °C to 15 °C. A culture at 20 °C is not recommended due to the impairment between growth and survival. This study is the first study of this kind to show interactions between rearing temperatures and novel feed ingredients for this species, further steps (e.g., other under- used sidestreams from agriculture) need to be taken to optimize biomass gain in addition to survival. In particular, this study shows that *G. locusta* can be a potential candidate for applying circular economy principles by which sidestreams such as carrot leaves can be used as the main feed for gammarid culture and potential biomass generation. The aim of this study was also to get independent from marine resources, such as macroalgae, that can be costly or not sustainable to use for feeding gammarids. Thus, showing that at least carrot greens are providing sufficient nutrients for a successful culture of *G. locusta* gives important directions in terms of sustainability. Moreover, under the present experimental conditions tested herein, diet has been established as the main modulator of FA profiling in *G. locusta*. However, a combination of agricultural and aquaculture sidestreams, along with other modulating conditions such as salinity, should be considered in order to set the best environment for large-scale gammarid production. Aside from the strong phenotypic effect of diet on the final FA profile, the presence of LC-PUFA like EPA and DHA, detected on *G. locusta* fed on diets devoid of these compounds, points towards unknown mechanisms of trophic upgrading beyond the theoretical endogenous biosynthetic capacity of the species.

4.5. References

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5.

Transcriptomic analysis and differential gene expression of the effects of diet and temperature in *Gammarus locusta* fed alternative terrestrial feeds

Abstract

Gammarids are arising as promising candidates for fishmeal (FM) and fish oil (FO) substitution in aquaculture feeds due to their feeding habits and nutritional profiles. The presented study aimed to elucidate whether different environmental conditions such as diet and temperature can affect growth performance and nutritional profiles, particularly LC-PUFA, in the marine gammarid *Gammarus locusta* from a molecular perspective. The gammarids were fed on three different diets with different LC-PUFA content and reared at four different temperatures. Subsequently, RNASeq analysis was conducted and the resulting transcriptome was assembled for differential gene expression (DGE) analyses. The results from the DGE analyses suggest that diets lacking LC-PUFA, particularly when combined with elevated temperatures, can impair growth by modulating the expression of genes involved in the moulting pathway of gammarids. Moreover, gammarids fed on LC-PUFA depleted diets showed a distinct gene expression pattern associated with reduced fatty acid catabolism, leading to a subsequent higher accumulation of fatty acids, presumably in the triacylglyceride fraction. Amongst all the conditions tested, lower temperatures, and diets closer to marine origin, emerged as optimal choices for culturing gammarids without drastically compromising their growth performance and LC-PUFA profiles.

5.1. Introduction

The significant growth of global aquaculture in the recent years has created new economic and ecological sustainability challenges (FAO, 2022). With regard to intensive fish farming, such challenges have been mostly linked to guarantee the supply of raw materials for feed manufacture that help to alleviate the dependence upon marine ingredients, namely fishmeal (FM) and fish oil (FO). Both FM and FO have been widely used in aquafeeds due to their good nutritional profiles, that satisfy the physiological requirements of all essential nutrients for fish. In particular, FM and FO are regarded as unique sources of long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) in aquafeeds, physiologically essential nutrients for animals that include arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Tocher, 2015; Shepherd *et al*., 2017). In addition, to ensure normal growth and development of farmed fish, inclusion of FM and FO in aquafeed formulations guarantees the accumulation of the health promoting "omega-3" (ω3) EPA and DHA, nutritionally relevant LC-PUFA with beneficial effects in preventing cardiovascular and metabolic diseases, and improving neural and visual development (Swanson *et al*., 2012; Calder, 2014, 2018). FM and FO are finite resources that are mostly produced from feed-grade marine fish and, with the abovementioned expansion of intensive aquaculture, pressure over fisheries exploited for FM and FO production has increased in the last decades, reaching, and often exceeding, their ecological sustainability limit. Non-marine raw materials from terrestrial animals and plants are nowadays part of commercial aquafeed formulations (Gasco *et al*., 2018; Aas *et al*., 2019, 2022c; Hardy and Brezas, 2022). However, the use of non-marine ingredients has resulted in a loss of nutritional value of fish farming products due to lowered contents of ω3 LC-PUFA (Bell *et al*., 2001; Mourente and Bell, 2006; Sprague *et al*., 2016, 2020; De Roos *et al*., 2017). Novel ω3 LC-PUFA sources in the form of oils such as those from genetically modified (GM) oilseed crops (Betancor *et al*., 2015; Napier *et al*., 2020) and microalgae (Sprague *et al*., 2017; Santigosa *et al*., 2021), and meals from marine invertebrates (Payne *et al*., 2001; Drillet *et al*., 2006; Baeza-Rojano *et al*., 2010; Dhont *et al*., 2013), are becoming more common in aquafeeds, although issues related to public opposition (GM oilseed crops), high production cost (microalgal oil), and ecological impact (exploitation of krill fisheries), are still limiting their more widespread use. The production of raw materials derived from low trophic level marine invertebrates cultivated on low cost, widely available substrates, arise as a promising strategy for making aquaculture a more environmentally sustainable and economically viable industry.

Gammarids (Order Amphipoda) are low trophic level crustaceans that are abundant in benthic communities in virtually all aquatic environments (Odum and Heald, 1972; Costa and Costa, 2000; Dauby *et al*., 2001; Väinölä *et al*., 2008). Moreover, several studies have reported that gammarids can inhabit and colonise a wide range of geographical regions and ecosystems (Kelly *et al*., 2002; Neuparth *et al*., 2002; Costa *et al*., 2009; Kunz *et al*., 2010; Whiteley *et al*., 2011). Gammarids are characterised by having a wide variety of feeding habits, and include detritivorous species, which have the ability to feed on a wide range of waste materials (sidestreams) from bio-based industries such as aquaculture, forestry and agriculture (Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020; Ribes-Navarro *et al*., 2022). Such feeding plasticity has made gammarids be regarded as excellent candidates to apply circular economy principles by which sidestreams can be utilised for production of biomass with high nutritional value. Previous studies have shown that gammarids' nutritional profiles are typically characterised by low levels of carbohydrates and high contents of proteins and lipids including ω3 LC-PUFA (Kolanowski *et al*., 2007; Baeza-Rojano *et al*., 2014; Jiménez-Prada *et al*., 2018).

Abundance of lipids in general, and ω3 LC-PUFA in particular, in animal tissues, depends upon their acquisition through diet, as well as endogenous anabolism and catabolism pathways. While the impact of dietary lipids in gammarids' lipid composition has been well documented (Makhutova *et al*., 2011; Alberts-Hubatsch *et al*., 2019; Jiménez-Prada *et al*., 2020; Ribes-Navarro *et al*., 2022), lipid metabolism is poorly understood in gammarids, with studies often inferring the activation of certain lipid metabolic pathways from lipid profiling datasets (Nelson *et al*., 2001; Baeza-Rojano *et al*., 2014; Alberts-Hubatsch *et al*., 2019). Yet, the omics era offers a unique opportunity to gain insight into fatty acid metabolism in amphipods including gammarids. Indeed, several transcriptomes and genomes from the amphipod *Hyalella azteca* (Poynton *et al*., 2018), and also gammarids (Cogne *et al*., 2019; Drozdova *et al*., 2019; Lipaeva *et al*., 2021), have become available in recent years, providing valuable resources that help to acquire understanding of the metabolic and physiological processes of these low trophic crustaceans in response to environmental factors (Chen *et al*., 2019; Neuparth *et al*., 2020b). Examination of publicly available transcriptomes has allowed us to confirm that gammarids have limited LC-PUFA biosynthetic capacity, with presence of fatty acyl elongases, enzymes involved in expanding fatty acid chain length, but lack of fatty acyl desaturases, enzymes that introduce double bonds (unsaturations) (Ribes-Navarro *et al*., 2021, 2023). These results suggested the occurrence of yet unknown alternative mechanisms by which gammarids are able to maintain relatively high levels of LC-PUFA even in the absence of dietary input (Alberts-Hubatsch *et al*., 2019; Ribes-Navarro *et al*., 2022)

Along dietary composition, lipid metabolism in general and fatty acid synthesis in particular, are greatly influenced by temperature in poikilotherms including crustaceans, which have been often reported to remodel cell membrane composition to accommodate for temperature-driven changes in fluidity (Boyen *et al*., 2020). Moreover, temperature markedly determines growth performance as recently shown in the marine gammarid *Gammarus locusta* (Ribes-Navarro *et al*., 2022). Central for crustacean growth is ecdysis ("moulting") (Dall *et al*., 1990), which has been established to be affected by temperature in gammarids (Pöckl, 1992). Interestingly, several investigations have reported on the relationship between lipid and fatty acid accumulation and moulting, especially during premoult stages where most crustaceans stop feeding and need to obtain energy from the nutrients already stored in their organism, especially lipids (Cuzin-Roudy *et al*., 1999; Kuballa *et al*., 2011; Tao *et al*., 2014; Jordão *et al*., 2015; Kaczmarek and Boguś, 2021; Qin *et al*., 2022). Although several studies have focused on the environmental effect on lipid accumulation and growth in crustaceans (Sutcliffe and Carrick, 1981; Pöckl, 1992; Chen *et al*., 2014, 2016; Tao *et al*., 2014; Wittmann *et al*., 2018; Lemos and Weissman, 2021; Qin *et al*., 2022), the underlying mechanism (at a molecular level) by which temperature is able to modulate that lipid accumulation and subsequent moulting stages in gammarids is yet unknown.

The main goal of the present study was to gain insight into the molecular mechanisms that allow the marine gammarid *G. locusta* to either synthesize or accumulate lipids, particularly fatty acids, when fed on diets devoid LC-PUFA. Moreover, this study aimed to elucidate how different diets and temperatures modulate, independently, the expression of genes involved in lipid and fatty acid metabolism, as well as other processes related with overall growth and survival in gammarids. As a preliminary goal, a *de novo* assembly transcriptome from *G. locusta* was built as a reference using the wild-caught specimens described in the experiment of Ribes-Navarro *et al*. (2022). Next, we examined the regulatory effect of diet and temperature on genes involved in fatty acid metabolism and growth using a set of analyses based on differential gene expression (DGE). This approach aimed at investigating the mechanism underlying the crosstalk between moulting and lipid metabolism in *G. locusta* reared at different temperatures. The results presented here are helpful to understand the physiological responses of *G. locusta* to metabolise dietary lipids, to accumulate high nutritional value LC-PUFA, as well as to fine tune the best culturing conditions regarding growth and survival for their further use as ingredient for aquafeeds.

5.2. Materials and Methods

5.2.1. Experimental design

The gene expression profile of selected individuals fed diets of different LC-PUFA content (Experiment 1), and reared at different temperatures (Experiment 2), were analysed. Full details of the experimental setup can be found in Ribes-Navarro *et al*. (2022). Briefly, to investigate the effects of diet, *G. locusta* juveniles (n=20/container) randomly allocated to 1 L buckets (n=3, one per dietary treatment) filled with 500 mL of freshly filtered seawater, were fed three different diets for 21 d. A "marine" diet consisting of thalli of the seaweed *Fucus* spp (hereafter referred to as "Fucus") was selected as a control based upon its content of LC-PUFA. Two further "non-marine" diets lacking LC-PUFA consisted of carrot leaves (hereafter referred to as "Carrot"), and coconut flesh (hereafter referred to as "Coco"). These represented an agricultural sidestream high in short-chain polyunsaturated fatty acid ($<C_{20}$, PUFA) content, and a potential sidestream rich in saturated fatty acids (SFA) respectively (Figure. 5.1). In order to investigate the effects of temperature (Experiment 2), *G. locusta* juveniles (density and replicates as described above for Experiment 1) fed on the Fucus diet were cultured at 5, 10, 15, and 20 °C for 21 d. At termination of both experiments, *G. locusta* individuals (n=4, one per treatment) were collected, washed and preserved in RNA*later*™ (Thermo Fisher Scientific, Waltham, MA, USA) for further analysis.

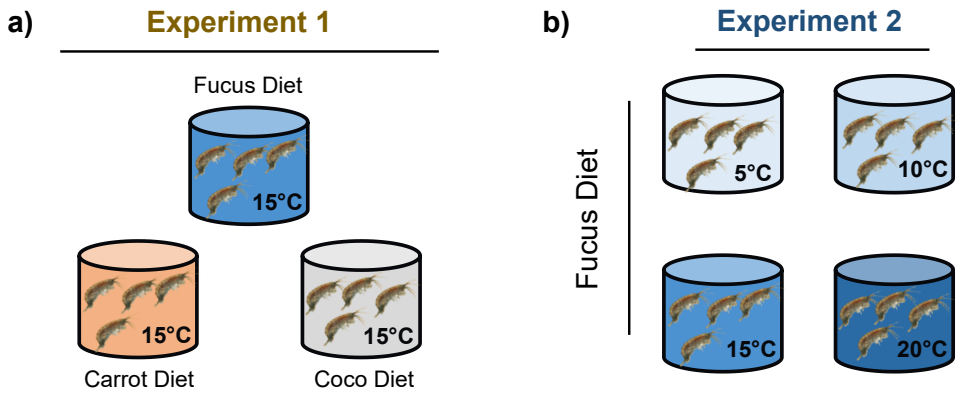

Figure 5.1. Experimental design. a) Experiment 1 evaluated the responses of *G. locusta* when fed on different diets (Fucus, Carrot, Coco) and reared at the same temperature (15 °C). **b)** Experiment 2 evaluated the responses of *G. locusta* fed on the Fucus diet when reared at different temperatures (5, 10, 15, and 20 °C). Each experimental condition was tested in triplicate 1 L buckets containing 20 individuals each. Both experiments were run simultaneously and therefore the control treatments in each experiment (*G. locusta* fed the Fucus diet and reared at 15 °C) consisted of the same samples.

5.2.2. RNA extraction, library preparation and Illumina sequencing

Four whole *G. locusta* male individuals from each condition of the two experiments described above were collected and assigned for further RNA extraction (Supplementary Table S5.1: MixS Descriptors). Notably, the condition "*G. locusta* fed on Fucus and reared at 15 °C is common to both experiments. Thus, total RNA from four males per experimental condition (*G. locusta* fed either on Fucus, Carrot, or Coco all reared at 15 °C, and *G. locusta* fed on Fucus and reared either at 5, 10, or 20 °C, n = 24) was extracted independently using the Illustra RNAspin Min RNA Isolation Kit (GE Healthcare, Chicago, IL, USA) according to manufacturer's instructions. A preliminary RNA sample integrity test was carried out by running a subsample (~500 ng) on an agarose gel electrophoresis, and determining the 260/280 and 260/230 quality ratios using a NanoDrop (ThermoFisher Scientific, Waltham, MA, USA). The best three samples per condition were subsequently used for RNASeq (n = 18). Thereafter, a subsample of 200 ng of total RNA was used for RNA sequencing using Illumina HiSeq2500 paired-end (PE) (2 x 150) (Novogene Europe, Cambridge, UK). These 18 samples were further analysed as described below and used for subsequent gene enrichment and DGE analyses. Besides, *G. locusta* collected from the wild (two

males, and one female) were used as internal controls for RNA-Seq analysis and further used for transcriptome generation and assembly (Supplementary Table S5.2: Samples processing).

5.2.3. *In silico* clean-up of raw datasets and *de novo* transcriptome assembly of *G. locusta*

The large amount (702.7 M) of paired end (PE) sequenced reads generated were analysed according to previous guidelines reported by Neuparth *et al*. (2020a) to assess the quality of the raw reads and correct potential sequencing errors. Briefly, the FastQC (v.0.11.8) software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was applied as a tool for quality control (QC) analysis. Next, the Rcorrector (v.1.0.3) (Song and Florea, 2015) was applied for error correction with default settings. To perform the *G. locusta* reference transcriptome assembly, the *de novo* assembly method Trinity (v.2.12.2) (Grabherr *et al*., 2011; Haas *et al*., 2013) was applied using RNASeq reads obtained from the *G. locusta* wild captured samples.

5.2.4. Decontamination and validation of transcriptome assembly

The transcriptome assembly underwent decontamination, redundancy removal, and validation through three distinct procedures. To decontaminate, we executed blast searches against NCBI databases, employing the same software, versions, and thresholds as outlined in Machado *et al*. (2020). For redundancy removal, we utilized the Corset tool (v.1.0.9) (Davidson and Oshlack, 2014; Machado *et al*., 2020). In this phase, each read dataset was aligned with the decontaminated transcriptome assembly using Bowtie2 (v. 2.3.4.2) (Langmead and Salzberg, 2012), employing specific settings (--no-mixed --no-discordant --end-to-end --all --score-min L, − 0.1, − 0.1), and the results were then filtered using Corset. Transcripts with a minimum of 10 mapped reads were retained and grouped based on shared reads and expression patterns. Subsequently, only the longest transcript in each cluster was preserved to eliminate redundancy.

The validation process was conducted using Trinity and TransRate scripts (v.1.0.3) (Smith-Unna *et al*., 2016). It involved comparisons with databases of conserved orthologous genes through the Benchmarking Universal Single-Copy Orthologs software (BUSCO v.5.2.2) (Simão *et al*., 2015) using the arthropoda_odb10 dataset, which consists of 1013 single-copy orthologs specific to arthropods. Additionally, the rate of reads mapping back to the transcriptome (RBMT) was calculated for both assembly versions. The RBMT analysis employed the Bowtie2 tool (version 2.3.5) and was performed in two stages. Firstly, it was executed with all twenty-one datasets

concatenated and applied to both transcriptome assemblies to evaluate the read content before and after the redundancy removal step. Subsequently, it was used to map all samples individually to the non-redundant Transcriptome assembly, confirming the reliability of this reference for gene expression analyses. As a result, two versions of the transcriptome assembly were generated: the first version, known as the Decontaminated Transcriptome Assembly (Transcriptome V0), and the second version, known as the Non-Redundant Transcriptome Assembly (Transcriptome V1).

5.2.5. Transcriptome annotation

The transcriptome annotation of the V1 assembly was performed using the methodology detailed in Gomes-dos-Santos, A *et al*. (2022). Briefly the prediction of open reading frames (ORFs), was done with the TransDecoder software (v.5.3.0) (Haas *et al*., 2013), following the author guidelines (https://transdecoder.github.io/). The .gtf file produced by the TransDecoder was processed by the Gtf/Gff Analysis Toolkit (AGAT) (version 0.8.0) (Dainat *et al*., 2021), and the names properly uniformized and formatted. Functional annotation was carried out utilising the InterProScan tool (version 5.44.80) (Jones *et al*., 2014), alongside with Blast-n, Blast-p, and Blast-x searches with NCBI Blast-n tool (Camacho *et al*., 2009) and the Blast-x and p tool of DIAMOND software (version 2.0.13) (Buchfink *et al*., 2015). These searches were conducted against the NCBI-nt database and the protein databases of NCBI, including NCBI-RefSeq (Reference Sequence Database) (Pruitt *et al*., 2007) and NCBI-nr (non-redundant database of NCBI) (Database resources of the National Center for Biotechnology Information., 2016). Given that few genomic resources are available for the Crustacea subphylum, annotated genomes such as *Daphnia magna*, *Daphnia pulex*, *Lepeophtheirus salmonis,* and *Tigriopus californicus* were used to perform the correct identification and validation of *G. locusta* transcripts. Subsequent to this stage and before executing the analyses for differential gene expression, the annotation details were merged with the previously computed counts using the Corset software.

5.2.6. Differential gene expression analysis

The DGE analyses were carried out in the Degust platform (v.4.1.1) (Powell, 2019) using the edgeR package (v.3.26.8) of R (v.3.6.1). Briefly, the gene count file generated by Corset was inputted into the Degus platform, and normalization was carried out in counts per million (CPM). Genes with fewer than one count per million mapped reads in at least three samples were excluded. Previous studies have reported that the standard culture conditions for *G. locusta* include temperature around 18 °C, and feeding on natural marine sources such as seaweeds (Costa and Costa, 2000; Neuparth *et al*., 2002). Since 20 °C is a temperature that leads to an increased mortality in gammarids (Neuparth *et al*., 2002; Ribes-Navarro *et al*., 2022), *G. locusta*

fed on the seaweed *Fucus* spp. and reared at 15 °C was set as control condition in both experiments. For DGE analysis of Experiment 1 (dietary effects), two conditions, namely *G. locusta* reared at 15 °C and fed on either Carrot or Coco, were compared with the control condition (*G. locusta* reared at 15 °C and fed on Fucus). For Experiment 2 (temperature effects), DGE analysis was performed by comparing three temperatures, namely *G locusta* fed on Fucus and reared at either 5, 10 or 20 °C, with the control (*G. locusta* fed on Fucus and reared at 15 °C). All genes with a False Discovery Rate (FDR) with corrected *p* value < 0.1 and log2FoldChange (FC) ≥ 2 were regarded as being differentially expressed. Additionally, a hierarchical clustering heatmap of the differentially expressed genes was performed using the Heatmapper tool (Babicki *et al*., 2016) with the clustering method (Average Linkage), and the Distance measurement method (Pearson) applied both to rows and columns of the dendrogram.

5.2.7. Enrichment and network analyses

Transcripts that successfully passed the DGE filters described above and were appropriately annotated to match existing genomic data for any of the crustaceans used for identification were subsequently included in the pipeline for gene ontology (GO) and enrichment analyses. The ShinyGO (v.0.77) online tool (http://bioinformatics.sdstate.edu/go/) (Ge *et al*., 2020) was employed for these analyses, utilizing a false discovery rate (FDR) cutoff with p < 0.05 and a maximum of 40 KEGG pathways, as determined by the KAAS server (Moriya *et al*., 2007).

5.2.8. Statistics

Differences in the DGE relative to the control group (*G. locusta* Fucus, 15 °C) were tested by using One Way Analysis of Variance (ANOVA) followed by Dunnett's *post-hoc* test ($p \leq 0.05$). Data analysis was performed using SPSS (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp).

5.3. Results

5.3.1. Sequencing, transcriptome assembly, and annotation of *G. locusta*

The sequencing process yielded approximately 702.7 million PE sequenced reads amongst all conditions analysed. Following stringent quality control procedures, a minor subset of reads was excluded from the initial dataset, attesting to its overall high quality (refer to Supplementary Table S5.2 for sample processing details). Subsequent to this, transcriptome assembly was conducted using samples obtained from three wild specimens (F1, M1, M2). Post-decontamination of the initial

transcriptome assembly, we derived Transcriptome V0, comprising 324,003 transcripts with an N50 length of 2099 base pairs (bp). The mean sequence length measured 1316 bp, with sequence lengths ranging from 283 bp to 26,444 bp. Through a meticulous redundancy removal process, the transcript set was refined to 115,543 transcripts, resulting in Transcriptome V1, characterized by an N50 length of 1843 and a mean length of 1301 bp. Despite the notable reduction, only a marginal percentage of gene content, approximately 0.3%, was discarded during this refinement (Total percentage of BUSCO genes found in the Arthropoda database, V0: 97.5%, V1: 97.2%) (see Supplementary Table S5.3 for assembly and annotation). Furthermore, when aligning the reads back to the transcriptome, a substantial proportion exhibited matches to both transcriptome versions (RBMT, V0: 95.68%, V1: 94.97%), underscoring the significant preservation of read content after redundancy removal (see Supplementary Table S5.3 for assembly and annotation details). Additionally, in a meticulous effort considering that the transcriptome was constructed from data obtained from only three out of the twenty-one sequenced individuals, we individually mapped all samples to the V1 transcriptome. This analysis confirmed a consistently high mapping rate across all samples, ranging between 91-95%, thereby confirming the suitability of the V1 assembly for conducting differential gene expression analyses (see Supplementary Table S5.4 for specific details on sample validation). The subsequent annotation process successfully identified 45,845 ORFs in Transcriptome V1, with gene symbols attributed to 36,761 transcripts. (For comprehensive assembly and annotation statistics, please refer to Supplementary Table S5.3).

5.3.2. Differentially expressed genes in Experiment 1 (effect of diet)

To identify genes with altered expression patterns in Experiment 1, *G. locusta* reared at 15 °C and fed either Carrot or Coco were compared to the control group (*G. locusta* fed on Fucus and reared at 15 °C). The transcriptomic analyses revealed a collective total of 610 genes exhibiting differential expression in *G. locusta* fed with either the Carrot or Coco diets, as compared to the control (FDR $p < 0.1$ and log2FC ≥ 2). Specifically, *G. locusta* individuals fed the Carrot diet exhibited 610 differentially expressed genes relative to the control diet (Fucus), while those fed the Coco diet showed 607 differentially expressed genes compared to the control (Figure 5.2a, Table 5.1). Examining the distribution of up- and down-regulated genes, gammarids fed Coco displayed the lowest and highest numbers of upregulated (97) and downregulated (510) genes, respectively. Conversely, *G. locusta* fed the Carrot diet demonstrated a total of 170 upregulated genes and 440 downregulated genes (Table 5.1). Comparison between gammarids fed either Carrot or Coco indicated a shared

total of 607 differentially expressed genes (99.51%), with only three genes showing differential expression exclusively in *G. locusta* fed on Carrot (Figure 5.2a).

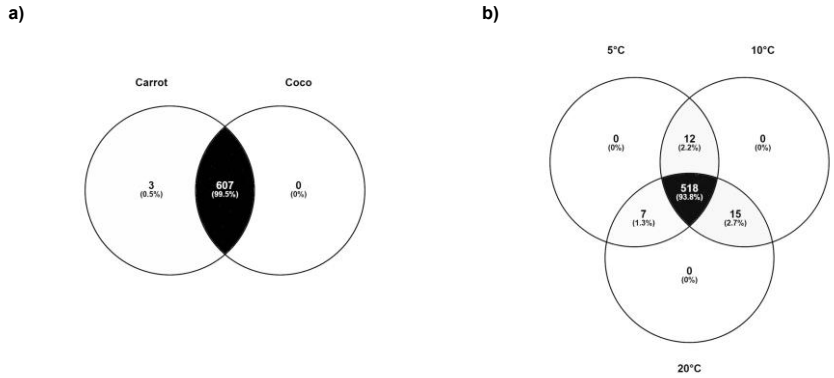

Figure 5.2. Venn diagrams of the DE genes (FDR, $p < 0.05$ and log2FC ≥ 2) in *G. locusta* fed different diets (Experiment 1) (a), and temperatures (Experiment 2) (b). Graphs were developed using the Venny online tool (Oliveros, 2007).

Table 5.1. Number of differentially expressed genes (DEG) in *G. locusta* fed on different diets (Carrot or Coco, Experiment 1) and reared at different temperatures (5, 10, and 20 °C, Experiment 2). All data have been expressed as number of DEG compared to control (*G. locusta* fed on Fucus and reared at 15 °C).

	Condition	Total of DEG	Upregulated	Downregulated
Experiment 1	**Carrot**	610	170	440
	Coco	607	97	510
Experiment 2	**5 °C**	537	302	235
	10 °C	545	323	222
	20 °C	540	284	256

Consistent with the findings presented in Table 5.1, the heatmap (Figure 5.3a) revealed the highest number of downregulated genes in the Coco column. Additionally, Fucus exhibited the highest number of upregulated genes, with the majority being downregulated in Coco. Furthermore, the hierarchical clustering heatmap, illustrating the grouping pattern, demonstrated that *G. locusta* feeding on

either Carrot or Coco resulted in a largely similar gene expression pattern. This pattern notably differed from the control, as evident from the grouped columns in the dendrogram (Figure 5.3a). Moreover, the heatmap results identified five sub-clades (Figure 5.3a, orange dots) encompassing all differentially expressed genes detected (FDR $p < 0.1$ and log2FC ≥ 2).

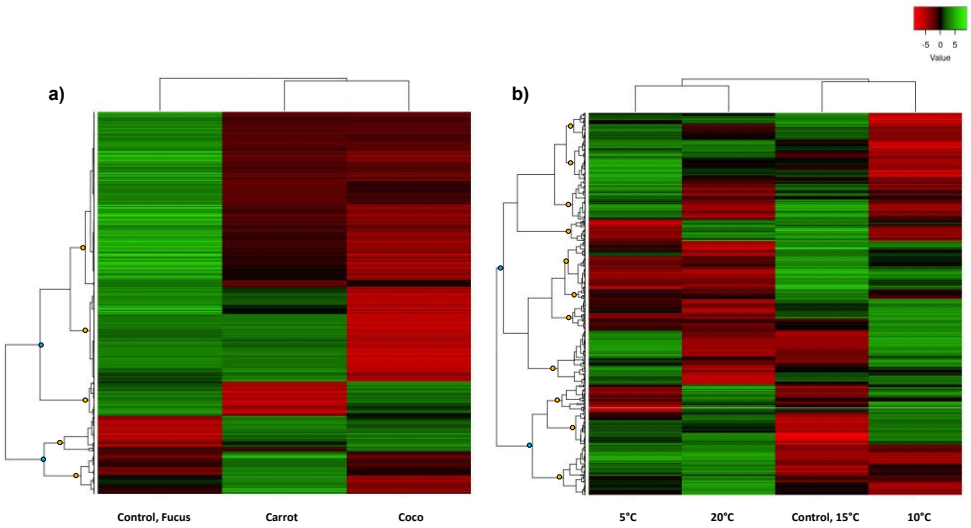

Figure 5.3. Hierarchical heatmap of differentially expressed genes among *G. locusta* cultured under different conditions. a) Heatmap corresponding to gammarids reared at 15 °C and fed either on Fucus, Carrot or Coco. **b)** Heatmap corresponding to gammarids fed on Fucus and reared at different temperatures (5, 10, 15, and 20 °C). Major clades are highlighted by blue dots, whereas sub-clades are highlighted by orange dots.

Pathway enrichment analyses, based on the KEGG database, revealed that in Experiment 1, "Metabolic Pathways" exhibited the highest number of enriched genes (773 genes, FDR $p < 0.001$, Fold Enrichment = 2.74) (Supplementary Table S5.4: Enrichment Experiment 1). Subsequently, our focus shifted to pathways specifically associated with fatty acid metabolism and growth, aiming to elucidate the findings on fatty acid composition and growth reported for *G. locusta* fed the investigated diets (Ribes-Navarro *et al.*, 2022). The selected pathways for fatty acid metabolism included: "Fatty acid metabolism" (38 genes, FDR $p < 0.001$, Fold Enrichment = 2.92), "Fatty acid degradation" (31 genes, FDR $p < 0.001$, Fold Enrichment = 3.17), "Fatty acid biosynthesis" (10 genes, FDR $p < 0.001$, Fold Enrichment = 3.08), "Biosynthesis of unsaturated fatty acids" (11 genes, FDR $p < 0.05$, Fold Enrichment = 1.88), "Fatty

acid elongation" (11 genes, FDR $p < 0.05$, Fold Enrichment = 1.95), and "Arachidonic acid metabolism" (15 genes, FDR $p < 0.01$, Fold Enrichment = 1.82). As for growth, the enriched pathways were: "Insect hormone biosynthesis" (8 genes, FDR $p < 0.01$, Fold Enrichment = 2.64), and "TGF-β signalling pathway (22 genes, FDR $p < 0.001$, Fold Enrichment = 2.74) (Figure 5.4a) (Supplementary Table S5.4).

