Aquaculture
January 2024, Volume 578, Pages 740080 (10p.)
https://doi.org/10.1016/j.aquaculture.2023.740080
https://archimer.ifremer.fr/doc/00853/96522/

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Examination of the effects of highly diluted bioactive compounds on gametogenesis in relation to energy budget and oocyte quality in mussel (*Modiolus capax*) broodstock

García-Corona José Luis [1, 2], Arcos-Ortega Guadalupe Fabiola [1], Rodríguez-Jaramillo Carmen [1], López-Carvallo Jesús Antonio [1, 3], Mazón-Suástegui José Manuel [1, *]

[1] Centro de Investigaciones Biológicas del Noroeste (CIBNOR, S.C.). Av. IPN 195 Col. Playa Palo de Santa Rita Sur, La Paz, Baja California Sur 23096, Mexico
[2] Institut Universitaire Européen de la Mer, Laboratoire des Sciences de l'Environnement Marin (UMR6539 CNRS/UBO/IFREMER/IRD) Technopôle Brest-Iroise, Plouzané 29280, France
[3] Departamento de Acuicultura, Facultad de Ciencias del Mar, Campus Guayacán, Universidad Católica del Norte, Coquimbo 1781421, Chile

* Corresponding author : José Manuel Mazón-Suástegui, email address : jmazon04@cibnor.mx

Jose.Corona@univ-brest.fr ; farcos04@cibnor.mx ; jaramilo04@cibnor.mx

Abstract :

A critical bottleneck for bivalve hatcheries is the availability of mature broodstock with high-quality gametes to ensure the production of healthy spat. The use of highly diluted bioactive compounds (HDBC) has emerged as a novel and prophylactic strategy to improve gonad development, reproduction bioenergetics, and oocyte quality of marine bivalve broodstock. This study evaluates the effect of HDBC formulas to enhance the laboratory-controlled breeding capacity of Modiolus capax adult mussel, selected as a model organism because of its aquaculture potential as an alternative seafood resource. The experimental design included three replicates of three HDBC treatments at 31Centesimal (1×10^{-31}) dynamizations using distilled water as the final excipient (TR1: SiT, CaS, HeS; TR2: PhA, FeP, ZnP; and TR3: ViP, ViA, ViT), and two control treatments: C+ hydro-alcoholic solution at 1% (Usual HDBC vehicle) and C- (distilled water) during a 30-day period. As a result, TR1 enhanced energy reserves (lipids and carbohydrates) in all tissues; TR2 promoted an increase in lipid and carbohydrate content in the gonad and their transfer into developing oocytes; TR3 significantly improved gonad development and reproductive capacity of the mussels with the highest proliferation of mature oocytes, enhancing food-nutrient assimilation and energetic reserve transfer from the somatic tissues to the ovary. Additionally, all HDBC formulas recorded a lower ($P < 0.05$) number of atretic oocytes compared to the untreated animals. This evidence reflects a simultaneous improvement of gametogenesis and energy budget of M. capax broodstock, ensuring sexual maturation and higher-quality oocytes when HDBC are used.

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Highlights

► The use of HDBCs reflects a simultaneous improvement of gametogenesis and energy storage of *M. capax* broodstock. ► An acceleration of sexual maturation and higher-quality oocytes were observed when HDBCs are used. ► The use of HDBCs is an innovative strategy to improve the current gonad conditioning protocols in the laboratory. ► HDBCs have proven to have quantifiable effects on the reproductive capacity of a native marine bivalve with high aquaculture potential.

Keywords : Modiolus capax, highly diluted formulas, gonad development, bioenergetics of reproduction, oocyte yield.

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1. Introduction

Bivalve shellfish aquaculture is one of the most profitable and sustainable seafood-production activities today (van der Schatte Olivier, 2020; FAO, 2020). Unfortunately, the natural banks of the most economically valuable bivalve species in the Baja California peninsula have been depleted over the years due to the intense fishing effort on their populations (Soria *et al.,* 2014; Ruiz-Verdugo *et al.,* 2016). Notwithstanding, other bivalve species with high nutritional value that are not commercially exploited are available in the region, which represents a potential alternative for fisheries and aquaculture.

The horse-mussel *Modiolus capax* is a native species from the Gulf of California that offers high potential to develop a sustainable aquaculture industry for commercial production, given its abundance and wide distribution on the Eastern Pacific coast (Farfán *et al.,* 2007). Recently, García-Corona *et al.* (2018) developed a detailed work on the reproductive biology of this species in La Paz Bay, Baja California Sur (BCS), México, offering knowledge about seasonal energy storage dynamics and oocyte quality during gametogenesis of wild populations. Although *M. capax* seems to maintain breeding capacity throughout the year (García-Corona *et al.,* 2018), its production cannot be completely based on the exploitation of its natural beds, which represent an important ecological spot for the recruitment of wide marine biodiversity (van der Schatte Olivier, 2020). Hence, the development of sustainable management strategies for hatchery seed production of this species is encouraged. Nevertheless, a limiting factor during bivalve spat production is the unavailability of mature broodstock on an annual basis with high-quality gametes to ensure healthy seed yields (Mazón-Suástegui *et al.,* 2005, López-Carvallo *et al*., 2017; Mazón-Suástegui *et al.*, 2022).

The use of artificially enriched microalga diets with carbohydrate-rich supplements (e.g. cereal flours) has been proposed as an effective strategy to meet the energy demands during gametogenesis of hatchery-conditioned *M. capax* broodstock outside the main breeding

season (López-Carvallo *et al.,* 2017). These kinds of enriched diets seem to be reliable in fueling gonad development in *M. capax* broodstock. Nonetheless, the authors reported prolonged sexual maturation periods (~ 60 days). These zootechnical protocols in aquaculture facilities can be costly when delays in gonad development occur, as they usually do in tropical bivalves (Mazón-Suástegui *et al.,* 2008; Arellano-Martínez *et al.,* 2011; Rodríguez-Jaramillo *et al.,* 2017).

The use of highly diluted bioactive compounds (HDBC) has recently emerged as a useful technique to improve survival, growth, immunomodulatory response, stress reduction, energetic metabolism, and reproduction of numerous marine species (mollusc, shrimp, and fish), of interest for aquaculture activity (Ortiz-Cornejo *et al.,* 2017; Mazón-Suástegui *et al.,* 2018a, López-Carvallo *et al*., 2021). The prophylactic use of HDBC has also improved nutrition, overall condition, and increased marine organism survival after challenges against pathogens (López-Carvallo *et al.,* 2020, 2021). Since HDBC are innocuous, inexpensive, and easy to use by immersion or ingestion, these formulas represent a sustainable alternative to improve marine organism production under hatchery conditions (López-Carvallo *et al.,* 2019, 2020, 2021; García-Bernal *et al.* 2020; Mazón-Suástegui *et al.,* 2020, 2022).

Despite these kinds of compounds have been scarcely applied in marine species, significant progress has been made in the last five years regarding the understanding of the biological effects of using HDBC on fish, crustaceans, and especially marine bivalves (Ortiz-Cornejo *et al.,* 2017; López-Carvallo *et al.,* 2021). There is experimental evidence that the use of HDBC based on *Silicea terra* (SiT), and *Phosphoricum acid* (PhA) improves the overall physiological condition in shrimp *Penaeus vannamei* (Mazón-Suástegui *et al.,* 2018b). Additionally, HDBC have also shown beneficial effects on the digestive activity of the mackerel *Seriola rivoliana* (Mazón-Suástegui *et al.,* 2019), and the survival and antioxidant response of *P. vannamei* challenged against the pathogenic bacteria *Vibrio parahaemolyticus*

(Mazón-Suástegui *et al.,* 2018b), while the commercial formula Passival® (PaV) has shown to increase the energy reserves and immune response of the marine snapper *Lutjanus gutattus* infested with parasites (Rosero-García *et al.,* 2019).

