Cestodes change the isotopic signature of brine shrimp Artemia hosts: implications for aquatic food webs

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ABSTRACT
To reach the final host (greater flamingos), the cestode *Flamingolepis liguloides* alters the behaviour of its intermediate host, the brine shrimp *Artemia parthenogenetica*, causing them to spend more time close to the water surface. In summer 2010 we showed that the prevalence of this cestode was consistently higher at the top of the water column in the Odiel salt pans in south-west Spain. We used stable nitrogen (N) and carbon (C) isotope analysis to test the hypothesis that cestodes also alter resource use by *Artemia*. In early summer, we compared stable isotopes in infected hosts at the surface with those from uninfected hosts at the bottom of the water column. In late summer, we compared infected and uninfected *Artemia* from the bottom. $\delta^{15}N$ was consistently enriched in infected individuals compared to uninfected hosts, especially in *Artemia* with multiple infections of *F. liguloides* (family Hymenolepididae) and those with mixed infections of *F. liguloides* and cestodes of the family Dilepididae. Infected individuals from the surface were enriched in $\delta^{13}C$ compared to uninfected ones from the bottom, but the opposite was found when comparing uninfected and infected *Artemia* from the same depth. This may be caused by the increase in lipid concentration in infected *Artemia*. Isolated cysticercoids of *F. liguloides* were significantly enriched in $\delta^{13}C$ compared to infected hosts, but surprisingly were not enriched in N. Our findings illustrate the way cestodes can alter food webs and highlight the importance of considering the parasitic status of prey in studies of trophic ecology in saline wetlands.

Keywords: C and N stable isotopes, *Artemia*, cestodes, intermediate host, parasitism, trophically transmitted parasite
1. Introduction

Parasites have often been ignored in aquatic food web studies (Lafferty et al. 2008). However recent theoretical and empirical data suggest that parasites affect trophic interactions and energy flow within ecosystems in a diversity of ways (Thompson et al. 2005; Lafferty et al. 2008). Trophically transmitted parasites, which depend on predation of their intermediate hosts to complete their life cycle, have the potential to strongly impact trophic interactions and food webs. Many parasites using trophic transmission have evolved sophisticated mechanisms to modify the behaviour and morphology of their intermediate hosts to increase the probability of predation by the final hosts (Moore 2002, Poulin 2007). However, the ecological consequences of such manipulations at different trophic levels remain largely unexplored.

Artemia spp. (Branchiopoda; Anostraca) are keystone species and dominant macrozooplankton in hypersaline ecosystems (Wurtsbaugh and Gliwicz 2001) and can control phytoplankton density via grazing (Lenz, 1987; Wurtsbaugh, 1992; Gliwicz et al. 2010). Many species of aquatic birds depend on brine shrimps as a food source (Sánchez et al. 2007a, Varo et al. 2011, Vest & Conover 2011). Moreover Artemia is the intermediate host for at least 15 species of cestodes which depend on predation by avian hosts (Georgiev et al. 2005, 2007). Brine shrimp become infected by ingesting cestode eggs released into the water with the feces of the avian definitive host. The newly hatched oncosphere (tapeworm embryo) penetrates into the hemocoel, where it develops into a cysticercoid (the larval stage). Birds become infected via trophic transmission when they feed on infected Artemia. Adult tapeworms develop and reproduce in the small intestine of birds, concluding their life cycle. A particularly abundant cestode in brine shrimps is Flamingolepis liguloides (Cyclophyllidea, Hymenolepididae), a parasite of flamingos. *F. liguloides* alters the size, fecundity, coloration,
physiology and behaviour of Artemia (Amat et al. 1991, Sánchez et al. 2006a, 2007b, 2009a, Amarouayache et al. 2009). Previous studies show that behavioural changes (positive phototactism) cause alterations in the spatial distribution of Artemia, with infected shrimps being more abundant at the surface of the water and uninfected ones mainly occupying the bottom of the water column (Gabrion et al., 1982; Thiéry et al., 1990; Sánchez et al. 2007b).

The aim of the present work is to explore potential trophic consequences of infection by cestodes in Artemia using C and N stable isotope analysis (hereafter $\delta^{13}C$ and $\delta^{15}N$ respectively); $\delta^{15}N$ signature can inform about the trophic position, owing to enrichment through the trophic levels; $\delta^{13}C$ signature provides information about the dietary source of carbon. Stable isotopes have been increasingly used in studies of avian diet (Hobson and Clark 1992a, b; Thompson and Furness 1995; Podlesak and McWilliams 2006, Gómez-Díaz and Figuerola 2010) and constitute a powerful tool for studies of food webs (Post 2002). However to date, studies of host-parasites relationships using C and N stable isotopes are still scarce (Doi et al. 2008, Dubois et al. 2009, Gómez-Díaz and González-Solís 2010) and have usually suggested that parasites have no effect on the isotopic signature of their hosts (but see Miura et al. 2006).

