

# Effect of the alveolate parasite *Perkinsus olseni* infection on sexual maturation and spawning efficiency of the clam *Ruditapes decussatus*

## Research Article

**Cite this article:** Fernández-Boo S *et al.* (2023). Effect of the alveolate parasite *Perkinsus olseni* infection on sexual maturation and spawning efficiency of the clam *Ruditapes decussatus*. *Parasitology* **150**, 321–328. <https://doi.org/10.1017/S0031182023000082>



Received: 3 August 2022  
 Revised: 17 January 2023  
 Accepted: 19 January 2023  
 First published online: 1 February 2023

### Keywords:

Alveolata; bivalve infection; bivalve reproduction; host–pathogen interaction; larvae production; oxidative stress

### Author for correspondence:

Sergio Fernández-Boo,  
 E-mail: [sboo@ciimar.up.pt](mailto:sboo@ciimar.up.pt)

Sergio Fernández-Boo<sup>1</sup> , Catarina Duarte<sup>2,3</sup>, Eric Guévelou<sup>2</sup>, Joana Sousa<sup>2</sup>, Rosa Freitas<sup>4</sup>, Sandra Joaquim<sup>3,5</sup>, Benjamin Costas<sup>1,6</sup>, Luisa Magalhães<sup>4</sup> , Domitília Matias<sup>3,5</sup> and Andreia Cruz<sup>2</sup>

<sup>1</sup>Animal Health and Aquaculture (A2S), CIIMAR – Interdisciplinary Centre of Marine and Environmental Sciences, University of Porto, Matosinhos, Portugal; <sup>2</sup>Oceano Fresco S.A, Porto de Abrigo, 2450-075 Nazaré, Portugal; <sup>3</sup>IPMA – Instituto Português do Mar e da Atmosfera, Av. 5 outubro s/n, 8700-503 Olhão, Portugal; <sup>4</sup>CESAM, Departamento de Biologia, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal; <sup>5</sup>Aquaculture and Seafood Safety, CIIMAR – Interdisciplinary Centre of Marine and Environmental Sciences, University of Porto, Matosinhos, Portugal and <sup>6</sup>ICBAS – Instituto de Ciências Biomédicas Abel Salazar, University of Porto, Porto, Portugal

### Abstract

The effect of *Perkinsus olseni* infection on the reproduction ability of clams has been underestimated so far. Although some studies found evidence of reduction of egg production and delay in gonad maturation after infection, the total effect of the infection is still unclear. In this study, *Ruditapes decussatus* clams from a naïve population were injected with two different doses of *P. olseni* parasites, a low dose leading to a light infection and a high dose leading to a heavy infection. Clams were maintained during 2 months for maturation, and at the end of the experiment, the spawning was induced, the number of larvae release and mortality were evaluated. During the maturation period, infection level, gonadal stage, condition index, gross biochemical composition and oxidative status of progenitors were evaluated at days 0, 30 and 60 post-injection. The effects of *P. olseni* infection on clams showed alterations on biochemical parameters, namely lipid peroxidation, a significant mortality and a delayed gonad maturation, with a greater effect in the highly infected individuals. The reproductive capacity of the clams was impaired in both infected groups showing a lower production and a higher mortality rate of larvae. Finally, this study indicates that the production of natural beds with a high prevalence of *P. olseni* could be compromised by a deregulation of the natural reproduction cycle and a decrease in larvae production by infected animals, probably due to a combination of lower egg production and lower lipid reserves in larvae from infected clams.

### Introduction

The effects on the reproduction capacity of bivalves under pathological infections have been extensively studied since it is a major cause of production and recruitment decrease in natural marine and estuarine beds (Dittman *et al.*, 2001; Villalba *et al.*, 2004). Parasites' impacts can vary from deleterious effects on animal reproduction to complete neutering, as it is usually the case in bivalves infected by trematodes (Kabat, 1986; Brian and Aldridge, 2020; Magalhães *et al.*, 2020). Other parasites have a lower effect on reproduction, but they can also greatly impact the host's performance. In the case of Haplosporidian parasites such as *Haplosporidium nelsoni*, a delay in gametogenesis development of the host and also a lower egg production were described (Ford and Figueras, 1988), while the effect of other parasites of the same group, such as *Bonamia ostreae* and *B. exitiosa*, on gametogenesis is not clear (da Silva *et al.*, 2009; Heenan, 2020). Intraheamocytic parasites such as *Perkinsus marinus* can cause a delay in the gonad development of the oyster *Crassostrea virginica* but reproductive capacity and spawning seem to be unaffected (Kennedy *et al.*, 1995; Dittman *et al.*, 2001).

*Perkinsus olseni* is an alveolate parasite that causes deleterious effects on several species of bivalves. First described in the gastropod *Haliotis rubra* (Lester and Davis, 1981), it is now a major concern for clam production worldwide (Villalba *et al.*, 2004; Choi and Park, 2010). This parasite can provoke severe effects on the host's physiology, leading to an energetic imbalance, a delay in gonad maturation and impacts on reproduction that could lead to massive mortality or infection by other pathogens due to a reduction in the immune defence capacity (Park *et al.*, 2006; Villalba *et al.*, 2011; Casas and Villalba, 2012; Soudant *et al.*, 2013). Mass mortality in *Ruditapes decussatus* clams associated with *P. olseni* infection was reported in several countries: Spain with up to 100% mortality in Delta de L'Ebre (Santmartí *et al.*, 1995), Portugal with 50–85% mortality observed in Ria Formosa (Ruano *et al.*, 2015) and Italy with *P. olseni* prevalent in moribund individuals (Ramilo *et al.*, 2015). Mortality impacting *Ruditapes philippinarum* has also been reported in Italy and Korea (Choi and Park, 2010; Pretto *et al.*, 2014), illustrating the magnitude of the problem for clam production worldwide.

© The Author(s), 2023. Published by Cambridge University Press. This is an Open Access article, distributed under the terms of the Creative Commons Attribution-NonCommercial licence (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original article is properly cited. The written permission of Cambridge University Press must be obtained prior to any commercial use.

The effects of *Perkinsus* spp. on host physiology were largely evaluated. In *C. virginica* oysters infected with *P. marinus*, it was reported that a large number of parasites can consume more energy than the oyster is able to produce, leading to a negative energy balance of the host (Choi *et al.*, 1989). The same effect was measured in *R. decussatus* infected with *P. olseni* but with no clear effect on spawning efficiency or fecundity (Casas, 2002; Villalba *et al.*, 2011; Casas and Villalba, 2012). This energetic imbalance resulted in the affected individuals' slower growth and a lower condition index (CI) (Burreson and Ragone Calvo, 1996; Leite *et al.*, 2004; Casas and Villalba, 2012). If the infection invades the gonad and the surrounding follicles, occupying their space, the reproductive capacity of the host can also be compromised. In *R. philippinarum* infected with *P. olseni*, egg production was shown to be reduced in heavily infected clams, resulting in poorer spawning efficiency and clam recruitment (Park *et al.*, 2006).

