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Infestation of the cupped oysters *Crassostrea angulata, C. gigas* and their first-generation hybrids by the copepod *Myicola ostreae*: differences in susceptibility and host response

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Abstract:

We studied the prevalence and intensity of the parasitic copepod Myicola ostreae in 2 closely related oysters Crassostrea angulata and C. gigas and their F1 hybrids. The effects on host and host reaction were also analysed to better understand host-parasite relationships between copepods and bivalve molluscs. Full reciprocal crosses were carried out between C. angulata and C. gigas and the progenies were reared in the wild in Ria Formosa Lagoon (Portugal), allowing natural infestation by M. ostreae. Prevalence and intensity were significantly higher in C. angulata than in C. gigas. The parasite level of F1 hybrids was similar to C. angulata and significantly higher than in C. gigas. The results of our study support a hypothesis of dominantly inherited susceptibility to M. ostreae infestation. Moreover, copepods were observed on the gill surface of C. gigas engulfed by a capsule-like structure. Histological analyses revealed that the copepods were surrounded by a massive agglomerate of haemocyte-like cells encircled by a thin layer of fibroblast-like cells. This encapsulation response was not observed in C. angulata or in F1 hybrids. These results suggest that the differential susceptibility to M. ostreae between C. angulata and C. gigas may be ascribed to host defence factors.

Keywords: oysters; copepods; hybridization; parasitism; dominance inherence; host-parasite relationship; host reaction; encapsulation

INTRODUCTION

Approximately one-third of the known copepod species are parasites or commensals of invertebrates or fish (Humes, 1994; Ho 2001). Some parasitic copepods of fish species, such as the members of the family Caligidae, often referred to as sea lice, are responsible for serious disease problems in fish farming (Pike and Wadsworth, 1999). The biology of parasitic copepods of invertebrates is much less known than that of fish (Kabata, 1981). The best-documented parasitic copepods of bivalve molluscs belong to the order Poecilostomatoida (Ho, 2000). Most of them are ectoparasites that are usually found in the mantle cavity of their hosts but some cases of endoparasites have also been described (Lauckner, 1983). It is presumed that the majority of these copepods are not really harmful to their hosts (Lauckner, 1983). The damage caused by some of these copepods to their bivalve hosts is thought to be mainly caused by their appendages used for attachment and crawling. Nevertheless, some parasitic copepods have been associated with high mortality rates in bivalve molluscs (Ho and Zheng, 1994). It is not known if the variation of resistance or tolerance to parasitic copepods in bivalves is under genetic control and relatively little is also known about the defence mechanisms of bivalves to parasitic copepods.

The parasitic copepod *Myicola ostreae* belongs to the family Myicolidae and was originally described as a parasite of the Pacific oyster *Crassostrea gigas* in Japan by Hoshina and Sugiura (1953). Several bivalve hosts have been reported in Europe for *M. ostreae* after the introduction of *C. gigas* namely, the Portuguese oyster *C. angulata* (Comps, 1972), the European flat oyster *Ostrea edulis* (His, 1979) and the razor clam *Sinonovacula constricta* (Kim, 2004). Gill lesions caused by *M. ostreae* have been

observed in *C. angulata* and *C. gigas* and it is presumed that high infestation intensity may have some impact on the host (Comps, 1972; His, 1979). This copepod is presumed to be native from Asia as it is known to be present in Japan and Korea, countries where *C. gigas* is endemic (Hoshina and Sugiura, 1953; Kim, 2004). *M. ostreae* was probably accidentally introduced in France with the mass introduction of *C. gigas* from Japan in the 1970's (His, 1979). Since then this copepod has been observed in several European countries (Streftaris *et al.* 2005). Little is known about the biology of *M. ostreae* but it is presumed to share common feature with other members of the family Myicolidae having six nauplial and five copepodid stages before they reach the adult stage (Ho, 2000). The infective stage is the first copepodid where individuals seek a suitable host.

