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Do vertebrate-type steroids play a role in bivalve reproduction? A case study across two reproductive cycles of *Magallana gigas*

Ana Rato [a,b,*], Sandra Joaquim [b,c], José P. Da Silva [a], Catarina Anjos [b], Domitília Matias [b,c], Peter C. Hubbard [a]

[a] Centro de Ciências do Mar (CCMAR), University of Algarve, Campus de Gambelas, Faro, 8005-139, Portugal
[b] Department of Sea and Marine Resources, Portuguese Institute for Sea and Atmosphere (IPMA, I.P.), Av. 5 de Outubro s/n, Olhão, 8700-305, Portugal
[c] Interdisciplinary Centre of Marine Environmental Research (CIIMAR), University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, Matosinhos, 4450-208, Portugal

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ABSTRACT

In bivalves, reproduction is regulated by a complex interaction between endogenous and environmental factors, including temperature and food availability. However, hormonal control of the reproductive cycle in bivalves is poorly understood. There is a general belief that vertebrate-type steroids (testosterone, progesterone and 17β-oestradiol) are involved in the reproductive cycle of bivalves, even though the necessary enzymes and receptors for such steroids are apparently lacking in the molluscan genome. To investigate the potential role of vertebrate-type steroids in reproduction, the current study characterized two consecutive reproductive cycles of the Pacific oyster (*Magallana gigas*), integrating biochemical composition, reproductive indices, environmental parameters, and steroid levels. Gonadal index (GI), gonadosomatic index, and condition index exhibited clear seasonal fluctuations. Protein, glycogen, and total lipid displayed distinct seasonal patterns, with protein exhibiting structural functions, whereas glycogen and total lipid acted as energy reserves supporting gametogenesis. Both progesterone (0.69-142.33 ng g^{-1}) and testosterone progesterone (2.55-262 ng g^{-1}) levels fluctuated during sampling; progesterone showed correlations with GI, temperature, and protein content, whereas testosterone was mainly associated with environmental factors. These patterns suggest that progesterone may reflect gonadal tissue development and energetic investment rather than acting as a classical sex steroid. The absence of sex-specific steroid patterns, together with the lack of classical steroid receptors in the molluscan genome, supports the hypothesis of an environmental origin and/or an eco-physiological rather than an endocrine role for vertebrate-type steroids in oysters. These findings demonstrate that hormonal studies in bivalves should integrate biochemical, reproductive, and environmental data to understand reproductive strategies.

1. Introduction

In vertebrates, the reproductive endocrine system and the mechanisms of action of the hypothalamic-pituitary-gonadal (HPG) axis are well characterized. In molluscs, including bivalves, reproduction is also mediated by an endocrine system; however, this is still poorly understood (Fodor et al., 2020). Nonetheless, there is a general belief that vertebrate-type steroids (testosterone, progesterone and 17β-oestradiol) are involved in the reproductive cycle of bivalves (*e.g.* Gauthier-Clerc et al., 2006; Martínez-Pita et al., 2012; Matsumoto et al., 1997; Smolarz et al., 2018). However, their endocrine system is morphologically distinct from that of vertebrates, with no clear indication of endogenous steroid synthesis, no analogous vertebrate pituitary gland, and no evidence of the presence of gonadotropins or nuclear receptors for vertebrate-type steroids (Fodor et al., 2020, 2025). The apparent presence and hormonal role of vertebrate-type steroids in molluscs remains, therefore, controversial (Scott, 2012).

The presence of vertebrate-type steroids in bivalves has been reported in several studies (*e.g.* Croll and Wang, 2007; Gauthier-Clerc et al., 2006; Ketata et al., 2007; Smolarz et al., 2018; Zabrzańska et al., 2015), with these being detected mainly in the gills, digestive gland and gonads. The highest levels were often found in the gonads, suggesting that these organs may be involved in steroid synthesis (Lavado et al., 2006; Scott, 2012). This, together with their levels

* Corresponding author. Centre of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8005-139, Faro, Portugal.
E-mail address: ana.rato@ipma.pt (A. Rato).

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appearing to fluctuate along the cycle of sexual maturation (Croll and Wang, 2007), has led researchers to assume that bivalves do contain these steroids and are able to synthesize them (Scott, 2012). Nevertheless, due to filter-feeding nature of bivalves, these steroids could be absorbed from the environment, and be retained in the gonadal tissue due to its high fat content (Scott, 2012; Smolarz et al., 2018).

When considering the classic biosynthetic pathways of steroids, genomic studies have suggested that CYP11A1 – an enzyme of the Cytochrome P450 family (Fodor et al., 2020; Scott, 2012) responsible for the first key step in steroidogenesis (cholesterol side-chain cleavage) – has only evolved in vertebrates (Markov et al., 2009, 2017). This could explain the failure of the conversion of cholesterol into pregnenolone in *Mytilus edulis* observed by De Longcamp et al. (1974). Similarly, the gene encoding CYP19A (aromatase) has not yet been identified in any molluscan genome (Reddy et al., 2018) and it only appears in a direct ancestor of the chordates (Scott, 2012).

In vertebrates, the presence of appropriate sex steroid receptors is necessary for the HPG axis to function. The main receptors are the oestrogen receptor (ER), the progestogen receptor (PR) and the androgen receptor (AR), with both membrane and nuclear forms involved in reproduction (Fodor et al., 2020; Levin and Hammes, 2016). The classical nuclear receptors for progestogens (nPR) and androgens (nAR) are believed to have evolved from an ancestral oestrogen-binding receptor (nER) after the divergence of the vertebrates (Scott, 2012). Hence, only the nER gene would be expected to occur in molluscan genomes (Fodor et al., 2020; Scott, 2012). Indeed, there is an analogue of the vertebrate nER; however, despite being structurally similar to the vertebrate nER, it is non-functional, as it does not bind to oestrogens – its ability to recognize oestrogens may have been lost through evolutionary history – and it is usually referred to as a 'constitutive receptor' (Scott, 2012).

In the absence of functional classical sex steroid receptors, an alternative explanation for the presence of vertebrate-type steroids in bivalves must be considered. Due to their filter-feeding nature, bivalves have an extraordinary capacity to absorb chemicals, such as vertebrate-type steroids, directly from the environment (Schwarz et al., 2017a, 2017b, 2018). Once absorbed, these steroids may be stored for long periods through conjugation with fatty acids – esterification (Gooding and LeBlanc, 2001) – that, due to their lipophilic nature, are retained in fat tissue (Scott, 2018). This ability to absorb vertebrate-type steroids from the water and esterify them has been shown in several laboratory experiments (Janer et al., 2004; Labadie et al., 2007; Peck et al., 2007; Schwarz et al., 2017a, 2017b, 2018). Therefore, these steroids may be of exogenous rather than endogenous origin (Scott, 2012, 2018).

Despite the widespread detection of vertebrate-type steroids in bivalve tissues (Fernandes et al., 2011; Lafont and Mathieu, 2007; Smolarz et al., 2018), a key question arises: if the key enzymes and receptors are apparently lacking from the molluscan genome, and considering that these steroids can be absorbed from the environment, can these compounds truly be assumed to have an endogenous origin? And, if so, do they play a functional role in bivalve reproduction?

Bivalve reproductive cycles are highly dynamic and strongly influenced by environmental conditions and associated energy allocation strategies (Joaquim et al., 2023). In temperate ecosystems, reproduction is typically seasonal, with gametogenesis and spawning closely linked to fluctuations in seawater temperature and food availability (Matias et al., 2025). Periods of increasing temperature and abundant phytoplankton supply are commonly associated with gonadal development and reproductive activity, reflecting the high energetic costs of gamete production (Matias et al., 2013). Seasonal changes in biochemical composition, such as glycogen, lipid and protein reserves, as well as in condition and gonadal indices, further demonstrate that reproductive cycles in bivalves are closely associated to energy storage and environmental variability (Pérez-Camacho et al., 2003).

