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Phoretic interaction between the kangaroo leech *Marsupiobdella africana* (Hirudinea: Glossiphoniidae) and the cape river crab *Potamonautes perlatus* (Decapoda: Potamonautidae) ☆



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ABSTRACT

The South African leech *Marsupiobdella africana* is a temporary ectoparasite of the amphibian *Xenopus laevis*, has a phoretic association with a freshwater crab *Potamonautes perlatus*, and exhibits advanced parental care by incubating its offspring in a brood pouch. Because phoretic associations are usually regarded to favor the phoront's dispersion, its occurrence within the biology of a parasitic species reflects an intimate context of interactions. In addition to phoresy, attachment to the crab may confer other advantages pertaining to offspring development and predator avoidance, dispersion and the parasitic life cycle. Two ponds where amphibian and crab hosts co-occur were sampled twice a month for a period of 1 year. The population dynamics of the leeches and their use of specific microhabitats as attachment sites on the crabs were also investigated. Results indicate a direct relationship between intra-specific variation in the sex ratio among captured crab hosts and the number of leeches recruited over time. The attachments to specific microhabitats on the hard surfaces of the host suggest a proximal proximate anti-predatory strategy. Finally, the importance of oxygen accessibility for the offspring development has been investigated experimentally. Results revealed a remarkable network of interactions linking all partners of this system raising the question as to whether the crabs merely act as a vehicle or play a role within the parasitic life cycle.

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1. Introduction

Phoresy is defined as an intimate and temporary association when an individual, the phoront, is transported by another of a different species, the host, by the creation of ephemeral bridges between patchy habitats (Binns, 1982; Saul-Gershenz and Millar, 2006). This relationship between dispersal and phoresy has been extensively documented in arthropods (Binns, 1982; Tizo-Pedroso and Del-Claro, 2007; Krishnan et al., 2010; Sabagh et al., 2011). A typical example is the case of nematodes that attach preferentially to winged female wasps allowing dispersal between figs while wingless males are avoided (Krishnan et al., 2010). When the attachment to the host fails to provide all resources necessary for the phoronts' survival, phoretic interactions may diverge towards

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amensal or parasitic habits (Holte et al., 2001; Balashov, 2006; Saul-Gershenz and Millar, 2006). Before such evolution takes place, phoresy may become an interaction where attachment to the host is life-stage specific, temporary and/or optional but still provides significant advantages with regard to predation avoidance, dispersal and/or developmental success to the phoront. Recently, phoretic association was documented in free-living Oligochaeta (Lopez et al., 2005), but Maia-Carneiro et al. (2012) reported an unusual case of a leech transported by a vertebrate amphibian host without any deleterious observations. In this context, the case of the brooding leech *Marsupiobdella africana* (Glossiphoniidae) brings new insights to the role played by phoresy within the network of living interactions, especially when the carrier host is suspected to favor the development and parasitic transmission of the phoront. Within the Class Hirudinea (leeches), two independent adaptive trends influenced the diversification of their reproductive strategies and the kind of intimate interactions they may experience with other living partners. On the one hand leeches have undergone an adaptive diversification of their feeding strategies, i.e. from scavengers to parasitic blood feeders (Siddall and Bureson, 1998; Light and Siddall, 1999). An example is the kangaroo leech *M. africana*, which temporarily infects the amphibian host *Xenopus laevis* (see Van Der

