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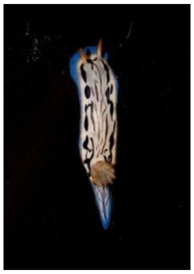

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Effects of the Parasite *Loxothylacus texanus* on the Agonistic Behavior of the Crab *Callinectes rathbunae*

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Abstract: This study presents the changes in the agonistic behavior of the crab *Callinectes rathbunae* parasitized by *Loxothylacus texanus*, collected in Alvarado Lagoon, Mexico. Alive parasitized crabs were observed in the laboratory for their behavior. Hepatopancreas of the parasitized crabs were fixed for examination. Both crabs with virgin and mature externa were monitored, and their degree of aggressiveness compared. The aggressiveness of the parasitized crabs decreases according to the parasite development. A possible relation with behavior and hepatopancreas infestation is given. In this study it is observed that the crabs with virgin externa are more aggressive than organisms with mature externa. Also, the agonistic behavior of the host decreases according to the development of the parasite. This is the first report where behavioral changes of parasitized crabs caused by a rhizocephalan, degree of development of the parasite and hepatopancreas atrophy are described.

Keywords: Aggressiveness, Behavior, Histology, *Loxothylacus*, Parasitism, Rhizocephala, Hepatopancreas, *Callinectes rathbunae*

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Introduction

The Rhizocephala is a group of uniquely specialized parasites infesting a range of other Crustacea, mainly decapods. The adult parasite consists of an external reproductive body that connects through a stalk with a more or less extensive system of rootlets inside the host (Høeg, 1992; Høeg and Lützen, 1995; 1996). It is significant that the root system invades the neuroendocrine organs of the host before invading the digestive system (Høeg, 1995; Bresciani and Høeg, 2001). A female cypris infects the recently molted host by penetrating the soft exoskeleton, giving rise to an internal phase named ‘‘interna” that develops into a net of fine rootlets that will anchor and nurture the reproductive body of the parasite known as the ‘‘externa”. After a variable growing period, when it reaches sexual maturity, the externa emerges, breaking the soft tegument of the internal surface of the host's abdomen. The externa is then fertilized by a male cypris and starts growing until the gonads contained in it mature and start releasing larvae (Høeg, 1992; Høeg and Lützen, 1995).

In the coastal lagoons of the Gulf of Mexico, one of the most important biotic factors that affect populations of crabs of the genus *Callinectes*, is the parasitism caused by rhizocephalan parasites (Crustacea: Rhizocephala) (Andersen *et al.*, 1990; Alvarez and Calderón, 1996).

The parasitic castration, cease of shell shedding and hyperfeminization are some effects that this parasitic barnacle causes to its host (Høeg and Lützen, 1995; 1996). The parasite inhibits reproduction and moulting and in males a feminized behavior is shown (Alvarez and Calderón, 1996). Also, Vázquez-López *et al.* (2006 b) reported paralysis, blindness and behavioral changes in *C. rathbunae* parasitized by *L. texanus*. The effects caused by *L. texanus* in *C. rathbunae* have been least studied. Based on what was found in the literature on the parasitic-host relationship (Rhizocephala-crabs), we believe that the agonistic behavior of crabs is altered according to the development of the root system of the parasite, within the hepatopancreas of the host, which is also affected. Keeping this in view, this work was aimed to describe the agonistic behavior of *C. rahbunae* with different degree of parasitism caused by *L. texanus*.

Materials and Methods

In the lagoon system of Alvarado, Veracruz, *C. rathbunae* parasitized by *L. texanus* were collected from commercial catches. In the sampling location, crabs with different levels of parasitism were fixed (virgin externa, parasites that do not produce larvae and mature externa, parasites that produce larvae) following the classification used by Wardle and Tirpak (1991) and Alvarez and Calderón (1996) for *Callinectes sapidus* parasitized by *L. texanus*. In addition an adult male and female which recently molted (to ensure that they were healthy organisms) were obtained from a local producer, serving as control organisms. All organisms were fixed by using an injection filled with a formaldehyde solution (Humason, 1972; Hammersen and Sobotta, 1980; Johnson, 1980; Paniagua and Nistal, 1983; Bell and Lightner, 1988; Bancroft and Gamble, 2008), in order to preserve the hepatopancreas and were transported to the Laboratory of Invertebrates, Faculty of Sciences at the

National Autonomous University of Mexico (UNAM). All organisms were measured and sexed.

