

Avian cestodes affect the behaviour of their intermediate host *Artemia parthenogenetica*: An experimental study

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Abstract

The brine shrimp *Artemia parthenogenetica* (Crustacea, Branchiopoda) is intermediate host for several cestode species whose final hosts are waterbirds. Previous field studies have shown that brine shrimps infected with cestodes have a bright red colour and are spatially segregated in the water column. However, the ethological mechanisms explaining such field observations are unknown. Changes in appearance and behaviour induced by trophically transmitted parasites have been shown to increase the risk of predation by the final host. In this experimental study, we compared the behaviour of uninfected *Artemia* and those infected by avian cestodes. We found that parasitised individuals behave differently from unparasitised ones in several ways. In contrast to uninfected individuals, infected brine shrimps were photophilous and showed increased surface-swimming behaviour. These observations suggest that the modified behaviour (in addition to the bright red colour of the majority of the infected individuals) results in infected brine shrimps becoming more vulnerable to avian final hosts, which facilitates parasite transmission. We discuss our results in terms of the adaptive nature of behavioural changes and their potential implications for the hypersaline ecosystem.

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1. Introduction

The parasite manipulation of host behaviour is currently a widely studied phenomenon because of its ecological and evolutionary significance (see Combes, 1991, 2001; Poulin, 1995, 1998; Moore, 2002; Thomas et al., 2005; Jog and Watve, 2005). Recent studies emphasise the importance of gathering empirical data from diverse host–parasite systems (Thomas et al., 2005; Klein, 2005; Hurd, 2005; Rigaud and Haine, 2005; Poulin et al., 2005; Ponton et al., 2006). Cases when two or more trophically transmitted parasites co-occur in the same host population and one or more of them manipulate host behaviour are of particular interest because of the complexity and the diversity of interspecific interactions (e.g. Thomas et al., 1998; Cézilly et al., 2000; Babirat et al., 2004; Haine et al., 2005; Rigaud and Haine, 2005; Kostadinova and Mavrodieva, 2005). Parasite-induced alterations in the host behaviour may have major implications at the

ecosystem level (Combes, 1996; Thomas et al., 1999; Thomas and Renaud, 2001; Mouritsen and Poulin, 2005; Thompson et al., 2005) but this remains a largely unexplored area.

Among the trophically transmitted avian helminths, most previous studies on parasite-induced behavioural modifications in invertebrate intermediate hosts have focussed on trematodes (e.g. Helluy, 1983, 1984; Thomas et al., 1995; Babirat et al., 2004; Kostadinova and Mavrodieva, 2005) and acanthocephalans (e.g. Hindsbo, 1972; Moore, 1983; Cézilly et al., 2000). Studies on parasite manipulation in invertebrates infected with larval cestodes are few (Graham, 1963; Hurd and Fogo, 1991; Yan et al., 1994).

Brine shrimps of the genus *Artemia* (Branchiopoda: Anostraca) act as intermediate hosts for 13 species of avian cestodes; their parasitic transmission depends on predation by birds (see review in Georgiev et al., 2005). Cestodes have been reported to induce changes in colour and spatial distribution of infected brine shrimps in the field; these alterations are suspected to be associated with behavioural modifications following infection (Gabrion et al., 1982; Thiéry et al., 1990; Sánchez et al., 2006a). However, the effect of larval cestodes on *Artemia* behaviour has never been assessed. In this study, we explore possible causes of

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parasite-induced spatial segregation and colour change observed in the field by examining the behaviour of naturally infected and uninfected brine shrimps in the laboratory. Since making the upstream host more visible to predators is believed to be a major mechanism facilitating parasite transmission along food chains (Combes, 2001), we quantify the light/dark microhabitat preference and measure time spent at the water surface to test the prediction that infected brine shrimps become more vulnerable to aquatic birds. We also test the hypothesis that the association between the high cestode burden and the red colour of brine shrimps is due to the increased time spent at the surface (resulting in accumulation of photoprotective carotenoids, see Sánchez et al., 2006a). In addition, cestodes have been suggested to increase the buoyancy of infected *Artemia* by increasing their lipid content (Amat et al., 1991) and therefore surfacing behaviour could easily be confounded with negative geotaxis. Thus, we designed an experiment to separate the effects of phototaxis and geotaxis and to evaluate whether photophily or changes in buoyancy are responsible for surfacing behaviour; for this purpose, we used light stimuli to drive the movement of brine shrimp in the water column. Finally, we discuss the potential effect of cestodes on salt pan communities through their influence on *Artemia* behaviour.