Understanding reproductive physiology and seasonal reproductive dynamics is of high relevance from both aquaculture and environmental perspectives. Knowledge of the timing and environmental drivers of gametogenesis and spawning may support the identification of suitable periods for broodstock collection and hatchery conditioning, as well as improve predictions of natural spat settlement periods (Azpeitia et al., 2017; Joaquim et al., 2023; Matias et al., 2013; Philippart et al., 2003). Additionally, due to their key role as ecosystem engineers contributing to habitat formation, biodiversity support and water filtration, oysters are fundamental constituents of coastal ecosystems (Beck et al., 2011; Grabowski et al., 2012). Hence, understanding the environmental regulation of reproduction may also provide valuable insights for oyster reef restoration and coastal ecosystem management under changing environmental conditions (Beck et al., 2011).

Therefore, the aim of this study was to address these questions by characterizing two consecutive reproductive cycles of the Pacific oyster (*Magallana gigas*) – an economically important bivalve species – and by assessing the potential involvement of these steroids throughout the reproductive cycle in relation to biochemical composition and environmental parameters.

2. Materials and methods

2.1. Sampling

Adult Pacific oysters (*Magallana gigas*, formerly known as *Crassostrea gigas*) (9.55 ± 1.07 cm total length and 83.51 ± 14.94 g total weight, mean ± SD) were collected monthly, over a 24-month period, from November 2020 to October 2022 ($n = 10$ individuals per month) in Ria de Alvor, southern Portugal (37° 07′ 50″ N 8° 37′ 38″ W). Seawater temperature was recorded *in situ* at the time of collection using a multiparametric probe, and monthly averaged chlorophyll *a* (Chl *a*) concentrations were obtained from the Copernicus Sentinel 2 satellite remote sensing database (https://data.marine.copernicus.eu). After collection, oysters were transported to the Molluscan Experimental Station of Tavira of the Portuguese Institute for Sea and Atmosphere, and maintained overnight in clean, filtered, and aerated seawater. On the following day, oysters were opened and gonadal development was evaluated by macroscopic observation according to a gametogenic scale adapted from Vilela (1972) (Table 1). Sex was determined by microscopic examination of gametes following a small incision in the gonads.

To calculate the gonadal index (GI), a numerical ranking was assigned for each of the gonadal stages: stage 0 = 0; stage I = 2; stage II = 3; stage III = 5; stage IV = 4; stage V = 1. The mean GI, ranging from 0 (all individuals are at sexual rest) to 5 (all individuals are mature), was then calculated as suggested by Seed (1976): GI = [(∑ ind. Each stage × stage ranking)/total ind. Each month].

Afterwards, the entire soft tissue of each oyster ($n = 10$ per month) was separated from the shell and placed on absorbent paper to remove excess water (~5 min). Then, after weighing soft tissues, the visceral mass (including gonads and digestive gland) was separated from the

Table 1
Macroscopic scale for evaluation of the gonadal stages. Adapted from Vilela (1972).

Stage	Description
0	Sexual rest, gonads barely visible or absent, immature gonad, translucent or greyish appearance; not possible to distinguish sexes (individuals are classified as Undetermined).
I	Immature gonad, slight increase in gonadal volume, translucent or greyish appearance. It is possible to distinguish males and females.
II	Gonad almost mature, increase in gonadal volume, pale colour (creamy white or light beige). Firm texture
III	Ripe individuals. Mature, well-developed and voluminous gonad with milky appearance.
IV	Partial spawning. Gonads still visible but less engorgement. Irregular appearance with stretch marks.
V	Spawning completed. Flaccid and reduced gonads, with translucent stretch marks.

remaining tissues and weighed. The gonadosomatic index was calculated as suggested by Deudero et al. (2017): GSI (%) = [(weight of the visceral mass (g)/weight of the total soft tissue (g)) × 100].

After homogenization of the total soft tissue in an ice bath with an Ultra-turrax homogenizer (IKA: https://www.ika.com/en), the homogenate was separated for the different analyses.

2.2. Condition index

Condition index (CI) was calculated as the ratio between the ash-free dry weight of soft tissue (g) and shell dry weight (g) (Walne and Mann, 1975), according to the following formula: CI = [(Ash-free dry weight (AFDW) of soft tissues/shell dry weight) × 100].

Both shell and soft tissue (±1 g) were dried at 80°C (24 h) and weighed. Dried soft tissue was subsequently incinerated in a muffle furnace at 450°C for 24 h and reweighed to determine ash-free dry weight.

2.3. Biochemical parameters

The biochemical composition – proteins, glycogen and total lipid – was analysed by standard methods. Proteins were precipitated by cold 5 % trichloroacetic acid (TCA), and the precipitate washed in warm 1.0 N sodium hydroxide (NaOH). Protein content was determined according to the method of Lowry et al. (1951) modified by Bensadoun and Weinstein (1976) and Hess et al. (1978), at 750 nm using serum albumin as standard. Total lipid content was extracted from fresh homogenate in chloroform/methanol (Folch et al., 1957) and spectrophotometrically estimated, at 375 nm using cholesterol as standard, after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). From dried homogenate (80°C for 24 h), glycogen content was determined using anthrone as reagent (Viles and Silverman, 1949), at 620 nm. Biochemical composition results are expressed as total organic ash-free dry weight (μg mg^{-1} of AFDW).

2.4. Extraction for hormonal analysis

The vertebrate-type steroids (testosterone, progesterone and 17β-oestradiol) potentially present in the tissue were extracted following an adaptation of the extraction method described by Schwarz et al. (2017a). Briefly, 0.5 g of homogenized tissue was extracted with methanol (2 ml), vortexed for 30 s, following the addition of ethyl acetate (3 ml) and subsequent agitation (1000-1500 rpm; 5 min). After centrifugation (2500 x *g*, 15 min), supernatants were collected and evaporated to dryness under nitrogen. The extraction step with ethyl acetate was repeated once, and the combined extracts were dried under nitrogen. Dried extracts were rinsed with 4 × 250 μl of ethanol, transferred to autosampler vials, evaporated to dryness, and reconstituted in 0.5 ml ethanol.

This procedure allowed the extraction of the total of vertebrate-type steroids present in the tissue, including free, sulphated and esterified forms. Given that the free form is biologically active – the LC-HRMS analysis (see next section) focused only on the targeted analysis of the free form of these steroids.

2.5. LC-HRMS analysis

Chromatographic separation was performed on a Thermo Scientific *ultimate* 3000 UHPLC (Thermo Fisher Scientific: https://www.thermofisher.com/en). The column was a Thermo Scientific Accucore RP-18 (2.1 × 100 mm, 2.6 μm). The mobile phase composition was prepared with water (A) and acetonitrile (B), both containing 0.1 % formic acid. The gradient (in v/v %) started with 20 % of B for 1 min, increased linearly to 60 % of B during 7 min and to 100 % of B during 14 min. This composition was maintained for 2 min, returned to 20 % of A in 0.5 min and then maintained at this composition for 4 min before the next run. The flow rate was 0.3 ml/min. The injection volume was 10 μl.