Concerning marine bivalves, in *Argopecten ventricosus*, Mazón-Suástegui *et al.* (2017) improved the survival, growth, immune response, and antioxidant defense of the spat treated with the SiT and PhA formulas and subsequently challenged against *V. alginolyticus*. Moreover, López-Carvallo *et al.* (2019, 2020) attained positive effects on the immunomodulatory response, antioxidant enzyme production, general condition index, and energy storage in juveniles of *A. ventricosus* when using three different HDBC, including *Vibrio* lysates, the scorpion venom VidatoX® (ViT), and the compounds based on SiT and PhA. Finally, recent evidence suggests that the use of HDBC formulated by SiT, ViT, and PaV optimizes *A. ventricosus* gonad ripening, food-nutrient management, and oocyte quality and reduces the time required to achieve gonad ripening (accelerating gametogenesis) of the treated organisms (Mazón-Suástegui *et al.,* 2020).

To our knowledge, no study has analyzed the potential use of HDBC formulas as a therapeutic strategy to improve the reproduction of mussels, which could be an alternative for the aquaculture industry. Therefore, the main objective of this research was to assess under laboratory conditions, three HDBC complexes to measure whether they could improve *M. capax* broodstock gametogenesis, energy budget, and oocyte quality, by using a carbohydrate-enriched diet proposed by López-Carvallo *et al.* (2017) to achieve gonad maturation for this species.

2. Materials and methods

2.1. Mussel acquisition and management

The 450 adult mussels (7.4 ± 0.2 cm shell length; 41 ± 5.2 g total weight) used in this study were collected from a natural bed in La Paz Bay, Baja California Sur on the western coast of the Gulf of California, Mexico (24° 17' 02.6 " N; 110° 20' 05.2" W). The collection of the organisms was carried out in early October 2014, which corresponds to the end of this species gametogenic cycle in La Paz Bay (García-Corona *et al.*, 2018).

Once in the laboratory, the mussels were scrubbed to remove epibionts and placed in a 1500 L fiberglass tank with a continuous flow of filtered seawater (1 µm) at 19 ± 1 °C and salinity of 35 UPS to emulate the environmental conditions from the collection site. Continuous aeration and food supply of a blend of the microalgae *Tisochrysis lutea* and *Chaetoceros calcitrans*, 1:1, at a concentration of 2.3×10^9 cells mussel^{-1} day^{-1}, was provided. The broodstock was maintained under these conditions for 72 h before they were subjected to sequential thermal shocks of 18 and 28 °C (30 min each), until spawning of the residual gametes in the gonads to restart the gametogenesis process. Ten mussels were sampled after spawning induction for histological purposes to verify the immaturity of the organisms before the experiment began.

2.2. Experimental design and drug formulation

The experimental conditions of this work were based on the gonad-conditioning protocol previously described by López-Carvallo *et al.* (2017) for *M. capax* with modifications. Briefly, the broodstock was randomly placed in 15 fiberglass maturation units of 80 L. Each unit was considered as a replicate and held 30 adult mussels. All treatments were set in triplicate. The maturation units were adapted with a running-flow system of filtered (1 µm) ultraviolet (UV)-sterilized seawater (35 UPS, pH 7.1, 24 °C) and maintained with constant aeration. All the mussels were daily feed with a carbohydrate-rich diet composed of a 1:1 blend of the microalgae *T. lutea* and *C. calcitrans* at 4% of the soft tissue dry weight, mixed

with 3% of the tissue dry weight in wheat flour (Espuma de Chapala®, Zapopan, Mexico). Water changes of 60% of the total volume of each unit were performed daily. The conditions described above were defined by Lopez-Carvallo *et al.* (2017) to achieve *M. capax* gonad maturation under controlled conditions in less than 60 days.

Three HDBC treatments were formulated from hydroalcoholic (87°) commercial drugs at potency 30 Centesimal (1×10^{-30}) dynamization. TR1 consisted of centesimal (C) preparations (1:100 dilution/succussion) of the commercial drugs *Silicea terra* (SiT, Similia®, CDMX, Mexico), *Calcarea sulphurica* (CaS, Similia®, CDMX, Mexico), and *Hepar sulphuris* (HeS, Similia®, CDMX, Mexico); TR2 consisted of centesimal (C) preparations (1:100 dilution/succussion) of the commercial drugs *Phosphoricum acid* (PhA, Similia®, CDMX, Mexico), *Ferrum phosphoricum* (FeP, Similia®, CDMX, Mexico), and *Zincum phosphoricum* (ZnP, Similia®, CDMX, Mexico); TR3 consisted of centesimal (C) preparations (1:100 dilution/succussion) of HDBC developed at Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico from *Vibrio parahaemolyticus* (ViP, CIBNOR, Mexico) and *V. alginolyticus* (ViA, CIBNOR, Mexico) lysates, and the centesimal (C) preparation (1:100 dilution/succussion) of the commercial drug VidatoX® (ViT, Labiofam®, Habana, Cuba), based on the venom of the scorpion *Rhopalurus junceus*. All working solutions were diluted in distilled water to have a final potency of 31C (1×10^{-31}) and prepared according to Mazón-Suastegui *et al.* (2020). Distilled water was used as the final dilution vehicle to prevent potential ethanol side effects. Additionally, two control treatments were assessed, positive control (C+) hydro-alcoholic solution at 1% (Usual HDBC vehicle), and distilled water as negative control (C-). Treatments that consisted of three HDBC formulations and controls were supplied directly to seawater in each experimental unit (100 $\mu L\ L^{-1}$). The water and food flow were cut 3 h a day to ensure treatment uptake through the epithelial tissues of the animals while food reservoir tanks were cleaned and refilled.

Broodstock mussels were kept under experimental conditions for 30 days before being sampled. Because López-Carvallo *et al*., 2017 assessed *M. capax* maturation for 60 days, remanent organisms were maintained under the same experimental conditions until day 60. Nonetheless, after day 35, sequential spawning was observed in all replicates of the animals treated with HDBC. Therefore, only the animals sampled on day 30 were analyzed. Randomly sampled animals were placed on crushed ice to reduce metabolism and sensitivity during dissections. Afterward, the whole meat was carefully excised from the shell and ~300 mg of gonad tissue was stored at -80 ± 1 °C for biochemical analyses. The remaining soft-body was fixed in Davidson solution (Kim *et al.*, 2006) for histological examination. As *M. capax* females exhibit the highest reproductive effort within wild populations of this species (García-Corona et al., 2018), only females were selected for subsequent analyses.

2.3. Quantitative histology and histochemistry

Transversal sections (~ 5 mm) of the mid visceral mass from 30 mussels of each experimental condition were dehydrated in progressive series of ethanol and cleared with xylene. The samples were then embedded in paraffin (Paraplast X-Tra; McCormick Scientific, San Diego, CA) and thin-sectioned (4 µm) using a microtome (Leica RM 2155, Leica Microsystems, Switzerland). Afterward, a series of three consecutive tissue sections from each individual were mounted on glass slides and stained either with (1) hematoxylin & eosin (H&E) for general gametogenesis description; (2) Periodic acid-Schiff (PAS) for the visualization of neutral glycoconjugates in magenta tones; and (3) Sudan black (SB) for detection of neutral lipids in black-bluish hues.

