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Metabolomic profiling of blood and seminal plasma in the small-spotted catshark (*Scyliorhinus canicula*): A comparative study of aquarium and wild populations in the Valencia region

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ABSTRACT

In chondrichthyans, despite males in *ex situ* conservation programs exhibiting good sperm quality, offspring production is confined to specific species. This study hypothesizes that the aquarium's environmental footprint may contribute to this loss of reproductive capacity. Consequently, the research initially focused on comparing the blood metabolomic profiles of the Small-Spotted Catshark (*Scyliorhinus canicula*) in both an aquarium environment at Oceanogràfic of Valencia and in wild populations along the Valencia Region coast (Spain). Furthermore, the study compared the blood metabolomic profiles with those of seminal plasma. This comparison aimed to identify potential variations in seminal plasma composition and its relationship with *in vitro* seminal characteristics. Samples were collected from *S. canicula* present in the aquarium (n = 7) and wild captured individuals (n = 12). Despite conventional semen quality assessments showing no significant disparities in semen volume and sperm concentration, considerable differences were observed in sperm motility and viability rates. Specifically, aquarium specimens showed slightly reduced metrics. The untargeted metabolic study in blood revealed significant differences between aquarium and wild individuals, with 158 semi-polar and 233 non-polar differential metabolites. A parallel analysis of seminal plasma similarly revealed substantial differences, with 2 semi-polar and 74 non-polar differential metabolites. Additionally, the analysis showed 104 semi-polar and 188 non-polar metabolites shared between the blood and seminal plasma of both animal groups. A further targeted analysis of 28 metabolites in blood identified significantly lower concentrations of phenylalanine, tyrosine, lysine, taurine, arginine, N-acetyl-L-glutamate, and creatinine in aquarium sharks. In seminal plasma samples, aquarium animals exhibited

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elevated levels of 7-Dehydrodesmosterol but reduced levels of phenylalanine, pantothenic acid (Vit B5), and spermine. In conclusion, these findings highlight alterations in both the blood and seminal plasma metabolomic profiles between aquarium-housed and wild animals, demonstrating the impact of the aquarium's environmental footprint, which could partly explain the reduced reproductive success of sharks in captivity.

1. Introduction

Metabolomics has been applied across all biological sciences, including studying the environment and its species in aquatic systems (Dumas et al., 2022). The comprehensive analysis of small molecules (metabolites), indeed, allows for the investigation of dynamic metabolic responses in living systems due to genetic or environmental factors (Alfaro and Young, 2016; Huang et al., 2019; Roques et al., 2020). Furthermore, this approach can lead to the identification of metabolic biomarkers, which can provide valuable biological insights by elucidating potential pathways that indicate physiological changes (Yoshida et al., 2014; Stevens et al., 2019), sometimes associated with health (Huang et al., 2018). Metabolites are intricately linked to physiological events through complex biochemical networks (Fukusaki, 2014; Guijas et al., 2018), and contribute to defining an individual's phenotype (Guijas et al., 2018). Additionally, metabolites play significant roles in the reproduction, growth, and survival of all biological organisms (Muhamadali et al., 2023).

In fish quality assessment, blood sampling is commonly used to monitor the physiological status (Carbajal et al., 2019; Krick et al., 2021). Considering that blood can reflect physio-pathological conditions as it circulates through organs (Wishart, 2019; Aru et al., 2021), and that the interrelationship between biological fluids in response to a stimulus is well-established (Ivanova et al., 2021), we propose that the seminal plasma metabolome may function as a central nexus connecting metabolic biomarkers related to the underlying adaptations to the environment with semen quality and male potential fertility. In chondrichthyans, although males display high sperm quality conducive to successful fertilization (Dzyuba et al., 2019a:b:c; García-Salinas et al., 2021; Muñoz-Baquero et al., 2021; Wyffels et al., 2020), the actual success in *ex situ* offspring production has largely remained anecdotal, to date (Masuda et al., 2003; Daly and Jones, 2017; Wyffels et al., 2021). As a result, profiling fertility-related biomarkers in sperm or seminal plasma can be helpful in predicting overall fertility. This is especially important because sperm evaluation methods based on conventional microscopic techniques do not provide information about the functional competence of spermatozoa (Johnson et al., 1990; Petrunkina et al., 2007) or offer a clear explanation of the subcellular factors associated with limited reproductive success (Selvam et al., 2019).

For an extended period, the considerable importance of the functions of seminal plasma factors has been undervalued, often restricted to the transport and protection of spermatozoa. However, it is noteworthy that significant advancements have been made in dissecting seminal plasma components, revealing new insights into multiple aspects of sperm function, as well as fertilization and pregnancy outcomes in recent years (Wang et al., 2020). Now, seminal plasma is considered a superior sample for metabolomic analysis when assessing male fertility, compared to other biofluids such as serum or urine (Courant et al., 2013; Zhang et al., 2014; Chen et al., 2015; Qiao et al., 2017; Wang et al., 2019). In fact, seminal fluid contains a variety of metabolites, including amino acids, lipids, nucleosides, minerals, electrolytes, and steroid hormones (Egea et al., 2014; Cheng et al., 2015; Zhang et al., 2021), which reflect the state of the sperm and individual metabolic status (Zhang et al., 2021). Indeed, metabolites influence sperm energy production, motility, and other aspects of sperm function (Goodson et al., 2012; Zhang et al., 2021). There is growing evidence that spermatozoa metabolize a wide range of exogenous substrates that directly or indirectly regulate signaling pathways involved in sperm motility, hyperactivation, capacitation, acrosome reaction, and sperm-oocyte fusion (Odet et al., 2013). While seminal plasma metabolomic characterization has been intriguing in recent years, not only in human medicine (Zhang et al., 2015; Wang et al., 2019; Xu et al., 2020) but also in veterinary medicine, such as yaks (Zhu et al., 2019), boars (Zhang et al., 2021), donkeys (Yu et al., 2022), bulls (Velho et al., 2018; Sun et al., 2022), poultry (Long et al., 2020) and fishes (Dietrich et al., 2019). In fishes, numerous proteins and metabolites have been identified, providing valuable information for conducting a comprehensive analysis of seminal plasma and spermatozoa components and their functions (Dietrich et al., 2019). In recent years, studies utilizing metabolomic approaches have revealed potential fertility biomarkers in the seminal plasma of various mammal species, including humans (Qiao et al., 2017; Tang et al., 2017), stallions (Wood et al., 2016), and bulls (Kumar et al., 2015; Velho et al., 2018; Menezes et al., 2019). However, there is a limited number of metabolomic studies in fishes, and significant knowledge gaps remain regarding metabolite identities, their concentrations in seminal plasma, and their molecular mechanisms involved in fertility. To date, seminal plasma metabolomic fingerprinting in chondrichthyan species has not been explored. However, its study is of special interest in chondrycthyan conservation due to the possible effect of environmental factors on seminal parameters, which could relate with a possible affection in the general reproductive status, both in the wild and aquarium sharks. While the small-spotted catshark is not considered threatened by the IUCN, it serves as a model chondrycthyan species. Therefore, metabolomic modifications or disorders in their reproductive fluids, alongside with other omic approaches, could enhance our holistic understanding of their reproduction, enabling researchers to develop specific tools to assess its overall reproductive fitness (Egea et al., 2014; Cheng et al., 2015; Zhang et al., 2021).