In this paper we tested the following hypotheses: 1. Parasite-induced microhabitat segregation affects $\delta^{15}N$ and $\delta^{13}C$ values of infected compared to uninfected Artemia. 2. The extent of this modification depends on parasite load and taxa. We considered the effect of dilepidid cestodes (Cyclophyllidea, Dilepididae) in coinfection with the dominant F. liguloides. Given that both groups of parasites show different site preferences within the host’s body likely to reflect different feeding strategies (authors, unpublished), and may cause different behavioural and physiological changes in their host, we tested whether $\delta^{13}C$ and $\delta^{15}N$
signatures were different between individuals infected with *F. liguloides* and those coinfected with dilepidids. Owing to the dominance of *F. liguloides*, we were unable to study *Artemia* infected only with dilepidids. 3. Isolated *F. liguloides* cysticercoids will be enriched in $\delta^{15}\text{N}$ compared to hosts, because parasites are assumed to feed on host tissue.

2. Material and methods

*Artemia* sampling was conducted in a saltpan of the Odiel marshes (Huelva, SW Spain, 37°15′29″N, 6°58′25″W), a site of international importance for waterbirds (Sánchez et al. 2006b). The study area (1,118 ha) is a complex of ponds through which the sea water is circulated and evaporated until salt precipitation. The Odiel brine shrimps are one of many clonal populations in the Old World, often grouped under the binomen *A. parthenogenetica* (Amat et al. 2005, Muñoz et al. 2010). Samples were collected in an evaporation pond of moderate salinity (E18, salinity = 110-120g/l) during Summer 2010. This pond (labelled as I6 in Sánchez et al. 2006c) of constant depth (24-27cm) is a simple environment with no structure from e.g. rocks or macrophytes (absent owing to the high salinity).

2.1 Distribution of infected and uninfected *Artemia* in the water column

In order to examine the proportion of infected and uninfected *Artemia* at the surface and depth of the water column, we collected samples (between 0900 h and 1200 h) at 0-5cm from the surface and 0-5 cm from the bottom during five different days from 30 June to 3 August 2010 at a fixed point 140 m from the shoreline. Samples were taken with an aquarium net (0.5 mm mesh) at a constant, slow speed for 1 minute at depth (higher density) and 5 minutes at surface. In the laboratory each individual was mounted in a temporary glycerol mount and examined under a microscope for parasites (after Georgiev et al. 2005).
2.2. Early summer sampling for isotope analysis: infected from the surface vs uninfected from the bottom

In order to test if changes in the spatial distribution of *Artemia* induced by parasites had trophic consequences, we collected samples for isotope analysis in early-summer (5 occasions from 14 June to 5 July 2010 to reach an adequate sample size). No variations in food availability and biochemical composition of *Artemia* likely to affect isotopic composition were expected to occur during such a short sampling period. We collected infected *Artemia* from within 5 cm of the surface and uninfected ones from within 5 cm of the bottom with a 0.1 mm mesh plankton net. In the laboratory, brine shrimps were immediately placed in a tank filled with filtered (45 µm sieve) water from the same pond, during 24h to remove all food particles and to allow digestion of previously ingested food. Individual *Artemia* were then inspected under the stereomicroscope for the presence of parasites. More than 500 specimens where checked on each sampling date so as to reach a minimum of 475 individuals infected only with *F. liguloides* (mixed infection with other cestodes is very frequent, Georgiev et al. 2007). We always selected adult individuals of the same size range to minimize age variation (8-10mm, measured from the anterior margin of the head to the end of caudal furca).

Four categories were selected for isotope analysis on the basis of their abundance in the field: no parasites NP (n = 18 samples), infected with 1 *F. liguloides* (1FL, n = 7 samples), infected with more than 1 *F. liguloides* (>1FL, 2-14 cysticercoids per shrimp, n = 32 samples) and infected with both *F. liguloides* + Dilepidiae (FL+DIL 1-2 cysticercoids of *F. liguloides* and 1-2 cysticercoids of Dilepididae, n = 12 samples). Each sample was necessarily made from 10-20 individuals so as to provide enough material for isotope analysis. It was not practical to divide the *Artemia* with multiple infections of *F. liguloides* (between 2 and 14 cysticercoids) into more categories, owing to the very low number of individuals of intermediate categories.
recorded, and the need to pool several individuals to achieve an acceptable weight for isotope analysis (25 mg). We chose the categories of 1FL and >1FL because our unpublished data suggest that host phenotype is affected by an increase from one to two \textit{F. liguloides} cysticercoids, but not by a further increase (e.g. red coloration becomes significantly more intense with an increase between 1 and 2 cysticercoids, but not with a further increase).