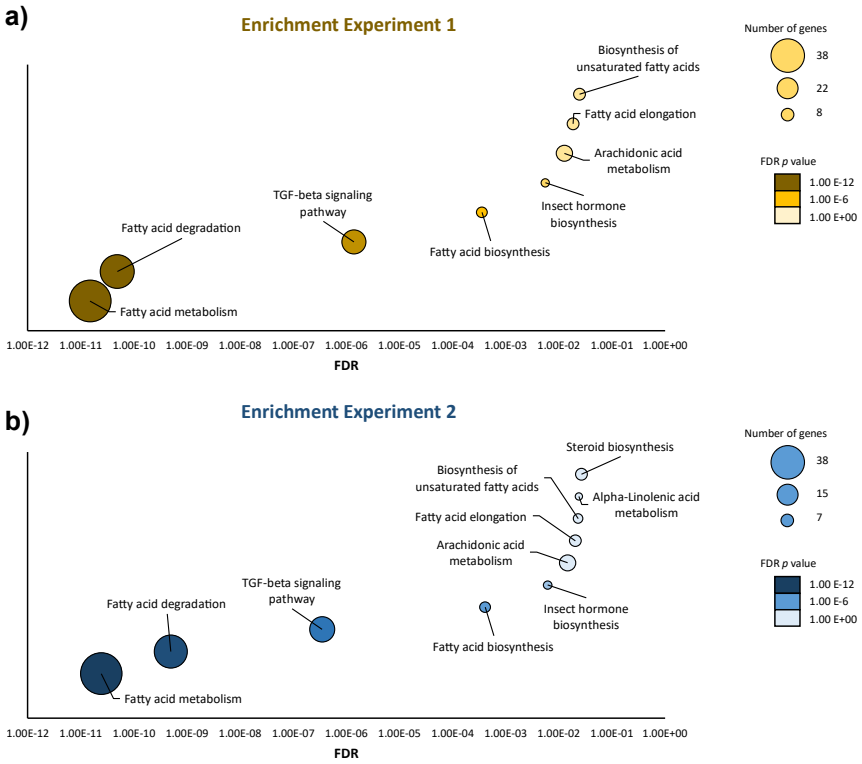

Figure 5.4. Pathway enrichment analysis was conducted independently for both Experiment 1 (a) and Experiment 2 (b). In the visual representation, circle size corresponds to the number of genes enriched in each pathway. Additionally, circle colour indicates the False Discovery Rate (FDR) with corrected p-value, providing a comprehensive overview of pathway significance.

5.3.3. Differentially expressed genes in Experiment 2 (effect of temperature)

For the DGE analyses in Experiment 2, *G. locusta* individuals, fed on Fucus and reared at three distinct temperatures (5, 10, and 20 °C), were compared to the control

condition, which involved *G. locusta* fed on Fucus and reared at 15 °C. Based on the number of differentially expressed genes, a cumulative total of 552 genes met the statistical threshold criteria (FDR $p < 0.1$ and log2FC ≥ 2) in *G. locusta* reared under any of the three temperature conditions. Individually, a total of 537, 545, and 540 genes exhibited differential expression in *G. locusta* cultured at 5, 10, and 20 °C, respectively, when compared to the control treatment (Table 5.1). The condition corresponding to 10 °C displayed the highest number of upregulated genes (323) and the lowest number of downregulated genes (222) when compared to the control (Table 5.1). Conversely, the condition at 20 °C exhibited the opposite pattern, with the highest number of downregulated genes (256) and the lowest number of upregulated genes (284) compared to the control (Table 5.1). Among all the genes detected in the comparison of different temperatures, 518 genes (93.84%) were found to be differentially expressed across all three temperature conditions (5, 10, and 20 °C), while no genes were specifically differentially expressed in only one condition (Figure 5.2b).

The heatmap, illustrating the DGE patterns in *G. locusta* reared at different temperatures, revealed that those cultured at 5 and 20 °C exhibited a similar gene expression pattern, while those reared at 10 °C displayed an expression pattern closer to the control group (15 °C) (Figure 5.3b), as indicated by the dendrogram showing column distribution. Furthermore, the heatmap corresponding to Experiment 2 demonstrated that all differentially expressed genes detected were grouped into 11 distinct sub-clades, as delineated by the row dendrogram (Figure 5.3b, orange dots). Additionally, the Control column exhibited a notable pattern in the upregulated vs downregulated genes, reflected by two major clades in the row dendrogram (Figure 5.3b, blue dots).

Pathway enrichment analyses, based on the KEGG database, demonstrated that in Experiment 2, "Metabolic Pathways" exhibited the highest number of enriched genes (786 genes, FDR $p < 0.001$, Fold Enrichment = 2.74) in (Supplementary Table S5.5: Enrichment Experiment 2). Similar to Experiment 1, only specific pathways were further analysed from the overall enrichment pathways (Figure 5.4b, Supplementary Table S5.5). In particular, the pathways chosen for fatty acid metabolism were: "Fatty acid metabolism" (38 genes, FDR $p < 0.001$, Fold Enrichment = 2.88), "Fatty acid degradation" (30 genes, FDR $p < 0.001$, Fold Enrichment = 3.03), "Fatty acid biosynthesis" (10 genes, FDR $p < 0.001$, Fold Enrichment = 3.03), "Biosynthesis of unsaturated fatty acids" (11 genes, FDR $p < 0.05$, Fold Enrichment = 1.85), "Fatty acid elongation" (11 genes, FDR $p < 0.05$, Fold Enrichment = 1.92), "Alpha-Linolenic acid metabolism" (7 genes, FDR $p < 0.05$, Fold Enrichment = 2.27), and "Arachidonic acid metabolism" (15 genes, FDR $p < 0.05$, Fold Enrichment = 1.79). Regarding growth processes, the enriched pathways selected were as follows: "Steroid biosynthesis" (9

genes, FDR $p < 0.05$, Fold Enrichment = 2.04), "Insect hormone biosynthesis" (8 genes, FDR $p < 0.01$, Fold Enrichment = 2.59), and "TGF-β signalling pathway (23 genes, FDR $p < 0.001$, Fold Enrichment = 2.83) (Figure 5.4b, Supplementary Table S5.5).

5.3.4. Differential expression of genes with roles in fatty acid metabolism

To offer molecular understanding to the compositional responses of *G. locusta* fed different diets and cultured at different temperatures (Ribes-Navarro *et al*. 2022), we analysed the regulation of a selection of genes with roles in fatty acid metabolism using our RNASeq datasets. More specifically, we examined the expression of the following genes: elongation of very long-chain fatty acids protein 6 and 1/7-like (*elovl6*, *elovl1/7*-like), stearoyl-CoA desaturase (Δ9 desaturase, *d9d*), fatty acid synthase (*fas*), fatty acid binding protein (*fabp*), diacylglycerol-acyltransferase (*dgat*), carnitine palmitoyltransferase 1A (*cpt1a*), acyl-CoA synthetase (*acs*), acyl-CoA thioesterase (*acot*), and acetyl-CoA carboxylase (*acc*). Moreover, two transcription factors with major roles in fatty acid and lipid metabolism, namely sterol regulatory element-binding protein (*srebp1*) and the nuclear factor kappa b (*NFκB*), were also analysed. All data were expressed as relative expression with respect to the control (*G. locusta* fed on Fucus and reared at 15 °C). Concerning Experiment 1, gammarids fed on either Carrot or Coco had similar expression patterns of all selected genes, except for *acot*, that was downregulated in gammarids fed on the Carrot diet, and *fabp*, which was upregulated in *G. locusta* fed the Carrot diet (Figure 5.5a). Concerning the two transcription factors, both *srebp1* and *NFκB* were upregulated in gammarids fed either on Carrot or Coco (Figure 5.5a). Regarding Experiment 2, *G. locusta* reared at 5 °C displayed upregulation in all the fatty acid metabolic genes considered herein compared to the control, except for *elovl1/7*-like, which was downregulated (Figure 5.5b). Interestingly, gammarids reared at 10 °C demonstrated the same expression pattern as those reared at 5 °C, except for *acot* and *dgat*, both of which were downregulated when gammarids were reared at 10 °C (Figure 5.5b). As for 20 °C, gammarids reared at this temperature showed upregulation in all the genes analysed except for *elovl6* and *dgat*, which were both downregulated (Figure 5.5b). Conversely, the transcription factor *srebp1* was upregulated in all tested temperatures, both above and below 15 °C (control), whereas *NFκB* was downregulated in both 5 and 10 °C, and upregulated at 20 °C (Figure 5.5b).

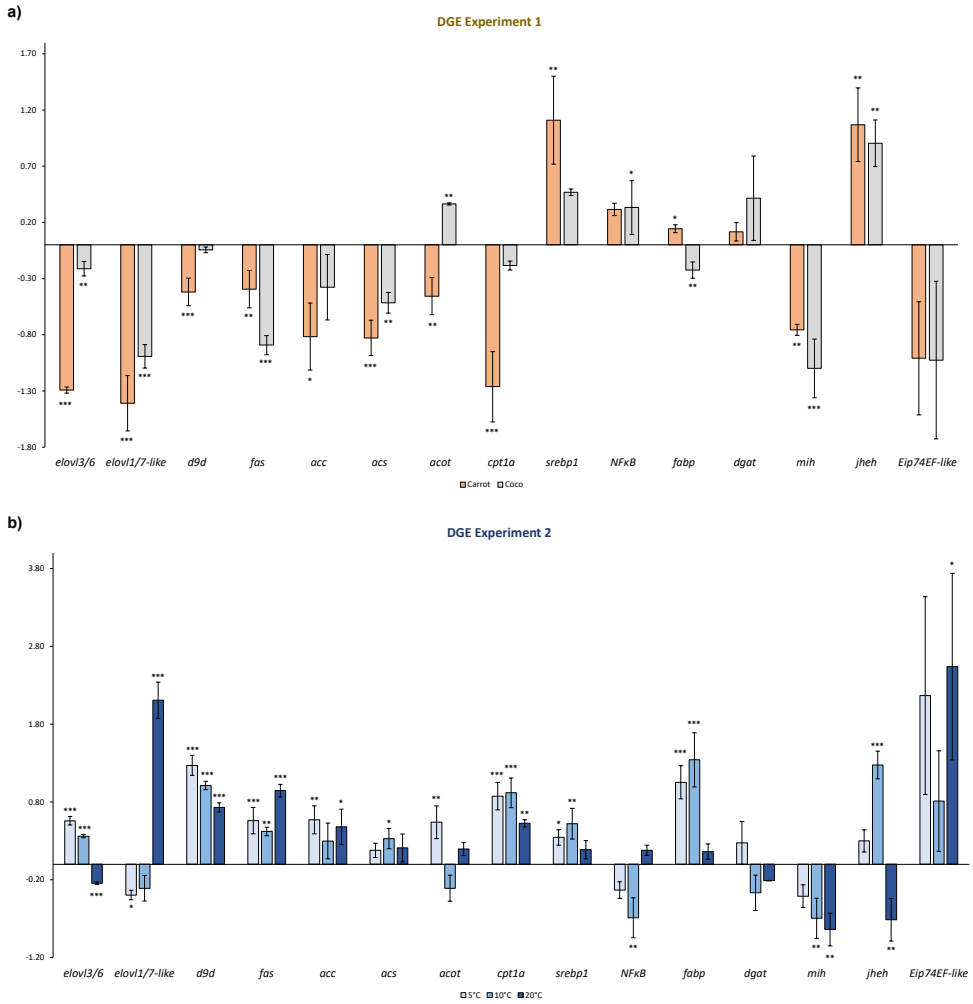

Figure 5.5. Graphical representation of the selected genes and transcription factors that are differentially expressed (FDR, $p < 0.05$ and log2FC ≥ 2) in *G. locusta* fed on different diets (a) and reared at different temperatures (b). Statistical differences (Dunnett's multiple comparisons, *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) are indicated by asterisks above or below their corresponding bars.

5.3.5. Differential expression of genes with roles in moulting

Ribes-Navarro *et al*. (2022) showed differences in growth of *G. locusta* fed on different diets and reared at different temperatures. Since moulting is a major

developmental event in crustaceans with a key impact in growth and survival, we further used the RNASeq analyses to investigate the regulation of genes involved in moulting to gain insight into potential interactions between the moulting process itself and lipid metabolism and accumulation (Kuballa *et al*., 2011; Tao *et al*., 2014; Jordão *et al*., 2015; Lemos and Weissman, 2021). Particularly, the following genes were considered: moult inhibiting hormone (*mih*), juvenile hormone epoxide hydrolase (*jheh*), and the transcription factor ecdysone-induced protein 74EF-like (*Eip74EF*-like). As stated above, all data were expressed as relative expression to the control (*G. locusta* fed on Fucus and reared at 15 °C). Regarding Experiment 1, both *mih* and *Eip74EF*-like were downregulated in gammarids fed either on Carrot or Coco, although *Eip74EF*-like results were not statistically significant ($p > 0.05$) (Figure 5.5a). On the contrary, *G. locusta* fed on either Carrot or Coco showed an upregulation of the *jheh* gene (Figure 5.5a). As for Experiment 2, the results showed that gammarids reared at 5, 10, and 20 °C had *mih* downregulated (Figure 5.5b). Particularly, those reared at 5 and 10 °C displayed upregulation in *jheh*, whereas those reared at 20 °C exhibited the opposite pattern (Figure 5.5b). As for *Eip74EF*-like expression, significant differences were observed only in gammarids reared at 20 °C compared to the control, with an upregulation observed in individuals reared at this temperature (Figure 5.5b).

5.4. Discussion

The present study investigated the effects of different diets and temperatures in the expression of key metabolic genes involved in fatty acid metabolism in the marine gammarid *G. locusta*. Besides, the transcriptomic data provided molecular insight into other developmental processes such as moulting. The final version of the transcriptome enabled the accurate annotation of approximately 31.82% of transcripts, matching (Naumenko *et al*., 2017; Neuparth *et al*., 2020a; Lipaeva *et al*., 2021) and in some cases improving previous results from other gammarid transcriptomes (Gismondi and Thomé, 2016; Cogne *et al*., 2019; Drozdova *et al*., 2019; Caputo *et al*., 2020).

5.4.1. Effect of different diets in the expression of key metabolic genes of fatty acid metabolism and moulting

Gammarids fed on either the Carrot or Coco diets showed the highest number of downregulated genes. It is likely that several of these downregulated genes are related to metabolic processes that enhance development and promote survival, since it has been reported that gammarids fed on those diets showed impaired growth and higher mortality as compared to those fed on Fucus (Ribes-Navarro *et*

al., 2022). Indeed, the results for the enrichment analyses support that many enriched genes were involved in several developmental processes such as the synthesis of moulting hormones, as well as fatty acid metabolism, which are directly related to proper growth and development (Swanson *et al*., 2012; Lee *et al*., 2018; Tocher *et al*., 2019). This hypothesis was further supported by the DGE analysis performed on selected genes confirming downregulation in gammarids fed on both the Carrot and Coco diets as compared to Fucus.

Regarding the expression of genes involved in fatty acid biosynthesis (Monroig *et al*., 2022), only *fas*, *d9d*, *elovl6,* and *elovl1/7*-like, were found to be differentially expressed. Intriguingly, no front-end or methyl-end desaturase sequences were identified in the transcriptome of *G. locusta*. This absence has also been reported in other marine gammarids such as *E. marinus* (Ribes-Navarro *et al*., 2021) and in other crustaceans like *Artemia franciscana* (Ramos-Llorens et al., 2023b). This raises a paradoxical observation that, despite not having desaturases, gammarids still maintain moderate levels of LC-PUFA such as EPA and DHA in their lipid profile even when fed on diets devoid of these compounds like the herein investigated Carrot and Coco (Kolanowski *et al*., 2007; Baeza-Rojano *et al*., 2014; Ribes-Navarro *et al*., 2022). Thus, the detection of these compounds in the lipid profile of *G. locusta* is due to other factors and mechanisms, such as the retention from pre-experiment diet and/or selective retention as demonstrated in the freshwater amphipod *Pallaseopsis quadrispinosa*, which stores essential fatty acids such as EPA before periods of reduced food availability (Taipale *et al*., 2021). Exogenous sources of LC-PUFA that could have also contributed to the *G. locusta* lipid profile include the consumption of microzooplankton with high LC-PUFA content present in the water, such as copepods and, to a lesser extent rotifers (Miller *et al*., 2008; Dhont *et al*., 2013; Boyen *et al*., 2020; Kabeya *et al*., 2021). Microbiota has been suggested as a potential contributor of LC-PUFA in other crustaceans such as the seasermid crab *Parasesarma erythodactyla* (Bui and Lee, 2015), and more recently in the mud crab *Scylla olivacea* (Lau *et al*., 2022). In the latter, bacteria from *Shewanella* and *Vibrio* genera have been identified in the microbiota of crabs fed with a LC-PUFA poor diet. These microbial species are known to be natural *de novo* producers of LC-PUFA through anaerobic pathways (Metz *et al*., 2001; Nichols and McMeekin, 2002). On the other hand, gammarids fed on either Carrot or Coco showed an upregulation of the *srebp1* transcription factor, which is related to an increase in lipid biosynthesis, particularly triacylglycerides (TAG) (Walker *et al*., 2011; Xu *et al*., 2016). Moreover, in gammarids fed on those diets, there was also an upregulation of the *NFκB* transcription factor, known for downregulating the lipid and fatty acid catabolism (Huang and Freter, 2015; Minegishi *et al*., 2015; Aboonabi *et al*., 2020). Consistently, the DGE analyses showed a downregulation of *cpt1a*, which codifies for the enzyme responsible for the

transport of fatty acids into mitochondria and subsequent *β*-oxidation (catabolism) (Schulz, 1991; Houten and Wanders, 2010), and an upregulation of the *dgat* gene that codifies for a protein responsible for TAG biosynthesis (Koch *et al*., 2023). Furthermore, in gammarids fed on Coco there is a downregulation of a fatty acid binding protein (FABP), a protein family present mainly in the adipose tissue and responsible for the transport of fatty acids away from that tissue for further catabolism (Furuhashi *et al*., 2014, 2016; Floresta *et al*., 2017; Furuhashi, 2019). Interestingly, *G. locusta* fed on Carrot showed the opposite pattern regarding *fabp* expression. This may be attributed to the higher amount of linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) of this diet (Ribes-Navarro *et al*., 2022), as these fatty acids are the preferred ligands for FABP4, therefore promoting its gene expression (Furuhashi *et al*., 2016; Furuhashi, 2019). Thus, the Coco diet impairs the transport of fatty acids, and promotes their accumulation in *G. locusta*, presumably in the TAG fraction, resulting in a reduced catabolism.

In a previous experiment (Ribes-Navarro *et al*., 2022), *G. locusta* showed impaired growth and lower survival when fed specially with Coco, even though less biomass generation was also detected when fed with Carrot. This is consistent with the findings in the present study, especially regarding the expression of genes related to moulting processes, such as *mih* and *jhej*. The *mih* downregulation was much more pronounced in those gammarids fed on Coco than on those feds on Carrot. Interestingly, Coco diet is very rich in SFA, known to induce obesity and increase fatty acid and lipid accumulation, especially TAG (Hariri *et al*., 2010; Phillips *et al*., 2012; Zhou *et al*., 2020). Since moulting pre stages are related with an accumulation of TAG (Cuzin-Roudy *et al*., 1999; Kuballa *et al*., 2011; Jordão *et al*., 2015; Fuertes *et al*., 2018; de Oliveira *et al*., 2019; Lemos and Weissman, 2021), it is likely that excess of TAG and cholesterol due to obesity lead gammarids to enter earlier into moult stages by decreasing *mih* expression, and thus, preventing the release of the moult inhibiting hormone (Chung and Webster, 2005; Nakatsuji *et al*., 2009; Mykles, 2021). Several cases of premature moulting due to dietary cholesterol have already been reported in other crustaceans such as the swimming crab *Portunus trituberculatus* (Han *et al*., 2015), the Chinese mitten crab *Eriocheir sinensis* (Tao *et al*., 2014) and, more recently, in the red swamp crayfish *Procambarus clarkii* (Tian *et al*., 2020). On the other hand, the *jheh,* which codifies for the enzyme responsible for the degradation of the juvenile hormone (JH) and, thus the acceleration of the moulting processes (Tusun *et al*., 2017), could be potentially upregulated in gammarids fed either Carrot and Coco, especially in the last one. This is in agreement with the hypothesis that Coco diet can lead gammarids to accelerate their development, resulting in earlier mortality.

5.4.2. Effect of different temperatures in the expression of key metabolic genes for fatty acid metabolism and moulting

G. locusta reared at extreme temperatures (5, and 20 °C) exhibited a similar gene clustering pattern, as indicated by the column dendrogram displayed in the hierarchical heatmap. Interestingly, lower temperatures (5 °C) have been shown to induce a state of hibernation ("diapause") in gammarids that results in a lowered metabolism (Axenov-Gribanov *et al*., 2016; Lipaeva *et al*., 2021). Conversely higher temperatures (20 °C) lead to an acceleration of the life cycle and growth, therefore increasing metabolic rates (Neuparth *et al*., 2002; Ribes-Navarro *et al*., 2022). However, some researchers have stated that some poikilothermic animals compensate the decreased enzymatic activity due to cold temperatures by enhancing the expression of genes related with ribosome biogenesis, mRNA processing and DNA and RNA binding and replication (Gracey *et al*., 2004; Ronges *et al*., 2012; Lipaeva *et al*., 2021) (Table S2). This compensatory up-regulation is one of the reasons of the similar gene clustering within 5 and 20 °C, despite the opposite metabolic rates. In order to study these metabolic differences within temperatures, particularly in fatty acids (Ribes-Navarro *et al*., 2022), and growth rates, genes related to these processes were regarded as the main focus. Consistently, enrichment analyses showed similar results to those observed with diets, with genes related to fatty acid metabolism and moulting found to be differentially expressed.

Regarding the DGE of genes responsible for fatty acid metabolism, the same set of genes as in the diets was detected and further analysed following the same pipeline. In this context, *elovl6* showed an upregulation at cold temperatures (5, and 10 °C) and a downregulation when gammarids were reared at 20 °C. In gammarids, this gene has been reported to be an ancestral orthologue of both *elovl6* and *elovl3* from vertebrates (Ribes-Navarro *et al*., 2021), being the latter directly related with the biosynthesis of saturated and monounsaturated very long-chain fatty acids (>C_{24}, VLCFA), and their further accumulation in the brown adipose tissue (BAT) of mice (Tvrdik *et al*., 1997; Westerberg *et al*., 2006). Interestingly, studies in mice have shown that *elovl3* expression is modulated by temperature, resulting in an upregulation when animals are exposed to cold temperatures and need a higher neat heat production in the BAT (Tvrdik *et al*., 1997; Westerberg *et al*., 2006; Jörgensen *et al*., 2007). Consistently, the *cpt1a* expression was highest when gammarids where reared at 5 °C, supporting the hypothesis of higher metabolic rates and increased energy release at lower temperatures, since *cpt1a* upregulation results in an increased fatty acid catabolism (Schulz, 1991; Houten and Wanders, 2010; Tang *et al*., 2013). Another result that reinforces the previous hypothesis is that *fabp* is upregulated in *G. locusta* reared at cold temperatures (5, and 10 °C), which implies an enhanced fatty acid and

lipid transport and further catabolism, as it also happens in rodents (Yamashita *et al*., 2008; Vergnes *et al*., 2011) and birds (Zhang *et al*., 2018). On the contrary, *elovl1/7*-like showed the opposite pattern to *elovl3/6*, with downregulation at 5 and 10 °C and upregulation at 20 °C. Since Elovl1/7-like shares an ancestor with both Elovl1 and Elovl7, which emerged after duplication in vertebrates, and mimics the function of those enzymes and the roles of Elovl3 (Guillou *et al*., 2010; Deák *et al*., 2019), a similar gene expression pattern should be expected, but it was not the case in this work. However, it has been recently reported that in species such as the naked carp (*Gymnocypris przewalskii*), that inhabit stressful environments, a turnover on the expression of this genes can occur in order to maintain fatty acid and lipid homeostasis for a proper fluidity of the cell membrane (Liu *et al*., 2022a). Regarding *d9d*, *fas*, *acc*, and *acs* genes, their expression was upregulated in all the temperatures tested, indicating a higher fatty acid biosynthesis (Wakil and Abu-Elheiga, 2009; Ellis *et al*., 2010; Guillou *et al*., 2010; Tang *et al*., 2013; Monroig *et al*., 2018). Additionally, in gammarids reared at any of the temperatures tested, there was also an upregulation of the *srebp1* transcription factor, related to an increase in lipid biosynthesis, particularly triacylglycerides (TAG) (Walker *et al*., 2011; Xu *et al*., 2016).

Regarding the DGE of genes with functional roles in moulting, the expression of *mih* was downregulated in all the temperatures tested. Previous studies have reported faster growth at higher temperatures for gammarids such as *G. locusta* (Neuparth *et al*., 2002; Ribes-Navarro *et al*., 2022) and *G. roeselii* (Pöckl, 1992). Interestingly, a recent research has found that there is a progressive increase in moulting from 5 to 20 °C in the Dungeness crab *Metacarcinus magister* (Wittmann *et al*., 2018). This finding is in agreement with the gradual downregulation of *mih* of *G. locusta* reared at either 5, 10 or 20 °C, with the expression of *mih* at 20 °C being significantly lower than at the other temperatures. This downregulation explains the increased and faster growth reported previously by Ribes-Navarro *et al*., (2022). Conversely, *jheh* was downregulated only in gammarids reared at 20 °C. The downregulation of *jheh* is echoed by an increased mortality, as observed in a previous work by Ribes-Navarro *et al*., (2022), due to the incapability for gammarids to progress into later developmental stages caused by a lack of JH degradation (Tusun *et al*., 2017). On the contrary, *G. locusta* reared at 10 °C also enter earlier into moulting stages, but the upregulation of *jheh* in this condition is not sufficient to interrupt the cycle, and does not alter their survivability. Upregulation of *Eip74EF* expression when *G. locusta* were reared at 20 °C is associated with an increase in growth and development, since Eip74EF-like and other ecdysone-induced proteins induce arthropods into metamorphosis/moulting and subsequent growth and development (Fletcher *et al*., 1995; Guo *et al*., 2016; Wang *et al*., 2021; Wu *et al*.,

2022). Moreover, this finding correlates as well with the higher growth found in *G. locusta* reared at 20 °C (Ribes-Navarro *et al*., 2022).

5.5. Conclusions

In summary, the present study sheds light on the modulating effects of diet and temperature in the expression of genes involved in fatty acid metabolism and moulting in the marine gammarid *G. locusta*. More specifically, the DGE results indicate that when *G. locusta* is fed on LC-PUFA depleted diets, especially Coco, there is a reduction in the fatty acid catabolism and transport, leading to a higher accumulation of fatty acids, likely in the adipose tissue and TAG fraction. Furthermore, when gammarids are reared at 20 °C there is a downregulation of *jheh* expression that results into a faster growth and increased mortality caused by premature moulting that results incomplete due to an impairment in the JH degradation. Overall, the findings from this study provide a molecular insight into the pathways that modulate fatty acid metabolism and development in gammarids. These insights are important for optimizing culture conditions, especially in terms of LC-PUFA accumulation and growth, thereby enhancing the potential of these crustaceans for their application as aquaculture feeds.

5.6. Data availability

The unprocessed datasets have been submitted to the NCBI Sequence Read Archive as part of BioProject PRJNA1034037. For the additional data, it has been made available on figshare (temporary reviewer link: https://figshare.com/s/214745bd0376cfe7b589). Specifically, the files shared on figshare encompass various elements, including different versions of transcriptome assemblies, transcript files, messenger RNA files, open reading frame predictions, as well as annotation files. All software, versions, and parameters can be found in the Materials and Methods section. Software applications without associated parameters were employed with their default settings.

5.7. References

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6.
Discusión general

La acuicultura se ha establecido durante los últimos años como una fuente cada vez más importante de alimentos derivados de organismos acuáticos (APROMAR, 2021; FAO, 2022). Los productos de acuicultura son, junto a los de la pesca, la principal fuente de ácidos grasos poliinsaturados de cadena larga (C_{20-24}, LC-PUFAs) n-3 en la nutrición humana. Estos nutrientes son de suma importancia a nivel fisiológico, asegurando el correcto crecimiento y desarrollo en fases tempranas de vertebrados; además, su ingesta trae consigo efectos beneficiosos, entre otros, a nivel de la salud cardiovascular y de enfermedades de tipo inflamatorio (Swanson *et al*., 2012; Calder, 2014, 2018). En un escenario de expansión de la acuicultura, sobre todo a través de la producción de especies carnívoras, y de limitación de materias primas ricas en LC-PUFAs n-3 (harinas y aceites de pescado) para la manufactura de piensos, es imperativo buscar nuevas fuentes de estos compuestos que permitan garantizar su aporte tanto en las primeras fases de cría, para prevenir deficiencias nutricionales que comprometen el correcto desarrollo de las especies cultivadas, como en la fase de engorde, para que los productos acuícolas sigan siendo fuentes importantes de LC-PUFAs n-3 para el consumidor.

Esta Tesis Doctoral ha tenido por objetivo principal investigar la capacidad biosintética de LC-PUFAs n-3 en gamáridos, para poder determinar si el cultivo de estos crustáceos con materiales de desecho de diversas industrias y de escaso valor nutricional, permite obtener biomasas ricas en LC-PUFAs n-3 que, en última instancia, puedan ser procesadas como ingredientes para piensos de acuicultura. La elección de los gamáridos se ha sustentado en sus perfiles nutricionales, con niveles de LC-PUFAs razonablemente altos (Kolanowski *et al*., 2007; Baeza-Rojano *et al*., 2010, 2013, 2014; Jiménez-Prada *et al*., 2018), así como en su fácil cultivo, rápido crecimiento y hábitos principalmente detritívoros que permiten su alimentación con productos de composición variada (Costa y Costa, 2000; Baeza-Rojano *et al*., 2014; Harlıoğlu y Farhadi, 2018). Para alcanzar el objetivo previsto, en primer lugar, se ha determinado la dotación y funciones de las enzimas que componen las rutas de biosíntesis de LC-PUFAs existentes en gamáridos (Capítulos 2 y 3). En segundo lugar, con el fin de optimizar la producción de biomasa y el valor nutricional de ésta (contenido en LC-PUFAs n-3), se ha estudiado el efecto modulador que ejercen la composición de la dieta y la temperatura sobre el metabolismo de ácidos grasos, crecimiento y supervivencia de estos organismos (Capítulos 4 y 5). Los siguientes apartados abordan la discusión integrada de los resultados obtenidos en los experimentos realizados.