The objective of this study was to utilize metabolomic profiling to assess and compare the blood and seminal plasma of *Scyliorhinus canicula* (*S. canicula)* from both an aquarium (Oceanogràfic of Valencia) and wild populations from the Valencia Region coast, with a focusing on identifying potential metabolic differences and their correlation to semen quality.

2. Material and methods

2.1. Ethics

The experimental portion of the current study involving aquarium animals was approved by the Ethical Animal Experiments Committee at Oceanogràfic Valencia (Reference number: OCE-18-–19) for the protection of animals used for scientific purposes in compliance with European Community Directive 2010/63/EU and its transposition into national legislation through the Royal Decree 53/2013. The study complied with the regulations and policies of Oceanogràfic-Valencia and adhered to the Canadian Council on Animal Care documents. Wild individuals involved in the experiment were obtained from local fisheries as a result of accidental captures.

2.2. Biofluids and sperm samples collection

Animal sampling was conducted in February 2022. Samples were collected from all *S. canicula* individuals housed in the Oceanogràfic of Valencia aquarium (n = 7), and from wild individuals captured off the Eastern coast of the Mediterranean Sea (n = 12). The wild individuals were donated from local fisheries and were a part of accidental captures intended for commercial fisheries ports in Valencia (39°26′45″N 0°19′12″O). Mediterranean Sea water parameters, as measured by the Valencia buoy (39°52′N 0°20′E), included temperatures between 14.6 and 19°C and salinity ranging from 34 to 37 g/ (http://www.puertos.es/es-es/oceanografia/Paginas/portus.aspx). The individuals under human care were housed at Oceanogràfic of Valencia, Spain, in a closed and recirculation system under controlled conditions. These conditions included monitored water quality (17–21 °C, 5.1 mg/L oxygen, 36 g/L salinity, and pH levels between 7.6–8.2), a fixed photoperiod of 12:12 h, and disinfection using UV light and ozone. All animals were isolated from females for at least 1 year before the experiment started, to prevent natural mating, avoiding the lack of seminal fluid inside the male reproductive tract. To mitigate the effects of the processes triggered by death on the post-mortem plasma parameters (Wosnick et al., 2017), the selected wild animals exhibited signs of recent death. Samples were collected immediately upon the fishing boat's arrival at the port, typically within one hour of capture at sea.

The aquarium animals diet was based on frozen-thawed whole fish, including herring (*Clupea harengus*), mackerel (*Scomber scombrus*), and squid (*Loligo sp*). For wild animals, there is a lack of information regarding the diets they consume. Nonetheless, they are considered generalist species and opportunistic mesopredators, feeding on a variety of invertebrates and small fish, including those from *Teleostei, Chondrichthyes, Crustacea, Cephalopoda, Annelida, Echinodermata*, phanerogams and macroalgae, depending on the accessibility of prey present in the area (Ford, 1921; Eales, 1949; Lyle, 1983; Kousteni et al., 2017).

All animals included in the study were classified as adults, displaying calcified claspers that ensured reproductive maturity (Kousteni et al., 2010). Their health was confirmed through physical examination, body condition assessment, and *in vitro* sperm quality testing (Muñoz-Baquero et al., 2021). Aquarium individuals were also determined to be healthy based on their clinical history. Biometric data were recorded for all individuals on each sampling day, with weights ranging from 174 to 370 g (mean ± SD of 309.9 ± 31.3 g). Specimen maturity was determined by their size, gonad development, and clasper calcification (Ebert and Dando, 2020). Lengths ranged from 41.5 to 45.8 cm (mean ± SD of 45.1 ± 1.9 cm), and clasper lengths ranged from 3.6 to 4.1 cm (mean ± SD of 3.9 ± 0.2 cm). Wild individuals had weights ranging from 183 to 319 g (mean ± SD of 258.2 ± 34.8 g). Lengths ranged from 40.6 to 46.5 cm (mean ± SD of 44.5 ± 1.8 cm), and clasper lengths ranged from 3.2 to 4.5 cm (mean ± SD of 3.7 ± 0.4 cm).

Peripheral blood and seminal fluid samples were collected from both aquarium and wild individuals. While following the veterinarian's instructions to collect samples from the aquarium individuals, *S. canicula* specimens from local fishery were also obtained. Peripheral blood was collected from the ventral coccygeous vein through caudal venipuncture using 21-gauge needles and transferred to lithium-heparin tubes (Muñoz-Baquero et al., 2021). Seminal fluid samples were collected through the application of direct pressure on the ampulla of the vas deferens by stripping, using a 5 mL syringe (Muñoz-Baquero et al., 2021). Before collection, the cloaca was rinsed with sterile elasmobranch's Ringer solution (22 g/L urea and 9 g/L NaCl) (Mylniczenko and Clauss, 2017) to clean the surface and reduce contamination. Any samples polluted with urine or feces were discarded. After sample collection, individuals were released back into the quarantine tank, and their recovery was monitored by the aquarium veterinarian. Samples from aquarium individuals were transferred to the laboratory at 4 °C in a dark container immediately after collection. Wild individuals' samples were also transferred to the laboratory under the same conditions, within a maximum of 1 hour after fishing.

Both blood and seminal plasma samples were centrifuged at 3000*g* and 7400*g*, respectively, for 10 minutes at a refrigerated temperature of 4 °C. After centrifugation, the seminal fluid fraction was verified under a microscope to ensure the absence of spermatozoa. All plasma samples were flash-frozen in liquid nitrogen and stored at −80 °C until laboratory processing.

2.3. Seminal quality assessment

The examined semen quality variables included semen sample volume, sperm concentration, total sperm count, total sperm motility, and viability, as previously described by Muñoz-Baquero et al. (2021). The volume of each semen sample was measured using an automatic micropipette. Aliquots from each sample were diluted 1:100 in shark Ringer's solution to determine sperm concentration. Subsequently, 10 μL of the resulting solution was placed in a 10 μm deep Makler counting chamber. Aliquots of 5 μL from each semen sample were diluted 1:20 with sterile elasmobranch's Ringer's solution for immediate assessment of total motility. Semen samples were analyzed using a phase-contrast microscope at 200× magnification (Nikon E 400).

Total motility was estimated by calculating the percentage of motile sperm, which included spermatozoa that were vibrating

without moving forward (Wyffels et al., 2020). The same sample was also examined for the percentage of live and dead spermatozoa using the LIVE/DEAD sperm viability kit (ThermoFisher). This kit consists of two DNA-binding fluorescent stains: a membrane-permeant stain, SYBR-14, and a conventional dead-cell stain, propidium iodide (PI). SYBR-14 stained the nuclei of living sperm in bright green, with an absorption spectrum of 488 nm and an emission spectrum at 518 nm when bound to DNA. In contrast, PI only stained spermatozoa that had lost their membrane functionality. To obtain this dye, 1 μL of SYBR and 1 μL of PI were mixed with 100 μL of the diluted semen sample. The evaluation was conducted under a fluorescent microscope at 400× magnification (Nikon E 400), examining at least 100 cells per sample.