In a field sample collected near the surface on 30 June and 5 July (N = 259) and quantified, the proportions of \textit{Artemia} in the four study categories were 29% NP, 18.1% 1FP, 32% >1FL and 4.2% FL+DIL; 16.6% corresponded to other categories of infected shrimps (i.e. presence of additional cestode species, and 74.4% of these individuals were coinfected with \textit{F. liguloides}). We were not able to distinguish between the three Dilepididae species infecting \textit{Artemia} (\textit{Eurycestus avoceti}, \textit{Anomotaenia tringae} and \textit{Anomotaenia microphallos}) because this would require dissection which would cause loss of most haemolymph. Cysticercoids of \textit{F. liguloides} are easily recognizable without dissection (Georgiev et al. 2005).

From samples taken on the same dates, we also isolated cysticercoids of \textit{F. liguloides} from the body of \textit{Artemia} (n = 10 samples, each sample containing 2000 cysticercoids). These cysticercoids were extracted from many samples of \textit{Artemia} from the top of the water column, which were then minced and sieved to extract the relatively hard cysticercoids. However, hosts were not conserved after cysticercoid removal as most haemolymph was lost in the dissection. Although a given sample may have contained multiple cysticercoids from the same individual host, each host could not be represented in more than one sample, so avoiding non-independence. The isotopic signatures of cysticercoids were compared to those of intact uninfected and infected hosts. Cysticercoids of Dilepididae are much smaller and
show low prevalence in *Artemia* (Georgiev et al. 2005, 2007) so isolation of an adequate quantity was not feasible.

2.3 Late summer sampling for isotope analysis: infected from the bottom vs uninfected from the bottom

In late summer (8 September 2010) we collected an additional sample of *Artemia* from the bottom in order to compare C and N ratios of infected and uninfected individuals at the same depth. The aim was to test the consequences of parasitism for isotope signature irrespective of those caused by microhabitat segregation. Host categories were similar to early summer except that there were insufficient individuals of the FL+DIL category for analysis. Hence we obtained NP (*n* = 25 samples, each made of 5 individuals), FL (*n* = 11 samples) and >1FL (*n* = 10 samples). In the laboratory the samples were processed as described above for early summer.

Prior to isotope analysis, no lipid extraction was carried out. Each sample was dried at 60°C for 24h and ground to a fine powder using a vibratory ball mill at 30 s⁻¹ for 25s. See “Supplementary Data S1” for detailed information on methods for isotope analysis.

2.4. Statistical analysis

We used Chi-square tests to compare the proportions of *Artemia* infected with *F. liguloides* between the surface and the bottom of the water column (vertical distribution).

Comparisons of $\delta^{15}$N between uninfected and different categories of infected *Artemia* were performed with one-way ANOVA, followed by Tukey's HSD post-hoc tests. Kruskall Wallis tests followed by Mann-Whitney U-tests were used to compare $\delta^{13}$C between categories in
early summer when normality assumptions were not met, even after log and other data
transformations. Similarly, comparisons of isolated cysticercoids and *Artemia* were made with
tests for $\delta^{15}$N, and Mann-Whitney U-tests for $\delta^{13}$C. P values were always adjusted for
multiple comparisons via false discovery rate (FDR, Benjamini and Hochberg 1995). All
statistical analyses were conducted using Statistica 6.0 (StatSoft 2001).

3. Results

3.1. Vertical distribution analysis

The proportion of *Artemia* infected with *F. liguloides* was consistently greater at the surface
than below in all sampling dates (Figure 1). These differences were statistically significant in
three of the five dates (Figure 1).

3.2. $\delta^{15}$N analysis - early summer

The range of $\delta^{15}$N varied among the different groups studied (Table 1). We found statistically
significant differences in $\delta^{15}$N between non infected *Artemia* (NP, $n = 18; 7.76 \pm 1.11\% \text{\textup{o}},$
mean $\pm$ SE), *F. liguloides*-infected *Artemia* ($n = 39; 8.72 \pm 0.06\% \text{\textup{o}}$) and specimens infected
with both *F. liguloides* and Dilepidae (FLD, $n = 12; 9.09 \pm 0.15\% \text{\textup{o}}$) (one way ANOVA: $F_{2, 66}$
$= 41.90, P = 0.000001$). Post-hoc Tukey’s HSD tests (corrected for multiple testing) revealed
statistically significant differences between NP and *F. liguloides*-infected *Artemia* and
between NP and FLD ($P < 0.001$), but not between both categories of infected individuals.

When we repeated analyses differentiating between individuals with only one cysticercoid of
*F. liguloides* (1FL, $n = 7$) and those with more than one (>1FL, 2-14 cysticercoids, $n = 32$),
values of $\delta^{15}$N also varied among groups (one-way ANOVA: $F_{3, 65} = 37.43, P = 0.000001$).
\( \delta^{15}N \) was significantly higher for >1FL (8.83 ± 0.054‰, mean ± SE) compared to those 1FL (8.23 ± 0.167‰) (Tukey HSD, \( P < 0.01 \), Figure 2, Table 2). All other pairwise comparisons were statistically significant, except between >1FL and FLD, and between NP and 1FL (Figure 2A, Table 2).