Each slide was first examined using a light microscope (Olympus BX50, Olympus Optical, Japan) for the description of the overall gonad development under the different treatments. The oocytes were classified according to their developmental stages: oogonias,

previtellogenic, vitellogenic, postvitellogenic, and atresic oocytes (García-Corona *et al.*, 2018). Their frequencies were assessed in three randomly selected regions of the ovary in a predetermined area of 1.44 mm^2 (magnification 20×) (Rodríguez-Jaramillo *et al.,* 2008). The gonad coverage area (GCA) was determined using three randomly selected images of mussel ovaries digitalized at high resolution (600 DPI, 7.9 mm^2, 4×) and processed using an image system analyzer (Image-Pro Premier v.9.0 software, Media Cybernetics, Bethesda, MD, USA). The software relies on automatic area calculations (μm^2) occupied by the gonad, based on the segmentation of pixels of the image in relation to the intensity of the specific colors of the H&E staining, and the total area of the image (Rodríguez-Jaramillo *et al.,* 2008). GCA was assessed as follows:

$$GCA\% = \frac{area\ occupied\ by\ the\ gonad}{total\ area\ of\ the\ image} \times 100$$

The gonad development index (GDI) was calculated as the ratio of postvitellogenic oocytes in relation to the total number of oocytes present in the gonad (Rodríguez-Jaramillo *et al.,* 2017).

To assess the energy budget of the broodstock during gametogenesis, the tissue sections stained with PAS and SB were digitized in triplicate (1.44 mm^2, 20×) and analyzed using the software Image-Pro Premier v.9.0. The analysis is based on automatic calculations of the area (μm^2) occupied by neutral lipids and neutral carbohydrates from the segmentation of image pixels in relation to the specific color intensity of the technique used to stain the tissues. The lipid (LI), and carbohydrate (CHI) indices of the main tissues (gonad, digestive gland, and muscle) related to the reproduction bioenergetics of this species (García-Corona *et al.,* 2018) were measured according to Rodríguez-Jaramillo *et al.* (2022) as follows:

$$\tau_{index} = \frac{\tau}{\beta} * 100$$

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Where τ represents the coverage area of each specific stain and β is the total area of the image.

Calculations of theoretical diameter (TD), nucleus/cytoplasm ratio (N/C), as well as lipid and carbohydrate index in oocytes were used as gamete-quality indicators (García-Corona *et al.*, 2018). For this purpose, three random digital images per ovary were digitalized and processed with Image-Pro Premier v.9.0. The oocytes TD was determined at 20×. Approximately 20–30 oocytes from each category with a visible nucleus were measured per image; then, TD was calculated from the total area (A) of each oocyte (Saout *et al.*, 1983):

$$TD = \sqrt{4A/\pi}$$

The N/C was automatically calculated with the Sigmascan software (Systat Software, San Jose, CA, USA) as follows:

$$N/C = \frac{area\ of\ the\ nucleus}{total\ area\ of\ the\ oocytes} \times 100$$

The ovary images (20×) from PAS and SB stain were used to quantify the area occupied by lipid droplets (LIo), and carbohydrates granules (CHIo) within a total number of ~325 vitellogenic, postvitellogenic and atretic oocytes per treatment (García-Corona *et al.*, 2018):

$$LIo = \frac{area\ occupied\ by\ lipid\ droplets\ within\ oocytes}{total\ area\ of\ the\ oocytes} \times 100$$

$$CHIo = \frac{area\ occupied\ by\ carbohydrate\ granules\ within\ oocytes}{total\ area\ of\ the\ oocytes} \times 100$$

2.4. Biochemical analyses

Quantification of the total lipids, carbohydrates, and proteins was carried out according to Arcos-Ortega *et al.* (2015) in the ovaries of 30 organisms per experimental condition. The frozen tissue samples were lyophilized and homogenized individually in a cold saline buffer

(0.05 M Tris, 0.5 M NaCl, 5 mM ethylenediamine tetra-acetic acid, pH 7; 3:1 w/v, tissue: buffer). Then, the extracts were centrifuged at 10,000 *g* at 4 °C for 15 min, and the supernatant was stored at –80 °C for quantification of (1) total lipids, according to the sulfo-phospho-vanillin method (Barnes & Blackstock, 1973); (2) total carbohydrates, by the anthrone method (Roe, 1955); and (3) total proteins, determined according to Bradford (1976) after digestion with 0.1 N NaOH, and using the BCA reagent (Sigma-Aldrich, St Louis, MO, USA) and bovine serum albumin (Sigma-Aldrich, USA) as standard. All the biochemical components were quantified colorimetrically in small microplates at wavelengths of 540, 630, and 562 nm, respectively.

2.5. Statistical analyses

All data analyses were computed using command lines in the programming environment R (R v. 4.2.2, R Core Team, 2020). Shapiro–Wilk and Bartlett's tests were applied to confirm normality and homogeneity of variances of the residuals, respectively (Hector, 2015). When needed, data were transformed ($\log$, $1/\chi$, or $\sqrt{\chi}$) prior to the analysis to meet *a priori* assumptions. Separate unifactorial analyses of variance (ANOVA) were applied to each response variable as a function of the five experimental conditions. As needed, *a posteriori* Tukey's honestly significant difference (HSD) test was applied between means (Zar, 2010). Prior to analysis, an angular transformation (arcsine $\sqrt{P}$) was applied to the values expressed in percentages, but all data are reported untransformed as the mean ± standard error (SE). The principal component analysis (PCA) was performed using the FactoMineR package with the factoextra package for data visualization into smaller factorial clusters within a 95% confidence interval. All data matrices were auto-scaled before the PCA analysis. The corrplot package was run to calculate the correlation coefficients and their significance between

variables in their given PCs. The level of statistical significance was set at $\alpha = 0.05$ for all the analyses (Zar, 2010).

3. Results

3.1. Gonad development

The observations of gonad tissue showed that broodstock mussels treated with HDBC (TR1, TR2, TR3) yielded the highest overall reproductive condition in terms of gonad ripening, energy storage, and oocyte quality compared to the control groups C+ and C- (Fig 1). In the ovaries of HDBC-treated mussels, large ovarian follicles with abundant pedunculated vitellogenic oocytes (Vto) attached to the follicular epithelium were observed. Postvitellogenic oocytes (Psvo) with a characteristic polyhedral morphology, rich-ovoplasm amounts, and conspicuous nucleus were predominant in the lumen of the follicles, while few atretic oocytes (Ato) with an irregular morphology were found in the follicular lumen of treated organisms (Fig 1A-C). As shown in Fig. 1D-E, the ovarian follicles of the control mussels were large but with wide empty areas due to the lowest number of Vto and Psvo, with a high presence of atretic oocytes.

The cytoplasm of Vto and Psvo of the animals from TR1, TR2, and TR3 had a higher content of carbohydrate granules compared to those observed in the control treatments (Fig. 1F-H). In the mussels from C+ and C- the Vto and Psvo had poor amounts of ovoplasm; the connective tissue surrounding the ovarian follicles was abundant, and the staining of glycogen was mainly localized in the reabsorbed Ato (Fig. I-J). Finally, as observed in Fig. 1K-M, both Vto and Psvo were rich in lipid droplets within their cytoplasm in HDBC-treated females, whereas the dyes corresponding to lipids were scarce in both connective and germinal tissues of the non-treated animals (Fig 1N-O).