In the laboratory hepatopancreas dissection was performed on each specimen: a male and a female recently molted, a feminized male, four crabs with virgin externa and five with mature externa. The hepatopancreas were processed using standard techniques and subsequently 10 µm thick tissue sections were obtained by using a manual microtome (American Optical). The sections were stained were with hematoxylin-eosin. The resulting slides were analyzed and images were taken from a compound microscope Olympus BX.

In the laboratory, the alive crabs were individually kept under culture conditions within recirculation water systems, with an initial salinity of 5‰, which then was gradually increased until it reached 15‰. Acclimation period lasted one week. Temperature at the laboratory was always at 27 C and represented an average of the temperatures recorded at field, being the minimum 25 C and the maximum 30 C. Salinity and temperature were daily measured. They were fed *ad libitum* every other day at different times, with fish fillet and/or chicken liver. The food remains were removed to avoid decomposition.

At the end of the acclimation period, the behavior of 113 parasitized crabs was monitored for six months, 93 with virgin externa and 20 with mature externa. The observations were made from 9:00 to 20:00 by the focal subgroups method, following the criteria proposed by Altmann (1974), Backwell *et al.* (2006) and De la Cruz-Manjarrez and Vázquez-López (2015).

The categories of agonistic behavior considered in the present investigation were submission and threat and/or aggression (attack) (Jachowski, 1974; McGlone, 1986; Innocenti *et al.*, 1998, 2003). The first (both sexes) were those who didn´t show any sign of threat, aggression or defense or which were slow in their responses. The aggressive crabs (both sexes) were the ones that showed aggressiveness or defense. A Chi-square test (X^2) was used to compare frequencies of the number of externa. Another Chi-square test (X^2) was used to compare behavior categories (Sokal and Rohlf, 1981; Statistica Spreadsheet of Excel).

Results

The minimum carapace width from hosts was 5.8 cm and the maximum 9.67 cm, with an average of 7.73±2.73 cm.

The chi-square test (number of mature externa X20=17.86, *p*>0.05 X20.05,6=12.59; number of virgin externa X20=44.69, *p*>0.05 X20.05,6=12.59) showed that the number of externa is related to the behavior of the parasitized crabs.

The chi-square test (mature externa vs virgin externa $X^2=35.21$; df=1, $p<0.001$) showed that the agonistic behaviors submission and threat and/or aggression (attack) are related to the development of the externa.

The hepatopancreas of unparasitized crabs consisted of an arrangement of tubules in close proximity, each one with a lumen with the shape of an X and an interstitial space between them (Fig. 1). The tubules presented an average length of 169.06 ±40.18 µm. Roots from *L. texanus* were observed in those slices from crabs with a virgin externa. They were positioned both transversally and logitudinally, infiltrated among the tubules, separating them, but despite this, the structure of the hepatopancreas was still recognized (Fig. 2 A). In those slices from crabs with a mature externa the tubules were more separated because of the roots from *L. texanus* (Fig. 2 B-G).