2.4. Semi-polar and non-polar metabolome extraction

Semi-polar metabolites were extracted from 100 μL of each plasma samples following a published method with a slight modification (Yu et al., 2021). Briefly, samples were dissolved in 200 μL of cold aqueous methanol (75 %), and 200 μL of acetronitrile (75 %), spiked with 10 μg/mL formononetin as internal standard. Then, the mixture was centrifugated at 20,000 xg for 15 min at 4 °C. The supernate (200 μL) was gained, and dried under low-temperature vacuum (Thermo Scientific, USA). The samples were redissolved and resuspended in 100 μL of methanol (10 %) and transferred to HPLC tubes and an aliquot of 3 μL was injected for the analysis. Non-polar metabolites were extracted from 50 μL of each plasma samples following a published method with a little modification (Yu et al., 2021). Briefly, samples were dissolved in 300 μl of cold aqueous methanol (100 %), spiked with 50 μg/mL alpha-tocopherol acetate as internal standard. Then, the mixture was swirled for 120 seconds, and 900 μL MTBE and 250 μL ultrapure water were added. After vortexed for 15 mins, the mixture was placed 30 min at 4 °C. Then, the supernatants (900 μL) were gained, and dried under low-temperature vacuum (Thermo Scientific, USA). The samples were redissolved and resuspended in 600 μL of acetonitrile–isopropanol mixture and transferred to HPLC tubes and an aliquot of 3 μL was injected for the analysis.

2.5. LC-ESI-HRMS and LC-APCI-HRMS analysis

Untargeted and targeted liquid chromatography-electrospray ionisation-high resolution mass spectrometry (LC-ESI-HRMS) analyses of the semi-polar metabolome were performed as previously described (Lorenzo-Rebenaque et al., 2022), while targeted and untargeted liquid chromatography-atmospheric pressure chemical ionisation-high resolution mass spectrometry (LC-APCI-HRMS) analyses of the non-polar metabolome were carried out as reported previously (Garcia-Dominguez et al., 2020). Untargeted metabolomics was performed using the Compound Discoverer software (Thermofisher Scientific). After detection of the features (the *m/z* and rt for each peak), and chromatogram alignment, the data generated were normalized with respect to internal standard. Principal components analysis biplot (PCA-biplot) of untargeted metabolomes were created with Past4 software. Metabolites were quantified in a relative way by normalisation on the internal standard (formononetin and DL-α-tocopherol acetate) amounts. This data is available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org where it has been assigned Project ID PR001535. The data can be accessed directly via it's Project DOI: 10.21228/M88T5J.

Targeted analysis was performed to quantify 28 known metabolites, in a relative way, of the seminal plasma metabolisms (Garcia-Dominguez et al., 2020; Mumcu et al., 2020; Alipour et al., 2021; Mateo-Otero et al., 2021; Zhang et al., 2021). Targeted metabolite analysis was performed by comparing chromatographic and spectral properties with authentic standards (if available) and reference spectra, in-house database, literature data, and on the basis of the *m/z* accurate masses, as reported in the PubChem database (http://pubchem.ncbi.nlm.nih.gov/) for monoisotopic mass identification, or on the Metabolomics Fiehn Lab Mass Spectrometry Adduct Calculator (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/MS-Adduct-Calculator/) in the case of adduction detection. The targeted metabolites were categorized in: i) metabolites related to amino acid metabolism (n = 10; Glutamine, Lysine, N-acetyl-L--glutamate, Valine, Taurine, Tyrosine, Arginina, Phenylalanine, D-aspartic acid, and Creatinine); ii) Metabolites related to lipid, phospholipid, and cholesterol metabolism (n = 11; Glycerophosphocholine, Taurochenodeoxycholate, Palmitic acid, Stearic acid, Calcitriol [Vit D], Secalciferol [Vit D], 24-Methylenecholesterol, Lathosterol, 7-Dehydrodesmosterol, Cholesterol, Oleic acid); and iii) Metabolites related to Krebs cycle, energy, and nucleotide metabolism (n = 8; Inosine 2′-Deoxycytidine-5′-monophosphorate, Glucuronolactone, Glucosamine 6-phosphate, Pantothenic acid [Vit B5], Pyridoxine [Vit B6], Spermine).

2.6. Statistical analysis

A generalized linear model (GLM) was conducted to examine the effect of individuals origin as a fixed predictor on the semen quality. Populations were treated as a categorical factor with two levels: aquarium and wild. Semen quality data as volume (mL), sperm concentration, total concentration, total motility (%) and viability (%) are expressed as the mean ± standard error of the mean (SEM). The level of significance was set at $p < 0.05$. The statistical analysis was performed using SPSS software (version 27). The effect size for comparative tests based on Cohen's d was performed using the 'effsize' R package (Torchiano, 2020).

Statistical analysis of seminal fluid and blood plasma metabolome composition was performed using the following methodology. No outlier samples were identified using a principal component analysis with the dataset without zeros, so all samples remaining in the datasets. Metabolites with almost 20 % zeros within each treatment were removed (Bijlsma et al., 2006). The remaining zeros were replaced by half of the minimum value detected for each metabolite. A total of 652 semi-polar and 510 non-polar metabolites were identified in blood fluid, along with 614 semi-polar and 413 non-polar metabolites in seminal plasma, across 24 samples for each, which are retained in the datasets. Datasets were transformed using the additive log-ratio (ALR) transformation following:

$$ALR(j|ref) = \log(x\_j/x\_ref) = \log(x\_j)\text{-}\log(x\_ref) \quad (1)$$

where j is the total number of variables in the dataset, x_j is the values for the genera or metabolite j, and x_ref is the reference variable used to transform the data. The lack of isometry was checked using Procrustes correlation (Greenacre et al., 2021). ALRs were auto-scaled with mean of 0 and standard deviation of 1.

A partial least square-discriminant analysis (PLS-DA) were used to identify the genera and metabolites that allow to classify or discriminate among the treatments. PLS-DA models were computed with the mixOmics packages in R (Rohart et al., 2017), using the treatments as the categorical vector y, and the ALR dataset for genera or metabolites as the matrix X. The balance error rate (BER) for the Mahalanobis distance, computed by a 4-fold cross-validation repeated 100 times was used to select the optimal number of components of the model in each iteration process. In each iteration, variables with a variable importance prediction (VIP) lower than 1 were removed from the X matrix because are not informative for the classification among the treatments (Galindo-Prieto et al., 2014). After variable selection a new PLS-DA model was computed. Variable selection and PLS-DA model computation were done until the lowest BER was achieved. The prediction performance of the final PLS-DA model was checked with the construction of a confusion matrix and a permuted-confusion matrix using a 4-fold cross-validation repeated 10,000 times. The former allows to determine the ability of the model to predict each treatment according to the variables selected by the PLS-DA. The latter determines if the performance achieved is due to a spurious selection of variables throughout the PLS-DA iterations. The prediction performance was considered spurious when the percentage of true positives for each treatment was far from their random probabilities (33 % for three categories and 50 % for two categories).