3.2. Reproductive capacity

The quantitative histological analysis of the ovary sections allowed an accurate estimation of the gonad development during the gametogenesis of the mussels after the experiment. As shown in Fig. 2A, the proliferation of oogonia (Oo) in the ovarian tissue was significantly higher in the organisms treated with TR1 (22.8 ± 3.1 oocytes/2.88 mm^2), whereas the highest frequency of Pvo occurred in the treatments TR1 and TR3 (~7 oocytes/2.88 mm^2; P <0.05). Notwithstanding, no significant differences were found in the Vto frequencies between the treatments and controls. The number of Psvo significantly peaked in the females that received the TR3 complex (48.7 ± 2.2 oocytes/2.88 mm^2) followed by those of TR1 and TR2 (~37 oocytes/2.88 mm^2). The lowest frequency of Psvo was found in the mussels of the control groups (~28 oocytes/2.88 mm^2; P <0.05). Additionally, the mussels of both control groups showed the highest number of Ato per area (~16 oocytes/2.88 mm^2; P <0.05) compared to organisms treated with HDBC (~6 oocytes/2.88 mm^2).

The gonad development measurements (Fig. 2B) showed a significantly greater gonad coverage area (GCA) obtained with TR2 (70.5 ± 0.9%), followed by TR3 and TR1 (68 ± 0.5%, and 66.4 ± 0.6%, respectively). Notably, the gonad development index (GDI) revealed that the gametogenic yield was significantly higher in the females from treatments TR3 (59.1 ± 1.9 %) and TR2 (57.1 ± 2.5 %). The gonad development of the mussels was significantly lower in the controls for both measurements, GCA and GDI (~59%, and ~42%, respectively).

3.3. Bioenergetics of reproduction and oocyte quality

Changes in energy management (from the histochemical and biochemical analyses) in the main tissues related to reproduction according to the use of HDBC are shown in Table 1. In

the ovary, the highest neutral lipid index (LI) occurred in the mussels treated with TR1 and TR3 (P <0.05) compared to both control groups, without significant differences in the neutral carbohydrate index (CHI) between HDBC-treated and control mussels. Although the highest amounts of total lipids (TL) and total proteins (TP) contents were recorded in TR3 organisms, and the maximum quantity of total glycogen (TG) content was recorded in the mussels from TR2, no significant differences were observed in the budget of these reserves in the gonads of HDBC-treated *vs* control females.

In the digestive gland, the LI was significantly higher in the organisms from TR3, with the lowest values (P <0.05) in mussels from both control groups. CHI showed the highest amounts of neutral carbohydrates in the mussels from TR1 and the lowest amount in females from the control groups. Furthermore, a significantly higher LI was found in the muscle of the animals that received TR1 compared to all the experimental conditions, while the mussels from TR1 and TR2 showed a higher CHI (P < 0.05) in this tissue compared to the organisms from both control groups.

Table 2 summarizes several oocyte-quality features evaluated according to the treatment and the oocyte developmental stage. The significantly highest TD was recorded for Vto in the organisms treated with the three HDBC, while the mussels treated with TR3 yielded the highest (P < 0.05) TD for Psvo. The significantly lowest TD values for Vto and Psv were observed in the mussels from the control groups. The amount of ovoplasm in the oocytes, measured as the N/C ratio, was higher (P < 0.05) in the Vto of the TR1 females compared to the rest of the treatments and controls. The proportions of energetic substrates (LIo and CHIo) in the ovoplasm of Vto and Pso significantly peaked in females treated with TR2 formula. Whereas the lowest (P < 0.05) LIo and CHIo in Psvo were observed in females from C- (non-treated mussels).

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3.4 Integrative analyses compiling reproductive and bioenergetic response to HDBC

The principal component analysis (PCA) was computed to summarize the differences between treated and control conditions using all the response variables measured in this study (Fig. 3). The PCA described more than a third (38.6%) of the total variance of the data along the first two principal dimensions. For the whole data set, clustering-PCA provided a clear distinction between treatments and controls (Fig. 3A). In the scatter plot, TR1 was separate from the rest of the experimental conditions. TR2 and TR3 have similar scores on the principal components and were different to TR1 and both control groups. Both control groups (C+ and C-) were similar to each other and grouped apart from all the HDBC treatments (Fig. 3A). As shown in Fig. 3B, dimension 1 (25.1% of the total variance) mainly explained the lipid index in the gonad (GONLI) and digestive gland (DGLI), as well as Oo proliferation, the GCA, and the TD of the oocytes. In this PCA, the GONLI and DGLI were strongly and positively correlated to the GCA (r =0.6; $P < 0.05$) and the TD of oocytes (r =0.5; $P < 0.05$), while Ato frequency was negatively correlated (r =-0.4; $P < 0.05$) to these variables. In this analysis, GDI, lipid content in the muscle (MLI), as well as the carbohydrates in the gonad (GONCHI) and digestive gland (DGCHI) were the strongest correlated variables to dimension 2 (13.5% of the explained variance). As observed in Fig. 3, TR1 was positively associated with higher GONCHI, DGCHI, and MLI, while TR2 and TR3 were positively associated with lower CHI in the ovary and digestive gland. However, a higher amount of lipids in these two tissues was observed, as well as the highest gametogenic yield (GCA and GDI). In contrast, the controls (C+ and C-) were positively associated with high Ato frequencies and low energy budget during gametogenesis.

4. Discussion

The evaluation of HDBC in aquaculture has widely spread in the last decade (Ortiz-Cornejo, *et al.*, 2017; García-Bernal *et al*., 2020; Mazón-Suástegui *et al*., 2017, 2018a, 2018b, 2019, 2020, 2022; López-Carvallo *et al.*, 2019, 2020, 2021). This kind of prophylactic treatment has been reported to modulate mostly the general condition of aquatic organisms including the immune system, enzymatic machinery related to the antioxidant response, and digestive activity (Mazón-Suástegui *et al.*, 2018a; López-Carvallo *et al.*, 2020). Furthermore, the recent evidence on the application of the HDBC obtained from minerals, toxins, plants, and inactivated pathogens have shown to successfully increase energetic reserves, growth, survival, food-nutrient management and enhancement. Additionally, the maturation process of aquatic animals has been improved in the laboratory facilities when using HDBC (Mazón-Suástegui *et al*., 2018a), including oysters (García-Bernal *et al*., 2020), scallops (Mazón-Suástegui *et al.*, 2020), fish (Mazón-Suástegui *et al*., 2019; Rosero-García *et al.*, 2019), and shrimp (Mazón-Suástegui *et al.*, 2018b).

Mazón-Suástegui *et al.* (2020) found that the SiT-based treatment contributed to greater lipid and carbohydrate storage in the ovaries and digestive gland of *A. ventricosus* broodstock. An increase of the energetic reserves in this species has been also reported by López-Carvallo *et al.* (2019), where the energy substrate accumulated in juveniles of *A. ventricosus* has been attributed to the use of SiT and PhA. The use of SiT and PhA treatments has been reported to raise glycogen accumulation in the liver of *L. guttatus* (Rosero-García *et al.*, 2019), as well as the assimilation of nutrients, digestion, growth, and survival in juvenile *Octopus bimaculoides* (Ibarra-García *et al.*, 2018). The aforementioned coincide with the results of this research, since the highest amount of lipids in the ovary and muscle, as well as the maximum amounts of carbohydrates in the digestive gland and muscle were obtained in the mussels that received the TR1 complex (SiT, Cas, HeS). Thus, TR3 (ViA, ViP, ViT) also boosted a higher lipid accumulation in the ovary and the digestive gland of the organisms, as well as the greatest

theoretical diameter of postvitellogenic oocytes; while TR2 (PhA, FeP, ZnP) led to the highest index of lipids and carbohydrates in mature postvitellogenic oocytes. These results were attributed to the presence of SiT, ViP-ViA, and PhA, respectively.