2. Material and methods

2.1. The host–parasite system

Brine shrimps *Artemia* are well-studied crustaceans with a near worldwide distribution inhabiting extreme hypersaline environments such as salt lakes, coastal lagoons and salt pans (Persoone et al., 1980; Abatzopoulos et al., 2002). *Artemia* tends to be the dominant invertebrate in number and biomass in the places where it occurs (e.g. Sánchez et al., 2006b). In our study area, *Artemia* is intermediate host for 8 species of cyclophyllidean tapeworms; their adults infect several species of waterbirds, including shorebirds, flamingos, gulls and grebes (Georgiev et al., 2005). The prevalence of the cestode infection in brine shrimps is av. 27% (Georgiev et al., 2005), locally reaching up to 90% (unpublished data). Brine shrimps become infected by consuming cestode eggs (containing a larva termed an oncosphere) released into the water with the faeces of the definitive hosts. Brine shrimps are non-selective filter feeders consuming particles in suspension (Reeve, 1963a,b) ranging 6.8–27.5 μm (Fernández, 2001). The oncosphere (20 μm in diameter for *Flamingolepis liguloides*, see Robert and Gabrion, 1991) penetrates through the intestine wall into the hemocoel where it develops into a cysticeroid (cestode larval stage containing scolex). The life cycle continues when an infected *Artemia* is predated by the final host.

2.2. Sampling, rearing and determination of infection status

Based on the observation that red colour in *Artemia* is associated with cestode parasitism (Thiéry et al., 1990; Robert and Gabrion, 1991; Amat et al., 1991; Sánchez et al., 2006a), we selected 50 red and 50 transparent *Artemia parthenogenetica*

in the field in order to compare the behaviour of infected versus non-infected individuals. *A. parthenogenetica* are frequently reared in the laboratory in conditions free of parasites (Hontoria and Amat, 1992), when the intense red colour of infected individuals found in the field has never been observed (F. Amat, personal communication). We selected adult individuals of the same size in order to control for age as these two variables are correlated (F. Amat, personal communication). We collected the samples with a net of 0.1 mm mesh from an evaporation pond of intermediate salinity at an industrial salt pan in the Odiel Marshes (SW Spain, 37°17'N, 06°55'W) in November 2003 (see Sánchez et al., 2006b, for details of the study area).

In the laboratory, on the day of collection, brine shrimps were introduced into a tank, 50 (length) \times 10 (height) \times 30 (width) cm, filled to within 1 cm of the top with water from the pond and under a natural photoperiod. After 24 h, we conducted a series of three different behavioural tests (see below) on each of the 100 individuals, one by one, alternating groups of 10 red and 10 transparent individuals. Once all the tests were finished, brine shrimps were killed by heating to 80 °C and preserved in 70% ethanol for identification of parasites (see Georgiev et al., 2005 for methods of cestode species identification).

2.3. Behavioural tests

2.3.1. Experiment 1: light/darkness choice

This experiment was carried out with a petri dish divided into dark and light halves. We used black plastic to cover one half of the dish and illuminated the other half with a cold light source to prevent a thermal gradient. Each *Artemia* was individually placed in the middle of the dish and after 1 min we recorded its position. We then repeated the experiment covering the opposite side of the petri dish in order to confirm the light/dark preference. In cases in which the response of brine shrimp changed between the two trials (only 4 of 100 cases), we repeated the experiment. The light/dark preference was scored as a dichotomous variable. We used χ^2 tests to compare the response to light between transparent uninfected, transparent infected, and red infected individuals. When over 20% of the expected values in the contingency table were less than 5, we performed Fisher exact tests.

2.3.2. Experiment 2: time at water surface

This experiment was carried out in a white shallow (50 (length) \times 10 (height) \times 30 (width) cm) tank that allowed light to penetrate the entire water column from above. Each *Artemia* was introduced individually into the tank. After 1 min, we recorded the time (in seconds, s) spent within 1 cm of water surface during the following 30 s. We used Kruskal–Wallis test for comparisons of median time at the water surface between transparent uninfected, transparent infected and red infected brine shrimps. For determining which pairs of groups were different, we performed multiple-comparison post hoc tests (Siegel and Castellan, 1988).