6.1. Capacidad biosintética de LC-PUFAs en gamáridos

Los estudios previos a la realización de la presente Tesis Doctoral habían sugerido que algunas especies de gamáridos tenían cierta capacidad para biosintetizar LC-PUFAs (Sushchik *et al*., 2003; Maazouzi *et al*., 2007; Alberts-Hubatsch *et al*., 2019) y, de esta manera, eran capaces de contribuir al enriquecimiento trófico ("*trophic upgrading*") en los ecosistemas acuáticos (Arts *et al*., 2009). Estas conclusiones sobre la potencial capacidad de gamáridos para producir LC-PUFAs se sostenían en evidencias indirectas basadas en análisis de ácidos grasos, pero en ningún caso se apoyaban de argumentos sólidos y directos de tipo bioquímico y/o molecular que demostraran tal capacidad metabólica. Los estudios sobre caracterización molecular y funcional de los genes de desaturasas y elongasas existentes en un organismo están considerados como la metodología más adecuada para estudiar la capacidad de biosíntesis de LC-PUFAs de dicho organismo ya que, al contrario que en otras aproximaciones experimentales como los ensayos bioquímicos con sustratos marcados, obvia la posible contribución de otros organismos (p. ej., microorganismos, simbiontes, etc.) a la actividad biosintética medida. Ésta ha sido la motivación que ha impulsado el abordaje metodológico usado en los Capítulos 2 y 3 de la presente Tesis Doctoral.

La búsqueda de secuencias que codifican para desaturasas *methyl-end* (ωx-des), desaturasas *front-end* (Fed) y elongasas de ácidos grasos (Elovl) realizada en los transcriptomas de gamáridos disponibles en bases de datos públicas, ha mostrado un patrón de genes aparentemente muy distinto entre especies marinas y dulceacuícolas (Capítulos 2 y 3). Estudios realizados en peces han mostrado que, en determinadas familias de teleósteos, existe una mayor capacidad de biosíntesis de LC-PUFAs en especies dulceacuícolas en comparación con especies marinas (Ishikawa *et al*., 2019; Matsushita *et al*., 2020), aludiendo a la necesidad de los primeros por conservar mecanismos de producción endógena de LC-PUFAs en ambientes con escasa abundancia de estos nutrientes esenciales (Colombo *et al*., 2016; Twining *et al*., 2021). Sin embargo, no deja de ser sorprendente observar un patrón de genes de desaturasas y elongasas tan distinto entre gamáridos marinos y dulceacuícolas, más si cabe cuando, en muchos casos, tales diferencias tienen lugar entre especies del mismo género (p. ej., *Gammarus*), que son difíciles de explicar desde el punto de vista evolutivo. Nuestros análisis moleculares han permitido aclarar esta cuestión al demostrar que la dotación de genes implicados en la biosíntesis de LC-PUFAs de gamáridos, independientemente del hábitat que ocupan, está muy conservada y se limita exclusivamente a elongasas (*elovl*), mientras que las desaturasas (*ωx*-des, *fed*) están ausentes en este grupo biológico.

Los gamáridos poseen tres elongasas diferentes. Su caracterización molecular y relación filogenética con respecto a los correspondientes ortólogos de vertebrados (Monroig *et al*., 2022) nos ha llevado a nombrarlas Elovl4, Elovl1/7-like y Elovl6. Este número y tipología de Elovl está conservado en otros crustáceos como los decápodos (Lin *et al*., 2018; Mah *et al*., 2019; Sun *et al*., 2020; Ting *et al*., 2020). La caracterización funcional de las elongasas de gamáridos ha revelado que, por un lado, Elovl4 y Elovl1/7-like juegan papeles importantes en la biosíntesis de LC-PUFAs, tal y como se desprende de su capacidad para elongar sustratos PUFAs, mientras que Elovl6 no parece tener una función relevante en la biosíntesis de LC-PUFAs. Según las rutas que rigen la biosíntesis de LC-PUFAs en animales (Figura 1.7), las funciones detectadas en las Elovl4 y Elovl1/7-like de gamáridos cubren todas las reacciones de elongación necesarias que exige la conversión de los precursores de C_{18}, ácido linoleico (LA, 18:2n-6) y α-linolénico (ALA, 18:3n-3), a LC-PUFAs n-6 y n-3, respectivamente (Monroig y Kabeya, 2018; Monroig *et al*., 2022) (Capítulo 2). No obstante, a pesar de dicha capacidad de elongación, la ausencia de genes de desaturasas, tanto ωx-des como Fed, permite concluir que los gamáridos no pueden biosintetizar LC-PUFAs, al menos a través de los mecanismos de producción endógenos que se conocen en la actualidad, y que se aceptan como válidos en el Reino Animal (Figura 1.7). Esta conclusión contradice en parte los resultados obtenidos en otras investigaciones sobre la aparente capacidad biosintética de los gamáridos (Sushchik *et al*., 2003; Maazouzi *et al*., 2007; Alberts-Hubatsch *et al*., 2019). Aparentemente, el estudio que afirma de forma más categórica que los gamáridos pueden biosintetizar LC-PUFAs es el de Alberts-Hubatsch *et al*. (2019). En este trabajo, se concluye que los gamáridos *Gammarus locusta* y *Echinogammarus marinus* tienen la capacidad para realizar *trophic upgrading* en base a la presencia de ciertos LC-PUFAs como el ácido eicosapentaenoico (EPA, 20:5n-3) y docosahexaenoico (DHA, 22:6n-3) en los lípidos de animales alimentados con dietas carentes de estos compuestos. Sin embargo, el estudio no muestra datos sobre el contenido de estos nutrientes al comienzo del experimento y, por tanto, no es posible asegurar que existe una producción neta de EPA y DHA como requería tal conclusión. Para demostrar que un animal puede biosintetizar un compuesto (p. ej., EPA, DHA) debe realizarse un balance de masas, para el que es necesario conocer los contenidos de éste en el animal, tanto al comienzo como al final del periodo experimental y, además, la dosis del compuesto en el alimento ingerido durante el experimento. Esta aproximación metodológica, que permite el cálculo de los denominados en inglés "*fatty acid production value*" o "*whole body mass balance method*", se ha utilizado para establecer, por ejemplo, la capacidad biosintética de LC-PUFAs en peces de cultivo como el salmón del Atlántico (*Salmo salar*) (Stubhaug

et al., 2007; Turchini *et al*., 2007; Sprague *et al*., 2019), pero no en gamáridos ni en ningún otro invertebrado acuático.

Como se ha indicado a lo largo de esta Tesis Doctoral, las rutas bioquímicas que permiten a los animales biosintetizar LC-PUFAs dependen de la acción coordinada de desaturasas y elongasas según una serie de reacciones que han sido establecidas con numerosos estudios en vertebrados y, más recientemente, en invertebrados (Apartado 1.4.1; Figura 1.7). A pesar del consenso que existe en la comunidad científica al considerar éstas como las únicas rutas que explican la biosíntesis de LC-PUFAs en animales, no se puede descartar completamente la existencia de mecanismos alternativos que son, a día de hoy, desconocidos. En este sentido, no es extraño encontrar reseñas en la bibliografía sobre especies de crustáceos que, como los gamáridos, carecen de desaturasas de los tipos aquí estudiados (ωx-des y Fed) y que, a pesar de ello, pueden producir PUFAs incluidos ciertos LC-PUFAs (Farkas *et al*., 1981; Schauer y Simpson, 1985; Ito y Simpson, 1996). Este es el caso, por ejemplo, del cladócero *Daphnia* y del branquiópodo *Artemia*, en los que se ha concluido que pueden biosintetizar LC-PUFAs como el EPA, a partir tanto de sustratos de cadenas más cortas como el LA, como de ácidos grasos saturados (p.ej., el ácido palmítico; 16:0), a pesar de que en ninguno de estos organismos se haya podido demostrar la presencia de desaturasas ωx-des y Fed (Ramos-Llorens *et al*., 2023b). Hasta la fecha tampoco se ha podido confirmar la existencia de desaturasas ωx-des y Fed en malacostráceos, clase de crustáceos donde se encuentran los decápodos, y en los que se centran varias investigaciones relevantes en este contexto. En estudios llevados a cabo en especies de interés comercial como *Cherax quadricarinatus* (Wu *et al*., 2018), *Scylla paramamosain* (Lin *et al*., 2017), *Macrobrachium nipponense* (Luo *et al*., 2018), *Litopenaeus vannamei* (Chen *et al*., 2017) y *Eriocheir sinensis* (Yang *et al*., 2013), se ha descrito la presencia de un tipo de desaturasa que, por su denominación "Δ6", parece corresponder a una Fed. Sin embargo, ninguna de estas desaturasas de decápodos ha sido caracterizada funcionalmente, y aunque su expresión parece estar regulada de forma similar a la de otras Fed (Luo *et al.*, 2018), no se puede asegurar su participación en la biosíntesis de LC-PUFAs. Cabe señalar en relación a esta Tesis Doctoral que los gamáridos también tienen este tipo de desaturasas "Δ6" descritas en decápodos y sin función conocida hasta el momento. Sin embargo, se descartó la realización de análisis funcionales en base a, por un lado, la falta de evidencias que apoyaran su actividad desaturasa en los estudios disponibles hasta el momento (Yang *et al*., 2013; Chen *et al*., 2017; Lin *et al*., 2017; Luo *et al*., 2018; Wu *et al*., 2018) y, por otro, porque los resultados de caracterización molecular mostraron una composición aminoacídica de cajas de histidina propia de "desaturasas de esfingolípidos" más que de Fed (Hashimoto *et al*., 2008). No obstante, cabe la posibilidad de que las desaturasas "Δ6" de crustáceos puedan reconocer algún

sustrato de desaturación en una forma distinta a la que utilizan las Fed (ácidos grasos en forma de acetil-CoA) y contribuir de este modo a biosintetizar LC-PUFAs (Sushchik *et al*., 2003; Maazouzi *et al*., 2007; Alberts-Hubatsch *et al*., 2019). Esta hipótesis sobre el posible rol de las desaturasas "Δ6" en la biosíntesis de LC-PUFAs de crustáceos se apoya en parte en los resultados de un estudio reciente en el silenciamiento del gen de la desaturasa "Δ6" del decápodo *Penaeus vannamei*, el cual resulta en una disminución en los niveles de EPA y DHA en su hepatopáncreas (Chen *et al*., 2024).

Otro factor que puede explicar las observaciones sobre la hipotética capacidad de biosíntesis de LC-PUFAs de gamáridos (Sushchik *et al*., 2003; Maazouzi *et al*., 2007; Alberts-Hubatsch *et al*., 2019), que no son respaldadas por los resultados de caracterización molecular y funcional llevados a cabo en esta Tesis Doctoral, es la posible contribución de la microbiota del tracto digestivo a la producción de estos nutrientes esenciales. Investigaciones previas han señalado a la microbiota como responsable de la producción de LC-PUFAs como el EPA y el DHA en los cangrejos *Parasesarma erythodactyla* (Bui y Lee, 2015) y *Scylla olivacea* (Lau *et al*., 2022). Como se indica en el Apartado 1.4.1, algunas bacterias como las del género *Shewanella* (Metz *et al*., 2001) pueden biosintetizar LC-PUFAs a través de rutas anaeróbicas en las que el complejo enzimático policétido sintasa (PKS) juega un papel central (Qiu, 2003). El PKS permite la síntesis de LC-PUFAs mediante una serie de reacciones de condensación-reducción-deshidratación-reducción a partir del malonil-CoA, que resulta en la producción de diferentes LC-PUFAs n-3 (Metz *et al*., 2001; Hauvermale *et al*., 2006; Remize *et al*., 2021). El análisis de la microbiota llevado a cabo en *S. olivacea* muestra la presencia de bacterias de los géneros *Shewanella* y *Vibrio* (Lau *et al*., 2022), ambos caracterizados por producir EPA y DHA mediante la ruta del PKS (Metz *et al*., 2001; Nichols y McMeekin, 2002). Además, en este mismo trabajo con *S. olivacea* se observa que, en cangrejos alimentados con dietas ricas en ácidos grasos de 18 carbonos, existe un incremento en el número de secuencias que codifican para la ceto-acil sintasa (CS), dominio involucrado en la biosíntesis de LC-PUFAs mediante el PKS, y que este número disminuye cuando a la misma dieta se le añade antibiótico, debido a una reducción en la microbiota de los géneros mencionados anteriormente. Otro estudio que apoya la producción de DHA por la microbiota es el de Bui y Lee (2015), que muestra que el cangrejo *P. erythodactyla* tiene niveles menores de DHA derivado de ácido palmítico marcado (^{13}C), cuando las dietas se suplementan con antibióticos, en comparación a los valores de DHA en cangrejos alimentados con dietas sin antibiótico. La bibliografía científica no contiene información sobre la composición del microbioma de gamáridos, hecho que dificulta valorar la posible contribución de géneros bacterianos a la producción de LC-PUFAs n-3 en estos crustáceos y que justifiquen, al menos en parte, el aporte de estos compuestos por fuentes diferentes a la dieta a mecanismos propios de biosíntesis. Estudios futuros

deben centrarse en aclarar esta cuestión para poder determinar esta posible contribución.

Resulta interesante remarcar que el complejo PKS también se encuentra en animales y, aunque caracterizado con funciones diferentes, comparte ciertos dominios con el PKS microbiano implicado en la biosíntesis de LC-PUFAs n-3 (Remize *et al*., 2021). El complejo PKS de animales está involucrado en numerosos procesos biológicos que van desde la producción de péptidos denominados "nemamidas" que alargan la vida de fases larvarias en el nemátodo *Caenorhabditis elegans* (Gurvitz, 2009; Nivina *et al*., 2019), hasta la formación del otolito en peces (Hojo *et al*., 2015), pasando por la producción de pigmentos ("*echinochrome-A*") (Calestani *et al*., 2003; Service y Wardlaw, 1984), y la de espículas del exoesqueleto (Castoe *et al*., 2007; Hojo *et al*., 2015) en equinodermos. Se desconoce si, entre las funciones tan especializadas que el PKS tiene en animales, se encuentra la de producir LC-PUFAs n-3. En este sentido, un estudio sobre el PKS del periquito común *Melopsittacus undulatus* ha permitido demostrar que este está implicado en la biosíntesis de "polienos altamente insaturados" consistentes en ácidos grasos conjugados de 14, 16 y 18 carbonos, y que constituyen los sustratos para la formación de aldehídos que componen los pigmentos de las plumas de estas aves (Cooke *et al*., 2017). Los resultados de expresión diferencial de genes obtenidos a partir de las investigaciones presentadas en el Capítulo 5 de la presente Tesis Doctoral han detectado la presencia de algunos de los dominios característicos del complejo PKS en el gamárido marino *G. locusta*, los cuales son regulados a través de la dieta y de la temperatura, de una forma similar a la que cabría esperar de genes implicados en la biosíntesis de LC-PUFAs, es decir, aumentado su expresión cuando aumenta la demanda de LC-PUFAs por parte del organismo (dietas con bajo aporte de LC-PUFAs y baja temperatura). Obviamente, estos resultados acerca de un posible rol del PKS de *G. locusta* en la biosíntesis de LC-PUFAs no son concluyentes, pero unidos a los de los estudios citados anteriormente donde se sugiere cierta capacidad de biosíntesis de LC-PUFAs n-3 en gamáridos (Sushchik *et al*., 2003; Maazouzi *et al*., 2007; Alberts-Hubatsch *et al*., 2019), hacen del complejo enzimático PKS de estos organismos, un candidato para futuros estudios.

6.2. Los gamáridos dulceacuícolas tienen asociados rotíferos capaces de biosintetizar LC-PUFAs

Un resultado importante, aunque también inesperado y sorprendente, derivado de esta Tesis Doctoral ha sido la confirmación de que muchos de los transcriptomas de gamáridos dulceacuícolas disponibles públicamente en bases de datos como, por ejemplo, las del *National Center for Biotechnology Information* (NCBI), están

contaminadas con material genético de rotíferos bdeloideos. Muchas especies de rotíferos bdeloideos son conocidas por ser epibiontes de gamáridos dulceacuícolas (Ricci y Melone, 2000; De Smet y Verolet, 2016), colonizando varias regiones externas de su cuerpo (apéndices), y en ocasiones llegando a colapsar las branquias, y provocando su muerte al impedir la respiración del animal (Bojko *et al*., 2017; Bojko y Ovcharenko, 2019). La presencia sistemática de material genético de rotíferos en las bases transcriptómicas de gamáridos dulceacuícolas explica la presencia de genes de desaturasas (*ωx*-des y *fed*) y elongasas (*elovl2/5*) que no se encuentran en transcriptomas de gamáridos marinos, y aclara los resultados de análisis preliminares que apuntaban a una dotación de estos genes muy diferente entre especies marinas y dulceacuícolas que, como se menciona en el Apartado 6.1, carece de sentido desde el punto de vista evolutivo.

La caracterización de las desaturasas ωx-des y Fed y de la elongasa Elovl2/5 de rotíferos bdeloideos ha arrojado resultados muy reveladores en el ámbito de la ecología trófica, evolución y bioquímica de ácidos grasos (Capítulo 3). En cuanto a los análisis funcionales, se ha concluido que los rotíferos bdeloideos son capaces de biosintetizar *de novo* PUFAs como LA y ALA y, a partir de ellos, LC-PUFAs de hasta 20 carbonos como ARA y EPA. No obstante, los resultados no han permitido demostrar si los rotíferos bdeloideos pueden biosintetizar DHA. Parece claro que, en ausencia de Fed con actividad Δ4 desaturasa, la biosíntesis de DHA a través de la "ruta Δ4" (Figura 1.7) no es factible en estos organismos. Cabe la posibilidad de que los rotíferos bdeloideos puedan realizar la biosíntesis de DHA a través de la ruta alternativa denominada "ruta de Sprecher", menos eficiente que la anterior ya que, entre otros motivos, implica transporte de ácidos grasos entre distintos orgánulos celulares (Sprecher, 2000). Se requiere de futuras investigaciones que aclaren estos extremos. Cabe destacar que, los análisis filogenéticos, junto con los resultados obtenidos de estudios moleculares adicionales como la estructura y organización génica en el genoma del rotífero bdeloideo *Adineta steineri*, han determinado que dos (*fed3*, *fed4*) de los siete genes descritos en rotíferos bdeloideos presentan características compatibles con su adquisición mediante transferencia horizontal ("HGT" del inglés). Particularmente, para el caso de la *fed3* los resultados obtenidos muestran que este gen tiene su origen en el Reino Protista, más concretamente de la clase *Kinetoplastea*, mientras que los resultados de la *fed4* han permitido concluir que este gen es de origen fúngico. Este proceso de HGT está ampliamente documentado en rotíferos como un mecanismo de adaptación por su parte frente a situaciones ambientales extremas como, por ejemplo, la desecación, de forma que el organismo adquiere capacidades nuevas que le permiten sobrevivir en condiciones desfavorables (Gladyshev *et al*., 2008; Boschetti *et al*., 2012; Flot *et al*., 2013; Eyres *et al*., 2015; Hespeels *et al*., 2015; Debortoli *et al*., 2016). Así pues, en

ambientes dulceacuícolas donde el aporte de LC-PUFAs es mínimo (Arts *et al*., 2009; Colombo *et al*., 2016), los rotíferos habrían adquirido estos genes a partir de otros organismos que habitan en el mismo ecosistema, como ciertos hongos y protistas (Arndt *et al*., 2000; Gozashti *et al*., 2022), para poder complementar su equipo enzimático endógeno (*fed1, fed2, ωx1, ωx2*, *elovl2/*5) y mejorar su capacidad biosintética. Ciertos tipos de hongos, como los de las divisiones *Mucuromycota* y *Rozellomycota* donde se ha descrito la presencia de elementos que promueven la HGT (Van Der Burgt *et al*., 2012; Gozashti *et al*., 2022), y de los cuales es originaria la secuencia nucleotídica *fed4* detectada en rotíferos bdeloideos, son abundantes en ecosistemas acuáticos tanto de forma saprótrofa sobre materia orgánica en descomposición de la cual se alimentan los gamáridos (Chamier y Willoughby, 1986), como de parásitos de los mismos (Bojko *et al*., 2017; Bojko y Ovcharenko, 2019) y también de rotíferos (Wilson y Sherman, 2010). Por otro lado, los kinetoplástidos, de donde procede la secuencia *fed3,* también son abundantes en ambientes dulceacuícolas tanto en forma libre (Arndt *et al*., 2000), como parásitos de un gran abanico de organismos, incluidos gamáridos (Bojko *et al*., 2017; Bojko y Ovcharenko, 2019) y también rotíferos (Gómez y Skovgaard, 2015; Cachon y Cachon, 1987), y se caracterizan por ser potenciales donantes de genes a rotíferos bdeloideos mediante HGT (Gladyshev *et al*., 2008; Eyres *et al*., 2015; Debortoli *et al*., 2016). Los resultados obtenidos a partir de los análisis funcionales tanto de *fed3* como de *fed4* suponen otro argumento a favor de una adquisición de estos genes mediante HGT por parte del rotífero. La caracterización funcional de Fed3 exclusivamente como Δ6 desaturasa coincide con las funciones de otras Fed de kinetoplástidos como *Leishmania major* (Tripodi *et al*., 2006), las cuales contrastan con la doble actividad Δ6 y Δ8 desaturasa coexistentes en este tipo de desaturasas cuando son de origen animal (Park *et al*., 2009; Monroig *et al*., 2011, 2022; Kabeya *et al*., 2021). Por otro lado, la ausencia de actividad desaturasa de la Fed4 de rotíferos bdeloideos observada en nuestros ensayos con levaduras es consistente con una pérdida de funcionalidad que experimentan a menudo ciertos genes transferidos horizontalmente debido, entre otras causas, a cambios en los patrones de lectura, o también a una divergencia funcional causada por una duplicidad génica en el organismo receptor del gen (Vogan y Higgs, 2011; Husnik y McCutcheon, 2018).

Es interesante valorar qué implicaciones conlleva la demostrada capacidad biosintética en LC-PUFAs de los rotíferos bdeloideos. En primer lugar, resultan obvias las ventajas ecológicas para el propio rotífero derivadas de la adquisición de mecanismos endógenos que permiten la provisión de nutrientes esenciales como los LC-PUFAs en ambientes donde estos compuestos escasean. Es curioso señalar que, mientras que los rotíferos bdeloideos son propios del bentos de ecosistemas dulceacuícolas (Segers, 2007), otras clases como los monogonontes, entre los que se

encuentran especies marinas y planctónicas (Segers, 2007), carecen de ωx-des y Fed según se desprende de búsquedas en bases de datos genómicas y transcriptómicas de especies del género *Brachionus* (Han *et al.*, 2019; Kang *et al*., 2020). Esta distribución de equipos enzimáticos completos en clases específicas dentro del filo *Rotifera*, indica que, en efecto, la disponibilidad de LC-PUFAs ha podido ejercer una presión selectiva por conservar una alta capacidad de biosíntesis de LC-PUFAs en rotíferos dulceacuícolas y su pérdida en marinos similar a como ocurre en algunos peces teleósteos (Ishikawa *et al*., 2019; Matsushita *et al*., 2020). Al margen de las ventajas ecológicas para el rotífero, la capacidad de producción de LC-PUFAs de rotíferos bdeloideos plantea la cuestión sobre un posible beneficio para el gamárido, ya que este último tendría fácil acceso a una fuente rica en LC-PUFAs en ambientes pobres en estos nutrientes. A pesar de que esta posible transferencia de LC-PUFAs desde el rotífero al gamárido no ha podido demostrarse en esta Tesis Doctoral, existen precedentes en la literatura que apoyan la existencia de estos eventos desde simbiontes al huésped. Tal es el caso de las zooxantelas (dinoflagelados unicelulares) al coral *Montipora digitata* mediante endosimbiosis (Papina *et al*., 2003), así como también en sentido contrario (Imbs *et al*., 2014). Otro caso de transferencia de LC-PUFAs a partir de asociaciones ecológicas, es el de los gasterópodos del género *Elysia* (Cruz *et al*., 2020; Cartaxana *et al*., 2021), donde se ha propuesto la posibilidad de que estos organismos obtengan sustratos para la producción de LC-PUFAs (p. ej., otros PUFAs o incluso malonil-CoA) a partir de cloroplastos que han obtenido previamente de macroalgas e incorporado los tejidos del animal, conservando su función en un proceso conocido como cleptoplastia (Rumpho *et al*., 2011). A pesar de estos indicios sobre una posible transferencia de ácidos grasos entre especies, existen una serie de limitaciones y dificultades técnicas que impiden afirmar categóricamente que este proceso ocurra en la naturaleza y, si lo hace, en qué grado. En el caso particular de los gamáridos dulceacuícolas aquí estudiados sería necesario discernir la fuente real de la que proceden los LC-PUFAs, determinando si el propio gamárido obtiene estos nutrientes del rotífero o de otras fuentes como microorganismos que habitan en ecosistemas dulceacuícolas (Chamier y Willoughby, 1986; Bojko *et al*., 2017; Bojko y Ovcharenko, 2019), o incluso su propia microbiota.

6.3. Efecto de la dieta y la temperatura en el metabolismo de ácidos grasos y el crecimiento de *G. locusta*

Los gamáridos se caracterizan por ser invertebrados con un gran potencial de adaptación a todo tipo de ecosistemas acuáticos y climas (Costa y Costa, 2000; Väinölä *et al*., 2008; Whiteley *et al*., 2011). Por tal motivo, la limitada capacidad biosintética de LC-PUFAs en gamáridos demostrada en esta Tesis Doctoral, plantea

una serie de retos relacionados con la adquisición de estos nutrientes esenciales. Al margen de la posible adquisición de LC-PUFAs a través de la microbiota, de rutas endógenas no convencionales y/o de rotíferos bdeloideos con los que cohabitan en medios dulceacuícolas (Apartados 6.1 y 6.2), cabe plantearse otros mecanismos alternativos por los que los gamáridos podrían satisfacer sus requerimientos de LC-PUFAs en condiciones de alta demanda y que explicaran, por ejemplo, la presencia de estos nutrientes en *G. locusta* alimentados con dietas sin estos compuestos como las aquí denominadas "Carrot" y "Coco" (Capítulos 4 y 5). En efecto, los análisis de las muestras de *G. locusta* alimentados con dietas carentes de LC-PUFAs muestran niveles bajos, pero medibles, de estos compuestos incluyendo EPA y DHA (Capítulo 4; Tablas 4.2 y 4.3), a pesar de la aquí demostrada incapacidad de estos animales para poder biosintetizarlos a través de rutas aeróbicas convencionales como las descritas en la Figura 1.7. A este respecto, es importante mencionar que los gamáridos utilizados en el experimento del Capítulo 4 se alimentaron previamente con la macroalga *Fucus* sp. cuyos lípidos contienen niveles moderados de EPA y DHA y que, por tanto, pudieron ser retenidos de manera selectiva hasta el final del experimento. De acuerdo con esta hipótesis, un estudio reciente realizado con el anfípodo dulceacuícola *Pallaseopsis quadrispinosa* sugiere la retención de LC-PUFAs a partir de dietas previas para poder adaptarse a períodos de escasez de dichos nutrientes (Taipale *et al*., 2021). En el mencionado experimento, se estudió la composición lipídica de *P. quadrispinosa* tras un período de ayuno y posterior realimentación con dos dietas diferentes basadas en algas verdes (*Acutodesmus* sp.) o en diatomeas (*Diatoma tenuis*). Para ello, se calculó la proporción de ácidos grasos que provenía directamente del animal, y la que derivaba de la dieta, mediante diversos algoritmos, y se observó que los ejemplares cultivados en condiciones de ayuno presentaron niveles de EPA y DHA similares a los de los recolectados directamente del medio natural, tras un período de realimentación de tan sólo siete días, con cualquiera de las dietas descritas (Taipale *et al*., 2021). Los mecanismos implicados en la retención/deposición selectiva de EPA y DHA son desconocidos en la mayoría de invertebrados, aunque estudios en mamíferos apuntan a que, para el caso del DHA, es la presencia de un doble enlace en la posición Δ4 la que explica su escaso valor como sustrato energético para la β-oxidación en las mitocondrias, el cual trae como consecuencia el depósito de DHA (Madsen *et al*., 1998). Además, también ha sido demostrado el papel de la acil-CoA sintetasa de cadena larga 6 (Acsl6) en la deposición selectiva de DHA en mamíferos (Fernandez *et al*., 2018) y en peces (Chen *et al*., 2022). Los análisis realizados en el transcriptoma de referencia de *G. locusta* construido en el marco de esta Tesis Doctoral (Capítulo 5), así como en otros transcriptomas de gamáridos, han permitido detectar la existencia en estos crustáceos de varias secuencias similares a las Acsl de vertebrados. No obstante, se

precisan de futuros estudios que permitan esclarecer si alguna de las Acsl de gamáridos puede ser la responsable de la posible retención selectiva que explica los niveles de EPA y DHA en *G. locusta* alimentados con dietas carentes de estos compuestos.

Es bien conocido que la dieta y la temperatura son factores que determinan directamente procesos metabólicos fundamentales en organismos poiquilotermos como los invertebrados y, entre los que destacan el crecimiento, el normal desarrollo y la supervivencia (Neuparth *et al*., 2002; Azad *et al*., 2011; Camargo-Cely y Collin, 2019; Malzahn *et al*., 2023). Nuestros resultados muestran que, de estos dos factores, la dieta ejerce un efecto más notable que la temperatura en el perfil de ácidos grasos de *G. locusta* ya que, en general, la composición en ácidos grasos de la dieta se refleja de forma muy evidente en los perfiles lipídicos de los gamáridos (Capítulo 4). Estos resultados, además de corroborar las conclusiones alcanzadas en esta Tesis Doctoral sobre la limitada capacidad de estos crustáceos para biosintetizar LC-PUFAs (Apartado 6.1), también indican que los gamáridos muestran una notable capacidad para acumular lípidos dietarios. Investigaciones previas demuestran que los invertebrados acuáticos, especialmente los bentónicos, tienden a acumular triacilglicéridos (TAGs) ricos en LC-PUFAs durante periodos de alta disponibilidad de estos nutrientes, para así poder adaptarse a situaciones de bajas reservas de los mismos (Napolitano y Ackman, 1989; Hill *et al*., 1992; Arts *et al*., 2009, Taipale *et al*., 2021). Los resultados de expresión génica de *G. locusta* alimentados con dietas carentes de LC-PUFAs sugieren una activación en las rutas metabólicas que permiten la acumulación de ácidos grasos en forma de TAGs, así como también una inhibición en su degradación (Capítulo 5). En particular, en las condiciones mencionadas anteriormente, el incremento en la expresión de *srebp1* y *dgat* indica una mayor biosíntesis de TAGs y acumulación de los mismos (Xu *et al*., 2016; Koch *et al*., 2023), mientras que expresiones reducidas de *cpt1a* sugieren una reducción en el catabolismo de ácidos grasos (Tang *et al*., 2013; Luo *et al*., 2018). La acumulación de TAGs es una estrategia utilizada comúnmente por crustáceos, incluidos los gamáridos, como preparación para el ciclo de muda, un proceso crucial en su desarrollo y que implica un largo periodo de inanición para el que se requieren reservas considerables de nutrientes y de energía (Cuzin-Roudy *et al*., 1999; Kuballa *et al*., 2011; de Oliveira *et al*., 2019). Cabe señalar que los ejemplares de *G. locusta* alimentados con la dieta Coco presentaron altos contenidos en TAGs (datos no publicados). Tal acumulación de TAGs puede deberse, por un lado, al alto contenido de estos compuestos en la propia dieta y que favorecen su depósito, o también como resultado de procesos biosíntesis de éstos. Los análisis composicionales de la dieta Coco muestran un elevado contenido lipídico, particularmente en ácidos grasos saturados (SFAs) (Capítulo 4) y en TAGs (datos no publicados) que concuerdan con

lo expuesto en otras investigaciones (Figueiredo-Silva *et al*., 2012; Dayrit, 2015). Además, los resultados de expresión génica diferencial correspondientes a *G. locusta* alimentados con la dieta Coco (Capítulo 5) sugieren una reducción en el catabolismo de ácidos grasos en comparación a otros tratamientos, evidenciado por un incremento en la expresión de *NFκB* y *dgat,* y una reducción en los niveles de expresión de *cpt1a* y *fabp* (Storch y Thumser, 2010; Tang *et al*., 2013; Minegishi *et al*., 2015; Xu *et al*., 2016). En relación con la muda, los resultados de esta Tesis Doctoral muestran que la dieta Coco tiene asociada una alteración en el patrón de este proceso y en el desarrollo del gamárido, según se desprende de los valores de expresión de genes relacionados con la entrada al ciclo de muda (*mih*) y con el paso a fases más adultas (*jheh*) (Capítulo 5). Estas observaciones podrían ser las causantes de la mayor mortalidad observada con la dieta Coco (Capítulo 4), posiblemente debido a sus altos contenidos lipídicos. En este sentido, estudios realizados en crustáceos han destacado que un exceso de lípidos en la dieta influye negativamente en su crecimiento, desarrollo y supervivencia debido a una baja capacidad de asimilación de lípidos en estos animales, así como también una limitada eficiencia para su utilización como sustratos energéticos (catabolismo) (Zhang *et al*., 2013; Huo *et al*., 2014; Sun *et al*., 2020a).