The isotopic signature of isolated cysticercoids of *F. liguloides* ranged between 7.67 and 8.66‰ (Table 1). Cysticercoids were not significantly enriched in \( \delta^{15}N \) compared to NP (8.00 ± 0.09 and 7.76 ± 0.11‰, mean ± SE respectively, t-test: \( t = -1.52, P = 0.14 \); Figure 2). Cysticercoids were significantly depleted in \( \delta^{15}N \) with respect to >1FL (\( t = 7.58, P = 0.000001 \)) and FLD (\( t = 6.062, P < 0.001 \)), but not 1FL (\( t = 1.28, P = 0.22 \), Figure 2A).

3.3. **\( \delta^{13}C \) analysis - early summer**

\( \delta^{13}C \) values were much more variable in all the groups than their respective N values (Table 1). The \( \delta^{13}C \) values differed among NP (-26.08 ± 0.42‰, mean ± SE) and *F. liguloides*-infected Artemia (-25.33 ± 0.22‰) and also FLD (-24.20 ± 0.45‰) (Kruskal Wallis test, H (2, N = 69) = 18.35, \( P = 0.0001 \)). Pairwise comparisons revealed statistically significant differences between NP and *F. liguloides*-infected Artemia (\( U = 167, P < 0.01 \)), between NP and FLD (\( U = 29, P < 0.001 \)) and between both categories of infected individuals (\( U = 109, P < 0.01 \)). Parasitized Artemia were enriched in \( \delta^{13}C \) compared to NP (Figure 2B).

When we repeated analyses differentiating between 1FL and >1FL individuals, the average values of \( \delta^{13}C \) were almost identical between these two categories (-25.453 ± 0.63 for 1FL and -25.303 ± 0.227‰ for >1FL, mean ± SE; \( U = 92, P = 0.464 \); Figure 2). All other pairwise comparisons were statistically significant (Figure 2), except between 1FL and NP individuals (-26.81 ± 0.424‰, mean ± SE; \( U = 34, P = 0.079 \); Table 2, Figure 2).
$\delta^{13}C$ values for isolated cysticercoids varied from $-20.99$ to $-23.73\%$. Cysticercoids were significantly enriched in $\delta^{13}C$ with respect to NP ($-23.256 \pm 0.256\%$ and $-26.081 \pm 0.424\%$ respectively, mean $\pm$ SE, $U = 167$, $P = 0.0016$). Cysticercoids also showed higher values of $\delta^{13}C$ compared to 1FL ($-25.453 \pm 0.637\%$, $U = 9$, $P = 0.011$) and $>1FL$ ($-25.305 \pm 0.227\%$, $U = 36$, $P = 0.00025$). However, there was no significant difference between cysticercoids and FLD ($U = 31$, $P = 0.06$).

3.4. $\delta^{15}N$ analysis - late summer

The pattern of $\delta^{15}N$ for host groups in late summer (infected and uninfected from the bottom) was equivalent to that observed in early summer (infected from the surface and uninfected from the bottom). Different host groups varied in their range of $\delta^{15}N$ (Table 1), infected individuals being enriched compared to NP. We found statistically significant differences in $\delta^{15}N$ between NP ($n = 25; 6.556 \pm 0.047\%$, mean $\pm$ SE), 1FL ($n = 11; 7.115 \pm 0.092\%$) and $>1FL$ ($n = 10; 7.322 \pm 0.051\%$; one-way ANOVA: $F (2, 43) = 44.134$, $P = 0.000001$). $\delta^{15}N$ was significantly higher for infected Artemia (both with multiple and simple infection) compared to NP (Tukey HSD, $P < 0.001$; Figure 1A, Table 2) but not between 1FL and $>1FL$.

3.5. $\delta^{13}C$ analysis - late summer

Contrary to the pattern in early summer (when infected Artemia came from the surface) individuals infected with $>1FL$ showed the most negative $\delta^{13}C$ values ($-27.438 \pm 0.210\%$, mean $\pm$ SE), followed by 1FL ($-26.982 \pm 0.398\%$) and NP ($-26.462 \pm 0.118\%$, mean $\pm$ SE) (Table 1, Figure 2B). The differences between categories were statistically significant (one-way ANOVA: $F (2, 43) = 5.261$, $P = 0.009$). Pairwise comparisons revealed statistically significant differences between NP and $>1FL$ (Table 2).
4. Discussion

The present study reveals strong differences in the isotopic signatures of C and N between infected and uninfected Artemia. Using C isotopes, Miura et al. (2006) found that trematode infection changed the habitat use and trophic ecology of marine gastropods, which moved to the lower intertidal zone, where they were more susceptible to predation by their final hosts (fish). However, other studies have reported no differences in stable isotopes of invertebrate hosts when infected by trematodes (Doi et al 2008, Dubois et al 2009).