2.3.3. Experiment 3: ability to descend in the water column

This was designed to separate the effects of phototaxis and geotaxis (and also the effects of the negative geotaxis and the

Table 1
Presence of cestode species (i.e., number of infected *Artemia* individuals) and number of them participating in simple and multiple infections

Cestode species	Presence	Simple infection	Multiple infection															
			2 species				3 species				4 species							
<i>Flamingolepis liguloides</i>	64	15	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	
<i>Flamingolepis flamingo</i>	6	0	×							×	×					×		
<i>Confluaria podicipina</i>	42	3		×					×	×	×		×	×	×	×	×	
<i>Eurycestus avoceti</i>	10	0			×								×				×	
<i>Anomotaenia microphallos</i>	13	1				×						×		×			×	
<i>Anomotaenia tringae</i>	4	0					×		×								×	
<i>Gynandrotaenia stammeri</i>	10	0						×		×				×	×			
No. of <i>Artemia</i> individuals infected	72	19	1	15	4	3	1	4	2	1	3	1	4	7	4	1	1	

The position of crosses in each column indicates the precise nature of multiple species infections observed, with *n* in the bottom row.

possible increased buoyancy), using light stimuli to drive the movement of brine shrimp in the water column. We used a graduated cylinder of 5 cm length filled to the top with water and placed the brine shrimp gently into the top of the column. After 1 min, we laterally illuminated the top of the tube with a cold light. The position of the *Artemia* individual in the column (top or bottom half) was scored after a further 30 s (time estimated for stabilization). This way we evaluated the ability of photophobic individuals to go to the bottom. Then we placed the tube in the dark and, after 1 min, we repeated the procedure but illuminated the bottom of the tube. In this way, we evaluated the ability of photophilous individuals to go to the bottom. The response to the light stimulus was scored as a dichotomous variable.

We revealed the effect of light on geotactic behaviour by combining the response under the previous tests. Thus, we identified individuals whose response was mainly determined by light (when their depth was determined by the position of the light in relation to the water column) and individuals whose position was mainly determined by gravity (when their depth was the same regardless of whether the water column was illuminated from above or below).

Chi-square tests were performed for two-way comparisons between transparent uninfected, transparent infected and red infected individuals, comparing both the ability to swim to the bottom and the effect of light on the geotactic response. For the analyses, we excluded 16 individuals that showed evidence of not being in a good condition at the time of carrying out the experiment. For the analysis of the effect of light on the geotactic response, we also removed a further 3 individuals that did not respond clearly (they stopped in the middle of the water column).

In order to explore possible causes for the differences between transparent infected and red infected *Artemia*, we compared the mean intensity of infection (number of cysticercoids per individual) between these two groups using Mann–Whitney *U*-tests. In the same way, we compared the prevalence of the different cestode species between red and transparent infected *Artemia*.

Non-parametric statistical tests were carried out using the Statistica 6.0 package (StatSoft Inc., 2001). Since repeated experiments and tests were carried out on the same individuals, correction for multiple testing is appropriate (García, 2004). All significant results for behavioural experiments remained

significant after strict Bonferroni correction unless otherwise stated.

3. Results

We identified seven species of tapeworms belonging to three different families (Hymenolepididae, Dilepididae and Progynotaeniidae) in the sample studied (Table 1). Cysticercoids of up to 4 different species were registered in a single *Artemia* and simple infection was recorded in only 26% of the infected individuals (Table 1). As most parasitised individuals showed multiple infections, we were unable to isolate the effect of the different species of cestodes on behaviour and colour in a satisfactory manner.

The experiments were conducted on 72 infected and 28 uninfected individuals, as some of the 50 transparent brine shrimps sampled proved to be parasitised. Therefore, we performed our analysis on 3 different groups: transparent uninfected ($n=28$), transparent infected ($n=22$) and red infected brine shrimps ($n=50$).