Mass analysis was performed on an Orbitrap Elite (Thermo Scientific) mass spectrometer with a Heated ElectroSpray Ionization source (HESI-II). HR-MS and MS/MS data were acquired using the following ionization parameters: spray voltages, 5.0 kV (positive ESI); sheath gas, 30 arbitrary units; auxiliary gas, 10 arbitrary units; heater temperature, 350°C; capillary temperature, 325°C; S-Lenses RF level, 64.4 %. Acquisition was performed under full scan and Multi Reaction Monitoring (MRM) using Collision-Induced Dissociation (CID). Transitions used were the following: 273 → 245, 255 (17β-oestradiol); 289 → 253, 271 (testosterone); 315 → 279, 297 (progesterone) (Table 2 and Fig. S1). LC-HRMS profiles were analysed using Xcalibur 4.1 (Thermo Scientific). Quantification was achieved using the full scan and MRM profiles. In the former case, the accurate mass extracted ion chromatograms (AM-XIC) were first obtained by taking *m/z* 273.1849 (17β-oestradiol), 289.2162 (testosterone) and 315.2319 (progesterone) from the full scan profiles using a ±5 ppm window. The signals were then integrated and calibration curves prepared. The quantification from the MRM profiles was obtained after integration of the signals corresponding to the transitions of each compound. Quantification was performed by comparing the area of samples with the results of the calibration curve obtained for the corresponding matrix. As the quantification based on MRM profiles provided better selectivity and lower limits of detection (LODs) and limits of quantification (LOQs), the samples were analysed and the compounds quantified using this acquisition mode.

Matrix effects (see section 2.6.2) were corrected by using calibration curves prepared in the extracts. Matrix-matched calibration curves with five concentration points (5, 10, 20, 40 and 60 ng/ml) spiked in the matrix were prepared. The matrix consisted of extracted homogenized oyster soft tissue from individuals at different maturation stages over the sampling period.

2.6. Validation of the developed methods

2.6.1. Recovery rate, limits of detection (LOD) and quantification (LOQ)

The recovery rates were obtained through a spike-and-recovery experiment using a reference tissue matrix. Six sets of different samples were prepared in triplicate: i) non-spiked samples; ii) post-extraction spiked samples, obtained by processing the reference matrix and spiking the resulting extracts with a mixed standard solution of the selected compounds; iii) pre-extraction spiked samples, in which the same mixed standard was added to the reference matrix prior to extraction. All samples were spiked with a constant concentration of 10 ng/ml. Samples were analysed by LC-HRMS under the same instrumental conditions, and peak areas were recorded. Recovery rate was calculated by comparing the responses of pre-extraction spiked with those of post-extraction spiked samples, according to: Recovery rate (%) = [(Peak area of C – Peak area of A)/(Peak area of B – Peak area of A) × 100], where C corresponds to the pre-spiked samples, A to the non-spiked sample and B to the post-extraction spiked samples.

Limits of detection (LOD) and quantification (LOQ) were calculated

Table 2
Mass spectrometric and chromatographic parameters of the analysed compounds, including precursor ion mass-to-charge ratio (m/z), fragmentation ions, relative fragment ion intensities (%), and retention times (min).

Analyte	*m/z*	Fragmentation ions	Relative intensity (%)	Retention time (min)
Testosterone	289.216	253.19	83.8	9.92
$C_{19}H_{28}O_2$		271.17	100.0	
Progesterone	315.232	279.21	30.3	13.02
$C_{21}H_{30}O_2$		297.22	100.0	
17β-oestradiol	273.185	245.10	16.4	9.71
$C_{18}H_{24}O_2$		255.13	100.0	

according to the guidelines of Eurachem (2014). Five replicate injections of matrix samples spiked at the second-lowest concentration (10 ng/ml) were analysed, and the standard error of the mean (SEM) was determined. The LOD and LOQ were estimated as 3 × SEM and 10 × SEM, respectively.

2.6.2. Matrix effects

The average matrix effect (ME) was evaluated following the approach described by Matuszewski et al. (2003). Calibration curves were prepared both in ethanol (solvent) and in samples of matrix extracts spiked after extraction. The ME (%) was calculated as the ratio between the slope of matrix-matched calibration curve and the slope of the calibration curve prepared in ethanol, multiplied by 100. An ME value close to 100 % indicates no matrix effects, whereas values below 100 % indicate ion suppression and values above 100 % indicate an enhancement of the signal.

2.7. Data treatment and statistical analysis

Differences between sexes, sampling years and maturation stages were evaluated using Kruskal-Wallis non-parametric tests, since the assumptions of normality and homogeneity of variances failed. When significant differences were detected, multiple pairwise comparisons were performed using Dunn's post-hoc test. Comparisons between sampling years for each month were performed using Student's *t*-test or Mann–Whitney *U* test (data shown in Supplementary Table S1). To control for false discoveries arising from multiple testing, *p*-values were adjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). Adjusted *p*-values $\leq$0.05 were considered statistically significant.

Since more than 60 % of the LC-HRMS data were below the limit of quantification (LOQ; left-censored values), hormonal concentrations were modelled using Regression on Order Statistics (ROS) to obtain unbiased estimates of the censored data for subsequent statistical analyses (dos Santos et al., 2023; Helsel, 2006). This is a semi-parametric method widely applied in environmental and biological studies to estimate censored observations while preserving the original data distribution. It assumes that uncensored values follow a normal or lognormal distribution and uses a regression model to estimate the missing censored values based on the available data (dos Santos et al., 2023). The use of ROS minimizes bias associated with values below LOQ and allows consistent integration of hormonal variables with other continuous measurements in the dataset (Antweiler and Taylor, 2008).

Pearson or Spearman correlation analyses were conducted, depending on the normality of the data, to determine the degree of association between parameters. These were performed using data separately by year and by sex. Matrixes of both Pearson and Spearman correlations combined are shown in the supplementary data (Tables S2 and S3). Results are expressed as mean ± SD. The significance level was set at $p \leq 0.05$. ROS analysis was implemented in *R* (RStudio) using the NADA2 package. Statistical analysis was performed using software Sigmaplot (version 15).

3. Results

3.1. Temperature and chlorophyll a

The monthly variation in temperature and chlorophyll *a* (Chl *a*) over the two-year sampling period is represented in Fig. 1. Temperature exhibited distinct seasonal patterns between the two sampling years, with a higher thermal variation observed during the second year. During the first year, from November 2020 to October 2021, the lowest temperature (15.0°C) occurred in March 2021 while the maximum temperature (23.3°C) was registered in September 2021. In contrast, during the second sampling year, the lowest temperature (15.0°C) was recorded in February 2022 followed by a steady increase towards the summer months, reaching a maximum of 24.2°C in July 2022.

Chlorophyll *a* concentration also showed contrasting patterns between the two sampling years. During the first sampling year, Chl *a* ranged from 2.84 mg m^{-3} in March 2021 to 4.87 mg m^{-3} in May 2021. A peak of Chl *a* was observed during spring months (May and June 2021), reaching values between 4.87 mg m^{-3} and 4.84 mg m^{-3}, respectively, and coinciding with unusually low temperatures for the spring months. In contrast, during the second year, the lowest Chl *a* concentration was observed in December 2021 (2.25 mg m^{-3}), while the highest concentration occurred in August 2022 (4.48 mg m^{-3}). Several smaller Chl *a* peaks were observed throughout the second year, particularly in January (3.78 mg m^{-3}), April (4.02 mg m^{-3}), June (4.33 mg m^{-3}) and August (4.48 mg m^{-3}). Overall, a significant positive correlation was found between temperature and Chl *a* concentration (tables S2A and S3A). This relationship was stronger during the second year (tables S2C and S3C).

3.2. Gonadal development

Throughout the sampling period, the reproductive cycle showed a clear seasonal pattern (Fig. 2), which was reflected by the gonadal index (GI), with no significant differences between sexes or between years (GI: Mann-Whitney, U = 287.00, $p = 0.984$; 1st year: U = 68.00, $p = 0.817$; 2nd year: U = 69.50, $p = 0.885$).

