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From hormones to habitat: A new framework for assessing maturity in the Central-Eastern Mediterranean blue shark (*Prionace glauca*) population

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ABSTRACT

The blue shark (*Prionace glauca*) is a widely distributed pelagic species, yet its Mediterranean population remains understudied despite being classified as critically endangered in the region. This study provides new insights into the reproductive features, physiological status, and spatial distribution of blue sharks at different stages of maturity in the Central-Eastern Mediterranean, specifically in the Southern Adriatic Sea. First, we evaluated the reproductive anatomy of 77 blue sharks and demonstrated significant changes across maturity stages, highlighting the importance of considering such anatomical shifts to improve maturity assessments. We then examined the potential of combining morphological, hormonal and lipid-based indicators as a non-lethal method for maturity classification. Principal component analysis revealed distinct morphological/physiological profiles between maturity stages in both sexes, and a supervised machine learning model (random forest classifier) achieved high classification accuracy (100 % for females and 75 % for males), confirming the predictive strength of these indicators. Converting the model outputs into a continuous maturity index enabled us to spatialize maturity patterns for males using geographic coordinates and environmental covariates in a generalised additive model. The results revealed a clear ontogenetic shift: mature males were predominantly located offshore, while immature individuals were found closer to the shore. This integrative approach supports the use of blood-based physiological profiling combined with morphological traits as a promising non-lethal method for assessing maturity and identifying potential geographic structuring between maturity stages in the Adriatic Sea. By linking physiology, maturity and environment, this study establishes a framework for future research and monitoring of vulnerable elasmobranch populations.

1. Introduction

Effective management and conservation of marine biodiversity depend on a solid understanding of species' biological and ecological parameters. However, for many threatened species, this knowledge has traditionally been obtained through invasive or lethal sampling, which carries considerable biological and ethical costs. This issue is particularly pertinent for species of conservation concern, as collecting reproductive or demographic information often necessitates the sacrifice of rare individuals.

Cartilaginous fish (Chondrichthyes), including sharks, rays and chimaeras, are among the oldest vertebrate lineages and play essential ecological roles as predators and mesopredators. However, they are also among the most threatened, with an estimated one-quarter of species at risk of extinction, while only around one-third are considered "least concern" (Dulvy et al., 2014, 2021). The situation is especially severe in the Mediterranean Sea, where more than half of shark and ray species are classified as threatened, primarily due to intense fishing pressure and habitat degradation (Ferretti et al., 2008). A key knowledge gap concerns the assessment of sexual maturity. Current methods of assessing maturity stages involve examining the claspers of males, but typically require lethal methods for females, such as macroscopic examination of the ovaries and uterus. As these approaches are invasive or lethal, they are problematic when working with protected species or monitoring bycatch in fisheries (Awruch et al., 2008; Mourier et al., 2024; Sulikowski et al., 2005). Therefore, developing non-lethal

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methods is a priority for improving biological knowledge while minimising harm to vulnerable populations.

Recent research has explored several non-lethal approaches. In recent decades, the number of studies on the reproductive endocrinology of chondrichthyans has increased (Gelsleichter et al., 2012). Endocrinological profiling, particularly the measurement of plasma steroid hormones such as testosterone, progesterone, 17β-estradiol, and 11-ketotestosterone, has shown strong potential for maturity classification in sharks (Awruch et al., 2008, 2014; Mourier et al., 2024; Sulikowski et al., 2005). Conversely, despite their importance for reproductive performance, energy metabolism indicators are rarely evaluated. However, these markers could help to distinguish between stages, as demonstrated in other species (e.g. (Rangel et al., 2021a; Hammerschlag et al., 2018a; Rangel et al., 2021b). Indeed, shark species are thought to be primarily capital breeders, involving the storage of food reserves to fuel the energetic requirements of reproduction (Hammerschlag et al., 2018a). Variations in lipids, for example, could indicate different conditions in individuals, but could also reflect energy requirements during reproduction (Valls et al., 2016). The most commonly studied lipid classes are triglycerides, non-esterified fatty acids and cholesterol (Gallagher et al., 2017). Taking into account both sexual and energetic parameters could enable a more accurate assessment of reproductive maturity.

In this context, the blue shark (*Prionace glauca*, Linnaeus, 1758) is a key species of concern. This species is caught in substantial quantities worldwide, primarily as bycatch in gill nets and longlines (Nakano and Stevens, 2008). The International Union for Conservation of Nature (IUCN) Red List considers the species to be nearly threatened globally, and the population in the Mediterranean Sea is specifically classified as critically endangered. This oceanic, epipelagic species has a very wide geographic distribution, spanning temperate and tropical waters (Nakano and Stevens, 2008). It is highly migratory with complex movement patterns and its distribution is notably influenced by food availability, reproductive cycle, and seasonal environmental variations (Nakano and Stevens, 2008; da Silva et al., 2021; Poisson et al., 2024). The reproductive mode of blue shark is placental viviparity, with a gestation period typically lasting nine to twelve months (Nakano and Stevens, 2008). Reproduction appears to be seasonal, with births typically occurring in spring and summer in the Atlantic, although there is significant geographic variability. Migrations, including those for reproductive purposes, have been studied in the Atlantic and Pacific Oceans (Nakano and Stevens, 2008; Renshaw et al., 2023; Mas et al., 2023), but little information is available for the Mediterranean Sea. However, a better understanding of the reproductive biology of blue shark populations is essential for establishing conservation management plans, including information on sizes corresponding to the different maturity classes, reproductive features, and temporal and spatial reproductive patterns (Awruch et al., 2008). The spatial distribution of maturity could also reflect environmental variations, information that are critical for identifying essential habitats, such as mating or pupping grounds. It should be noted that the movement patterns of blue sharks have recently been described in the Western Mediterranean Sea, along with the identification of mating, parturition and nursery grounds (Poisson et al., 2024; Mancusi et al., 2023). However, the absence of connectivity with the other areas of Mediterranean basin (Poisson et al., 2024), along with significant genetic variation between Eastern and Western Mediterranean blue shark populations (Leone et al., 2024), suggest that further research on blue sharks in the Central-Eastern Mediterranean is required, as area-specific characteristics may arise. From a fisheries perspective, the blue shark is the most commonly caught species by Mediterranean surface longline, representing over 70 % of the chondrichthyan catch, followed by the shortfin mako shark (*Isurus oxyrinchus*) (Bradai et al., 2012; Carbonara et al., 2023). Bycatch rates are particularly high in the Alboran and Adriatic Seas, making these regions focal points for ecological and management concerns (Carbonara et al., 2023; FAO, 2016; Megalofonou et al., 2009; Megalofonou et al., 2005; Carbonara et al., 2024).