Among the 72 infected brine shrimps, 69% were red (the prevalence of infection in red individuals was 100%), whereas all 28 (100%) uninfected individuals were transparent ($\chi^2 = 36.16$, d.f. = 1, $P < 0.001$). The mean intensity of infection was twice as high in red (4.28 ± 0.33 , mean \pm S.E.) than in infected transparent *Artemia* (2.45 ± 0.48) (Mann–Whitney test, $U = 241.5$, $P = 0.00013$). The maximum number of cysticercoids was 11 in transparent and 13 in red brine shrimps. There were no clear differences in cestode species composition between infected individuals of different colour. However, the prevalence for two species was significantly higher for red *Artemia* (Table 2).

In general, parasitised individuals looked more vigorous than non-parasitised ones and resisted the experimental manipulations better. Among 14 individuals that died during the experiments, 13 were transparent and only one was red ($\chi^2 = 10.05$, d.f. = 1, $P = 0.002$).

The proportion of individuals that actively moved towards light in Experiment 1 was highest for red infected (0.92), followed by transparent infected (0.73) and finally by transparent uninfected individuals (0.54). We found significant differences between transparent uninfected and red infected individuals ($\chi^2 = 13.378$, $P < 0.001$). However, we found no significant differences between transparent *Artemia* of different parasitic

Table 2
Prevalence of cestode species in transparent infected and red infected individuals and comparison between them

	Prevalence (%)		U	P
	Infected transparent (n=22)	Infected red (n=50)		
<i>Flamingolepis liguloides</i>	77.3	96	447	0.014*
<i>Flamingolepis flamingo</i>	13.6	6	508	0.283
<i>Anomotaenia tringae</i>	4.5	6	542	0.805
<i>Confluaria podicipina</i>	36.4	68	376	0.012*
<i>Gynandrotaenia stammeri</i>	0	20	440	0.024
<i>Eurycestus avoceti</i>	13.6	14	548	0.967
<i>Anomotaenia microphallos</i>	9.1	22	479	0.192

* These tests remain significant when corrected for multiple testing with a sequential Bonferroni-type procedure (Benjamini and Hochberg, 1995).

status ($\chi^2 = 1.192$, d.f. = 1, $P = 0.275$) or between infected *Artemia* of different colours (Fisher exact test, $P = 0.058$). When comparing all infected with all uninfected *Artemia*, we found that a significantly higher proportion of infected individuals moved towards light ($\chi^2 = 10.29$, d.f. = 1, $P < 0.001$).

In Experiment 2, we recorded statistically significant differences in the time spent at the surface between groups (Kruskal–Wallis test, $H(2, 100) = 23.39$, $P < 0.001$). Red individuals spent more time at the water surface (26.30 ± 1.29 s, mean \pm S.E.) than transparent ones (infected 12.21 ± 2.64 s, uninfected 12.21 ± 2.63 s) and these differences were statistically significant. However, there was no significant difference between transparent individuals of different parasitic status. When comparing all infected with all uninfected *Artemia*, we found that infected individuals spent significantly more time at the surface (Mann–Whitney test, $U = 627.0$, $P = 0.001$).

The results of Experiment 3 did not support the hypothesis that increased surface-swimming behaviour was due to physical impediment (high floatability) of infected individuals. Most of the *Artemia* individuals (more than 83% in the three different groups) swam into the bottom half of the water column during the experiment, with no significant differences between groups in the proportions doing so ($P > 0.6$ in all cases). Brine shrimp position in the water column (top or bottom half) was modified by light position in 61% of uninfected transparent, 60% of infected transparent and 78% of red *Artemia*, there being no significant differences between groups in these proportions ($P > 0.22$ for all comparisons). Therefore, light (phototaxis) was the most important stimulus determining the final position of *Artemia* (both infected and uninfected) in the water column, gravity (geotaxis) having a secondary role. Among individuals whose position was not affected by light, we found 60% of red infected ($n = 10$), 43% of infected transparent ($n = 7$) and only 14% of uninfected individuals ($n = 7$) to use the top half of the water column (i.e., to be negatively geotactic). However the small sample size was not enough to detect statistically significant differences ($P > 0.16$ in all the cases). For the same reason, when comparing all infected with all uninfected *Artemia*, we did not find a significant difference in the proportion of infected individuals showing negative geotaxis (Fisher exact test, $P = 0.172$).