In both sampling years, the development of gametogenesis occurred in February for females and March for males, as evidenced by the increase in gonadal index and the concomitant decrease in the proportion of undetermined individuals. During the first sampling year, two reproductive peaks were observed in May and August 2021, when oysters had fully mature gonads (stage III) or were undergoing partial

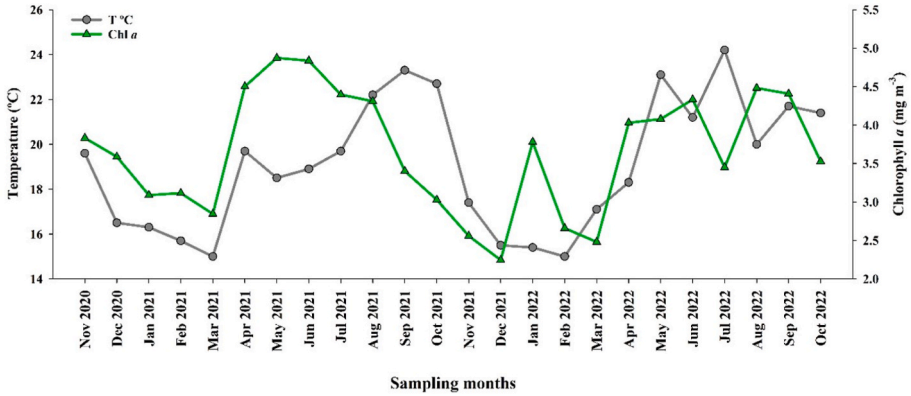

Fig. 1. Monthly variation of temperature (°C) and chlorophyll *a* (mg m^{-3}) along the two-year sampling period (November 2020 to October 2022).

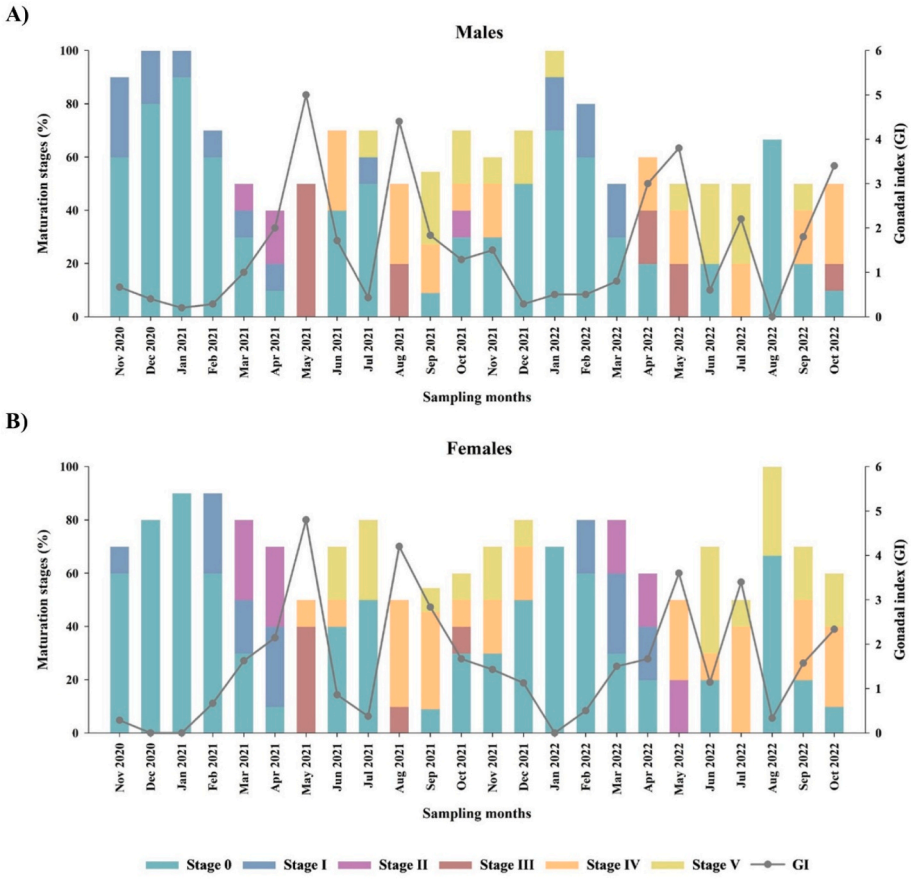

Fig. 2. Gonadal development (maturation stages; bar graph) and mean gonadal index (GI; grey lines) over the two-year sampling period for (**A**) males and (**B**) females.

spawning (stage IV). These peaks coincided with the highest GI (males: 5.0 and 4.4; females: 4.8 and 4.2, respectively), indicating an increase in gonadal investment. In contrast, during the second sampling year, reproductive activity was less pronounced, with three lower peaks in May 2022 (GI = 3.8 for males and 3.6 for females), July 2022 (GI = 2.2 for males and 3.4 for females) and October 2022 (GI = 3.4 for males and 2.3 for females). Gonadal development was more irregular, with increased heterogeneity of gonadal stages, mainly during spring, and no clear prevalence of fully mature gonads. Instead, partial spawning (stage IV; Table 1) followed by spent stage (stage V; Table 1) was frequently observed.

Interestingly, during the first year, the onset of the gametogenic cycle did not coincide with the increase of temperature, resulting in a temporal discrepancy between reproductive and temperature peaks. In contrast, in the second year, both reproductive and temperature peaks were synchronous. Periods of lower temperature were associated with reduced gonadal values, corresponding to sexual rest stages. When correlating by years, only the GI of the second year showed a positive correlation with temperature (tables S2C and S3C). No correlation was found between GI and Chl *a*.

3.3. Gonadosomatic index and condition index

The variation over the two-year sampling period of gonadosomatic

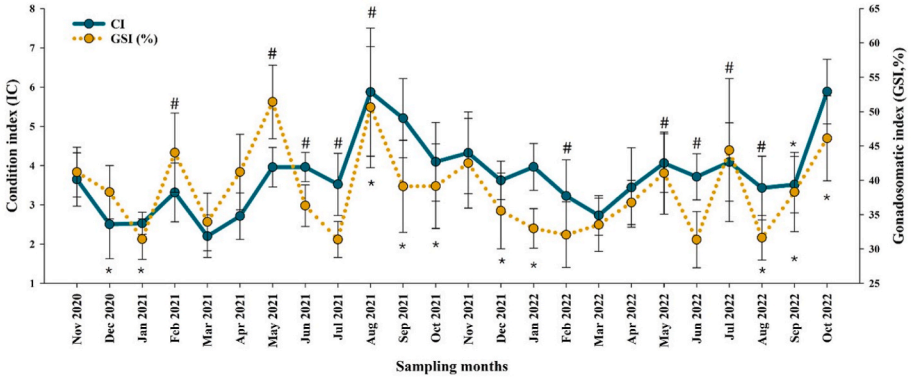

Fig. 3. Condition index (CI; green line) and gonadosomatic index (GSI; yellow dotted line) over the two-year sampling period (mean ± SD). Asterisk (*) and hash symbols (#) indicate significant differences in CI and GSI, respectively, between corresponding months of different years ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

index (GSI), which reflects the gonadal volume, and condition index (CI), an indicator of the physiological condition, showed no significant differences between males and females for either index (CI: KW, $H = 0.028$, $d.f. = 1$, $p = 0.866$; GSI: KW, $H = 0.045$, $d.f. = 1$, $p = 0.832$). Therefore, data from both sexes were pooled and plotted together (Fig. 3). Both indices were positively correlated with each other for both sexes (tables S2A and S3A); however, when separating by years and sexes, CI was positively correlated with GSI in the first year for females (table S3B) and in the second year for males (table S2C).

Significant differences in GSI values were observed between corresponding months of different years, with February, May, June and August (*t*-test, $p < 0.05$; Table S1), showing significantly higher GSI peaks in the first year (February: 44.03 ± 5.77 %; May: 51.41 ± 5.36 %; August: 50.65 ± 8.21 %) compared with the second year (May: 41.04 ± 5.97 %; July: 44.38 ± 10.42 %; October: 46.12 ± 6.20 %).

Similarly to GI, the highest GSI values were coincident with the reproductive peaks. The release of gametes (spawning) was reflected by a decrease in GSI values. In females, GSI was positively correlated with GI in the first year (Table S3. B), and in males in the second year (table S2C). No correlation was found between GSI and temperature or Chl *a*.

