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Relative helminth size in crustacean hosts: *in vivo* determination, and effects of host gender and within-host competition in a copepod infected by a cestode

Claus Wedekind[1], Mira Christen[2], Lukas Schärer[3] and Nathalie Treichel[4]
Abteilung Verhaltensökologie, Zoologisches Institut, Universität Bern, CH-3032 Hinterkappelen, Switzerland; Present addresses: [1] *Institute of Cell, Animal and Population Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, Scotland, UK (Fax: ++44 131 650 65 64; E-mail claus.wedekind@ed.ac.uk);* [2] *Max-Planck Institute of Limnology, Department of Evolutionary Ecology, 24306 Plön, Germany;* [3] *Université Pierre et Marie Curie, Laboratoire d'Ecologie - UMR 7625, 7 Quai Saint Bernard - Case 237, F-75252 Paris Cedex 05, France;* [4] *Institut für Biochemie, ETH-Zentrum, CH-8092 Zürich, Switzerland*

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Abstract

Crustaceans are important hosts for a number of helminth parasites, and they are increasingly used as models for studying the physiology, ecology and evolution of parasite-host interactions. In ecological studies, this interaction is commonly described only in terms of prevalence and number of larvae per infected host. However, the volume of helminth parasites can vary greatly, and this variation can potentially give important insights into the nature of a parasite-host relationship. It may influence and be influenced, for example, by within-host competition, host size, growth, and life history. Here we present a simple method that allows rapid approximation of the absolute and relative volumes of cestode larvae within copepod hosts of various developmental stages (nauplii, copepodites and adults). The measurements are taken *in vivo* without much disturbance of the animals, i.e. the technique allows study of growth and development of the parasites in relation to that of their hosts. The principles of this technique can be adopted to other helminth parasites and other crustacean hosts. Using this method in the copepod *Macrocyclops albidus* infected with the cestode *Schistocephalus solidus*, we found that the relative parasite size (= 'parasite index') ranged from 0.5% to 6.5% of host size 14 days after infection. It was greater in male than in female hosts. With increasing number of parasites per host, the total parasite volume increased while the mean volume of the individual parasites decreased. The magnitude of the observed parasite indices, the large variation that was found within a sample of 46 infected adult copepods, and the observed correlates suggest that this new index can indeed be an important measure of parasite success and its pathogenecity.

Introduction

Many helminth parasites and many of their crustacean hosts can be kept and studied in the laboratory under fairly controlled conditions. This is a reason why these taxa are frequently used as models for studying co-evolutionary processes and physiological or ecological aspects of parasite-host interaction. Some recent examples include the works of Dupont & Gabrion (1987), Poulin et al. (1992), Nie & Kennedy, (1993), Pasternak et al. (1995), Urdal et al. (1995), Ashworth et al. (1996), Wedekind & Milinski (1996), Bakker et al. (1997) and Wedekind (1997). Despite the fact that many helminths can grow relatively large in their copepod hosts (e.g., Clarke, 1954; Guttowa, 1961; see examples below), most studies on helminth infection in crustaceans only report the prevalences or the number of parasites per individual host as measures of

parasite load. The few studies that include measures of parasite size and growth mostly use only relative measures (e.g., length of a cestode procercoid) instead of absolute size (e.g., procercoid volume) that could be compared to the absolute size of the host. This is in contrast to studies on helminth infection in vertebrate hosts where size and growth of the parasite is often studied in relation to host size (as, e.g., in *Schistocephalus solidus* infecting three-spined sticklebacks, see Clarke, 1954; Meakins & Walkey, 1973). This paper describes a method that allows to study growth and development of the procercoid larvae in relation to host size and development. The method is demonstrated with the pseudophyllidean cestode *Schistocephalus solidus* infecting its first intermediate host, the cyclopoid copepod *Macrocyclops albidus*. The principles of this method can be applied to many other helminth parasites and host species. We further report examples of actual parasite indices in male and female *M. albidus* that indicate the importance of size measurement in our model species.