Lande and Tinsley, 1976). *M. africana* develops to maturity over a period of 2–3 weeks while attached to the host *X. laevis*. They then detach from the host to find a mate and copulate. Embryos are transferred externally to the brood pouch where up to 50 young develop over a period of up to 4 weeks. If the leech makes contact with a *Xenopus*, the young are discharged explosively on the surface of the host (Van Der Lande and Tinsley, 1976). The second adaptive trend involves the deposition of encapsulated eggs by the parental leech on the shells of arthropods and is described as a temporary and life stage specific phoretic strategy to protect the eggs from predation by snails (Sawyer, 1971; Sloan et al., 1984). Leech parental care evolved further from the direct protection of the cocoon by the parental body towards advanced stages of brooding behavior (Sawyer, 1971; Kutschera and Writz, 2001). The ultimate step in this evolutionary trend is reached in *M. africana*, where the parental leech transfers embryos from the female gonopore to a ventral brood pouch and incubates them until the final stages of their development (Van Der Lande and Tinsley, 1976). This evolution should have negated the need for phoretic strategies selected among related species to prevent egg predation. However, a phoretic relationship is maintained between the adult leeches of *M. africana* and a freshwater crab (Dick, 1959). This suggests that this relationship may confer advantages other than protection of the offspring, including improving parasite transmission. Studies on the biology of crab species revealed major seasonal variations in their spatial patterning and behavior depending on their sex and/or size (Trott, 1998; Unno, 2008; Smith et al., 2010; Di Virgilio and Ribeiro, 2012). These variations result in heterogeneity between individuals regarding their suitability for dispersion of leeches. Because most parasitic characteristics are largely governed by their ability to adapt transmission opportunities (Poulin, 1996; Combes, 2001), the choice of a parasitic leech for attachment/detachment to the crab host may result in several spin-offs. These include offspring development, predation avoidance, and dispersion.

This study focuses on the distribution of leeches on the Cape river crab *Potamonautes perlatus*. Whereas the role of the crab has previously been restricted to that of a vehicle for the leech (Dick, 1959; Van Der Lande and Tinsley, 1976), we tried to determine its importance within the “life cycle” of the leech and especially the nature of the factors governing attachment and detachment. We investigated whether seasonal variations in the dynamics of the crab population could impact on the survival and transmission of the leech species. The use, by the leech, of specific microhabitats on the crab was also considered. Ultimately the environmental factors that may account for the detachment of leeches from the crab were explored experimentally.

2. Materials and methods

2.1. Study site

Two artificial ponds in the botanical garden of the North-West University in Potchefstroom (North-West Province, South Africa), that support large populations of *P. perlatus* and *X. laevis*, were selected for this study. The bottom of the first pond (15 m × 6 m) sloped gradually, to reach a maximum depth of 0.9 m. Mosquito fish (*Gambusia affinis*), a potential predator of *M. africana*, and tadpoles of the common river frog (*Amietia angolensis*) were abundant in this pond. The second pond was round (3 m diameter) and sloped rapidly to a depth of 80 cm. Fish and *Amietia* tadpoles were absent from this pond. In both ponds mud and organic debris, up to 30 cm deep, covered the bottom. After heavy rains, overflow from the first pond sometimes reached the second. A preliminary survey showed that the pH, conductivity and temperature variations were very similar in both ponds.

2.2. Host and phoront sampling

Twice a month between March 2011 and March 2012, the two ponds were sampled at night (8–10 pm). Chicken liver in a gauze bag attached to a line was used as bait and lowered to a depth of 20–30 cm in the water. Bait bags were randomly distributed around the ponds. Due to the difference in the size of the two ponds, 12 and 4 bait bags were used for ponds 1 and 2, respectively to equilibrate sampling efforts. Crabs were collected using a dipnet and transferred to a bucket containing pond water. Crabs were sexed, the largest width of the upper carapace was measured and the number of leeches per crab counted by naked eye for each segment of the crab. The presence of lesions that could have been associated with the occurrence of leeches was checked under a dissecting microscope. All captured crabs were released after the last one had been caught in order to avoid overnight pseudo-replication. A sub-sample of 21 crabs was used to determine which microhabitats on the surface of the host are utilized by the leeches. A group of 15 individuals was also used in the lab experiment carried out during November 2011.

2.3. Experimental test for oxygen dependence

We tested the hypothesis that dissolved oxygen acts as a factor driving the interaction between crabs and leeches. As a preliminary condition, the borehole water used in this experiment was boiled to remove dissolved oxygen and then allowed to cool for one day. Three groups of 5 crabs were maintained individually at 20 °C for four days in identical tanks containing 7 L of this water. Due to the dimensions of the tanks (30/20/20 cm), the depth of the water (12 cm) was enough to prevent the crabs from reaching the surface, where both crabs and leeches would have been directly exposed to oxygen. The first group was maintained in continuously aerated water using air stones. The second group was maintained in water that was aerated for only one day prior to the experiment and then left without an external air supply, to simulate oxygen depletion by the host individual's consumption. The third group of crabs was maintained in water without any aeration. At 24 h intervals following the onset of the experiment, the leeches that detached from the crab were counted and removed. After 96 h of exposure the remaining leeches on the crabs were counted.