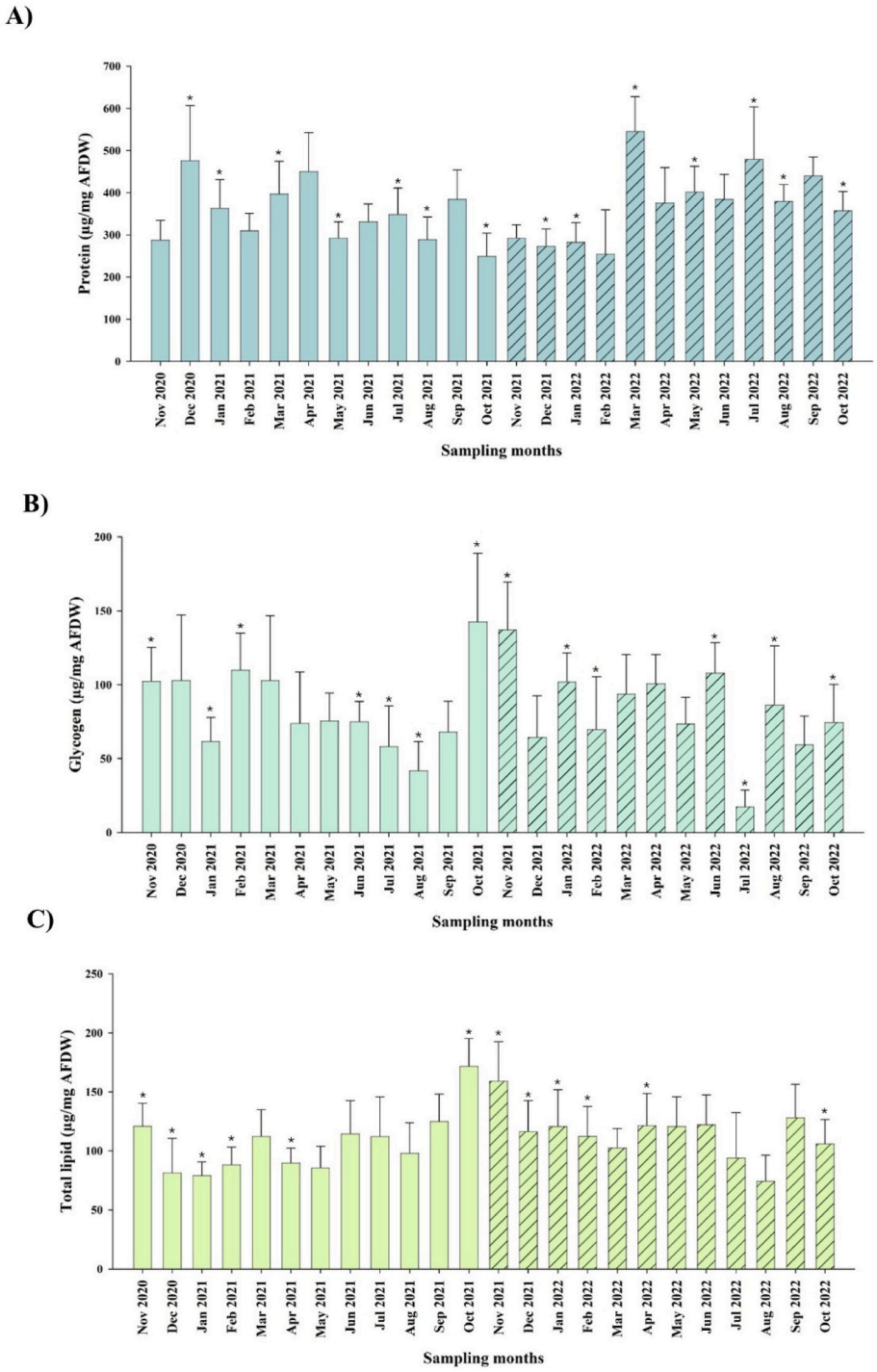

Fig. 4. Monthly variation (mean ± SD) in biochemical composition (**A**) protein, (**B**) glycogen and (**C**) total lipid (µg mg^{-1} AFDW) during the sampling period (1st year: November 2020 to October 2021 (coloured bars); 2nd year: November 2021 to October 2022 (coloured and striped bars). Asterisk (*) indicates significant differences between corresponding months of different years ($p < 0.05$).

Oysters were in better physiological condition (CI) during August 2021 (5.87 ± 1.63, mean ± SD) and October 2022 (5.88 ± 0.82), whereas the lowest CI was observed in March 2021 (2.20 ± 0.55). Overall, individuals had higher CI values during the second year. Significant differences were observed between corresponding months of different years, with December, January and October (*t*-test, $p < 0.05$; Table S1) showing significantly lower CI values in the first year than those of the second year. On the contrary, August and September 2021 (*t*-test, $p < 0.05$; Table S1) had significantly higher CI values than the corresponding month in 2022.

In addition to its correlation with GSI, CI was also positively correlated with GI and temperature, only during the first year (tables S2B and S3B). In general, when analysing by sex, CI was positively correlated with GI (tables S2A and S3A) and temperature (tables S2A and S3A) in both sexes. No correlation was detected between CI and Chl *a*.

3.4. Biochemical composition

Protein was the predominant compound, followed by total lipids and then glycogen (Fig. 4). No significant differences were observed between males and females in protein and glycogen contents (proteins: KW, H = 0.342, *d. f.* = 1, $p = 0.558$; glycogen: KW, H = 0.0774, *d. f.* = 1, $p = 0.781$). Although, total lipid content differed significantly between sexes, with females showing higher values than males (KW, H = 6.993, *d. f.* = 1, $p = 0.008$), this difference was expected due to the higher lipid requirements associated with oocyte formation. Therefore, data from both sexes were pooled and plotted together (Fig. 4).

In the first year, the highest protein content was recorded in December (476.48 ± 130.26 μg mg^{-1} AFDW) and the lowest in October (250.03 ± 54.50 μg mg^{-1} AFDW). Conversely, the highest protein content during the second year was observed in March (545.81 ± 82.32 μg mg^{-1} AFDW) and the lowest in February (255.29 ± 104.34 μg mg^{-1} AFDW). Significant differences were observed between corresponding months of different years. Protein content recorded in December 2020, and January 2021 (1st year) (*t*-test or Mann-Whitney, $p < 0.05$; Table S1) was significantly higher than those recorded in the second year. In contrast, protein content observed in March, May, July, August and October 2021 (*t*-test, $p < 0.05$; Table S1) was significantly lower than the corresponding months in 2022. When analysing data separately, protein displayed a negative correlation with CI in males (table S2A) and, during the second year, a positive correlation with temperature (table S2C) (, mostly due to male contribution (table S2C).

Glycogen content exhibited slight fluctuations during sampling period. In the first year, the lowest content was recorded in August 2021 (42.04 ± 19.33 μg mg^{-1} AFDW), whereas the highest glycogen content was in October 2021 (142.55 ± 46.40 μg mg^{-1} AFDW). In contrast, during the second year, the highest glycogen content was observed in November 2021 (137.33 ± 32.09 μg mg^{-1} AFDW) and the lowest in July 2022 (17.71 ± 10.86 μg mg^{-1} AFDW). Comparisons of corresponding months from different years revealed significant differences. Glycogen values recorded in November 2020, January 2021, June 2021 and August 2021 were significantly lower (*t*-test, $p < 0.05$; Table S1) than those recorded in the second year. Conversely, glycogen contents registered in February 2021, July 2021 and October 2021 were significantly higher (*t*-test, $p < 0.05$; Table S1) than those recorded during the second year. No correlations were found between glycogen and the aforementioned parameters.