Methods

The animals

Copepods of the species *Macrocyclops albidus* were used. They originally stem from a pond in Bielefeld (Germany) and are maintained in laboratory cultures following techniques described by Orr & Hopkins (1969). To collect the parasites, naturally infected three-spined sticklebacks (*Gasterosteus aculeatus*, the second intermediate host of this parasite) were caught from a pond in Bochum (Germany) in early summer and brought to the laboratory. The plerocercoids (*Schistocephalus solidus* larvae in their fish host) were aseptically removed from the fish and put into an *in vitro* system that simulates the final host (a fish eating bird). The technique used is modified from Smyth (1954) and described in detail in Wedekind (1997). After a few days in the *in vitro* system, eggs were collected and kept at 20 °C until they hatched. Before infestation, several hundred copepods of different stages and without egg sacs were filtered from the culture tanks and transferred singly to wells of ELISA-plates (water volume ca. 2 ml). Six coracidia (= free swimming first larva of *S. solidus*) were caught with a micropipette and added to each individual copepod. Copepods were fed one day after exposure and thereafter every two or three days with freshly hatched *Artemia salina*.

Measurements of the copepods and conversion factors

Twenty-seven nauplii of the first five nauplius stages, six female copepodites in their fifth copepodite stage, eight adult female copepods and eight adult males were each anaesthetised with carbonated water, transferred with a glass pipette to a glass slide with little water and measured under a compound microscope. The glass pipette we used had an enlarged opening to avoid damage to the copepod. The microscope was connected to a video camera via a c-mount. The camera was connected to the built-in frame grabber of a Macintosh Quadra 840AV. Pictures of the copepods' lateral and dorsal view (see Figures 1 and 2) were taken and analysed using the public domain NIH Image software (developed at the U.S. National Institute of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

To estimate the body volume of the copepodites and the adult copepods, their shape was simplified to one large ellipsoid plus one ellipsoid cylinder plus one truncated cone plus two truncated cones (Figure 1b). The corresponding 12 measurements of the copepods (Figure 1b) were taken to the nearest 0.01 μm. This way, the body volume of the simplified copepodite and adult was calculated by the formula

$$V = \frac{4\pi abc}{3} + h_1\pi r_1 r_2 + \frac{h_2\pi}{3}(r_3^2 + r_4^2 + r_3 r_4) + 2\frac{h_3\pi}{3}(r_5^2 + r_6^2 + r_5 r_6). \quad (1)$$

All the measurements taken on the copepodites and the adults correlated well with each other (mean Pearson's correlation coefficients: $r = 0.78$, range = 0.28–0.99, $n = 22$, mean $p < 0.001$). The Pearson's correlation coefficients between the four largest measurements, $2a, 2b, 2c$ and h_2 (see Figure 1), i.e., the measurements that are expected to have the smallest relative measurement error and therefore to have the strongest influence on the calculation of the volume, ranged from $0.90 < r < 0.97$ ($n = 22$, p always $\ll 0.001$).

The largest length measured, i.e., the distance from the base of first antenna to the end of the 4th thoracic segment (Figure 1c), turned out to be a good predictor of copepod volume as approximated here (Figure 3a). Copepod volume was also highly correlated to the next three largest measurements that were taken from the lateral view ($0.93 < r < 0.98$, p always $\ll 0.001$). Therefore, the volume estimation was simplified by measuring only the length as indicated in Figure 1c and then converting this measure by the formula