In this context, this study had four objectives: (i) to collect information on the reproductive features of individuals from the Southern Adriatic Sea; (ii) to characterise variations in sex steroids and lipid-based physiological indicators by sex and across maturity stages; (iii) to evaluate the predictive potential of these indicators as a non-lethal method for maturity classification; and (iv) to develop a methodology that can be used to spatially map maturity stage in order to evaluate spatial aggregations, as well as to evaluate potential correlations with relevant environmental variables. By integrating morphology, endocrinology, and energy metabolism, this study contributes to the development of non-invasive maturity assessment tools for sharks, while generating much-needed knowledge on a critically endangered Mediterranean population. Together, this work may help support more informed and spatially explicit future conservation and management strategies for this vulnerable population.

2. Material and methods

The animals used in this study were obtained as incidental bycatch from authorised commercial fisheries. The sampling design and handling methods were reviewed and approved by the Committee on the Ethics of Animal Experiments of COISPA (Italian Ministry of Health 17/2022-UT).

2.1. Sample and data collection

As part of the sampling for the SafeShark and MedBycatch project, a total of 77 blue sharks found already dead at the hauling (47 males and 30 females caught as by-catch) were collected from the Southern Adriatic Sea and taken to our laboratory. Various morphological and reproductive parameters were recorded. Total length (cm), weight (g) and gonadal weight (g) were taken for all individuals. The developmental stage was assessed using the MEDITS protocol (Anonymous, 2017). For the males, we measured different reproductive indicators: total gonad weight (g), average (left and right side) testis weight (g), average (left and right side) epididymis weight (g), epigonal organ weight (g) and average (left and right side) clasper length (mm). For the females, we measured the total gonad weight (g), the average (left and right side) oviductal gland weight (g), the epigonal organ weight (g), the uterine weight (g) and the average oocyte diameter (mm).

In addition, between 2020 and 2023, morphological data and blood samples were collected from 58 other blue sharks as part of the regular monitoring of commercial pelagic longline fisheries in the Adriatic Sea (Carbonara et al., 2024) (Table 1), as well as the SafeShark and MedByCatch tagging projects. These sharks were either caught alive, sampled, and then released, or caught dead. They were all different individuals to the dead sharks used for measuring morphological reproductive parameters.

The fishing operations (pelagic longline targeting swordfish and blue fin tuna) started early in the afternoon (3–4 pm), and the 30-40 km-long longline was placed and was in position in about 3 h. The longline haul back was completed around 11 a.m.-12 p.m. The hooks were left at sea for a total of 10–20 h (i.e. the interval between the final hook being dropped at sea and the first hook being recovered). Following the methodology of Poisson et al. (Poisson et al., 2012), the sharks were blinded with a wet towel once on board, which has a mild sedative effect (Bruce and Bradford, 2012). A tube was then placed in their mouths and sea water was poured in to maintain proper gill oxygenation. Total length (cm) was measured for all individuals and 2.5 ml of blood were drawn by caudal venipuncture using heparinised syringes as soon as the animal was placed on the ship's deck. The maturity stage of each individual was assessed in accordance with the MEDITS maturity scale (Anonymous, 2017). For males, examination of the clasper and the presence or absence of sperm was sufficient to classify all individuals. For females, maturity stage was determined based on size and direct

Table 1
Characteristics of the blue sharks used in this study.

Capture period	Number of sharks	Sex	Maturity class	Total length range (cm)
August 2020	9	4 males 5 females	Immature: 1 Maturing: 4 Mature: 3 Regenerating: 1	139.5–174.7
September 2020	16	9 females 7 males	Immature: 6 Maturing: 5 Mature: 3 Regenerating: 2	83.5–195.5
October 2020	11	3 females 8 males	Immature: 2 Mature: 8 Regenerating: 1	132–219
August 2021	14	6 females 8 males	Immature: 4 Maturing: 4 Mature: 6	138–223.7
November 2021	1	Female	Mature	202.3
August 2022	4	4 females	Immature: 2 Mature: 1 Regenerating: 1	126.6–181.2
September 2022	1	Female	Immature	108
August 2023	2	1 female 1 male	Immature Mature	126.6 180

observation of ovaries for dead individuals. The geographic coordinates of each catch were recorded.

2.2. Sample processing

After plasma extraction, six parameters were measured. Testosterone and 17β-estradiol (E2) were measured using BT LAB commercial ELISA kits (EA0000FI and E0021FI, respectively). Progesterone was measured using a FineTest ELISA kit (EU0398). Cholesterol and triglycerides were measured using commercially available diagnostic kits (Colorimetric enzymatic CHOD-PAP and GPO-PAP, respectively) on the Cobas Pure e303 analyser (Roche Diagnostics). The concentration of non-esterified fatty acids (NEFA) in shark plasma was measured using an enzymatic colorimetric method (Randox Laboratories Ltd; acyl-CoA synthetase, acyl-CoA oxidase, TOOS) on the Cobas Pure e303 analyser (Roche Diagnostics). All ELISAs were performed following the manufacturer's instructions. Samples and standards were measured in duplicate and intra- and inter-assay coefficients of variation were below 20 %. Each plate included blank wells and a standard curve. A subset of samples was measured across different plates to monitor assay performance and minimize batch effects.

2.3. Reproductive features analyses

To investigate the relationship between body size/weight and reproductive features across maturity stages, biometric data were plotted in R software (version 4.3.1 (R Core Team and Team RC, 2023);) for 77 blue sharks. Scatter plots were generated using the ggplot2 library to visualise the relationship between total weight or length and each reproductive metric. Linear regressions or second-order polynomial fitting were applied to illustrate trends. The adjusted coefficient of determination (R^2) for each regression was displayed on the plots using the ggpmisc package.

2.4. Sex and maturity variations in reproductive hormones and lipid indicators

Some values outside the ELISA calibration curve were estimated using modelling ($n = 11$ for progesterone, $n = 2$ for testosterone and $n = 2$ for E2). To verify that the general findings remained unchanged, all analyses that are presented in the following sections were performed using both the dataset containing only individuals without missing values and the dataset containing imputed values. Diagnostics values resulting from the analyses of the two datasets were compared (more details in Section 2.5).

Boxplots of the different sex steroids and lipid parameters were created to illustrate the variations observed between sexes and maturity stages. Univariate statistics were performed using Kruskal–Wallis tests to assess statistical differences in physiological variables between maturity stages within each sex. As all hormones potentially act together, multivariate statistics were performed using principal component analysis (PCA (Jolliffe, 2002);) to obtain a comprehensive global perspective. After scaling the data, PCA was performed for each sex using the FactoMineR library. The nFactors package was used to identify the number of principal components to retain for analysis. The PCA results were visualised using the factoextra library and the variables contributing most to each PCA component, and particularly to differentiation between maturity stages, were identified using the FactoMineR library. Correlations were assessed between variables and dimensions, with a significance threshold of $P < 0.05$. In addition to total length, all physiological variables were included in the PCA. To highlight the distribution of individuals within each maturity class, 95 % confidence ellipses were added around each maturity group using the factoextra package. The ellipse size corresponds to the 95 % confidence region around the group centroid in the PCA space. To test for global differentiation among maturity classes, we performed a multivariate analysis of variance (PERMANOVA; vegan R package) based on permutations, using the same variables as those used for the PCA.