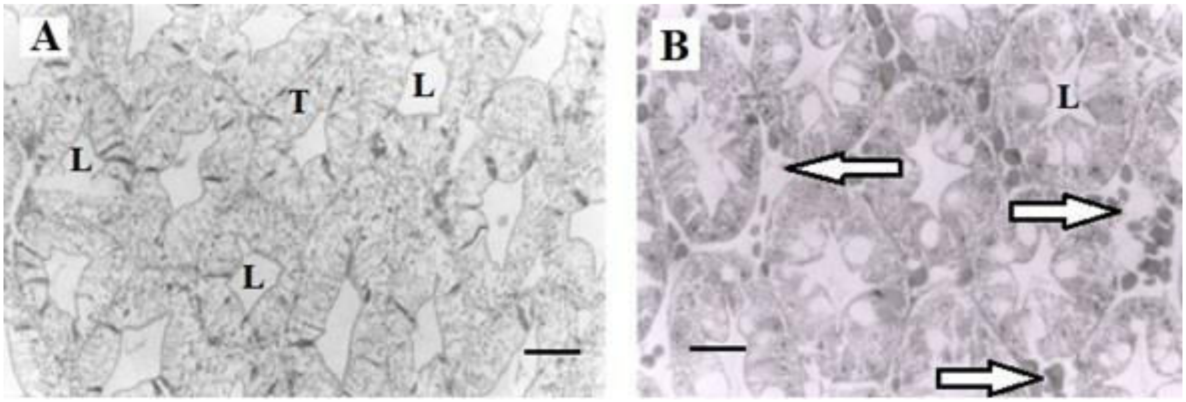

Fig. 1 (A): Healthy hepatopancreas from a female crab *C. rathbunae* that has recently shedded its shell. L= lumen, T= tubule. Scale bar 50 µm. (B) Healthy hepatopancreas from a male crab *C. rathbunae* . L= lumen, the arrows indicate the interstitial spaces. Haemotoxylin and eosin. Scale bar 50 µm.

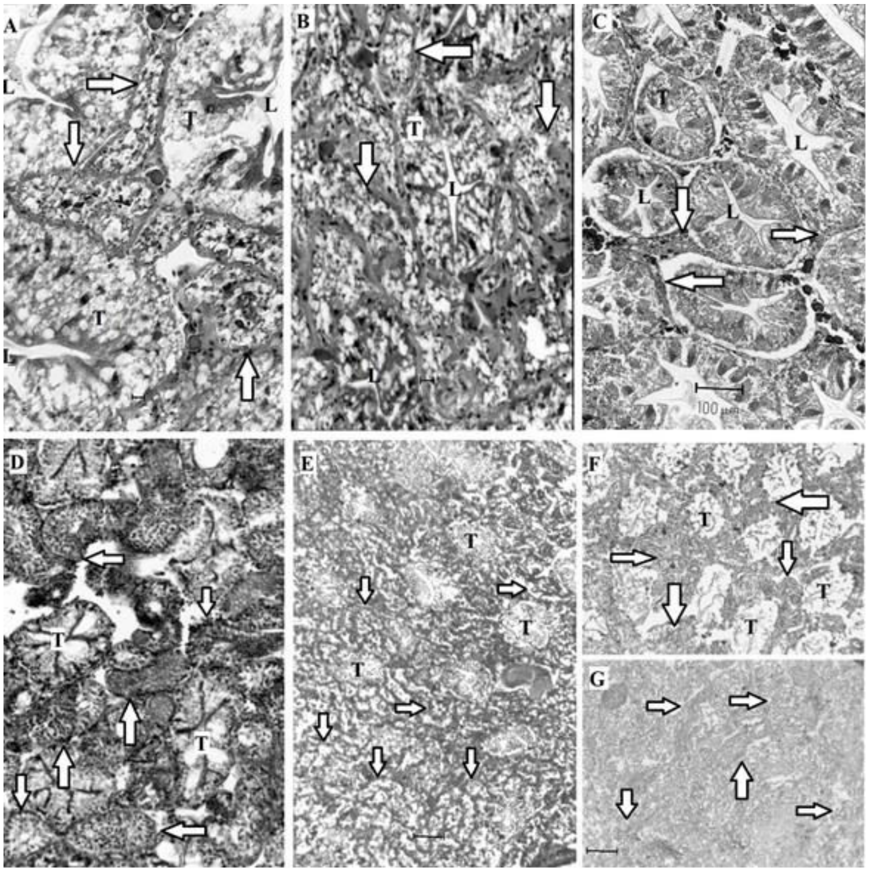

Fig. 2: Slices of the hepatopancreas of C. rathbunae affected by *L. texanus*. (A) male with 1 virgin externa, (B) female with 1 mature externa, (C) male with 1 mature externa, (D) female with 2 mature externae, (E) female with 3 mature externae, (F and G) male with 4 mature externae. T= tubule, L= lumen, the arrows show the roots from *L. texanus*. Scale bar: A and B 10 µm, C 100 µm, D-G 50 µm.

Crabs with virgin externa:

Hosts with one and four externae were found. The specimens with one and two virgin externas (51 females and 31 males) showed aggression and attack as the one observed in healthy crabs from the genus *Callinectes*. These crabs, independently of their sex, displayed a defensive position when being in front of another individual, and when a person or object approached them, they would immediately attack.