We interpret our results in a similar manner to Miura et al. (2006), and suggest that infection by cestodes has direct trophic consequences for their brine shrimp host, at least partly because the parasite induces movement towards the water surface. As expected from existing literature, prevalence of infected Artemia (with F. liguloides) was higher at the surface of the water. A. parthenogenetica undergo diel vertical migrations and typically spend most of the daytime in the bottom 25% of the water column and most of the night in the other 75% (Britton et al. 1986). Each individual does not stay at a fixed depth throughout the daily cycle, and cestode infection changes the proportion of the time spent at different depths (Sánchez et al. 2007b). The change in Artemia trophic ecology appears to be one of a suite of interdependent host characters changed by infection, including colour, behavior and lipid accumulation (Sánchez et al. 2006a, 2007b, 2009a). The differences found between infected and uninfected shrimps collected together from the bottom of the water column are most likely to be explained by the infected shrimps having spent more of their time at the top of the water column than the neighbouring uninfected shrimps (and hence having consumed different proportions of available food items), even though they were close together at the moment of sampling.
An alternative explanation for the link between infection and isotopic signature would be that it is an *effect* of inherent differences between individual shrimps in trophic ecology, not a *cause*; i.e. that some individuals feed at a higher trophic level in such a way that they are more likely to ingest cestode eggs and become infected. We cannot eliminate this possibility without conducting experimental infections in the laboratory, but we consider it unlikely. In any case, by comparing infected and uninfected shrimps from the same depth, we have shown that infection status is a more important determinant of isotopic signature than position in the water column at the time of sampling.

Stable isotope ratios of a parasite-host system can potentially shift because of the direct effect of parasite biomass (‘mass-balance’ shift, Duboi et al. 2009). Changes in isotope ratios between healthy and parasitized hepatopancreas of freshwater snails have been attributed to the high proportion of trematode biomass (up to 3.4%) within this organ (Doi et al. 2008). Given our inability to remove cysticercoids from *Artemia* without losing host fluids, we estimated the contribution of the parasite to the total host biomass to evaluate whether the presence of parasites have an important effect on the isotopic signature of *Artemia*. We determined the average fresh weight of 60 infected *Artemia* within the size range used in our study, and estimated the average mass of cysticercoids using the dimensions reported by Georgiev et al (2005). Thus, we estimated that 1 cysticercoid would represent only 0.788 × 10^{-4}% of the total *Artemia* biomass. Individuals with multiple infections usually had 2 to 4 cysticercoids, with a maximum of 14. Thus, even when infestation load is highest, the proportion of the host constituted by the parasite would not exceed 0.01%. Thus, the parasite biomass makes a negligible contribution to the overall isotopic signature of the host.
Observed variations in N isotope ratios indicate that infected and uninfected *Artemia* may occupy different trophic positions, as $\delta^{15}N$ increases at higher trophic levels (Bearhop et al. 2004; Boecklen et al., 2011). According to $\delta^{13}C$ values, infected and uninfected individuals used different carbon sources within the food web. We consistently found the strongest differences between non-infected *Artemia* and those with either multiple or mixed infections, suggesting a minimum number of parasites are required to make changes in stable isotope signature most evident. This dose-dependent effect of parasites in their host is a common phenomenon in nature in a variety of host-parasite associations (Blair and Webster 2007).

*Artemia* is considered a non-selective filter feeder (Provasoli and Shiraishi, 1959; Dobbeleir et al., 1980), and is likely to feed on whatever microorganisms are present at the top or bottom of the water column. At the salinity of our study pond, a diversity of autotrophic and heterotrophic bacteria and eukaryotes are found in salt ponds, especially cyanobacteria, purple bacteria, diatoms and protozoans (Pedrós-Alió et al. 2000, Estrada et al. 2004, Oren 2005). These taxa are likely to vary greatly in their isotope signatures. *Artemia* are also known to feed on detritus (Eardley, 1938; Conte and Conte, 1988; Wear et al., 1986, Savage and Knott 1998). Differences in $\delta^{15}N$ values between early and late summer indicate that, as well as the influence of cestode infection on diet, there was also important seasonal variation in *Artemia* diet likely to reflect a change in the relative abundance of different food items (Figure 2A). Higher $\delta^{15}N$ values in infected *Artemia* and in early summer may reflect a relatively greater reliance on primary consumers (microzooplankton such as small ciliates and flagellates, and heterotrophic bacteria) than on primary producers (phytoplankton such prokaryotic and eukaryotic microalgae, and phototrophic bacteria). They may also reflect the consumption of *Artemia* exoskeletons shed during moult which float at the water surface and are known to be
ingested by adult brine shrimps (Gliwicz et al. 2010), this being equivalent to cannibalism in terms of isotopic enrichment.