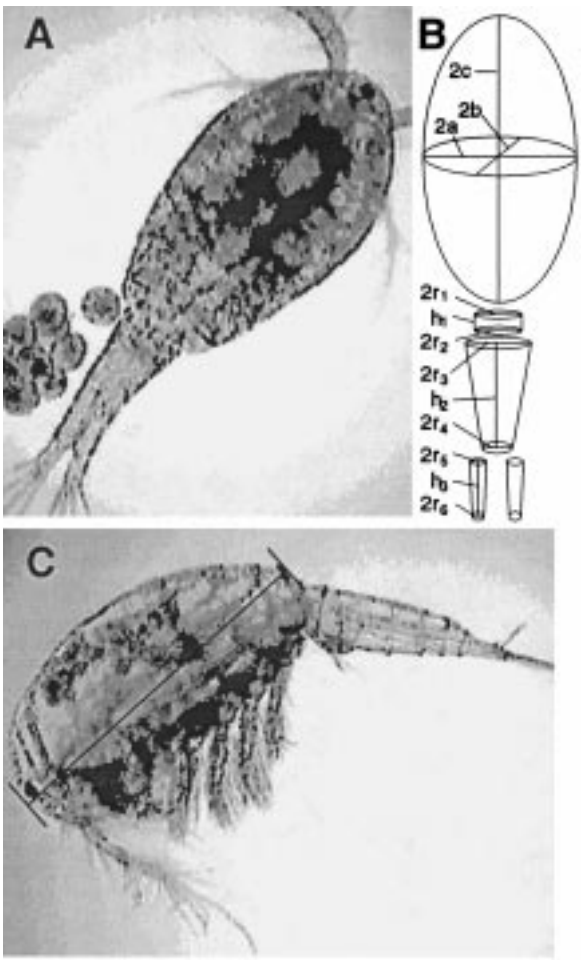

Figure 1. Video pictures of an adult female *M. albidus* and simplified bodies for the approximation of copepod volume. (A) Dorsal view of a female (with some eggs); (B) the geometrical bodies used to simplify the copepod's shape (see text and equation 1) (C) lateral view with length measurement used to estimate the copepod's body volume (see equation 2 in text). The little white cross indicates the ventral point that was used to estimate body depth.

$$\text{copepodite or adult volume [mm}^3\text{]} = e^{-4.485} \times \text{length [}\mu\text{m]}^{\,3.383} \times 10^{-9}. \quad (2)$$

The exponents of Equation (2) are taken from the equation of the regression line in Figure 3a.

The body volume of nauplii (Figure 2a) was simplified to a flattened ellipsoid (Figure 2c; $V = 4\pi abc/3$). NIH Image allows measurement of areas by encircling the area of interest with the mouse. The program then automatically reports the long and the short axes of an ellipse that approximates the measured area best. The first two axes of this flattened ellipsoid were therefore obtained by measuring the

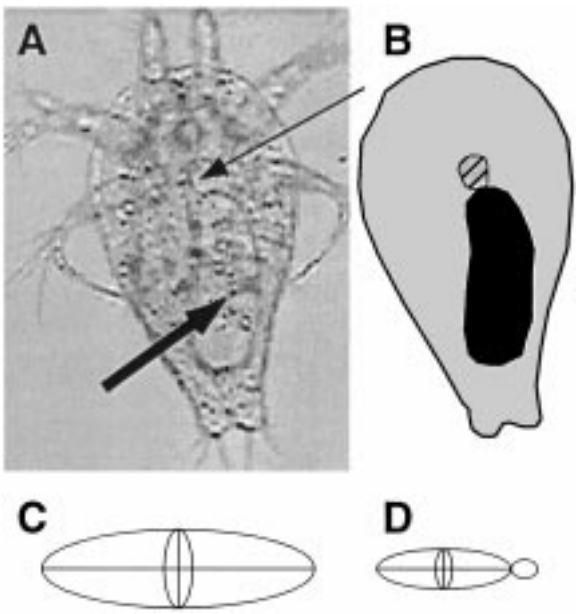

Figure 2. (A) Video picture of an infected *M. albidus* nauplius. The thick arrow points to the procercoid, the thin arrow to its cercomer. (B) The area of the nauplius (grey and black) and of the procercoid without its cercomer (black only) can be used to estimate the body volume of the nauplius (see Equation (3) in text) and of the procercoid, respectively (see Equations (4) and (5) in text). The location of the cercomer is indicated by the hatched area. (C) The bodies of nauplii were simplified to a flattened ellipsoid for determination of the volumes, and (D) the procercoids were simplified to two ellipsoids.

area of the nauplii's bodies (without extremities) as seen in the ventral or dorsal view in the microscope (Figure 2b). The third axis of the flattened ellipsoid was measured from pictures of the lateral view.

The area of the nauplii's bodies (without extremities) as seen in the ventral or dorsal view was used as a predictor of the nauplius volume by the formula

$$\text{nauplius' volume [mm}^3\text{]} = e^{0.135} \times \text{area [}\mu\text{m}^2\text{]}^{\,1.393} \times 10^{-9}. \quad (3)$$

The exponents in Equation (3) were taken from the equation of the regression line in Figure 3b.