The present study aimed to evaluate the effect of *P. olseni* on the reproduction of *R. decussatus*, a highly important commercial clam species from the Mediterranean and Northeast Atlantic regions. For that, naïve clams were injected with two doses of parasites, low and high infection under laboratory conditions. Their maturation, CI, biochemical performance, spawning capacity and larvae viability were evaluated during a 2-month trial. This research is included in a broader project which aims to develop a robust protocol for seed yield and increase the production capacity of native species.

## Materials and methods

### Clam collection and *P. olseni* injection and diagnosis

Around 720 adult *R. decussatus* clams ( $43.95 \pm 2.89$  mm) were collected in March 2021 from a *P. olseni*-free natural bed in Testal beach, Noia (42.791184, -8.914513; Spain). They were transported at 15°C in freezer bags to Oceano Fresco S.A. facilities [Nazaré (39.581626, -9.076963), Portugal] and conditioning for 3 weeks ( $18 \pm 0.5^\circ\text{C}$ , 35 ppm) before the start of the experiment. The parasite-free status was further verified on 15 clams using the Ray's Fluid Thioglycolate Method (RFTM) method (Ray, 1952; Villalba *et al.*, 2005), which established the infection scale: level 0 – absence of parasite, 1 – very slight infection, 2 – slight infection, 3 – moderate infection, 4 – intensive infection, 5 – very intensive infection. For the experimental challenge, clams were notched in the shell next to the anterior adductor muscle (Fig. 1). Then, clams were randomly divided into 3 groups and injected with 100 µL of *P. olseni*  $5 \times 10^4$  suspension ( $5 \times 10^3$  cells per clam) for the low infection (LI) group, and 100 µL of *P. olseni*  $5 \times 10^6$  suspension ( $5 \times 10^5$  cells per clam) for high infection (HI) group. Controls (CTL) were injected with 100 µL of filtered sea water (FSW) according to the protocol described in Garcia *et al.* (2022).

After injection, clams were transferred to 50 L tanks (63 clams per tank), 3 tanks per condition (CTL, LI, HI), with aerated 1 µm FSW, at  $19 \pm 1^\circ\text{C}$  with a mixed feeding of *Diacronema lutheri*, *Isochrysis galbana* clone T-iso, *Chaetoceros gracilis* and *Tetraselmis chuii*, in a proportion of 1:1:1:1 based on size terms. This conditioning is commonly used in hatcheries to create the optimal conditions for bivalve gonad maturation and to obtain larvae out of the natural spawning season. A total of 30 clams per condition (10 clams per tank) were sampled on days 0, 30 and 60 after challenge (Fig. 1). Fifteen clams per condition (5 clams per tank) were frozen directly at  $-20^\circ\text{C}$  for CI and gross biochemical parameters evaluation such as protein, glycogen and lipid content. The remaining 15 sampled clams (5 clams per tank) were used for histological evaluation of gonadal development, oxidative stress parameters evaluation and diagnosis

of *P. olseni* infection intensity by RFTM method using one hemigill according to Villalba *et al.* (2005). Mortality was recorded during the whole experiment.

### Condition index and whole-body composition

For condition index (CI) determination, the dry meat and shell weight of 15 clams per condition (5 clams per tank) from each sampling period were determined after oven drying at  $80^\circ\text{C}$  for 24 h. Meat samples were then turned into ash at  $450^\circ\text{C}$  for 24 h in a muffle furnace, ash weight determined and organic matter weight calculated as the ash-free dry meat weight (AFDW). The CI was calculated according to Walne and Mann (1975):  $[\text{AFDW (g)}/\text{dry shell weight (g)}] \times 100$ .

For gross biochemical composition determination, briefly, for each specimen, the protein content was determined using the modified Lowry method (Shakir *et al.*, 1994), glycogen content was determined from dried homogenate ( $80^\circ\text{C}$  for 24 h) using the anthrone reagent (Viles and Silverman, 1949) and total lipids were extracted from fresh homogenized material in chloroform/methanol (Folch *et al.*, 1957) and estimated spectrophotometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). Duplicate determinations were performed in all cases and values were expressed as a percentage of AFDW.

### Histological analysis

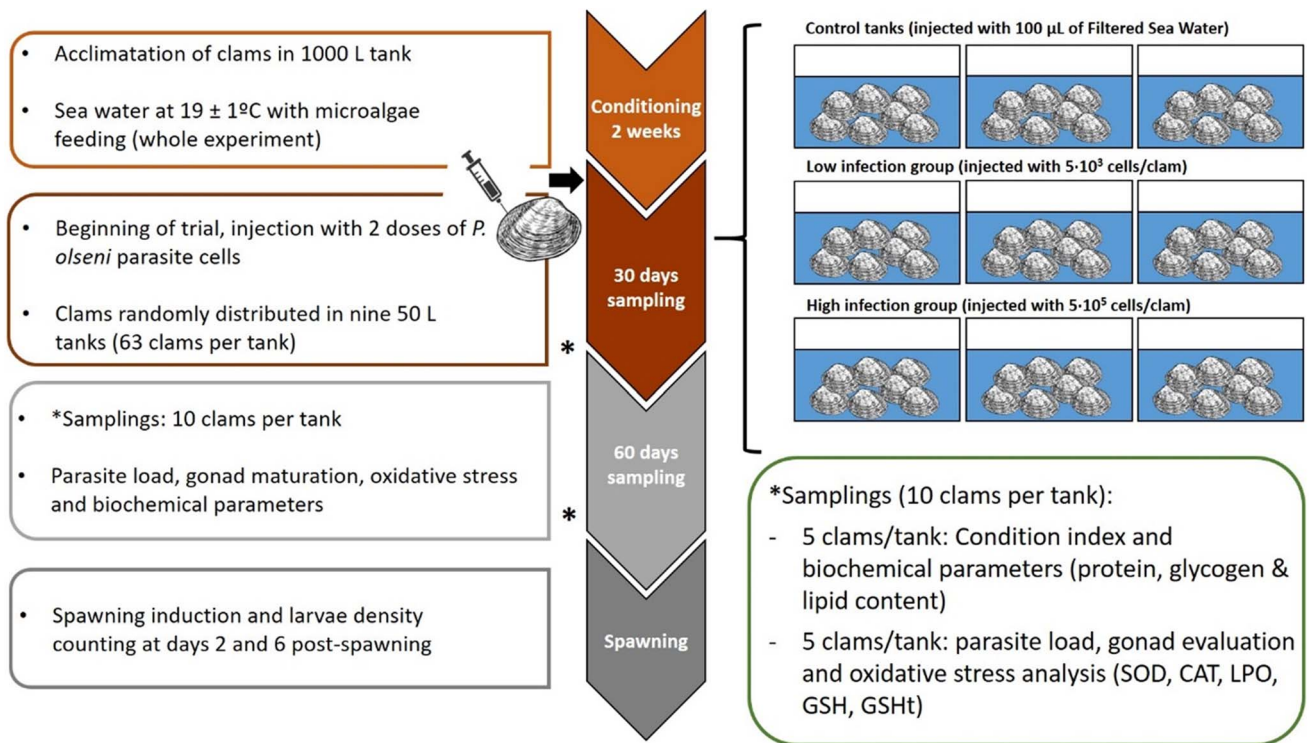
Fifteen individuals per condition from each sampling period were examined histologically to determine the gametogenic stage. A small section of the visceral mass was separated from siphons and gills and fixed in Davison solution for 48 h, then transferred to 70% ethyl alcohol for storage. Tissues from these samples were dehydrated with serial dilutions of alcohol and embedded in paraffin. Sections ( $6\text{--}8\ \mu\text{m}$ ) were cut on a microtome and stained with haematoxylin and eosin. The histologically prepared slides were examined and each specimen was assigned to a sex and gonadal stage according to Delgado and Pérez-Camacho (2007). When more than one developmental stage occurred simultaneously within a single individual, the assignment of a stage criteria decision was based upon the condition of the majority of the section according to Matias *et al.* (2013).