C. angulata and *C. gigas* are two closely related edible cupped oysters of high commercial importance. They are presumed to be native to the North West Pacific region and were introduced into many countries (Ruesink *et al.* 2005). *C. angulata* was accidentally introduced in Europe from Asia, probably soon after the genesis of global shipping routes (Boudry *et al.* 1998). *C. gigas* was voluntarily introduced in Europe in the late 1960's and early 1970's (Grizel and Héral, 1991). *C. angulata* and *C. gigas* interbreed readily in the laboratory (Menzel, 1974) as they are genetically very closely related as revealed by allozymic makers (Buroker *et al.* 1979). Hybrids are fully fertile and no hybridization barrier has yet been demonstrated in the laboratory (Huvet *et al.* 2001; 2002). There is also evidence that the two taxa can hybridize in the wild namely in Ria Formosa lagoon, Portugal (Huvet *et al.* 2004) where the present study was performed. Nevertheless, genetic (O'Foighil *et al.* 1998; Huvet *et al.* 2000; Leitão *et al.* 2007), morphological (Batista *et al.* 2008) and physiological (Haure *et al.* 2003; Batista *et*

al. 2007) differences have been observed between the two taxa. In the present work we performed a common-garden experiment that allowed us to describe the infestation level of *M. ostreae* in the closely related cupped oysters *C. angulata*, *C. gigas* and their F1 hybrids. The current study allowed us to propose a genetic basis for resistance mechanisms to *M. ostreae* in cupped oysters. Moreover, histopathological analyses were also performed to examine host response mechanisms and impact on host.

MATERIAL AND METHODS

Experimental procedure

Crassostrea angulata and *C. gigas* adults were collected from wild populations in Sado river (Portugal) and Seudre estuary (France), respectively. Full reciprocal crosses were carried out using 20 *C. angulata* individuals (15 females and 5 males) and 18 *C. gigas* individuals (10 females and 8 males) in order to produce the following groups: *C. angulata* (AA), *C. gigas* (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA). Larval rearing was carried out in the shellfish hatchery of IFREMER Genetic and Pathology Laboratory (La Tremblade, France) and the juvenile rearing was performed in the shellfish hatchery of IPIMAR/INRB in Tavira (Portugal) as described by Batista *et al*. (2007). Four mesh bags with 50 oysters each per group were transferred from the Tavira shellfish hatchery to the Cacela-Velha site in Ria Formosa Lagoon (Portugal) on the 3rd of March of 2004. At the beginning of the experiment, the oysters from the 4 groups had approximately the same mean shell height and live weight and were 204 days old. Ria Formosa is a tidal lagoon system located on the southern coast of Portugal with a mean depth of 4 m and does not receive permanent fresh water streams. The Cacela-Velha site is in a mid intertidal zone and the sea floor is a mixture of sand and mud. Salinity at Cacela-Velha site during the present study was generally high (around 35 ppt) and temperature ranged between 12 and 25 °C. Oyster bags were placed off-bottom (ca. 40 cm) on iron frame tables. The oysters were collected on the 26th of November of 2004. A total of 280 oysters were examined by macroscopic observation (between 11 and 22 individuals per bag) for the presence of parasitic copepods in their soft tissues and the

number of copepods present in each individual was counted for 84 of them. The copepods observed were identified based on the description by Hoshina and Sugiura (1953). The parasite level in the oysters was assessed using two parameters: prevalence (percentage of infested hosts) and intensity (number of parasites per infested host). The dry shell weight of both valves of the oysters analyzed was recorded to a precision of 0.001 g. For histological examination, a transverse section (ca. 5 mm thick) through the body, containing mantle, gonad, digestive gland and gills was done for each specimen. The tissues were then fixed in Davidson's solution for 48 h and preserved in 70 % ethanol. Sections were cut 3-4 μm, deparaffinized, and stained with hematoxylin and eosin following standard techniques.

Data analysis

Data were analysed using one-way analysis of variance (ANOVA) to determine if there were significant differences among groups for dry shell weight, prevalence and intensity of *M. ostreae* using the oysters bags as the replicates (n = 4). The Cochran test was used to assess heteroscedasticity. Pairwise comparisons between groups were performed using Student-Newman-Keuls (SNK) tests when significant differences were revealed by ANOVA.

