RESEARCH ARTICLE

Impact on reproductive performance and body condition in a small limpet parasitized by a large castrator pea crab

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Abstract

Parasitic castrators utilize the energy reserves that the host allocates on reproduction resulting in sterilization of the host. However, whether other processes (e.g., growth) are also impaired depends on the balance between the castrator's energy requirements and the available resources that the castrated host does not use in reproduction. We investigated whether the castrator pea crab *Calytraeotheres garthi* alters body condition, reproductive performance, and occurrence of copulation in its limpet host *Bostrycapulus odites*. For this purpose, we examined the anatomy of the ovary, the seminal receptacles, and the body condition of parasitized and unparasitized limpets. The histology of the female gonad of parasitized limpets revealed the ovarian tubules are smaller and characterized by a greater proportion of intertubular and intratubular free space compared with non-parasitized individuals. The body condition of female limpets from all sizes (during summer) and those larger than ~16 mm (during spring and autumn) were impacted by the pea crab. These results are in contrast to that previously reported in the comparatively larger limpet species *Crepidula cachimilla,* in which the same pea crab species does not alter or even increase the host body weight. We concluded this pea crab species could drastically impair its host's reproduction and body condition although deleterious effects are species-specific and likely depend on limpet body size. The histology of seminal receptacles revealed an uncommon disposition of spermatozoa (i.e., excessive debris and acrosomes detached from epithelium) in seminal receptacles of some parasitized limpets. However, this analysis failed to determine whether sperm derived from present matings or previous pea crab infection. Further studies are needed to address whether pea crabs interfere with the mating behavior of limpets and if the alterations in sperm disposition are a consequence of castration.

KEYWORDS
body size, Calyptraeidae, ovary appearance, Pinnotheridae, slipper limpet

1 | INTRODUCTION

Parasitic castrators selectively appropriate the energy reserves that the host allocates on reproduction, resulting in a severe reduction or cessation of embryo production of hosts (Hurd, 1993; Lafferty & Kuris, 2009). In diverse taxa (e.g., mollusk snails infected by trematode species—Lafferty & Kuris, 2002), castrators do not only impede the hosts' reproduction but also induce an enhanced growth (Bonds, 2006). This occurs because, once sterilized, the host redirects its energy resources into other processes, such as growth that can, in turn, benefit the parasite (Ebert et al., 2004). Indeed, parasites living in a larger host individual may achieve higher fecundity, larger life

span, and/or maximum body sizes (Lafferty & Kuris, 2009), whereas the host becomes merely the extended phenotype of the parasite (Sorensen & Minchella, 2001). The increased growth mainly depends on the balance between the extra energy resources resulting from castration and the parasite's energy requirements (Ebert et al., 2004). The host will increase in size when unused resources (due to the absence of reproduction) exceed the energy that both fighting the infection and/or nutritionally sustaining the parasitic demands. However, if the energy loss imposed by the parasite exceeds the hosts' savings by not reproducing, the expected outcome might be a reduction in host body condition.

Parasitic castration is pervasive in the marine realm, highly prevalent in some taxa, for example, mollusks, echinoderms, and crustaceans (Poulin, 2007), and typically involves invertebrates as both parasite and host (Lafferty & Kuris, 2002). Among crustaceans, parasitic castration has independently evolved in several taxa, including copepods (e.g., Tunnicliffe et al., 2008), bopyrid and entoniscid isopods (Williams & Boyko, 2012), and rhizocephalan barnacles (Høeg et al., 2005). However, within the ecologically diverse Decapoda (e.g., shrimps, crabs, and lobsters), parasites acting as castrators have been solely reported in few species in the family Pinnotheridae. The Pinnotheridae (vulgarly known as pea crabs) contain commensals or parasites of a wide diversity of invertebrates, such as gastropods, bivalves, ascidians, holothurians, echinoids, and sea urchins (Baeza et al., 2018). Pea crabs commonly inflict deleterious effects upon host individuals (e.g., Bierbaum & Shumway, 1988; Cuesta et al., 2020; Stauber, 1945), and therefore, they are increasingly considered parasites rather than commensals (De Bruyn et al., 2009). Pea crabs pertaining to the genus *Calyptraeotheres* deserve special attention because of the peculiar mechanism to impair reproduction of their hosts. Pea crabs from this genus exclusively parasitize limpet snail species in the family Calyptraeidae (Campos, 1999). These limpets brood their egg masses in a body space (a sort of incubatory chamber) that the *Calyptraeotheres* pea crab fill and occupy when present (Figure 1a,b). The space available within this chamber determines whether spawning occurs or not (Chaparro et al., 2001). Thus, the presence of a pea crab results in host individuals halting reproduction (Chaparro et al., 2001). Consequently, this castration is a "physic castration" given crabs (particularly those large females) exert a steric interference that impedes the spawning and/or brooding process (Ocampo et al., 2014).

Additionally to castration, the ingestion of food by *Calyptraeotheres* is at the expense of the limpet host. Crabs take away pieces of phytoplankton-rich mucous the filter-feeding limpets accumulated with the gill (Ocampo et al., 2014). Competition for food between host and parasite may trigger multiple deleterious effects on the host, including reduced energy intake (Polak, 1996). Nevertheless, an increment in the body condition of limpets *Crepidula cachimilla* parasitized by *C. garthi* is often observed, at least during the reproductive season of this host (Ocampo et al., 2014). The energy resources the limpet does not use for reproduction (due to the castration) is thought to exceed the energy it loses by the food deprivation imposed for the pea crab feeding activity (Ocampo et al., 2014). Whether this enhancement of body condition is
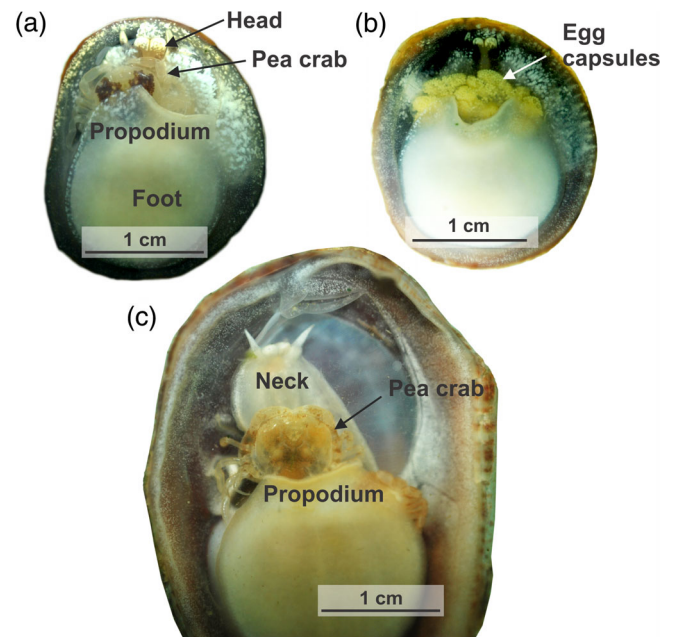