Measurements of the procercoids and conversion factors

Copepods that we had exposed to the parasites were examined for infection under the microscope. Pictures of 70 procercoids were taken (as in Figure 2a) and measured with NIH Image. To estimate the volume of the procercoids, their form was simplified to a large and a small ellipsoid (Figure 2d; $V = 4\pi ab^2/3$). The area of the longitudinal section of the procercoids'

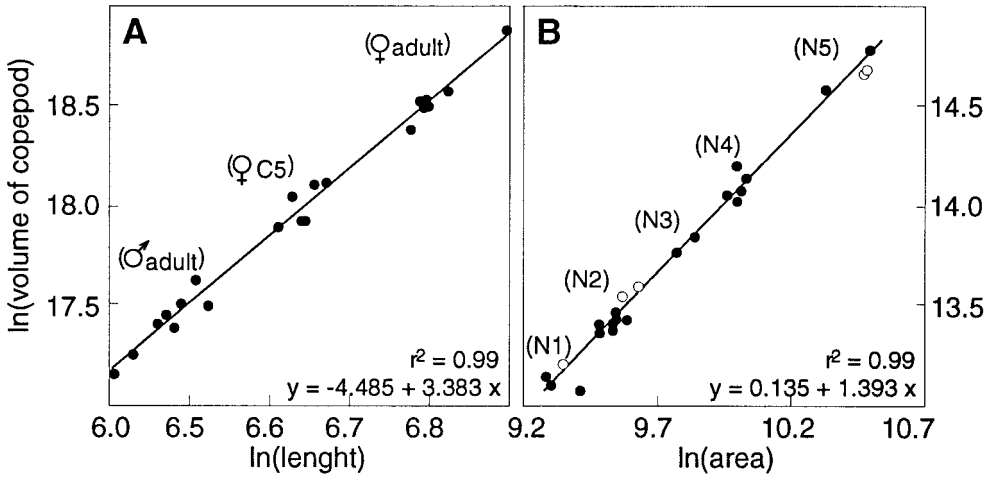

Figure 3. Relationship between (A) the length measurement (see Figure 1c) and the determined body volume of stage five female copepodites, adult males and adult females, (B) the area of nauplii (see Figure 2b) and their determined volume. The figures also give the r^2 and the equations for the regression lines. The sizes of the copepods typically cluster according to the larval stage and the sex of the copepods, as indicated in the graphs, 'C5' means fifth copepodite stage, 'N1–N5' means the first five nauplius stages (five nauplii are included that were not classified for certain, indicated as open points).

body and cercomer was measured as described for the estimation of nauplius volumes (the cercomer is a structure that forms around the time when procercoids become infective to the fish). The two axes of the approximated ellipsoid that the program provided were used to calculate the volumes of the two ellipsoids.

Procercoid volume and cercomer volume correlated well (Figure 4a). Therefore, the estimation of the volume of a procercoid was simplified by measuring only the maximal area of the longitudinal section of its body as seen in the microscope. This measurement was converted by the formula

$$\text{procercoid volume } [\text{mm}^3] = e^{0.533} \times \text{area } [\mu\text{m}^2]^{\,1.363} \times 10^{-9}, \quad (4)$$

if the procercoid had developed a cercomer (see also Figure 4b). For procercoids that had not yet developed their cercomer the formula was

$$\text{procercoid volume } [\text{mm}^3] = e^{0.279} \times \text{area } [\mu\text{m}^2]^{\,1.385} \times 10^{-9}. \quad (5)$$

The approximated parasite and host volumes can be used to calculate a 'parasite index', which is the percentage of the volumes of all parasites in a copepod relative to the body volume of its host.

Repeatability of the measurements

For estimating the repeatability of our measurements we used a new sample of infected *M. albidus*. We measured the representative length indicated in Figure 1c to determine the copepod volumes with Equations (2) of eight adult females, seven adult males, and seven females in the fifth copepod stage. The representative area indicated in Figure 2b and Equation (3) was used to measure the volumes of six nauplii in the second or the third nauplius stage. We further determined the number of procercoids and the mean procercoid volume per copepod (with Equations (4) and (5)). Within two days, each of the four authors took these measurements directly from the living animals, i.e., not from stored video images, and without knowing the measurements of the others. The measurements were highly correlated, which indicates that our method was repeatable: the mean Pearson's correlation coefficient of all pairwise comparisons ($n = 6$) for the determined copepodit and adult volumes was: $r = 0.99$ (range 0.99–1.0, $n = 22$, p always $\ll 0.001$); for the volume of the nauplii: $r = 0.93$ (range 0.83–0.98, $n = 6$, p always < 0.01); for the mean procercoid volume as measured *in vivo*: $r = 0.93$ (range 0.86–0.97, $n = 9$, p always < 0.001); for the number of procercoids per copepod: Spearman's $r_s = 0.97$ (range 0.94–0.99, $n = 28$, p always $\ll 0.001$); and for the parasite index of the infected copepods, measured *in vivo*: $r = 0.91$ (range 0.83–0.97, $n = 9$, p always < 0.002).