2.5. Prediction of maturity stage based on sex steroids and energetic indicators

Our aim here was to evaluate whether maturity could be predicted based on sex steroids and lipid indicators. Initial exploratory strategies considered classical dimensionality reduction techniques such as PCA, but these methods were ultimately unsuitable for our purposes. While these techniques can summarise multivariate variation effectively, the reduced-dimension axis typically requires the retention of multiple dimensions and therefore often lacks biological interpretability. To address this issue, we adopted a supervised learning strategy involving a random forest (RF) classifier (Breiman, 2001), which was used to model the relationship between physiological features and discrete maturity stages that had been assigned in advance. RFs are a type of ensemble machine learning method that operate by building a large collection of decision trees during the training phase. Each decision tree asks a series of yes/no questions about the input data and uses the answers to split the data into groups that correspond to different maturity stages, based on class probabilities. Random forests are particularly powerful because they introduce randomness in two ways: firstly, each tree is trained on a bootstrapped sample of the data; secondly, at each decision point, only a random sample of the available features is considered (Fawagreh et al., 2014). This randomness ensures the forest is more robust and less likely to overfit.

In this study, subsequent to feature collinearity check (Supplementary Material 1), we used the following indicators to predict sexual maturity stage: testosterone, E2, progesterone, NEFA, triglycerides and cholesterol. Following initial testing, total length was also added as a predictor to improve the classification. Albeit the effective sample size for mature females (three mature females, five regenerating females) and immature males (two immature males) is small, Random Forests can still be trained in such cases because each tree is fitted on bootstrap subsamples and does not require a minimum number of examples per class. However, such small groups limit generalisation and increase variance in model estimates. To mitigate this, we simplified the classification into two categories: "immature" (comprising the "immature" and "maturing" classes) and "mature" (comprising the "mature" and "regenerating" classes). To fit the data, we trained an RF classifier using a 5-fold cross-validation scheme, specifying 200 trees and allowing each tree to use 70 % of the features each time. To account for class imbalance, we applied class weights that were inversely proportional to class frequencies, ensuring that the model gave appropriate importance to minority classes. We evaluated model performance using the mean across folds for accuracy, ROC-AUC (discrimination between maturity classes), PR-AUC (balance between precision and recall under class imbalance) and the Brier score (calibration of predicted probabilities). Additionally, we estimated uncertainty in model accuracy through 1000 bootstrap resamples. All analyses were conducted in Python (Van Rossum and Drake, 1995) using the machine learning library scikit-learn.

2.6. Development of a methodology to spatialize maturity index taking into account environmental features

Building on the RF classifier described above, we transformed the output class probabilities into a continuous maturity index. Instead of using the model for discrete classification, we computed a weighted average of the predicted class probabilities to produce a smooth, biologically meaningful index that is bounded between 0 and 1. The aim of this section is exploratory in nature: we are not aiming to validate the model's predictions, but rather to develop a methodological framework for spatialising maturity patterns from sex steroid, lipid and total length data. Due to the small sample size and the exploratory nature of the analysis, we retrained the RF classifier using the entire dataset and then made predictions using the same dataset. This approach generated a comprehensive maturity index without introducing classification uncertainty. This approach minimises the risk of mislabelling individuals, which is critical given the scarcity of data, and ensures that the resulting spatial model is based on the best available estimate of each shark's maturity status.

The next step consisted of modelling the spatial and environmental patterns of maturity using geographical and oceanographic covariates. Surface temperature, salinity and depth data were extracted from the Copernicus Marine Service Information for the Mediterranean Sea, specifically from the Mediterranean Sea Physics Reanalysis (Nigam et al., 2021) and the Mediterranean Sea Physics Analysis and Forecast datasets (Copernicus, 2025). Chlorophyll and dissolved oxygen were extracted from the Mediterranean Sea Biogeochemistry Reanalysis (Cossarini et al., 2021) and Analysis and Forecast (Salon et al., 2019) datasets. For all variables, monthly values corresponding to the dates of the blue shark catches were extracted. All analyses were performed using the R software. Collinearity between all parameters was checked prior to modelling. As our response variable is continuous and constrained within the interval [0, 1], we employed a generalised additive model (GAM) with a beta distribution family (betar in the mgcv package), as beta regression is the natural choice for modelling bounded continuous data (Geissinger et al., 2022). This approach is particularly appropriate for proportion-like responses, offering robustness to heteroskedasticity and greater flexibility than linear models. Smoothing parameters were estimated by restricted (residual) maximum likelihood, which provides more reliable smoothing parameter estimates and avoids undersmoothing. Additional penalties were imposed on each smooth that targets the null space of the original penalty to shrink non-informative smooth terms to zero, by means of the select method as TRUE and gamma parameter equal to 1.4 (Wood, 2020). Convergence, concurvity and diagnostic plots (including Q-Q plots, histograms of residuals, residuals versus fitted values and response versus fitted values) were examined for all models to assess model performance. Additionally, the predictive accuracy of the GAMs was assessed by visually comparing their predicted values to the observed values in the dataset.

For males, depth was excluded due to its high collinearity with sea surface temperature. Attempts to include both salinity and chlorophyll as smooth terms resulted in extreme concurrency (0.99 for both), which made their joint inclusion infeasible. Consequently, only salinity and temperature were retained as the environmental covariates in the final model. Concurvity check revealed that the included smooth terms exhibited low levels of concurvity, with worst-case values below 0.4 (Supplementary Material 2). This suggests that the model terms were not overly redundant.

Attempts to model spatial or environmental gradients in females resulted in high concurvity for any smoothed term included, unless only one covariate was used. Linear covariates were also highly concurved with smooth terms, which made interpreting GAMs for females unreliable (Supplementary Material 3). For these reasons, we only modelled the male maturity index. The final best model used for males was as follows:

$$\begin{aligned}&\textit{Maturity index} \sim ti(\textit{temperature}, \textit{salinity}, k = 3)\\&\quad + ti(\textit{easting}, \textit{northing}, k = 3) + 0\end{aligned}$$

3. Results

3.1. Reproductive indicators

For females, the strongest relationship was found between the average uterine weight and total weight/length (Fig. 1). A strong relationship was also found between average weight of the oviductal glands and total weight/length (Fig. 1). Larger/longer individuals exhibited greater variability, corresponding to females at the regenerating stage, for the average oocyte diameter and gonad weight relative to total length and total weight (Fig. 1). Conversely, epigonal organ weight was more variable in maturing females than in regenerating females (Fig. 1). Immature females ranged from 116 to 136.5 cm (5300–9200 g), maturing females from 132 to 168.5 cm (6960–17900 g) and regenerating females from 166 cm to 219 cm (13710–38500 g).