RESULTS

The morphologic features of the female copepods observed were consistent with the characters proposed by Hoshina and Sugiura (1953) for *M. ostreae*, namely: female body cylindrical, with prosome swollen in ovigerous specimens, caudal ramus slender, with setae much shorter than ramus, the first antenna with 7 segments, the second antenna with 3 segments and maxilliped absent. The egg sacs were multiseriate. The mean length of the specimens observed was 2.1 mm and ranged between 1.8 and 2.5 mm (not including setae on caudal rami). The main morphological features that allowed us to distinguish the copepods observed in the present study from other species of the genus *Myicola* were: (1) size of ovigerous females (between 1.8 and 2.5 mm); (2) number of eggs in egg sac (about 40 eggs); (3) caudal ramus of females (length/width=5.0); and (4) second segment of leg 5 of females (length/width=1.9). The copepods were observed in the gills of the oysters and no other organ appeared to be affected. All copepods observed were females that were carrying eggs. Some of the females carried eggs that when disturbed hatched free-swimming nauplius with a length of approximately 150 µm.

Significant differences in the prevalence of *M. ostreae* were observed among groups [$F_{(3, 12)} = 40.4$, $p < 0.001$]. In the *C. angulata* group (AA), the mean prevalence of *M. ostreae* was 71 % whereas in AG and GA hybrids a slightly lower prevalence was observed (60 and 59 %, respectively), but not significantly different from AA group (SNK test; $p > 0.05$). A highly significantly lower mean prevalence (SNK test; $p < 0.001$) was observed in *C. gigas* progeny (GG; 23 %) compared to AA, AG and GA groups (Fig. 1A). The number of *M. ostreae* per oyster in *C. angulata* progeny ranged from 1 to 13, and in both AG and GA hybrids the number of copepods ranged between 1 and 8. In *C.*

gigas progeny the number of parasitic copepods per host ranged between 1 and 5. Significant differences in the mean intensity were observed among groups [$F_{(3, 12)} = 11.1$, $p < 0.001$]. Pairwise comparisons revealed no significant differences in intensity between AA, AG and GA groups (SNK test, $p > 0.05$). However, the infestation intensity was significantly lower (SNK test, $p < 0.05$) in *C. gigas* in comparison with the other groups (Fig. 1B).

The area in the gills where *M. ostreae* was attached had a yellow/green aureole which could be associated with a hemocyte infiltration. The histological examination revealed copepods in the gills that where interpreted as being *M. ostreae* based on the macroscopic observations. Occasionally, hemocytic infiltrations and gill lesions were observed near the region where the copepods were attached and tissue debris was observed surrounding the copepods (Fig. 2). Some *M. ostreae* attached to gills of *C. gigas* specimens (in ca. 20% of the individuals analyzed) where involved by a massive agglomerate of hemocyte-like cells (Fig. 3). The external part of this formation was covered by a thin layer of fibroblast-like cells, similar to a capsule. No fibers were observed in the agglomerate of cells surrounding the copepods. This encapsulation response was only observed in *C. gigas* individuals.

The shell dry weight (DSW) was significantly different among groups ($F_{(3, 12)} = 52.7$, $p < 0.001$). The DSW of GG group was significantly higher (SNK test; $p<0.01$) than AA and AG groups, but not from GA group (Fig. 3). The DSW of AA group was significantly lower than AG, GA and GG groups (SNK test; $p<0.01$). No significant differences were observed between AG and GA groups (SNK test; $p>0.05$).

DISCUSSION

A higher prevalence and intensity of *M. ostreae* was observed in *C. angulata* than in *C. gigas*. We also reported that F1 *C. angulata* X *C. gigas* reciprocal hybrids had a pattern of susceptibility to *M. ostreae* that resemble the susceptible parent, which constitute the first example to date of such type of response (dominance for susceptibility) on hybrid animals (Fritz *et al.* 1999; Wolinska *et al.* 2007). A putative defence mechanism, involving the encapsulation of the parasitic copepods at the gill surface, was only observed in *C. gigas* individuals. These results lead us to hypothesise that the differential susceptibility to *M. ostreae* between *C. angulata* and *C. gigas* may be ascribed to host defence factors.

Parasite level in C. angulata *and* C. gigas

The prevalence of *M. ostreae* in *C. gigas* observed in the present study (23 %) was similar to the one observed by Hoshina and Sugiura (1953) of 10-30 % and within the interval reported by His (1979) of 4-40 %. We also observed an infestation intensity in *C. gigas* similar to the one reported by His (1979) of 1 to 4 copepods per host. This suggests that the parasite levels observed in Cacela-Velha site where within the values previously observed in *C. gigas*. However, parasite levels observed in *C. angulata* were significantly higher than in *C. gigas*. Since there are no published studies about the prevalence and intensity of *M. ostreae* in *C. angulata* it is not possible to tell if the levels observed were abnormally high for this species or not.