The treatments based on PhA and SiT have been reported as growth promoters, as well as accumulation boosters of the energy reserves stored in different tissues of *A. ventricosus* (López-Carvallo *et al.*, 2019; Mazón-Suástegui *et al.*, 2020). In addition, these two compounds have been shown to increase the activity of digestive enzymes, such as trypsins and lipases in *S. rivoliana* juveniles, which are important for the digestive tract maturation and the correct assimilation of food nutrients in this species (Mazón-Suástegui *et al.*, 2019). Moreover, the use of micro-particulate silica powder has been reported to increase the activity of peptidases in *L. vannamei*, improving digestion and subsequent nutrient assimilation (Phromkunthong, 2015). This result suggests that SiT and PhA may be acting as enhancers for food assimilation, which means that a higher energetic budget can be channeled to the gametogenesis process in females treated by TR1 and TR2.

Notwithstanding, the effect of the TR3 complex (ViA, ViP, ViT) on the gonad development and reproduction bioenergetics of a marine bivalve had not been proven so far. However, its positive effect on the increment of energetic reserves and gonad quality in *M. capax* may be influenced by the activation of the biological processes related to nutrient intake. In *S. rivoliana* the use of ViP and ViA has been related to the increment of Chymotrypsin (Mazón-Suástegui *et al.*, 2019), a hydrolytic enzyme related to peptide degradation (Blow, 1976). In shrimp, the same HDBC have reported to increase weight gain in *P. vannamei* juveniles when prophylactically treated with ViA and ViP (García-Bernal *et al.*, 2020). Additionally, other non-measured biological processes and mechanisms may be regulated. According to the literature, HDBC act under the principles of the hormesis theory where a compound with cytotoxic effects at high concentrations is beneficial when used in high dilutions (Dei y

Bernardini, 2015). Therefore, *Vibrio* lysates and scorpion venom would be expected to have an inverse positive effect on the organisms treated with these HDBC. The main clinical signs observed in *L. guttatus* (Reyes-Becerril *et al.,* 2016) and the Spanish seabream *Sparus aurata* (Reyes-Becerril *et al.,* 2017) juveniles infected by Vibriosis were progressive anemia and physiological stress. This result may also explain the positive effect of HDBC formulated by ViP and ViA on the reproductive condition of *M. capax* reported in this study. Additionally, Decker *et al.* (2011) reported an infectious affinity of *Vibrio sp.* to gonad tissues with a significant deterioration in the quality of developing and mature oocytes in adult oysters *Crassostrea gigas*. If the hormesis theory is assumed, ViP and ViA should improve oocyte quality in treated mussels as observed in this study.

The HDBC used in this study allowed reducing the time required for the organisms to achieve gonad maturation. Most tropical marine bivalve species have wide seasonal variability in their gonadal maturation cycles with long periods of intense gametogenesis and short episodes of partial or total spawning (Arcos-Ortega *et al.,* 2015 Rodríguez-Jaramillo *et al.,* 2017). This variability is due to temperature and food availability, which are the main factors driving bivalve reproduction in the natural environment (Fabioux *et al.,* 2005; Barber & Blake, 2016). For *M. capax* particularly the highest gametogenic activity can extend throughout the spring and almost all the summer before the most intense spawning peak of wild females in early autumn (García-Corona *et al.,* 2018). In this work, less than 35 days were enough to achieve gonadal development of the treated mussels compared to the 60 days reported by López-Carvallo *et al.* (2017) for this species only using carbohydrate-rich diets, which demonstrates that the use of HDBC accelerates gametogenesis, reducing the time required for the organism to attain gonad ripening. Similar results were obtained by Mazón-Suastegui *et al.* (2020) where gonad development of adult scallops was completed in only 21 days using HDBC,

against the 3-4 weeks required for organisms to become mature when traditional methods such as dietary and temperature manipulation are employed (Mazón-Suástegui, 2005).

When the gametogenesis process is accelerated and intense, reproductive activity is triggered in marine bivalves. However, this activity is accompanied by high frequencies of atresia and degeneration in oocytes (Beninger, 2017; Rodríguez-Jaramillo *et al.*, 2022) as it occurs in *M. capax* wild populations during gonad ripening peaks (García-Corona *et al.*, 2018). A similar phenomenon was observed by Mazón-Suástegui *et al.* (2020) in *A. ventricosus* broodstock when using the ViT formula where organisms registered the highest proliferation of vitellogenic and postvitellogenic oocytes, but also the highest frequency of degenerating atretic oocytes. These results suggest that the use of ViT accelerates gametogenesis compromising the quality of the oocytes in the scallops. Nevertheless, all the treatments in this study allowed accelerating the gametogenesis process accompanied by a significantly lower atresia frequency compared to the mussels from the control groups. This result suggests the reliability of using these compounds (HDBC), including ViT in combination with ViA and ViP to accelerate gametogenesis without affecting oocyte viability in *M. capax*.

The natural reproductive strategy of *M. capax* relies on the accumulation of neutral carbohydrates and lipids obtained from recently ingested food and subsequently used as a primary source of energy to fuel gametogenesis (López-Carvallo *et al.*, 2017; García-Corona *et al.*, 2018). In this research, the amounts of neutral carbohydrates accumulated in the somatic tissues of the mussels that received HDBC were higher than in the organisms of the control groups, which suggests that the primary needs for this energy substrate were largely met in treated mussels. It is important to highlight that the use of HDBC of TR3 led to a twofold increase in lipid storage in the ovary with respect to the amount previously reported for this species in the natural environment (García-Corona *et al.*, 2018). It is equally important to mention that the TR2 compounds also doubled the incorporation of this energetic substrate

into late-developing oocytes compared to values reported in wild females (García-Corona *et al.*, 2018), and laboratory-conditioned mussels (López-Carvallo *et al.*, 2017). The storage of neutral lipids in developing oocytes is crucial to provide internal energy reserves to sustain embryonic and onset-larval development (Palacios *et al.*, 2007; Mazón-Suástegui *et al.*, 2008; Arcos-Ortega *et al.*, 2015). This shows that HDBC contributes to substantially improved yields of high-quality oocytes compared to controls and the natural reproductive strategy of *M. capax* broodstock.

Although the amounts of total lipids, glycogen, and proteins measured by the biochemical analyses were not significantly different among the treatments and controls after 30 days of sexual maturation using HDBC, these values were similar to the quantities reported by López-Carvallo *et al.* (2017) after 60 days of gonad conditioning only using carbohydrate-enriched diets. This result corroborates the high variability of reproduction bioenergetics in this species and the importance of innovating the zootechnical protocols available to boost the gonad ripening of this marine bivalve.

Thus, all discussed above and the results of this research suggest that the use of TR1 enhances energy accumulation in all the tissues involved in reproduction, probably through activating the digestive enzymatic battery of *M. capax*. Further investigation into transcriptomics and proteomics is necessary to corroborate this hypothesis. The TR2 and, particularly, TR3 formulas improved the gonad development and reproductive capacity of the mussels. The GCA and GDI increased and the highest proliferation of late-developing oocytes enhanced food-energy assimilation and nutrient mobilization from the somatic tissues to the ovary, which favored lipid accumulation in the latter. Finally, TR2 seems to promote the incorporation of the energetic substrates stored in the gonad into the developing oocytes. All these advantages were evident in the enhancement of gametogenesis and energy budget in the mussel broodstock, thereby ensuring the maturation of higher-quality oocytes within this

species. These findings are crucial for the advancement of current *M. capax* gonad conditioning protocols in laboratory settings and for guaranteeing the supply of high-quality spat to the aquaculture industry.

5. Conclusions

The results of this study revealed that the use of these bioactive formulas has quantifiable effects on *M. capax* broodstock. Significant differences were observed in gonad development, energy storage, and oocyte quality among the treatments by half the time required in other protocols to ensure sexual maturation of this species. Since the zootechnical management (temperature and food) of mussels was identical under laboratory conditions, the differences mentioned above can be directly related to the HDBC applied to the experimental groups.