FIGURE 1 Ventral view of the limpets (a) *Bostrycapulus odites* and (c) *Crepidula cachimilla* harboring stage-V female pea crab of *Calyptraeotheres garthi*. (b) Ventral view of a limpet of *B. odites* brooding egg masses

species-specific or would occur in all limpets parasitized by *Calyptraeotheres* crabs is unknown. The most relevant peculiarity of limpets in *C. cachimilla* is its large body size, which is double that of other co-distributing limpet snails, such as *Bostrycapulus odites* (Figure 1a,c). The pea crab mass that an individual of *B. odites* must carry (and nutritionally maintains) is notably larger than a parasitized limpet of *C. cachimilla* has to support. Therefore, *C. garthi* is expected to negatively affect the body condition of limpets of *B. odites*.

However, like in many other parasitic castrations (e.g., Kuris et al., 1980), the castration of limpets infested by *Calyptraeotheres* is temporary. The ovary of castrated limpets remains filled with oocytes during the breeding season (Chaparro et al., 2001) and, after experimental extraction (and presumably natural death) of crabs, the hosts recover their spawning capacity (Chaparro et al., 2001; Ocampo et al., 2014). Resuming reproduction, however, seems not to be possible for all limpet individuals after infections by pea crabs. Although some individuals of the limpet *C. cachimilla* spawned shortly after being stripped of pea crabs, more than half of these experimental limpets failed to deposit eggs (Ocampo et al., 2014). In addition, resuming reproduction may need a variable time, even several weeks, as observed in the limpet host *Crepipatella fecunda* after removing pea crabs of *Calyptraeotheres* sp. (Chaparro et al., 2001). A possible explanation of the individual disparity in effectively resuming reproduction, or the delay some limpets show to recover spawning activity after infection, might be that the pea crab impedes limpet copulation. Limpets in the family Calyptraeidae are protandrous hermaphrodites, the fertilization is internal and mobile spermatozoa ejaculated by individuals acting as males are stored in a female reservoir organ (= seminal

receptacle) of the copulation partner (Beninger et al., 2016). During copulation, the small limpet acting as a male extends the penis through the pallial cavity and inserts it into the female genital pore, located exactly where the parasitic crab is positioned. If crabs block limpet copulation, the resultant lack or limitation of spermatozoa may impede the fertilization of eggs. Thus, it is expected that limpets that lost the parasite will need to mate (again) to be able spawning a new offspring cohort.

Two main objectives are analyzed in the present study. First, we examined the body condition of limpets of *B. odites* to look for differences induced by the presence of the pea crab *C. garthi*. Although the absence of reproduction may result in extra energy resources that limpets could redirect into growth, the crabs' feeding activity implies a cost that would affect the limpet body condition. Considering the relatively small body size of *B. odites*, we hypothesize that the cost of the crab could be detrimentally high for its host. Thus, we predict that the presence of this crab will affect any energy-demanding processes such as growth and/or gamete production. The latter is herein explored by analyzing the histology of the ovary of infected limpets. Second, we analyzed the histology of seminal receptacles in parasitized female limpets to determine whether individuals harboring pea crabs contain spermatozoa. Considering female pea crabs occupy the space where the limpet male introduces its penis, we hypothesized that crabs may affect limpet reproduction (inhibiting the copulation) resulting in empty or content-reduced seminal receptacles.

2 | MATERIAL AND METHODS

2.1 | Collection of limpets and crabs

The limpet host *Bostrycapulus odites* Collin, 2005 and the parasitic pea crab *Calyptraeotheres garthi* (Fenucci, 1975) were collected at the intertidal zone of San Antonio Oeste (S 40°43′, W 64°55′), located in San Matías Gulf, North Patagonia, Argentina. *Bostrycapulus odites* occurs mainly attached to small-sized rocks and it is exposed to a semidiurnal macro-tidal regime; limpets are submerged from 0.5 to 9 m depth during low and high tide, respectively. Specimens were sampled during low tides at end of March 2014 (austral summer), November 2014 (austral spring), and February 2015 (austral autumn) by hand. As the frequency of occurrence of pea crabs on *B. odites* is low (Ocampo et al., 2012), to obtain a considerably high number of crabs without sacrificing many limpets we actively collected specimens harboring crabs or brooding egg masses. Limpets were detached from rocks and in situ inspected for pea crabs and limpets' egg masses, which are both of them located between the ventral side of the neck and an anterior fold of the limpet's foot (see Figure 1a,b). Most crab-free and embryo-free limpets were then carefully re-attached to substrates and deposited into the habitat. Before releasing them, the shell length (SL) and the number of crab-free limpets were recorded to calculate the frequency of occurrence of crabs and the proportion of incubating limpets. Individuals smaller than 13 mm (i.e., the smallest size of mature females in this species, see Cledón

et al., 2016) were excluded from calculating the proportion of incubating limpets. The limpets and crabs we kept for weighing and measuring were individually fixed and stored in 70% ethanol. Both crabs and limpets were handled and sacrificed according to ethical standards required by local authorities.

In pea crab species, including *C. garthi*, the ultimate instar in females is named stage V. Female crabs in this stage are larger than all other previous stages, they are obligated symbiotic, and during this stage female individuals start spawning eggs (Baeza et al., 2018; Ocampo et al., 2016). Stage-V female crabs are reported to negatively impact hosts (Bierbaum & Shumway, 1988; Cuesta et al., 2020; Møller Christensen & McDermott, 1958). Considering the above, in this study we exclusively analyzed the effect of stage-V female crabs on limpets of *B. odites*.

For histological procedures, limpets collected during February 2015 were dissected and soft tissues were fixed for 48 h in Davidson solution and then individually stored in 70% ethanol. The SL of each limpet was measured with calipers to the nearest 0.1 mm and sexed based on the presence or absence of a penis (Cledón et al., 2004). The carapace width (CW) of each crab was measured under the stereomicroscope equipped with a calibrated ocular micrometer (precision = 0.01 mm). Male crabs were classified as pre-hard or hard, and female crabs as pre-hard, hard, stage II, III, IV, or V according to their size, external shape, and morphology of pleopods (Ocampo et al., 2016).