Although the infestation mechanism of *M. ostreae* is not known it may be assumed that it is similar to the one observed in other parasitic copepods of bivalves in

which a free-swimming copepodid enters the host by the inhalant current (Gee and Davey, 1986). The infestation success is therefore generally associated with the amount of water filtered by the host and consequently by its size and age. At the end of the experiment, the dry shell weight of *C. gigas* individuals was significantly higher than *C. angulata* individuals (Fig. 4). Furthermore, it was shown that the mean filtration rate in *C. gigas* is equal or higher to the one in *C. angulata* (His, 1972; Haure *et al.* 2003). Hence, the higher parasite level observed in *C. angulata* when compared with *C. gigas* is unlikely to be due to differences in size and/or filtration rate. A possible explanation for the observed higher parasite level in *C. angulata* is that European populations of this taxon have not been in contact with *M. ostreae* since it was introduced in Europe from Asia (O'Foighil *et al.* 1998) and hence they could be seen as a naive host. Indeed, *M. ostreae* was not observed in Taiwan (Lin and Ho, 1999) where *C. angulata* is known to be present and from where *C. angulata* is presumed to have been introduced into Europe (Boudry *et al.* 1998).

Parasite level in hybrids

Several studies have been conducted on parasitism of plant and animal hybrids (Fritz *et al.* 1999; Moulia, 1999; Wolinska *et al.* 2007). Different patterns have been observed which imply different genetic mechanisms. Hybrids can be more susceptible (hybrid susceptibility pattern) or more resistance (hybrid resistance pattern) to parasites compared to parental taxa. Moreover, hybrids may resemble one of the parental taxa in resistance characteristic (dominance pattern). For this pattern, two different scenarios can be observed: (1) hybrids may resemble the susceptible parent (dominance for susceptibility);

or (2) hybrids may resemble the resistant parent (dominance for resistance). Parasite levels in hybrids can also be intermediate between parental taxa (additive pattern). It seems that the hybrid susceptibility pattern is the most common one in animal hybrids and that the dominance pattern is the most unusual one (Fritz *et al.* 1999). In the present study, the parasite level of reciprocal *C. angulata* x *C. gigas* F1 hybrids was similar to *C. angulata* but significantly higher than in *C. gigas*. These results suggest that susceptibility to *M. ostreae* is inherited as a dominant character in the F1 hybrids. To our knowledge all studies conducted with animal hybrids until present observed the dominance to resistant pattern (Fritz *et al.* 1999; Moulia, 1999; Wolinska *et al.* 2007). On the contrary, the results of the present study support a hypothesis of dominantly inherited susceptible traits.

A study conducted with two closely related blue mussels revealed that susceptibility was related to a single parental genome (Coustau *et al.* 1991). It was observed that *Mytilus edulis* and introgressed individuals with a predominantly *M. edulis* genome were more susceptible to the trematode parasite *Prosorhynchus squamatus* than *M. galloprovincialis*. Since the putative different type of hybrids sampled in the wild (*i.e.*, F1s, F2s, backcrosses or later-generation hybrids) were pooled together this prevents a direct comparison with our results. Nevertheless, a similar pattern was observed in the present study in which individuals with *C. angulata* genes had a higher parasite level than pure *C. gigas* individuals. One explanation for the lower parasite level observed in *C. gigas* in comparison with *C. angulata* and F1 hybrids might be that some host-specific factors required by *M. ostreae* are not present in *C. gigas*. Another explanation might be

that putative anti-parasite factors produced by *C. gigas* are silenced or reduced in the hybrids leading to the higher parasite levels observed in the present study.

Effect on host and host response

In the present study, gill lesions and yellow/green aureoles were observed in the regions where *M. ostreae* copepods were attached. Comps (1972) also reported gill lesions and yellowish aureoles in the regions of attachment of *M. ostreae* in *C. angulata* individuals. In some cases histological examination revealed heavy hemocytic infiltration in the gills most probably caused by copepods. The gill lesions caused by *M. ostreae* were apparently not severe. Although the gill lesion caused by *M. ostreae per se* apparently have little impact on the host maybe they can reduce host fitness and/or allow the introduction of pathogens especially when infestation intensity is high. Indeed, it has recently been suggested that ectoparasites can immunosuppress their invertebrate host (Yang and Cox-Foster, 2005).