### Oxidative stress parameters determination

After histological sampling, remaining soft tissues were manually homogenized with liquid nitrogen, using a mortar and a pestle, and separated into subsamples (3 subsamples with 0.5 g) for further analysis. For each parameter, extraction was performed with a specific buffer in a 1:2 (w/v) ratio with the previously homogenized tissue as described in Magalhães *et al.* (2018). Protein (PROT) content, catalase (CAT) and superoxide dismutase (SOD) activities, lipid peroxidation (LPO) level, Electron transport system (ETS), total glutathione (GSHt) and reduced glutathione (GSH) contents were determined according to Magalhães *et al.* (2018).

### Spawning and larval viability

After 60 days of the experiment, 73 clams from the CTL, 74 from LI and 65 from HI groups were induced to spawn by thermal stimulation, through five cycles of a rapid increase of temperature from  $15$  to  $25 \pm 1^\circ\text{C}$  over a 1 h interval, recovery for 30 min at  $15 \pm 1^\circ\text{C}$  and repeat the increasing temperature following the protocol previously established by Matias *et al.* (2008). Embryos from each treatment were incubated in 450 L tanks, with 1 µm-filtered seawater, maintained at  $19 \pm 0.5^\circ\text{C}$ , at a density of around 20 embryos per mL. After 48 h, the number of D-larvae



**Fig. 1.** Experimental design of the experience. Three groups were evaluated (control, low and high infected) and two samplings were done during experience at days 30 and 60 post-injection. Spawning was induced after 60 days; fecundity and larval viability were evaluated.

was determined. D-larvae were then reared in 450 L tanks. During larval rearing (around 10 larvae per mL), water was renewed 3 times a week ( $19 \pm 0.5^\circ\text{C}$ ). Before the water change (days 2, 4 and 6 post-spawning), to estimate the survival, larvae were counted in 5 10 mL samples. After each water renewal change, all treatments received an equal volume of phytoplankton cells. The larval diet constituted 40 cells per  $\mu\text{L}$  of a microalgae mix composed of *D. lutheri*, *I. galbana* clone T-iso, *C. gracilis* and *T. chuii*, in equal proportion based on size terms.

### Statistical treatment of data

All statistical analyses were performed using SPSSv.26 statistical analysis software (IBM, USA). Cumulative mortality of progenitors was evaluated using a Kaplan–Meier curve with a log-rank test for group comparisons.

Results were expressed as mean  $\pm$  standard deviation (S.D.). After verifying normality and variance homogeneity, a one-way analysis of variance was carried out. Tukey's Honest Significant Difference (HSD) *post hoc* test was performed to identify significant differences among groups with a level of significance of  $P < 0.05$ .

## Results

### Infection intensity and mortality

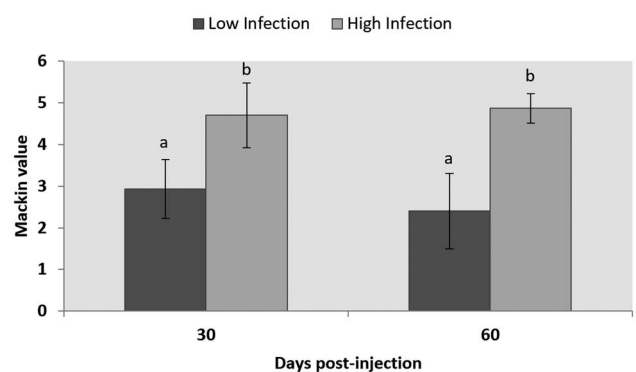
The evaluation of the infection level of *P. olseni* by the RFTM technique showed no infection in control groups at any sampling point, while, at day 60, an average of  $2.4 \pm 0.9$  and a  $4.86 \pm 0.35$  level were observed in LI and HI groups, respectively. No significant differences were observed between samples of the same group between days 30 and 60 post-injection, but significant differences were detected between LI and HI groups ( $P < 0.001$ ) (Fig. 2).

During the 60 days of the experiment, mortality occurred under all treatments, especially during the first 30 days of maturation. The control group presented a cumulative mortality of 9.5%

while the LI group reached 21.2% and the HI group 16.2% (Fig. 3). Statistically significant differences were found between the infected groups and the control group (LI vs CTL,  $P = 0.020$ ; HI vs CTL,  $P = 0.018$ ), but no significant differences were found between low and high infected groups ( $P = 0.431$ ).

### Condition index and whole-body gross biochemical composition

CI showed significant differences among groups and the sampling period. The maximum CI was found in control clams at time 0, before conditioning, with a value of 11.28. However, these values decreased significantly in the first month with a minimum CI value measured at LI\_T1 group with 8.31 (Table 1), which was slightly lower than clams from control and high infected group at the same time, but without significant differences. At the end of the experiment, CI varied from 8.73 in the control group to 9.68 in the HI group, however there were no significant differences among treatments.



**Fig. 2.** Infection level according to the Mackin scale of LI and HI groups after 30- and 60 days post-injection. Results are shown as mean  $\pm$  s.d. Letters show statistically significant differences among groups ( $P < 0.05$ ).