Al margen de los efectos de la dieta sobre la supervivencia y el crecimiento descritos anteriormente, la temperatura tiene también implicaciones importantes en estas variables. Mientras que los Capítulos 4 y 5 abordan la interpretación de los resultados desde enfoques diferentes, concretamente biométrico y composicional en el Capítulo 4 y molecular en el Capítulo 5, existen aspectos transversales que requieren de un análisis conjunto. Son varios los estudios realizados en crustáceos en general (Wittmann *et al*., 2018; Azra *et al*., 2019, 2020; Vigliano Relva *et al*., 2024), y en anfípodos en particular (Sutcliffe y Carrick, 1981; Pöckl, 1992; Chaumot *et al*., 2020), que demuestran cómo la temperatura, además de afectar al perfil lipídico del animal, influye en su desarrollo alterando los procesos de muda y reproducción. La conclusión que se extrae a partir de estos estudios es que un aumento en la temperatura provoca un mayor crecimiento del animal y una maduración más rápida, desde la fase embrionaria (puesta y desarrollo en el huevo) hasta la adulta, debido a unos tiempos de incubación más cortos y a un aumento tanto en número de ciclos de muda como en la velocidad a la que se completan. El gen *mih* codifica para la hormona inhibidora de la muda (MIH), y su función radica la correcta regulación para la entrada en este proceso y su posterior finalización (Chung y Webster, 2005; Nakatsuji *et al*., 2009; Mykles, 2021). La baja expresión del gen *mih* a temperaturas elevadas parece indicar un aumento en el número de ciclos de muda en estas condiciones, asociado a un mayor crecimiento y posterior aumento en las tasas de mortalidad (Wittmann *et al*., 2018), que explicaría los datos de baja supervivencia y

alto crecimiento obtenidos para *G. locusta* a temperaturas altas (Capítulo 4). En efecto, los resultados del Capítulo 4 muestran que las temperaturas altas de cultivo (20 °C) inducen una mayor mortalidad y un aumento en las tasas de crecimiento específicas (SGR) en *G. locusta*, independientemente de la dieta considerada. Las mayores tasas de supervivencia, aunque también con tasas de crecimiento menores, se obtuvieron a temperaturas entre 5 y 15 °C, más en consonancia con las condiciones que *G. locusta* tiene en el medio natural (Costa y Costa, 2000; Neuparth *et al*., 2002). Los invertebrados, al igual que otros organismos acuáticos (Hazel, 1984; Sanchez Granel *et al*., 2019), pueden ajustar la proporción de ácidos grasos en sus membranas lipídicas para adaptarse a los cambios de temperatura mediante un proceso conocido como "adaptación homeoviscosa", el cual conlleva un aumento del grado de insaturación de las cadenas de ácidos grasos para favorecer la fluidez de las membranas celulares a bajas temperaturas (Moyes y Ballantyne, 2011; Ernst *et al*., 2016). Parte del incremento en el grado de insaturación y, por tanto, en la fluidez de membrana, es consecuencia de la acción de enzimas como la estearoil-CoA desaturasa (Scd), conocidas también como "Δ9 desaturasas", que introducen el primer doble enlace en sustratos saturados, dando lugar a la biosíntesis de ácidos grasos monoinsaturados (MUFAs) (Miyazaki y Ntambi, 2003; Castro *et al*., 2011). Sin embargo, los datos de expresión génica del Capítulo 5 no son concluyentes con respecto a la posible activación de la Scd de *G. locusta* a bajas temperaturas ya que, por un lado, la expresión de Scd sí aumenta a temperaturas menores al control (15 °C) como cabría esperar, pero también lo hace a temperaturas superiores. Como las Scd suelen ser desaturasas que no participan en la biosíntesis de LC-PUFAs, esta Tesis Doctoral no ha abordado la caracterización de estas enzimas en gamáridos. Además de las Scd, las desaturasas de tipo Fed y ωx-des también contribuyen a aumentar la fluidez a las membranas y, por tanto, a la adaptación homeoviscosa en condiciones de baja temperatura (Sanchez Granel *et al*., 2019). En un escenario en el que organismos como los gamáridos tengan desaturasas como la Scd pero carezcan de otras como las Fed y ωx-des, se suscita la pregunta de si éstos son capaces de adaptar la fluidez de la membrana en condiciones extremas como las que combinan bajas temperaturas con alto aporte de lípidos dietarios altamente saturados como en la dieta Coco. Los resultados obtenidos en el Capítulo 4 indican que la supervivencia de los gamáridos alimentados con la dieta Coco no varía a temperaturas entre 5 y 15 °C. Por tanto, nuestros resultados permiten concluir que, a pesar de las limitaciones en la capacidad de desaturación señalados anteriormente, los gamáridos pueden adaptarse con relativa viabilidad a temperaturas bajas incluso cuando son alimentados con dietas muy saturadas.

6.4. Conclusiones finales y perspectivas futuras

Esta Tesis Doctoral ha abordado la pregunta de si los gamáridos tienen la capacidad de biosintetizar LC-PUFAs para su aprovechamiento como ingrediente de alto valor nutricional en piensos de acuicultura. Los resultados obtenidos han permitido demostrar que los gamáridos tienen una dotación de elongasas que cubre todas las necesidades de elongación requeridas para la biosíntesis de LC-PUFAs. Sin embargo, los datos recogidos durante este trabajo de investigación muestran que estos animales no tienen desaturasas ωx-des o Fed que, junto a las elongasas, también son necesarias en la biosíntesis de LC-PUFAs. Estos resultados permiten concluir que los gamáridos no pueden biosintetizar LC-PUFAs según las rutas metabólicas conocidas a día de hoy, y que dicha limitación se debe principalmente a la escasa capacidad desaturasa. Por otro lado, las secuencias de genes que codifican para elongasas y desaturasas halladas exclusivamente en transcriptomas de gamáridos dulceacuícolas no son propias de estos animales, sino de rotíferos bdeloideos que son epibiontes de éstos en ecosistemas de agua dulce. Estos resultados ponen en evidencia la necesidad de realizar un tratamiento adecuado de las muestras antes de su uso para metodologías moleculares, al tiempo que recomiendan que el análisis de bases de datos transcriptómicas sea cauto y, cuando sea posible, apoyado de análisis confirmatorios en genomas y en estudios filogenéticos para discernir la identidad de las secuencias. Los resultados de caracterización funcional de las secuencias de rotíferos bdeloideos han permitido establecer a estos organismos como productores de LC-PUFAs y plantean la hipótesis sobre la posible transferencia de LC-PUFAs de los rotíferos a los gamáridos dulceacuícolas. Futuros estudios son necesarios para aclarar estos extremos, así como la existencia de rutas alternativas de biosíntesis de LC-PUFAs en gamáridos y la contribución de la microbiota a la provisión de estos nutrientes esenciales, que expliquen la presencia de LC-PUFAs en los lípidos del animal cuando no hay aporte desde la dieta.

Adicionalmente, esta Tesis Doctoral ha tenido como uno de sus objetivos el estudio de los efectos de la dieta y la temperatura sobre el crecimiento y el metabolismo de ácidos grasos en el gamárido marino *G. locusta*, abordando aspectos fisiológicos, composicionales y moleculares. Por un lado, se plantea la posibilidad de que los porcentajes de EPA y DHA detectados en *G. locusta* alimentados con dietas carentes en estos compuestos sean debidos a una acumulación de dietas previas. Por ende, se hipotetiza sobre la existencia de mecanismos de retención selectiva de LC-PUFAs presentes en la dieta que les permitan sobrevivir durante periodos de limitada o nula biodisponibilidad de estos nutrientes. Respecto al efecto de la dieta, se ha observado que dietas carentes de LC-PUFAs, pero ricas en SFAs, resultan en una acumulación de ácidos grasos en el

animal, probablemente en la fracción de TAGs. Este fenómeno está asociado con la modificación en la expresión de genes tales como *cpt1a*, entre otros, y factores de transcripción como *srebp1*. Además, la combinación de dietas carentes de LC-PUFAs y temperaturas elevadas, influye negativamente en la supervivencia del animal al verse afectadas vías del desarrollo como la muda, efecto que no se observa a temperaturas bajas. Estos resultados indican que *G. locusta*, y probablemente también otros gamáridos de latitudes altas, pueden acomodarse a bajas de temperatura mediante mecanismos como la adaptación homeoviscosa, entre otros, a pesar de que carecen de varios tipos de desaturasas implicadas en estos procesos. Estos resultados abren una interesante línea de investigación dedicada a esclarecer los mecanismos subyacentes a la retención selectiva de LC-PUFAs en gamáridos para poder desarrollar estrategias de cultivo adecuadas que permitan maximizar la biomasa y su valor nutricional. Esta estrategia sería más eficiente en organismos que, además de tener una alta capacidad de retención de LC-PUFAs, como los gamáridos, también tuvieran una alta capacidad para producirlos endógenamente, por lo que futuros estudios deben valorar el potencial de otros grupos de crustáceos o incluso de otros invertebrados acuáticos.

6.5. Bibliografía

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7.
Conclusiones

1. Los gamáridos tienen tres genes de elongasas (*elovl4*, *elovl6* y *elovl1/7*) cuyos enzimas pueden mediar todas las reacciones de elongación implicadas en las rutas de biosíntesis de LC-PUFAs desde los precursores de 18 carbonos.
2. Los gamáridos carecen de genes de desaturasas "*front-end*" y "*methyl-end*", lo que les impide complementar a las elongasas y, en última instancia, los incapacita para biosintetizar LC-PUFAs según las rutas conocidas en la actualidad.
3. Los genes de desaturasas "*front-end*" y "*methyl-end*", así como el de la elongasa *elovl2/5* identificados exclusivamente en los transcriptomas de gamáridos dulceacuícolas, pertenecen a rotíferos bdeloideos y no a gamáridos. La caracterización funcional de estos enzimas demuestra que los rotíferos bdeloideos pueden sintetizar LC-PUFAs, por lo que estos organismos se postulan como productores primarios de LC-PUFAs en hábitats dulceacuícolas.
4. Algunas de las desaturasas "*front-end*" de rotíferos bdeloideos deben su presencia en el genoma de estos organismos al fenómeno de transferencia horizontal génica desde microorganismos pertenecientes a los grupos *Protista* y *Fungi*.
5. La dieta y la temperatura afectan al crecimiento, el desarrollo y la supervivencia del gamárido marino *Gammarus locusta*. El efecto de la dieta sobre los perfiles de ácidos grasos de *G. locusta* es mucho mayor que el de la temperatura, llegando incluso a enmascarar cualquier potencial efecto de esta última.
6. Los *G. locusta* alimentados con dietas carentes de LC-PUFAs, pero ricas en LA y ALA, exhiben valores de crecimiento y de supervivencia comparables a los alimentados con una dieta de macroalgas típica de su hábitat natural.
7. La composición lipídica de la dieta determina los perfiles de ácidos grasos de *G. locusta* y, por tanto, su valor nutricional. Sin embargo, *G. locusta* muestra cierta capacidad de retención de LC-PUFAs, tal y como muestra la presencia de dichos compuestos en individuos alimentados con dietas carentes de ellos.
8. La supervivencia de *G. locusta* es mayor a temperaturas de cultivo bajas (5 °C y 15 °C), aunque estas condiciones llevan asociadas menores tasas de crecimiento específico.

9. Una temperatura de cultivo elevada conlleva una alta mortalidad en *G. locusta*, la cual está relacionada con una disrupción en el proceso de muda del exoesqueleto evidenciado por alteraciones en los niveles de biomarcadores génicos tales como *mih* y *jheh*.
10. El desajuste en el proceso de muda, y el consiguiente impacto negativo en la supervivencia, también tiene lugar cuando *G. locusta* es alimentado con dietas ricas en lípidos altamente saturados.
11. *G. locusta* muestra una notable capacidad para acumular lípidos dietarios. Los resultados de expresión génica evidencian que en este proceso se combina, por un lado, la activación de rutas anabólicas que favorecen la síntesis de lípidos de reserva (triglicéridos) y, por otro, la inhibición de rutas catabólicas que repercuten en una baja eficiencia en el uso de ácidos grasos dietarios como sustratos energéticos.

ANEXOS

marine drugs

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Article

Biosynthesis of Long-Chain Polyunsaturated Fatty Acids in Marine Gammarids: Molecular Cloning and Functional Characterisation of Three Fatty Acyl Elongases

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Abstract: Long-chain ($C_{20–24}$) polyunsaturated fatty acids (LC-PUFAs) are essential nutrients that are mostly produced in marine ecosystems. Previous studies suggested that gammarids have some capacity to endogenously produce LC-PUFAs. This study aimed to investigate the repertoire and functions of elongation of very long-chain fatty acid (Elovl) proteins in gammarids. Our results show that gammarids have, at least, three distinct *elovl* genes with putative roles in LC-PUFA biosynthesis. Phylogenetics allowed us to classify two elongases as Elovl4 and Elovl6, as they were bona fide orthologues of vertebrate Elovl4 and Elovl6. Moreover, a third elongase was named as "Elovl1/7-like" since it grouped closely to the Elovl1 and Elovl7 found in vertebrates. Molecular analysis of the deduced protein sequences indicated that the gammarid Elovl4 and Elovl1/7-like were indeed polyunsaturated fatty acid (PUFA) elongases, whereas Elovl6 had molecular features typically found in non-PUFA elongases. This was partly confirmed in the functional assays performed on the marine gammarid *Echinogammarus marinus* Elovl, which showed that both Elovl4 and Elovl1/7-like elongated PUFA substrates ranging from C_{18} to C_{22}. *E. marinus* Elovl6 was only able to elongate C_{18} PUFA substrates, suggesting that this enzyme does not play major roles in the LC-PUFA biosynthesis of gammarids.

Keywords: *Echinogammarus marinus*; elovl enzymes; LC-PUFA biosynthesis; functional characterisation; gammarids

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1. Introduction

Long-chain ($C_{20–24}$) polyunsaturated fatty acids (LC-PUFAs), including arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are physiologically essential compounds required for normal growth and development of vertebrates [1]. Moreover, the n-3 (or omega-3) LC-PUFAs EPA and DHA have beneficial roles in human health [2]. Since n-3 LC-PUFAs are nearly exclusively produced in marine ecosystems by low trophic marine organisms, such as microbes [3–5] and certain invertebrates [6,7], marine products are regarded as unique sources of EPA and DHA in the human diet [8]. Products derived from marine aquaculture have traditionally guaranteed the supply of health-promoting n-3 LC-PUFAs due to the fact that aquafeeds were formulated with high inclusion levels of the so-called "marine ingredients" fishmeal (FM) and fish oil (FO), naturally rich in n-3 LC-PUFAs [9,10]. FM and FO are mostly produced from feed-grade fish species and, with the rapid expansion of aquaculture worldwide, pressure on fisheries exploited for FM and FO production has remarkably increased, reaching and,

on occasion, exceeding their ecological sustainability limit. This prompted interest in exploring alternative ingredients for aquaculture and, currently, raw materials derived from land animals or plants are commonly used to partly or totally replace FM and/or FO in aquafeeds [11–13]. With respect to FO alternatives, oils derived from microalgae and transgenic oilseed crops have become available in recent years, representing promising sources of the n-3 LC-PUFAs [14–16]. Currently, the use of vegetal oils (VOs) as substitutes for FO in fish feed has become a widely extended practice [9,17,18]. However, the use of non-marine ingredients is often associated with a loss of nutritional value of farmed fish products mainly due to decreased contents of LC-PUFAs, especially EPA and DHA, which are absent or at low levels in non-marine ingredients. Biomasses derived from low trophic marine crustaceans, such as krill and copepods, as well as the ingredients derived from their processing, have been demonstrated to be excellent sources of essential nutrients including n-3 LC-PUFAs and thus arise as promising alternative raw materials for aquafeed formulation [19–21]. However, the exploitation of wild stocks for the production of crustacean-derived ingredients poses the same ecological sustainability issues alluded to above for reduction fisheries. Alternatively, mass production of marine crustaceans appears to be a reasonable strategy to address such a sustainability hurdle, particularly with species of low trophic level with high nutritional value (i.e., high n-3 LC-PUFAs) and culture performance output.

Gammarids, crustacean amphipods, are aquatic invertebrates that are abundant in benthic communities in virtually all aquatic environments [22,23]. Previous studies have shown that gammarids' nutritional profiles are characterised by a high protein content, low levels of carbohydrates, and relatively high contents of n-3 LC-PUFAs [24–29]. Such characteristics, along with the possibility to establish high density cultures [25,30,31], have prompted interest for using gammarids in aquaculture [32]. Importantly, some investigations reported on the ability of gammarids to be used in a wide range of sidestreams, in bioindustries such as aquaculture, forestry and agriculture [28,30,31,33]. Consequently, gammarids arise as promising candidates to apply circular economy principles by which sidestreams can be utilised for the production of biomass with high nutritional value. Moreover, in the scenario of a limited availability of marine ingredients alluded to above, it is important to elucidate whether marine gammarids are able to bioconvert fatty acids (FAs) present in sidestreams into the high-value physiologically essential LC-PUFAs.

The ability of animals for endogenous production (biosynthesis) of LC-PUFAs depends upon the gene repertoire and function of two types of enzymes [6,34]. On one hand, front-end desaturases (Fad) introduce double bonds (unsaturations) into polyunsaturated fatty acid (PUFA) substrates; on the other hand, elongation of very long-chain fatty acid (Elovl) proteins (or commonly known as "elongases") catalyse the usually rate-limiting reaction (condensation) in the FA elongation pathway and results in the extension of the pre-existing FA in two carbons [35]. To the best of our knowledge, no studies have reported on the molecular and functional characterisation of *fad* and *elovl* genes from gammarids, and the LC-PUFA biosynthetic capacity of gammarids has been only inferred from feeding experiments assessing the impact of dietary FA on body FA composition [24–26,28,33]. While the presence of Fad-like enzymes in crustaceans remains to be clarified [6], *elovl* genes with roles in LC-PUFA biosynthesis have been recently reported in crabs such as the mud crab *Scylla paramamosain* [36], the orange mud crab *Scylla olivacea* [37,38] and the swimming crab *Portunus trituberculatus* [39]. To date, three different elongases named Elovl4, Elovl6 and Elovl7 have been reported in crustaceans [36–39]. While the vertebrate Elovl4 has been demonstrated to elongate PUFA substrates, Elovl6 and Elovl7 have not been reported to play a role in LC-PUFA biosynthesis of vertebrates [1,35], suggesting functional diversification of animal Elovl [6]. Our overall aim is to elucidate the LC-PUFA biosynthetic pathways in gammarids in order to identify species with a high capacity to convert short-chain FA compounds available in various sidestreams potentially used as feed into high nutritional value LC-PUFAs. Specifically, the present study aimed to characterise molecularly and functionally the set of *elovl* genes identified in *Echinogammarus marinus*, a

gammarid species in which capacity for LC-PUFA endogenous production was previously reported [33]. We here provide compelling evidence showing that *E. marinus* possess three *elovl* genes, named Elovl4, Elovl6 and Elovl1/7-like, with roles in LC-PUFA biosynthesis.

2. Results

2.1. Phylogeny of the E. marinus Elovl

Our search strategy enabled us to identify three *elovl* sequences from *E. marinus*, which were homologous to the three Elovls found in the amphipod reference genome from *Hyalella azteca* [40]. The newly cloned *E. marinus elovl4* cDNA (deposited in GenBank database with accession number MW660836) has an open reading frame (ORF) of 960 base pairs (bp), encoding a putative protein of 319 amino acids (aa). The *E. marinus elovl6* cDNA has an ORF of 1029 bp (MW659697) that translates to a putative protein of 342 aa residues, whereas *elovl1/7*-like contains an ORF of 1077 bp (MW659696) that encodes for a putative protein of 358 aa. The putative aa sequences deduced from the *E. marinus elovl* have some of the distinctive characteristics of fatty acyl elongases, such as the conserved domains KXXEXXDT, NXXXHXXMYXYY and TXXQXXQ, a histidine box (HXXHH) and a specific number of transmembrane-spanning regions (Figure 1). More specifically, prediction of transmembrane-spanning domains showed that the deduced aa sequences of the three *E. marinus* Elovls contained seven transmembrane-spanning regions (Figure S1A–C). The histidine box (HXXHH), a common feature shared with desaturase and hydrolase enzymes, and crucial for the coordination of electron exchange during FA elongation [41,42], was found in all three *E. marinus* Elovls (Figure 1). However, the sequence of the N-terminal side of the histidine box of the three *E. marinus* Elovls presented some noteworthy differences. On one hand, Elovl4 and Elovl1/7-like from *E. marinus* had, respectively, a glutamine (Q) and a histidine (H) at position -5 preceding their histidine boxes; on the other hand, the *E. marinus* Elovl6 had a proline (P) at position -5 and a lysine (L) at position -4 (Figure 1). Subcellular location analysis revealed that these three elongases are located in the endoplasmic reticulum (ER) membrane (Table S1A,B).

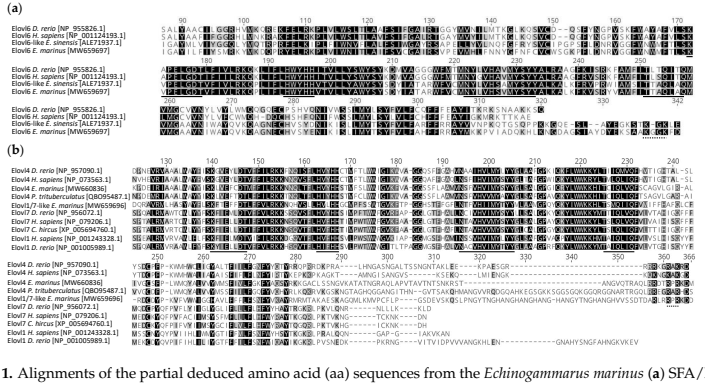

Figure 1. Alignments of the partial deduced amino acid (aa) sequences from the *Echinogammarus marinus* (**a**) SFA/MUFA like elongase *elovl6* (MW659697) and (**b**) PUFA-like elongases *elovl4* (MW660836) and *elovl1/7*-like (MW659696), with several elongases already characterised from vertebrates and crustaceans including the swimming crab *P. trituberculatus*. Conserved domains KXXEXXDT, NXXXHXXMYXYY and TXXQXXQ, and the histidine box (HXXHH) are underlined (solid line). Additionally, aa residues corresponding to putative endoplasmic reticulum retention signal have a dotted underline.

In order to elucidate the orthology of the newly cloned *elovl* from *E. marinus*, a phylogenetic tree was constructed to compare them with Elovl proteins from a variety of animal

groups, including both invertebrates and vertebrates (Figure 2). Phylogenetic analysis of the aa sequences showed that the putative *E. marinus* Elovl4 grouped together with Elovl4 from the crustaceans *P. trituberculatus* and *S. olivacea*, as well as vertebrate Elovl4. The *E. marinus* Elovl6 protein grouped closely to Elovl6 sequences from other crustaceans such as the Chinese mitten crab *E. sinensis* and more distantly from their vertebrate orthologues. With respect to the phylogeny of the Elovl1/7-like retrieved from *E. marinus*, the putative protein clustered together with a group of as yet unnamed putative Elovl proteins from other crustaceans like *P. trituberculatus* and *S. paramamosain*, themselves being closely related to *H. azteca* (XP_018024127.1) and *S. olivacea* (AWM30548.1) that were originally annotated as "Elovl7-like". Three *elovl2/5*-like sequences were identified from freshwater gammarids including *Echinogammarus berilloni*, *Gammarus waitieri* and *Gammarus pulex*, but no homologous sequences of this *elovl* type could be found in transcriptomic databases from either *E. marinus* or *G. locusta*.

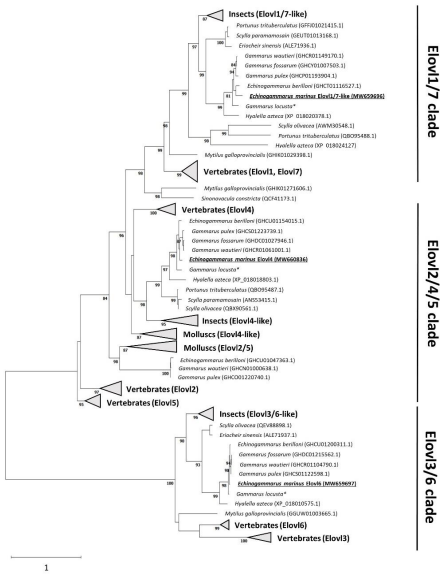

Figure 2. Phylogenetic tree comparing gammarid Elovl with several elongases retrieved from other crustaceans, as well as molluscs and vertebrates. There are three different clades that grouped the majority of the sequences analysed, namely Elovl1/7 clade, Elovl2/4/5 clade and Elovl3/6 clade. Two sequences that do not correspond to any of the previously defined clusters are PUFA elongases found in *Mytilus galloprovincialis* and *Sinonovacula constricta* [43]. The protein evolutionary model used was LG4X [44]. The tree was constructed using the maximum likelihood method by randomized axelerated maximum likelihood (RAxML) within the CIPRES external server. Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations. The transfer distance bootstrap support value (%) is given in each node. Values lower than 80% are not shown. Accession numbers according to the NCBI database are given for each sequence. * Elovls from *Gammarus locusta* were retrieved from blasting the transcriptome assembled by Neuparth et al. [45].

All putative Elovl proteins found in *E. marinus* were further confirmed to exist in other gammarids including both freshwater and marine species from the *Gammaridae* family (Figure 2). Given the close taxonomic relationship between *Echinogammarus* and *Gammarus* species, the Elovl proteins from the different gammarid species shared a high homology and therefore clustered together in the tree (Figure 2).

2.2. Roles of the E. marinus Elovl4, Elovl6 and Elovl1/7-like in LC-PUFA Biosynthesis

The role of the *E. marinus* Elovl proteins in LC-PUFA biosynthesis was investigated by expressing their ORFs in yeast. Transgenic yeast expressing each of the *E. marinus elovl* sequences were grown in the presence of exogenously supplied PUFA substrates including linoleic acid (LA, 18:2n-6), α-linolenic acid (ALA, 18:3n-3), γ-linolenic acid (GLA, 18:3n-6), stearidonic acid (SDA, 18:4n-3), ARA (20:4n-6), EPA (20:5n-3), docosatetraenoic acid (DTA, 22:4n-6), docosapentaenoic acid (DPA, 22:5n-3) and DHA (22:6n-3). The elongase conversions are shown in Table 1. For the *E. marinus* Elovl4, the results showed that this elongase has the ability to elongate all PUFA substrates to the corresponding 2-carbon longer products (Table 1). Conversions of C_{18} and C_{20} substrates were all above 1%, whereas conversions towards C_{22} were below 1% (Table 1). A second elongation product was detected when 18:4n-3, 20:5n-3, 20:4n-6 and 22:5n-3 were used as substrates, resulting in the production of 22:4n-3, 24:5n-3, 24:4n-6 and 26:5n-3 (Table 1). The *E. marinus* Elovl6 was able to elongate only C_{18} PUFA, with conversions to the corresponding C_{20} products being below 0.4 % in all cases (Table 1). Functional characterisation of the *E. marinus* Elovl1/7-like revealed that, like Elovl4, this enzyme was able to elongate all substrates assayed (Table 1). It is worth mentioning that conversions towards the C_{20} substrates EPA and ARA were relatively high (13.17 and 5.75 %, respectively), and also a second elongation product was detected in both cases (Figure 3). However, unlike Elovl4, the *E. marinus* Elovl1/7-like did not produce 26:5n-3.

Table 1. Functional characterisation of the *Echinogammarus marinus* Elovl4, Elovl6 and Elovl1/7-like. Conversions of exogenously supplied fatty acid (FA) substrates were calculated according to the formula [areas of all products with longer chain than substrate/(areas of all products with longer chain than substrate + substrate area)] × 100.

FA Substrate	FA Product	Elovl4	Elovl6	Elovl1/7-Like	Activity
18:3n-3	20:3n-3	2.67	0.22	0.29	C18→C20
18:2n-6	20:2n-6	1.32	0.11	0.19	C18→C20
18:4n-3	20:4n-3	1.82	0.33	1.46	C18→C20
	22:4n-3	0.02	nd	0.02	C20→C22
18:3n-6	20:3n-6	1.21	0.35	0.87	C18→C20
20:5n-3	22:5n-3	2.52	nd	13.17	C20→C22
	24:5n-3	0.05	nd	0.17	C22→C24
20:4n-6	22:4n-6	1.61	nd	5.75	C20→C22
	24:4n-6	0.02	nd	0.09	C22→C24
22:5n-3	24:5n-3	0.92	nd	2.16	C22→C24
	26:5n-3	0.09	nd	nd	C24→C26
22:4n-6	24:4n-6	0.46	nd	0.72	C22→C24
22:6n-3	24:6n-3	0.38	nd	0.54	C22→C24

nd, not detected.