2.4. Statistical analyses

We performed a GLM (Poisson error and log link function) to investigate the relationship between the mean number of leeches per host individual and the host gender across the different ponds and months of sampling. A similar analysis was performed to compare the variations in the size of host individuals. ANOVA and post hoc testing (HSD Tukey) was performed to investigate the variations in the size of carrier hosts across gender for each month of sampling. Linear Pearson and nonlinear correlations between leech numbers, crab size and the host sex ratio were also tested. The proportions of leeches recovered from parts of the body and segments of the appendages, were compared between male and female hosts using a chi-square test. Finally, non-parametric ANOVA (Friedman test) was performed to test the differences in the number of leeches detached from host crabs depending on experimental conditions for each day of counting.

3. Results

3.1. Distribution of leeches on crabs and in ponds

In this study, leeches were counted on 462 crabs (223 females, 239 males) between March, 2011 and March, 2012. No wounds or evidence that leeches had pierced the shell of the host or had fed from it, were observed.

There was a positive linear correlation between the size of the crabs and the number of leeches. This correlation is significant for males from the two ponds analyzed together ($R^2 = 0.3235$, $p < 0.0001$), but not for females ($p = 0.9766$). However, females in pond 1 (65.4 ± 6.3 mm), which are significantly larger than males (60.9 ± 9.7 mm) (GLM-norm; $p < 0.0001$), carry significantly fewer leeches (11.8 ± 10.5 ; $n = 138$) than males (16.2 ± 16.8 ; $n = 137$) (GLM-poisson; $p < 0.0001$); similarly, a significant difference between the number of leeches on female (21.1 ± 20 ; $n = 85$) and male (51 ± 59.8 ; $n = 102$) crabs was observed in pond 2 (GLM-poisson; $p < 0.0001$). These results also demonstrate the substantial differences between the two ponds in the number of leeches on both female and male crabs (GLM-poisson; $p < 0.0001$). Finally, the size of female ($F_{222} = 0.9830$; $p = 0.4662$) and male ($F_{238} = 0.8819$; $p = 0.5662$) hosts did not vary across the months of sampling.

These data are highlighted by the relationship between the sex ratio of the crabs captured and the number of leeches per male host for each month of sampling. These two parameters appear firmly linked by an exponential relationship with R^2 of 0.7 and 0.8 for pond 1 and 2, respectively (Fig. 1).

3.2. Seasonal variation

For analysis of seasonal variation, the year can be divided into three time periods. Firstly, March and April, when females were more frequently collected from both ponds and carried 13 and 15 leeches each while 50% of the males carried fewer than 2 leeches (Fig. 2). The second period extends from May to October

when the mean number of leeches increased continuously for male crabs until it reached the maximum recorded value for this study, 64 ± 22 and 152 ± 80 leeches per male crab in pond 1 and 2, respectively. In October, a large male host (72 mm) in pond 2 was captured with a total of 330 leeches attached to it. The beginning of this period is also characterized by a drop in the mean number of leeches per female crab until the lowest value recorded for females, 3 ± 3 and 3 ± 5 for pond 1 and 2, respectively, in May (Fig. 2). Thereafter, this number increased continuously until October and November for pond 1 and 2, respectively. During this second period, which corresponds to the mid-autumn, winter and early spring, with the first rainfalls in October, male crabs accounted for 71% and 80% of captures in pond 1 and 2, respectively. The third period extends from November 2011 to March 2012 and encompasses the breeding season of the crabs and *X. laevis* during the hottest nights of the summer in December and January. This period shows a return to the initial situation observed at the beginning of our study. The number of leeches per male crab decreases rapidly with 50% of males carrying less than 2 leeches, while the number of leeches attached to the female crab drops rapidly then remains stable at 10 ± 3 and 18 ± 20 leech per female crab in March 2012 for pond 1 and 2, respectively (Fig. 2).