The highest total lipid values were observed in October 2021 (1st year, 171.90 ± 23.26 μg mg^{-1} AFDW) and November 2021 (2nd year, 159.29 ± 33.29 μg mg^{-1} AFDW), whereas the lowest contents were recorded in December 2021 (1st year, 81.60 ± 29.16 μg mg^{-1} AFDW) and August 2022 (2nd year, 74.71 ± 21.65 μg mg^{-1} AFDW). Significant differences were observed between corresponding months of different years, with November, December, January, February and April of the second year showing higher (*t*-test, $p < 0.05$; Table S1) values than those of the first year, while October showed significantly lower total lipid content (*t*-test, $p < 0.05$; Table S1) in the second year. Total lipid content showed a positive correlation with glycogen, mostly due to males in the second year (table S2C). Also in males, there was a general negative correlation with proteins (table S2A). For females, a positive correlation was found between total lipid and temperature during the first year (table S3B).

3.5. Validation of the developed methods

The recovery rate (%), the matrix effect (%), and the limits of detection and quantification (LOD and LOQ) for testosterone and progesterone are shown in Table 3. Fig. 5 shows the AM-XIC chromatograms of the target compounds showing the corresponding retention times. Under the analytical conditions used, the LOD and LOQ values for 17β-oestradiol were more than tenfold higher than those for testosterone and progesterone. Therefore, recovery rates were not evaluated for this compound, as the concentration range required for quantification exceeded expected biological levels and was not representative of biologically relevant concentrations (Hallmann et al., 2016).

The extraction procedure yielded a recovery rate of 108.8 ± 9.6 % for testosterone and 108.9 ± 12.9 % for progesterone. The LOD was determined as 3.92 ng g^{-1} for testosterone and 3.65 ng g^{-1} for progesterone, whereas the LOQ was 13.09 ng g^{-1} for testosterone and 12.18 ng g^{-1} for progesterone. The variability observed in the matrix effect, which ranged from 40.4 % to 140.9 % for testosterone, and 14.2 % to 116.1 % for progesterone, reflects differences in the sample composition. Each sample set contained homogenized tissue from oysters at different maturation stages, resulting in variations in tissue composition, particularly in lipid content, which in turn influenced matrix effects. These effects have been associated with compounds present in the matrix that co-elute with the targeted molecules (Costa et al., 2022). In addition, a distinct calibration curve was prepared for each sample set. All calibration curves had a correlation coefficient (r) > 0.99 (supplementary data, Table S4).

3.6. Steroid quantification

Only 38 % of the LC-HRMS measurements for testosterone and progesterone were above the quantification limit (LOQ).

Over the two sampling years, both steroids exhibited distinct fluctuations in males and females, with no clear relationship with the reproductive cycle. The temporal pattern was similar between sexes, except for progesterone in May 2021. In both sexes, a sharp increase in both steroids was observed at the end of the second year (July to October 2022) (Fig. 6).

During the first year, testosterone concentrations ranged from 2.55 ng g^{-1} of wet weight (ww) (ROS-estimated) in September 2021 (undetermined individuals) to 61 ± 55.15 ng g^{-1} ww in November 2020 (males). In the second year, concentrations ranged from 15.27 ± 0.38 ng g^{-1}ww in November 2021 to 262 ± 33.94 ng g^{-1}ww in October 2022, both recorded in males. Progesterone levels, during the first year, varied from 0.69 ng g^{-1} ww (ROS-estimated) in November 2020 (females) to 108.25 ± 24.51 ng g^{-1} ww in May 2021 (males). In the second year,

Table 3
Method validation parameters, including recovery rate (%), limits of detection (LOD) and quantification (LOQ) (mean ± SD), and matrix effect range (%) of testosterone and progesterone.

Analyte	Recovery rate (%)	Matrix effect (%)	LOD (ng g^{-1})	LOQ (ng g^{-1})
Testosterone $C_{19}H_{28}O_2$	108.8 ± 9.6	40.4 – 140.9	3.92	13.09
Progesterone $C_{21}H_{30}O_2$	108.9 ± 12.9	14.2 – 116.1	3.65	12.18

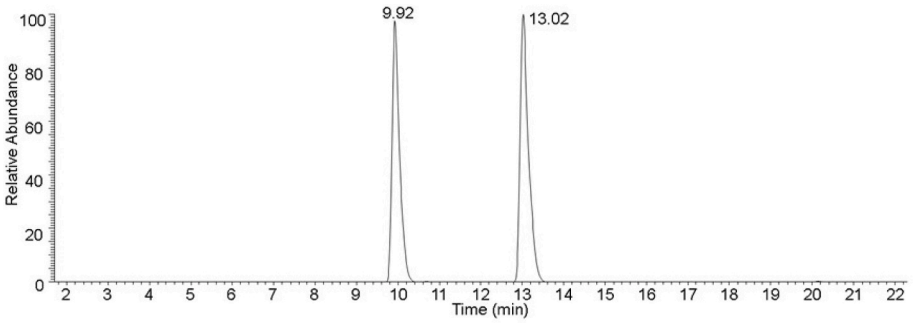

Fig. 5. Exact mass extracted ion chromatograms (AM-XIC, ±5 ppm) of testosterone (m/z 289.216, 9.92 min) and progesterone (315.232,13.02 min). AM-XIC chromatograms were taken from a standard solution containing 50 ng/ml of testosterone and progesterone.

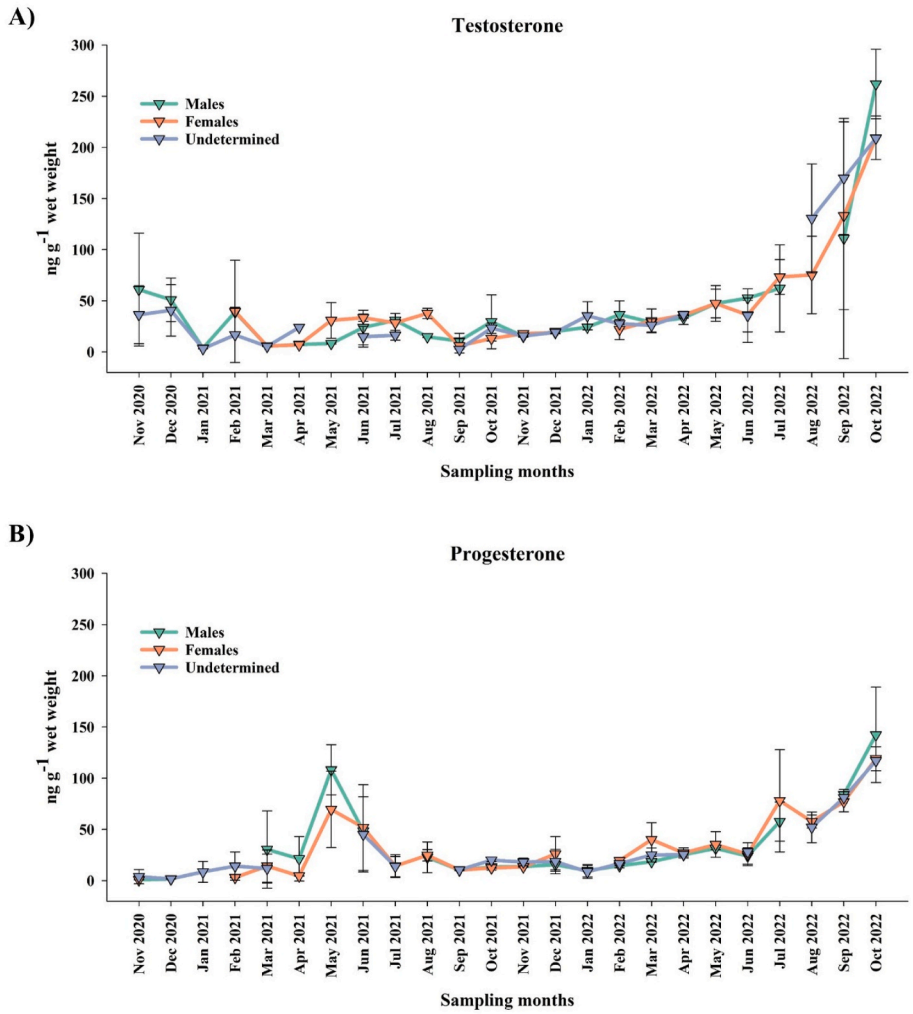

Fig. 6. Monthly variation (mean ± SD) of (**A**) testosterone and (**B**) progesterone (ng g^{-1}) during the two-year sampling period for males (green line), females (orange line) and undetermined (blue line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