As in Experiment 1, the proportion of brine shrimps showing positive phototaxis (excluding those individuals whose response was mainly determined by gravity) in Experiment 3 was much

higher amongst red infected *Artemia* (75%, $n = 36$) than transparent ones (36%, $n = 11$, for uninfected transparent and 40%, $n = 10$, for infected transparent). The difference between uninfected and red infected shrimps was significant (Fisher exact test, $P = 0.029$) but not after Bonferroni correction. When comparing all infected with all uninfected *Artemia*, we found no significant difference (Fisher exact test, $P = 0.086$).

4. Discussion

4.1. General comments

Our results show pronounced differences in behaviour and colour of brine shrimps when infected by cestodes. Infected brine shrimps exhibited positive phototaxis, spent more time swimming at the surface of the water and usually displayed a bright red colouration. These results explain previous field observations of differential distribution of infected individuals of *Artemia* in the water column (Gabrion et al., 1982).

We observed the strongest differences between the red infected and the transparent uninfected *Artemia*. In experiments 1 and 3, the behaviour of transparent infected *Artemia* was intermediate between that of the other two groups. Infection intensity may explain our observations, as the mean number of cysticercoids was nearly twice as high in red than in transparent infected *Artemia*. Manipulation of hosts by helminth parasites is likely to be related to parasite load as shown for acanthocephalans (Maynard et al., 1998) and trematodes (Webber et al., 1987). Although one parasite individual seems to be enough to induce host modifications in some systems, a positive correlation exists between the number of parasites and the extent of the modification (Trabalon et al., 2000). In contrast, the effect of larval acanthocephalans on amphipod hosts appear to be dependant on their presence only, not on the intensity (Cézilly et al., 2000; Bauer et al., 2000). Other studies suggest that the degree of maturation of larvae determines the extent of behavioural modification (Valkounova, 1983; Williams et al., 2004).

We were unable to isolate the effect of each cestode species on *Artemia* phenotype, as multiple infections are extremely frequent in the field. As our experiments rely on naturally infected *Artemia*, they do not in themselves rule out the possibility that a given behaviour was a cause of increased exposure to parasites

rather than a consequence of it. However, existing knowledge of normal, adaptive host behaviour supports a causal relationship between infection and behaviour.

4.2. The normal behaviour of *Artemia*

Artemia has no sophisticated antipredator structures and is an easy and nutritional prey for birds, fishes and aquatic invertebrates. Its defence against predators is to avoid them, (1) by living in hypersaline habitats, which are too extreme for most predatory invertebrates and fish and (2) undergoing daily vertical migration (DVM) (Lenz, 1980; Forward and Hettler, 1992). Uninfected *Artemia* ascend in the water column at sunset to feed near the surface and descend at sunrise to avoid visual avian predators (Forward and Hettler, 1992). In doing so, uninfected brine shrimp shows a strong negative phototaxis (Lenz, 1980; Bradley and Forward, 1984) and positive diurnal geotaxis. Additionally, DVM constitutes an efficient ecological protection from harmful ultraviolet light (Rhode et al., 2001). In *Artemia*, carotenoid pigments are diverse and reach high concentrations (Nelis et al., 1984, 1988); they are believed to have, inter alia, photoprotective functions (Amat et al., 1991) and to cause the red colouration of brine shrimps (see also Sánchez et al., 2006a). However, similar colouration was reported to increase predation risk for copepods (Hairston, 1979).

4.3. Parasite-induced alterations in *Artemia* behaviour

Trophically transmitted parasites have been shown to increase their transmission success by adaptively manipulating the behaviour of their intermediate host (Moore, 2002; Thomas et al., 2005). The observed phenotypic changes (surface-swimming behaviour and red appearance) seem to increase the exposure and visibility of *Artemia* to surface-feeding avian predators.

Increased surface activity has been reported for a variety of invertebrates parasitised by nematodes (McCurdy et al., 1999), trematodes (Crowden and Broom, 1980; Mouritsen and Jensen, 1997), acanthocephalans (Hindsbo, 1972) and cestodes (Lester, 1971; Yan et al., 1994). In most cases, the underlying mechanism is unknown. Problems of buoyancy have been reported for sticklebacks parasitised by cestodes, causing them to remain closer to the surface (Arme and Owen, 1967; Giles, 1983; LoBue and Bell, 1993; Ness and Foster, 1999). Our results do not support the hypothesis that extra buoyancy derived from the higher lipid content of parasitised *Artemia* (Amat et al., 1991) is the proximate cause for the surfacing behaviour, as parasitised individuals were able to swim towards the bottom when stimulated by light. Likewise, altered host behaviour does not seem to be due to infection-induced host debilitation.