When food was brought to them, the crabs exhibited fast movements from both the antennules and the mouth appendages, going straight towards it. Other recorded behaviors were constant grooming as healthy organisms do, however, they would raise their abdomen repeatedly to introduce a pereiopod (second, third or fourth) and seemed as they were cleaning the externa, and they would also perform movements as if they were burying sediment just as healthy crabs do.

Those crabs with three or four externae (nine females and two males) were observed raising their abdomen two or three times during the observation period and would introduce a pereiopod (second, third or fourth), occasionally displaying movements as if they were burying sediment. When food was given to them, they showed the same behavior as other crabs with one and two externae, displaying fast movements from the antennules and mouth appendages, moving towards the food as well as showing an aggressive behavior. One behavior observed in all 93 organisms, was that once they were fed, they would stay away from the food and start grooming themselves. It was noted that the swimming appendages fold upwards so that the animals lose the ability to swim.

Crabs with mature externa:

Host crabs presented one to four externae. The crabs with a mature externa (20) showed a completely different behavior from those crabs with a virgin externa. The organisms with one and two externae (eight females and nine males) were less aggressive, being more evident in those crabs with three and four externae. Even in some cases, the researcher could put his hand near these animals without getting any type of aggressive response. On the other hand, crabs with a virgin externa (20), tended to be more voracious, feeding more than once during the observation period.

During the feeding process, the movement of the antennules and the mouth appendages were not as vigorous; the individuals consumed more food but ingested it slower than those crabs with a virgin externa. The organisms with three and four externae moved around the entire tank until they could find food.

Another difference was that the frequency of grooming was much less, even the crabs with three and four externae did not clean their carapace even when accumulated sediment was observed on them. Those 20 crabs did not display any attempt to bury themselves in the sediment and remained longer periods of time without moving from the same place, showing constant movement from the abdomen as females with an ovigerous mass do when they are oxygenating it, they kept themselves leaning on the tank´s wall, raising the posterior part of their bodies. They were also seen cleaning the externae with one of the pereiopods (second, third or fourth). No mobility was observed in the swimming pair of legs of these 20 crabs.

Throughout the experiment some of the crabs with three or four externa died when they were placed in the tanks with different salinities of 15 ups, which was not recorded with those crabs that had a virgin externa.

Discussion

Jachowski (1974) used healthy crabs from the species *Callinectes sapidus* Rathbun, 1896 during the intermolt cycle. He performed some experiments in order to keep track of their agonistic behavior, observing that males were aggressive and fought among themselves for food and individual space, and displayed this behavior even with non-receptive females.

Same author observed that both sexes fight over food and when one of these organisms (both sexes) holds an object in one of its claws and feels threatened by another individual it fights vigorously using the other claw. This author also mentions that crabs are more aggressive with rivals from the same size and are less aggressive with smaller crabs because they can dominate them easily. The level of aggression decreases in those individuals that are about to start the molt cycle and females also are less aggressive just after having shedded their shells, some of them even get closer to males in order to mate.

Other researchers have observed that juvenile organisms of *Callinectes sapidus* are aggressive, react to external stimuli, such as foreign objects and they are cannibals, and these behaviors persist in adult stages (Hines and Ruiz, 1995; Clark *et al.*, 1999; Hines, 2007; Reichmuth *et al.*, 2011).

Pedetta *et al.* (2010) observed the agonistic behavior of adult males of *Chasmagnathus granulatus* (*Neohelice granulata* Dana, 1851) during the intermolt cycle, describing it as very aggressive. These same authors observed very dynamic combats between individuals and also reported that isolation increases aggressiveness in crabs.

It was observed that in those parasitized crabs, the agonistic behavior decreased consistent with the maturation process of the externa. In the corresponding histologic slices it was observed that in the hepatopancreas affected by three or four mature externas, the tubules were separated from each other by the presence of many roots and in some cases even the tubule´s shape was lost, which did not occurred in those hepatopancreas affected by a virgin externa.