Bayesian statistics were used complementary to the PLS-DA to measure the relevance of the differences in the metabolites abundance between the aquarium and wild individuals. A model with a single effect of "treatment" and flat priors was fitted. The estimation of the marginal posterior distribution of the unknows were done with MCMC using four chains of 50,000 iterations, with a burn-in of 1000 and a lag of 10. The mean of the marginal posterior distribution of the differences between the control and each one of the two types of phage administration were used to estimate the posterior mean of the differences in genera or metabolites between the aquarium and wild individuals. These estimates were reported as unit of standard deviations (SD) of each variable. The differences in the mean abundance of the metabolites between groups were considered relevant when these differences were higher than 0.5 units of SD, and the probability of the differences (Blasco, 2017) being higher (if the difference is positive) or lower (if negative) than 0 (P0) was higher than 0.9.

3. Results

3.1. Comparison of semen parameters between aquarium and wild populations

The results for the semen parameters, including volume, concentration, motility, and viability, are presented in Table 1. No significant differences were observed between aquarium and wild individuals in terms of sperm volume ($p = 0.072$; Cohen $d = -0.49$ [95 % CI (-1.51–0.52)]) and concentration ($p = 0.072$; Cohen $d = 0.21$ [95 % CI (-0.79–1.22)]). However, significant differences were found in both motility rate ($p = 0.006$; Cohen $d = -1.67$ [95 % CI (-2.92 to −0.41)]) and viability rate ($p = 0.006$; Cohen $d = -2.08$ [95 % CI (-3.42 to −0.74)]), with the aquarium *S. canicula* exhibiting lower values compared to the wild individuals.

Different superscript letters (a, b) in the same column indicate differences ($p < 0.05$).

4. Metabolomic profiling

Metabolomic analyses was conducted on both peripheral blood and seminal plasma using a non-targeted approach, covering both semi-polar and non-polar fractions in both positive and negative ionization modes. A total of 652 and 510 semi-polar and non-polar metabolites were identified in blood plasma samples. In addition, 614 and 413 semi-polar and non-polar metabolites were identified in seminal plasma samples.

A multivariate data analysis was used to analyze *S. canicula* samples collected at different habitats. In addition, a principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) classification model was established. The exploratory analysis conducted with PCA showed that Component 1 explained more than 95 % of the variance in all sample types (Fig. 1). These results showed a clear distinction between the samples (blood plasma and seminal plasma) from the aquarium and the wild animals.

To define which compounds were influential metabolites in the PLS-DA model, a feature selection based on VIP scores was performed. For blood plasma, the analysis identified 158 semi-polar and 233 non-polar relevant metabolites in the final model for the

Table 1
Sperm quality comparison of aquarium-housed and wild-caught small-spotted catshark (*Scyliorhinus canicula*). Data are expressed as the mean ± standard deviation.

Groups	n	Volume (μL)	Concentration (10^6/mL)	Total sperm (10^6)	Motility (%)	Viability (%)
Aquarium	7	714.2 ± 201.07	140.4 ± 29.60	97.3 ± 39.58	76.2 ± 2.95^b	70.8 ± 4.0^b
Wild	12	975.0 ± 153.57	123.7 ± 22.60	131.12 ± 30.23	89.9 ± 2.25^a	92.3 ± 3.1^a