Strong relationships were found between gonad weight and average testis weight relative to total weight/length in males (Fig. 2). A strong relationship was also observed between average clasper length and total length/weight (Fig. 2). Larger, more mature males exhibited greater variability in the average weights of their epididymis and epigonal organ relative to their total weight/length (Fig. 2). Immature males ranged from 83.5 to 123.6 cm (1396–6580 g), maturing males from 122 to 155.4 cm (6140–11840 g) and mature males from 141 cm to 223.7 cm (8020–43120 g).

3.2. Maturity variations in sex steroids and lipid indicators by sex

The patterns were not very clear when analysed using univariate statistics, and no difference between the maturity stages could be seen (Supplementary Material 4). However, when a PCA that considers all factors together was used, a distinction between maturity stages could be seen both for females (Fig. 3A) and males (Fig. 3B).

Regarding females, the first two principal components were retained, accounting for 48.6 % of the total variance in the dataset (the first component explaining 29.09 %, and the second component 19.51 % of the total variance; Fig. 3A; Supplementary Material 5A). The contribution of the variables differed across the two components (Fig. 3A–Supplementary Material 5B. The first component was significantly associated with cholesterol ($r = 0.74$, $P < 0.001$), progesterone ($r = -0.58$, $P < 0.001$), E2 ($r = -0.56$, $P < 0.01$), triglycerides ($r = 0.56$, $P < 0.01$), testosterone ($r = -0.54$, $P < 0.01$), and NEFA ($r = 0.48$, $P < 0.01$). The second principal component was primarily associated with total length ($r = 0.82$, $P < 0.001$), testosterone ($r = 0.61$, $P < 0.001$), and

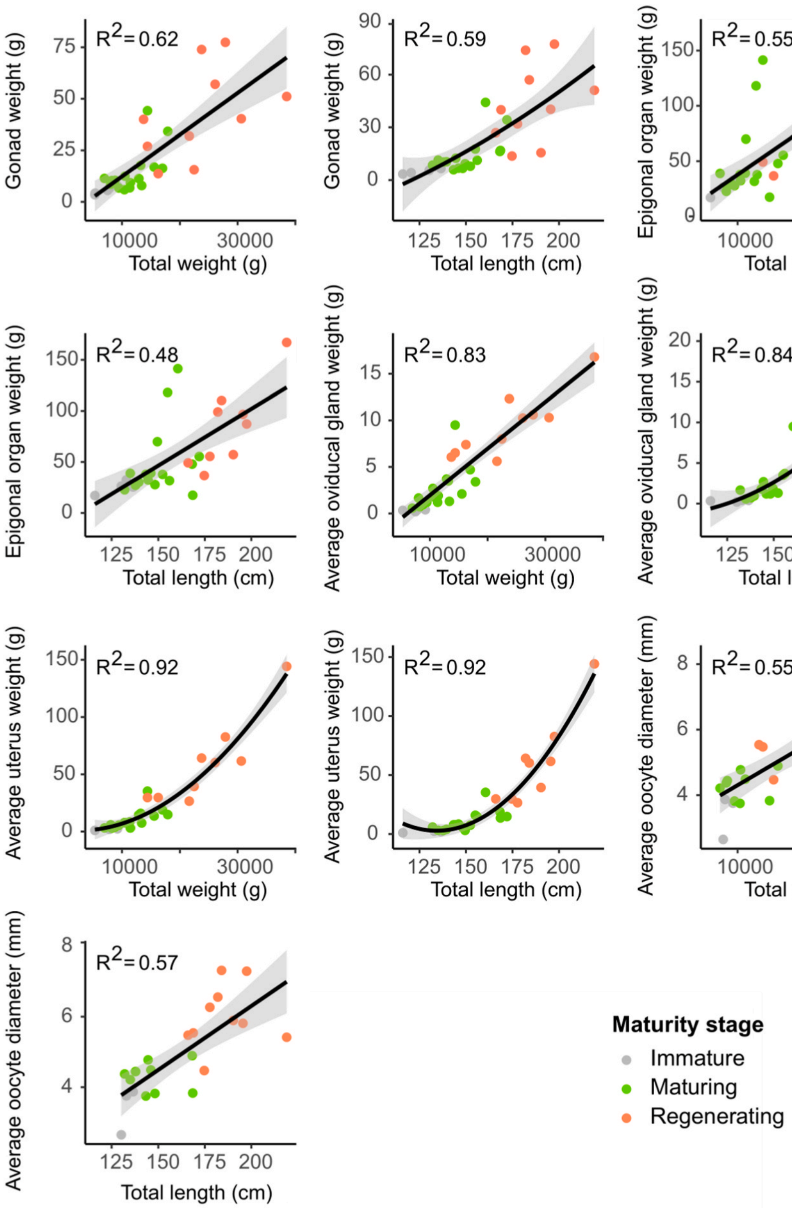

Fig. 1. Scatter plots showing the relationship between various reproductive features (gonad weight (g), epigonal organ weight (g), average oviductal gland weight (g), average uterine weight (g) and average oocyte diameter (mm)) and either total weight (g) or total length (cm) in blue shark females.

E2 ($r = -0.49$, $P < 0.01$). On a global scale, maturing and regenerating females tended to present higher cholesterol, triglycerides and NEFA and lower E2, progesterone and testosterone than immature and mature females (Fig. 3A). Immature females also tended to display higher E2 and lower testosterone and total length than mature females (Fig. 3A). Maturing individuals also tended to display higher E2 and lower testosterone and total length than regenerating females (Fig. 3A). To statistically support group differences, we applied PERMANOVA on the same variables used in the PCA, confirming significant separation among maturity classes ($R^2 = 0.262$, $F = 3.08$, $P < 0.05$).Regarding males, the first two principal components were retained, accounting for 47.6 % of the total variance in the dataset (the first component explaining 25.68 %, and the second component 21.86 % of the total variance; Fig. 3B; Supplementary Material 5C). The contribution of the variables also varied across the two components (Supplementary Material 5D). The first component was significantly associated with triglycerides ($r = 0.86$, $P < 0.001$), cholesterol ($r = 0.65$, $P < 0.001$), E2 ($r = -0.48$, $P < 0.01$), NEFA ($r = 0.46$, $P < 0.05$) and progesterone ($r = -0.45$, $P < 0.05$). The second principal component was primarily associated with testosterone ($r = 0.63$, $P < 0.001$), progesterone ($r = -0.59$, $P < 0.001$), total length ($r = 0.56$, $P < 0.01$) and NEFA ($r = 0.50$, $P < 0.01$). On a global scale, mature males tended to present higher testosterone, total length and NEFA and lower progesterone than immature and maturing males (Fig. 3B). Immature males also tended to display higher E2 and progesterone and lower triglycerides, cholesterol and NEFA than most maturing males (Fig. 3B). The PERMANOVA test was, however, not supporting differences across maturity classes ($R^2 = 0.10$ $F = 1.33$, $P = 0.26$).