Crabs of species *Callinectes sapidus* parasitized with virgin externae of *L. texanus* have an already heavily infiltrated hepatopancreas with the rhizocephalan rootlets, most of which are solid and with a mean diameter of 20-60 µm. At this stage of parasitization the hepatopancreatic tubules start to become separated by the numerous rootlets. The intertubular connective tissue appears still somewhat organized. The hepatopancreas of a crab carrying a mature externa of *L. texanus* shows a greater separation between hepatopancreatic tubules. Most of the parasite’s rootlets have developed a lumen and there are disperse remnants of connective tissue in the intertubular spaces. In the tubules many binucleate cells can be seen (Bortolini and Alvarez, 2008).

Histological changes were routinely observed in the structure of hepatopancreas of infected crabs while gills showed no apparent changes from parasitization. The hepatopancreas from apparently healthy crabs consists of tubules separated by haemal spaces which contain connective tissue and circulating haemocytes (Powell and Rowley, 2008), meanwhile the hepatopancreas or perigastric organ from healthy organisms is a tubular gland that occupies most of the cephalothorax, being enclosed inside a thin membrane. It is highly compressed, with small spaces among the tubules and each tubule´s epithelium consists of five cell types called: embryonic, fibrillar, midget, reserve and secretory cells, based on the size and each tubule shows a lumen with an X shape (Ramadevi *et al.*, 1990; Cuartas *et al*,. 2002; Bortolini and Alvarez, 2008; Franceschini-Vicentini *et al.*, 2009; Cervellione *et al.*, 2017).

Sacculinids such as *L. texanus* have the most extensive root systems in the Rhizocephala. The system lacks any main parts and the randomly ramifying rootlets can penetrate into every conceivable part of the body, including many internal organs and all or most of the appendages (Høeg and Lützen, 1985; Bresciani and Høeg, 2001).

The degree to which the roots proliferate through the host varies extensively, in most Rhizocephala, including all Kentrogonida, the roots infiltrate the hepatopancreas, but apart from this the ventral nerve cord represents the only other organ frequently reached by roots. This corresponds to the fact that the root system has two principal functions, first, absorption of nutrients and second, host control affected through perturbations to the neuroendocrine system of the host (Høeg, 1995). On the other hand, the external sac of the parasite masquerades as the egg mass of their host and the parasite often changes the behavior of male hosts to make them less aggressive and more like pre-ovigerous females (Shields, 2012).

Bishop and Cannon (1979), observed the behavior of crabs *Portunus pelagicus* (L.), parasitized by *Sacculina granifera* Boschma, 1973, finding that the parasitized males were more active while being alone during the feeding process. They also mention that general integument cleaning behavior was identical for both normal and parasitized crabs, although parasitized crabs, having more epizoics than normal crabs, sac-grooming behavior was found to vary in intensity in relation to the type of stance and appendages used and the duration of grooming during a 30 min observation. During low intensity sac-grooming behavior, the body was raised 2 or 3 cm above the substratum and the cephalothorax was held nearly horizontal to the substratum. While the abdomen was extended slightly away from the externa, the sac and surrounding area were lightly prodded with the dactyls of only the third walking leg. In more than half of the observations the other walking legs were concurrently used for body care, the abdomen was varyingly extended and abdominal flapping was frequently accompanied by medial movement of the pleopods.

Ritchie and Høeg (1981) reported that porcelain crabs parasitized by *Lernaeodiscus porcellanae* Müller, 1862 helped the parasite in delivering nauplii larvae, by holding on to a rock and rising and shaking their bodies energetically against the current. Høeg (1995) reported that crabs *Carcinus maenas* parasitized by *Sacculina carcini* groom themselves less than those organisms capable of burying in the sediment and that the crabs with a mature externa tend to raise their bodies, moving the abdomen with back and forth motions.

Charybdis (*Goniohellenus*) *longicollis* Leene, 1938, parasitized by *Heterosaccus dollfusi* Boschma, 1960 clean their externa with the second, third and fourth periopod as they contract their abdomen, keeping the externa clean and oxygenated, just like ovigerous females do. The parasitized crabs with a mature externa, do not show movement from their last periopods. A common behavior observed in unparasitized crabs is burying themselves in the sediment, which does not occur in parasitized crabs because it is difficult for them to do it, as they cannot coordinate their movements (Innocenti *et al.,* 1998).

Regardless of their sex, *C. rathbunae* are less aggressive as they develop their externa, being less aggressive those organisms that present three or four mature externa. Those crabs with mature externa lose their ability to bury; they do not groom themselves and display vigorous abdomen movements in order to oxygenate the externa. The parasitized individuals lose their ability to swim as their swimming legs lose their mobility (Vázquez-López *et al.*, 2006 a, b; 2012; Vázquez-López, 2010).