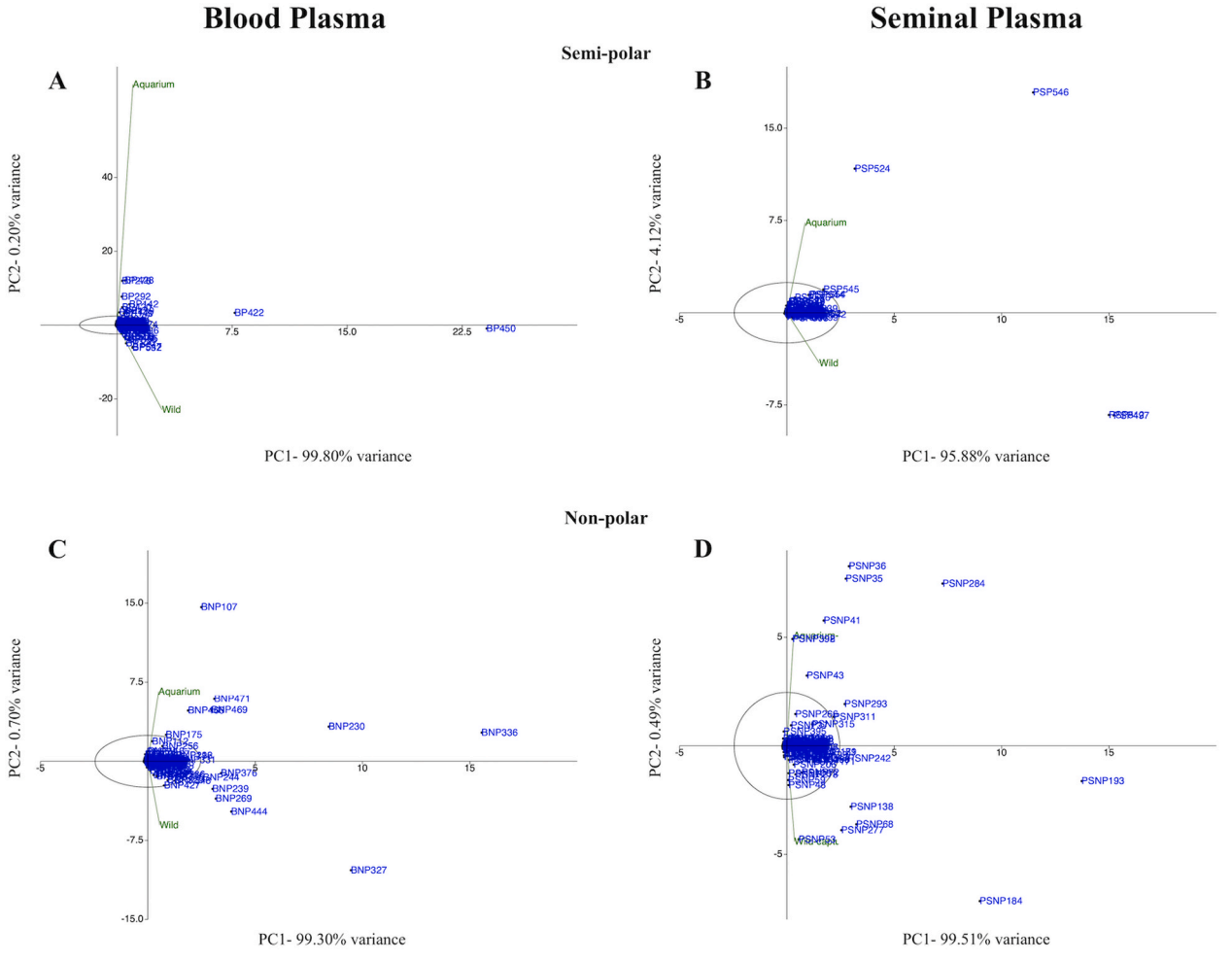

Fig. 1. Unbiased metabolomic data (untargeted study) of peripheral blood plasma and seminal plasma comparing aquarium-housed and wild-caught small-spotted catshark (*Scyliorhinus canicula*) specimens. Biplots derived from principal component analysis illustrate the distribution of metabolites within the peripheral blood plasma and seminal plasma of the *S. canicula*. Specifically, these biplots display (A) semi-polar and (C) non-polar metabolites in peripheral blood plasma, as well as (B) semi-polar and (D) non-polar metabolites in seminal plasma.

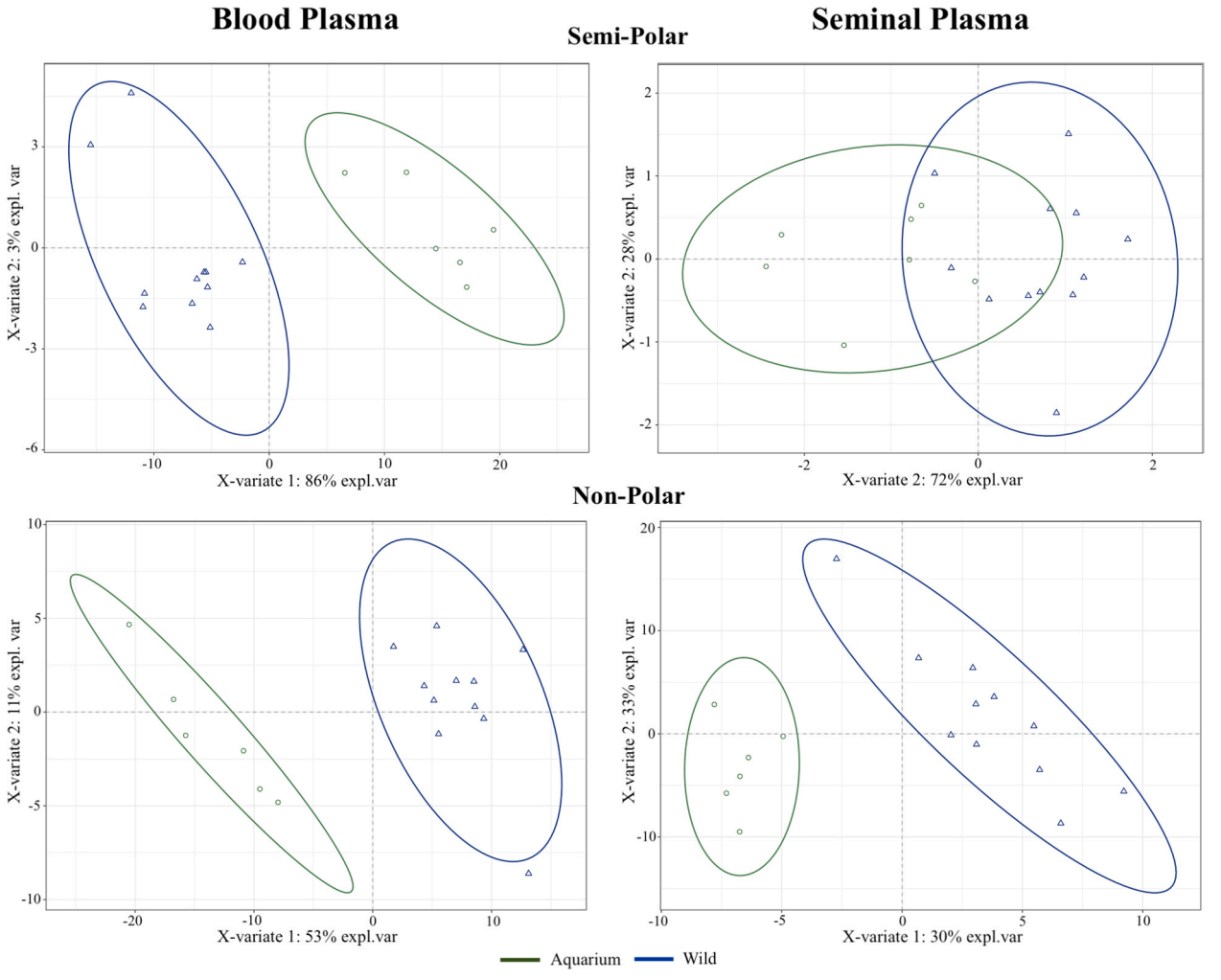

Fig. 2. Dissimilarities in the seminal plasma and peripheral blood plasma metabolome compositions of the small-spotted catshark (*Scyliorhinus canicula*), both aquarium-housed (green) and wild-caught (blue), are represented through the first (X-variate 1) and second components (X-variate 2) of the identified relevant variables (metabolites) in the final Partial Least Squares Discriminant Analysis (PLS-DA) models: semi-polar and non-polar metabolites of peripheral blood plasma, and semi-polar and non-polar metabolites of seminal plasma.

classification among the two habitats. Using these relevant metabolites and a number of components equal to two, the predictive capacity of the model was determined by calculating the percentage of false positives, false negatives, and true positives identified. The final PLS-DA model exhibited high classification performance for both semi-polar fraction (aquarium = 100.00 % and wild = 99.97 %) and non-polar fraction (aquarium = 100.00 % and wild = 100.00 %). In this line, in Fig. 2, the PLS-DA score plots showed a clear separation between the groups, indicating significantly different metabolic profiles among the groups for peripheral blood plasma. In seminal plasma, the analysis identified 2 semi-polar and 133 non-polar relevant metabolites in the final model for the classification among the two habitats. The final PLS-DA model also showed good classification performance for both semi-polar fraction (aquarium = 85.62 % and wild = 85.15 %) and non-polar fraction (aquarium = 100.00 % and wild = 100.00 %). In this line, in Fig. 2, the PLS-DA score plots exhibited a clear separation between the groups, indicating markedly distinct metabolic profiles in the non-polar seminal plasma, while they were closer in the semi-polar, suggesting that the metabolic composition of both groups was more similar.

Moreover, the relevance of the metabolites identified by PLS-DA was validated through Bayesian statistical analysis. For peripheral blood plasma, the analysis demonstrated that, out of the initial 158 identified variables for the semi-polar fraction and 233 for the non-polar fraction, all had a posterior mean of the differences of at least 0.5 times the standard deviation of the variable, with a probability (P0) higher than 0.90 of differences being higher or lower than 0. In seminal plasma, the analysis showed that out of the initial 2 identified variables for the semi-polar fraction and 74 for the non-polar fraction, all had a posterior mean of the differences of at least 0.5 times the standard deviation of the variable, with a probability (P0) higher than 0.90 of differences being higher or lower than 0.