In general, parasitised individuals looked more vigorous than non-parasitised ones and resisted the experimental manipulations better. Since one function of carotenoids is lipid storage, carotenoids may be responsible for such differences. Lipid content is believed to be a good predictor of copepod survival (Franz and Kurtz, 2002). Increased lipid reserves in infected *Artemia* (Amat et al., 1991) may function as a parasite strategy to increase

the longevity of infected individuals (thus enhancing the probability of encounter with the final host) or may increase the profitability of the prey in order to augment its attractiveness for the final host.

We are confident that surface-swimming behaviour was not an incidental consequence of the infection caused by an increased oxygen (Smith and Kramer, 1987; Lester, 1971) or energy demand (Milinski, 1985; Godin and Sproul, 1988) imposed by the parasites, as we provided no oxygen or food gradient in the shallow water used in our experiments. Moreover, at least for *F. liguloides*, infection has been shown not to affect the respiration rate in *Artemia* (Varó et al., 2000). The water column was uniformly lit, and neither can increased attraction of parasitised *Artemia* to light explain our results. One alternative explanation is that the position in the water column may be altered in *Artemia* because the parasites reverse the normal positive geotaxis. We showed light to be the most important factor driving the movements of *Artemia* in the water column. However, among the individuals whose response were not influenced by light, 86% of uninfected *Artemia* showed positive geotaxis (bottom behaviour) whilst 53% of infected *Artemia* were negatively geotactic (surface-swimming behaviour). Negative geotaxis and positive phototaxis have been reported in amphipods infected by trematodes (Helluy, 1983).

The association observed between the presence of cysticercoids and red colour in *Artemia* agrees with previous studies (Thiéry et al., 1990; Robert and Gabrion, 1991; Amat et al., 1991; Sánchez et al., 2006a). We suggest that cestodes induce red colouration in *Artemia* through an indirect mechanism mediated by the alteration in photoresponse. Photophilous individuals are more exposed to UV radiation, which stimulates synthesis of photoprotective carotenoids. Accumulation of carotenoids has been reported for copepods exposed to high levels of UV radiation, resulting in intensely red crustaceans (Hairston, 1979). But the definite proof for the induction of a red coloration by surfacing behaviour should come from an experiment specifically testing it by forcing uninfected shrimps and transparent infected shrimps to stay at the surface (caged shrimps in the field). One less parsimonious explanation is that parasites induce red colour in brine shrimps by acting directly on the control of carotenoid synthesis. Whatever the mechanism, red colour is likely to increase the visibility of infected *Artemia* to visual predators (Hairston, 1979) as confirmed in experiments using waders in captivity (Sánchez et al., in preparation). If changes in behaviour are initiated prior to colour change, the parasite is more likely to be mature enough when transmission to avian hosts occurs.

4.4. Consequences of parasite-induced behavioural changes

Our study demonstrates that parasite-induced behavioural alterations result in spatial segregation in host populations, which supports the view that parasites may influence dynamics, distribution and gene flow in host populations (Wellnitz et al., 2003). However, there are also immediate ecological consequences for the host (and for the parasites) due to the different

environmental conditions among microhabitats (light, oxygen, temperature, nutrients, predators, etc.). Living in algae-rich surface water increases predation risk, but *Artemia* can also benefit from nutrient acquisition, which may explain the larger lipid reserves of infected brine shrimps (Amat et al., 1991). *Artemia* is a keystone species in hypersaline habitats and a major food resource for waterbirds (Cooper et al., 1984; Britton et al., 1986; Verkuil et al., 2003; Sánchez et al., 2006b). By increasing the vulnerability and profitability of brine shrimps to avian predators and by reducing their fecundity (Amat et al., 1991), cestodes are likely to be major determinants of predator–prey dynamics and predator habitat selection. At the ecosystem level, cestodes are embedded in the food web and may have a strong effect on energy flow and community structure in salt pans. More research is required to elucidate the effects of parasites on *Artemia* populations and on hypersaline ecosystems.

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