The highly depleted values of $\delta^{13}C$ exhibited by non-infected individuals in early summer and all Artemia in later summer (Figure 2B), from the bottom of the water column, suggest a diet dependent on detritus and/or on benthic species that feed on detritus of terrestrial influence (Fry and Sherr, 1984; Bouillon et al., 2002, Tavares et al. 2008). The detritus may originate partly from halophytic Chenopodioideae that surrounds the study pond, since chenopod detritus has similar depleted values (Cre´ach et al., 1997). In contrast, the higher C values for infected individuals from the top of the water column suggest a marine contribution to the diet (Fry and Sherr, 1984; Peterson et al., 1985), perhaps due to plankton and other C sources entering with seawater pumped into the salt pond. Additional studies of gut contents of infected and uninfected Artemia, e.g. by metagenomic analysis using New Generation Sequencing (Boyer et al. 2012), are required to clarify the difference in diet between parasitized and unparasitized individuals, and those at different depths.

A potential cause of shifts in the pattern of $\delta^{13}C$ for hosts from late summer (Figure 2B), with infected individuals showing lower values than uninfected ones from the same depth is parasite-induced host alteration of metabolic and physiological functions (pathological effects). Modifications of host metabolism can involve changes in biochemical composition of tissues or the whole animal (Thompson 1983 for review, Lauckner 1983, Plaistow et al. 2001), and different biochemical compounds have different stable isotope signatures (DeNiro & Epstein 1977, Fantle et al. 1999, Pakhomov et al. 2004, Bodin et al. 2007). Changes in isotope signatures between healthy and infected invertebrates caused by disturbances in host metabolism have previously been reported (Dubois et al. 2009). In our study system, F.
*liguloides* increases the concentrations of lipids in *Artemia* (Amat et al 1991, Sánchez et al 2009a) which may cause shifts in isotope values since lipids are $\delta^{13}$C depleted (DeNiro and Epstein 1977; Lorrain et al. 2002; Bodin et al. 2007) and were not extracted from host tissues prior to analysis. Reallocation of energy from reproduction to growth (due to parasite induced host castration as reported in *F. liguloides*, Amat et al. 1991), may also contribute to our results.

Cysticercoids of *F. liguloides* were significantly enriched in $\delta^{13}$C relative to the host. This result is consistent with the classical pattern that $\delta^{13}$C increases slightly up the food chain (DeNiro and Epstein 1978, 1981, see also Gómez-Díaz and González-Solís 2010). However an unexpected result was that isolated cysticercoids of *F. liguloides* did not show significant enrichment of $\delta^{15}$N relative to their host, but rather the opposite trend. The consumer (parasite) is usually expected to be enriched in N signatures with respect to its diet (host), such enrichment typically being much higher than for C (Peterson and Fry 1987; DeNiro & Epstein 1978, 1981, Hobson & Welch 1992, Post 2002; Checkley & Entzeroth 1985). For endoparasites such as cestodes, there is typically a $^{15}$N-enrichment from their hosts because the parasites feed mainly on host tissues and fluids (Boag et al., 1998; Doucett et al., 1999; Power and Klen, 2004; O’Grady and Dearing 2006). Nonetheless, our results are not unique (Lafferty et al 2008; Dubois 2009), and the lack of $\delta^{15}$N enrichment in Greenland cod cestodes relative to hosts was attributed to the trophic transfer of common dietary sources (Power and Klein 2004). The inability of some endoparasites to synthesize amino acids and the need to take them from their host (Deudero et al. 2002) has also been proposed to explain slight or no $\delta^{15}$N trophic enrichment of parasites (Dubois et al. 2009). No enrichment in N may also be explained by the abbreviated metabolic pathways of parasites and the low rate of excretion, tegument diffusion and respiration (Dubois et al. 2009). All these explanations may
apply to our system. Owing to the need to pool large numbers of cysticercoids from different
hosts to have enough sample for analysis, we do not know the exact infection types to which
these cysticercoids belonged (but see methods for the relative abundance of different infection
types). Hence, although they were significantly depleted compared to *Artemia* with multiple
and mixed infections, it remains unclear whether they were depleted compared to their hosts
on an individual basis.

We also found substantial differences in the level of enrichment of C and N among *Artemia*
infected with *F. liguloides* and those co-infected with metacestodes from three species of the
family Dilepididae. The isotope enrichment of *Artemia* with mixed infection (*F. liguloides* +
*Dilepididae*) compared to individuals infected with *F. liguloides* may be due to a dose-
dependent effect since a host with a mixed infection harbours at least two parasites.
Nevertheless, it is possible that cestodes from different families uses different resources in
their host, or alter host metabolism in a different way. While Dilepidid cysticercoids are
mainly found in the adult thorax, *F. liguloides* cysticercoids are predominantly located in the
abdomen of adult *Artemia* (authors’ unpublished data, Amarouayache et al. 2009, Redón et al.
2010).