The blood plasma of aquarium *S. canicula* exhibited a significant decrease in the accumulation of all semi-polar metabolites when compared to samples collected from wild individuals (Fig. 3). An equal number of significantly accumulated non-polar metabolites, both up and down, was observed. Regarding the seminal plasma samples, a similar number of significant metabolites (both semi-polar and non-polar) exhibited accumulation in both directions (Fig. 3).

Among the 28 candidate metabolites, blood in the aquarium *S. canicula* presented significantly lower concentrations of phenylalanine, tyrosine, lysine, taurine, arginine, N-acetyl-L-glutamate and creatinine when comparing to samples collected from the wild individuals (Table 2).

On the contrary, in seminal samples, high concentration of 7-Dehydrodesmosterol was observed in the aquarium individuals, while low concentration of phenylalanine, pantothenic acid (Vit B5) and spermine was found in the aquarium *S. canicula* individuals when comparing to samples collected from the wild individuals (Table 3).

5. Discussion

The main objective of this study was to enhance the understanding of the reproductive biology of sharks in aquarium settings, using *S. canicula* as a model to explore how sperm quality and seminal fluid composition differ between captive and natural populations, for further application in conservation programs This research was driven by the need to understand the factors contributing to the observed reduction in reproductive capacity among aquarium-housed animals. In this sense, metabolomic analysis has been applied to investigate the biochemical composition of blood and seminal plasma. The comparative metabolomics approach has demonstrated its effectiveness in discerning the metabolic status and unique characteristics of shark species in a small-scale study and might serves as a valuable tool for monitoring *S. canicula*, by assessing environmentally induced metabolic changes, and understanding their effects on seminal fluid.

Blood metabolites exert systemic effects while circulating through body organs (Dai et al., 2015; Han et al., 2022). Nevertheless, for

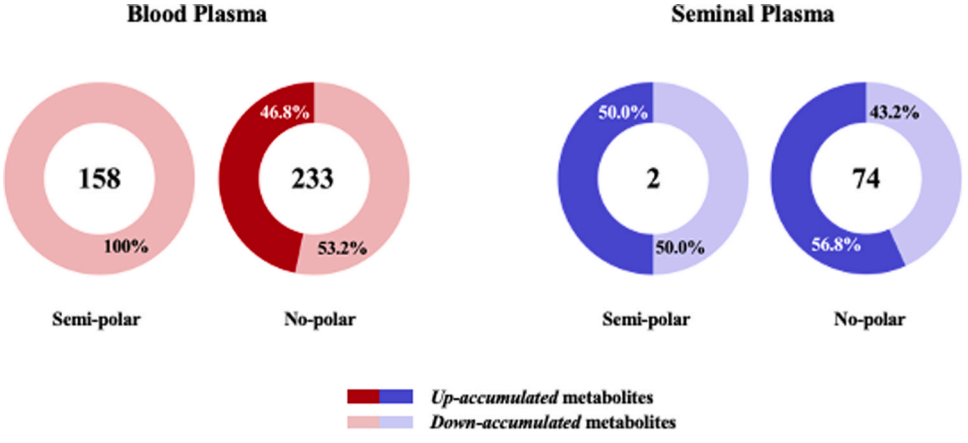

Fig. 3. Characterization of seminal plasma and peripheral blood plasma metabolome features in aquarium-housed and wild-caught small-spotted catshark (*Scyliorhinus canicula*) specimens. Pie charts depicting the percentages of up- and down-accumulated metabolites between aquarium-housed and wild-caught specimens. Compounds have been identified as biologically significant through Partial Least Squares Discriminant Analysis (PLS-DA) and Bayesian statistical analysis. Diagram compares metabolites found in peripheral blood plasma and seminal plasma across semi-polar and non-polar fractions from both aquarium-housed and wild-caught specimens.

Table 2
Targeted/identified metabolites in peripheral blood plasma of aquarium-housed and wild-caught small-spotted catshark (*Scyliorhinus canicula*).

Targeted metabolites	P0	HDP95	D
Metabolites related to amino acid metabolism			
Phenylalanine*	100.00*	[-2.31,-0.96]	-1.65
Tyrosine*	99.92	[-2.29,-0.71]	-1.48
Lysine*	99.91	[-2.28,-0.64]	-1.44
Taurine*	99.14	[-2.10,-0.24]	-1.18
Arginine*	98.32	[-2.02,-0.08]	-1.07
N-acetyl-L-glutamate*	95.73	[-1.93,0.11]	-0.89
Creatinine*	91.66	[-1.80,0.37]	-0.74
Valine	81.48	[-1.58,0.64]	-0.48
D-aspartic_acid	89.59	[-0.44,1.78]	0.68
Glutamine	76.03	[-1.54,0.71]	-0.38
Metabolites related to lipid, phospholipid, and cholesterol metabolism			
Stearic acid	99.98	[-2.33,-0.90]	-1.60
Palmitic acid	99.97	[-2.31,-0.85]	-1.58
Oleic_acid	99.62	[-2.15,-0.38]	-1.29
Cholesterol	99.40	[0.30,2.15]	1.24
Taurochenodeoxycholate	99.12	[-2.09,-0.22]	-1.19
7-Dehydrodesmosterol	97.81	[0.02,2.050]	1.04
Calcitriol (Vit D)	97.62	[-2.01,0.00]	-1.03
Secalciferol (Vit D)	97.37	[-2.01,0.03]	-1.02
24-Methylenecholesterol	96.23	[-0.16,1.95]	0.94
Lathosterol	87.22	[-0.54,1.69]	0.62
Glycerophosphocholine	nd	nd	nd
Metabolites related to Krebs cycle, energy, and nucleotide metabolism			
Inosine	99.95	[-2.26,-0.72]	-1.51
Glucuronolactone	98.01	[-2.03,-0.06]	-1.05
Glucosamine 6-phosphate	97.56	[-0.03,1.97]	1.01
2'-Deoxycytidine-5′-monophosphorate	96.87	[-1.98,0.07]	-0.96
Pantothenic acid (Vit B5)	90.47	[-1.78,0.40]	-0.70
Pyridoxine (Vit B6)	nd	nd	nd
Spermine	nd	nd	nd

nd: no detected metabolite. HPD95 = The highest posterior density region at 95 % of probability. P0 = Probability of the difference (Daquarium-wild) being greater than 0 when Daquarium-wild > 0 or lower than 0 when Daquarium-wild < 0. D = Mean of the difference - aquarium-wild (median of the marginal posterior distribution of the difference between the control group aquarium-wild group). Statistical differences were assumed if | Daquarium-wild | surpassed R value and its P0>0.90. * Asterisk denotes Relevant metabolites by PLS-DA and Bayesian.