progesterone concentrations varied from 8.68 ± 6.31 ng g^{-1} ww (ROS-estimated) in January 2022 (undetermined) to 142.33 ± 46.61 ng g^{-1} ww in October 2022 (males). No significant differences in testosterone levels were detected among sexes in either sampling year (1st year: KW, H = 1.405, *d. f.* = 2, $p = 0.495$; 2nd year: KW, H = 2.472, *d. f.* = 2, $p = 0.290$). In contrast, progesterone levels differed significantly between the sexes, with undetermined oysters being significantly different from males and females during the first year (KW, H = 12.336, *d. f.* = 2, $p = 0.004$ and $p = 0.027$, respectively), and from females during the second year (KW, H = 9.228, *d. f.* = 2, $p = 0.007$).

Regardless of sex, no significant differences were found for testosterone among maturation stages in either of the years (1st year: KW, H = 4.387, *d. f.* = 5, $p = 0.495$; 2nd year: KW, H = 7.646, *d. f.* = 5, $p = 0.177$). In contrast, progesterone levels differed significantly among maturation stages, with higher gonadal stages (III and IV) showing significantly higher values than stages 0 and I, mainly during the first sampling year (KW, H = 42.081, *d. f.* = 5, $p < 0.001$). During the second year, progesterone levels in stage IV were significantly higher than those

in stage 0 (KW, H = 13.50, *d. f.* = 5, $p = 0.014$).

Sex-specific analyses revealed distinct correlation patterns. In males, progesterone was positively correlated with temperature and GI (table S2A). When separating by years, during the first year, only progesterone and GI were positively correlated (table S2B). In the second year, testosterone was positively correlated with temperature, with Chl *a*, and progesterone (table S2C). Additionally, progesterone was positively correlated with protein content and temperature, and negatively correlated with total lipid content (table S2C).

In females, testosterone and progesterone were positively correlated with each other, while progesterone was also correlated with GI (table S3A). Throughout the first year, only progesterone exhibited correlations with CI, with protein content and with GI (table S3B). In contrast, in the second year, testosterone and progesterone were strongly correlated with each other and both were positively correlated with proteins (table S3C). Testosterone was also correlated with Chl *a* (table S3C).

4. Discussion

In bivalves, reproduction is strongly influenced by the interaction between endogenous and exogenous factors, such as temperature (Matias et al., 2013), which also indirectly affects food availability (Matias et al., 2025). The distinct pattern observed in temperature variation across the two sampling years, with a greater thermal range in the second year was, in general, reflected in the reproductive status of the oysters. This was evident in both gonadal index (GI), which indicates the stages of gonad maturation, and the gonadosomatic index (GSI), reflecting the investment in reproductive tissue. Interestingly, the highest GI values were observed during the first year, which may be partly explained by a higher food availability then, despite the lower-than-usual spring temperatures. This combination of colder waters and enhanced primary productivity may be indicative of an upwelling event, typically characterized by nutrient-rich waters that promote phytoplankton blooms (Loureiro et al., 2005), thereby supporting reproductive investment despite less favourable thermal conditions. Conversely, in the second year, temperature strongly influenced gonadal development, leading to greater heterogeneity of maturation stages during the spawning season. Nevertheless, the results of this study strongly support the occurrence of two spawning events within each reproductive season, mainly during the first year, as previously suggested for this species (Massapina et al., 1999). These results also indicate that the reproductive cycle follows a seasonal pattern in accordance with temperature and chlorophyll *a* variation. However, during the second year, spawning occurred in successive partial events that were less pronounced, which may be explained by the correspondingly weaker temperature and chlorophyll *a* peaks.

Condition index (CI) reflects the physiological condition of the oysters, and is closely linked to reproductive activity (Castro and de Vido de Mattio, 1987; Massapina et al., 1999; Ojea et al., 2004). In this study, CI showed clear seasonal fluctuations, as observed for other bivalves, where CI varies in close association with environmental factors and with reproductive cycle (Ojea et al., 2004). The strong correlation between CI and temperature highlights temperature as one of the main environmental factors mediating physiological condition and reproductive cycle, as suggested for a wide variety of bivalves (Delgado and Pérez Camacho, 2007; Gomes et al., 2014; Massapina et al., 1999). However, the less consistent and significant correlations observed in the second year, may indicate interannual differences in physiological responses to environmental factors, due to variability in food availability and differences in the temperature range. The correlation between CI and reproductive parameters (GI and GSI) indicates that individuals with better physiological condition are likely to show higher gonadal development and, consequently, higher reproductive investment. This strongly suggests the condition index as a good indicator of reproductive condition in bivalves (Yildiz et al., 2011).

The close relationship between physiological condition and gonadal development suggests that reproductive processes are strongly dependent on energy acquisition and allocation (Matias et al., 2013; Ojea et al., 2004). In bivalves, a seasonal cycle of energy storage and mobilization linked to sexual maturation is commonly observed (Joaquim et al., 2023; Sühnel et al., 2012). Typically, energetic reserve storage increases before the reproductive peak and decreases after spawning due to the mobilization of such reserves for gonadal development and gamete release (Matias et al., 2013). Energy supply may result from either previously stored reserves, in the form of protein, glycogen and lipids (Ojea et al., 2004), or from the ingestion of available food, which is closely reflected in the biochemical composition (Pérez-Camacho et al., 2003). Bivalve species can be characterized according to different reproductive energy allocation strategies. Some bivalves store glycogen when food is abundant and gametogenesis occurs when food availability is low, thus allowing the first spawning as temperature increases – this is a conservative strategy. In other species, gamete production occurs from spring onward, linked with the first phytoplankton bloom – opportunistic strategy (da Costa et al., 2012). In the current study, bivalves have employed an opportunistic strategy, in which the onset of gametogenesis was coupled with several phytoplanktonic blooms, allowing to a restoration of the reserves after spawning and, consequently, a constant gonadal regeneration (Massapina et al., 1999; Matias et al., 2013, 2025).

Protein was the predominant biochemical component throughout the sampling period, followed by total lipid and glycogen. This pattern is commonly reported for bivalves and reflects the structural and metabolic importance of proteins in somatic and gonadal tissues (Joaquim et al., 2011; Matias et al., 2025). Protein content remained relatively constant throughout the sampling period, and the absence of significant correlations with GI, GSI and CI strongly supports the role of proteins as a structural component rather than an energy reserve (Albentosa et al., 2007; Beninger and Lucas, 1984). Similar patterns have been observed in other bivalves (Joaquim et al., 2008; Matias et al., 2011, 2013). Glycogen is the preferential form of energy reserve in oysters (Pogoda et al., 2013) and, together with total lipid, constitutes the main energetic reserve for gametogenesis (Matias et al., 2016). After an initial period of storage, glycogen is simultaneously used with food as energy support for gametogenesis (González-Araya et al., 2011; Zhicui et al., 2006). Glycogen content exhibited moderate seasonal fluctuations and interannual differences, which is consistent with its role as a rapidly mobilized energy reserve (Gómez-Valdez et al., 2021). The lower glycogen levels observed during summer months, in particular during the second year, probably reflect its utilization to support reproductive activity, as suggested for other bivalves (Joaquim et al., 2014, 2023; Li et al., 2009). Total lipid also plays a crucial role in reproduction, being associated with gamete formation (Benomar et al., 2010; Delgado et al., 2004; Mouneyrac et al., 2008). The positive correlation between total lipid and glycogen content, mainly in males during the second year, may suggest that both components act synergistically as energy reserves for reproductive processes.