3.3. Host microhabitats used by leeches

The 21 crabs sub-sampled for a detailed examination of the microhabitat used by the leeches consisted of 11 females and 10 males carrying 26 ± 8 and 24 ± 7 leeches, respectively. No significant differences were found between male and female crabs in the distribution of leeches over the different microhabitats ($\chi^2_8 = 0.01705$, $p = 0.8961$) (Fig. 3). The majority of leeches (55%) were recorded on the main body of the crab and the remaining (45%) were attached to the podites. Surprisingly, the largest surface of the crab, i.e. the upper carapace, was only used by 3% while the pterygostomial region harbored 49% of the leeches. Of the various podites, the merus segment hosted the most leeches (20%).

3.4. Oxygen depletion and the detachment of the phoront

In this experiment, 15 crabs carrying 29 ± 18 leeches were individually exposed to one of three different conditions simulating the presence, depletion, and absence of dissolved oxygen in water (Fig. 4). For crabs maintained in permanently aerated water, only 5% of the leeches detached within 96 h. On the contrary, 65% of the leeches detached from the crab within 24 h and 95% within 96 h with depleted oxygen. When a depletion of oxygen is simulated, 6% of the leeches detached from the crab within 24 h and 60% after 96 h.

4. Discussion

In the lack of any deleterious effect on the crabs, results of this study suggest that the association between the leech and the crab do not belong to any kind of host-parasite relationship. A similar ambiguous interaction was recently depicted from a leech that is only transported by an amphibian host (Maia-Carneiro et al., 2012). In addition, Van Der Lande and Tinsley (1976) were able to rear several generations of *M. africana* in laboratory conditions with the sole presence of the amphibian host, *X. laevis*. As a consequence, *M. africana* must be considered to exhibit an opportunistic and phoretic interaction with an invertebrate while remaining a strict ectoparasite of a vertebrate. In this context, life history traits of the leech species should reflect the cyclic colonization of suitable and dispersed intermediate (phoretic) or definitive (parasitic) host resources.

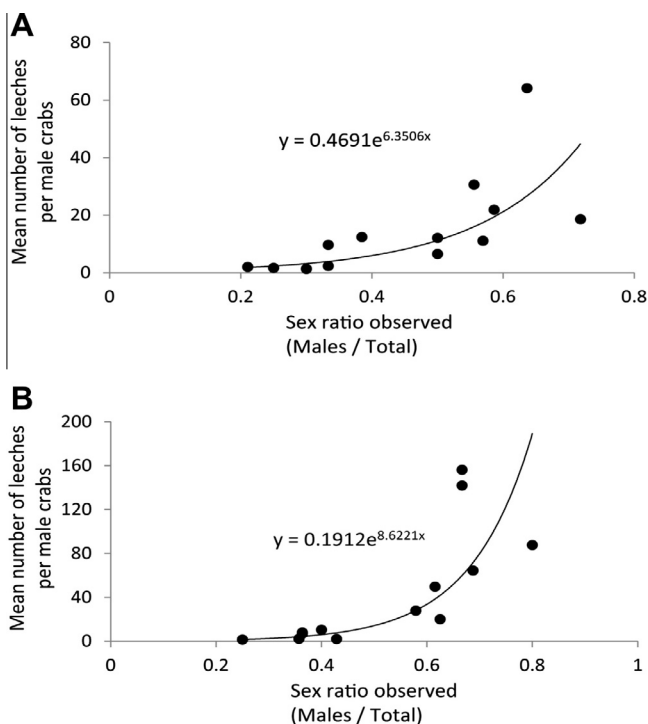


Fig. 1. Exponential correlation between the mean number of leeches per male crab host and the sex ratio as observed for the 12-month of sampling for pond 1 (A) and pond 2 (B).