an accurate interpretation of blood analysis, it is necessary to consider a multitude of variants, such as the reproductive cycle, age, sex, feeding behavior, stress, nutritional status, water quality, and the species' habitat (see review Burgos-Aceves et al., 2019). In this study, it has been posited that blood plasma metabolites are mobilized as part of the environmental response (Tort, 2011; Burgos-Aceves al., 2019), thereby enabling the demonstration of significant physiological differences between wild animals and those under human care. Growing evidence supports the direct influence of environmental conditions on the blood metabolome (Nassan et al., 2021). Studies analyzing the blood metabolomic profiles between aquarium and wild animals are scarce. A limitation of this study was the lack of controls (i.e., fish subjected to each parameter), as well as the feeding, making it difficult to ascertain which parameter has the predominant impact. However, to avoid bias in this research, a homogeneous population of animals with similar weights and sizes were included in the study to minimize interindividual variability. Indeed, the biometric similarity between the experimental groups validates the direct comparison. In summary, the biometric data for weight (269.5 vs. 309.8 g for wild vs. aquarium), length (45 vs. 45 cm for wild vs. aquarium), width (10.9 vs. 13.4 cm for wild vs. aquarium), and clasper length (3.8 vs. 4.0 cm for wild vs. aquarium) corroborate the results. Another biases effect is the post-mortem effect, which other authors suggest can lead to alterations in some plasma Another bias effect is the post-mortem effect, which other authors suggest can lead to alterations in some plasma parameters such as glucose, lactate, and potassium levels (Wosnick et al., 2017). For this, samples were taken from animals displaying signs of recent death, within an hour of being captured at sea.

Relevant to this study, untargeted metabolomics identified 158 relevant metabolites in the polar fraction (approximately 50 %) and 233 in the non-polar fraction (approximately 67 %) of blood samples that reflect the modulation of host metabolism based on environmental factors. Metabolomics has proven to be an efficient and accurate technique for studying the impacts of environmental changes on the physiological metabolism of aquatic animals, providing a comprehensive understanding of how they respond to stressors (Kullgren et al., 2013; Goode et al., 2020). The potential of metabolomics lies in its high sensitivity (i.e., the ability to measure low levels of metabolites), high assessment efficiency (i.e., the capability to measure a broad range of metabolites without prior knowledge), and comprehensive evaluation (i.e., it provides an overview of molecular effects, reflecting the physiological status of the organism) (Dumas et al., 2022). While the application of environmental metabolomics in investigating molecular effects and activity in marine organisms is highly promising for risk assessment, in this study, its use has been directed to elucidate how wild animals adapted to aquarium conditions exhibit notable physiological differences (Dumas et al., 2022).

Subsequently, targeted metabolomics was exploited, by compiling a database of 28 compounds implicated in blood and seminal

Table 3
Targeted/identified metabolites in seminal plasma of aquarium-housed and wild-caught small-spotted catshark (*Scyliorhinus canicula*).

Targeted metabolites	P0	HDP95	D
Metabolites related to amino acid metabolism			
Phenylalanine*	96.62	[-1.83,0.09]	-0.89
Tyrosine	72.51	[-1.38,0.75]	-0.32
Lysine	54.95	[-1.02,1.12]	0.06
Taurine	63.04	[-1.23,0.92]	-0.18
Arginine	65.20	[-0.86,1.29]	0.20
N-acetyl-L-glutamate	nd	nd	nd
Creatinine	50.89	[-1.15,1.16]	0.01
Valine	58.42	[-1.21,0.97]	-0.11
D-aspartic_acid	nd	nd	nd
Glutamine	89.24	[-1.66,0.4]	-0.62
Metabolites related to lipid, phospholipid, and cholesterol metabolism			
Stearic acid	86.08	[-1.63,0.53]	-0.58
Palmitic acid	83.83	[-1.63,0.57]	-0.53
Oleic_acid	56.28	[-1.06,1.24]	0.09
Cholesterol	99.60	[0.40,2.18]	1.29
Taurochenodeoxycholate	91.65	[-1.76,0.30]	-0.70
7-Dehydrodesmosterol*	99.75	[0.51,2.22]	1.35
Calcitriol (Vit D)	69.89	[-1.45,0.83]	-0.29
Secalciferol (Vit D)	50.70	[-1.15,1.16]	-0.01
24-Methylenecholesterol	96.66	[-0.08,1.96]	0.94
Lathosterol	95.52	[-0.18,1.88]	0.89
Glycerophosphocholine	92.91	[-1.71,0.30]	-0.73
Metabolites related to Krebs cycle, energy, and nucleotide metabolism			
Inosine	70.68	[-1.34,0.78]	-0.28
Glucuronolactone	nd	nd	nd
Glucosamine 6-phosphate	88.98	[-0.44,1.63]	0.62
2'-Deoxycytidine-5′-monophosphorate	76.17	[-1.43,0.67]	-0.37
Pantothenic acid (Vit B5)*	99.60	[-2.03,-0.32]	-1.20
Pyridoxine (Vit B6)	84.98	[-1.57,0.50]	-0.53
Spermine*	99.60	[-2.09,-0.38]	-1.24

nd: no detected metabolite. HPD95 = The highest posterior density region at 95 % of probability. P0 = Probability of the difference (Daquarium-wild) being greater than 0 when Daquarium-wild > 0 or lower than 0 when Daquarium-wild < 0. Daquarium-wild = D = Mean of the difference - aquarium-wild (median of the marginal posterior distribution of the difference between the control group aquarium-wild group). Statistical differences were assumed if | Daquarium-wild | surpassed R value and its P0>0.90. * Asterisk denotes Relevant metabolites by PLS-DA and Bayesian.

plasma metabolism and quality. They included amino acids, lipids and metabolites taking part in primary metabolism pathways (e.g. Krebs cycle, energy and nucleotide metabolism). Among the 28 target metabolites examined in this study, distinctive differences in amino acid metabolism were observed between aquarium-held and wild *S. canicula*. Levels of arginine, taurine, lysine, tyrosine, and phenylalanine were found to be lower in the serum plasma of aquarium animals compared to their wild counterparts. Specifically, aquarium blood plasma levels of arginine and N-acetyl-L-glutamate, which are essential for synthesizing urea via the urea cycle, were significantly lower than those in wild specimens. The urea cycle, which converts ammonia into urea in many aquatic animals, requires N-acetylglutamate (NAG), an essential allosteric activator of carbamyl phosphate synthetase III (CPSIII) (Haskins et al., 2008). In sharks, skates, and rays, urea serves as an osmolyte (Goldstein and Forster, 1971a:b; Anderson et al., 1980; Hong et al., 1994; Ip et al., 2003; Tam et al., 2003; Chew et al., 2006). Fish NAGS enzymes are known to be partially inhibited by arginine (Haskins et al., 2008). Highlighting the importance of arginine in spermatogenesis, previous studies have linked an arginine-deficient diet with a decrease in sperm count and motility (Mumcu et al., 2020).