Biochemical dynamics appeared to differ between sexes, with sex-specific patterns observed in the relationships among biochemical components and environmental variables. In males, a negative correlation between total lipid and protein content was observed when both sampling years were analysed together; however, this relationship was not maintained when years were analysed separately. Therefore, the observed pattern may reflect interannual variability rather than a physiological association between these biochemical components. In contrast, the positive correlation between lipids and temperature found in females during the first sampling year may reflect an increase in energetic demands for oogenesis (Gómez-Valdez et al., 2021). The observed variation in biochemical composition emphasises the influence of both environmental variability (temperature) and sex on reproductive energetic processes. These findings highlight the importance of an integrated approach combining biochemical composition, reproductive indices, and environmental factors to better understand and fully

characterize the energetic demands, constraints, and adaptive strategies underlying bivalve reproductive cycles.

However, the characterization of the reproductive cycle in this species, although already well-established (*e.g.* Fabioux et al., 2005; Massapina et al., 1999), was primarily intended to provide a biological context to address the question: "Do vertebrate-type steroids play a role in bivalve reproduction?". Firstly, it is noteworthy that, despite the complexity of the oyster homogenate matrix, the recovery rate was within the acceptable ranges for complex matrixes (European Commission, 2021). The variability observed within the matrix effects reinforce that quantification requires a careful evaluation of the matrix effects. The fact that it was not possible to quantify 17β-oestradiol, similar to Gust et al. (2010), this does not necessarily mean that it is not present; this may represent an additional limitation to the interpretation of results, due to its importance in reproduction (*e.g.* Gauthier-Clerc et al., 2006; Ketata et al., 2007). Future work should focus on a better optimization of the methods, both LC-HRMS and the extraction, in order to "clean" the matrix. This would be crucial to obtain clearer results, with less variability, which could increase the percentage of values above LOQ, which in this study was approximately 38 % for both progesterone and testosterone.

Although both steroids fluctuated over the sampling period, with levels in accordance with what was reported for other bivalves (Martínez-Pita et al., 2012; Smolarz et al., 2018; Zabrzańska et al., 2015), there was no clear association with the reproductive cycle. Nevertheless, some correlations between hormones and reproductive parameters were observed, warranting further consideration.

From a general perspective, the positive correlation observed between progesterone levels and gonadal index (GI), which reflects the gonadal development, may suggest that progesterone is linked to reproductive activity. In contrast, no correlation was found between testosterone and GI, thereby indicating that this hormone may not be directly associated with gonadal maturation. Moreover, the similar patterns observed in both sexes suggest that these steroids may be more closely related to the overall physiological status than to sex-specific reproductive processes.

In the current study, temperature showed positive correlations with both steroids, predominantly during the second year and exclusively in males. This pattern may be explained by the higher thermal amplitude observed during the second year, which may have acted as a physiological threshold, particularly in males, in which spermatogenesis is, in general, faster and more sensitive to environmental stimuli (Galtsoff, 1930). Both steroids may be indirectly responding to the increase of metabolic and reproductive activity induced by temperature (Smolarz et al., 2018; Zapata-Restrepo et al., 2019).

Furthermore, the association observed between chlorophyll *a* and testosterone, exclusively during the second year, may be indicative of an accumulation of testosterone via filtration and particle ingestion. In fact, it has been suggested that phytoplankton can bioaccumulate testosterone from the water and biodegrade it (Fu et al., 2019; Gong et al., 2014). Phytoplankton blooms can also be a source of natural endocrine disrupting chemicals, such as testosterone, therefore contributing to the hormonal levels in seawater and, due to the filter-feeding-nature of bivalves, bioaccumulate in the food web (Gong et al., 2014).

If considering an exogenous origin of these vertebrate-type steroids, as suggested by Scott (2012), and the lack of functional classical receptors and key enzymes in the molluscan genome (Fodor et al., 2020), the above-mentioned correlations, most likely, reflect patterns of environmental exposure and bioaccumulation in fat tissues (Scott, 2012) rather than endocrine regulation. This is further supported by the correlation detected mainly between progesterone and protein content, predominantly during the second year. This correlation, together with its association with GI, temperature and the fact that higher maturation stages (III and IV) showed higher progesterone levels, may suggest that progesterone is linked to gonadal tissue development and overall energetic investment. However, this relationship does not correspond to the behaviour expected for a classical sex steroid hormone, as such hormones do not typically fluctuate in parallel with tissue protein content (Mani et al., 2012; Wierman, 2007). Instead, progesterone may act as a physiological or metabolic marker associated with reproductive condition rather than as a direct regulator of gonadal maturation.

Taken together, these results, although potentially indicative of an association between progesterone and the reproductive cycle of bivalves, should be interpreted with caution. Progesterone accumulation in gonadal tissues likely reflects its affinity for lipophilic tissues (McManus et al., 2019; Scott, 2018), rather than a classical endocrine role in reproductive regulation. This could explain the higher concentrations observed in advanced maturation stages. Similarly, the absence of meaningful correlations involving testosterone suggests that this is primarily influenced by environmental factors.

Furthermore, the lack of a clear sex-specific pattern in steroid levels suggests that, in bivalves, the endocrine regulation may not be sex-specific and may involve non-steroid hormones, differing from that of vertebrates. This is further supported by the lack of evidence for functional and classical steroid receptors and key enzymes in the molluscan genome (Fodor et al., 2020; Scott, 2012). Nevertheless, a potential role of 'enzyme promiscuity' (Atkins, 2015; Lathe et al., 2015) should be considered, as it may enable the biotransformation of different dietary sterols through non-canonical pathways into compounds structurally analogous to testosterone and progesterone (Fodor et al., 2025; Scott, 2012). This may explain the results obtained by several other researchers (*e.g.* Gauthier-Clerc et al., 2006; Ketata et al., 2007; Martínez-Pita et al., 2012; Siah et al., 2002; Zhu et al., 2018), who observed a clear association with the reproductive cycle by employing different techniques (ELISA or RIA). One of the major limitations of these techniques is the presence of matrix effects and cross-reactions, in which the apparent concentration of the steroid may be affected by compounds extracted along with the steroids (Scott, 2012). Additionally, such assays are usually developed for use with human or other mammalian fluids; often, they are not validated for use with invertebrate fluids. In addition, steroid antibodies are not 100 % specific, and structurally similar compounds may compete with the labelled steroid for the antibody binding site, potentially binding in its place with equal or lower affinity (Scott, 2012).

Moreover, Scott (2012) suggested that these hormones can be absorbed from the environment and be retained in the fat tissues. This is strongly supported by our results, which suggest an exogenous origin, while also reflecting regulation by bivalve physiology. In this respect, it is pertinent to add that the peak of both steroids observed in both sexes during the second year (2022), starting in June, was not seen in the first year (2021). This highlights interannual variability in environmental exposure, suggesting that these steroids may originate from natural sources, such as fish and birds, or from anthropogenic inputs.

This study contributes to the growing body of evidence suggesting that vertebrate-type steroids in bivalves should be interpreted within an eco-physiological context rather than a classical endocrine one, meaning that steroid levels reflect a combination of environmental exposure, physiological condition, and energetic status rather than direct endocrine control. To our knowledge, this is the first study analysing two consecutive reproductive cycles together with the steroidal pattern.

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CRediT authorship contribution statement

Ana Rato: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Sandra Joaquim:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing. **José P. Da Silva:** Methodology, Writing – review & editing. **Catarina Anjos:** Methodology, Writing – review & editing. **Domitília Matias:** Conceptualization, Funding acquisition, Writing – review & editing. **Peter C. Hubbard:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing.

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.2026.108202.

Data availability

Data will be made available on request.

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