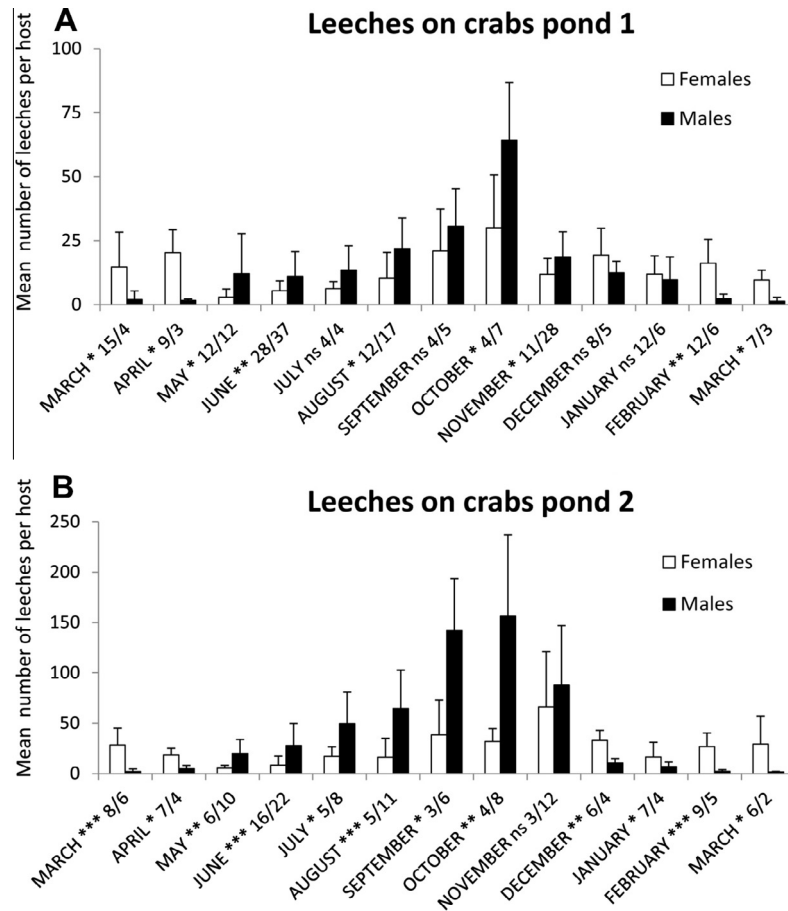


Fig. 2. Monthly variations (with standard deviation) of the mean number of leeches per host individual for both genders in pond 1 (A) and pond 2 (B). The time line starts in March 2011 and ends in March 2012. Legend for test of significance between host genders: significant (* $p < 0.05$). Highly significant (** $p < 0.01$). Extremely significant (***) $p < 0.001$). The number after each month of the X axes refer to the number of female and male hosts captured separated by a slash.

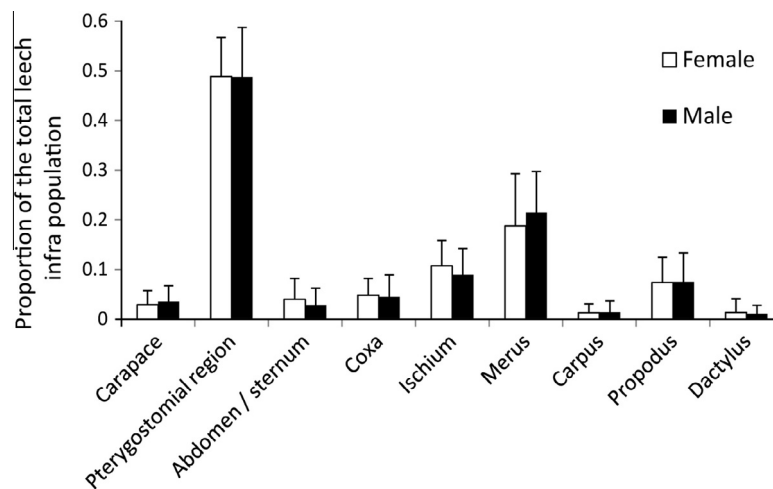


Fig. 3. Distribution of leech individuals over the various microhabitats used on the body of the crab host. Coxa to dactylus sections correspond to podites segments in order.

