

Chytrid infection reduces thoracic beat and heart rate of *Daphnia pulicaria*

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Abstract Zooplankters are hosts to numerous endo- and ectoparasites, some of which have dramatic impacts on their hosts. Epizootics on zooplankton are probably more widespread in lake systems than it is currently known, and few studies have explored the direct and indirect importance of parasitism in aquatic food webs. In addition, our understanding of the sublethal effects of parasitic infections on host

organisms and populations is limited. We used a novel electro-chemical based technique to measure in the outflow of the feeding current changes in the beat rate of the thoracic appendages in female *Daphnia pulicaria*. We observed simultaneously the heart rates and compared chytrid infected animals with uninfected gravid and non-gravid ones. We found in uninfected animals a thoracic beat rate of 3.81 ± 0.18 Hz and a heart rate of 4.67 ± 0.42 Hz. Gravid daphnids had a 14% lower thoracic beat rate (3.27 ± 0.30 Hz) than non-gravid females while the heart rate did not significantly differ (4.48 ± 0.28 Hz). In contrast, infected animals showed a 22% lower thoracic beat rate (2.96 ± 0.47 Hz) and a 36% lower heart rate (2.98 ± 0.5 Hz) when compared with uninfected non-gravid females. We discuss the ways *Daphnia* are affected by *Polycaryum laeve* infections on the individual and population level.

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A Passion for Plankton: a tribute to the life of Stanley Dodson

Stanley I. Dodson died on 23 August 2009.

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Introduction

Planktonic animals are host to numerous endo- and ectoparasites, including bacteria, helminths, fungi, and protists, some of which have dramatic individual and population-level impacts on their hosts (Metschnikoff, 1884; Green, 1974; Wedekind & Jakobsen, 1998; Fels et al., 2004; Wolinska et al., 2004;

Skovgaard & Saiz, 2007). In aquatic ecosystems, zooplankton community composition and dynamics are traditionally thought to be controlled by a combination of predation pressure and resource availability (Carpenter et al., 1987; Dodson & Frey, 1991). However, predation and food levels alone are often not enough to explain the observed fluctuations in zooplankton populations (Luecke et al., 1992; Mehner et al., 1998; Hulsmann & Weiler, 2000; Johnson et al., 2009), and there is growing recognition of the important contribution of parasites and diseases in determining zooplankton dynamics (Yan & Larsson, 1988; Ebert, 1995; Ebert et al., 2001; Johnson et al., 2009). Disease epidemics probably occur far more frequently among lake systems than is currently known, and more studies are needed that explore the direct and indirect importance of parasitism in aquatic food webs.

Most studies of parasitism focus on the density-mediated effects of parasitic infections on zooplankton communities but ignore the sublethal effects of parasitism at the individual level. Parasites negatively affect the growth, survival, and reproductive capacity of their hosts (Wedekind & Jakobsen, 1998; Baldauf et al., 2007), but can also enhance vulnerability to predators and alter migratory and grazing behavior (Johnson et al., 2006b). Parasitism increases predation risk as some endoparasitic infections make the organisms more opaque leading to a higher incidence of predation (Bakker et al., 1997; Ebert et al., 2000; Decaestecker et al., 2005; Johnson et al., 2006a; Baldauf et al., 2007). Most studies dealing with zooplankton diseases have focused on changes in survival and reproduction rates (Stirnadel & Ebert, 1997; Ebert et al., 2000; Wolinska et al., 2004; Rice et al., 2006), yet the sublethal repercussions of infection on physiology and metabolism warrant further study.

Our study organism, *Daphnia pulicaria*, is a common large-bodied cladoceran found in many North American lakes and is known to be an obligate host for numerous endoparasites (Green, 1974; Dodson & Frey, 1991). Daphnids are an important component of pelagic food webs both as efficient grazers of phytoplankton and as food source of small planktivorous and larval fishes (Rudstam et al., 1993). The recently re-described chytrid, *Polycaryum laeve*, is a pathogenic fungus that causes a sharp reduction in *D. pulicaria* growth and survival and leads to a complete cessation of reproduction (Stempell, 1903;

Johnson et al., 2006a, b). As *P. laeve* infections spread throughout the population it leads to a marked reduction of *D. pulicaria* in the water column immediately following an epidemic (>2 orders of magnitude, Johnson et al., 2006a). Its mode of transmission remains unclear, but there are indications that it could be horizontally transferred (Johnson et al., 2006a).