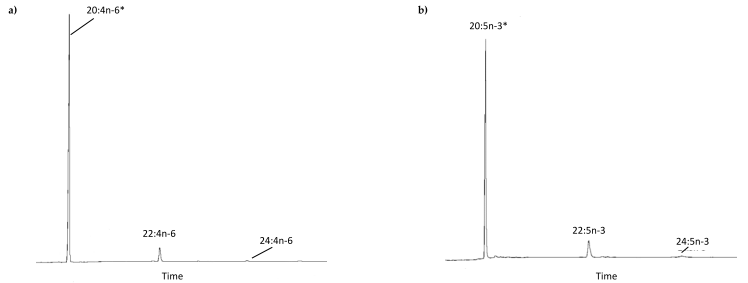

Figure 3. Functional characterisation of the *E. marinus* Elovl1/7-like activity towards C_{20} PUFA substrates. Yeast (*S. cerevisiae*) expressing the *E. marinus elovl1/7*-like gene was grown in the presence of (**a**) arachidonic acid (ARA) (20:4n-6) and (**b**) eicosapentaenoic acid (EPA) (20:5n-3). Substrates (*) and their corresponding elongation products are indicated in each panel.

3. Discussion

There exists a growing interest in using gammarids in aquaculture associated with their nutritional profiles, characterised by relatively high contents of n-3 LC-PUFA [24,25]. Moreover, gammarids have shown an ability to be grown on a varied range of sidestreams from bio-based industries [28,30,31,33]. Recently, Alberts-Hubatsh et al. [33] reported that the gammarids *E. marinus* and *G. locusta* had some capacity for "trophic upgrading", implying that these crustaceans can bioconvert FA present in sidestreams from agriculture into high nutritional value LC-PUFA [33]. However, the actual enzymes accounting for such FA conversions remained to be elucidated and, therefore, this study contributes to investigating the role that endogenous Elovls play in gammarids' trophic upgrading capacity. We herein report on three elongases with putative functions in the LC-PUFA biosynthesis of gammarids, and demonstrate that the relatively high LC-PUFA contents of gammarids grown on agriculture sidestreams can account for the endogenous enzymatic capacity existing in gammarids themselves.

Our phylogenetic analysis revealed a well-conserved pattern among gammarids, consisting of three Elovl proteins that have been here referred to as Elovl4, Elovl6 and Elovl1/7-like. The phylogenetic analysis clearly established that both *elovl4* and *elovl6* found in multiple gammarids are closely related to the Elovl4 and Elovl6 present in higher metazoans [1,35], and previously characterised in other non-gammarid crustaceans like the mud crab *S. paramamosain* [36], the swimming crab *P. trituberculatus* [39] and the orange mud crab *S. olivacea* [38]. Interestingly, we named a further *elovl* as "*elovl1/7*-like" since the phylogenetic analyses illustrated that this elongase grouped closely with the vertebrate Elovl1 and Elovl7 subfamilies. Moreover, our results showed that this elongase is closely related to an Elovl named as "Elovl7-like" for *S. olivacea* [37]. It is important to note that some invertebrates such as molluscs possess an elongase named Elovl2/5 [43,46–48], which represents an ancestor elongase that gave rise to the Elovl2 and Elovl5 protein families present in vertebrates [49]. Like Elovl2 and Elovl5 [35], the mollusc Elovl2/5 has well-established roles in the elongation of PUFAs [43,46–48]. Our *elovl*-like sequence search strategy identified three putative *elovl2/5* sequences from freshwater gammarids, namely *Echinogammarus berilloni*, *Gammarus waitieri* and *Gammarus pulex*. However, multiple attempts to isolate a partial sequence of this elongase from marine gammarids including *E. marinus* and *G. locusta* did not result in any conclusive result, suggesting that these elongases could be absent from genomes of these species or, alternatively, are expressed at relatively low levels compared to those of the elongases characterised in this study.

We further characterised the sequences of the *elovl* genes present in the marine gammarid *E. marinus*, a species in which capacity for trophic upgrading of dietary FA had been previously reported [33], and is now also confirmed to be a valid representative of marine gammarids as it possesses at least one copy of the *elovl4*, *elovl6* and *elovl1/7*-like genes. The newly cloned *elovl4*, *elovl6* and *elovl1/7*-like from *E. marinus* have all the distinctive characteristics shared by Elovls [41] and were further confirmed to exist in orthologues from *S. paramamosain*, *S. olivacea* and *P. trituberculatus* [36,38,39]. Such Elovl characteristic traits include the conserved domains KXXEXXDT, NXXXHXXMYXYY, TXXQXXQ, a histidine box (HXXHH) and a specific number of transmembrane-spanning regions. However, the above-described differences in the residues located in the N-terminal side of the histidine box between the *E. marinus* Elovl4 and Elovl1/7-like and Elovl6 suggest different putative functions according to the categorisation of PUFA vs. non-PUFA elongases proposed by Hashimoto et al. [42]. Clearly, our results established that both Elovl4 and Elovl1/7-like from *E. marinus* and other gammarids, such as *G. locusta*, have the diagnostic residues in the surrounding region of the histidine box that are a common feature of PUFA elongases [42]. On the contrary, the gammarid Elovl6 contains the diagnostic residues that are typically found in Elovls involved in elongation of saturated and monounsaturated fatty acids [42]. The predicted subcellular localisation results showed that all three Elovls are located in the ER, the cellular site where LC-PUFA biosynthesis takes place [1,35,41]. Retention in the ER is crucial for Elovl to exert its function and, consistently, the *E. marinus* Elovl4 and Elovl1/7-like, but not Elovl6, have the RXR motif that is also associated with ER retention [50] and predicted in the *P. trituberculatus* Elovl4 [39]. Collectively, the molecular characterisation results showed that the *E. marinus* Elovl4 and Elovl1/7-like have distinctive features with respect to Elovl6, which can partly account for the substrate specificities revealed in our functional assays in yeast.

The functional characterisation of the herein studied *E. marinus* Elovls revealed that these enzymes, particularly Elovl4 and Elovl1/7-like, play roles in the LC-PUFA biosynthesis of gammarids, which likely account for the trophic upgrading capacity of these crustaceans [33]. Indeed, the gammarid Elovl4 and Elovl1/7-like were able to utilise a variety of C_{18-22} PUFAs as substrates and convert them into longer products, including multiple LC-PUFAs. On the contrary, the chain length of PUFA substrates recognised by the *E. marinus* Elovl6 was limited to C_{18} and with relatively low conversions throughout. While our results do not allow us to completely rule out a putative role of the *E. marinus* Elovl6 in PUFA elongation, the restricted elongation capacity towards C_{18} PUFA, along with the abovementioned sequence characteristics of non-PUFA Elovl [42], suggests that Elovl6 does not play a relevant role in LC-PUFA biosynthesis in gammarids. Importantly, the elongation abilities contained within the gammarid Elovl4 and Elovl1/7-like enable these crustaceans to catalyse all elongation reactions involved in the LC-PUFA biosynthetic pathway, even in the absence or low expression of the PUFA elongase *elovl2/5* in marine species such as *E. marinus* and *G. locusta*, as pointed out above. The ability of the gammarid Elovl4 and Elovl1/7-like to produce 24:5n-3 from 22:5n-3, but also from 20:5n-3, suggests that these elongases can contribute to the biosynthesis of DHA via the Sprecher pathway, requiring 24:5n-3 as an intermediate for a Δ6 desaturase to produce 24:6n-3, which is subsequently β-oxidised to DHA (22:6n-3) [51]. This key metabolic pathway is found in some mammals [51], fish [52] and molluscs [43], where Δ6 desaturases that are able to convert 24:5n-3 into 24:6n-3 have been reported. However, to the best of our knowledge, no front-end desaturases from gammarids having such capacity have been yet reported in the literature. While further studies aiming to characterise the gene complement and function of front-end desaturases from gammarids are needed, the current evidence suggest that it is unlikely that DHA can be produced via the Sprecher pathway in gammarids. Likewise, biosynthesis of other physiologically important LC-PUFA such as EPA and ARA appears to not be possible either, unless the existence of front-end desaturases, particularly Δ5 desaturases, is demonstrated to exist in gammarids. Rather than biosynthetic products, however, EPA and ARA appear to be metabolic precursors in gammarids, according to

the results of the yeast assays showing that these compounds can be efficiently elongated to the corresponding C_{22} elongation products. Conversion of EPA to 22:5n-3 (DPA) was particularly high (13.17%) for Elovl1/7-like, and considering the low concentration of DPA in detritus that gammarids naturally feed on, it is tempting to speculate that part of the DPA found in wild caught *E. marinus* [33] derives from biosynthesis.

Beyond its role in LC-PUFA biosynthesis, the gammarid Elovl4 can also participate in the biosynthesis of the so-called very long-chain (>C_{24}) PUFAs (VLC-PUFAs). Our functional characterisation assays showed that transgenic yeast expressing the *E. marinus elovl4* were able to produce 26:5n-3 when 22:5n-3 was supplied as a substrate. Such elongation capacity was found to be unique among the three gammarid elongases characterised in the present study, since neither the *E. marinus* Elovl6 nor Elovl1/7-like were able to synthesise VLC-PUFAs. These results are consistent with those reported previously for the crab *P. trituberculatus* Elovl4 [39] and illustrate putative roles of crustacean Elovl4 in the biosynthesis of VLC-PUFAs as occurs for vertebrates [1,41,52,53], as well as other invertebrates such as molluscs [43,46,54]. Vertebrate Elovl4 is widely expressed in the central nervous system (CNS) and photoreceptors of the retina [53] and, in fish, pineal gland [55], and hence it is believed to play pivotal roles in brain functioning and photoreception by providing VLC-PUFAs that guarantee normal function. Analysis of VLC-PUFAs from natural samples is technically challenging [56,57] and it can be only accurately performed on lipid samples prepared from specific polar lipid fractions collected from key tissues such as the CNS or retina. For that reason, it was not possible to clarify whether the ability of the gammarid Elovl4 for the biosynthesis of 26:5n-3 shown here occurs in vivo.

In conclusion, the research described in this work demonstrates that gammarids possess at least three distinct *elovl* genes, namely *elovl4*, *elovl6* and *elovl1/7*-like, with putative roles in the biosynthesis of LC-PUFAs. Molecular and functional characterisation of the set of *elovl* sequences from the marine gammarid *E. marinus* revealed that gammarids' Elovl4 and Elovl1/7-like are PUFA elongases with affinity towards PUFA substrates ranging from C_{18} to C_{22}, and account, by themselves, for all the elongation reactions required for LC-PUFA biosynthesis from C_{18} biosynthetic precursors. On the contrary, the gammarid Elovl6 sequence contained characteristics typically found in non-PUFA elongases which, along with its elongase capacity being restricted to C_{18} PUFA substrates, indicated that this enzyme does not play major roles in LC-PUFA biosynthesis in gammarids. Elovl4 was the sole Elovl found in gammarids with the ability to produce PUFAs of up to 26 carbons. Overall, the present study provides insight into the endogenous machinery enabling gammarids to bioconvert dietary fatty acids into high nutritional value LC-PUFAs.

4. Materials and Methods

4.1. Molecular Cloning of Full-Length cDNAs of Three Elovls from E. marinus

Several *E. marinus* individuals collected from the wild in the intertidal zone in Trondheim, Norway, were preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and sent to the facilities of the Instituto de Acuicultura Torre de la Sal, Spain, for further analysis. Total RNA was extracted from one single whole individual using the Maxwell® instrument and the Maxwell® 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. First strand complementary DNA (cDNA) was synthesised from 2 µg of total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega) following the manufacturer's instructions.

The strategy to retrieve the *elovl* sequences from *E. marinus* was established by considering that this species possesses the same *elovl* gene complement as *Hyalella azteca*, a closely related amphipod species with a reliable genome annotation (www.hgsc.bcm.edu/arthropods/hyalella-azteca-genome-project (accessed on 10 September 2019); www.ncbi.nlm.nih.gov/bioproject/243935 (accessed on 10 September 2019)). Searches for *elovl* homologues within the *H. azteca* genome resulted in the identification of four sequences: 1) gb|XM_018163314.1|, *H. azteca* elongation of very long-chain fatty acids protein 4-like; 2) gb|XM_018155086.1|, *H. azteca* elongation of very long-chain fatty acids protein 6-like;

3) gb|XM_018168638.1|, *H. azteca* elongation of very long-chain fatty acids protein 7-like; and 4) gb|XM_018164888.1|, *H. azteca* elongation of very long-chain fatty acids protein AAEL008004-like. Preliminary analysis of the sequences suggested that sequence 3) did not translate to any potentially functional putative elongases in *E. marinus*, nor in any other gammarid. Therefore, only sequences 1) "*elovl4*"; 2) "*elovl6*"; and 4), which we hereafter refer to as "*elovl1/7*-like", were used for further analyses. The *H. azteca elovl*-like sequences were used as queries to run BLAST searches for homologous sequences within the Transcriptomic Shotgun Assembly (TSA) database of the *Gammarus* BioProject (www.ncbi.nlm.nih.gov/bioproject/PRJNA497972 (accessed on 10 September 2019)) as follows. First, *H. azteca elovl4* (gb|XM_018163314.1|) was used to search homologous sequences from gammarid species available at NCBI (www.ncbi.nlm.nih.gov (accessed on 10 September 2019)), which included several gammarid species such as *Echinogammarus berilloni* (GHCU01154015.1), *Gammarus fossarum* (GHDC01027946.1) and *Gammarus pulex* (GHCS01223739.1). Alignment of these sequences (Geneious Prime Software, Version 2019.0.3, www.geneious.com (accessed on 10 September 2019)) [58] enabled the design of the degenerate primers EM_ELO4_F1 and EM_ELO4_R2 on conserved regions (Table 2). A polymerase chain reaction (PCR) using a high-fidelity polymerase (Phusion Green High-Fidelity DNA Polymerase, Thermo Fisher Scientific) was performed in order to amplify the first DNA fragment of the *E. marinus* putative *elovl4.* Primer sequences and PCR conditions are given in Table 1. A PCR product of the expected size (~350 bp) was obtained and then purified on a 1% (*w*/*v*) agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega). The identity of the peak was confirmed by DNA sequencing (DNA Sequencing Service, IBMCP-UPV, Valencia, Spain). Subsequently, gene-specific primers based on the first fragment sequence of the *E. marinus* putative *elovl4* were designed in order to obtain the full-length open reading frame (ORF) sequence by rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE Kit, Thermo Fisher Scientific). A two-round (nested) PCR using the *Taq* Polymerase GoTaq®G2 Green Master Mix (Promega) was performed. Briefly, the first round of the 3′ RACE PCR was run with the gene-specific primer EM_ELOVL4_F3 (Table 2) and the primer 3′ RACE Outer (provided by the kit), and *E. marinus* 3′RACE cDNA as a template. The second-round PCR was performed using the gene-specific primer EM_ELOVL4_F5 (Table 2) and the primer 3′RACE Inner (provided by the kit), and the first-round PCR product as a template. A 3′ RACE PCR product was obtained, which was subsequently purified and sequenced as above.

In order to obtain the 5′ end of the *E. marinus elovl4* ORF, a BLASTn (Megablast) was performed using the partial *elovl4* cDNA generated previously as a query against the TSA database of the *Gammarus* BioProject. We identified a high identity sequence from *Marinogammarus marinus* (GHCW01145927.1), a former species name for *E. marinus* [59]. This sequence (GHCW01145927.1), containing part of the 5′ untranslated region (5′UTR) and the 5′ end of the ORF, aligned with the 3′ end of the partial first fragment *elovl4* sequence obtained in previous steps. Gene-specific primers designed in the 5′UTR (EM_ELOVL4_ORF_U5F) and the 3′UTR (EM_ELOVL4_ORF_U3R) were used to obtain a 1246 bp product by running a PCR (Phusion Green High-Fidelity DNA Polymerase), which encompassed the ORF of the putative *E. marinus elovl4.* The PCR product was purified and sequenced as described above. The ORF sequence was predicted using the NCBI ORF Finder tool (www.ncbi.nlm.nih.gov/orffinder (accessed on 17 September 2019)).

Table 2. Primer sets and corresponding PCR conditions used in the cloning of the *E. marinus elovl4*, *elovl6* and *elovl1/7*-like genes.

Gene	Aim	Primer Name	Primer Sequence	Cycles	Tm	Extension
ELOVL4	1st fragment generation	EM_ELOVL4_ F1	TCTACAACCTTGCTGTCATG	35	55 °C	72 °C (1 min)
		EM_ELOVL4_ R2	TGCACAAAGCTGTTCATCAT			
	3'RACE PCR	3'Outer_Primer	GCGAGCACAGAATTAATACGACTCACTATAGGT12	35	55 °C	72 °C (75 s)
		3'Inner_Primer	CGCGGATCCGAATTAATACGACTCACTATAGGT12			
		EM_ELOVL4_F3	CACGTGTATCACCACTCGAC			
		EM_ELOVL4_F4	GGATTGGAGTCAAGTTTGTGG			
		EM_ELOVL4_F5	CCTGGCGGCAATGATGAACA			
	Full ORF	EM_ELOVL4_ORF_U5F	ATGAATTTAGTGAACAACAA	35	62 °C (10 cycles) 58 °C (25 cycles)	72 °C (1 min)
		EM_ELOVL4_ORF_U3R	CAAGATGCCTGAACTCCCGGT			
	Functional characterisation	EM_FW_ELOVL4_HindIII EM_RV_ELOVL4_XbaI	CCCAAGCTTACAATGGCTGCCTCTGTT CCGTCTAGACTACATGTCCTTTCGAGG	35	62 °C (10 cycles) 58 °C (25 cycles)	72 °C (45 s)
ELOVL6	1st fragment generation	EM_ELOVL6_ F1 EM_ELOVL6_ R2	GGCTTCTGGAACTGGATGTT TTCATGTAGGCACGACGGAA	35	55 °C	72 °C (1 min)
	Full ORF	EM_ELOVL6_ORF_U5F EM_ELOVL6_ORF_U3R	CTTTACCACGTTTTACTGGG ACTGGTAGTTTTGTATGCAT	35	62 °C (10 cycles) 58 °C (25 cycles)	72 °C (1 min)
	Functional characterisation	EM_FW_ELOVL_HindIII EM_RV_ELOVL_XbaI	CCCAAGCTTACGATGGCCCTCTCGGAC CCGTCTAGATTAATCGAACTTCCCTCCCT	35	62 °C (10 cycles) 58 °C (25 cycles)	72 °C (45 s)
ELOVL1/7-LIKE	1st fragment generation	EM_ELOVL_ F1	GTCATCCACCACGGATGCATG	35	55 °C	72 °C (1 min)
		EM_ELOVL_ R1	GCCTTCACGTAGAAGTTGGAG			
	Full ORF	EM_ELOVL_ORF_U5F	AAAAACGTGTTCTCGGCCAG	35	62 °C (10 cycles) 58 °C (25 cycles)	72 °C (1 min)
		EM_ELOVL_ORF_U3R	GAGGCTTAACTAAAACGAAC			
	Functional characterisation	EM_FW_ELOVL_HindIII EM_RV_ELOVL_XbaI	CCCAAGCTTAAGATGGCGGGTACAGCA CCGTCTAGATCAGTCGTCCTTCCGAGG	35	62 °C (10 cycles) 58 °C (25 cycles)	72 °C (45 s)

For the cloning of the *E. marinus elovl6* and *elovl1/7*-like ORF, we identified contig assemblies containing full-length sequences of the ORF by searching within the *M. marinus* (or *E. marinus*) BioSample (SAMN10259948). Briefly, the *H. azteca* XM_018155086.1 (*elovl6*) and XM_018164888.1 (*elovl1/7*-like) sequences were translated to their corresponding protein sequences and used as queries for TBLASTn searches against the *E. marinus* BioSample (SAMN10259948). These searches allowed the identification of the highly homologous sequences GHCW01079894.1 and GHCW01079198.1, containing, respectively, the putative *elovl6* and *elovl1/7*-like from *E. marinus*. Due to the large size of these sequences (~5.7 to 6.5 Kb), a preliminary analysis was performed in order to predict the ORF sequences for the putative elongases (NCBI ORF Finder). The predicted proteins were subsequently analysed using the Pfam online tool (https://pfam.xfam.org/ (accessed on 15 October 2019)) and those containing the complete "ELO" domain (pfam 01151) were selected as putative Elovls and considered for further analysis. Primer pairs for each target gene (*elovl6* or *elovl1/7*-like) were designed in the putative 5′ and 3′UTR (Table 2), and subsequently used for PCR amplification as described previously to obtain PCR products of 1405 bp for *elovl6*, and 1233 bp for the *elovl1/7*-like.

4.2. Sequence and Phylogenetic Analysis of the E. marinus Elongases

The putative aa sequences of the three newly cloned *elovl* from *E. marinus* were predicted using the ORF Finder tool available from NCBI. The transmembrane regions and protein domains of the *E. marinus* Elovl were predicted using the TMHMM online tool (www.cbs.dtu.dk/services/TMHMM/ (accessed on 18 October 2019)). Moreover, the subcellular location of the newly cloned elongases was predicted using the DeepLoc 1.0 online tool (www.cbs.dtu.dk/services/DeepLoc/ (accessed on 29 March 2021)). A phylogenetic analysis comparing the Elovl aa sequences from model vertebrates such as *Danio rerio* and *Mus musculus*, as well as Elovl protein sequences from crustacean species such as Elovl4 from the swimming crab *P. trituberculatus* (QBO95487.1), and Elovl4 (QBX90561.1), Elovl6 (QEV88898.1) and Elovl7 (AWM30548.1) from the orange mud crab *S. olivacea*, was performed. In addition, several putative Elovl protein sequences from other crustacean species, including gammarids from the genus *Gammarus* and other amphipods, were retrieved and also included in the phylogenetic analysis. Briefly, 1) orthologues for the *E. marinus* Elovl sequences were retrieved by tblastn (NCBI) within the *Gammarus* TSA database (PRJNA497972), 2) putative Elovl sequences were analysed using the Pfam online tool and the ones containing the "ELO" domain (pfam 01151) were selected as candidates to be included in the phylogenetic analysis. The phylogenetic tree and all the corresponding analyses were performed using the CIPRES platform [60] (https://www.phylo.org/ (accessed on 29 March 2021)). Briefly, first, a MAFFT alignment and subsequent trimming (TrimAl) were performed. Then, a model test was run in order to select the best evolutionary model for aa substitution. Finally, randomized axelerated maximum likelihood (RAxML) was performed in order to build the phylogenetic tree and the LG4X was used as an evolutionary model [44]. Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations, following the transfer distance bootstrap approach [61,62].

4.3. Functional Characterisation of the E. marinus Elovl by Heterologous Expression in Yeast

The ORF sequences of the three *E. marinus elovl* sequences were cloned into the yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, USA). Briefly, primers containing *Hind* III (forward) and *Xba* I (reverse) restriction sites were designed for amplification of the whole ORF of the three *E. marinus elovl* genes (Table 2). The PCRs were performed using the high-fidelity polymerase Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific), with conditions used for each gene detailed in Table 1. Then, PCR products were purified as above and subsequently digested with the corresponding restriction enzymes before being ligated into similarly restricted pYES2. Ligation reactions were transformed into the *E. coli* competent TOP10^{TM} strain (Invitrogen) and positive colonies were grown

overnight in Luria–Bertani (LB) broth containing ampicillin (100 μg/mL). Next, plasmids were purified using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instructions prior to DNA sequencing (IBMCP-UPV) in order to confirm the sequence correctness of the inserts.

The plasmid constructs pYES2-Elovl4, pYES2-Elovl6 and pYES2-Elovl (Elovl1/7-like) were transformed into *Saccharomyces cerevisiae* competent cells (strain INVSc1) (Invitrogen). The recombinant yeast were selected on *S. cerevisiae* minimal medium minus uracil ($SCMM^{-ura}$) agar plates for 3 d at 30 °C. The culture of recombinant yeast was carried out as described in detail by Jin et al. [63]. Briefly, one single recombinant colony from each transformation (*E. marinus elovl* pYES2 constructs) was grown in $SCMM^{-ura}$ broth for 2 d at 30 °C to produce a bulk culture with an OD600 of 8–10. Subsequently, an appropriate volume of the yeast bulk cultures was inoculated in 5 mL of $SCMM^{-ura}$ broth contained in individual 150 mL Erlenmeyer flasks to provide an OD600 of 0.4. The recombinant yeast in each Erlenmeyer flasks was grown for 4 h at 30 °C and under constant shaking (250 rpm) until they reached an OD600 of ~1, at which point the transgene expression was induced by supplementing the culture media with galactose at 2% (*w*/*v*). Moreover, in order to test the ability of the *E. marinus* Elovl to elongate PUFA substrates, recombinant yeast expressing the *E. marinus elovl4*, *elovl6* and *elovl1/7*-like were supplemented with one of the following PUFA substrates in the growth media: α-linolenic acid (18:3n-3), linoleic acid (18:2n-6), stearidonic acid (18:4n-3), γ-linolenic acid (18:3n-6), eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6), docosapentaenoic acid (22:5n-3), docosatetraenoic acid (22:4n-6) and docosahexaenoic acid (22:6n-3). Final concentrations of the exogenously supplied PUFA substrates were 0.5 mM (C_{18}), 0.75 mM (C_{20}), 1.0 mM (C_{22}) as uptake efficiency decreases with increasing chain length [64]. A control consisting of yeast transformed with the empty pYES2 was run exactly as described above for the three *E. marinus* elongases. Except stearidonic acid (18:4n-3), all FA substrates (>98–99% pure) used for the functional characterisation assays were obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA). Yeast culture reagents including galactose, nitrogen base, raffinose, tergitol NP-40, stearidonic acid (>99%) and uracil dropout medium were obtained from Sigma-Aldrich (St. Louis, MO, USA). The yeast cultures were maintained at 30 °C and under constant shaking (250 rpm) for 2 d until yeast was harvested by centrifugation at 1500 g for 2 min. Yeast pellets were washed twice with 5 mL of ddH_2O, homogenised in 6 mL of 2:1 (*v*/*v*) chloroform:methanol containing 0.01% (*w*/*v*) butylated hydroxytoluene (BHT, Sigma-Aldrich) as antioxidant and stored at −20 °C for a minimum of 24 h in an oxygen-free atmosphere until further analysis.

4.4. Fatty Acid Analysis

The FA composition of yeast containing either the ORF of the *E. marinus* elongases (i.e., transformed with either pYES2-Elovl4, pYES2-Elovl6 or pYES2-Elovl) or empty pYES2 (control) was determined using the method described by Monroig et al. (2013) [65]. Briefly, total lipids were extracted from the homogenised yeast samples using the Folch method [66]. Subsequently, total lipids were used to prepare fatty acid methyl esters (FAME), which were analysed using gas chromatography coupled with mass spectrometry (GC–MS) [55]. The elongation of exogenously supplemented PUFA substrates by the *E. marinus* Elovl4, Elovl6 and Elovl1/7-like was calculated by the proportion of substrate FA converted to elongated FA product(s) as [areas of all products with longer chain than substrate/(areas of all products with longer chain than substrate + substrate area)] × 100.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/md19040226/s1, Figure S1: Predicted transmembrane-spanning regions of *E. marinus* elongases, Table S1: Subcellular localisation prediction of the three Elovl proteins from *E. marinus* and Elovl type determination.

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Data Availability Statement: The data presented in this study are included in the corresponding sections throughout the manuscript. Sequences of the newly characterised *Echinogammarus marinus elovl* genes were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/) (accessed on 10 September 2019).

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Effects of diet and temperature on the fatty acid composition of the gammarid *Gammarus locusta* fed alternative terrestrial feeds

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The fast and remarkable growth of global aquaculture in recent years has created new challenges, such as guaranteeing a sustainable supply of raw materials used for aquafeed formulation. Gammarids are low-trophic crustaceans with an increasing interest in aquaculture due to their high nutritional profiles and their capacity to grow under high-density conditions. Moreover, gammarids have the ability to thrive on a wide range of sidestreams while accumulating relatively high levels of long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA). In the present study, juveniles of the marine gammarid *Gammarus locusta* were cultured at four different temperatures (5°C, 10°C, 15°C, and 20°C) for 21 days and fed three diets, including the seaweed *Fucus* sp. as control, and carrot leaves and coconut flesh representing two agri-food industry sidestreams. Our results indicate that both the survival and biomass of *G. locusta* were highly affected by diet, with coconut showing the lowest growth performance. The temperature had no effect on biomass, although high temperature (20°C) resulted in a decrease in survival. The effects of temperature on the gammarid fatty acids were not evident, with diet being the main modulator of the profiles. Furthermore, the results also reveal that the *Fucus* sp. diet was associated with relatively high percentages of n-3 and n-6 LC-PUFA. Interestingly, essential LC-PUFA such as eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids were detected in gammarids fed on either *Fucus* sp. or any of the sidestreams irrespectively of their presence in the diets. These results suggest an ability of *G. locusta* for LC-PUFA biosynthesis (trophic upgrading) and/or retention, making this species a promising candidate for the production of high-value ingredients for aquafeeds.

KEYWORDS

agri-food sidestreams, circular economy, *gammarus locusta*, long-chain polyunsaturated fatty acids, novel marine ingredients

Introduction

Rapidly expanding aquaculture worldwide has created new economic and ecological challenges (FAO, 2020). With regard to fish farming, such challenges have been mostly linked to modifying the supply of raw materials used for feed formulation in order to reduce the current usage of finite resources such as the so-called marine ingredients fishmeal (FM) and fish oil (FO). FM and FO are regarded as major sources of essential nutrients for aquafeeds, including long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) (Tocher, 2015; Shepherd et al., 2017). The current production of FM and FO largely relies on feed-grade species fisheries and, with the expansion of aquaculture worldwide, pressure on these fisheries has grown to an extent that they may not sustainably fulfill the increasing demand (Naylor et al., 2009; FAO, 2020).

Several efforts have been made to find alternative feeding sources to alleviate the abovementioned pressure on fisheries and reduce the dependence upon FM and FO in finfish aquaculture (Naylor et al., 2009; Turchini et al., 2011b; Jannathulla et al., 2019). Up to the present, the use of raw materials derived from animals or plants has become a widely extended practice (Turchini et al., 2009; Jannathulla et al., 2019; Galkanda-Arachchige et al., 2020). However, the replacement of FM and FO with nonmarine ingredients has often been associated with decreased nutritional value of fish farming products, including reduced levels of the health-promoting n-3 LC-PUFA eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), as well as suboptimal growth (Bell et al., 2001; Mourente and Bell, 2006; Turchini et al., 2011a; Yıldız et al., 2018; Romano et al., 2020). Therefore, high-quality alternative ingredients are needed in order to successfully replace traditional sources of FM and FO in aquafeed while maintaining growth performance and nutritional value (i.e., levels of n-3 LC-PUFA) of farmed fish (Alberts-Hubatsch et al., 2019). Indeed, the search for novel aquatic ingredients for aquafeed is a priority within the EU, as is their production through Integrative Multi-Trophic Aquaculture (IMTA) strategies (Guerra-Garcia et al., 2016).