McGlone (1986) mention that the agonistic behavior is based on three major criteria: physiological mechanisms, motor patterns shown during agonistic behavior, and the environmental situation. So if it is known that the roots of the interna affect the neuroendocrine organs, it is possible that the presence of *L. texanus* also affects the host's hormonal levels. Although hormonal levels were not measured in the present investigation, because it is a mainly descriptive work and is not the main objective, it is important to mention the following, since it can reinforce what was observed: Different types of parasites modify their hosts´ behavior, combined with the manipulation; the parasites create deep effects in their hosts. Most of the studies demonstrate that the presence of parasites decrease the ability of the host´s performance (Moore, 2013; McElroy and de Buron, 2014).

Parasite-induced modifications of host behavior are known from a wide range of host-parasite associations, in many cases, these behavioral changes are thought to be adaptive and benefit the parasite by increasing its probability of successful transmission (Poulin, 1994, 2013). The evolutionary origins of host manipulation by parasites may go all the way back to the establishment of complex life cycles (Smith, 1980; Moore, 1984; Poulin, 1994), but the mechanisms underlying ethological changes are by no means well characterized.

Evidence is accumulating that the biogenic amine serotonin (5-hydroxytryptamine, 5-HT) is involved in several host-parasite systems (Helluy and Thomas, 2003). 5-HT is present in the crustacean nervous system (Livingstone *et al.,* 1981; Elofsson *et al.,* 1982; Laxmyr, 1984) It may be that 5-HT occurs in all animal kingdom and has an important role in the regulation of a very wide range of physiological processes (Walker, 1984).

There is evidence that shows changes in the levels of serotonin in a range of hosts, from crustaceans to mammals, which are infected by a wide diversity of parasites like protozoans and helminths. Recent studies reveal that when crustaceans and other parasitized and healthy invertebrates are injected with serotonin, there is a change in their behavior, an increase in their aggressiveness and a trend to fight with dominant individuals, so it has been concluded that 5-HT plays an important role in the behavior. It has been observed that in a big pair of neurons found in the first abdominal node in crustaceans there are serotonin neurosecretions that seem to act over the exoskeleton muscles, the bending and over the motor system which has to do with the fight or flight response (Edwards and Kravitz, 1997; Huber *et al.,* 1997; Helluy and Thomas, 2003; Wu and Cooper, 2012). The serotonin and dopamine (DA) are neurotransmitters in crustaceans that control and modulate many physiological functions, including reproduction and ovarian maturity, whereas the serotonin can stimulate ovarian development triggering the release of gonadal inhibition hormone (GIH). DA can suppress reproduction, triggering the release of a suppressing gonad hormone from X-organ-sinus-gland complex, as well as inhibit GSH (Sarojini *et al.,* 1995 a, b; Khornchatri *et al.,* 2015).

Rojas and Ojeda (2005) inoculated crabs of *Hemigrapsus crenulatus* H. Milne-Edwards, 1837 with the acantocephalus *Profilicollis antarcticus* Zdzitowiecki, 1985, revealing an increase in the levels of DA circulating in the hemolymph and an absence of 5-HT. These authors mention that this benefits the parasite, as crabs are less aggressive they become an easy target for gulls, which are the final host. Sneddon *et al.* (2000) conducted fights between healthy crabs of the species *Carcinus maenas*. No crabs were fed and they were individually isolated to encourage aggressive behaviour. Only blood samples were taken from the control crabs and they were not disturbed further. On the other hand, blood samples from the crabs that were used in the fights were taken before and after each fight. These authors mention that within the hemolymph samples from the control individuals only DA was detected, while in the samples from the fighting crabs, 5-HT, octopamine and DA were registered. DA levels were significantly different among winners and losers during the 7th and 9th day of isolation and lower levels of DA were detected in those crabs that had lost the fights.

Most of rhizocephalan parasites infiltrate their roots into the hepatopancreas, the stomach and the ventral nervous cord; in saculinids, the ventral ganglion mass represent the main objective for the roots, whereas the infiltration of gonads, brain and androgenous gland are facultative and the extent of this infiltration is variable and it rarely reaches the ocular stems where the sinus glands are located. It is significant that the root system invades the neuroendocrine organs of the host before invading the digestive system.