In Wisconsin lakes, *P. laeve* infections can reach high prevalence (80%) by late winter/early spring, and estimates of chytrid sporangia in an infected individual range from light (1–500 sporangia) to severe (>1,000 sporangia) (Johnson et al., 2006a). *P. laeve* sporangia densities of up to 2,500 sporangia in a single individual have been observed, but at this level most host individuals are dead. Severe infections markedly alter the appearance of infected daphnids, filling the body with thousands of darkly colored fungal sporangia that are conspicuous among living as well as preserved *Daphnia* (Johnson et al., 2006b). In addition to increasing the predation risk, these *P. laeve* infections also induce metabolic stress, as parasites consume the organism's resources (e.g. lipid storage) (Schwartz & Cameron, 1993; Lemly, 1996). Furthermore, as the infection spreads it reduces the quality of the host as a food resource as their overall length is not only smaller, but they contain less nitrogen and phosphorus and are lower in several important fatty acids (Forshay et al., 2008).

Our study site, Allequash Lake in northern Wisconsin, is known to harbor *P. laeve* infected *D. pulicaria* during late winter/early spring (Johnson et al., 2006a). This is also true of a nearby lake, Devil's lake, where a 15-year study of revealed only *D. pulicaria* to be consistently infected by *P. laeve* (Johnson et al., 2009). Although *P. laeve* can infect other *Daphnia* spp., we have rarely seen infections in other zooplankton species within our field studies.

The goal of this study was to evaluate the sublethal effects of a *P. laeve* infection with two important *Daphnia* physiological metrics: the thoracic beat rate and the heart rate. Previous studies have shown that *Daphnia* clearance and feeding rates change according to metabolic stress due to natural ecological and physiological conditions, such as oxygen levels (Pirow et al., 2001), temperature (Burns, 1969; Lamkemeyer et al., 2003), and body size (Burns, 1969). Clearance and feeding rates can also be altered by algae size and concentration (DeMott, 1982; Porter et al., 1982). We replaced measuring the clearance rate with a correlated

metric and applied a novel technique that determines the beat rate of the thoracic appendages in the outflow of the feeding current in near real-time (Peñalva-Arana et al., 2008). Heart rates can vary according to both ecological and metabolic demands, such as temperature and oxygen levels (Meijering, 1972; Paul et al., 2004), and the number of offspring in the brood chamber (Ingle et al., 1937; Campbell et al., 2004). In our experiments, we controlled oxygen concentration, temperature, and body size. We hypothesized that as daphnid winter lipid reserves are diminished when infected by *P. laeve* (Johnson et al., 2006a; Forshay et al., 2008), there would be a corresponding increase of flow through the carapace, and we would observe an increase of the thoracic beat rate. This change would be required to increase food intake in order to renew lipid reserves. We also hypothesized that this increased activity would lead to higher heart rates. We tested these hypotheses by comparing the rates of three types of animals, uninfected ones, uninfected ones that are gravid, and infected ones. The inclusion of gravid females allowed us to investigate whether or not the infection affects the animal differently than carrying off-springs.

Materials and methods

Daphnia collection and preparation

Allequash Lake is a 172 ha lake located in Vilas County, Wisconsin (46.041°N, 89.628°W). It is one of eleven lakes in the North Temperate Lakes Long Term Ecological Research (LTER) program run by the University of Wisconsin- Madison. Detailed information on the physicochemical and biological features of this lake is available at <http://lter.limnology.wisc.edu>. In February 2006, during complete ice cover, zooplankters were collected using an 80 μm Wisconsin net from the deepest point in the lake. Zooplankton samples were diluted in 4-l bottles containing filtered lake water (Whatmann GF/F, 0.7 μm) and were brought back to the laboratory to gradually acclimate the animals to room temperature ($20 \pm 2^\circ\text{C}$) over a 24 h period in dim light. All samples were supplemented with a 1–3 mg C l⁻¹ of cultured alga (*Scenedesmus acutus*).