3.3. Maturity predictions from total length, sex steroids and energetic indicators

The random forest classifier generated class probabilities for each maturity stage (mature and immature) and achieved high average cross-validated accuracy and diagnostics scores both in males and in females (Table 2). Given the small sample size and class imbalance, the apparent

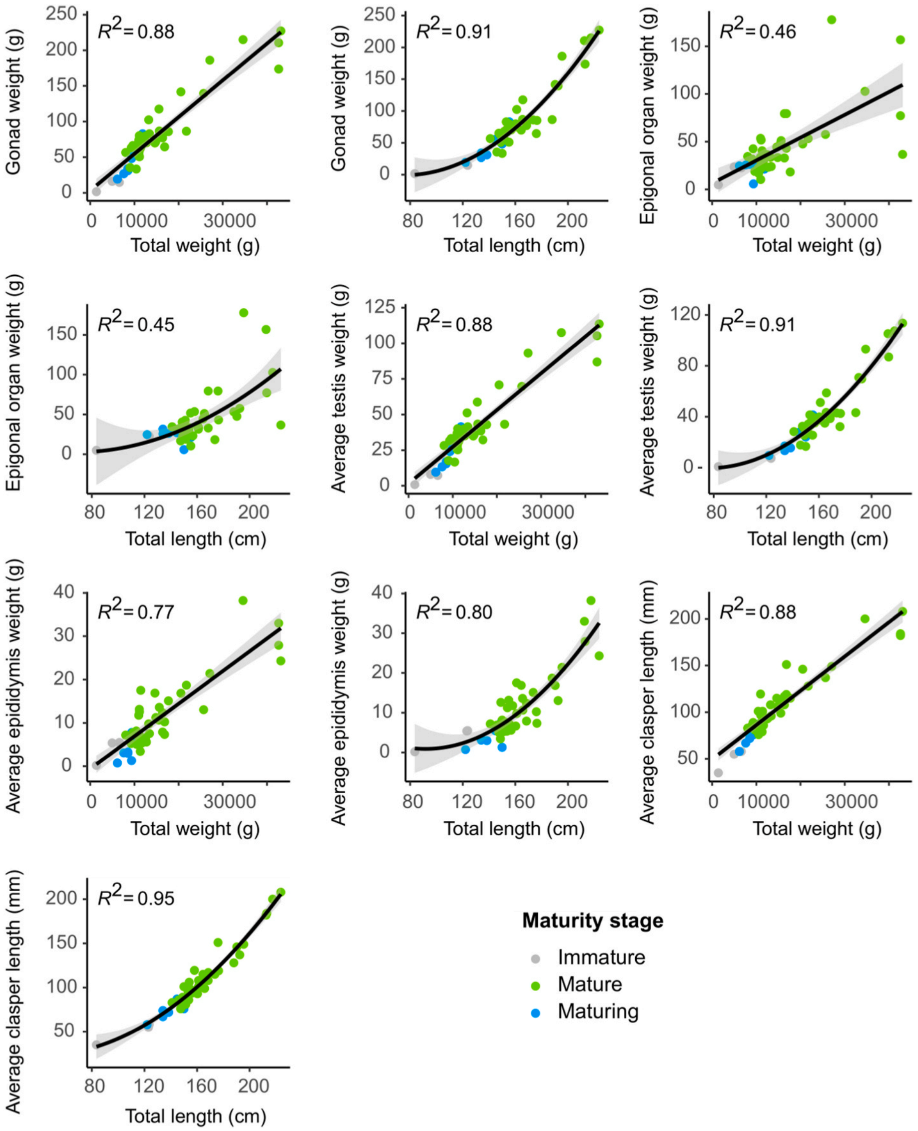

Fig. 2. Scatter plots showing the relationship between reproductive features (gonad weight (g), epigonal organ weight (g), average testis weight (g), average epididymis weight (g) and average clasper length (mm)) and total weight (g) or total length (cm) in blue shark males.

perfect accuracy for females required further investigation. Hence, to quantify uncertainty and assess robustness we calculated bootstrap confidence intervals with 1000 resamples, evaluating model performance on out-of-bag samples at each iteration. This yielded a 95 % CI of [0.86, 1.00] (Supplementary Material 6), highlighting the highly separability of the datasets likely due to the small effective sample size. Learning curve analysis (Supplementary Material 7) showed that the model consistently achieved perfect training scores, whereas test accuracy improved gradually with increasing sample size, indicating some overfitting and sensitivity to small sample fluctuations. Threshold sensitivity analysis (Supplementary Material 8) further illustrated the trade-off between precision and recall at different decision thresholds. Overall, the near-perfect discrimination observed in females likely reflects high within-sample separability rather than genuine generalisability. The limited sample size and class imbalance constrain the model's ability to capture broader biological variability.

To provide a direct view of prediction outcomes, aggregated confusion matrices across the five folds are reported in Supplementary Material 9, summarizing the number of correct and incorrect classifications for each sex. Feature importance was assessed via bootstrapped SHAP analysis (n = 100) and it indicated that total length was the most influential predictor for both sexes. For females, total length had a mean absolute SHAP value of 0.591 ± 0.143, followed by progesterone (0.050 ± 0.031) and testosterone (0.035 ± 0.027). For males, total length remained dominant (0.406 ± 0.135), with testosterone (0.091 ± 0.064) and E2 (0.066 ± 0.047) as the next most important features (Supplementary Material 10). Sensitivity analyses are available in Supplementary Material 11 and show almost no impact from the inputation of hormone values on results.

3.4. Maturity index spatial patterns

Spatial modelling of the continuous maturity index for males revealed clear geographical and environmental structuring. The final GAM explained 63.8 % of the deviance and had an adjusted R^2 of 0.27, indicating its moderate ability to explain spatial and environmental patterns in male maturity. Higher maturity probabilities were predominantly observed in offshore areas farther from the Italian, Croatian and Montenegrin coastlines (Fig. 4). Coastal zones generally exhibited lower maturity index values, indicating a greater probability of the presence of immature individuals closer to shore (Fig. 4). Deeper waters were also consistently associated with higher maturity indices, highlighting a