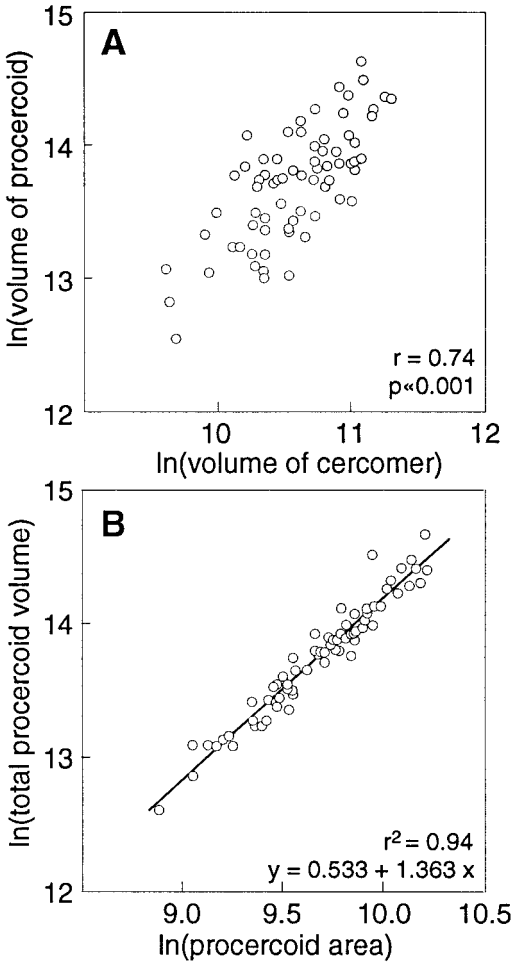

Figure 4. (A) Correlation between volume of the cercomer and volume of the procercoid body without cercomer. (B) Relationship between the area of the procercoid as seen in the microscope (without cercomer) and the determined volume of the total procercoid (including cercomer). The equation describes the regression line.

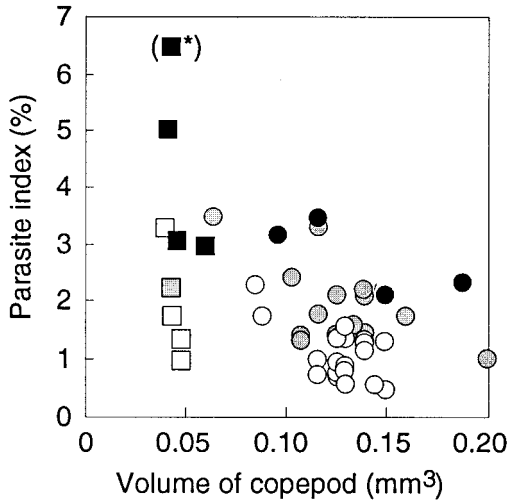

Figure 5. Parasite indices (i.e., the total volume of all parasites as a percentage of copepod volume) of adult *M. albidus* 14 days after infection in males (squared symbols) and females (round symbols), plotted against copepod volume. Copepods contained either one (open symbols), two (grey symbols), three (black symbols) or six procercoids (black symbol marked with an asterisk). See text for statistics.

Examples of parasite indices, sex effects and effects of parasite number

We exposed another sample of copepods to coracidia of *S. solidus* and found 14 days after exposure nine adult male and 37 adult female copepods to be infected with one to six procercoids. All these proceroids were infective for the next host, i.e. they had developed a cercomer (Clarke, 1954). The mean parasite volume (i.e., the total volume of all parasites per copepod divided by their number) ranged from 0.00046 mm^3 to 0.00204 mm^3 and decreased with increasing number of parasites per host, i.e., the individual parasites were smaller in multiple infections (Spearman's rank correlation coefficient $r_s = -0.271$, $p < 0.05$, directed, see Rice & Gaines, 1994). The parasite index of the copepods, i.e. the volume of all their parasites as a percentage of copepod volume, ranged from 0.5% to 6.5% (Figure 5). It increased with increasing number of procercoids per infected host ($r_s = 0.734$, $p \ll 0.001$).