Phenylalanine, an essential amino acid, must be obtained through the diet. It is metabolized via two pathways: oxidation into tyrosine and transamination into phenylpyruvate, integral to the classic metabolic pathways (Shafik et al., 2014). Therefore, the observed difference in this amino acid may be directly linked to the variability in amino acid content of the diet, which could result in differences in physiological and cellular effects on metabolism (Salamanca et al., 2020). Tyrosine is a direct precursor of catecholamine hormones and plays a role in thyroid hormone synthesis. Additionally, it can be catabolized into hydroxyphenylpyruvate, contributing to energy metabolism. Tyrosine is implicated in the overall antioxidant capacity of seminal plasma and has been associated with deviations in stillbirths per litter (Mateo-Otero et al., 2021). Furthermore, phenylalanine and tyrosine are precursors to critical hormones and neurotransmitters involved in stress response, such as adrenaline, noradrenaline, and dopamine (Zehra and Khan, 2014).

Taurine is one of the major intracellular amino acids in elasmobranch erythrocytes and is often used for cell volume regulation due to its metabolically inert nature (Boyd et al., 1977; King et al., 1980; Fincham et al., 1987). It also plays an essential role in antioxidative and membrane-stabilizing functions, which contributes to its significant role as a capacitating agent and a factor in sperm motility (Alipour et al., 2021). Therefore, low levels of seminal plasma taurine have been implicated in male infertility (Alipour et al., 2021). Lysine is vital for protein synthesis and is considered one of the first limiting essential amino acids in diets (Furuya et al., 2023), and its levels have been noted in studies of infertile men (Mumcu et al., 2020). Creatinine, a byproduct of creatine phosphate breakdown in muscle, may be elevated in wild *S. canicula* due to strenuous activity during escape behaviors at capture (Volfinger et al.,

1994). Given the remarkable differences observed (in untargeted and targeted metabolome) between aquarium and wild animals, it is relevant to emphasize that, taken together, these findings suggest an inherent metabolic adaptation to the underlying changes in the aquarium environment. Consequently, it is reasonable to postulate that the environment may influence reproductive physiology, extending its impact to the levels of metabolites in seminal fluid.

One notable finding from this study was the significant difference in semen parameters between the two experimental groups. Although there were no significant variations in semen volume and sperm concentration, aquarium *S. canicula* exhibited lower sperm motility and viability rates compared to their wild counterparts. These findings align with previous research that highlights the potential influence of environmental factors, including captivity, on semen quality in various species (Wyffels et al., 2020; Beirão et al., 2019; Caldeira et al., 2018; Skjæraasen et al., 2009). While this may seem contradictory to previous results obtained (Muñoz-Baquero et al., 2021), in the current study, wild animals donated by fishermen from the port of Valencia were analysed, ensuring that the samples were processed within less than one hour after collection. In contrast, the previous study included individuals from the port of Jávea, where there was a time lapse of 4–8 hours between retrieval of the animals and assessment of the samples. It is well-documented that post-mortem storage time can significantly alter sperm motility characteristics when using sperm from sacrificed fish (Dietrich et al., 2005; Routray et al., 2006).

The disparities in sperm quality and *in vitro* parameters may be attributed to the different environmental conditions and stressors experienced by the aquarium individuals, such as nutrition, photoperiod, temperature cycles, confinement, and limited physical activity, which are challenging to replicate (e.g. vitamin D-related metabolites) (Wyffels et al., 2020; Clavere-Graciette et al., 2022). Despite the challenges in fully understanding the marked decline in reproductive potential associated with the observed reductions in motility and viability in aquarium animals, the analysis performed in this study of seminal plasma metabolites illuminates the underlying metabolic factors contributing to this decline. Among the 28 target metabolites examined, it has been observed significant differences in the concentrations of phenylalanine, pantothenic acid, spermine, and 7-dehydrodesmosterol between the aquarium *S. canicula* and their wild counterparts. Notably, phenylalanine was the only metabolite that displayed reduced concentrations in both blood and seminal plasma of aquarium *S. canicula*. In seminal plasma, this metabolite has been linked to sperm capacitation and acrosomal exocytosis in humans (Houston et al., 2015; Mateo-Otero et al., 2021) and with post-thaw sperm viability in cattle (Ugur et al., 2020; Mateo-Otero et al., 2021).

Pantothenic acid plays a significant role in cellular protection against oxidative damage, serving as a precursor to glutathione, one of the most potent intracellular antioxidants. The seminal plasma antioxidant system includes both enzymatic and non-enzymatic components, such as vitamins. Furthermore, pantothenic acid is essential for testicular endocrinology and sperm motility in male rats (Yamamoto et al., 2009). Spermine, produced in the prostate gland, plays a critical role in protecting and nourishing spermatocytes. Its antioxidant and nutritional properties are crucial for maintaining sperm health and viability, contributing to successful fertilization and reproduction (Lefèvre et al., 2011). Spermine has also been described as a male pheromone that attracts ready-to-mate females in sea lampreys (Scott et al., 2019).

As for 7-dehydrodesmosterol, which exhibits higher concentrations in aquarium *S. canicula*, it is a sterol intermediate in steroid biosynthesis, particularly in the synthesis of cholesterol. Cholesterol is vital for maintaining cell membrane stability, permeability, and fluidity, as well as playing a regulatory role in membrane fluidity, sperm motility, capacitation, the acrosome reaction, and the fertilization process (de Neergaard et al., 2018; Dasgupta et al., 2022). Interestingly, this study suggests that aquarium *S. canicula* exhibit higher cholesterol concentrations according to Bayesian statistics. In humans, there is a described positive association between cholesterol in seminal plasma and sperm counts (de Neergaard et al., 2018). Moreover, lipids and cholesterol may have a protective effect against environmental changes, especially temperature variations, which occur when fish semen is released (Bozkurt et al., 2008). These factors could be associated with the consistent feeding regimen in the aquarium facility, as opposed to the variability in food intake observed under natural conditions. This variability may be influenced by factors such as the animals' prey capabilities, food availability, or seasonal variations (Valls et al., 2011; Bengil et al., 2019).

6. Conclusion

The findings obtained in this study confirm a decrease in seminal quality among aquarium-housed *S. canicula*, consistent with previous research conducted on other marine species. In addition, our findings highlight alterations in both the blood and seminal plasma metabolomic profiles between aquarium-housed and wild animals, demonstrating the potential impact of aquarium environmental conditions on the reproductive success of captive sharks. Moreover, the identification of biomarkers in seminal plasma could serve as indicators of habitat suitability, providing insights into the environmental conditions affecting shark reproduction.

CRediT authorship contribution statement

Marta Muñoz-Baquero: Writing – review & editing, Supervision, Investigation. **Gianfranco Diretto:** Writing – review & editing, Software. **Sarah Frusciante:** Writing – review & editing, Software, Conceptualization. **Daniel García-Párraga:** Writing – review & editing, Conceptualization. **Clara Marin:** Writing – review & editing, Conceptualization. **Francisco A. García-Vázquez:** Writing – review & editing, Conceptualization. **Francisco Marco-Jiménez:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Lorenzo-Rebenaque:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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