Animals were observed and measured under a dissection microscope with an ocular ruler calibrated

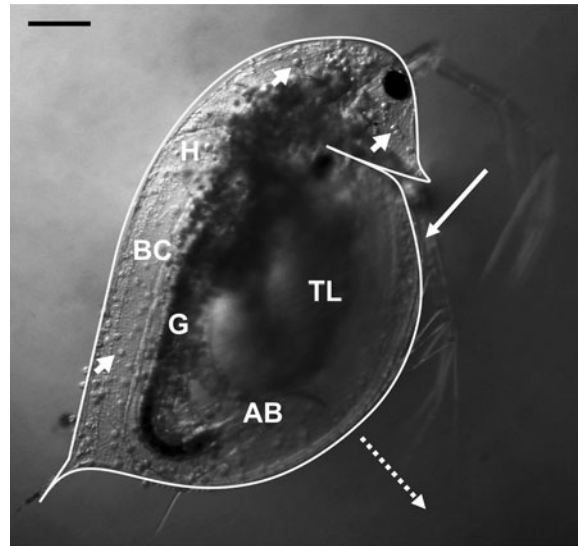


Fig. 1 A *Daphnia* infected with *P. laeve*. Note spots throughout body (short arrows parasitic spores/sporangia; Bar = 250 μm). H heart, TL thoracic limbs, BC brood chamber, G gut, AB abdominal claw. Path of thoracic appendage generated flow indicated by straight and dashed arrows

to the given magnification. Individual adult *D. pulex* were sorted according to sex, size, and infection status. Uninfected mature females were further sorted into non-gravid and gravid females. As the chytrid infection has a castrating effect, those females carrying eggs were considered uninfected. We selected females of similar size (>2 mm), and we used a semi-quantitative estimate of infection as in Johnson et al. (2006b) to sort out individuals with similar mid-level infections (1000–1500 sporangia ind⁻¹) (Fig. 1), and controlled the gravid females for the number of eggs in the brood chamber (10–20 eggs ind⁻¹). Body size was defined as the length from the base of the tail spine to the edge of the carapace directly above the eye by forming a straight line with the ocular micrometer.

Measuring thoracic beat and heart rates

Individuals were isolated and placed in filtered lake water prior to observation. All recordings of thoracic beat and heart rates were made within 72 h of collection. Ten individuals were selected from each group (infected, uninfected gravid and uninfected

non-gravid females). The females were tethered to a squirrel hair and suspended in a 3-l vessel containing only filtered lake water. Individuals were allowed to acclimate to their new condition for 30 min prior to observation. No food was added in order to assess the rates correlated to only the infection status. After recording their thoracic beat and heart rates, each individual was re-assessed for infection status and number of eggs.

The method for measuring the thoracic beat rate (Peñalva-Arana et al., 2008) is briefly described here. As *Daphnia* moves its thoracic appendages, it creates a feeding current. The smooth inflow is drawn into the carapace, delivering suspended particles and oxygen. On passing through the abdominal cavity the smooth flow is broken up into distinct packages due to the oscillatory movement of the thoracic appendages. These packages of water are expelled as a series of waves, and the frequency of these waves correlates with the beat frequency of the thoracic appendages. A carbon microelectrode is placed near the animal into the outflow current of the individual *Daphnia* to record the flux of a tracer that is added to the inflow current. The mean resting thoracic beat rate of each individual is determined by combining the average number of peaks generated by the thoracic appendages from 20 s recordings taken twice per minute over a 10-min period (Fig. 2). In our study, mean resting thoracic beat rate for each individual was calculated without prior knowledge of the host's infection status.

The heart rate was estimated from video tape recordings taken while we measured the thoracic beat rate. We used a horizontally placed digital camcorder (SONY) equipped with an optical tube attachment resulting in a 10 \times magnification. The heart rate of each individual *Daphnia* was recorded at 30 frames s⁻¹, and subsequently played back to calculate individual heart rate as the mean number of beats in 120 s intervals. The mean heart rate for each individual was calculated without prior knowledge of the subject's infection status.