Changes in spatial distribution of *Artemia* induced by parasites appeared to change resource
consumption. Given the abundance of *Artemia* and their importance as food source for birds,
cestode parasites have the potential to alter food web properties, energy and mass flows in
hypersaline ecosystems. On the other hand, as major consumers of phytoplankton and organic
particulate matter in hypersaline waters, alterations of the diet in *Artemia* can potentially
cause strong cascading effects through alteration of grazing effects on phytoplankton and
bacterioplankton. Feeding by *Artemia* influences phytoplankton density and composition
(Mohebbi, F. 2010), although there is no previous information on the influence of parasites on
grazing. Higher grazing rates by infected individuals at the surface of the water may decrease phytoplankton abundance, thus increasing water clarity (Lenz 1987, Wurtsbaugh 1992, Gliwicz et al. 2010). Augmentation of light passing through the water column is likely to affect the algal-cyanobacteria community at the bottom of the water column. For example, it might favor phototrophic bacteria which are common beneath the cyanobacterial layers in hypersaline microbial mats (DasSarma and Arora 2001). Laboratory grazing experiments with infected and uninfected Artemia would provide further insight into the ecological implications of parasite-induced changes in diet of Artemia.

This study shows that cestodes can induce strong changes in isotopic signatures of their intermediate hosts, and that infected and uninfected invertebrates are not equivalent from a trophic point of view. Parasitic status of invertebrates should be checked and incorporated into aquatic food web research and studies of trophic ecology and diet of birds. Given that stable isotopes can be used to assess the importance of specific prey in the diet of birds (Alexander et al. 1996; Tavares et al 2009), this technique can evaluate the importance of infected and uninfected prey as a food source for birds. On the other hand, in studies of avian ecology, invertebrates are typically sampled as potential bird prey without considering the presence of helminth parasites and their potential effects on isotopic signatures. This may be an important source of error when determining the part that each invertebrate taxon plays in avian diet. As a consequence of their manipulations of host colour and behaviour, birds are often particularly likely to ingest invertebrates that have been parasitized (Sánchez et al. 2009b). The prevalence of parasites is likely to vary between those invertebrates ingested by birds and those collected by biologists as potential food items, since the sampling methods may be very different. Our work shows it is very important to take into account the parasite communities in those taxa, and how they can influence the isotope ratios of avian prey.
ACKNOWLEDGEMENTS

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Thompson, S. N. 1983 Biochemical and physiological effects of metazoan endoparasites on their host species. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, Volume 74, Issue 2, 1983, Pages 183-211*


Table 1. Range of δ¹⁵N and δ¹³C (‰) in non infected *Artemia* (NP), *Artemia* infected with 1 *F. liguloides* (1FL), *Artemia* infected with more than one *F. liguloides* (>1FL), infected by both *F. liguloides* and Dilepididae (FLD) and isolated cysticercoids of *F. liguloides* (Cy FL) in early summer (June-July). n = number of pooled samples, each of which is made of 10-20 individuals.

<table>
<thead>
<tr>
<th></th>
<th>Early summer</th>
<th></th>
<th>Late summer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>δ¹⁵N range (‰)</td>
<td>δ¹³C range (‰)</td>
<td>n</td>
</tr>
<tr>
<td><em>NP</em></td>
<td>18</td>
<td>(6.97, 8.32)</td>
<td>(-27.61, -21.79)</td>
<td>25</td>
</tr>
<tr>
<td><em>1FL</em></td>
<td>7</td>
<td>(7.55, 8.65)</td>
<td>(-26.80, -21.80)</td>
<td>11</td>
</tr>
<tr>
<td><em>&gt;1FL</em></td>
<td>32</td>
<td>(8.26, 9.65)</td>
<td>(-26.65, -22.28)</td>
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</tr>
<tr>
<td><em>FLD</em></td>
<td>12</td>
<td>(8.41, 10.03)</td>
<td>(-27.61, -21.79)</td>
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</tr>
<tr>
<td><em>Cy FL</em></td>
<td>10</td>
<td>(7.67, 8.66)</td>
<td>(-23.73, -20.99)</td>
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</tbody>
</table>
Table 2. Results of pair-wise comparisons for $\delta^{15}$N (Tukey HSD test) and $\delta^{13}$C (Mann-Whitney U test) between uninfected Artemia and different categories of infected individuals (see Table 1 for details). P values are showed. *p< 0.05, **p < 0.01, ***p < 0.001 after correction for multiple testing (FDR).

<table>
<thead>
<tr>
<th></th>
<th>$\delta^{15}$N</th>
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<th>$\delta^{13}$C</th>
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<td></td>
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<tr>
<td>NP</td>
<td>0.054</td>
<td>0.0002***</td>
<td>0.0002***</td>
<td>0.080</td>
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<tr>
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<td>0.0003***</td>
<td>0.22</td>
<td>0.464</td>
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<tr>
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<tr>
<td>Late summer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>0.0001***</td>
<td>0.0001***</td>
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<td>0.207</td>
</tr>
<tr>
<td>1FL</td>
<td>0.1333</td>
<td>...</td>
<td></td>
<td>0.428</td>
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</table>
FIGURE LEGENDS

Figure 1. Proportion of Artemia infected with Flamingolepis liguloides at the surface (dark) and below (light). Sample sizes are given above the bars. *p<0.01 after FDR correction.