4.1. Host factors and phoront recruitment

Of particular interest is the discovery of large variations in the proportion of each sex captured over the sampling period. Similar results for other crab species have been attributed to heterogeneity in behavioral variations over time between sexes (Trott, 1998; Gratwike, 2004; Unno, 2008). This behavioral heterogeneity is well known to reach advanced levels of social and territorial interac-

tions between and within crab genders (see Wada, 1993; Milner et al., 2010; Smith et al., 2010), and was also shown to alter the study of crab feeding strategies by skewing the results towards the most active gender (see Di Virgilio and Ribeiro, 2012). Therefore, our method of sampling based on attraction to food is expected to reflect true behavioral mechanisms for changes in the spatial and/or foraging activities of the two host genders across the year.

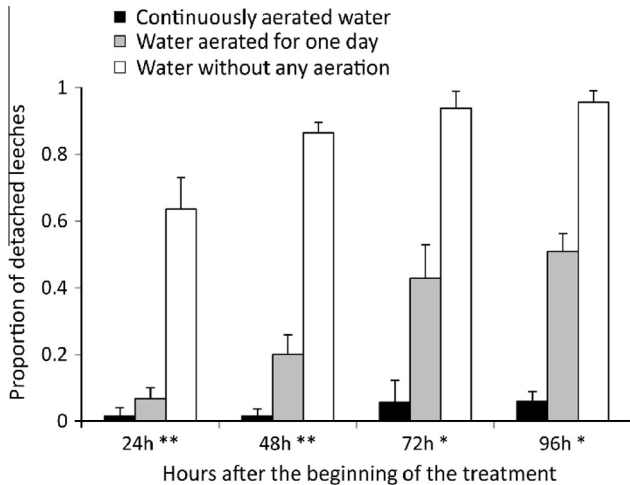


Fig. 4. Cumulative percentages of detached leeches from crab hosts contained in water with different oxygen supplies. Legend for test of significance between host genders: significant ($*p < 0.05$). Highly significant ($**p < 0.01$).

Considering that seasonal variations in the behaviour of crabs would be directly related to their spatial mobility, the number of leeches recovered from each crab would be expected to increase with the likelihood of capturing the most active gender for each month of sampling. In the present study, the behavioral ecology of the crab species is indirectly approached by the measure of the sex ratio of captured individuals for each month as well as the observation of a dedicated period for mating events in the hottest part of the year (December and January). This period delimits two sequences when female and male crabs were respectively more caught, *i.e.* more active (Fig. 2). Since we find a positive correlation between the sex ratio of sampled crab individuals and the number of leeches attached to them (Fig 1), it may be suggested that the asymmetric use of crab genders by leeches over the year results from different pre- and post-mating behavior of the hosts. These behaviors could involve the exploitation and/or the defense of territories as well as the period of extensive foraging. In other words, the seasonal variations in the mobility of female and male crabs influence their respective potential of leech recruitment by increasing their suitability as transporting hosts.

Our data also supports a positive relationship between the size of male individuals and the number of leeches recruited. This supports the hypothesis that heterogeneity in size (or age) of the host (Smith et al., 2010) is related to their suitability for leech attachment. Our finding that the correlation between host size and the number of leeches recruited applies only to males, may result from either a lack of small females in our samples, or a lack of female–female interactions. The relationship between host size and leech recruitment is consistent with previous findings, in that in some instances behavioural heterogeneity can be attributed to social and territorial conflicts between individual crabs of the same gender (Wada, 1993; Milner et al., 2010; Smith et al., 2010). Finally, since leeches are known to have well-developed sensory systems that allow them to respond to specific situations (Gaudry et al., 2010; Petersen et al., 2011), the differences observed between male and female hosts might also arise from an ability of the leech to recognize the gender of the host crab. This ability has not been demonstrated, however, and appears unlikely.

The observed variation in the number of leeches attached to a host crab might also depend on the reproductive and developmental rhythms of the leech itself. Most leech species, in which the annual life cycles are known, are restricted to a single generation per year (Elliot, 1973; Daniels and Sawyer, 1975; Peterson, 1983). In our study, the maximum number of leeches per crab

was reached in October, while the minimum occurred 6 months later, in April (Fig. 2). This was quite surprising because successive generations of *M. africana* were obtained every 7 weeks under laboratory conditions (Van Der Lande and Tinsley, 1976). While it is possible that several generations might be produced between August and September, this is not supported by our data. We consider it likely that brooding leeches attach to their amphibian host in summer and instantaneously release the juveniles which begin to feed on the host. Parents detach and die (Van Der Lande and Tinsley, 1976) while the new generation will attach to crabs in winter and incubate their own offspring until the following summer.