2.2 | Frequency of occurrence and effect of crabs on body weight and spawning of limpet hosts

We determined the overall frequency of occurrence of crabs in limpets and the frequency of each crab stage (i.e., female and male pre-hard and hard, and stage-V females) on hosts of different body sizes. Then, we evaluated whether *C. garthi* affects spawning in *B. odites* employing a two-way contingency table. In this table, we used the presence/absence of egg masses and the presence/absence of crabs (either male or female crabs) as categorical variables. We considered only sexually mature limpets, that is, larger than 13 mm of SL, the smallest limpet observed brooding eggs (see Cledón et al., 2016). The observed frequencies of occurrence were compared with expected frequencies calculated under the null hypothesis of independence between the presence of egg masses and crabs. Significant differences between the observed and expected frequencies were examined using a chi-square test of independence.

We evaluated whether the presence of stage-V female crabs affects the body condition of their hosts using *B. odites* limpets collected during three seasons; autumn (March 2014), spring (December 2014), and summer (February 2015). In *B. odites* separating the gonad from the visceral mass is quite complex and often results in broken structures and tissues. Therefore, we prefer to use the entire body mass (somatic tissues + gonad), and we evaluated separately the potential effects of the crab on host gonad using a histological approach (see below). In the laboratory, the soft body part of each limpet was carefully separated from its shell with forceps. Then, shell

and soft body were dried for 48 h at 60°C and weighed with an analytical balance (precision = 0.001 mg). Differences in the dry soft body weight between limpets harboring and not harboring crabs were analyzed with three general linear model tests (GLM), one per season. In the GLMs, we used the presence/absence of female crab and egg masses as the categorical factor. Shell length of limpets was used as the covariate and dry soft body weight as the response variable. Prior to the GLMs, the data set was graphically inspected for normality (Q–Q plots) and homogeneity of variances (plot of residuals versus fitted values) using the DHARMa package (Hartig, 2020). The interaction between the factor and the covariate was also tested to determine the homogeneity of slopes. An a posteriori Tukey test for multiple comparisons was applied when GLM detected an effect of the factor on the response variable. In case of heterogeneity of slopes, we first conducted an a posteriori Tukey test to determine differences in the slopes of the three levels of the factor (i.e., limpets with crabs, limpets brooding egg masses, limpets without crabs and eggs). Next, we used a Johnson-Neyman (J-N) test to identify those values of the covariate at which the elevation of the curves was significantly different from each other (Hunka & Leighton, 1997). The JN test is used when slopes are heterogeneous and it detects those values of the shell length at which there is and there is not a significant effect of the factor on the covariate (weight of the limpet).

All statistical analyses described in this and the next section were performed using R 3.3.1 (R Core Team, 2020).

2.3 | Histology of limpet

Histology was employed to evaluate: (1) whether the ovary of limpets infested by the pea crab contains oocytes and/or if it exhibits differences with respect to the ovary of crab-free limpets and (2) whether female limpets harboring pea crabs contain spermatozoa inside their seminal receptacles (SR). A total of 16 female limpets were used for histology; 6 hosted female pea crabs, 5 did not host crabs but brooded egg masses, and 5 individuals had neither crabs nor embryos when caught. Samples were dissected under the stereomicroscope and the seminal receptacle was separated from the visceral mass. Samples were dehydrated by transfer through a graded series of ethanol solutions with increasing concentrations. Then, samples were embedded in paraffin and sectioned with a microtome (Leica RM2165, Leica Microsystems, Germany) at 7 μm. The ovary was transversely sectioned while the SR was sectioned across different planes. Samples were stained with hematoxylin-eosin for tissue differentiation. Photographs were taken using an INFINITY1-3 (Teledyne Lumenera, Canada) camera attached to the microscope.

Histological photographs of the ovary were analyzed to look for differences in the proportion of the area occupied by oocytes (mature and immature), the unoccupied area in the lumen of the tubule (i.e., unoccupied tubule space), and the unoccupied space outside tubules (i.e., intertubular space) among limpets harboring crab, limpets brooding eggs, and limpets with neither crabs nor embryos. For this purpose, one representative photography per individual taken at

×100 was divided into a grid of 20 × 20 μm squares with the software ImageJ (Rueden et al., 2017). We used histology preparations that were sectioned at the same depth of the ovary. Four of the 20 × 20 μm squares per individual were randomly chosen and the area of free space and that covered by oocytes was measured. The grids containing deformations, breaks, or stretching of the structures were not used to avoid any bias in calculating the areas. The maximum length of mature oocytes with evident cytoplasm and nucleus was also measured. Differences in these areas and the mean size of oocytes among the three limpet categories were investigated with different one-way analysis of variances (ANOVAs). Prior to the ANOVAs, the data set was graphically inspected for normality and homogeneity of variances as described above. The a posteriori Tukey test for multiple comparisons was applied when the ANOVA detected an effect of the factor over the response variable.

Differences in the disposition of spermatozoa between crab-free limpets and those limpets harboring crabs (see results) made it impossible to stereologically analyze the SR as it was conducted for the ovary. Instead, we decided to describe differences in SR among limpet categories qualitatively.

3 | RESULTS

3.1 | Frequency of occurrence of the pea crab and impact on the limpet spawning

We collected a total of 177 limpets harboring the pea crab *C. garthi*, 46 sampled during March 2014, 71 in November 2014, and 60 in February 2015. The shell length of limpets harboring crabs varied between 4.5 and 25.4 mm with a mean (±*SD*) of 18.63 (2.96) (Figure 2). Crabs occurred in limpets with a mean frequency 3.66% (3.11% in March 2014, 4.06% in November 2014, and 3.82% in February 2015), indicating most limpets did not host crabs (Figure 2). The smallest host was a sexually undifferentiated juvenile limpet of 4.5 mm SL, which harbored a tiny first crab stage (i.e., the first stage after larval metamorphosis) of 0.62 mm CW. This individual was the only crab found within a juvenile limpet. Few male and female crabs were found inhabiting functional male limpets. By contrast, the greatest frequency of occurrence of crabs (ovigerous and non-ovigerous stage-V females, hard males, and co-occurrence of two crabs) was found among the largest functional female limpets (Figure 2). The most frequently observed crab stage was the stage-V female, followed by hard males. Hard females, immature stage II–IV females, and pre-hard crabs were also found but in low frequency (Figure 2). A total of 19 co-occurrences of two crabs within the same host individual were found among hosts. Most of these pairs (15) were stage-V females with hard males. The other four co-occurrences comprised one hard male and one immature female.