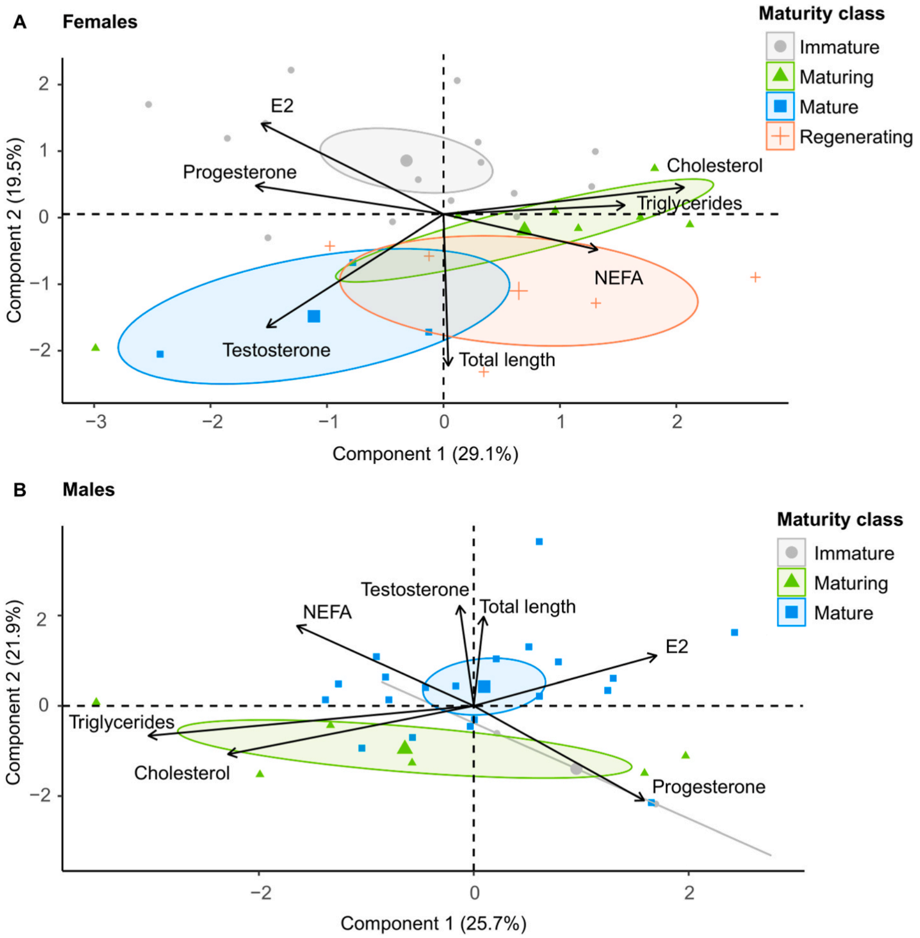

Fig. 3. Biplots showing the contribution of sex steroids, lipid variables and total length to components 1 and 2 of the principal component analysis, as well as the positioning of the individual sharks as a function of maturity class (immature in grey, maturing in green, mature in blue, and regenerative in orange) for (A) females and (B) males. Confidence ellipses with a confidence level of 0.95 were drawn around maturity classes.

Table 2
Mean score values across folds. ROC-AUC measures the classifier's ability to distinguish between mature and immature individuals across all possible thresholds, with values closer to 1 indicating better separation. PR-AUC emphasizes precision (the proportion of predicted positives that are correct) versus recall (the proportion of actual positives correctly identified), which is especially informative for imbalanced datasets. Brier score quantifies the accuracy of predicted probabilities, with lower values indicating that predicted probabilities closely match observed outcomes.

Sex	Accuracy	ROC-AUC	PR-AUC	Brier
Males	0.820 ± 0.107	0.850 ± 0.200	0.961 ± 0.049	0.143 ± 0.047
Females	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	0.020 ± 0.008

tendency for mature males to occupy offshore habitats (Fig. 4). The interaction between temperature and salinity was not significant ($\chi^2 = 1.54$, $p = 0.08$), but close to the significance threshold. It could suggest a trend for mature males to be more likely to be found in areas characterised by higher salinity and temperatures between 22 and 26 °C (Supplementary Material 12). The spatial tensor ti (easting, northing) was highly significant ($\chi^2 = 24.58$, $p < 0.001$), confirming the presence of strong spatial structure in maturity distribution.

4. Discussion

This study sheds valuable light on the reproductive biology and spatial ecology of the blue shark (*Prionace glauca*) population in the Central-Eastern Mediterranean Sea. Using a combination of sex steroid and lipid profiling, supervised machine learning, and spatial modelling, we presented new evidence that informs non-lethal assessments of sexual maturity and identifies maturity-associated physiological signatures. We also explored how the environment structures maturity patterns in this critically endangered population. Below, we relate these findings to known results in the literature and we explore the implications for blue shark ecology and conservation.

Our results provide novel insights into the maturity characteristics of blue sharks in the Adriatic Sea, with relevant implications for the assessment of the regional population, including an evaluation of the reproductive organs and variations in steroids and lipids, which can be used as a baseline for future studies on maturity. First, mature females were observed at smaller sizes than those previously reported in the Central Mediterranean (Megalofonou et al., 2009). This finding suggests potential geographic variation in growth or maturation dynamics and highlights the importance of including more regionally specific biological parameters in models advising for management. Such differences may reflect ecological or environmental factors specific to the Adriatic Sea, such as prey availability, habitat use or oceanographic conditions. Moreover, one particularly informative aspect of our findings is the relationship between the reproductive organ development and body size (length and weight). In males, gonad weight, average testis weight and clasper length all showed strong positive correlations with total body length and weight. This indicates that these parameters are reliable anatomical indicators of reproductive maturity, consistent with findings from other blue shark populations and related species (e.g. (Mas et al.,

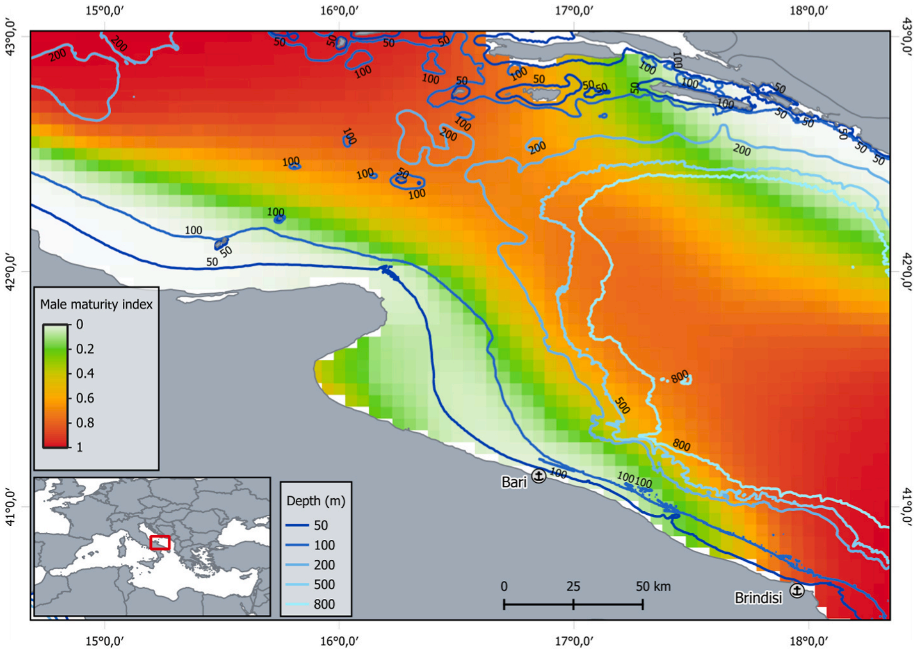

Fig. 4. Spatial GAM prediction mapping of the maturity index of blue shark males in the Southern Adriatic Sea.