The thoracic beat rate and heart rate differences among the three groups were assayed using one way analysis of variance followed by all pairwise multiple comparisons (post hoc testing), using the Holm-Sidak step down method ($P < 0.05$) (One-way ANOVA, SigmaPlot v.11, Systat Software).

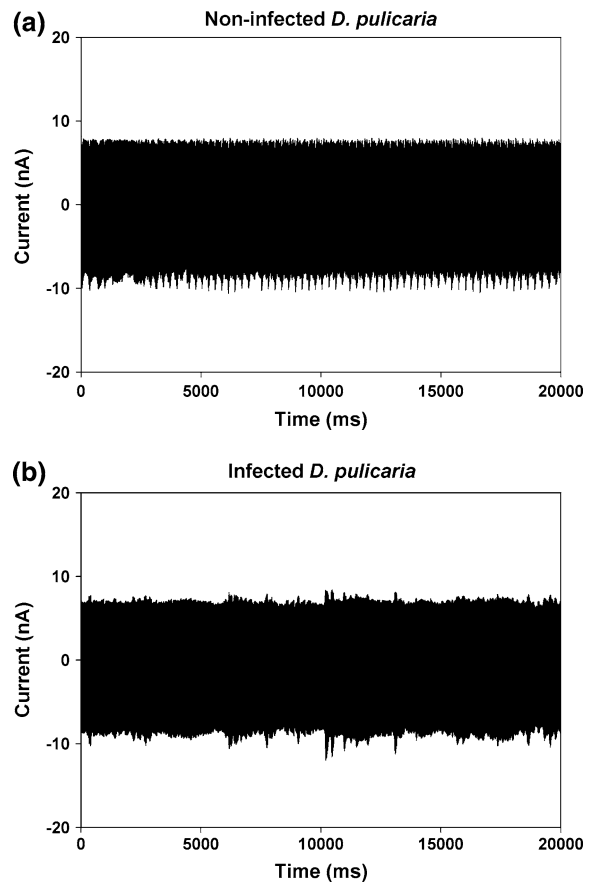


Fig. 2 Actual thoracic beat rate data collected using electro-chemical system. **a** Represents an uninfected non-gravid *D. pulicaria*'s individual filtering rate over 20 s, where each downward peak represents a wave generated by the thoracic limbs. **b** Same as in **a**, except for an infected *D. pulicaria*. Note the inconsistent rhythm and pattern of the outflow

Results

Mean size of the daphnids was 2.23 ± 0.16 mm ($n = 10$) for uninfected non-gravid, 2.32 ± 0.10 mm ($n = 10$) for gravid females, and 2.3 ± 0.014 mm ($n = 10$) for infected females (mean \pm SD). We did not find a statistically significant difference in size across treatments (ANOVA, $P = 0.274$, $F_{2,27} = 1.36$).

Thoracic beat rates

One way analysis of variance test between groups was significant for the thoracic beat rate data (One way ANOVA, $n = 10$, $P < 0.001$, $F_{2,27} = 16.15$),

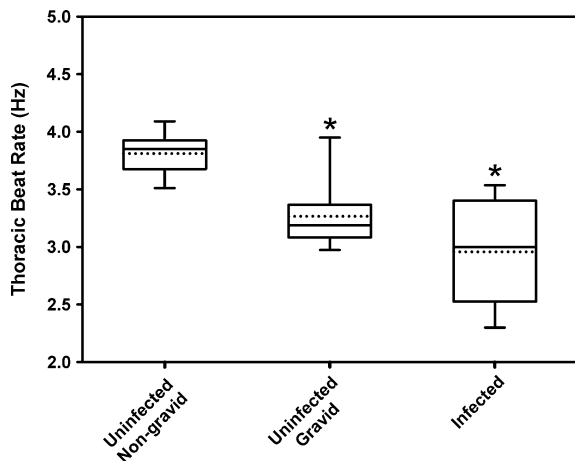


Fig. 3 Differences in thoracic beat rate according to occurrence of infection. The *boxes* are standard statistical box plots, with the box stretching from the 25th to 75th quartile, which identifies where 50% of the data lie, and a line at the median. The dotted line represents the mean. The *error bars* above and below each box reach to the maximum or minimum values or 1.5 times the size of the box, whichever is smaller. The *asterisks* represent the treatments that are statistically different than the control; in this case both non-infected gravid and infected daphnids (ANOVA, $df = 2$, $F_{2,27}$, Holm–Sidak $P = 0.003$ and $P < 0.001$, respectively)