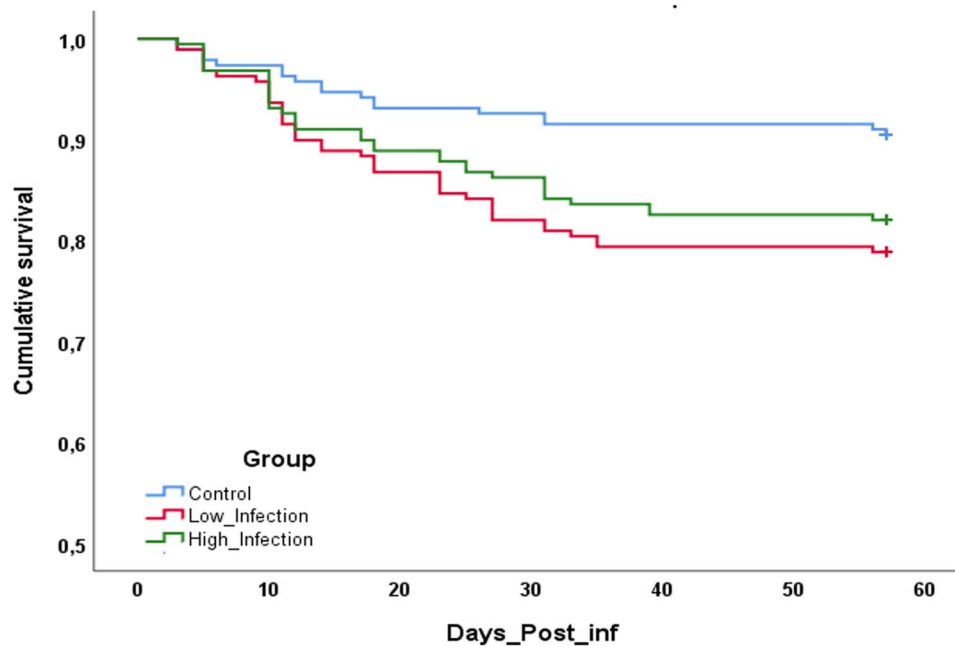


Fig. 3. Cumulative survival of clams after 60 days of experience. \*Indicates statistically significant differences against control condition ( $P < 0.05$ ).

Regarding histology, at T0 the gonads from all clams were on sexual rest. After 30 days of conditioning, most of the clams were already in full maturation in all treatments; however, some differences were found between the control and infected groups (Fig. 4). The control group was the most advanced one in sexual maturation with 85.71%, while 14 and 29% of the individuals were in stages IV (ripe) and V (partially spawned) of gonad development, respectively. Individual reaching stage V was only observed in the control group at 30 days post-infection. In low and high infected groups, the percentage of stage IV was 71.43 and 66.66%, respectively. Also, some clams were still in sexual rest in infected groups at 30 days post-infection (LI = 14.29%; HI = 6.67%). HI clams showed higher variability, what appears to be a delay in gonadal development. At the end of conditioning, 60 days post-infection, the proportion of sexually mature individuals decreased in control and LI clams, and partially spawning (stage V) increased in all groups (46.67% in the control and 26.67% in both infected groups). Also, a small percentage of 6.67% of clams in sexual rest still appeared in all treatments.

Regarding gross biochemical composition, protein, total lipid and glycogen contents showed differences among groups (Table 1; Fig. 5). Energy reserves, in glycogen form, were higher

at the beginning of the experiment ( $107.05 \pm 35.34 \mu\text{g mg}^{-1}$  AFDW). After 30 days, the infected clams showed lower glycogen content ( $59.47 \pm 27.42$  and  $52.55 \pm 15.60 \mu\text{g mg}^{-1}$  AFDW, for LI\_T1 and HI\_T1, respectively) compared with the individuals under control conditions ( $75.27 \pm 20.71 \mu\text{g mg}^{-1}$  AFDW) but differences were only significant between control and HI groups. At the end of the experiment, no significant differences were observed among conditions. The lowest protein and lipid content were present in control clams at time 0 in which all clams were in the sexual rest stage of gonad development. The maximum values were recorded in LI\_T2 and HI\_T2, when glycogen minimum values were found ( $36.52 \pm 8.42$ ,  $33.32 \pm 8.15$  and  $30.08 \pm 12.66 \mu\text{g mg}^{-1}$  AFDW, in C\_T2, LI\_T2 and HI\_T2, respectively).

#### Oxidative stress parameters

No differences were found in the ETS activity and reduced GSH content, regardless of the treatment and the sampling period tested. On the other hand, differences were found in LPO levels during the experimental period with a maximum value in Control\_T0 ( $203.36 \pm 43.02 \text{ nmol MDA g}^{-1}$ ) and a minimum in HI\_T2 ( $81.68 \pm 22.90 \text{ nmol MDA g}^{-1}$ ); while for a given sampling

Table 1. Values of condition index, protein, glycogen and lipid during conditioning

	Condition index	Protein	Lipids	Glycogen
Control_T0	$11.28^b \pm 2.8$	$322.60^a \pm 77.66$	$25.88^a \pm 5.71$	$107.05^d \pm 35.34$
Control_T1	$8.70^a \pm 1.33$	$347.20^{ab} \pm 48.72$	$33.47^{ab} \pm 8.76$	$75.27^c \pm 20.71$
LI_T1	$8.31^a \pm 1.28$	$393.93^{bc} \pm 48.05$	$54.70^{cd} \pm 14.03$	$59.47^{bc} \pm 27.42$
HI_T1	$9.71^{ab} \pm 1.2$	$372.22^{abc} \pm 46.78$	$37.46^{ab} \pm 11.05$	$52.54^{ab} \pm 15.6$
Control_T2	$8.73^a \pm 2.62$	$411.68^c \pm 48.1$	$42.46^{bc} \pm 10.13$	$36.52^a \pm 8.42$
LI_T2	$9.52^{ab} \pm 1.27$	$404.60^c \pm 71.95$	$54.70^{cd} \pm 10.23$	$33.32^a \pm 8.15$
HI_T2	$9.68^{ab} \pm 1.6$	$396.51^{bc} \pm 74.78$	$59.86^d \pm 13.46$	$30.07^a \pm 12.66$
P value	0.015	<0.001	<0.001	<0.001

Values of gross biochemical composition are expressed as  $\mu\text{g mg}^{-1}$  of ash-free dry meat weight (AFDW). Data are expressed in mean  $\pm$  s.d. Superscript letters represent significant differences among groups ( $P < 0.05$ ).