The 9%, 26.3%, and 34.6% of sexually mature limpets (i.e., specimens larger than 13 mm SL see Cledón et al., 2016) were found brooding egg masses when sampled during March 2014, November 2014, and February 2015, respectively; with a mean
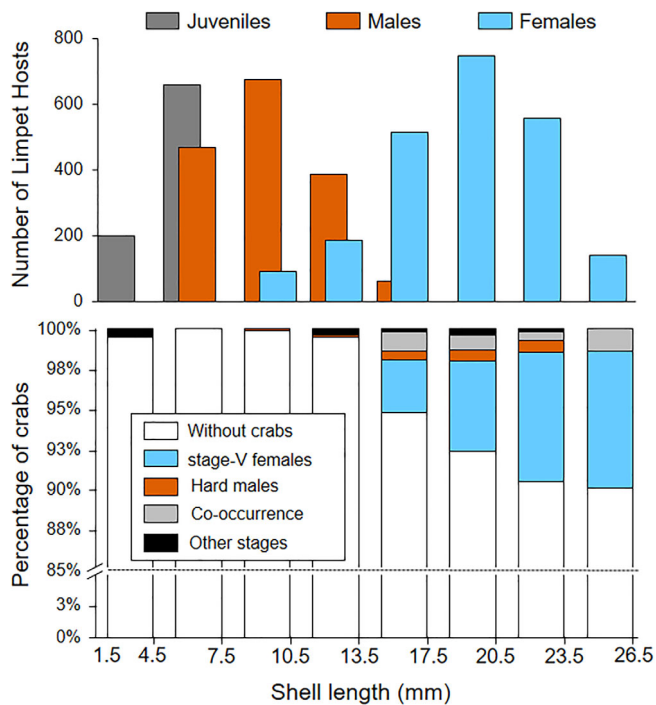
FIGURE 2 Population structure of the limpet host *Bostrycapulus odites* (above) and the percentage of occurrence of different demographic categories of pea crabs in limpets from different size classes (below)

frequency of 23.3% considering all seasons. None of the parasitized limpets were observed incubating egg masses. Accordingly, the coexistence of crabs and egg masses was lower than expected under the null hypothesis of independence (chi-square test of independence: $X^2 = 44.35$, $df = 1$, $p < .0001$).

Pooling all stage-V female individuals from the three sampling dates, the mean (± *SD*) dry body weight of these crabs was 0.007651 (0.002731) g, and it represents 1.07% of the mean dry body weight of limpets harboring crabs. In turn, pooling egg masses from limpets caught during the three dates, the mean (± *SD*) dry weight was 0.008651 (0.005467) g. On average, the egg masses represented 1.09% of the mean dry body weight of limpets brooding eggs.

3.2 | The impact of pea crabs on the limpet body weight

In the austral autumn and spring, the body condition of the limpet host *B. odites* varied between parasitized and non-parasitized limpets. The interaction term between the factor and the covariate was significant in these two seasons, indicating the heterogeneity of slopes (Table 1). The pairwise comparisons revealed differences in the slopes of the regression lines between parasitized and non-parasitized limpets (both limpets brooding and non-brooding eggs) (see Tukey test for autumn and spring in Table 1). However, no differences in the slopes were detected between non-parasitized limpets that brooded eggs and non-parasitized non-brooding limpets in both seasons

(Table 1). When we compared parasitized limpets with limpets brooding eggs, the J-N test detected differences in the elevation of the lines depicting the relationship between body weight and SL in individuals ≥15.86 and ≥ 16 mm SL in autumn and spring, respectively (Figure 3a,b). Similarly, the J-N test showed differences in the elevation of the regression lines between parasitized and non-brooding non-parasitized limpets ≥18.11 and ≥ 15.88 mm SL in autumn and spring, respectively (Figure 3a). The J-N test revealed no differences in the elevation of the regression lines between brooding limpets and non-brooding non-parasitized limpets during both seasons. Therefore, the J-N test indicated that female crabs of *C. garthi* impact the body condition of almost all (but not the smallest) female limpets in the population in autumn and spring. By contrast, the body condition of unparasitized limpets did not vary with the reproductive status (i.e., brooding or non-brooding) of the limpets during these seasons.

In the austral summer, the body condition of the limpet host *B. odites* also varied between parasitized and non-parasitized limpets. However, in contrast to that observed in other seasons, the interaction term of the GLM was not significant during summer (Table 1). The GLM showed a decrease in the line's elevation depicting the relationship between body weight and SL in parasitized limpets (Figure 3c). The Tukey test revealed differences in the mean body weight between parasitized and non-parasitized limpets (both limpets brooding and non-brooding eggs) (Table 1). However, no differences in the mean body weight were detected between non-parasitized brooding and non-parasitized non-brooding limpets (Table 1). Thus, GLM indicated all female limpet hosts in the population decrease their body weight when parasitized by female crabs of *C. garthi* in summer.

3.3 | Histology of the ovary and comparison of oocyte and cell-free areas

The ovary tubules in limpets of *B. odites* exhibit vitellogenic (mature) and developing (previtellogenic and early vitellogenic) oocytes (Figure 4). The mature oocytes are recognized by their large size, rounded shapes, often visible membrane, and vitellogenic drops disperse in the cytosol (Figure 4b,c,e,f). Developing oocytes are observed in the tubule walls and are characterized by their small size mainly occupied by nuclei (Figure 4b,c). The above cell categories were observed in all analyzed individuals, indicating that the ovary remains active in limpets parasitized by the pea crab. Vitellogenic oocytes dominate the ovary of parasitized and non-parasitized limpets, though previtellogenic cells were also often found in tubule walls (Figure 4a, d). Tubules dilated and full of vitellogenic oocytes characterize the histological sections of the ovary in unparasitized limpets (both in brooding and non-brooding specimens; Figure 4a). However, the ovary of limpets parasitized by female crabs has smaller tubules, a lower proportion of oocytes, and a higher proportion of unoccupied areas (Figure 4d,e). No differences in the maximum length of mature oocytes were observed among parasitized limpets (mean ± *SD*: 157.8 ± 24.6 μm), limpets brooding eggs (mean ± *SD*: 177.8 ± 32.9 μm), and non-parasitized non-brooding limpets (mean ± *SD*: 192.3 ± 34.1 μm)