and was followed by P values pairwise comparisons calculated by applying the Holm–Sidak pairwise multiple comparison step down method. Measurements following this method showed that the thoracic beat rate in the infected individuals was significantly lower (22% decrease) than in uninfected non-gravid individuals: 2.96 ± 0.47 versus 3.81 ± 0.18 Hz, respectively (Fig. 3, Holm–Sidak method, $P < 0.001$). However, the thoracic beat rate of infected individuals (all of which were non-gravid) did not significantly differ from that of uninfected gravid females (3.27 ± 0.30 Hz; Fig. 3, Holm–Sidak method, $P = 0.053$). A significant decrease (14%) in resting thoracic beat rates of uninfected gravid females was observed when compared with that of uninfected non-gravid females (Fig. 3, Holm–Sidak method, $P = 0.003$).

Heart rate

The one way analysis of variance test between groups was also significant for the heart rate data (One way ANOVA, $n = 10$, $P < 0.001$, $F_{2,27} = 49.37$), and

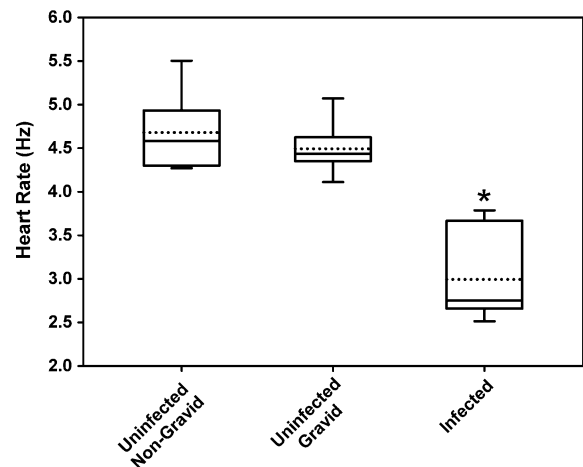


Fig. 4 Differences in heart rate according to occurrence of infection. The *boxes* are standard statistical box plots as explained in Fig. 3. The *asterisks* represent the treatment that is statistically different from the control; in this case only infected daphnids (ANOVA, $df = 2$, $F_{2,27}$, Holm–Sidak $P < 0.001$)

P values from pairwise comparisons were calculated by applying the Holm–Sidak step down method. The heart rate of uninfected non-gravid and gravid females did not significantly differ: 4.67 ± 0.42 versus 4.48 ± 0.28 Hz, respectively (average \pm SD) (Fig. 4, Holm–Sidak method, $P = 0.325$). However, average heart beat rate in infected daphnids (2.96 ± 0.47 Hz) was significantly lower (34–36%) than that of uninfected gravid and non-gravid females, respectively (Fig. 4, Holm–Sidak method, $P < 0.001$).

Discussion

We initially hypothesized that the resting thoracic beat and heart rate of infected daphnids would increase as they attempted to replenish their lipid reserves consumed by the parasite in the course of infection (Johnson et al., 2006a; Forshay et al., 2008). On the contrary, we found that the thoracic beat and heart rate are impaired by the *P. laeve* infection, leading to a corresponding decrease in both rates relative to uninfected non-gravid animals. On average, infected daphnids exhibited a 22% reduction in the thoracic beat rate and a 36% reduction in the heart rate when compared with uninfected non-gravid individuals. *D. pulicaria* females infected with