The sexes did not differ significantly in the number of procercoids per infected host (Mann–Whitney $U = 127$, $p = 0.23$). However, the volume of the parasites was larger in females than in males (Mann–Whitney U-tests on mean procercoid volume: $U = 309$, $p < 0.001$; on total procercoid volume per copepod: $U = 239$, $p = 0.045$, two-tailed). This may be because the mean size of the male copepods (mean volume = 0.046 mm^3, SE = 0.002, $n = 9$) was 2.76 times smaller than the mean size of the females (= 0.127 mm^3, SE = 0.004, $n = 37$). Nevertheless, the males appeared to suffer more from the infections as they had higher parasite indices than the females (Mann–Whitney U-test, $U = 80$, $p = 0.017$).

Discussion

The size of helminth parasites within their hosts may be a crucial measure in studies of the physiology and ecology of a parasite-host interaction. The simple method developed here allows to study cestode growth

in relation to copepod growth and development. The approximated parasite and host volumes can be used to calculate a parasite index. The parasite indices we observed in our sample of adult copepods did not reach the high values that are possible in three-spined sticklebacks infected with *S. solidus* where parasites can reach nearly twice the net weight of their fish host (Clarke, 1954; C. Wedekind, personal observation). Nevertheless, the observed indices were substantial, and varied over a large range. This suggests that this index can be an important measure of parasite success and pathogenecity. It therefore adds to the ones that are commonly used in ecological and evolutionary studies, namely the prevalence and the number of parasites per host.

The procercoid volume has been described as difficult to determine (Clarke, 1954; Nie & Kennedy, 1993). However, with a microscope connected to a normal desktop computer via a video system, and with the appropriate software (e.g., NIH Image which is available on the Internet for no cost), it is comparatively easy to get good measures of procercoid size. Moreover, the measurement techniques and the conversion factors described here allow for approximation of the body volumes of the parasites and the copepods by taking only one simple measurement each. Because these representative measurements are relatively large ones, the influence of measurement error is minimized, as suggested by our analyses of repeatability. The disturbance of the animals during *in vivo* measurements is also minimized since animal handling time can be relatively short (around half a minute) for the described techniques. This allows for repeated measurements on the same individuals and opens the possibility to study aspects like parasite growth, within-host competition, host growth in response to parasite growth, the severity of infection ('parasite index') which is likely to affect aspects like host anti-predator behaviour (Poulin et al., 1992; Wedekind & Milinski, 1996), host life history decisions (as discussed in Wedekind & Jakobsen, 1998) or hunger-induced micro-habitat choice (Jakobsen & Wedekind, 1998).

The copepod volumes as determined here are estimates that exclude the volume of legs and antennae. Therefore, they are likely to be slight underestimates of their actual values. Legs and antennae are difficult to measure, but we assume that they represent only a few per cent of the copepod body (see Figure 1). Therefore, our method may be sufficient for practical comparative purposes.

Recently, Barber (1997) described a method that allows the *in vivo* quantification of cestode plerocercoid burden in fish. He demonstrated the method using *S. solidus* infecting its second intermediate host, the three-spined stickleback, but the method is likely to be useful in other host-parasite systems, e.g., *Ligula intestinalis* in Cyprinidae. By combining Barber's (1997) and our methods, sequential parasite burdens of individual cestodes could potentially be followed over time.

Callot & Desportes (1934) found as many as 60 procercoids in a single *Cyclops viridis*. They did not determine the procercoids' volumes, but from their drawings we estimated the volumes of their infectious procercoids to range between 5×10^4 and 10×10^4 μm^3 (i.e., a bit larger than eggs of *S. solidus*, see Wedekind et al., 1998, for egg sizes). Since this is much smaller than the procercoid sizes we found here in copepods that contained only one to six procercoids, Callot & Desportes' observation corresponds well to our finding that procercoids are increasingly smaller with increasing number of competitors.

Wedekind & Jakobsen (1998) tested for possible gender effects in susceptibility to *S. solidus*. They found that the prevalence and the number of procercoid per infected copepod was higher in male than in female *M. albidus*. With the new measurement techniques described here, we found that males had on average a higher parasite index than females, although the number of parasites per infected copepod was not significantly different between the sexes in the present sample. If the parasite index correlates with the pathogenecity of a helminth infection, male *M. albidus* suffer more from infection than females. This example also shows the potential importance of size determinations in studies of helminth parasites.

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