GLMs	F	t ratio	JN
Autumn			
Slopes (interaction term)	**3.5483***		
Tukey pairwise comparisons			
Limpets harboring crabs versus limpets brooding eggs		**−2.660***	
Limpets harboring crabs versus non-parasitized non-brooding limpets		**−2.374***	
Limpets brooding eggs versus non-parasitized non-brooding limpets		−0.893	
Elevation		n.s.	
Limpets harboring crabs versus limpets brooding eggs			≥ 15.86
Limpets harboring crabs versus non-parasitized non-brooding limpets			≥ 18.11
Limpets brooding eggs versus non-parasitized non-brooding limpets			-
Spring			
Slopes (interaction term)	**3.3304***		
Tukey pairwise comparisons			
Limpets harboring crabs versus limpets brooding eggs		**−2.438***	
Limpets harboring crabs versus non-parasitized non-brooding limpets		**−2.593***	
Limpets brooding eggs versus non-parasitized non-brooding limpets		0.320 n.s.	
Elevation			
Limpets harboring crabs versus limpets brooding eggs			≥ 16
Limpets harboring crabs versus non-parasitized non-brooding limpets			≥ 15.88
Limpets brooding eggs versus non-parasitized non-brooding limpets			-
Summer			
Slopes (interaction term)	2.367 n.s.		
Elevation	**12.320*****		
Tukey pairwise comparisons			
Limpets harboring crabs versus limpets brooding eggs		**−3.317****	-
Limpets harboring crabs versus non-parasitized non-brooding limpets		**−4.260*****	-
Limpets brooding eggs versus non-parasitized non-brooding limpets		−0.893 n.s.	-

TABLE 1 Result of GLMs of soft body weight against shell length in limpet of *Bostrycapulus odites* harboring female crabs of *Calyptraeotheres garthi*, brooding egg masses, and without crabs and eggs (i.e., non-parasitized non-brooding limpets) during autumn, spring, and summer

Note: In case of heterogeneity of slopes, the Johnson–Neyman (JN) test provides the depth range (mm) in which elevations are different.
*p < .05, **p < .01, ***p < .001.

(ANOVA test: $F = 1.424$, $df = 2$, $p = .235$). In turn, significant differences in the space occupied by oocytes were detected among parasitized limpets, limpets brooding eggs, and non-parasitized non-brooding limpets (ANOVA test: $F = 30.444$, $df = 2$, $p < .0001$). The percentage of the area covered by oocytes was similar in non-parasitized non-brooding limpets (89.4%) and limpets brooding eggs (88.8%; Table 2). However, the portion covered by oocytes was smaller in parasitized limpets attaining, on average, 77.09% of the total area of the ovarian tubules (Table 2). Accordingly, significant differences were found when we compared the tubule (ANOVA test: $F = 10.45$, $df = 2$, $p = 0.00197$) and intertubular (ANOVA test: $F = 4.1495$, $df = 2$, $p = 0.04039$) cell-free areas between the three groups of limpets (Table 2). In parasitized limpets, the proportion of unoccupied intertubular space of the ovary (14.81%) was significantly higher than that in brooding limpets (7.96%) and non-brooding non-parasitized limpets (6.95%). Lastly, the portion of unoccupied space in the tubule lumen was also higher in parasitized limpets (8.10%) than in non-brooding crab-free limpets (3.65%) but not significant when compared with brooding limpets (Table 2).
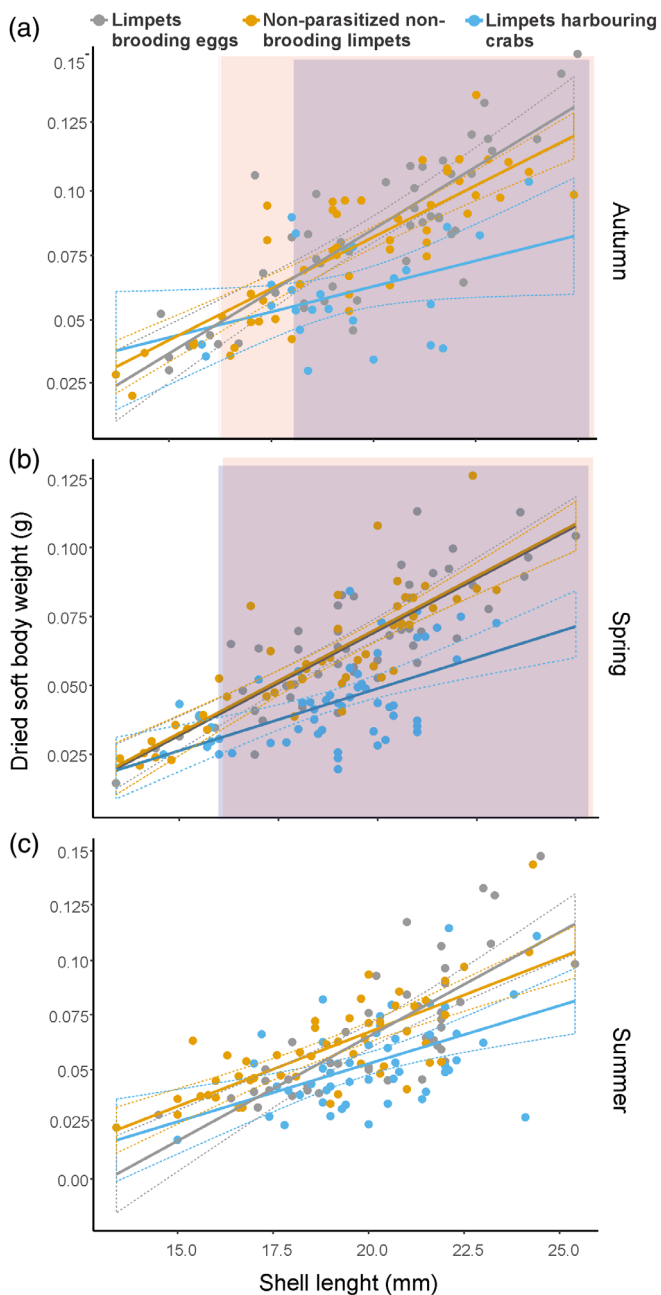
FIGURE 3 Relationship between dry soft body weight and shell length in limpets of *Bostrycapulus odites* brooding egg masses, harboring female pea crabs and non-parasitized non-brooding limpets. The size range in which there was a significant effect of the crab on the host condition respect to limpets brooding eggs is highlighted in light pink. The gray squares indicate the size range in which pea crabs impact host condition compared with limpets without crabs and eggs