Phagocytosis and encapsulation are two major mechanisms of the cellular response in bivalve molluscs. Encapsulation is the mechanism by which hemocytes isolate or remove foreign intruders through the formation of a capsule-like envelope around them (Cheng and Rifkin, 1970). This host defence reaction is generally used against intruders that are too large to be phagocytosed. In the present study, single copepods were observed in the gills of *C. gigas* engulfed by a capsule-like structure composed by multiple cells but no fibers were observed. Five distinct types of encapsulation were described in bivalve molluscs in response to metazoan parasites by Cheng and Rifkin (1970). The encapsulation complex observed in the present study

resembles the “Leucocytic encapsulation” type described by Cheng and Rifkin (1970) that involves the aggregation of hemocytes to form a tunic surrounding the parasite.

Figueras *et al.* (1991) observed in *M. galloprovincialis* mussels an unidentified copepod buried in the peripheral connective tissue of the digestive gland producing a strong haemocytic encapsulation response. A similar response was observed by Cáceres-Martínez and Vásquez-Yeomans (1997) in *M. galloprovincialis* and *M. californianus* mussels described as a granuloma-like structure engulfing *Pseudomyicola spinosus* copepods in which hemocytes were involved. Copepods encapsulated by hemocytes were also observed in connective tissue surrounding the digestive gland of *M. galloprovincialis* (Olivas-Valdez and Cáceres-Martínez, 2002). The encapsulation response described in the three previous studies is similar to the one observed in the present study though the capsule-like structures were observed outside of oyster tissues at the gill surface. A similar host reaction was observed in the polychaete *Spirographis spallanzani* to the copepod *Sabelliphilus sarsi* (Carton, 1967). Carton observed that the copepods were engulfed by an exudate of blood and coelomic cells leading to a reduction in the number of copepods infesting *S. spallanzani*. Other studies have also reported large differences in the encapsulation response between closely related taxa. (Knopf and Mahnke, 2004; Prévost *et al.* 2005).

The lower prevalence and intensity of *M. ostreae* observed in *C. gigas* than in *C. angulata* together with the observation of copepod encapsulation only in *C. gigas* lead us to hypothesize that the differential susceptibility to *M. ostreae* between the two oyster taxa may be ascribed to host defence factors. However, it remains unclear if, and how, the encapsulation response observed in *C. gigas* can eliminate adults of *M. ostreae*.

Further insights into the putative cellular host response of *C. gigas* and *C. angulata* to *M. ostreae*, might be obtained by experimental infestation studies. It is known that resistance of parental taxa and hybrids to parasites may be influenced by genetic and environmental factors as well as by the physiological condition and age of the host (Fritz *et al.* 1999). In the present study, the effects of these confounding factors were minimized since all animals were of the same age, were grown under identical conditions in a common-garden experiment, were naturally infested by the parasite and the genetic status of the hybrids was known.

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Figure 1. Mean prevalence (A) and intensity (B) of *M. ostreae* in *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrids (AG and GA). The error bars represent the standard deviation (n = 4). Groups with different letters are significantly different (SNK test, $p < 0.05$).

Figure 2. A section of the gills of *C. angulata* showing a *M. ostreae* attached to gills (arrowhead) and hemocyte infiltration near that region (arrow). Hematoxylin-eosin staining.

Figure 3. A section of (A) an adult female of *M. ostreae* observed in the gills of *C. gigas* involved with hemocyte-like cells, and (B) magnification of the cells surrounding the copepod. co, *M. ostreae*; eg, copepod eggs; ce, hemocyte-like cells; f, cell-like wall. Hematoxylin-eosin staining.

Figure 4. Mean dry shell weight of *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrids (AG and GA). The error bars represent the standard deviation (n = 4). Groups with different letters are significantly different (SNK test, $p < 0.05$).