2023; Segura et al., 2013; Montealegre-Quijano et al., 2014; Viducic et al., 2022)). The pronounced increase in clasper length, particularly following a polynomial pattern, is consistent with the morphological changes known to be associated with sexual maturity in elasmobranchs (see references above). Similar patterns were observed in females, where oviductal gland and uterine weight exhibited strong linear or polynomial relationships with body size (weight and length). It is notable that average oocyte diameter and gonad weight increased substantially in larger individuals, particularly among regenerating females, highlighting these features as useful proxies for maturity stage. These relationships also enable a clear differentiation among maturity stages, emphasizing the importance of gaining more knowledge on reproductive trait variation throughout sexual maturation, although the relatively small and uneven sample sizes, particularly regarding mature and regenerating females, restricted the statistical power and generalisability of some findings. Overall, these anatomical changes provide a morphological basis that strengthen maturity assessments, but they can only be conducted on dead individuals, while the hormonal/lipid assessment requires non-lethal blood sampling, representing a more practical solution for studying live sharks caught as bycatch.

In line with previous studies conducted on other species (e.g. (Mourier et al., 2024; Awruch et al., 2014; Rangel et al., 2021a)), the hormone profiles observed in our samples exhibited significant variations in accordance with reproductive stage. Although the analysis of steroid hormones in chondrichthyans has increased significantly over the last decade (Becerril-García et al., 2020), studies focusing specifically on blue sharks remain scarce. To our knowledge, only one study investigated the hormone levels in blue sharks from the Pacific area and was limited to females (Fujinami and Semba, 2020). Various hormones have been studied in different species, and the three steroids examined in this study, which are relevant to both male and female reproductive physiology, are among the most commonly used markers in shark reproduction research (Becerril-García et al., 2020). The range of variation observed for the different hormones in this study is consistent with that reported by Fujinami and Semba (2020). Testosterone appears to be involved in spermatogenesis and sperm motility and transportation, as well as the growth of reproductive features (e.g. testes) (Rangel et al., 2021a; Becerril-García et al., 2020), although its role in gonad development remains relatively unclear (Awruch, 2013). This is consistent with the trend for mature males to have higher testosterone levels. Higher testosterone levels were also observed in mature females, reflecting the role of this androgen in follicle development and its involvement in reproductive behaviour, particularly during the mating season (Becerril-García et al., 2020). Similar to what was observed in other species (Awruch et al., 2008), both mature and immature females exhibited higher progesterone levels. Progesterone acts as an antagonist of E2 in females, reducing liver vitellogenin synthesis. The higher progesterone levels observed in mature females, as opposed to those in maturing or regenerating females, are consistent with the hypothesis that ovulation and mating occur in summer, followed by an increase in progesterone as the pregnancy progresses (Fujinami and Semba, 2020; Koob and Callard, 1999). According to Fujinami and Semba (2020), both E2 and progesterone levels are high during ovulation and mating. Then, throughout autumn, progesterone levels remain high while E2 levels decrease, before the opposite pattern emerges at parturition (Fujinami and Semba, 2020). Sampling females in summer might therefore mask the more drastic differences that could be observed in blue shark females later in the year during pregnancy. High levels in immature sharks may indicate that females are preparing for their first mating (Hammerschlag et al., 2018b), or they may reflect progesterone's involvement in other biological functions. In males, the tendency to observe lower progesterone levels and higher E2 levels in mature individuals is consistent with the known or suspected roles of these hormones in male sharks. Indeed, E2 is linked to spermatogenesis, while progesterone is thought to play a role in testosterone synthesis and sexual maturation in male sharks (Becerril-García et al., 2020). Given the variability of hormonal levels even among individuals at the same stage of maturation (Mourier et al., 2024; Fujinami and Semba, 2020), it appears crucial to analyse a sufficient number of samples. In addition, considering other androgens (e.g. 5α-dihydrotestosterone), oestrogens (e.g. estriol) and relevant glucocorticoids (e.g. 1α-hydroxycorticosterone) would improve our understanding of the reproductive cycle (Becerril-García et al., 2020) and enable more accurate distinction between different maturity stages. Furthermore, we report lipid variations, which provide valuable complementary insights to steroid patterns. For example, the lower cholesterol and triglyceride levels observed in mature males compared to maturing males may reflect the energetic costs associated with reproduction (e.g. spermatogenesis, courtship or migration), as proposed in earlier studies on other elasmobranchs (Rangel et al., 2021a; Moorhead et al., 2021). Similarly, it has been demonstrated that female nurse sharks (*Ginglymostoma cirratum*) accumulate energy reserves in preparation for vitellogenesis, which, once initiated, mobilises lipids and depletes available reserves (Moorhead et al., 2021). The results of this study could indicate the capital breeding strategy of blue sharks, whereby they secure and store food resources prior to reproduction and then mobilise them to fuel offspring production (Hammerschlag et al., 2018b). Conversely, the trend of lower cholesterol and triglycerides in immature individuals could reflect the mobilisation of energy reserves for purposes other than reproduction, such as growth. It could also indicate lower foraging efficiency compared to experienced adults, as has been observed in other species (Moorhead et al., 2021). Future research should aim to increase the temporal resolution of hormone and lipid sampling. Seasonal or monthly sampling regimes would allow researchers to track dynamic endocrine profiles throughout the reproductive cycle, potentially enabling more precise and timely maturity classifications. Furthermore, integrating fatty acid analyses could help to distinguish the effects of reproductive investment from dietary variation, thereby clarifying the biological significance of lipid marker fluctuations.