3.4 | Histology of the seminal receptacle

The seminal receptacle is found close to the genital pore, located at the right extremity of the pallial cavity. The seminal receptacle consists of 5–8 small, elliptical lobes (Figure 5a), circular in cross-sections (Figure 5b). Each lobe is individually connected to the distal glandular part of the oviduct (most likely the capsular gland) through a long cylindrical duct (Figure 5a). The lobes are single structures, that is, no small subdivisions (= lobules) were found in our histological preparations. The lobes attain similar sizes in parasitized (mean maximum length ± SD: 0.31 ± 0.11, n = 5) and unparasitized (mean maximum length ± SD: 0.32 ± 0.12, n = 5) limpets. In crab-free limpets, long thin spermatozoa line the inner surface of each lobe (Figure 5c,d), while sperm debris is typically observed at the center of this structure (Figure 5a,b). The acrosomes are observed in close contact with the inner epithelium of the lobe, with flagella pointing toward the lobe lumen (Figure 5c,d). In two out of the five parasitized limpets analyzed, the general aspect of lobes and sperm arrangement resemble that described above for unparasitized limpets. Two parasitized limpets, however, showed higher proportions of spermatozoa debris. In the three other parasitized specimens, the seminal receptacle had an atypical histological profile (Figure 5e–h). In these individuals, the lobe lumen is almost entirely occupied by free sperm and/or spermatozoa debris (Figure 5e,g). Furthermore, although in some areas the spermatozoa are in contact with the inner epithelia (Figure 5e), most of the sperm appear to be detached from this tissue (Figure 5f,h). In some histological sections of these parasitized limpets, large empty spaces between the spermatozoa and the inner epithelia are observed (Figure 5e), and the epithelial cell layer is mostly degraded (Figure 5f,h).

4 | DISCUSSION

In the present study, we found the body condition of limpets of *Bostrycapulus odites* is negatively affected by stage-V female pea crabs of *Calyptraeotheres garthi*. For the three analysed seasons (austral spring, autumn, and summer), the soft body weight of castrated limpets was lower than that of unparasitized limpets. In the austral summer, female limpets of all sizes were negatively affected by pea crabs while only limpets larger than ~16 mm were impacted during the other seasons. Furthermore, the histology analysis indicates that *C. garthi* does not suppress gamete production but negatively affects host gametogenesis. The ovary of parasitized limpets showed vitellogenic and developing (previtellogenic and early vitellogenic) oocytes. Mature oocytes did not differ in size between parasitized and non-parasitized limpets. These results indicate the previtellogenic oocytes grow, accumulate vitellin droplets, and attain a similar size to those of unparasitized ones. Nevertheless, the histological sections also revealed that limpets harboring crabs had smaller ovarian tubules than non-parasitized individuals. A lower density of oocytes also characterizes the ovarian tubules of parasitized limpets. Accordingly, the ovaries of parasitized limpets showed an increment in both the unoccupied tubule space and the intertubular space. Therefore, as we expected, our results strongly suggest that female pea crabs impact the body condition of limpets and also reduce their capacity to produce oocytes. In addition, we did not find pea crabs and egg masses coexisting in the same host individual during the breeding period of *B. odites* which confirms the pea crab *C. garthi* does castrate this limpet species (see Ocampo et al., 2014). Lastly, we predicted if pea crabs
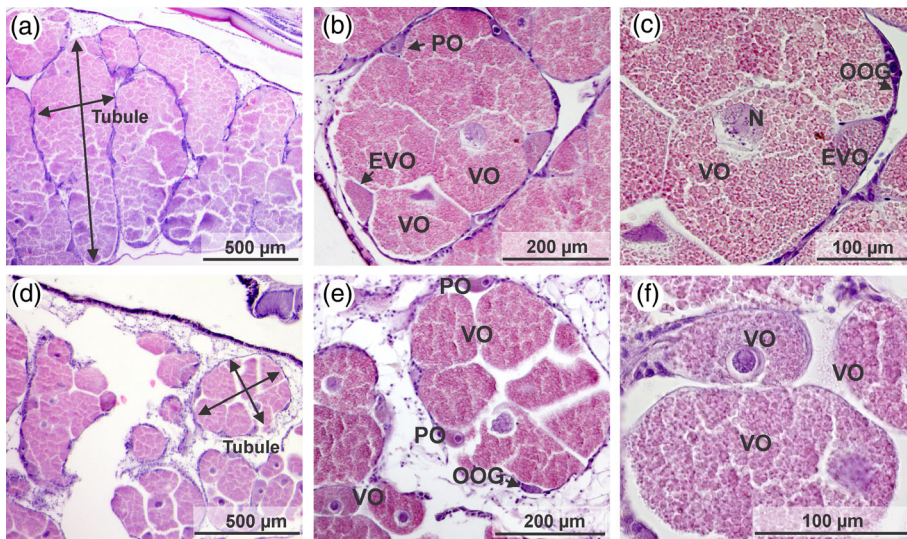
FIGURE 4 The ovary aspect of unparasitized limpets of *Bostrycapulus odites* (a, b, c) and those harboring Stage-V female pea crabs of *Calyptraeotheres garthi* (d, e, f). EVO, early vitellogenic oocytes; N, nucleous; OOG, oogonia; PO, previtellogenic oocytes; VO, vitellogenic (mature) oocytes

TABLE 2 Result of multiple comparison Tukey post hoc for oocyte area, intertubular space, and unoccupied tubule space

Comparisons	Tukey post hoc t ratio
Oocyte area	
Non-parasitized non-brooding limpets versus limpets brooding eggs	0.728 n.s.
Limpets harboring crabs versus non-parasitized non-brooding limpets	**7.005*****
Limpets harboring crabs versus limpets brooding eggs	**6.246*****
Intertubular space	
Non-parasitized non-brooding limpets versus limpets brooding eggs	0.596 n.s.
Limpets harboring crabs versus non-parasitized non-brooding limpets	**4.177****
Limpets harboring crabs versus limpets brooding eggs	**3.554****
Unoccupied tubule space	
Non-parasitized non-brooding limpets versus limpets brooding eggs	0.297 n.s.
Limpets harboring crabs versus non-parasitized non-brooding limpets	**2.798***
Limpets harboring crabs versus limpets brooding eggs	2.289 n.s.

*$p < .05$, **$p < .01$, ***$p < .001$.

interfere with copulation in *B. odites*, empty (or underfilled) seminal receptacles would be found in parasitized limpets. A lack of sperm could explain why some castrated limpets do not spawn eggs after the crabs were removed from the limpet (Ocampo et al., 2014). However, all limpet individuals here analyzed (i.e., parasitized and unparasitized) contained spermatozoa inside the lobes of the seminal receptacles.