If by any chance the defense system of the host fights the roots during growth, these will remain small (Høeg, 1995; Bresciani and Høeg, 2001), even though it has been reported that the roots of *Sacculina carcini* avoid the defense mechanism of *Carcinus maenas* and present immunity against the infectious agents which are thought to be present in crabs and that act as immune agents (Russell *et al.*, 2000).

Andrieux *et al.* (1981) injected a raw extract of parasitized *S. carcini* and hemolymph of parasitized *Carcinus maenas* into healthy crabs of *Carcinus mediterraneus* (*Carcinus aestuarii* Nardo, 1847) and *Carcinus maenas*, observing that both substances affected the protein composition from the hemolymph of healthy crabs and there were effects such as the cessation of moult, Vázquez-López (2010) mention that it can be deduced that the roots of *Loxothylacus texanus* possibly secrete some infectious agent that can act locally in the innervations of the natatory legs of *C. rathbunae* where it can permanently block the transmission of the synapses. Regarding this, Høeg (1995), Robles *et al.* (2002), Rojas and Ojeda (2005) and Vázquez-López *et al.* (2006 b) mention that parasites represent a high metabolic maintenance cost for the host and that the altered physiological conditions benefit the parasites. Alvarez *et al.* (2002) observed that *Loxothylacus texanus* affect significantly the osmoregulation process in *C. rathbunae*, although Reisser and Forward (1991), mention that *Loxothylacu spanopaei* Gissler, 1884 does not affect the osmoregulation of *Rhithropanopeus harrisii* Gould, 1841.

Robles *et al.* (2002) argue that *Loxothylacus texanus* during the virgin externa and immature externa phases do not affect the respiratory rate of *C. rathbunae*, but mature parasites (mature externa) do affect the breathing rate from 57 to 139%, increasing it despite the salinity.

Environmentally, trematodes *Leucochloridium* spp affect the shape, coloration and tentacle size of some species of snails as well as their behavior, in order to become easy targets for birds and to spread themselves. The parasitic protozoan *Toxoplasma gondii* affect rat behavior so they can be consumed by cats to complete their life cycle (Poulin, 2010). The acantocephalus *Profilicollis antarcticus* (Zdzitowiecki, 1985) causes phenotypic (changes in color) and behavioral changes (constant movements) in the crab *Hemigrapsus crenulatus* (Milne-Edwards 1837). This makes parasitized organisms an easy target for predators such as gulls, allowing these parasites to complete their life cycle and be able to spread themselves in the environment (Pulgar *et al.*, 1995; Haye and Ojeda, 1998).

Thompson and Kavaliers (1994) proposed that in studies of parasitism it is possible to integrate various levels of biological organization, allowing links among behavior, physiology and ecology. Haye and Ojeda (1998) mention that there exist a close relationship between behavior and physiology, which supports the hypothesis that alterations of host behavior are correlated with changes in metabolic rates.

Based on what was previously stated by other authors, it can be suggested that *Loxothylacus texanus* suppresses the synapsis in the pair of swimming pereiopods to avoid the damage of the externa, it also causes a decrease in the serotonin levels which reduces aggressiveness and thus prevents any type of harm to the host and to the externa, and it also hinders the motor coordination of the host so it cannot bury itself and crush the externa; with all these actions, the parasite increases its chances of survival and continuity.

Conclusion

We concluded that initially *L. texanus* affects the hepatopancreas of *C. rathbunae* as it feeds itself from this organ. This is supported by the high amounts of roots found inside the hepatopancreas, which make this organ lose its structure and functionality. In the molt prior to the emergence of the externa virgin, *L. texanus* infiltrates the ventral nerve cord.

Once the virgin externa has emerged there is a decrease of agonistic behavior which could possibly coincide with the decrease of serotonin levels in the hemolymph and with the presence of dopamine, keeping the parasitized individuals passive, which makes the host save energy for its own benefit which in turn will be used by the parasite. *L. texanus* represents a huge metabolic burden for its host. This is the first report where behavioral changes of parasitized crabs by a rhizocephalan, degree of development of the parasite and hepatopancreas atrophy are described.

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