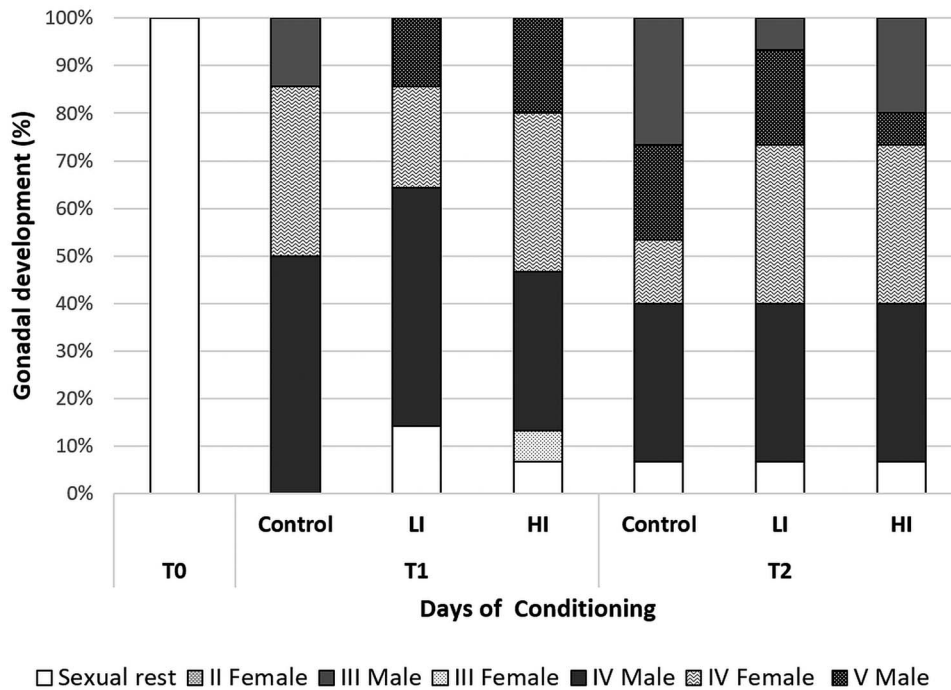


Fig. 4. Gonad development stage of clams at each condition and sampling time. T1: 30 days post-infection, T2: 60 days post-infection.

period, significant differences among groups (CTL, LI, HI) were only observed between control\_T1 and HI\_T1 (Table 2). An opposite trend was observed for GSht, with significantly lower values at the beginning of the experiment (Control\_T0,  $116.19 \pm 18.1 \text{ nmol g}^{-1} \text{ FW}$ ) in comparison to values recorded at HI\_T2 ( $184.79 \pm 47.31 \text{ nmol g}^{-1} \text{ FW}$ ) (Table 2). Nevertheless, no significant differences were observed among conditions at each sampling period. In CAT activity, results showed no significant differences among groups from the same sampling time and among sampling periods for the same group (Table 2). Finally, regarding the SOD activity, no significant differences were observed among groups for each sampling period, but some differences were found between LI clams from T1 and T2 (Table 2).

### Spawning and larvae viability

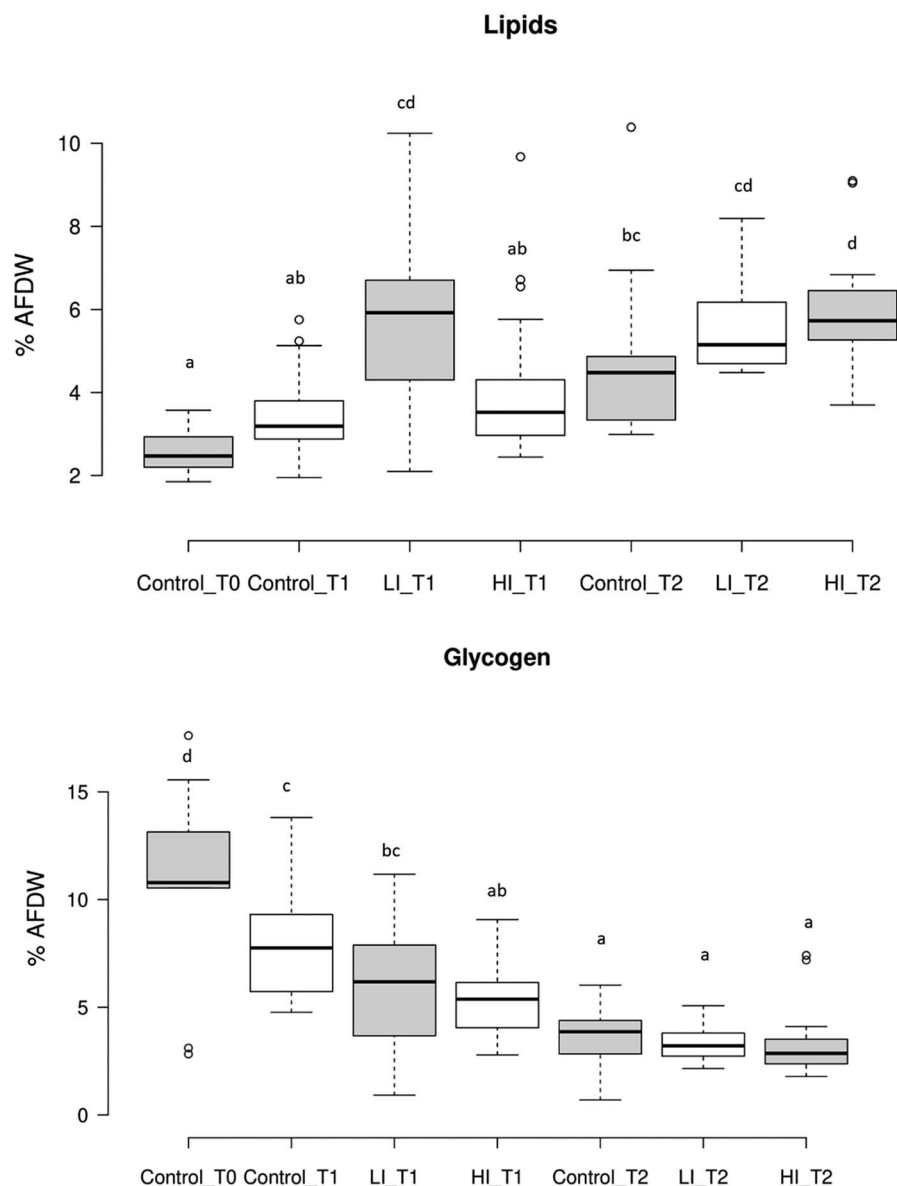
Two days after induction of spawning,  $12.8 \times 10^6$  larvae were obtained from control group clams,  $3.02 \times 10^6$  larvae from LI and  $3.55 \times 10^6$  from the HI group. High mortality of larvae was observed in each group on day 6 post-spawning; this was especially intense in the LI group with a 98.41% of mortality, while larvae from the control and HI group presented 31.67 and 78.37%, respectively (Fig. 6).

### Discussion

In the present paper, the effect of *P. olseni* on clam gametogenesis and fecundity was evaluated using several morphometric and biochemical tools. The injection protocol successfully provoked an intense accumulation of parasite cells in clam's gills and the level of infection remained stable during the 2-month trial. These results are in accordance with those previously obtained by Garcia *et al.* (2022) in which a moderate infection was measured in the LI group and a high infection in the HI group but without an increase of parasite cells during time. It was previously documented, that unlike juvenile clams, *R. philippinarum* adults are able to avoid a rapid proliferation of *Perkinsus* cells while maintaining a stable level of infection during time (Waki *et al.*,

2012) which seems that is happening here. The results showed that significantly higher mortality occurred in both infected groups in comparison with control. This result is incongruent with previous infection studies using the same methodology where no-significant mortality was observed using similar treatments and similar water temperature. These results suggest that animals were potentially in a weaker physiological condition than in the previous experiment. Although the animals came from the same area, the collection was made in a different season that could imply a different physiological condition and could have negative effect on survival.