Ingredients derived from biomasses of low-trophic marine crustaceans such as gammarids, krill, and copepods have been regarded as promising candidates for aquafeed formulations due to their balanced profiles of essential nutrients, including n-3 LC-PUFA (McKinnon et al., 2003; Suontama et al., 2007; Naylor et al., 2009; Dhont et al., 2013; Harlıoğlu and Farhadi, 2018). While the exploitation of wild populations of marine crustaceans poses negative ecological impacts similar to those of wild-capture fisheries alluded to above, biomass production using intensive aquaculture systems arises as an interesting strategy. Gammarids are abundant in benthic communities and inhabit practically all aquatic environments, which is reflected in their high diversity of feeding habits (Costa and Costa, 2000; Harlıoğlu and Farhadi, 2018). Moreover, gammarids are fish's natural prey, and their use as an alternative ingredient for fish feeding has been explored (Ashour et al., 2021). Gammarids can be grown and maintained in laboratory cultures and adapt well to different culture conditions and diets (Sexton, 1928; Costa and Costa, 2000; Neuparth et al., 2002; Ahyong and Hughes, 2016; Beermann et al., 2018; Alberts-Hubatsch et al., 2019). Interestingly, recent investigations have also demonstrated that gammarids can be fed on a wide range of sidestreams from bioindustries such as agriculture and aquaculture itself (Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020). Such sidestreams are characterized by being deprived or having low contents of LC-PUFA, but, intriguingly, gammarids fed on these sidestreams have shown relatively high levels of LC-PUFA, suggesting that gammarids have some capacity for trophic upgrading *via* endogenous lipid metabolism (Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020). Thus, applying circular bioeconomy strategies by which sidestreams derived from bioindustries are used for the production of high nutritional value gammarid biomasses as potential fish feed ingredients has been proposed (Jiménez-Prada et al., 2020). However, up to date, little is known about the optimal culture conditions for reliable large-scale cultures of marine gammarids (Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020). Several factors, including diet, temperature, and salinity can modulate LC-PUFA metabolism, growth, and survival of aquatic invertebrates (Neuparth et al., 2002; Monroig and Kabeya, 2018; Alberts-Hubatsch et al., 2019). Hence, it is necessary to fine tune the culture conditions for the production of LC-PUFA–rich gammarid biomass.

A previous study comparing the effects of several diets on two gammarid species, namely *Gammarus locusta* and *Echinogammarus marinus*, suggested that *G. locusta* is the best candidate regarding LC-PUFA composition, growth, and survival when fed sidestreams (Alberts-Hubatsch et al., 2019). However, to the best of our knowledge, information on the combined effects of diet and environmental factors, on survival, growth, and LC-PUFA content of marine gammarids is lacking. The main goal of this study was to elucidate the effects of three diets and four temperatures on the FA profile and growth performance of the marine gammarid *G. locusta*. For this purpose, cultures were carried out at 5°C, 10°C, 15°C, and 20° C, representing ambient temperatures in the wild, with *G. locusta* occurring in coastal areas of the North Atlantic (Costa and Costa, 2000). *Fucus* spp. was used as a natural marine diet, and carrot leaves and coconut flesh as two different nonmarine diets, mimicking agriculture sidestreams of different nutritional values.

Materials and methods

Animal collection and culture

All *G. locusta* specimens used in the experiments were obtained from a laboratory culture at the Alfred Wegener Institute, with stock cultures originating from the German Bight, North Sea. Specimens were reared in the laboratory prior to the experiment at temperatures of 18°C, a salinity of 33–34 ppt, and pH 8. Broodstock cultures and juvenile *G. locusta* were raised on a mixture of dried *Fucus* spp. and vegetable greens (mainly carrot greens and kale leaves). Juvenile *G. locusta* (28 days posthatch) were collected from the same batch culture, maintained at 10°C for 5 days, and starved for 24 h prior to the experiment.

Experimental setup

The juvenile gammarids (5.327 ± 1.54 mm SD, n = 960) were randomly transferred into white 1-L buckets, filled with 500 ml of freshly filtered seawater (5µm tube filter) at 33–34 ppt and equipped with a mesh (70 × 70 mm, 5 mm mesh size) and an oyster shell as substrate. Each container was stocked with 20 specimens at 10°C and randomly allocated to the respective temperatures (5°C, 10°C, 15°C, and 20°C) and diet in quadruplicates. The temperature was slowly adjusted by transferring the containers into water baths at the desired temperature. Three different diets were prepared: one natural marine food source, thalli of *Fucus* spp. containing LC-PUFA (hereafter referred to as Fucus), and two nonmarine diets: carrot leaves (hereafter referred to as "Carrot"), an agricultural sidestream, (high shorter-chain (<C20) PUFA content), and a diet that mimics a potential sidestream rich in saturated fatty acids (SFA) consisting on coconut flesh (hereafter referred to as "Coco"). All diets were rinsed in fresh water and dried at 55°C for 24 h. The temperature in the experimental containers was recorded twice a day, and water exchange with fresh seawater adjusted to the respective temperature was done every second day. Feeding was done *ad libitum* with remaining food items removed and replaced during water exchange. Dead individuals were removed on a daily basis. Gammarids were cultured at the corresponding diet vs. temperature combination for 21 days, until sexual maturity was reached in the higher temperature treatments.

Growth and survival

Initially, a subsample of 100 juvenile gammarids (average 5 mm) was analyzed. Total body lengths of gammarids were measured at the beginning and end of the experiment by analyzing pictures of each replicate taken on scale paper using Fiji ImageJ (vers. 1.53q, Schneider et al., 2012). Total lengths were measured from the basal point of the antennae to the third urosome segment. Specific growth rate (SGR, in % day^{-1}) was calculated as length gain per experimental duration for each pool as follows:

SGR = 100 × [ln (final length) − ln (initial length)/time interval]. Initial individual weights could not be taken at the beginning of the experiment due to the vulnerability of the early life stages. Therefore, at the end of the experiment, all remaining specimens were counted and wet biomass per replicate was recorded using an analytical scale (Sartorius Practum 213-S1, d = 0.001 g). The gammarids were rinsed in Milli-Q water twice, placed into Eppendorf tubes, and immediately frozen at −80°C. The frozen samples were then freeze-dried at −52°C within 2 weeks after the experiment and thereafter stored at −80°C until further analyses.

Fatty acid analysis

Total lipids and FA were analyzed from gammarid samples as well as from experimental diets. Briefly, total lipids were extracted from the homogenized samples using the Folch method (Folch et al., 1957). Subsequently, total lipids were used to prepare fatty acid methyl esters (FAME), which were analyzed using a Thermo Trace GC Ultra Gas Chromatograph (Thermo Electron Corporation, Waltham, MA, USA), equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 µm, Teknokroma, Sant-Cugat del Vallès, Spain), fitted with an on-column injection system, using helium as a carrier gas, and a flame ionization detector (FID). The analytical temperature was programmed from 50°C to 220° C. Chromatograms were integrated and analyzed with Azur Datlys (St Martin d'Heres, France) software. FAs were identified by comparison of retention times of each peak with those of well-characterized standards.

Statistical analysis

All data were subjected to statistical analyses using PAST (vers. 4.09) (Hammer et al., 2001). Prior to the analyses, data were tested for normality (Shapiro–Wilk) and homogeneity (Levene's test). After assuring that normality and homogeneity criteria were met, data were analyzed using analysis of variance (fixed effects two-way ANOVA) with Tukey's *post-hoc* test for multiple comparisons. Principal component analysis (PCA) was used to analyze and visualize the relationship between diet and FA profiles of gammarids. Differences in FA levels among treatments were tested by using a two-way permutational multivariate analysis of variance (PERMANOVA) with factors: "Temperature," four different conditions (5°C, 10°C, 15°C, and 20°C), and "Diet," three different treatments (Fucus, Carrot, Coco). One-way PERMANOVA was further used to compare the scores of the dietary groups. Ellipses were fitted to the scores

at 95% confidence. Additionally, the percentage of similarity analysis (SIMPER) was used to determine the FA responsible for dissimilarities between conditions within the dietary groups. Unless otherwise stated, statistical significance was tested at 95% confidence level ($p \leq 0.05$). The Unsaturation Index (UI) was used to establish a relationship between the FA levels of diets used and the FA levels of gammarids fed on the corresponding diets in order to determine how diet can affect the gammarids' FA profiles and to ascertain previous retention. The UI was calculated according to the following formula: $\sum$ [area of fatty acid * number of unsaturations].

Results

Growth and survival

The two-way ANOVA revealed that both temperature (F (3, 36) = 12.43, $p < 0.0001$) and diet (F (2, 36) = 18.08, $p < 0.0001$) had a significant effect on the survival of *G. locusta*, although no interacting effects of temperature and diet on survival were observed (F (6, 36) = 0.38, $p = 0.89$). Pairwise comparisons of the survival of gammarids at different temperatures revealed that the highest temperature (20°C) resulted in significantly lower survival rates, whereas no differences were observed between the temperatures (Table 1). Regarding the diets, Coco resulted in significantly lower survival of gammarids, whereas no differences between the effects of Carrot and Fucus were found. The highest mortality was obtained with the Coco diet at 20°C (Table 1).

Regarding total biomass, there was a significant interacting effect of temperature and diet as revealed by the two-way ANOVA (F (6, 36) = 3.17, $p = 0.01$). Here, our analysis shows no significance of temperature (F (3, 36) = 1.63, $p = 0.20$) but a highly significant effect of diet (F (2, 36) = 29.07, $p < 0.001$). Pairwise comparisons of the biomass output revealed highly significant differences ($p < 0.005$) between all diet groups, with the Fucus diet resulting in the highest (Table 1). No significant differences were found in the gammarid biomasses when comparing temperature groups.

The final length was not affected by the interaction of temperature and diet (F (6,35) = 1.47, $p = 0.22$). In this case, the diet had no effect on final length (F (2, 35) = 3.03, $p = 0.06$), in contrast to temperature, which strongly affected final length (F (3, 35) = 20, 73, $p < 0.001$). When looking at pairwise comparisons, it becomes evident that this effect was caused by the 20°C treatment, which resulted in significantly higher final lengths compared to all other temperature groups ($p < 0.001$), whereas no differences were found between the other groups (Table 1).

The SGR followed a similar pattern as the final length data, with temperature having a highly significant effect on SGR (F (3, 35) = 31.83, $p < 0.001$). This was also explained by the higher SGR in the 20°C treatment, which was significantly different from all other temperature treatments ($p < 0.001$) while the other temperatures were not different (Table 1). Diet had a significant effect on SGR (F (2, 35) = 5.26, $p = 0.01$), which was caused by the low SGR when regarding pairwise comparisons, in which Coco was significantly different from Fucus but not from Carrot (Table 1). This was biased by the very high mortality in this treatment group (see above). Similarly, a significant interaction effect of diet and temperature on SGR (F (6, 35) = 3.44, $p = 0.008$) was observed.

TABLE 1 Survival, total biomass, and total length of *G. locusta* in response to different diets and temperatures after the 21-day feeding trial.

	5°C	10°C	15°C	20°C
% Survival (± SD)				
Fucus	73.75 ± 10.31 a, A	65.00 ± 10.80 a, A	71.25 ± 14.93 a, A	45.00 ± 28.28 b, A
Carrot	71.25 ± 6.29 a, A	60.00 ± 10.80 a, A	66.25 ± 27.80 a, A	32.50 ± 10.41 b, A
Coco	47.50 ± 8.66 a, B	23.75 ± 23.94 a, B	47.50 ± 8.66 a, B	8.75 ± 4.79 b, B
Biomass (mg ± SD)				
Fucus	76.00 ± 11.86 a, A	122.50 ± 31.03 ab, A	121.25 ± 38.35 b, A	159.00 ± 36.39 ab, A
Carrot	67.50 ± 20.04 a, B	78.00 ± 26.89 ab, B	95.75 ± 41.10 b, B	86.50 ± 27.74 ab, B
Coco	64.00 ± 43.14 a, C	27.25 ± 9.00 ab, C	51.25 ± 16.66 b, C	27.25 ± 12.47 ab, C
Length (mm ± SD)				
Fucus	6.92 ± 0.85 a	8.28 ± 1.44 a	8.24 ± 0.1 a	11.59 ± 2.45 b
Carrot	6.85 ± 0.73 a	7.10 ± 0.32 a	7.62 ± 1.05 a	9.81 ± 1.73 b
Coco	7.26 ± 2.63 a	5.31 ± 1.57 a	6.75 ± 0.3 a	10.89 ± 8.0 b
Specific growth rate (SGR ± SD)				
Fucus	0.37 ± 0.12 a, A	0.89 ± 0.11 a, A	0.89 ± 0.13 a, A	1.68 ± 0.32 b, A
Carrot	0.53 ± 0.08 a, AB	0.71 ± 0.04 a, AB	0.74 ± 0.04 a, AB	1.29 ± 0.44 b, AB
Coco	0.43 ± 0.36 a, B	−0.10 ± 0.58 a, B	0.66 ± 0.22 a, B	1.48 ± 0.39 b, B

Fucus, Fucus spp.; Carrot, carrot leaves; Coco, coconut flesh. Data are expressed as mean of the different replicates per condition ± standard deviation (SD). Statistical differences (Tukey's pairwise comparisons, $p \leq 0.05$) are indicated by different letters: lowercase letters (a–d) indicate differences in temperature and uppercase letters (A–D) indicate differences in diet.

Fatty acid profiles of gammarids

The FA analysis of the diets revealed that Fucus was rich in oleic acid (18:1n-9) (29.8%), whereas α-linolenic acid (ALA, 18:3n-3) was the most abundant FA in carrot leaves (26.5%). On the other hand, the Coco diet showed high levels of SFA (90.4%), such as lauric acid (12:0) (48.7%) and myristic acid (14:0) (19.9%) (Table 2). Low levels of DHA (22:6n-3) were detected only in the Fucus diet (1.1%), whereas EPA (20:5n-3) was also present in Fucus (4.8%) and only in trace amounts (0.2%) in Carrot. Moreover, the Coco diet had the lowest UI (6.2) whereas the Fucus and Carrot diets showed similar levels (150.4 and 138.3, respectively), reflecting that the former is very poor in PUFA and MUFA and rich in SFA, as compared to the other treatments.

FA analysis of cultured *G. locusta* showed similar profiles when different temperature treatments were compared (Table 3). However, the two-way ANOVA of the FA analyses only showed significant differences when comparing the different dietary treatments (Table 4). One-way ANOVA of gammarids fed on the different diets (Table 5) did not show significant differences in EPA and DHA levels. The two-way PERMANOVA results did not show differences in FA among different temperatures ($F(3, 28) = 1.20$, $p = 0.29$), or the interaction temperature and diet ($F(6, 28) = 0.73$, $p = 0.74$). Moreover, the two-way PERMANOVA revealed diet as the main modulator of FA profiles ($F(2, 28) = 8.97$, $p < 0.001$). PCA revealed that the first component (PC1) accounted for the 60.81% of variance of this dataset, whereas the PC2 accounted for the 19.19% of variance (Figure 1). The PCA loading plot showed saturated fatty acids such as lauric acid (12:0) and myristic acid (16:0) are separated on the negative side of PC1 from unsaturated and polyunsaturated fatty acids including LC-PUFA such as arachidonic acid (20:4n-6, ARA), EPA (20:5n-3), docosapentaenoic acid (22:5n-3, DPA), and DHA (22:6n-3), which load on the positive side (Figure 1). On the other hand, 16:1n-7, LA (18:2n-6), and ALA (18:3n-3) load on the negative side of PC2. This variable distribution drives the scores segregation (Figure 1). The FA profiles of *G. locusta* fed on Coco are correlated with SFA (Figure 1). Those fed carrots are associated with LA and ALA, whereas those fed the Fucus diet are associated with LC-PUFA, among others. It is also interesting to note that the scores of the FA profiles of gammarids fed on the Fucus diet showed less dispersion than those fed the carrot leaves and coconut diets (95% ellipses, Figure 1). One-way PERMANOVA ($F = 9.38$, $p < 0.0001$), however, showed that the three dietary groups were significantly different (Supplementary Table S1). No significant differences were found for PC2. Additional SIMPER analysis (Supplementary Tables S2-S4) revealed that oleic acid (18:1n-9, OA) was the FA with more dissimilarity between the Fucus diet and the other diet groups (Carrot and Coco). On the other hand, the SIMPER analysis comparing Carrot vs. Coco showed that the SFA were the FA characteristic of the Coco diet.

Generally, *G. locusta* fed on Fucus showed FA profiles richer in LC-PUFA ($C_{20\text{-}24}$) than those fed on Carrot or Coco, whereas

TABLE 2 Relative FA composition (% of total FA) of diets used in the feeding trial.

	Fucus	Carrot	Coco
12:0	1.1	1.1	48.7
14:0	11.4	1.4	19.9
16:0	13.6	17.5	9.3
16:1n-7	1.5	0.2	0.1
18:0	0.7	1.3	3.5
18:1n-9 (OA)	29.8	2.1	4.7
18:2n-6 (LA)	8.8	14.3	0.7
18:3n-3 (ALA)	3.3	26.5	0.1
20:4n-6 (ARA)	12.0	0.4	nd
20:5n-3 (EPA)	4.8	0.2	nd
22:5n-3 (DPA)	nd	nd	nd
22:6n-3 (DHA)	1.1	nd	nd
SFA	27.7	23.2	90.4
MUFA	32.4	2.8	4.7
n-3 PUFA	11.6	26.7	0.1
n-6 PUFA	21.6	14.9	0.7
n-3 LC-PUFA	6.0	0.3	nd
n-6 LC-PUFA	12.8	0.6	nd
UI	150.4	138.3	6.2

Fucus, Fucus spp.; Carrot, carrot leaves; Coco, coconut flesh; OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; UI, unsaturation index; nd, not detected.

TABLE 3 Relative fatty acid (FA) composition (% of total FA) of samples of *G. locusta* fed different diets at four temperatures.

	5°C Fucus	5°C Carrot	5°C Coco	10°C Fucus	10°C Carrot	10°C Coco	15°C Fucus	15°C Carrot	15°C Coco	20°C Fucus	20°C Carrot	20°C Coco
12:0	0.2 ± 0.1	0.1 ± 0.08	6.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	11.6	0.7 ± 0.2	0.2 ± 0.1	8.2	0.2 ± 0.2	0.3 ± 0.1	8.7 ± 1.7
14:0	3.0 ± 0.4	0.7 ± 0.1	4.9 ± 0.1	3.4 ± 0.7	1.0 ± 0.2	12.9	2.8 ± 0.2	1.0 ± 0.3	9.1	2.4 ± 1.0	1.1 ± 0.5	7.3 ± 2.0
16:0	15.0 ± 0.3	17.2 ± 1.3	15.7 ± 0.8	16.2 ± 0.6	15.3 ± 1.5	31.8	16.6 ± 0.2	16.4 ± 0.6	37.0	18.9 ± 1.4	18.3 ± 2.6	19.7 ± 2.9
16:1n-7	2 ± 0.5	1.5 ± 0.5	1.7 ± 0.9	2.2 ± 0.5	2.5 ± 1.4	0.8	2.0 ± 0.6	3.4 ± 0.4	0.9	1.5 ± 0.2	2.4 ± 0.6	1.8 ± 0.7
18:0	2.5 ± 1.4	3.4 ± 0.6	3.9 ± 0.69	2.1 ± 0.2	2.8 ± 0.4	9.1	3.6 ± 0.9	3.3 ± 0.5	12.5	3.3 ± 1.0	5.6 ± 1.6	5.2 ± 0.7
18:1n-9 (OA)	29.7 ± 4.0	17.5 ± 2.5	26.2 ± 2.4	27.2 ± 3.4	17.6 ± 6.1	18.3	27.7 ± 3.0	14.0 ± 2.7	8.7	24.9 ± 2.6	16.1 ± 2.7	14.2 ± 4.9
18:2n-6 (LA)	7.3 ± 0.7	8.6 ± 1.7	4.5 ± 0.2	6.7 ± 1.1	10.0 ± 0.0	2.2	6.0 ± 0.6	13.9 ± 1.4	0.7	5.5 ± 0.8	7.8 ± 3.3	2.4 ± 0.1
18:3n-3 (ALA)	2.3 ± 0.7	5.4 ± 1.9	0.4 ± 0.0	2.6 ± 0.3	8.5 ± 2.4	nd	2.1 ± 0.6	12.0 ± 1.5	1.1	1.8 ± 0.4	5.9 ± 0.8	0.3
20:4n-6 (ARA)	10.1 ± 3.6	10.9 ± 2.6	13.2 ± 0.3	12.1 ± 0.8	8.3 ± 1.3	3.5	12.3 ± 1.3	4.8 ± 1.0	0.8	13.5 ± 1.9	4.1 ± 1.6	4.6 ± 0.1
20:5n-3 (EPA)	7.1 ± 3.5	7.5 ± 2.3	8.9 ± 0.3	9.9 ± 0.8	6.3 ± 1.1	1.7	10.8 ± 1.4	4.1 ± 0.7	1.4	12.6 ± 3.7	5.9 ± 0.8	9.7 ± 2.1
22:5n-3 (DPA)	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.5 ± 0.1	0.1 ± 0.2	nd	0.6 ± 0.1	0.2 ± 0.1	nd	0.7 ± 0.3	0.2 ± 0.1	0.3 ± 0.1
22:6n-3 (DHA)	2.3 ± 0.6	3.3 ± 0.9	2.3 ± 1.7	2.4 ± 0.7	3.0 ± 0.4	0.6	2.6 ± 0.9	1.7 ± 0.4	0.4	3.0 ± 0.8	3.0 ± 1.1	3.0 ± 0.6
SFA	20.9 ± 0.7	22.1 ± 1.6	32.0 ± 1.6	22.2 ± 0.9	22.3 ± 0.9	66.8	24.1 ± 0.2	22.3 ± 0.7	69.2	25.8 ± 3.7	24.6 ± 4.9	37.1 ± 11.4
MUFA	36.0 ± 4.0	23.7 ± 3.0	31.9 ± 0.2	35.7 ± 1.8	25.3 ± 8.8	22.4	34.2 ± 2.0	24.0 ± 3.8	12.5	30.9 ± 2.3	23.9 ± 1.9	23.7 ± 2.7
n-3 PUFA	16.6 ± 1.3	20.0 ± 1.2	12.8 ± 0.3	18.3 ± 1.6	20.2 ± 1.1	2.3	17.6 ± 1.5	21.8 ± 3.1	2.9	19.7 ± 4.1	21.1 ± 6.5	15.2 ± 1.9
n-6 PUFA	21.2 ± 0.3	23.0 ± 2.3	19.0 ± 0.1	20.8 ± 0.9	20.1 ± 1.0	6.0	19.6 ± 1.3	20.4 ± 2.0	1.6	20.5 ± 1.4	19.0 ± 1.9	10.8 ± 5.5
n-3 LC-PUFA	12.2 ± 0.4	13.8 ± 0.6	12.4 ± 0.3	13.6 ± 1.4	11.6 ± 1.1	2.3	14.3 ± 2.3	9.7 ± 4.0	1.8	16.5 ± 4.8	12.7 ± 6.2	13.2 ± 2.4
n-6 LC-PUFA	13.5 ± 0.3	12.6 ± 2.6	14.5 ± 0.1	13.6 ± 0.8	10.1 ± 1.0	3.8	13.8 ± 1.3	6.5 ± 1.4	0.8	15.0 ± 2.1	9.4 ± 3.3	5.3 ± 0.2
UI	184.0 ± 2.2	193.7 ± 9.1	165.0 ± 2.1	189.5 ± 9.9	165.3 ± 2.2	53.8	184.5 ± 9.6	162.1 ± 11.8	30.0	196.7 ± 26.6	184.4 ± 40.6	136.9 ± 19.3

Fucus, Fucus spp.; Carrot, carrot leaves; Coco, coconut flesh.; OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids; UI, unsaturation index; nd, not detected.

G. locusta fed on Carrot showed the highest levels of PUFA (<C_{20}) such as LA and ALA (Table 3). Notably, levels of EPA and DHA were detected in *G. locusta* fed on either Carrot or Coco regardless of these FA not being present in those diets. Regarding SFA, *G. locusta* fed on Coco showed higher percentages as compared to *G. locusta* fed on either Fucus or Carrot (Table 3). It is important to note as well that lower percentages of SFA were detected in cultured *G. locusta* fed on Coco in comparison with the Coco diet itself, while maintaining moderate LC-PUFA levels.

Discussion

The nutritional profiles of gammarids have been studied as alternative sustainable ingredients for aquafeeds (Kolanowski et al., 2007; Baeza-Rojano et al., 2010; Baeza-Rojano et al., 2013; Baeza-Rojano et al., 2014; Jiménez-Prada et al., 2018) due to the potential of these organisms to feed on a wide range of sidestreams (Harlıoğlu and Farhadi, 2018). However, only a few investigations have focused on culture strategies based on the use of aquaculture and/or agriculture waste as primary food (Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020), and up to date, there are no investigations reporting on the effect of food and different environmental conditions on the fatty acid profile, growth, and survival of these organisms. In the present study, juveniles of *G. locusta* survived and reached sexual maturity in all the conditions tested for both temperature and diet groups. Interestingly, the survival of *G. locusta* was significantly lower at 20°C than at the rest of the temperatures. This result supports the hypothesis that at 20°C there is an acceleration of growth and reduction of the life cycle of *G. locusta*, resulting in a faster length growth and therefore metabolism, which results in an increased mortality (Neuparth et al., 2002). Regarding the effect of different diets, survival was significantly lower in *G. locusta* fed on Coco,

TABLE 4 Two-way ANOVA results of the fatty acid composition of samples of *G. locusta* fed different diets at four temperatures.

	Temperature		Diet		Interaction	
	F-test (2, 38)	*p*-value	*F*-test (3, 37)	*p*-value	*F*-test (6, 40)	*p*-value
12:0	0.56	0.65	19.78	< 0.001	0.32	0.92
14:0	0.27	0.84	14.09	< 0.001	0.25	0.96
16:0	0.32	0.81	1.15	0.329	0.34	0.91
16:1n-7	0.63	0.60	1.34	0.275	1.38	0.26
18:0	0.75	0.53	9.24	< 0.001	1.02	0.43
18:1n-9 (OA)	1.23	0.31	32.13	< 0.001	0.89	0.51
18:2n-6 (LA)	1.59	0.21	8.79	< 0.001	1.84	0.12
18:3n-3 (ALA)	1.87	0.15	11.71	< 0.001	1.60	0.18
20:4n-6 (ARA)	1.19	0.33	8.96	< 0.001	1.79	0.14
20:5n-3 (EPA)	1.56	0.22	4.28	0.021	0.74	0.62
22:5n-3 (DPA)	1.07	0.37	8.82	< 0.001	0.45	0.84
22:6n-3 (DHA)	2.06	0.12	0.85	0.437	0.75	0.61
SFA	0.19	0.90	7.09	0.002	0.30	0.93
MUFA	0.80	0.50	25.03	< 0.001	0.54	0.78
n-3 PUFA	0.13	0.94	4.36	0.019	0.49	0.81
n-6 PUFA	0.84	0.48	7.38	0.002	0.76	0.60
n-3 LC-PUFA	1.19	0.33	2.93	0.065	0.61	0.72
n-6 LC-PUFA	1.14	0.35	8.72	< 0.001	0.99	0.44

OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids.

whereas no differences were found between those of gammarids fed on the Carrot and Fucus diets. The absence of physiologically essential compounds such as LC-PUFA in the coconut diet may explain this difference (Jump, 2002; Monroig et al., 2022). Indeed, it is tempting to hypothesize that the high content of SFA in the diet may be related to an

TABLE 5 Tukey's *post-hoc* test after ANOVA of the fatty acid composition of *G. locusta* fed different diets.

	p-value		
	Fucus vs. Carrot	Fucus vs. Coco	Carrot vs. Coco
12:0	0.95	<0.001	<0.001
14:0	0.25	<0.001	<0.001
16:0	0.52	0.34	0.90
16:1n-7	0.63	0.69	0.25
18:0	0.04	<0.001	0.13
18:1n-9 (OA)	<0.001	<0.001	0.45
18:2n-6 (LA)	0.08	0.08	<0.001
18:3n-3 (ALA)	<0.001	0.77	<0.001
20:4n-6 (ARA)	0.004	0.002	0.002
20:5n-3 (EPA)	0.033	0.07	1.00
22:5n-3 (DPA)	0.010	<0.001	<0.001
22:6n-3 (DHA)	0.94	0.41	0.61
SFA	0.58	0.002	0.023
MUFA	<0.001	<0.001	<0.001
n-3 PUFA	0.99	0.03	0.04
n-6 PUFA	0.49	<0.001	0.03
n-3 LC-PUFA	0.21	0.07	0.76
n-6 LC-PUFA	0.01	<0.001	0.62

OA, oleic acid; LA, linolenic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids.

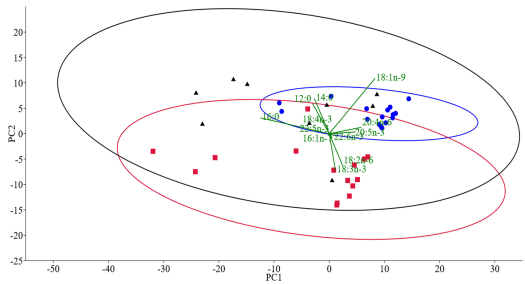

FIGURE 1
PCA of the fatty acid composition of *G. locusta* fed different diets and reared at different temperatures. Dots (blue), squares (red), and triangles (black) are the scores of *G. locusta* fed on *Fucus* spp., carrot leaves, and coconut, respectively. Fatty acids responsible for the grouping pattern are displayed in the biplot (green vectors); 95% confidence ellipses are shown. For the sake of clarity, and due to the lack of statistical significance, temperature labels are not shown.

impairment of the membrane composition and properties, ultimately resulting in an increase in mortality under the most stressful temperature conditions (Hazel and Eugene Williams, 1990; Hazel, 1995; Ernst et al., 2016; Laila, 2021; Zinoöcker et al., 2021). Noticeably, cannibalistic behavior was observed in gammarids fed on a coconut diet, suggesting a potential corrective mechanism towards the nutritional impairment. Although LC-PUFA was low in carrot leaves, the survival of gammarids fed on this diet was similar to that obtained with Fucus. As previously reported by Alberts-Hubatsch et al. (2019), this higher survival may be partially explained by the presence of other health-promoting nutrients like carotenoids that can thus compensate for suboptimal levels of LC-PUFA (Saini et al., 2015; González-Peña et al., 2021). Indeed, carotenoids are regarded as biomolecules that protect against unspecific and oxidant stress conditions such as suboptimal temperatures, which can ultimately cause increased mortality in gammarids (Agnew and Taylor, 1986; Neuparth et al., 2002; Wijnhoven et al., 2003). Significant differences in total gammarid biomass outputs were found when comparing different diets, and, regarding temperature, only significant differences were found between 5°C and 15°C, the latter having been reported as the optimal growth temperature for *G. locusta* (Neuparth et al., 2002). Interestingly, culturing gammarids at 20°C and fed on Fucus resulted in the highest output of biomass as well as final length and SGR. This may be due to the combined effects of the high nutritional value of the Fucus diet (Alberts-Hubatsch et al., 2019) as well as the increased growth performance of *G. locusta* when grown at 20°C (Neuparth et al., 2002) that compensates for the biomass loss associated with the abovementioned high mortality rates detected at 20°C.