Further research on using HDBC for the species gonad ripening, reproduction bioenergetics, and gamete quality should focus on the transcriptomic and proteomic effects of these formulas on the broodstock reproductive physiology to decipher the main mechanisms involved in the differences observed between treatments. The study of the transgenerational effect of HDBC on the hatchery-produced spat is also necessary.

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Acknowledgments

The authors are grateful to Delfino Barajas-Frías, Pablo Ormart, and Julián Garzón (from CIBNOR, Mexico) for their support during the *in vivo* experiments and production of microalgae, as well as Caroli Ibarra-Serrano and Antonia Rosero-García for their help during sampling; also thankful to Eulalia Meza-Chavez and Roberto Hernández-Herrera (CIBNOR, Mexico) for their technical assistance with histological and biochemical analyses, respectively; to Diana Fischer for English edition.

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.

Funding

This research was funded by CONACYT (in English, National Council for Science and Technology) through the budget program F003 ["Programas Nacionales Estratégicos de Ciencia, Tecnología y Vinculación con los Sectores Social, Público y Privado"], Basic Science Grant #258282 "Experimental evaluation of homeopathy and new probiotics in rearing mollusks, crustaceans, and fish of commercial interest" and PROINNOVA Grant #241777 under the academic responsibility of JMMS. JLGC was a master fellow (CONACYT 715583) under the academic co-direction of JMMS and GFAO and currently a recipient of a doctoral fellowship from CONACYT, Mexico (REF: 2019- 000025-01EXTF-00067). During a specific phase of this research, JALC received a Postdoctoral fellowship from the National Research Council (CONAHCYT), Mexico (CVU: 565306).

Data availability statement

The evidence and data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statements

The mussels (*Modiolus capax*) used in this study were transported and handled according to the International Standards for the Care and Use of Laboratory Animals. The number of

sampled organisms contemplated "the rule of maximizing information published and minimizing unnecessary studies". In this sense 450 mussels were considered the minimum number of organisms needed for this research.

Author contributions

Conceived the study and designed the experiments: JMMS, FAO, JLGC. Performed the experiments: JLGC, JALC. Sampled: JLGC, CRJ, JALC. Processed the samples: JLGC, CRJ. Analyzed the data: JLGC. Interpreted data: JLGC, CRJ, JMMS, FAO. Contributed with reagents/materials/analysis tools: JMMS, FAO, CRJ. Wrote the first draft of the manuscript: JLGC. Wrote – reviewed & edited: JMMS, JALC, FAO.

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Table 1. Mean histochemical (lipid index = LI; carbohydrate index = CHI) and biochemical (TL = total lipids; TG = total glycogen; TP = total proteins) changes in gonad and somatic tissues related to reproduction bioenergetics in the horse-mussel *Modiolus capax* female broodstock (n = 140) treated for 30 days with highly diluted bioactive compounds (HDBC).

Treatment/ target tissue	TR1	TR2	TR3	C+	C-	Statistical analysis	
Ovary							
LI (%)	34.8 ± 1.8^{a}	27.6 ± 1^{b}	33 ± 0.6^{a}	16.7 ± 1.3^{c}	12.6 ± 1.2^{c}	$F_{(df=4)} = 57.1,$	$P = 0.000$
CHI (%)	11.1 ± 0.8^{ab}	9 ± 1.2^{abc}	6.9 ± 0.1^{bc}	6.3 ± 0.6^{c}	11.3 ± 2.1^{a}	$F_{(df=4)} = 5.1,$	$P = 0.001$
TL (mg. g^{-1})	78.2 ± 19.4^{a}	70.9 ± 19.4^{a}	106.7 ± 20.9^{a}	81.8 ± 10.5^{a}	74.8 ± 12.2^{a}	$F_{(df=4)} = 0.7,$	$P = 0.586$
TG (mg. g^{-1})	97.6 ± 15.5^{a}	54.3 ± 14.3^{ab}	66.8 ± 10.1^{ab}	75.8 ± 9.9^{ab}	42.9 ± 7.2^{b}	$F_{(df=4)} = 5.1,$	$P = 0.016$
TP (mg. g^{-1})	489.5 ± 33.7^{a}	387.1 ± 52.6^{a}	551.8 ± 32.1^{a}	461.4 ± 33.9^{a}	430.1 ± 41.1^{a}	$F_{(df=4)} = 1.8,$	$P = 0.134$
Digestive gland							
LI (%)	29.9 ± 0.8^{b}	31.2 ± 1.4^{ab}	34.8 ± 0.5^{a}	19.5 ± 1.2^{c}	21 ± 1^{c}	$F_{(df=4)} = 40,$	$P = 0.000$
CHI (%)	13.7 ± 1.1^{a}	4.8 ± 0.2^{c}	7.8 ± 0.1^{b}	6.8 ± 0.3^{b}	8 ± 0.4^{b}	$F_{(df=4)} = 42.1,$	$P = 0.000$
Muscle							
LI (%)	20.2 ± 1^{a}	11.9 ± 1.2^{b}	6.4 ± 0.7^{c}	6.6 ± 0.6^{c}	6.5 ± 0.8^{c}	$F_{(df=4)} = 45.5,$	$P = 0.000$
CHI (%)	11 ± 0.9^{a}	13.8 ± 3^{a}	4.8 ± 0.3^{b}	3.2 ± 0.2^{b}	3.1 ± 0.6^{b}	$F_{(df=4)} = 13.8,$	$P = 0.000$

Data (mean ± standard error [SE]) were analyzed using the treatment (five levels) as independent variable in separate univariate analysis of variance (ANOVA). The *F* test statistic and degrees of freedom are reported. Different superscript letters denote statistically significant differences at $P < 0.05$.

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Table 2. Mean morphometric (theoretical diameter = TD; nucleus/cytoplasm index = N/C) and histochemical (lipid index = LIo; carbohydrate index = CHIo) changes in oocytes (n = 1950) in the horse mussel *Modiolus capax* female broodstock treated for 30 days with highly diluted bioactive compounds (HDBC).