Some differences were also found in the measure of stress parameters. Although no differences were found in SOD activity, a reduction of the detrimental effects on cell membranes when compared to controls (measured through LPO determination) was clear, especially in HI group. The limited ETS activity in infected clams compared with control ones could be associated with limited reactive oxygen species (ROS) production, explaining low LPO levels in the most stressful conditions, even under low antioxidant capacity. Here, organisms may be experiencing a limited antioxidant capacity resulting from an overall reduction in clams' metabolism, which is a common strategy associated with reduced filtration and respiration capacities, a behaviour frequently observed in bivalves exposed to stressful conditions (Lassudrie *et al.*, 2014; Magalhães *et al.*, 2018). Nevertheless, while CAT presented low activity with no clear pattern, we hypothesize that, although without any metabolic activation, the Glutathione peroxidase (GPx) alternative pathway was used to successfully prevent oxidative stress in infected clams using reduced glutathione (GSH) as a co-factor. Reduced glutathione levels did not differ significantly between treatments, but total glutathione content (composed by SH equivalents: GSH + GSSG) was significantly higher in infected clams compared to those not exposed to *P. olseni* and was also significantly higher over time (from T0 to T2). This trend reveals an increase in pro-oxidant status, i.e. lower GSH/GSSG ratio due to increased oxidated glutathione (GSSG) content, mainly in infected clams at the end of the experiment, which is consistent with the increasingly challenging status. Although the loss of redox balance was



**Fig. 5.** Results of lipid and glycogen content present as % of ash-free dry weight (AFDW) of meat (g)/dry shell weight (g). Letters show statistically significant differences among groups ( $P < 0.05$ ). T1: 30 days post-infection, T2: 60 days post-infection.

observed, especially in infected clams, no cellular damage was noticed.

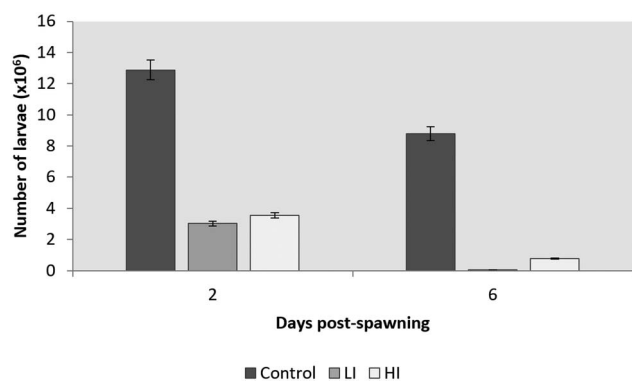
CI is generally considered to reflect reproductive activity, energy storage and exploitation strategy of bivalve species (Massapina *et al.*, 1999; Ojea *et al.*, 2004; Joaquim *et al.*, 2008). In this study, the maximum values were found before infection,

when clams were in sexual rest and the energetic reserves, in glycogen form, were higher. Glycogen is the main energy source stored in gonads as well as other tissues as an energetic reserve for gametogenesis and gonadal development in adult clams (Matias *et al.*, 2013). This observation demonstrated that clams were in the best physiological condition to initiate sexual

**Table 2.** Values of the different oxidative stress parameters are expressed in mean  $\pm$  standard deviation

	ETS	LPO	GSht	GSH	CAT	SOD
Control_T0	100.80 $\pm$ 28.60	203.36 <sup>c</sup> $\pm$ 43.02	116.19 <sup>a</sup> $\pm$ 18.10	14.50 $\pm$ 4.06	0.12 <sup>ab</sup> $\pm$ 0.04	0.13 <sup>a</sup> $\pm$ 0.07
Control_T1	108.69 $\pm$ 26.44	202.56 <sup>c</sup> $\pm$ 51.26	124.05 <sup>a</sup> $\pm$ 29.33	13.83 $\pm$ 4.37	0.11 <sup>ab</sup> $\pm$ 0.02	0.21 <sup>a</sup> $\pm$ 0.09
LI_T1	116.02 $\pm$ 23.59	154.87 <sup>bc</sup> $\pm$ 94.88	137.26 <sup>ab</sup> $\pm$ 44.95	16.22 $\pm$ 4.90	0.12 <sup>b</sup> $\pm$ 0.02	0.18 <sup>a</sup> $\pm$ 0.10
HI_T1	107.82 $\pm$ 27.99	110.24 <sup>ab</sup> $\pm$ 53.41	124.86 <sup>a</sup> $\pm$ 19.37	13.28 $\pm$ 3.00	0.10 <sup>ab</sup> $\pm$ 0.02	0.16 <sup>ab</sup> $\pm$ 0.08
Control_T2	127.87 $\pm$ 29.92	134.29 <sup>ab</sup> $\pm$ 42.33	165.59 <sup>bc</sup> $\pm$ 39.04	15.72 $\pm$ 2.82	0.10 <sup>a</sup> $\pm$ 0.02	0.20 <sup>ab</sup> $\pm$ 0.07
LI_T2	102.27 $\pm$ 41.58	119.52 <sup>ab</sup> $\pm$ 55.84	177.29 <sup>c</sup> $\pm$ 57.61	13.56 $\pm$ 3.26	0.11 <sup>ab</sup> $\pm$ 0.02	0.27 <sup>b</sup> $\pm$ 0.09
HI_T2	101.55 $\pm$ 31.60	81.68 <sup>a</sup> $\pm$ 22.90	184.79 <sup>c</sup> $\pm$ 47.31	16.23 $\pm$ 3.95	0.11 <sup>ab</sup> $\pm$ 0.02	0.19 <sup>ab</sup> $\pm$ 0.08
<i>P</i> value	0.116	0.000	0.000	0.141	0.046	0.000

ETS is presented in  $\text{nmol min}^{-1} \text{g}^{-1} \text{FW}$ ; LPO in  $\text{nmol MDA g}^{-1} \text{FW}$ ; GSH and GSht in  $\text{nmol g}^{-1} \text{FW}$  while CAT and SOD are expressed in  $\text{U mg}^{-1} \text{protein}$ . Significant differences among groups are indicated with superscript letters ( $P < 0.05$ ).



**Fig. 6.** Number of larvae (in millions) obtained at days 2 and 6 after spawning of clams from each group after 60 days of experiment.

maturation. In the first month of maturation, these values substantially decreased both in non-infected and infected groups, which reflected the advance of gonadal development and the consumption of glycogen reserves. At the end of the first month of the experience, most of the clams were already in full maturation in all conditions and a partial spawning started to occur. However, infected groups showed different behaviours being the control group the more advanced one. Otherwise, clams under high infection had a significant higher CI and showed higher variability in gonadal maturation stages than control and low infected clams, which can be a consequence of a delay in gonadal development, highlighting the effect of the *Perkinsus* infection on conditioning. This observation is consistent with the results obtained by Casas and Villalba (2012) where *Perkinsus* spp. infection was linked to delays in the gametogenesis in *R. decussatus*. Similar results were also obtained in oysters infected with *P. marinus* with a delay in the gametogenesis of infected animals in comparison with non-infected ones (Dittman *et al.*, 2001). During the maturation, the inverse relationship was found between decreasing glycogen and increasing total lipid values, reflecting the biosynthesis of glycogen in lipids during the formation of gametes (Matias *et al.*, 2013).