One of the most significant outcomes of this study is the validation of hormonal and lipid indicators, in combination with total length, as effective non-lethal predictors of reproductive maturity in blue sharks, although in this case limited to differentiating between immature and mature individuals. Although necessary for modelling purposes due to the small sample size, simplifying to two categories may have obscured biological differences between the maturing, mature and regenerating states. Incorporating endocrine and energetic parameters alongside traditional morphometrics into the analysis demonstrates yet that these physiological markers have good diagnostic value. Successfully applying a supervised machine learning approach (specifically, an RF classifier) to predict maturity stages represents a methodological advancement in shark reproductive research with promising applications for *in situ* monitoring and conservation. The RF model exhibited a very strong performance for female blue sharks, achieving 100 % classification accuracy. This suggests that the selected physiological indicators, particularly progesterone and testosterone, have strong discriminative power across maturity stages, in agreement with their assumed roles in reproduction, as discussed above. In males, the RF model achieved a classification accuracy of 75 %, which was slightly lower. Testosterone, triglycerides and progesterone were identified as the most informative variables. Testosterone was found to be at its highest level in mature individuals, which is consistent with its function in reproductive readiness. Conversely, triglycerides and cholesterol were inversely associated with maturity stage, with mature males exhibiting lower levels, likely reflecting the mobilisation of energy reserves for reproductive behaviours such as mating, competition or long-range movement, which aligns with findings in other shark species (Rangel et al., 2021a; Hammerschlag et al., 2018b). Expanding the dataset to include more individuals and additional steroid/lipid markers could improve the classification accuracy for males. While total length was found to be a dominant predictor in both sexes, accounting for roughly 45–55 % of feature importance, including hormonal and lipid markers in the model significantly enhanced its classification power, particularly in distinguishing individuals near the size thresholds for maturity. This represents a significant improvement on length-based assessments alone, which can be affected by factors such as individual variability, environmental influences or geographic differences in growth rates. Thus, physiological data supplement traditional metrics and offer insight into the internal state of individuals, which is especially valuable when visual gonadal assessment is not possible, for example with live or protected specimens. However, interpreting hormonal data requires careful consideration of the timing of sampling within the reproductive cycle. For example, E2 levels in females typically peak during vitellogenesis and decline post-ovulation. This means that static hormone measurements may not always correspond neatly to morphological maturity categories. Similarly, lipid indicators such as triglycerides and NEFA are influenced by recent feeding and energy expenditure, meaning that maturity assessments could be inaccurate if these factors are not considered alongside broader physiological or ecological data. Overall, these findings confirm that endocrine and energetic markers, when used alongside size metrics and machine learning, offer a robust, non-lethal approach to assessing reproductive status in blue sharks. The ability to predict maturity with high accuracy, even in individuals lacking external morphological cues, could represent a significant advancement in elasmobranch biology and provide scalable tools for monitoring vulnerable populations in the field. This is particularly relevant in the Mediterranean, where elasmobranchs face some of the highest extinction risks worldwide, and where conducting traditional maturity studies is constrained due to the species' protected status. This paves the way for ethical and scalable monitoring approaches that mitigate the biological impact of conservation research.

Finally, spatial modelling of male maturity index revealed clear, biologically meaningful geographic patterns within the Southern Adriatic Sea. Specifically, mature males were predominantly found in offshore areas farther from the Italian, Croatian and Montenegrin coastlines, while immature individuals were more frequently located in shallower, nearshore waters. This spatial segregation is consistent with ontogenetic habitat partitioning, a common ecological strategy among elasmobranchs whereby juveniles and adults occupy different habitats to reduce intraspecific competition and the risk of predation (Poisson et al., 2024). The correlation between higher maturity index values and deeper waters indicates that mature males may prefer offshore pelagic environments, which could be linked to mating behaviour or the availability of prey that can support the energy demands of reproduction. This result is consistent with a study of the Western Mediterranean showing that juvenile blue sharks tend to remain on the shelf in summer, while adults are mostly found in the open sea (Poisson et al., 2024). Male sharks are known to invest significant energy in reproductive activities, including spermatogenesis, searching for mates, and potentially undertaking long-distance migrations. The observed depletion of triglycerides and cholesterol in mature males further supports this hypothesis, suggesting a higher metabolic cost associated with reproductive activities in offshore habitats. Furthermore, Carbonara et al. (2024) recently found that the vertical movements typical of this species were influenced by size. Indeed, larger specimens were capable of reaching greater depths than smaller specimens. These data support the idea that large specimens are found further offshore than juveniles. Although environmental variables showed trends that aligned with maturity gradients, the tensor between temperature and salinity did not reach statistical significance within the current GAM framework, likely because they were partially incorporated in the geographical variability. The tendency of mature individuals to occur in warmer (22–26 °C) and more saline waters, despite the relatively small magnitude of the salinity change, may reflect environmental cues or preferences associated with reproductive readiness. These conditions may correspond to areas of higher primary productivity or prey abundance, which could indirectly support reproductive aggregations. However, the inability to detect significant statistical association is likely due to both limited temporal resolution and sample size. Many environmental parameters exhibit high temporal variability, and single-point measurements may fail to capture the dynamic nature of shark–environment interactions. Considering other relevant environmental variables over a longer temporal study period might also help to highlight the role of environmental conditions in reproductively linked spatial distribution more effectively. The absence of reliable spatial models for females further emphasizes the importance of collecting data across broader temporal and spatial scales, potentially by integrating telemetry, gonadal histology, and wider environmental datasets. Despite these limitations, the significance of the geographic tensor component in the GAM model confirms that geographic location alone accounts for a substantial proportion of variation in the maturity index in males. Spatial mapping of maturity stages could ultimately help delineate mating or nursery grounds, contributing to more spatially explicit conservation measures. For example, our finding about the distinct habitats uses, with mature males occupying offshore areas and juveniles remaining closer to the coast could be used to inform fisheries management. This could support the design of time-area closures, gear restrictions, or other spatial measures to mitigate bycatch (Carbonara et al., 2023). The reported presence of newborns in Montenegrin waters in May and July (Ćetković et al., 2019) suggests local parturition and highlights the need to monitor female maturity more closely in this area to identify potential nursery habitats. From this perspective, a large-scale satellite tagging programme could complement the data presented here to define the migrations of this species in the Central-Eastern Mediterranean more precisely (Carbonara et al., 2024). Together, these insights highlight the value of integrating physiological, ecological and spatial tools into a regional management framework for *P. glauca*.

5. Conclusion

This study paves the way for non-lethal maturity assessment in blue sharks by combining endocrine and energetic markers with machine learning. By aligning physiology with spatial ecology, we contribute to offering a solution to the ethical issues surrounding lethal sampling and the pressing requirement for region-specific biological data. In the Central-Eastern Mediterranean, a region of high fishing pressure and conservation concern, this integrative approach could enhance our ability to identify reproductive habitats, refine maturity schedules and develop targeted management strategies. Although further work is required to increase sample sizes, include females in spatial analyses and address seasonal dynamics, the present study demonstrates that an ethical, data-rich monitoring of elasmobranchs is feasible. Ultimately, integrating physiology, maturity and environment provide a way forward for the more sustainable management of blue sharks and could be applied to other threatened elasmobranchs worldwide. Continued efforts to expand these datasets and integrate them with broader ecological monitoring programmes (e.g. satellite tagging) are essential if we are to realise their full potential for conservation impact. By reducing the biological cost of knowledge, we can better align conservation science with its ultimate goal of securing the future of vulnerable marine predators.

CRediT authorship contribution statement

Pierluigi Carbonara: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Nazareno Campioni:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis. **Walter Zupa:** Writing – review & editing, Methodology, Formal analysis. **Andrea Bellodi:** Writing – review & editing, Investigation, Data curation. **Massimiliano Bottaro:** Writing – review & editing, Investigation, Data curation. **Cosmidano Neglia:** Writing – review & editing, Visualization, Formal analysis. **Maria Cristina Follesa:** Writing – review & editing, Investigation, Data curation. **Lola Toomey:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2025.107745.

Data availability

All data will be made available by the corresponding author upon reasonable request.

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