P. laeve exhibited asynchronous thoracic limb movements (as seen in Fig. 2b), which resulted in decreased thoracic beat rates when compared with uninfected non-gravid individuals. Although the reason for the decreased and asynchronous beat rate is unknown, we hypothesize that chytrid sporangia interfere with the mechanics of limb movement, and/or induce detrimental neurological effects, such as loss of muscle control or muscle degeneration. The possible presence of neurological effects was suggested for chytrid infected amphibians and daphnids, respectively (Johnson et al., 2006a; Retallick & Miera, 2007). As the infection spreads to different parts of the body, it may become progressively more difficult for daphnids to move and carry out their metabolic activity efficiently. *P. laeve* not only takes up space in the daphnids' body, which in itself offers a physical challenge, but it also eats away at the lipid reserves, and slowly degenerates both daphnid muscle and reproductive tissue (Johnson et al., 2006a). This inability to maintain a steady thoracic beat rate can lead to a reduction in the clearance rate, and therefore in feeding efficiency. This reduced feeding efficiency could explain the observed differences in stoichiometry (decreases in nitrogen and phosphorus) between uninfected and infected individuals (Forshay et al., 2008). In addition, with a reduced feeding current the supply of oxygen is reduced. Whether or not such reductions directly correlate with reduced growth, increased mortality, and behavioral changes will need further research.

Gravid females versus infected ones

One intriguing observation was the fact that there was no statistically significant difference between the mean thoracic beat rate of uninfected gravid and infected *Daphnia* (Fig. 3, Holm–Sidak method, $P = 0.053$). However, our results do show that thoracic beat rate is significantly different between gravid and non-gravid females (Fig. 2, Holm–Sidak method, $P = 0.003$). Therefore, we conclude that both, being chytrid infected and being gravid lead to a reduction in thoracic beat rate. In gravid females, this decrease in thoracic beat rate could be explained by the reduction in thoracic space, which is compressed when carrying a brood. The size of the brood then could have different effects on the mechanics of limb movement and affect the thoracic beat rate by

slowing it down. However, the pattern generated by the thoracic beat movements in gravid females is similar to, albeit slower, than the one of uninfected animals (Fig. 2a), while the pattern of the infected animals (Fig. 2b) is very different. Gravid females in other crustacean species, e.g., grass shrimp, are known to have reduced feeding and exhibit changes in foraging behavior (Guadagnoli & Reiber, 2005) and could explain our observed differences. However, one could also argue that the added volume of the developing embryos could alter metabolic demand on the individual, increasing oxygen and feeding requirements, and lead to an increase—not a decrease in thoracic beat rate. This observed reduction requires further investigation into *Daphnia* foraging and coping strategies, flow mechanics through the carapace, and how changes in the egg volume of gravid females affect their buoyancy and energetics.

Changes in heart rate

The heart rates of uninfected non-gravid and gravid females do not significantly differ. Meijering (1972) reported the same pattern while investigating the effect of temperature on the heart rate of *Daphnia* at different life stages. Meijering also demonstrated that heart rate decreased linearly with temperature decrease for various daphnid groups. As all our daphnids were acclimated to room temperature prior to observation, both heart and thoracic beat rates remained comparable across all treatments, and differences between them are attributed to infection and fecundity.

Further considerations

Research on the interaction between *Daphnia* and their parasites offers both short- and long-term insights into the consequences of infection. Ideally, studies of disease need to consider both the lethal as well as sublethal effects on hosts in order to understand the influence of an infection on the population and ecosystems dynamics. As stated previously, once infected, a daphnid's winter lipid reserves are diminished by the parasitic infection (Forshay et al., 2008). In addition, *P. laeve* is an "obligate-killer" parasite that can only spread once the host is killed (Johnson et al., 2006a). Considering

the timing of infections, *P. laeve* is most prevalent during winter months when water viscosity is higher due to colder temperatures; this higher viscosity requires more energy expenditure to produce a current through the carapace (Koehl & Strickler, 1981; Vogel, 1994). Therefore, winter's increased energy demands coincide with the demands of the infection—a recipe for disaster. At the ecosystem level, in early spring when incidence of disease reaches as high as 80%, it leads to a population crash by March, a time when predation is low and algae are abundant (Johnson et al., 2006a). Thus, infection does not allow the daphnid population to take advantage of these optimal conditions for survival, and thus the population growth will shift to later in the year. In summary, *P. laeve* affects *Daphnia* three ways, sub-lethal, lethal and long term.

Acknowledgments This manuscript was a work in progress and had been revised by SI Dodson prior to his untimely death. As such, we dedicate this manuscript to his memory, great work and everlasting inspiration. Stanley was a great friend, mentor and scientist. We also thank four anonymous reviewers and J. Barker, N. Keith, R. Sommer, C. Trincot for their helpful comments.

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