Finally, regarding fecundity, parasite-infected clams seem to produce a lower quantity of gametes due to the lower quantity of larvae obtained at day 2 post-spawning, which is consistent with the results obtained by Park *et al.* (2006) in *R. philippinarum* clams infected by *P. olseni* who demonstrated that infected individuals have a lower egg production capacity. Also, the higher mortality rate of larvae from the infected treatments on day 2 could be linked to a lower quantity of neutral lipid reserves in the eggs, which are the main source of energy for the larvae during the first 2 days of life, at the brief endotrophic phase of development (Matias *et al.*, 2011). But this theory should be confirmed in future experiments where the lipid content of the eggs should be evaluated between infected and non-infected clams.

## Conclusions

During the maturation period (2-month), no differences were found between low and high infected treatments concerning mortality, gonadal development and spawning efficiency, suggesting that even moderate infections could lead to a significant impact on natural populations. In this context, *R. decussatus* natural beds affected by *P. olseni* may experience decreased recruitment due to lower-quality larvae derived from infected animals, which can be detrimental to natural populations, especially if this problem is associated with other factors such as environmental changes, contamination or the introduction of new species with better performance. Additionally, the use of infected

broodstock for seed production in hatcheries could lead to a lower productivity of the facility given that a delay in sexual maturity, a reduction in fecundity and lower larval viability were observed.

**Data Availability Statement.** The authors confirm that the data supporting the findings of this study are available within the article. Raw data were generated from this study were generated at CIIMAR, University of Aveiro, IPMA and Oceano Fresco S.A. Derived data supporting the findings of this study are available from the corresponding author SFB on request.

**Acknowledgements.** The authors acknowledge Confradía de Pescadores San Bartolomeu de Noia (Galicia, Spain) and especially Liliana Solís for their help in the collection. The authors also thank the following Oceano Fresco hatchery team members for their assistance during the realization of the challenge: Ana Cerviño-Otero, Óscar Iglesias Baños, Ana Torres, Paulo Frias, Carlos Silva and Ricardo Luis.

**Financial support.** This article is a result of the project 'INOvAmêijoa: Da maternidade ao viveiro: Inovação no cultivo integral de amêijoa-boia (*R. decussatus*) e amêijoa-macha (*V. corrugata*)' ref: CENTRO-01-0247-FEDER-047018/ALG-01-0247-FEDER-047018, supported by Centro Regional Operational Programme (Centro 2020) and Algarve Regional Operational Programme (CRESC Algarve 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). This work was also supported by the project Tools4Breed with reference FA\_05\_2017\_025, co-financed by Fundo Azul and Minister of the Sea of the Portuguese Republic, by the project 'EUROCLAM – Unlocking the potential of Euro-native bivalves' (CENTRO-01-0247-FEDER-113950) co-financed by CENTRO 2020, PT2020 and ERDF and also by the project BISPECIAL: BivalveS under Polluted Environment and Climate chAnge (PTDC/CTA-AMB/28425/2017) funded by FEDER, through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/MCTES. This research was also supported by national funds 'through FCT – Foundation for Science and Technology' within the scope of UIDB/0443/2020 and UIDP/04423/2020. Sergio Fernández-Boo was financed by the FCT Scientific Employment Stimulus programme with reference CEECINST/00027/2021. Thanks are also due to FCT/MCTES for the financial support to CESAM (UIDP/50017/2020; UIDB/50017/2020; LA/P/0094/2020) through national funds.

**Conflict of interest.** None.

**Ethical standards.** Not applicable.

## References

- Brian JI and Aldridge DC (2020) An efficient photograph-based quantitative method for assessing castrating trematode parasites in bivalve molluscs. *Parasitology* **147**, 1375–1380.
- Burreson EM and Ragone Calvo LM (1996) Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. *Journal of Shellfish Research* **15**, 17–34.
- Casas SM (2002) *Estudio de la perkinsosis en la almeja fina, Tapes decussatus (Linnaeus 1758), de Galicia* (Ph.D. thesis). University of Santiago de Compostela, Spain.
- Casas SM and Villalba A (2012) Study of perkinsosis in the grooved carpet shell clam *Ruditapes decussatus* in Galicia (NW Spain). III. The effects of *Perkinsus olseni* infection on clam reproduction. *Aquaculture* **356–357**, 40–47.
- Choi KS and Park KI (2010) Review in the protozoan parasite *Perkinsus olseni* (Lester and Davis 1981) infection in Asian waters. In Ishimatsu A and Lie H-J (eds), *Coastal Environmental and Ecosystem Issues of the East China Sea*, pp. 269–281.
- Choi KS, Wilson EA, Lewis DH, Powell EN and Ray SM (1989) The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. *Journal of Shellfish Research* **8**, 125–131.
- da Silva PM, Fuentes J and Villalba A (2009) Differences in gametogenic cycle among strains of the European flat oyster *Ostrea edulis* and relationship between gametogenesis and bonamiosis. *Aquaculture* **287**, 253–265.
- Delgado M and Pérez-Camacho A (2007) Comparative study of gonadal development of *Ruditapes philippinarum* (Adams and Reeve) and