4.2. Microhabitat use

Our results show that the distribution of leeches among microhabitats on the host is independent of the sex of the crab host (Fig. 3). This suggests that gender-related differences in host morphology do not change the microhabitat preferences of *M. africana*, and perhaps other leeches. For *Notostomum cyclostoma*, another leech species that deposits its cocoons on the shells of crabs, microhabitat selection may be attributed to the avoidance of abrasive forces on the substrate, and also cheliped grooming by the crab (Sloan et al., 1984). Unlike *N. cyclostoma*, *M. africana* avoids the upper side of the carapace and prefers the pterygostomial region and the merus segment. During our experiment, we observed crabs eating detached or migrating leeches from the forelimbs. Similarly, detached leeches are predated by the amphibian host itself (Van Der Lande and Tinsley, 1976).

We propose several non-exclusive hypotheses to explain the distribution of leeches over the crab carapace. First, it may reflect a strategy to avoid predation, at least by the crabs themselves. Predation pressure from fish or amphibian tadpoles and adults might also influence the choice of specific sites. Since our study consisted of only two ponds, in which the overall population of leeches estimated for the site with potential fish predators was less than that of the site without fish predators (Fig. 2), there was insufficient evidence to support any hypothesis involving the effect of predation by fish. Also, the two ponds might differ in other parameters not considered in this study that influence the overall biology of crabs, amphibians, fish and leeches. Therefore, the importance of microhabitat selection in predation avoidance needs further investigation. Another possibility that cannot be excluded is that the leeches choose sites that are less exposed to desiccation when crabs reach the pond surface for grazing or migration.

4.3. Dependence on oxygen and parental care

In the present study, we show that, in experimental conditions, a shortage or absence of oxygen resulted in detachment of the leech from the crabs (grey and white boxes in Fig. 4). These results suggest that oxygen is a key factor driving the reversion of this phoretic association. A possible explanation is related to the oxygenation of the developing offspring. Indeed, *M. africana* was seen to perform waving movements during egg incubation without vibrations in the water due to potential hosts moving around (Van Der Lande and Tinsley, 1976). These movements, as well as the detachment from the host, might reveal the necessity for parental leeches and their incubated offspring to access oxygen resources as has been proposed for another leech species (see Peterson, 1983). Because the survival of the offspring of *M. africana* is directly linked to the choices made by the parent, the break-up of the phoretic association could manifest as an evolution of parental care just like all the other adaptations aimed at avoiding predation and increasing the probability of juveniles finding their first blood meal (Sawyer, 1971; Kutschera and Writz, 2001).

4.4. Concluding remarks

The description of this association between *M. africana* and *P. perlatus* species allows for discussion of semantic issues for ecological interactions. In concrete terms, a free-living stage of a temporary parasite is truly transported, without any apparent consequences on the host, until it detaches to complete its life cycle. Noticeably, the transport offered by the crab host is not directly associated with a spatial component as it has been described for most phoretic associations (see Binns, 1982; Saul-Gershenz and Millar, 2006). In fact, the journey of the leech on the crab corresponds to the incubation period of its offspring until it becomes infective for the amphibian host. Therefore, this case of phoresy corresponds to a time travel within the same environment where the different host species present overlapping ecological niches and behaviors (see Dick, 1959). On the other hand, the parasitic habit of the leech brings a new dimension to the phoretic association. In the field of parasitology, the status of definitive host is restricted to the host species in which sexual reproduction of the parasite occurs. Interestingly, copulation and insemination have been observed to occur only after the leech detaches from the amphibian host (Van Der Lande and Tinsley, 1976). If mating events occur once leeches are attached to the crab host, phoresy is then favoring the encounter, in part by increasing the density and diversity, of potential sexual partners for the leech. As a direct semantic consequence, the host would then become the definitive host for the parasitic component of the leech life cycle.

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