While few pea crabs were observed inhabiting small males and juveniles of *B. odites*, stage-V females were invariably found among large female limpets. Host colonization in pea crab species occurs early when the megalopae molt into the first juvenile crab (Møller Christensen & McDermott, 1958; Ocampo et al., 2016). Later, during an intermediate life stage, male and female crabs may shift hosts (Baeza et al., 2018), and thus the female crab recolonizes the host and molts several times attaining stage V (Ocampo et al., 2016). Female crabs appear to select exclusively large limpets during the recolonization, which may explain why we only find these crabs inhabiting large host individuals. Furthermore, the overall occurrence of pea crabs on limpets here reported is low (~3.66%) but increases with host size. Thus, the negative impact that the pea crabs inflict on hosts would particularly affect the largest individuals of *B. odites*. Given that large female limpets exhibit the highest fecundities (Cledón et al., 2016), the impacts exerted by pea crabs on the overall reproductive success of the population could be significant and worthy of evaluation in the future studies.

There are two possible explanations for the impairment in the ovary and the overall condition of limpets observed here. First, several pathogen species, either typical parasitic consumers or castrators, impact hosts' gamete production and/or body condition by exerting direct damage to tissues (Lauckner, 1980; Ramadan & Ahmad, 2010). For instance, trematode sporocysts have been reported to harm and even completely destroy the gonad of their mussel host *Perna perna* (Lasiak, 1993). Moreover, pea crabs species parasitizing bivalves can erode gills and other tissues and these damages result in reduced body weight and growth rate (Møller Christensen & McDermott, 1958; Stauber, 1945). Nevertheless, the pea crab *C. garthi* is not in contact with (nor can it access) any organ of the limpet reproductive system. Moreover, host tissues in contact with pea crab legs and claws (e.g., the food canal of the neck, the gill, and the foot) have been examined under the stereomicroscope in this work and while we conducted previous studies (Ocampo et al., 2014, 2016, 2017) and injuries were never detected. Therefore, it appears unlikely that the impact on body condition results from physical damages in this pea crab-limpet system. Second, the food deprivation that a parasite imposes may cause host-energy depletion that, in turn, alters other energy-demanding
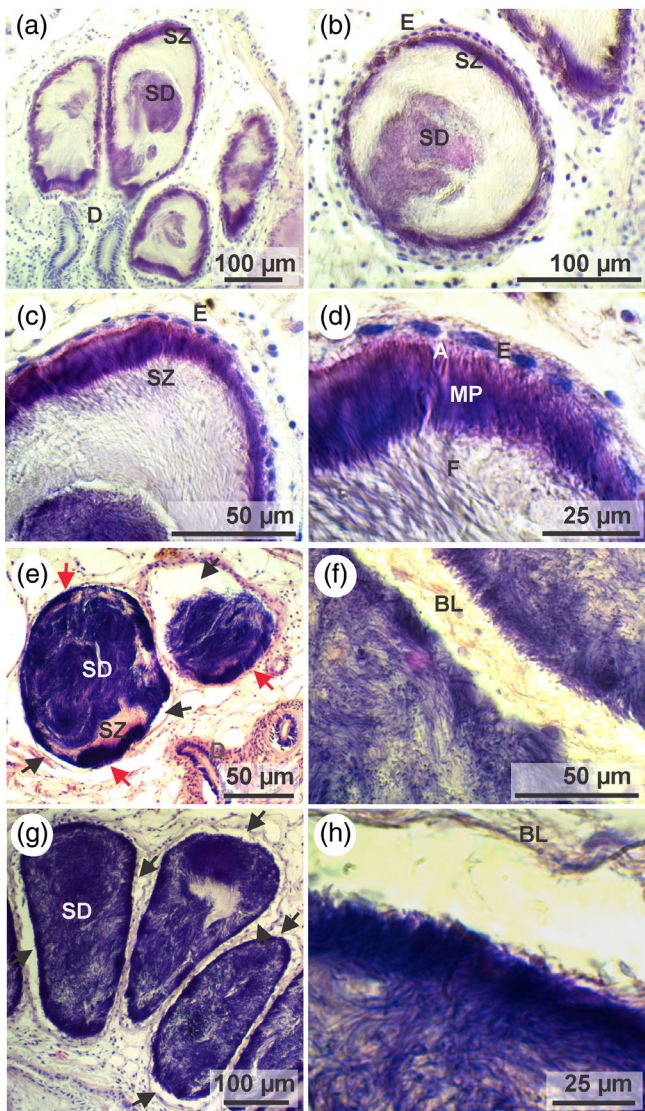
FIGURE 5 Seminal receptacle of unparasitized (a, b, c, d) and parasitized (e, f, g, h) limpets of *Bostrycapulus odites*. In (e) and (g) black arrows point free gaps between inner epithelium and spermatozoa. Red arrows point stretch contact zones between inner epithelium and spermatozoa. A, acrosome; BL, basal lamina; D, duct of the lobe; E, inner epithelium; F, flagelae, MP, mid-piece; SD, spermatozoa debris; SZ, spermatozoa

processes (Lettini & Sukhdeo, 2010; Robar et al., 2011). Limpet species obtain food by filtrating and then concentrating phytoplankton into a mucous cord that is then transferred to the limpet's mouth (Ocampo et al., 2014). Pea crabs are positioned between a fold of the foot (i.e., the propodium) and the ventral side of the neck. From this strategic position, the crab introduces the left claw underneath the neck of the limpet to extract pieces of phytoplankton-rich mucous (Ocampo et al., 2014). This klepto-parasitic feeding behavior of pea crabs could induce a nutritional stress in *B. odites*, resulting in the decrement of gametes production and/or body condition.

Observations made in pea crab parasitizing bivalves provide additional support for the later argument. As in *Calytraeotheres* crabs, pea crabs parasitizing bivalves take food from hosts (González-Ortegón et al., 2021) and this feeding mode is responsible for multiple deleterious effects. For instance, the pea crab *Zaos ostreum* captures nutritive particles from the gills of the *Mytilus edulis* reducing the host gonad area (O'Beirn & Walker, 1999). Similarly, the pea crab *Nepinnotheres novaezelandiae* steals food from the mussel *Perna canaliculus* causing weight loss in the host (Trottier & Jeffs, 2012).