Treatment/ Oocyte type	TR1	TR2	TR3	C+	C-	Statistical analysis
Vitellogenic						
TD (µm)	61.5 ± 0.4^{a}	60.9 ± 0.4^{a}	61.6 ± 0.3^{a}	56.8 ± 0.3^{b}	50.9 ± 0.4^{c}	$F_{(df=4)}$ = 113.9, P = 0.000
N/C (%)	55.6 ± 1^{a}	49.3 ± 0.8^{bc}	50.8 ± 0.8^{b}	47.1 ± 0.6^{c}	42 ± 0.9^{d}	$F_{(df=4)}$ = 27.2, P = 0.000
LIo (%)	48.7 ± 0.4^{b}	57.9 ± 0.9^{a}	40.8 ± 0.5^{c}	20 ± 1.1^{e}	35 ± 2^{d}	$F_{(df=4)}$ = 214.6, P = 0.000
CHIo (%)	31.1 ± 1.2^{b}	58.2 ± 1.3^{a}	14.8 ± 0.4^{d}	13.5 ± 0.6^{d}	21 ± 1.2^{c}	$F_{(df=4)}$ = 383.5, P = 0.000
Postvitellogenic						
TD (µm)	58.3 ± 0.4^{b}	58.1 ± 0.4^{b}	59.9 ± 0.3^{a}	55.7 ± 0.3^{c}	48.9 ± 0.4^{d}	$F_{(df=4)}$ = 93.5, P = 0.000
N/C (%)	66.4 ± 1^{a}	56.5 ± 1.1^{ab}	51.6 ± 6.8^{b}	55.8 ± 1^{ab}	46.7 ± 1.1^{b}	$F_{(df=4)}$ = 3.7, P = 0.005
LIo (%)	51.5 ± 0.5^{b}	59 ± 0.6^{a}	45 ± 0.6^{c}	14.7 ± 0.8^{e}	24.9 ± 1.5^{d}	$F_{(df=4)}$ = 581.3, P = 0.000
CHIo (%)	35.3 ± 1.1^{b}	53.8 ± 1.2^{a}	15.9 ± 0.5^{d}	11.3 ± 0.5^{e}	19.7 ± 1^{c}	$F_{(df=4)}$ = 437.4, P = 0.000
Atretic						
TD (µm)	51.9 ± 0.5^{a}	50.7 ± 0.4^{a}	47.8 ± 0.4^{b}	48.6 ± 0.3^{b}	43.9 ± 0.5^{c}	$F_{(df=4)}$ = 42.1, P = 0.000
N/C (%)	8.3 ± 0.4^{a}	5.5 ± 0.1^{bc}	6.3 ± 0.2^{b}	5.1 ± 0.5^{bc}	4 ± 1^{c}	$F_{(df=4)}$ = 10.8, P = 0.000
LIo (%)	46 ± 0.5^{a}	39 ± 1.4^{b}	48.2 ± 0.5^{a}	10.4 ± 0.4^{c}	9.6 ± 0.8^{c}	$F_{(df=4)}$ = 493.9, P = 0.000
CHIo (%)	32.5 ± 1.4^{b}	45.3 ± 1.3^{a}	28 ± 0.7^{c}	15.1 ± 0.6^{e}	20.2 ± 1.1^{d}	$F_{(df=4)}$ = 137.2, P = 0.000

Data (mean ± standard error [SE]) were analyzed using the treatment (five levels) as independent variable in separate univariate analysis of variance (ANOVA). The F test statistic and degrees of freedom are reported. Different superscript letters denote statistically significant differences at $P < 0.05$.

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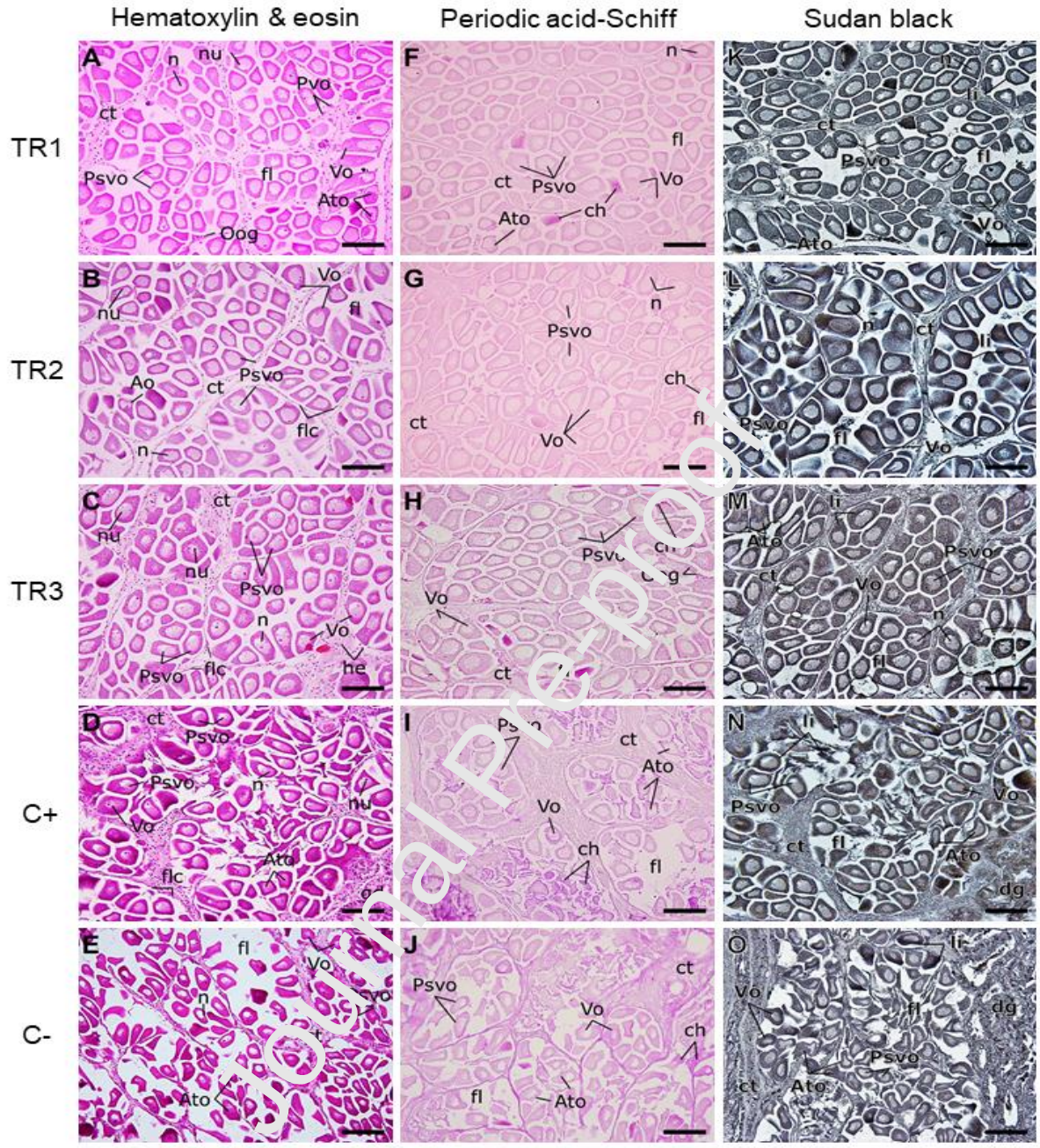

Figure 1. Ovary histology and histochemistry of *Modiolus capax* mussels treated for 30 days with highly diluted bioactive compounds (HDBC). Sections were stained with either Hematoxylin–eosin, Periodic acid-Schiff (carbohydrates), or Sudan black (lipids). Ch = carbohydrates; ct = connective tissue; dg = digestive gland; fl = ovarian follicles; flc = follicular cells; he = hemocytes; li = lipids; Psvo = postvitellogenic oocytes; n = nucleus; nu = nucleolus; Oog = oogonia; Pvo = previtellogenic oocytes; Ato = atretic oocytes; Vo = vitellogenic oocytes. Scale bar = 100 μm.