Under the present experimental conditions, *G. locusta* showed similar FA profiles regardless of the rearing temperature. Only slight differences among temperatures within the same diet were evident, indicating either an apparent lack of temperature effect or a much more predominant dietary phenotypic impact. The FA dietary fingerprint was noticeable in the Fucus treatment in the LC-PUFA of the gammarid's profiles, whereas Carrot treatment resulted in higher levels of LA and ALA, and Coco induced higher amounts of SFA such as lauric and myristic acids in the final biomass. Interestingly, *G. locusta* fed on either Carrot or Coco still showed EPA and DHA percentages similar to those of *G. locusta* fed on Fucus, regardless of the absence of these LC-PUFA in the diets. This finding is in agreement with the potential capacity of gammarids for trophic upgrading, as has been suggested by several authors (Baeza-Rojano et al., 2013; Baeza-Rojano et al., 2014; Jiménez-Prada et al., 2018; Alberts-Hubatsch et al., 2019; Jiménez-Prada et al., 2020), indicating that these animals could bioconvert the short-chain FA present in sidestreams into high nutritional value LC-PUFA *via* their biosynthetic pathway.

LC-PUFA biosynthesis relies especially upon the gene repertoire and in the coordinated action of two types of enzymes (Monroig and Kabeya, 2018; Monroig et al., 2022). These two enzymes, elongation of very long-chain-fatty acid proteins (commonly known as "elongases"), and front-end desaturases (also named "desaturases"), need to be present and active in the organism in order to efficiently biosynthesize LC-

PUFA such as EPA and DHA from dietary FA (Castro et al., 2016; Monroig and Kabeya, 2018; Monroig et al., 2022). A recent report on the three different elongases in the marine gammarid *E. marinus* (Ribes-Navarro et al., 2021) indicates the potential capacity for endogenous production of LC-PUFA in marine gammarids, but the presence and activity of desaturases remain to be elucidated. The presence of EPA and DHA can be due to reasons other than endogenous biosynthesis, such as the accumulation and retention of these FA from sources other than the diet provided, like the occasional and practically unavoidable presence of prey organisms such as copepods and rotifers in the culture tanks during the experiment. These organisms, particularly copepods, are characterized by having high LC-PUFA contents (McKinnon et al., 2003; Miller et al., 2008; Naylor et al., 2009; Nielsen et al., 2019; Boyen et al., 2020; Kabeya et al., 2021). It should be noted, however, that during the experiment, it was evident that gammarids fed actively on the vegetal substrates, and although impossible to rule out, the possible contribution of the above-mentioned accompanying fauna entering the system as well as that of the rapid development of biofilm seems at most testimonial. Other factors, like the fungal contribution described in the digestive process for the freshwater *G. pulex* (Chamier and Willoughby, 1986), can also be invoked to explain the presence of unexpected components in the final profiles, but this can only be clarified in experiments run in controlled conditions, far beyond the scope of the present experimental design.

In summary, the present study demonstrates that gammarids can be fed on agricultural sidestreams (carrot leaves) without drastically affecting their growth performance and survival when reared at temperatures ranging from 5°C to 15°C. A culture at 20°C is not recommended due to the impairment between growth and survival. This study is the first study of this kind to show interactions between rearing temperatures and novel feed ingredients for this species, further steps (e.g., other under-used sidestreams from agriculture) need to be taken to optimize biomass gain in addition to survival. In particular, this study shows that *G. locusta* can be a potential candidate for applying circular economy principles by which sidestreams such as carrot leaves can be used as the main feed for gammarid culture and potential biomass generation. The aim of this study was also to get independent from marine resources, such as macroalgae, that can be costly or not sustainable to use for feeding gammarids. Thus, showing that at least carrot greens are providing sufficient nutrients for a successful culture of *G. locusta* gives important directions in terms of sustainability. Moreover, under the present experimental conditions tested herein, diet has been established as the main modulator of FA profiling in *G. locusta*. However, a combination of agricultural and aquaculture sidestreams, along with other modulating conditions such as salinity, should be considered in order to set the best environment for large-scale gammarid production. Aside from the strong phenotypic effect of diet on the final FA profile, the presence of LC-PUFA like EPA and DHA, detected on *G. locusta* fed on diets devoid of these compounds, points towards unknown mechanisms of trophic upgrading beyond the theoretical endogenous biosynthetic capacity of the species.

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.

Ethics statement

All experiments were carried out under the European Directive 2010/63/EU (European Parliament, Council of the European Union, 2010) for the protection of animals used for experimentation and other scientific purposes, from which invertebrates other than cephalopods are excluded. The experiments were run bearing in mind the 3R guidelines.

Author contributions

Conceptualization: HA-H and JN. Methodology: AR-N, HA-H, JN, and ÓM. Formal analysis: AR-N, HA-H, JN, and ÓM. Investigation: AR-N, HA-H, JN, FH, and ÓM. Resources: HA-H. Writing—original draft preparation: AR-N, HA-H, and JN. Writing—review and editing: AR-N, FH, HA-H, JN, and ÓM. Project administration: FH, HA-H, JN, and ÓM. Funding acquisition: FH, HA-H, JN, and ÓM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.931991/full#supplementary-material

SUPPLEMENTARY TABLE 1
Calculated *p* values for the one-way PERMANOVA (Bonferroni corrected *p* values).

SUPPLEMENTARY TABLE 2
SIMPER analysis of the dietary groups Fucus *vs* Carrot. All FA detected were included in the analysis and those above the cumulative cutoff of 70% were not shown in the table, except for the first one.

SUPPLEMENTARY TABLE 3
SIMPER analysis of the dietary groups Fucus *vs* Coco. All FA detected were included in the analysis and those above the cumulative cut-off of 70% were not shown in the table, except for the first one.

SUPPLEMENTARY TABLE 4
SIMPER analysis of the dietary groups Carrot *vs* Coco. All FA detected were included in the analysis and those above the cumulative cut-off of 70% were not shown in the table, except for the first one.

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Examination of gammarid transcriptomes reveals a widespread occurrence of key metabolic genes from epibiont bdelloid rotifers in freshwater species

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Previous data revealed the unexpected presence of genes encoding for long-chain polyunsaturated fatty acid (LC-PUFA) biosynthetic enzymes in transcriptomes from freshwater gammarids but not in marine species, even though closely related species were compared. This study aimed to clarify the origin and occurrence of selected LC-PUFA biosynthesis gene markers across all published gammarid transcriptomes. Through systematic searches, we confirmed the widespread occurrence of sequences from seven elongases and desaturases involved in LC-PUFA biosynthesis, in transcriptomes from freshwater gammarids but not marine species, and clarified that such occurrence is independent from the gammarid species and geographical origin. The phylogenetic analysis established that the retrieved elongase and desaturase sequences were closely related to bdelloid rotifers, confirming that multiple transcriptomes from freshwater gammarids contain contaminating rotifers' genetic material. Using the *Adineta steineri* genome, we investigated the genomic location and exon–intron organization of the elongase and desaturase genes, establishing they are all genome-anchored and, importantly, identifying instances of horizontal gene transfer. Finally, we provide compelling evidence demonstrating Bdelloidea desaturases and elongases enable these organisms to perform all the reactions for de novo biosynthesis of PUFA and, from them, LC-PUFA, an advantageous trait when considering the low abundance of these essential nutrients in freshwater environments.

1. Introduction

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), and more specifically, the omega-3 (ω3 or *n*-3) LC-PUFA eicosapentaenoic acid (EPA, 20 : 5*n*-3) and docosahexaenoic acid (DHA, 22 : 6*n*-3), are of high relevance for animal health and development [1–5]. Aquati

c ecosystems, and particularly marine habitats, are main sources of *n*-3 LC-PUFA due to the abundance of low trophic organisms with the ability for their endogenous production (biosynthesis) [6]. Microorganisms such as

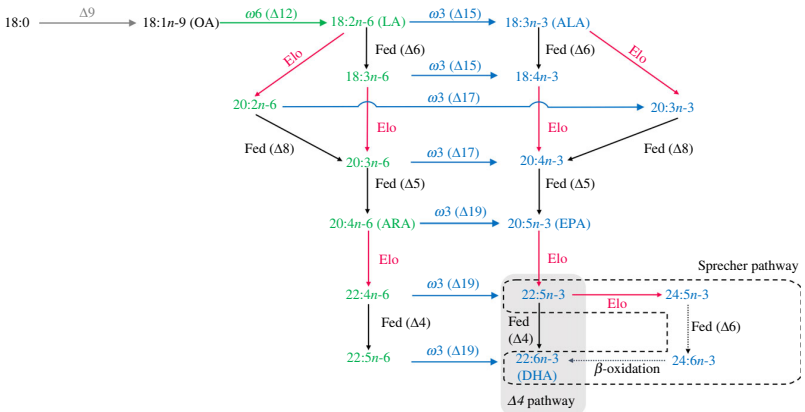

Figure 1. General overview of the LC-PUFA biosynthesis pathways in animals. Reactions catalysed by front-end desaturases are marked as 'Fed' along with their specific Δ*y* activity (*y* indicates position of double bond from the –COOH terminus of the fatty acid). Reactions catalysed by methyl-end desaturases are denoted as 'ω*x*' (*x* indicates position of double bond from the $-CH_3$ terminus of the fatty acid), and their specific Δ*y* activity. Reactions marked with 'Elo' are catalysed by elongation of very long-chain fatty acid (Elovl) proteins. Two distinct pathways enabling DHA biosynthesis are possible, namely the 'Δ4 pathway' (grey background) and the 'Sprecher pathway' (dashed line).

photosynthetic microalgae, heterotrophic protists and bacteria have been historically regarded as primary producers of *n*-3 LC-PUFA in the ocean [7–9]. However, more recently, certain invertebrates have been also shown to contribute to *n*-3 LC-PUFA production in both aquatic and terrestrial ecosystems [10–13]. Flux of *n*-3 LC-PUFA from primary producers to upper trophic levels is crucial for marine ecosystems since fish, as all vertebrates, depend upon provision of pre-formed health beneficial *n*-3 LC-PUFA in their diet [6]. Interest in understanding the mechanisms by which living organisms biosynthesize *n*-3 LC-PUFA has been prompted in recent years due, among other reasons, to the decrease in global availability of these essential nutrients predicted to occur because of climate change [6,14,15].

Microorganisms can biosynthesize *n*-3 LC-PUFA using either anaerobic or aerobic metabolic pathways, each of them involving different enzymatic components [16]. The anaerobic pathways occur mostly in prokaryotes and some eukaryotic organisms, and are mainly driven by the polyketide synthase (PKS) complex [17]. However, most eukaryotic organisms including animals operate aerobic pathways enabling LC-PUFA biosynthesis through the orchestrated action of three key enzymes, namely methyl-end desaturases (ω*x*), front-end desaturases (Fed), and elongation of very long-chain fatty acid (Elovl) proteins [10,11]. Briefly, the monoene oleic acid (OA, 18 : 1*n*-9), biosynthesized from stearic acid (18 : 0) by a Δ9 desaturase occurring in virtually all living organisms [18], can be converted into linoleic acid (LA, 18 : 2*n*-6) and α-linolenic acid (ALA, 18 : 3*n*-3), which represent the simplest cases of *n*-6 and *n*-3 polyunsaturated fatty acids (PUFA), respectively (figure 1). With some exceptions [19–22], the de novo biosynthesis of *n*-6 and *n*-3 PUFA (i.e. LA and ALA) is carried out by ω*x* enzymes (figure 1). The ω*x* responsible for the biosynthesis of LA from OA are commonly known as Δ12 or ω6 desaturases, whereas those enabling the conversion of LA into ALA are known as Δ15 or ω3 desaturases (figure 1). Interestingly, ω3 desaturases typically recognize a variety of *n*-6 PUFA as substrates, converting them into *n*-3 products (figure 1). LA and ALA have no major biological functions themselves but serve as precursors of the physiologically important LC-PUFA by the action of further enzymes including Fed and Elovl (figure 1). Like ω*x*, Fed introduce double bonds (unsaturations) into the pre-existing fatty acyl chains, but such insertion takes place in the 'front end' of the fatty acid acting as substrate, more specifically between an existing double bond and the carboxyl terminus (–COOH) [23,24]. Typical activities reported in Fed include Δ4, Δ5, Δ6 and Δ8 (figure 1) [10]. Besides, Elovl catalyse the condensation reaction in the fatty acid elongation pathway that results in the extension of an existing fatty acid in two carbons [10,23] (figure 1). Both invertebrates and vertebrates possess Fed and Elovl involved in the biosynthesis of LC-PUFA from C_{18} PUFA precursors (figure 1). However, a distinctive trait among animals' LC-PUFA biosynthetic capacity is that invertebrates, but not vertebrates, can have ω*x* enabling in them the de novo biosynthesis of PUFA (i.e. LA and ALA) described above (figure 1).

Evidence supporting the capacity of invertebrates for LC-PUFA biosynthesis had been mostly collected from compositional studies and biochemical assays aiming to determine *in vivo* bioconversions of labelled fatty acid substrates [25–31]. However, the ever-increasing availability of genomic data from multiple invertebrates now offers the opportunity to identify the specific genes encoding LC-PUFA biosynthetic enzymes, thus often enabling one to distinguish the invertebrate's enzymatic capacity from that of (micro)organisms that may inhabit their hosts [32]. Studies aiming to characterize LC-PUFA biosynthetic genes from invertebrates have helped to predict the gene complement and function in certain groups [10,12,33]. Crustaceans have been the focus of particular attention due to their central roles in trophic ecology of essential

fatty acids in aquatic ecosystems, as well as their commercial interest in aquaculture [34–39]. Current evidence suggests that both *ωx* and Fed enzymes occur in certain copepods [12,37,40], but are absent in other crustaceans [10]. Comparatively to desaturases, the repertoire of Elovl with functions along the LC-PUFA biosynthetic pathways in crustaceans is far more varied, with occurrence of multiple *elovl*-like genes, not only in copepods [37,40,41] but also in decapods [34,36,42,43], branchiopods [39] and gammarids [44]. Such an Elovl diversity has been hypothesized to compensate for the apparent absence of Elovl2/5 in crustaceans, a pivotal PUFA Elovl reported in molluscs and amphioxus [45–47]. Surprisingly, Ribes-Navarro *et al*. found putative *elovl2/5* sequences in freshwater gammarids, but not in marine species, an unexpected finding considering they are very closely related species, often from the same genus (e.g. *Gammarus*) [44].

The present study aimed to clarify the origin and occurrence of sequences of LC-PUFA biosynthesizing genes, such as *elovl2/5* and further biosynthetic gene components including Fed and *ωx* in freshwater gammarids. To do so, we conducted a systematic search of diagnostic motifs characteristic of each three LC-PUFA biosynthetic enzyme families within publicly available transcriptomes from both freshwater and marine gammarids [48–55]. Our results confirmed that sequences encoding putative Elovl2/5, Fed and *ωx* are widespread in transcriptomic databases from freshwater gammarids but absent in those from marine species. We further analysed the phylogenetic relationship of the retrieved desaturases and elongases. Strikingly, we unequivocally established that they were closely related to bdelloid rotifers. Using the genome of the freshwater bdelloid rotifer *Adineta steineri* as a proxy-model, we investigated the genomic location and exon–intron organization of all the desaturase and elongase genes used as diagnostic markers. We establish they are all genome-anchored and clarify some instances of horizontal gene transfer (HGT). Finally, we provide compelling evidence that the Bdelloidea desaturase and elongase sequences encode enzymes enabling all the reaction for de novo biosynthesis of PUFA and, from them, of LC-PUFA.

2. Material and methods

2.1. Retrieval of LC-PUFA biosynthesis genes in gammarid transcriptomes

Sequences from functionally characterized LC-PUFA biosynthetic desaturases and elongases from aquatic invertebrates including annelids, molluscs and arthropods were used as queries for BLAST searches (*tblastn*) within transcriptomes from a variety of gammarids, including freshwater and marine species [48–55]. Hits were only selected for further analysis when they contained the full-length open reading frame (ORF) and their predicted protein sequences (https://www.ncbi.nlm.nih.gov/orffinder) contained specific features according to Hashimoto *et al*. [56]. Briefly, Fed had to contain three diagnostic histidine boxes (H-box) 'HXXXH', 'HXXXHH' (or 'HXXHH') and 'QXXHH', and a heme binding motif (HPGG) in the cytochrome b_5 domain. Putative desaturase sequences with the third H-box 'HXXHH' instead of 'QXXHH' were not regarded as Fed based on evidence collected from other crustaceans suggesting these enzymes lack fatty acyl desaturation capacity [10,11]. Selection of *ωx* was made based on the presence of the three H-boxes as 'HXXXH', 'HXXHH' and 'HXXHH', and absence of cytochrome b_5 domain [56]. As for elongases, the deduced amino acid (aa) sequences had to contain the conserved domains 'KXXEXXDT', 'NXXXHXXMYXYY' and 'TXXQXXQ', and a H-box as 'HXXHH'.

2.2. Phylogeny of LC-PUFA biosynthesis genes retrieved from freshwater gammarid transcriptomes

The deduced aa sequences of the *elovl2/5*, *fed* and *ωx* retrieved from freshwater gammarid transcriptomes were used for phylogenetic analysis, along with a wide range of previously characterized genes from vertebrates and invertebrates [12,37,44]. Putative desaturase and elongase sequences from Rotifera transcriptomes (PRJNA295486-295489) were also included in the analysis [57]. Besides, for the Fed tree, several sphingolipid desaturases from crustaceans were used as outgroups. The resulting dataset was aligned with a MAFFT E-INS-i (*–maxiterate 1000 –genafpair*) [58]. Subsequently, trimming with a gap threshold of 95% was performed for each alignment (trimAl) [59]. Finally, next generation version of randomized axelerated maximum likelihood (RAxML-NG) [60] was performed in order to build the phylogenetic tree with LG + I + G4 as the best aa substitution model calculated by Modeltest-NG [61,62]. Confidence in the resulting phylogenetic tree branch topology was measured with all-in-one mode (*–all*). Auto bootstrapping (autoMRE, cutoff: 0.03) was converged after 350 replicates for the front-end desaturase tree, 650 replicates for the methyl-end desaturase tree and 450 replicates for the elongase tree.

2.3. Screening gammarid transcriptomes for 'contaminant' gene sequences from Rotifera symbionts

Since freshwater gammarids have often been reported to have bdelloid rotifers as epibionts [63–65], we analysed the occurrence of Rotifera in published transcriptomes from freshwater (*Gammarus pulex*, *Gammarus fossarum*, *Gammarus wautieri* and *Echinogammarus berilloni*) and marine (*Echinogammarus marinus*) wild caught gammarids [50]. We chose the dataset from Cogne *et al*. [50] not only because of its species coverage including both freshwater and marine species, but also because it is deposited as sequence read archive (SRA) enabling analysis and reproducibility of the raw sequencing data. Sequences retrieved from the above searches were introduced into a newly developed pipeline for the selection of the mitochondrial gene cytochrome c oxidase subunit 1 (*cox1*) to identify genetic contamination from the phylum Rotifera in the gammarid transcriptomes. Briefly, all the Metazoan complete mitochondrial (mtDNA) genomes available of NCBI (February of 2021) were retrieved and used as a reference to extract mitochondrial reads from the gammarid SRA, using the tool bbmap.sh and reformat.sh from BBMap v.37.77 (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbmap-guide/). The filtered mtDNA reads for each sample were assembled using rnaSPAdes v3.11.1 with default parameters [66]. Assembled contigs were blast searched against the nucleotide database of NCBI (NCBI-nt, Download; 24 August 2021), using the blastn

from BLAST + v. 2.11.0 [67], and all contigs with hits with the phylum Rotifera retrieved. The mtDNA gene *cox1* is used as a standardized molecular identification system in most metazoan [68], thus one of the most sequenced genes available on NCBI. Therefore, to identify the putative Rotifera species present within each sample, all *cox1* sequences from Rotifera were retrieved from NCBI and used as a reference database for a blast search (blastn) to identify Rotifera *cox1* containing contigs. All hits that resulted in DNA sequences with identity scores higher than 75% were considered as positive for genetic contamination.

2.4. Detection of LC-PUFA biosynthesis genes in gammarids collected from the wild and laboratory cultures

In order to validate the occurrence of rotifer-origin genetic material in gammarid transcriptomes, we developed a polymerase chain reaction (PCR) assay to determine the presence of LC-PUFA biosynthetic gene sequences in *G. pulex* adults collected from the wild and laboratory cultures. The herein referred to as 'wild-captured' *G. pulex* sample consisted of one sole whole individual collected from a small freshwater stream in northern Germany (53°29′52″ N, 8°46′08″ E), which was preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) until further analysis. The 'laboratory cultured' *G. pulex* sample consisted of one sole adult of an apparent same size as the wild-captured *G. pulex* sample, and derived from the second generation resulting from maintaining wild-captured *G. pulex* from the above location. The laboratory cultured *G. pulex* were maintained in fresh water at 18°C with coarse gravel as substrate and fed a mixed diet containing pellets made of dried carrot leaves, sugar beet and brewer grains. Furthermore, a second adult specimen of laboratory cultured *G. pulex* was dissected for collecting a hepatopancreas sample. Total RNA was extracted the three *G. pulex* samples (wild-captured, laboratory cultured and hepatopancreas) using the Illustra RNAspin Mini kit (Cytiva, Marlborough, MA, USA) following the manufacturer's instructions. The extracted RNA was treated with DNase I to avoid potential genomic DNA contamination. Subsequently, first strand complementary DNA (cDNA) was synthesized from 2 µg of total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega, Madison, WI, USA) and stored at −20°C until further analysis. Using the three cDNA samples (wild-captured whole individual, laboratory cultured whole individual, laboratory culture hepatopancreas) as templates, PCR (GoTaq®G2 Green Master Mix, Promega) were run using gene-specific primers targeting the sequences of *elovl2/5*, four front-end desaturases (*fed1-4*) and two methyl-end desaturases (*ωx1-2*) retrieved from *G. pulex* transcriptomes (electronic supplementary material, table S1). Moreover, a PCR with degenerate primers targeting a conserved region of the housekeeping gene *β-actin* (*actb*) for *Rotaria* species was also run to check for possible rotifer genetic material within the above three template cDNA samples (electronic supplementary material, table S1). Specific primers targeting the *G. pulex actb* were used for PCR as positive controls. Primer sequences and PCR conditions are indicated in electronic supplementary material, table S1. The resulting PCR products were purified on a 1% (w/v) agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega) and their identity confirmed by DNA sequencing (DNA Sequencing Service, IBMCP-UPV, Valencia, Spain) (electronic supplementary material, figure S1).

2.5. Sequence identity of the LC-PUFA biosynthesis genes among gammarid transcriptomes

The intra- and interspecific diversity of the LC-PUFA biosynthesis genes within gammarid transcriptomes was analysed by estimating the nucleotide (nt) identity scores (https://www.ebi.ac.uk/Tools/msa/clustalo/) as follows. First, we compared the elongase and desaturase nt sequences retrieved from transcriptomes of *Gammarus* species sampled at two geographical sites (intraspecific identity). The publicly available transcriptomes (table 1) enabled us to estimate the intraspecific identity for (1) *G. pulex*, comparing sequences occurring in transcriptomes built from samples collected in Germany (53°29′52″ N, 8°46′08″ E) and France (45°57′21″ N, 5°15′44″ E) (BioProject ID: PRJNA497972), and (2) *G. fossarum*, comparing sequences occurring in transcriptomes built from samples collected at two distinct French sites (47°53′31″ N, 6°58′53″ E, and 45°57′21″ N, 5°15′44″ E) (BioProject ID: PRJNA497972). Second, we determined the interspecific identity of the elongase and desaturase nt sequences retrieved from transcriptomes of two *Gammarus* species (*G. pulex* and *G. fossarum*) sampled at the same site, more specifically the Pollon River, Saint-Maurice de Rémens, France (45°57′21″ N, 5°15′44″ E) (BioProject ID: PRJNA497972). Finally, we analysed the sequence nt identity between two different gammarid species (*G. pulex* versus *G. lacustris*) sampled at very geographically distant locations, namely Germany (53°29′52″ N, 8°46′08″ E) and Russia (55°55′14.39″ N, 105°4′19.48″ E), respectively. Further details of the location and sample type can be found in table 1.

2.6. Genome location and exon–intron structure analysis of LC-PUFA biosynthesis genes in bdelloid rotifers

Expanding on the phylogeny results suggesting that sequences for LC-PUFA biosynthesis genes found in freshwater gammarid transcriptomes belong to Bdelloid rotifers, we identified the corresponding LC-PUFA biosynthesis gene sequences from *A. steineri* and studied their genome location (WGS Project: CAJNOJ01). In order to gain insight into the evolutionary origin of the LC-PUFA biosynthesis genes, we determined the exon–intron structure using the Exon-Intron Graphic Maker webpage (http://wormweb.org/exonintron, accessed 29 March 2023). Due to the exon–intron organizations of the *fed3* and *fed4* genes, as well as their unexpected location in the phylogenetic tree, we examined their genomic neighbourhood along the *A. steineri* genome in order to clarify whether these genes were not a spurious artefact of the genome assembly. We further addressed the evolutionary origin of the flanking genes with respect to the taxonomic distribution with blast (animal versus non-animal presence).

Table 1. Transcriptomic databases from gammarids mined for occurrence of key metabolic LC-PUFA genes (*elovl2/5*, *fed* and *ωx*). Details on species, geographical location of the sampling site and genes found (number in parentheses) are indicated. All databases were built from analysis of specimens collected from the wild except for *G. locusta* (PRJNA600472; laboratory culture), and correspond to whole individuals except for *G. pulex* hepatopancreas (PRJEB13055) and *G. fossarum* 'internal tissues' (PRJNA556212).

species name	BioProject ID	location	genes found
Gammarus pulex	PRJNA497972 [50]	Pollon River, Saint-Maurice de Rémens, France (45°57′21″N, 5°15′44″E)	*elovl2/5*, *fed* (4), *ωx* (2)
Gammarus pulex	PRJEB13055 [48]	Blanc-Gravier River, Liege, Belgium (50°34′60″N, 5°34′60″E)	None
Gammarus pulex	PRJEB13055 [48]	Vesdre River, Vaux-sous-Chevremont, Belgium (50°36′00″N, 5°37′58″E)	None
Gammarus fossarum	PRJNA497972 [50]	Pollon River, Saint-Maurice de Rémens, France (45°57′21″N, 5°15′44″E)	*elovl2/5*, *fed* (4), *ωx* (2)
Gammarus fossarum	PRJNA497972 [50]	Seebach River, Fellering, France (47°53′31″N, 6°58′53″E)	*elovl2/5*, *fed* (4), *ωx* (2)
Gammarus fossarum	PRJNA556212 [51]	Elgg, Switzerland (47°30′04.23″N, 8°51′09.40″ E)	*elovl2/5*, *fed* (2), *ωx* (1)
Gammarus wautieri	PRJNA497972 [50]	Galaveyson River, Le Grand Serre, France (45°16′27″N, 5°07′08″E)	*elovl2/5*, *fed* (4), *ωx* (2)
Echinogammarus berilloni	PRJNA497972 [50]	Saucats River, Saucats, France (44°39′34″N, 0°34′25″W)	*elovl2/5*, *fed* (4), *ωx* (2)
Gammarus lacustris	PRJNA660769 [53]	Lake no. 14, Baikal Lake, Irkutsk, Russia (51°55′14.39″N, 105°4′19.48″E)	*elovl2/5*, *fed* (2), *ωx* (2)
Gammarus lacustris	PRJNA505233 [55]	Lake no. 14, Baikal Lake, Irkutsk, Russia (51°55′14.39″N, 105°4′19.48″E)	*elovl2/5*, *fed* (2), *ωx* (2)
Hyalellopsis costata	PRJNA32160 [49]	Baikal, Bolshie Koty, across from ISU Biostation (51.90° N, 105.07° E)	*elovl2/5*, *fed* (2), *ωx* (2)
Echinogammarus marinus	PRJNA497972 [50]	sea coast, Portsmouth, UK (50°47′41″N, 1°01′50″W)	None
Echinogammarus marinus	PRJEB34316 [52]	intertidal mudflat, Saltash, UK (50°47′41″N, 1°01′50″W)	None
Gammarus locusta	PRJNA600472 [54]	south margin of the Sado estuary, Portugal (38°27′N, 08°43′W)	None

2.7. Functional characterization of LC-PUFA biosynthetic elongases and desaturases in yeast

Elongase and desaturase genes isolated from wild-captured *G. pulex* were functionally characterized by expressing their ORF in brewer's yeast *Saccharomyces cerevisiae*. Primers containing restriction sites for further cloning into the yeast expression vector pYES2 (Thermo Fisher Scientific) were used to amplify the ORF sequences using the high-fidelity polymerase Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific) (electronic supplementary material, table S1). The PCR template consisted of cDNA prepared from total RNA of wild-captured *G. pulex* as described above. Each of the PCR products was digested with the corresponding restriction enzymes (electronic supplementary material, table S1) and further ligated into a similarly restricted pYES2 plasmid using T4 DNA ligase (Promega). The pYES2 constructs containing the ORF of the genes of interest (pYES2-Elovl25, pYES2-Fed1, pYES2-Fed2, pYES2Fed3, pYES2-Fed4, pYES2-ωx1 and pYES2-ωx2) were individually transformed into INVSc1 *S. cerevisiae* competent cells (Thermo Fisher Scientific) using the *S.c.* EasyComp yeast transformation kit (Thermo Fisher Scientific). Selection and growth of recombinant yeast were carried out as described by Ribes-Navarro *et al*. [44]. To test the capacity of the elongase and desaturase enzymes, recombinant yeast expressing one sole LC-PUFA biosynthesis gene was supplemented with one of the following potential PUFA substrates selected according to LC-PUFA biosynthetic pathways (figure 1). For Elovl2/5, the assayed PUFA substrates included 18 : 3*n*-3, 18 : 2*n*-6, 18 : 4*n*-3, 18 : 3*n*-6, 20 : 5*n*-3, 20 : 4*n*-6, 22 : 5*n*-3, 22 : 4*n*-6 and 22 : 6*n*-3, whereas the exogenously PUFA supplied for transgenic yeast expressing front-end desaturases were 18 : 3*n*-3 and 18 : 2*n*-6 (Δ6 desaturation), 20 : 3*n*-3 and 20 : 2*n*-6 (Δ8 desaturation), 20 : 4*n*-3 and 20 : 3*n*-6 (Δ5 desaturation), and 22 : 5*n*-3 and 22 : 4*n*-6 (Δ4 desaturation). For methyl-end desaturases, the assayed PUFA included 18 : 2*n*-6, 18 : 3*n*-6, 20 : 2*n*-6, 20 : 3*n*-6, 20 : 4*n*-6, 22 : 4*n*-6 and 22 : 5*n*-6. Each PUFA substrate was supplemented as sodium salt at a final concentration of 0.5 mM (C_{18}), 0.75 mM (C_{20}), 1.0 mM (C_{22}) in order to compensate for the decreased uptake efficiency as chain length increases. All fatty acid substrates (greater than 98–99% pure) used for the functional characterization assays were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Immediately after addition of the PUFA substrates, further supplementation of galactose (2%, w/v) induced gene expression. Along with their activity towards exogenously supplied PUFA substrates, we tested the ability of the two *ωx* to desaturate yeast endogenous fatty acid as previously reported [37,69]. For that purpose, transgenic yeast transformed with pYES2-ωx1 or pYES2-ωx2, as well as yeast transformed with the empty pYES2 vector (control), were grown in the absence of exogenously added PUFA substrates. Gene expression was induced with galactose as described above. After induction, yeast cultures were maintained at 30°C and under constant shaking (250 rpm) for 2 d until yeast were harvested by centrifugation at 1500*g* for 2 min. Yeast pellets were washed twice with 5 ml of ddH_2O, homogenized in 6 ml of 2 : 1 (v/v) chloroform:methanol containing 0.01% (w/v) butylated hydroxytoluene (BHT, Sigma-Aldrich) as antioxidant and stored at −20°C for a minimum of 24 h in an oxygen-free atmosphere until further analysis. Yeast culture reagents including galactose, yeast nitrogen base without aa, raffinose, Tergitol NP-40, and uracil dropout medium were obtained from Sigma-Aldrich (St Louis, MO, USA).