The impact on body condition and gamete production in parasitized individuals of *B. odites* notably contrast to that previously observed in the limpet *Crepidula cachimilla* (Ocampo et al., 2014). In this case, *Calyptraeotheres garthi* does not affect or even improves the body condition of *C. cachimilla* limpets. In a parasitic castration, the host's energy resources are diverted from reproduction to other vital processes such as growth, maintenance, and/or survival (Lafferty & Kuris, 2009). Theoretically, both castrated limpet species (i.e., *B. odites* and *C. cachimilla*) contain an energy surplus that they saved from blocked reproduction activity (Ebert et al., 2004; Hall et al., 2007). Nevertheless, the cost of nutritionally sustaining the pea crabs may differ between these limpets due to the notable disparity of body sizes. Parasitized limpets in *C. cachimilla* attain 52.2 mm of shell length (Cledón et al., 2004). Parasitized *B. odites* here analyzed are substantially smaller, with a maximum shell length of 25.4 mm and a mean and maximum total body weight of 0.71 and 1.11 g, respectively. When the crab weight is normalized by the limpet weight, differences become more evident: the mass of female crabs represent, on average, 1.07% of *B. odites* and 0.38% of *C. cachimilla*. Models on parasitic castration propose that the pathogen virulence evolves toward the parasite demands fit the energy resources available that the host would have used for reproduction (Jaenike, 1996; O'Keefe & Antonovics, 2002). However, in cases, the energy appropriated by the castrator exceeds that saved by the host given the halt in reproduction, the consequence is an increment in host's body weight (Ebert et al., 2004). The energy resources the limpet *C. cachimilla* does not use for reproduction (due to castration) seem to exceed the energy demands of the pea crab. The reallocation of these surplus resources into growth may explain the weight gain that this species experiences when parasitized.

If the energy demands of a parasite overcome those that the host had allocated to its reproductive process, a possible consequence is that the host suffers a reduction in somatic growth and/or reproductive performance. This prediction appears to be met in *B. odites* parasitized by *C. garthi*. The mass of egg capsules of *B. odites* is similar to (or slightly higher than) the mass of pea crabs. Thus, producing, spawning, and brooding eggs for a non-parasitized individual could imply an equivalent cost to sustain the crab for a parasitized limpet. However, the spawning in *B. odites* occurs during intermittent periods in the breeding season and ceases throughout the resting time of winter and part of the autumn and spring. The parasitized limpet, by contrast, has to continuously maintain the pea crab that steals food from the gills and drains the nutritional resources of the host. Therefore, energy shunted away from reproduction may not compensate for the pea crab's energy requirements and would ultimately impact the host's body condition and reproductive performance.

On the other hand, based on two observations (i) that more than half of limpet individuals of *C. cachimilla* did not spawn egg masses after pea crabs were experimentally removed (Ocampo et al., 2014) and (ii) that some individuals of *Crepipatella fecunda* delayed their spawning after being stripped from *Calyptraeotheres* sp., we hypothesized that limpets could not contain sufficient sperm to fertilize oocytes when parasitized by pea crabs (Chaparro et al., 2001). Female of Calyptraeidae limpets store the sperm inside of a seminal receptacle (SR) compartmentalized into sub receptacles named lobes (Beninger et al., 2010). We found that all lobes of parasitized limpets of *B. odites* were filled with spermatozoa. There are two possible explanations for these results. First, contrary to our expectations, female pea crabs do not inhibit the copulation of limpets. Although crabs occupy the same space that the male's penis crosses to reach the female genital pore, copulation would still occur. Second, the spermatozoa observed in the SR would be derived from matings previously to the pea crab colonizing the limpet. Although the time that females of *B. odites* may store sperm is unknown, it has been reported that sperm remain viable for up to 1 year in the SR in some species (Hoagland, 1978). Thus, the observed spermatozoa could have been ejaculated before the pea crabs parasitized the limpets. Unfortunately, the present results do not allow us to discern between the above possibilities. Future experiments using male limpets and controlling the presence of pea crabs in female limpets could shed light on this aspect.

In general, the anatomy of the SR and the morphology of spermatozoa of *B. odites* are similar to those described in the limpet species *Crepidula fornicata* (Beninger et al., 2010, 2016): the lobes showed an elliptic appearance, the spermatozoa exhibited an overall filiform shape with acrosomes embedded in the inner epithelium of the lobes. The main difference with respect to *C. fornicata* is the absence of lobules (i.e., small compartments) in *B. odites* (see Beninger et al., 2016). Interestingly, we observed some differences in the disposition of spermatozoa inside the SR's lobes between non-parasitized limpets and some parasitized individuals. In the SR of parasitized limpets, a great content of spermatozoa debris was found in the center of each lobe. More importantly, in three out of the five parasitized individuals analyzed, free sperm and/or spermatozoa debris almost entirely covered the lobe lumen. In these three parasitized individuals, small areas of the inner epithelium were lined by sperm. Most of these areas, however, showed spermatozoa detached from the epithelia, exhibiting in some cases large free gaps between the spermatozoa and the tissue. The small number of analysed individuals and the disparity of these observations prevent us to make an adequate interpretation of this result. In the limpet *C. fornicata*, sperm are detached and released from the epithelium and then fertilize oocytes (Beninger et al., 2016). If spermatozoa from castrated limpets do not fertilize oocytes (given these limpets do not spawn), these spermatozoa could be accumulating inside the SR and forming the dense masses of debris that we observed in our histological preparations. Future studies using numerous parasitized and crab-free limpets could clarify this issue.

Our results cannot give more details of other aspects of the parasitism of *C. garthi* on *B. odites*. For instance, given oocytes of parasitized limpets grow and attain maturity, the observed decrease in ovary tubule size and the lower proportion of oocytes appear not to compromise future spawning events. Therefore, if a host eventually outlives the pea crab, this limpet would resume the reproduction activity during the breeding season. A reduction in limpet's fecundity could be expected in such cases, considering the impact that pea crabs exert on the reproduction performance of limpets. Analyzing reproductive parameters (e.g., number of eggs, size, and number of egg capsules) in limpets after experimentally retrieved from crabs would help to understand the above supposition. On the other hand, as the entire gonadosomatic weight of *B. odites* is evaluated in this study, we cannot indubitably state that somatic parts are reduced in parasitized limpets. The decrease in soft body weight could be solely a consequence of reduction in gonad area inferred from histological analyses. However, the gonadal tissues in this species constitute a small portion of the total soft body. Given the body condition of parasitized limpets is up to half of the body condition of unparasitized individuals, we suspect infection of *C. garthi* reduces both, somatic and gonadal conditions. Lastly, it would be worthy studying if negative effects of this crab are translated into population-level impacts in *B. odites*, such as reductions in recruitment rate and/or host fecundity compensation (i.e., increased investment in reproduction before infection/anticipated reproduction in smaller females; Ebert et al., 2004; Gilardoni et al., 2012).

AUTHOR CONTRIBUTIONS

Emiliano H. Ocampo and Tomás A. Luppi designed the research. Emiliano H. Ocampo sampled specimens, classified, measure, and weight the specimens, and partially conducted the histology. Emiliano H. Ocampo, Jesús D. Nuñez, and Macarena Pérez García performed the statistical analyses. Emiliano H. Ocampo, Jesús D. Nuñez, Macarena Pérez García, and Tomás A. Luppi wrote the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/jmor.21409.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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