- Ruditapes decussatus* (L.) (Mollusca: Bivalvia): Influence of temperature. *Scientia Marina* **71**, 471–484.
- Dittman D, Ford S and Padilla DJ** (2001) Effects of *Perkinsus marinus* on reproduction and condition of the eastern oyster, *Crassostrea virginica*, depend on timing. *Journal of Shellfish Research* **20**, 1025–1034.
- Folch J, Lees M and Sloane Stanley GH** (1957) A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of Biological Chemistry* **226**, 497–509.
- Ford SE and Figueras A** (1988) Effects of sublethal infection by the parasite *Haplosporidium nelsoni* (MSX) on gametogenesis, spawning, and sex ratios of oysters in Delaware Bay, USA.
- García A, Estêvão J, Costas B, Cruz A and Fernández-Boo S** (2022) Evaluation of the *Ruditapes decussatus* immune response after differential injected doses of *Perkinsus olseni*. *Journal of Invertebrate Pathology* **195**, 107849.
- Heenan S** (2020) Aspects of reproduction in a post-*Bonamia exitiosa* epidemic *Ostrea chilensis* fishery, Foveaux Strait, Southern New Zealand. University of Otago. <http://hdl.handle.net/10523/9974>.
- Joaquim S, Matias D, Lopes B, Arnold WS and Gaspar MB** (2008) The reproductive cycle of white clam *Spisula solida* (L.) (Mollusca: Bivalvia): implications for aquaculture and wild stock management. *Aquaculture* **281**, 43–48.
- Kabat AR** (1986) Effects of trematode parasitism on reproductive output of the bivalve *Tranennella tantilla*. *Canadian Journal of Zoology* **64**, 267–270.
- Kennedy VS, Newell RIE, Krantz GE and Otto S** (1995) Reproductive capacity of the eastern oyster *Crassostrea virginica* infected with the parasite *Perkinsus marinus*. *Diseases of Aquatic Organisms* **23**, 135–144.
- Lassudrie M, Soudant P, Richard G, Henry N, Medhioub W, da Silva PM, Donval A, Bunel M, Le Goïc N, Lambert C, de Montaudouin X, Fabioux C and Hégaret H** (2014) Physiological responses of Manila clams *Venerupis* (= *Ruditapes*) *philippinarum* with varying parasite *Perkinsus olseni* burden to toxic algal *Alexandrium ostenfeldii* exposure. *Aquatic Toxicology* **154**, 27–38.
- Leite RB, Afonso R and Cancela ML** (2004) *Perkinsus* sp. infestation in carpet-shell clams, *Ruditapes decussatus* (L.), along the Portuguese coast. Results from a 2-year survey. *Aquaculture* **240**, 39–53.
- Lester RJG and Davis GHG** (1981) A new *Perkinsus* species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. *Journal of Invertebrate Pathology* **37**, 181–187.
- Magalhães L, de Montaudouin X, Figueira E and Freitas R** (2018) Trematode infection modulates cockles biochemical response to climate change. *Science of the Total Environment* **637–638**, 30–40.
- Magalhães L, Freitas R and de Montaudouin X** (2020) How costly are metacercarial infections in a bivalve host? Effects of two trematode species on biochemical performance of cockles. *Journal of Invertebrate Pathology*, **177**, 107479. doi: 10.1016/j.jip.2020.107479
- Marsh JB and Weinstein DB** (1966) Simple charring method for determination of lipids. *Journal of Lipid Research* **7**, 574–576.
- Massapina C, Joaquim S, Matias D and Devauchelle N** (1999) Oocyte and embryo quality in *Crassostrea gigas* (Portuguese strain) during a spawning period in Algarve, South Portugal. *Aquatic Living Resources* **12**, 327–333.
- Matias D, Joaquim S, Leitão A and Massapina C** (2008) Effect of geographic origin, temperature and timing of broodstock collection on conditioning, spawning success and larval viability of *Ruditapes decussatus* (Linné, 1758). *Aquaculture International* **17**, 257.
- Matias D, Joaquim S, Ramos M, Sobral P and Leitão A** (2011) Biochemical compounds' dynamics during larval development of the carpet-shell clam *Ruditapes decussatus* (Linnaeus, 1758): effects of mono-specific diets and starvation. *Helgoland Marine Research* **65**, 369–379.
- Matias D, Joaquim S, Matias AM, Moura P, de Sousa JT, Sobral P and Leitão A** (2013) The reproductive cycle of the European clam *Ruditapes decussatus* (L., 1758) in two Portuguese populations: implications for management and aquaculture programs. *Aquaculture* **406–407**, 52–61.
- Ojea J, Pazos AJ, Martínez D, Novoa S, Sánchez JL and Abad M** (2004) Seasonal variation in weight and biochemical composition of the tissues of *Ruditapes decussatus* in relation to the gametogenic cycle. *Aquaculture* **238**, 451–468.
- Park K-I, Figueras A and Choi K-S** (2006) Application of enzyme-linked immunosorbent assay (ELISA) for the study of reproduction in the Manila clam *Ruditapes philippinarum* (Mollusca: Bivalvia): II. Impacts of *Perkinsus olseni* on clam reproduction. *Aquaculture* **251**, 182–191.
- Preto T, Zambon M, Civettini M, Caburlotto G, Boffo L, Rossetti E and Arcangeli G** (2014) Massive mortality in Manila clams (*Ruditapes philippinarum*) farmed in the lagoon of Venice, caused by *Perkinsus olseni*. *Bulletin European Association of Fish Pathology* **34**, 43–53.
- Ramilo A, Carrasco N, Reece KS, Valencia JM, Grau A, Aceituno P, Rojas M, Gairin I, Furones MD, Abollo E and Villalba A** (2015) Update of information on perkinsosis in NW Mediterranean coast: identification of *Perkinsus* spp. (Protista) in new locations and hosts. *Journal of Invertebrate Pathology* **125**, 37–41.
- Ray SM** (1952) A culture technique for the diagnosis of infection with *Dermocystidium marinum* Mackin, Owen and Collier in Oysters. *Science* **116**, 360–361.
- Ruano F, Batista FM and Arcangeli G** (2015) Perkinsosis in the clams *Ruditapes decussatus* and *R. philippinarum* in the Northeastern Atlantic and Mediterranean Sea: a review. *Journal of Invertebrate Pathology* **131**, 58–67.
- Santmartí MM, Garcia-Valero J, Montes J, Pech A and Durfort M** (1995) Seguimiento del protozoo *Perkinsus* sp. en las poblaciones de Tapes decussatus y Tapes semidecussatus del delta del Ebro. In Castelló F and Calderer A (eds), *Actas del V Congreso Nacional de Acuicultura*. Barcelona: Universidad de Barcelona, pp. 260–265.
- Shakir FK, Audilet D, Drake AJ and Shakir KMM** (1994) A rapid protein determination by modification of the Lowry procedure. *Analytical Biochemistry* **216**, 232–233.
- Soudant P, Fu-Lin EC and Volety A** (2013) Host-parasite interactions: marine bivalve molluscs and protozoan parasites, *Perkinsus* species. *Journal of Invertebrate Pathology* **114**, 196–216.
- Viles FJ Jr. and Silverman L** (1949) Determination of starch and cellulose with anthrone. *Analytical Chemistry* **21**, 950–953.
- Villalba A, Reece KS, Ordás MC, Casas SM and Figueras A** (2004) Perkinsosis in molluscs: a review. *Aquatic of Living Resources* **17**, 411–432.
- Villalba A, Casas SM, López C and Carballal MJ** (2005) Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). II. Temporal pattern of disease dynamics and association with clam mortality. *Diseases of Aquatic Organisms* **65**, 257–267.
- Villalba A, Gestal C, Casas SM and Figueras A** (2011) Perkinsosis en moluscos. Enfermedades de moluscos bivalvos de interés en acuicultura. Fundación Observatorio Español de Acuicultura, Madrid., 181–242.
- Waki T, Shimokawa J, Watanabe S, Yoshinaga T and Ogawa K** (2012) Experimental challenges of wild Manila clams with *Perkinsus* species isolated from naturally infected wild Manila clams. *Journal of Invertebrate Pathology* **111**, 50–55.
- Walne P and Mann R** (1975) Growth and biochemical composition in *Ostrea edulis* and *Crassostrea gigas*. In Barnes H (ed), *Ninth European Marine Biology Symposium*. Scotland: Aberdeen University Press, pp. 587–607.