2.8. Fatty acid analysis

The fatty acid composition of yeast collected from functional assays was determined using the method described by Monroig *et al*. [70]. Briefly, extracts of total lipids from yeast [71]

were used to prepare fatty acid methyl esters (FAME) that were analysed using gas chromatography coupled with a flame ionization detector (GC-FID) [72]. The GC-FID was equipped with a fused silica 30 m × 0.25 mm open tubular column (TRACE TR-WAX, film thickness: 0.25 µm; Thermo Fisher Scientific) fitted with an on-column injection system and using helium as a carrier gas. Conversions of the LC-PUFA biosynthesizing enzymes towards the exogenously supplied PUFA substrates were calculated according to the formula [all product areas/(all product areas + substrate area)] × 100. Where necessary, further confirmation of FA products was carried out using an Agilent 6850 GC equipped with a mass spectrometry (MS) detector (5975 Series) and a 30 m × 0.25 mm open tubular column (DB5-MS, film thickness 0.25 µm; Agilent, Santa Clara, CA, USA) and comparing the spectra against those from the NIST library (MS Search v.2.0).

3. Results

3.1. Phylogeny of the LC-PUFA biosynthesis gene sequences retrieved from gammarid transcriptomes

Full-length ORF sequences of putative PUFA elongases (*elovl2/5*), front-end desaturases and methyl-end desaturases could be only found in transcriptomes from freshwater gammarids but not marine species (table 1). Interestingly, no sequences encoding the above diagnostic LC-PUFA biosynthetic genes were found in the freshwater gammarid *G. pulex* transcriptome built using RNA extracted from hepatopancreas instead of whole individuals (PRJEB13055; table 1). Irrespective of the enzyme type, the phylogenetic analyses showed that the aa sequences retrieved from freshwater gammarid transcriptomes clustered closely (bootstrap > 90%) with their orthologues from rotifers, specifically from the Bdelloidea class (figure 2*a*; electronic supplementary material, figure S1). First, *elovl2/5* from the freshwater gammarids *G. pulex*, *G. fossarum*, *G. wautieri* and *E. berilloni* grouped together with putative *elovl2/5* from several *Rotaria* species, and more distantly with orthologues from other invertebrate groups including the functionally characterized Elovl2/5 from molluscs and amphioxus (figure 2*a*; electronic supplementary material, figure S1). Regarding front-end desaturases, the phylogenetic tree showed three well-differentiated clusters grouping all the gammarid sequences in two of these three clusters (figure 2*b*; electronic supplementary material, figure S2). The first cluster included two distinct sequences (Fed1 and Fed2) that could be consistently identified in transcriptomes from the freshwater gammarids analysed herein (figure 2*b*; electronic supplementary material, figure S2). These sequences clustered closely with front-end desaturase sequences belonging to the invertebrate Group A desaturases [73,74] and, more distantly, vertebrate sequences (figure 2*b*; electronic supplementary material, figure S2). Moreover, the first cluster included gammarid transcriptomic sequences termed as 'Fed4', which grouped along fatty acyl desaturases from several Fungi (figure 2*b*; electronic supplementary material, figure S2). The second cluster of front-end desaturases retrieved from gammarid transcriptomes (termed herein as 'Fed3') located closely to non-metazoan sequences from e.g. Kinetoplastea (figure 2*b*; electronic supplementary material, figure S2). Noteworthy, all Fed sequences retrieved from gammarid transcriptomes clustered closely to their rotifer orthologues. For methyl-end desaturases, all putative sequences retrieved from freshwater gammarid transcriptomes clustered within the so-called 'Clade 3' [12], along with methyl-end desaturases from Mollusca, Annelida, Arthropoda and Rotifera (figure 2*c*; electronic supplementary material, figure S3). Two distinct sequences termed herein as '*ωx1*' and '*ωx2*' were identified in each gammarid transcriptome from which methyl-end desaturases were retrieved (table 1). Importantly, the *ωx* sequences retrieved from freshwater gammarid transcriptomes grouped more closely to Rotifera sequences rather than other invertebrates including Arthropoda (figure 2*c*; electronic supplementary material, figure S3)

3.2. Occurrence of Rotifera mitochondrial genes in gammarid transcriptomes

Sequence homology searches of the *cox1* gene showed a very high identity with bdelloid rotifer *cox1* sequences (99.61% *Rotaria magnacalcarata*; 98.78% *Rotaria socialis*) within *G. pulex* RNASeq data (SRR8089720) (electronic supplementary material, table S2). Additionally, several other hits with a lower percentage of identity (79.4–86.6%) with other bdelloid rotifers were also identified. On the other hand, sequence homology searches of *cox1* within RNASeq from marine gammarids did not show any relevant identity with rotifer *cox1* sequences (electronic supplementary material, table S2).

3.3. Sequence identity of *elovl2/5*, *fed* and *ωx* retrieved from gammarid transcriptomes

The identity scores of the nt sequences from the LC-PUFA biosynthesis genes retrieved from transcriptomes of *Gammarus* species sampled at two geographical sites was determined to estimate the intraspecific diversity. The results showed that the identity scores of each homologous gene between *G. pulex* from Germany and France were all ≥ 96.9% (table 2). Similarly, comparison of sequences of each gene between *G. fossarum* from two distinct sites in France showed identity scores ranged between 96.9 and 99.5% (table 2). Subsequently, we analysed the interspecific identity of sequences encoding LC-PUFA biosynthetic enzymes from *G. fossarum* and *G. pulex* sampled at the same site in France (Pollon River, Saint-Maurice-de-Rémens). The results showed identity scores ranging between 98.7 and 99.9% (table 2). Finally, the results of the sequence comparison between *G. pulex* and *G. lacustris* sampled at very geographically distant locations (Germany and Russia, respectively) showed identity scores greater than 98% (table 2).

3.4. Occurrence of LC-PUFA biosynthesis genes in gammarids collected from the wild and laboratory cultures

We next confirmed the occurrence of rotifer genetic material in freshwater gammarids by detecting the *in silico* retrieved LC-PUFA biosynthesis genes by RT-PCR on cDNA prepared from laboratory cultured whole individual adult *G. pulex* (L), hepatopancreas from adult *G. pulex* (H), wild-captured adult

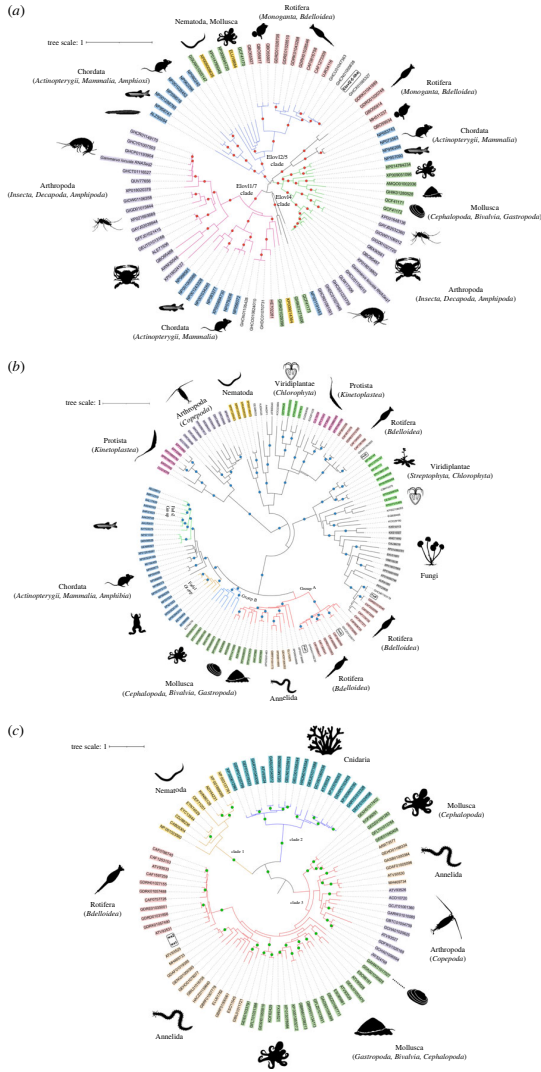

Figure 2. Condensed maximum likelihood phylogenetic trees of (*a*) elongases, (*b*) front-end desaturases, and (*c*) methyl-end desaturases. The protein evolutionary model was LG + I + G4. The tree was constructed using the maximum likelihood method by next generation version of randomized axelerated maximum likelihood (RAxML-NG). Confidence in the resulting phylogenetic tree branch topology was measured by auto-bootstrapping mode. Coloured dots on the nodes represent supporting values of 80% or above for RAxML. For each sequence, the accession numbers are given according to the information contained in the corresponding NCBI databases. Sequences subject to functional characterization in this study are rounded and highlighted in bold. Raw trees are available in the electronic supplementary material.

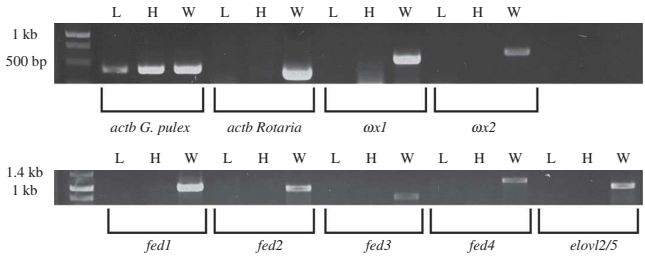

Figure 3. RT-PCR assays for detection of candidate genes (*ωx1*, *ωx2*, *fed1*, *fed2*, *fed3*, *fed4*, and *elovl2/5*) and housekeeping genes (*actb* from *G. pulex* and *Rotaria*) in laboratory cultured whole individual *G. pulex* (L), hepatopancreas (H) and wild caught whole individual *G. pulex* (W).

Table 2. Intra- and interspecific identity of the nucleotide sequences of selected LC-PUFA biosynthetic genes retrieved from transcriptomes of *Gammarus* spp. (*a*) Intraspecific comparisons made between sequences retrieved from *G. pulex* sampled in France (45°57′21″ N, 5°15′44″ E) and Germany (53°29′52″ N, 8°46′08″ E), and *G. fossarum* sampled at two French sites (47°53′31″ N, 6°58′53″ E and 45°57′21″ N, 5°15′44″ E). (*b*) Interspecific comparisons made between *G. pulex* and *G. fossarum* sampled at the same geographical location (Pollon River, Saint-Maurice de Rémens, France; 45°57′21″ N, 5°15′44″ E). (*c*) Interspecific comparisons made between *G. pulex* sampled in Germany (53°29′52″ N, 8°46′08″ E) and *G. lacustris* sampled in Russia (Lake no. 14, Baikal Lake, Irkutsk, 51°55′14.39″ N, 105°4′19.48″ E). *No gene found in the *G. lacustris* transcriptome (PRJNA660769), as indicated in table 1.

	% identity						
	elovl2/5	*fed1*	*fed2*	*fed3*	*fed4*	*ωx1*	*ωx2*
(*a*) intraspecific							
Gammarus pulex	99.6	99.9	98.4	99.8	97.3	99.6	99.7
Gammarus fossarum	97.9	96.9	98.5	99.5	97.5	98.2	99.5
(*b*) interspecific							
G. pulex versus *G. fossarum*	99.6	99.5	98.7	99.8	99.9	99.7	99.8
(*c*) interspecific							
G. pulex versus *G. lacustris*	98.1	99.7	99.4	*	*	99.5	99.5

G. pulex (W) (figure 3). RT-PCR targeting each of the candidate LC-PUFA biosynthetic genes were positive for W, but not L and H (figure 3). Similarly, only the W sample, but not L and H, showed a positive band when targeting the *Rotaria actb* chosen as diagnostic gene to detect the presence of epibiotic rotifers (figure 3; electronic supplementary material, figure S4). Moreover, sequencing results for the *Rotaria actb* band confirmed its identity as a rotifer gene. A positive control targeting the *G. pulex actb* was positive in all three cDNA samples (L, H and W) (figure 3).

3.5. Exon–intron organization of LC-PUFA biosynthesis genes from Bdelloidea rotifers

To confirm the results from the phylogenetic analysis suggesting that the LC-PUFA biosynthesis genes retrieved from gammarid transcriptomes belong to epibiotic bdelloid rotifers, we mapped them into the genome of *A. steineri* (WGS project: CAJNOM01; Assembly: GCA_905250015.1) and determined their exon–intron organization (figure 4*a*). Our results revealed that the seven LC-PUFA biosynthesis genes retrieved from gammarid transcriptomes, namely one elongase (*elovl2/5*), four *fed*, and two *ωx*, are genome-anchored in *A. steineri*. The analysis of their exon–intron organization showed that the front-end desaturase herein termed '*fed3*' is an intronless gene (figure 4*a*). Besides, the *fed4* sequence contained characteristic features of 'introner-like' elements [75], including a relatively long intron (third, 940 bp), conserved motifs with pyrimidine rich regions, and acceptor and donor sites for recognition by the spliceosome (figure 5). The particular exon–intron organization of *fed3* and *fed4*, along with their close phylogenetic relationship with sequences from kinetoplastids and fungi, respectively, suggested HGT as the evolutionary origin of these genes in bdelloid rotifers. To exclude the possibility that such kinetoplastid and fungi sequences might be a contamination in bdelloids, we further examined the genomic neighbourhood of both *fed3* and *fed4* along the *A. steineri* genome (figure 4*b*). The *A. steineri fed3* and *fed4* are both flanked by genes that have orthologues in other metazoan species, confirming that both *fed3* and *fed4* are real components of the *A. steineri* genome and not a putative contamination of kinetoplastids and fungi (figure 4*b*).

3.6. Functional characterization of LC-PUFA biosynthesis enzymes from bdelloid rotifers

The activities of the LC-PUFA biosynthetic enzymes encoded by the diagnostic genes from bdelloid rotifers were investigated by expressing their ORF in yeast. Transgenic yeasts expressing

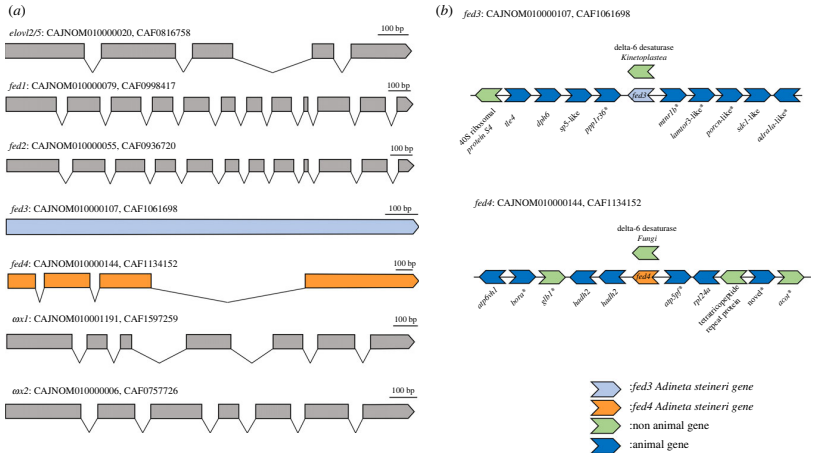

Figure 4. Exon–intron organization and genomic neighbourhood of LC-PUFA biosynthetic genes from *Adineta steineri*. (*a*) Exon–intron structure of candidate LC-PUFA biosynthetic genes (*elovl2/5*, *fed1*, *fed2*, *fed3*, *fed4*, *ωx1*, and *ωx2*) in the *A. steineri* genome. Introns are indicated by solid lines between exons (coloured boxes). All genes analysed have introns except for *fed3*, which is intronless. Besides, *fed4* has an exon–intron organization consistent with its HGT origin. (*b*) Distribution of the *fed3* and *fed4* genes across the *A. steineri* genome. Both genes are flanked by animal genes in the genome according to identity analyses results (BLAST). Moreover, BLAST results also revealed that *fed3* has a protist origin whereas *fed4* has fungi origin. *Identity scores less than 45%.

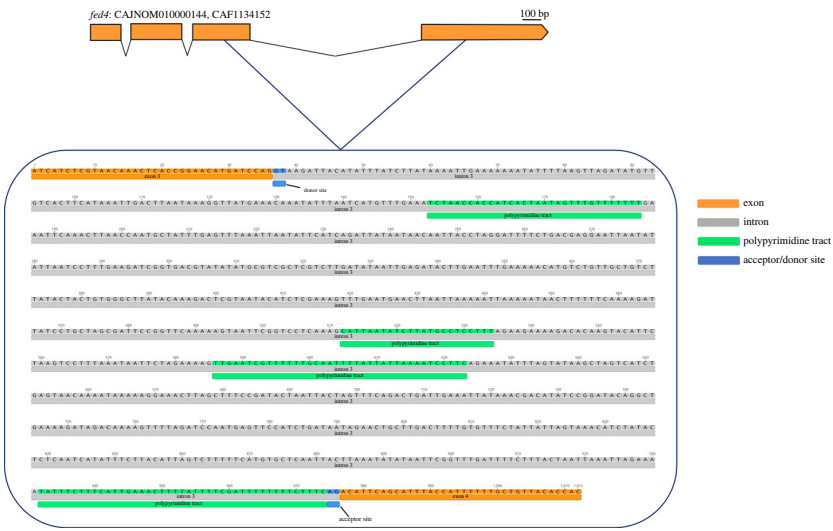

Figure 5. Nucleotide sequence of the *Adineta steineri fed4* genomic sequence. The *A. steineri fed4* genomic sequence (CAJNOM010000144, CAF1134152) has characteristic motifs of an intron-like sequence (ILE). Intron 3 (grey) is flanked by exons 3 and 4 (orange). The particular nucleotide sequences typical from regular spliceseosomal introns (RSI) are polypyrimidine tracts (green) and acceptor/donor sites (blue) for the spliceosome recognition and removal [75,97].

the *elovl2/5* were able to elongate both C_{18} and C_{20} PUFA substrates, but not C_{22}, to their corresponding C_{20} and C_{22} elongation products, respectively (electronic supplementary material, table S3). Regarding front-end desaturases, Fed1 showed Δ5 desaturase activity as denoted by the conversions of 20 : 4*n*-3 and 20 : 3*n*-6 into 20 : 5*n*-3 (EPA) and 20 : 4*n*-6 (ARA), respectively (electronic supplementary material, table S4). Functional characterization of Fed3 showed this enzyme is a Δ6 desaturase as it was able to convert 18 : 3*n*-3 and 18 : 2*n*-6 into 18 : 4*n*-3 and 18 : 3*n*-6, respectively (electronic supplementary material, table S4). Fed2 and Fed4 did not show activity toward any of the assayed substrates (data not shown). For methyl-end desaturases, both characterized enzymes showed dual Δ12 and Δ15 activities as they were able to desaturate 18 : 1*n*-9 to 18 : 2*n*-6 (Δ12 desaturation), as well as 18 : 2*n*-6 and 18 : 3*n*-6 to 18 : 3*n*-3 and 18 : 4*n*-3, respectively (Δ15 desaturations) (electronic supplementary material, table S5).

4. Discussion

Our sequence retrieval strategy has revealed a markedly different repertoire of LC-PUFA biosynthetic genes in transcriptomes from freshwater gammarids compared to marine species, with the former having a generally conserved number of *elovl2/5* (1 gene), *fed* (4 genes) and *ωx* (2 genes), and none in the latter. Instances of animal lineages with enhanced LC-PUFA biosynthetic capacity in species inhabiting freshwater ecosystems compared to that of marine counterparts have been previously reported in sticklebacks and American soles [76,77]. However, such an evolutionary innovation enabling colonization of freshwater environments by these teleosts occurred in one specific front-end desaturase gene, *fads2* [76,77], rather than an acquisition of multiple genes as suggested by the gammarid transcriptomic database searches. The present study provides compelling evidence demonstrating that the unexpected occurrence of key desaturase and elongase genes in freshwater gammarid transcriptomes, rather than gammarids themselves, is accounted for by the presence of bdelloid rotifers in the analysed samples.

Our phylogenetic analysis revealed that, with the exception of the herein termed *fed3* and *fed4*, the LC-PUFA biosynthetic genes used as diagnostic markers clustered together with orthologues from bdelloid rotifers, particularly *Rotaria* and *Adineta* species. These results strongly suggested that multiple transcriptomes from freshwater gammarids contain rotifers' genetic material contaminating those publicly available databases. Due to the fact that bdelloid rotifers are typical epibionts of freshwater gammarids associated with their branchial plates and appendages (e.g. 22 different species reported in *G. pulex*), their high prevalence (up to 80%), and their relatively small size (ranging from 150 to 700 µm approx.) [63–65,78], it is reasonable to speculate that the gammarid specimens used for RNA sequencing contained rotifers that were not removed prior to analysis. As opposed to whole individuals [50], the transcriptome built from *G. pulex* hepatopancreas [48] did not result in any positive hit against any of the LC-PUFA biosynthesis gene markers assessed, confirming that internal body parts of the gammarid are free from epibiotic rotifers. Unlike freshwater species, marine gammarids such as *E. marinus* [50,52] and *G. locusta* [54] did not have any of the diagnostic LC-PUFA biosynthesis genes, consistent with the fact that the above described epibiotic relationship between gammarids (host) and bdelloid rotifers (epibiont) is restricted to freshwater environments [64]. Our data further support that the abundance of rotifers in the gammarid samples used for RNA sequencing was not negligible according to the presence of a remarkable amount of rotifer-derived mitochondrial genes (*cox*) in some of the available RNA sequencing data [50]. It is also worth noting that, while pipelines for de novo transcriptome assembly typically imply filtering strategies [51,79–81], these appear not have been effective in removing rotifer contamination from the analysis. This probably explains why, despite not being the targeted species (gammarid), full-length sequences for all diagnostic LC-PUFA biosynthesis gene markers were found in most freshwater gammarid transcriptomes when such genomic platforms were built with freshly sampled wild-captured whole individuals. Laboratory cultured gammarids that have progressively lost epibionts after successive generations and/or moulting events, as well as internal tissues such as the abovementioned hepatopancreas [48], arise as safe, clean alternative samples, as supported by the PCR-based determinations of gene markers in *G. pulex*. As noted above, our phylogenetic analyses could only identify *Rotaria* and less probably *Adineta* as the most likely bdelloid genera contributing to the occurrence of LC-PUFA biosynthetic gene sequences in gammarid transcriptomes. Interestingly, only slight variations along the nucleotide sequences of the analysed genes were found, even when comparing sequences from samples collected from very distantly located sites like Central Europe (France, Belgium, Germany and Switzerland) and Baikal Lake (Russia). These results show that all epibiotic rotifers inhabiting the exoskeleton of freshwater gammarids are very closely related irrespectively of their geographical location and biotope.

Phylogenetics suggested that Fed3 and Fed4 are from a heterologous origin unlike other LC-PUFA biosynthesis genes investigated herein, since they clustered together with front-end desaturases from kinetoplastids (protists) and fungi, respectively. To clarify this point, we examined the genomic location of *fed3* and *fed4* within the genome of the bdelloid rotifer *A. steineri* [82], demonstrating both genes are genome-anchored. Similarly, other LC-PUFA biosynthetic genes, namely *elovl2/5*, *fed1*, *fed2*, *ωx1* and *ωx2*, were also confirmed to be genome-anchored, demonstrating that the complement of LC-PUFA biosynthetic genes in bdelloid rotifers goes well beyond *ωx1* and *ωx2* previously reported in *Adineta vaga* [12]. Importantly, in agreement with their unexpected location in the phylogenetic trees, we established that *fed3* and *fed4* likely originate via HGT, a well-documented evolutionary mechanism by which bdelloid rotifers have gained bacterial, protist and fungal genes [57,83–87]. Moreover, analysis of the exon–intron organization in *fed3* and *fed4* further supported these were likely instances of HGT. The *A. steineri fed3* is intronless, characteristic of horizontally transferred genes by RNA retrotransposition [88–90] likely from kinetoplastids that are often prevalent in the freshwater habitats occupied by bdelloid rotifers [91–94]. Unlike *fed3*, *fed4* contains introns suggesting a relatively more complex mechanism by which *fed4* has been transferred into the *A. steineri* genome, combining RNA retrotransposition as described for *fed3* and/or further intron gain likely occurring via the intron-generating transposons called 'introners' [95,96]. Consistently, the structure of the third intron of the *A. steineri fed4* includes typical features of 'introner-like' elements [75], particularly those belonging to the 'novel RNA-propagated elements' family,

which have been described in fungi [97]. Introners had been originally described in the picoeukaryote *Micromonas* [96], the pelagic tunicate *Oikopleura dioica* [98], and the dothideomycete fungus *Meloidogyne graminicola* [99]. However, it has been recently discovered that they are more widespread in eukaryotes than originally anticipated and, interestingly, disproportionately common in aquatic lineages [97]. While fungi, identified as potential donors justifying the origin of *fed4*, have been previously reported to explain HGT to bdelloid rotifers [83,87], no previous studies have reported HGT to bdelloid rotifers from kinetoplastids as suggested herein for *fed3*. However, abundance of kinetoplastids in aquatic ecosystems, particularly freshwater environments, makes them likely donors in a HGT of *fed3* to rotifers [92,93,100,101]. In agreement, the substrate specificity of kinetoplastid front-end desaturases shared features with that of the bdelloid rotifer Fed3-like desaturase characterized in our study, further supporing a common origin.

The bdelloid rotifer Fed3 was functionally characterized as a Δ6 desaturase and showed no activity as Δ8 desaturase. These results are in agreement with functions reported in one of the three front-end desaturases from the kinetoplastid *Leishmania major*, which was shown to have Δ6 activity but no Δ8 [102]. Such regioselectivity is markedly different from that of multiple animal front-end desaturases characterized from mammals [103], teleosts [104] and copepods [37] that, in addition to Δ6, have also Δ8 desaturase activity. Exceptions to the above pattern can be found in, for example, the bivalve mollusc *Sinonovacula constricta* Δ6 Fed [105], although this protein is phylogenetically unrelated to those from the cluster formed by kinetoplastid and bdelloid rotifer Fed3 [10]. A more widespread regioselectivity among invertebrates' Fed is Δ5 desaturase [10], herein demonstrated to occur in the bdelloid rotifer Fed1 but not Fed2 despite both genes being duplicates as our phylogenetics and exon–intron organization analyses in *A. steineri* clearly demonstrated. Consequently, since our yeast system did not show any detectable activity that would suggest neofunctionalization beyond Δ5 activity, it can be hypothesized that functional redundancy has driven loss of function in Fed2 [106,107]. Reasons accounting for the loss of function in Fed4 are less clear, although they could be related to their HGT origin as reported elsewhere in the literature [108,109]. Along the functions of front-end desaturases, characterization of the two methyl-end desaturases ($\omega \times 1$ and $\omega \times 2$) and Elovl2/5 demonstrate that bdelloid rotifers have complete enzymatic capacity that enable them to biosynthesize de novo LC-PUFA up to ARA and EPA (figure 1). Indeed, both $\omega \times 1$ and $\omega \times 2$ from bdelloid rotifers have Δ12 and Δ15 desaturase activities, enabling the conversion of OA to LA and, subsequently, ALA (figure 1). While missing the Δ17 activity also contained in the *A. vaga* methyl-end desaturases [12], the combined Δ12/Δ15 regioselectivities within the same enzyme demonstrated herein for both ωx desaturase enzymes is consistent with their bdelloid rotifer origin and contrasts that from non-rotifer methyl-end desaturases in which the gene complement typically includes also an ω3 desaturase [10]. Elovl2/5 exhibited activity towards C_{18} and C_{20} PUFA substrates, converting them into the corresponding C_{20} and C_{22} products, respectively. Such elongation abilities by Elovl2/5 account for all the elongations required to biosynthesize ARA and EPA (figure 1). However, DHA biosynthesis capacity in bdelloid rotifers appears to be limited in the apparent absence of a Δ4 front-end desaturase as our results suggest (figure 1). Potential DHA synthesis in bdelloid rotifers, if existing, would necessarily proceed via the so-called Sprecher pathway but, additionally to Elovl2/5, participation of further PUFA elongases with the ability to elongate 22 : 5*n*-3 to 24 : 5*n*-3 would be mandatory (figure 1). Importantly, the above results raise the question of whether bdelloid rotifers can supply their hosts (gammarids) with physiologically essential LC-PUFA originated via biosynthesis, which might be highly advantageous for the gammarids according to their limited biosynthetic capacity [44] and the relatively low availability of preformed LC-PUFA in freshwater environments [6,110].

In conclusion, the present study demonstrates that multiple transcriptomes from freshwater gammarids contain sequences that can be unequivocally assigned to bdelloid rotifers, well-established epibionts of gammarids. More specifically, we determined the systematic occurrence of key metabolic enzymes of LC-PUFA biosynthesis, and clarified that such occurrence is independent from the species and their geographical origin when transcriptomes are built from freshly sampled wild-captured whole individuals. Genomic location and exon–intron organization further demonstrated that the diagnostic elongase and desaturase genes are bona fide components of the *A. steineri* (and presumably other bdelloid rotifers) genome. Moreover, we provide compelling evidence demonstrating that two of the front-end desaturase genes, namely *fed3* and *fed4*, have been acquired via HGT. Our findings offer a critical example of the value of functional genomics and critical assessment of sequence data to infer ecological impacts in the age of *Omics*.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. All data produced in the present study are presented in the paper and associated electronic supplementary material.

The data are provided in electronic supplementary material [111].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. A.R.-N.: formal analysis, investigation, methodology, writing—original draft, writing—review and editing; N.K.: conceptualization, formal analysis, investigation, methodology, resources, writing—review and editing; L.F.C.C.: formal analysis, investigation, methodology, resources, writing—review and editing; A.G.-d.-S.: formal analysis, investigation; M.M.F.: formal analysis, investigation, methodology, writing—review and editing; H.A.-H.: formal analysis, investigation, resources, writing—review and editing; F.H.: funding acquisition, investigation, project administration, resources, writing—review and editing; J.C.N.: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, writing—original draft, writing—review and editing; Ó.M.: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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