Figure 1

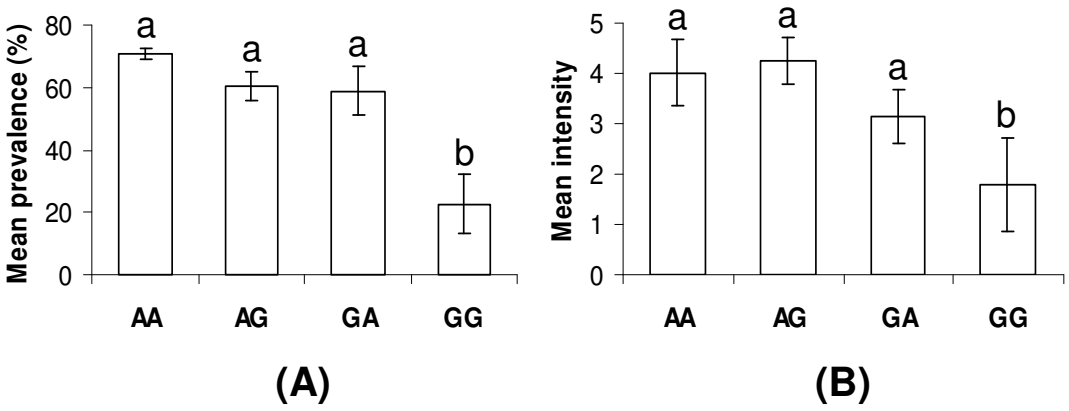
Mean prevalence (%)
80
60
40
20
0
a
a
a
b
AA
AG
GA
GG
(A)
Mean intensity
5
4
3
2
1
0
a
a
a
b
AA
AG
GA
GG
(B)

Figure 2

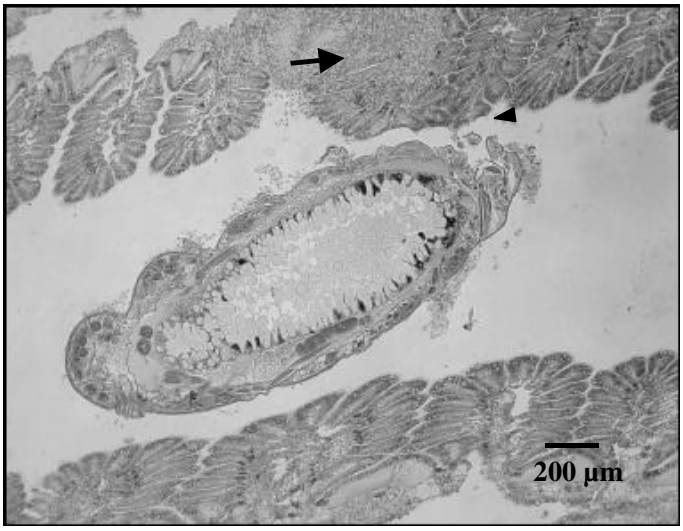

Figure 3

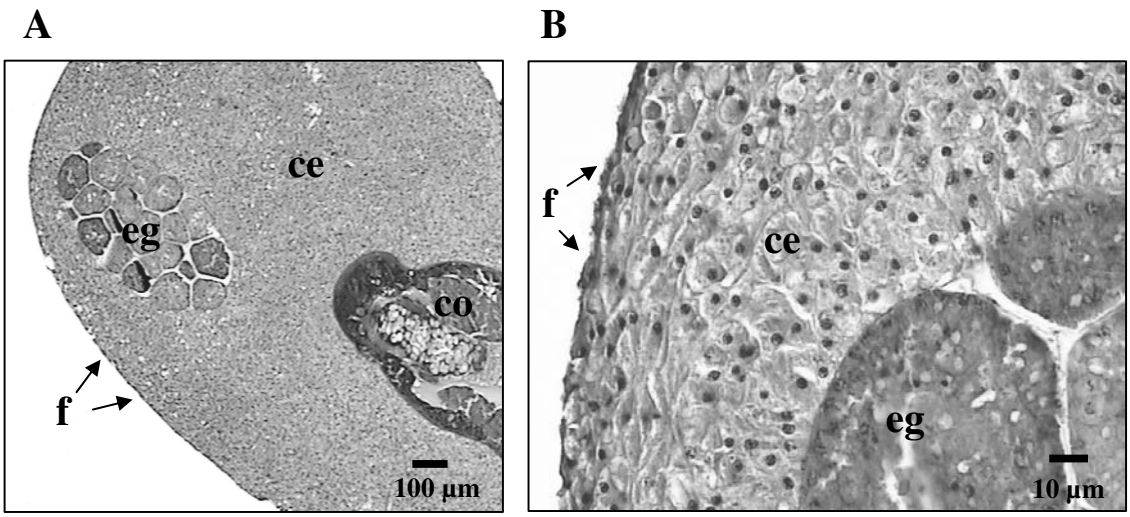

Figure 4