Figure 2. Mean (± SE) values for A) δ¹⁵N and B) δ¹³C in early and late summer for non-infected Artemia (NP), those infected with 1 cysticercoid of F. liguloides (1FL), infected with more than 1 cysticercoid of F. liguloides (>1FL), infected with both F. liguloides and Dilepididae (FL+DIL), and isolated cysticercoids of F. liguloides (C). Squares are samples from early summer, circles from late summer. Solid symbols are samples from the surface; empty symbols are samples from the bottom.
Fig. 1

Flamingolepis liguloides prevalence (%)

<table>
<thead>
<tr>
<th>Date</th>
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<th>Bottom</th>
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<tr>
<td>30-June</td>
<td>21</td>
<td>44</td>
</tr>
<tr>
<td>5-July</td>
<td>64</td>
<td>51</td>
</tr>
<tr>
<td>26-July</td>
<td>15</td>
<td>53</td>
</tr>
<tr>
<td>29-July</td>
<td>62</td>
<td>63</td>
</tr>
<tr>
<td>3-Aug</td>
<td>45</td>
<td>39</td>
</tr>
</tbody>
</table>

Surface vs. bottom prevalence from 30-June to 3-Aug.
Fig. 2

A) 

B)
Supplementary material

1. \( \delta^{15}N \) and \( \delta^{13}C \) Analyses

Carbon and nitrogen isotope analysis was undertaken by EA-IRMS (elemental analyzer isotope ratio mass spectrometry) by Isoanalytical laboratories (UK). In this technique, samples and reference materials are weighed into tin capsules, sealed, and then loaded into an automatic sampler on a Europa Scientific Roboprep-CN sample preparation module. From there they were dropped into a furnace held at 1000 °C and combusted in the presence of oxygen. The tin capsules flash combust, raising the temperature in the region of the sample to ~ 1700 °C. The combusted gases are swept in a helium stream over a combustion catalyst (\( \text{Cr}_2\text{O}_3 \)), copper oxide wires (to oxidize hydrocarbons), and silver wool to remove sulphur and halides. The resultant gases (\( \text{N}_2 \), \( \text{NO}_x \), \( \text{H}_2\text{O} \), \( \text{O}_2 \), and \( \text{CO}_2 \)) are swept through a reduction stage of pure copper wires held at 600 °C. This removes any oxygen and converts \( \text{NO}_x \) species to \( \text{N}_2 \). A magnesium perchlorate chemical trap removes water. A Carbosorb trap is used to remove \( \text{CO}_2 \) during nitrogen-15 analysis. Nitrogen or carbon dioxide is resolved by a packed column gas chromatograph held at an isothermal temperature of 100 °C. The resultant chromatographic peak enters the ion source of the Europa Scientific 20-20 IRMS where it is ionised and accelerated. Gas species of different mass are separated in a magnetic field then simultaneously measured on a Faraday cup universal collector array. For \( \text{N}_2 \), masses 28, 29, and 30 are monitored and for \( \text{CO}_2 \), masses 44, 45, and 46 are monitored.

The reference material used for \( \delta^{15}N \) and \( \delta^{13}C \) analysis was NBS-1577B (powdered bovine liver, \( \delta^{15}N_{\text{Air}} = 7.65 \) ‰, \( \delta^{13}C_{\text{V-PDB}} = -21.60 \) ‰). NBS-1577B, a mixture of IA-R045 (ammonium sulfate, \( \delta^{15}N_{\text{Air}} = -4.71 \) ‰) and IA-R005 (beet sugar, \( \delta^{13}C_{\text{V-PDB}} = -26.03 \) ‰) and a mixture of IA-R046 (ammonium sulfate, \( \delta^{15}N_{\text{Air}} = 22.04 \) ‰) and IA-R006 (cane sugar, \( \delta^{13}C_{\text{V-PDB}} = -11.64 \) ‰) were run as quality control check samples during sample analysis.
NBS-1577B, IA-R045 and IA-R046 are calibrated against and traceable to IAEA-N-1 (ammonium sulfate, $\delta^{15}N_{\text{Air}} = 0.40 \%$). NBS-1577B, IA-R005 and IA-R006 are calibrated against and traceable to IAEA-CH-6 (sucrose, $\delta^{13}C_{\text{V-PDB}} = -10.43 \%$). IAEA-CH-6 and IAEA-N-1 are inter-laboratory comparison standards distributed by the International Atomic Energy Agency (IAEA), Vienna.