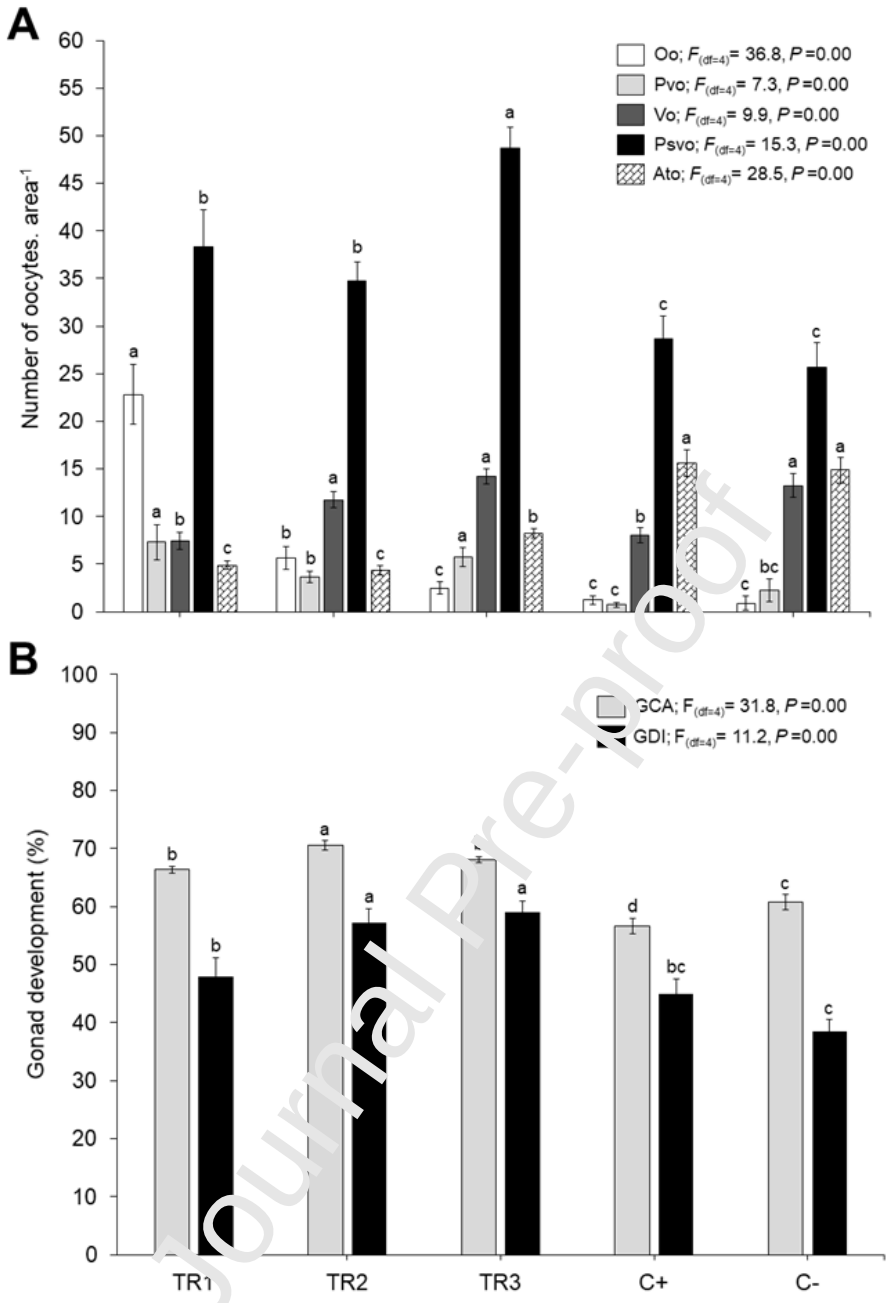

Figure 2. Reproductive capacity of the horse-mussel *Modiolus capax* (n = 140) treated for 30 days with highly diluted bioactive compounds (HDBC). (**A**) Mean number of oocytes per female (at 10×) and (**B**) Gonad development (gonad coverage area = GCA; gamete development index = GDI). Oocyte type is distributed in Oo, oogonia; Pvo, previtellogenic oocytes; Vo, vitellogenic oocytes; Pso, postvitellogenic oocytes; Ato, atretic oocytes. Data (mean ± standard error [SE]) were analyzed using the treatment (five levels) as independent variable in univariate analysis of variance (ANOVA) for each oocyte type. The F-test statistics and degrees of freedom (df) are reported. Different superscript letters denote statistically significant differences of each separate ANOVA at $P < 0.05$

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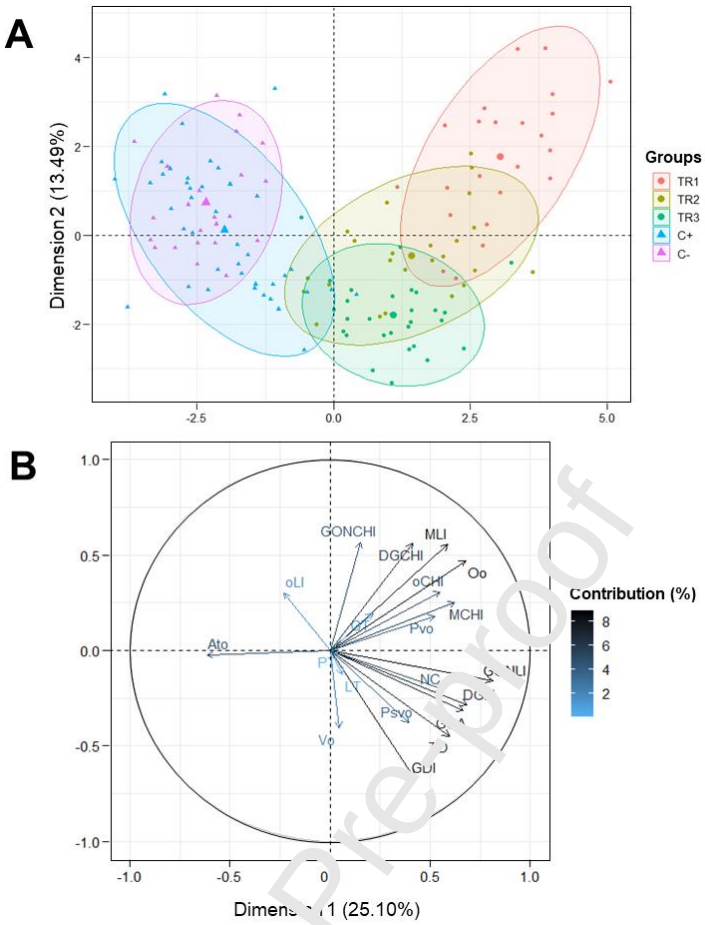

Figure 3. The results of the principal component analysis (PCA) of *Modiolus capax* (n = 140) mussels treated for 30 days with highly diluted bioactive compounds (HDBC). Dimension 1 and 2 together describe 38.6% of the total variance. (**A**) Factorial score plot of individuals from treated (circles) and control (triangles) conditions. Larger symbols are the barycenter of each group and the confidence ellipse level was fixed at $\alpha = 0.05$; (**B**) Variable contribution plot. The arrow direction shows the correlations of variables (GCA = gonad coverage area, GDI = gonad development index; DGLI = digestive gland lipid index; DGCHI = digestive gland carbohydrate index; GONLI = gonad lipid index; GONCHI = gonad carbohydrate index; MLI = muscle lipid index; MCHI = muscle carbohydrate index; TD = theoretical diameter of vitellogenic and postvitellogenic oocytes; NC = nucleous/cytoplasm ratio of vitellogenic and postvitellogenic oocytes; oLI = lipid index of vitellogenic and postvitellogenic oocytes; oCHI = carbohydrate index of vitellogenic and postvitellogenic oocytes; GT = total ovary *glycogen*; PT = total ovary proteins; LT = total ovary lipids; Oo = oogonia; Pvo = previtellogenic oocytes; Vo = vitellogenic oocytes; Psvo = postvitellogenic oocytes; Ato = atretic oocytes) with given PCs, and its color intensity shows their contribution (%) to the explained variance.

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May 08 2023

Declaration of interests

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights

- The use of HDBCs reflects a simultaneous improvement of gametogenesis and energy storage of *M. capax* broodstock.
- An acceleration of sexual maturation and higher-quality oocytes were observed when HDBCs are used.
- The use of HDBCs is an innovative strategy to improve the current gonad conditioning protocols in the laboratory.
- HDBCs have proven to have quantifiable effects on the reproductive capacity of a native marine bivalve with high aquaculture potential.

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