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Journal Article**Author(s):**

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Publication date:

2009

Permanent link:

<https://doi.org/10.3929/ethz-b-000021580>

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Originally published in:

Parasitology 136(8), <https://doi.org/10.1017/S0031182009006180>

Acanthocephalan size and sex affect the modification of intermediate host colouration

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(Received 5 January 2009; revised 10 February, 10 March and 16 March 2009; accepted 16 March 2009; first published online 19 May 2009)

SUMMARY

For trophically transmitted parasites, transitional larval size is often related to fitness. Larger parasites may have higher establishment success and/or adult fecundity, but prolonged growth in the intermediate host increases the risk of failed transmission via natural host mortality. We investigated the relationship between the larval size of an acanthocephalan (*Acanthocephalus lucii*) and a trait presumably related to transmission, i.e. altered colouration in the isopod intermediate host. In natural collections, big isopods harboured larger worms and had more modified (darker) abdominal colouration than small hosts. Small isopods infected with a male parasite tended to have darker abdominal pigmentation than those infected with a female, but this difference was absent in larger hosts. Female size increases rapidly with host size, so females may have more to gain than males by remaining in and growing mutually with small hosts. In experimental infections, a large total parasite volume was associated with darker host respiratory operculae, especially when it was distributed among fewer worms. Our results suggest that host pigment alteration increases with parasite size, albeit differently for male and female worms. This may be an adaptive strategy if, as parasites grow, the potential for additional growth decreases and the likelihood of host mortality increases.

Key words: Acanthocephala, *Asellus aquaticus*, cystacanth, host exploitation, host phenotype manipulation, intermediate host, larval life history, sexual dimorphism, trophic transmission.

INTRODUCTION

For trophically transmitted parasites, infectivity to the next host in the life cycle is only achieved at a certain developmental stage. Consequently, any parasite traits related to transmission, such as manipulation of host phenotype (reviewed by Moore, 2002; Thomas *et al.* 2005), should be expressed only after some degree of infectivity is achieved (e.g. Bethel and Holmes, 1974; Poulin *et al.* 1992; Pulkkinen *et al.* 2000; Seppälä *et al.* 2005; Franceschi *et al.* 2008). However, developing to an infective stage does not necessarily indicate that the probability of parasite establishment in the next host is at a fixed level. Invasion success typically varies among infective-stage individuals with larger parasites often faring better (Rosen and Dick, 1983; Steinauer and Nickol, 2003). Large larval parasites may also have other fitness advantages, such as a shorter developmental time to maturity or higher adult fecundity (Parker *et al.*

2003; Fredensborg and Poulin, 2005). Prolonged larval growth, however, has associated costs, such as an increasing likelihood of natural host mortality and thus failed transmission. This trade-off between transitional size and age is the basis for many models on life-cycle evolution (Rowe and Ludwig, 1991; Stearns, 1992; Berrigan and Koella, 1994; Abrams *et al.* 1996; Day and Rowe, 2002; Iwasa and Wada, 2006). If a large larval size is very advantageous, then continued growth may be worth the risk, perhaps even after parasites have reached an infective stage. Under these conditions, delayed host manipulation may be a favourable strategy.

In this study, we investigated the relationship between the larval size of an acanthocephalan (*Acanthocephalus lucii*) and the alteration of intermediate host colouration. Freshwater fishes are the definitive hosts of *A. lucii*, usually European perch (*Perca fluviatilis*). Parasites mate in the fish's intestine and eggs are released into the environment with the host's faeces. Intermediate hosts, freshwater isopods of the species *Asellus aquaticus*, become infected by ingesting eggs. Parasites develop in the body cavity of isopods for several weeks before they reach the infective cystacanth stage (Andryuk, 1979). Post-infectivity larval size can vary considerably in

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Table 1. Naturally collected isopods used in the analysis

(The isopods were collected in different seasons and exposed to different treatments in the laboratory.)

Block	Collection date	N (infected)	Experimental treatment	Reference*
1	Sept. 2005	148 (58)	Approximately 1 week at 17 °C, 18 h light	1
2	Sept. 2005	84 (42)	Less than 1 week at 17 °C, 18 h light	1
3	Oct. 2005	69 (27)	Eight weeks observation at 17 °C, 18 h light	1
4	May 2006	40 (16)	Two weeks observation at 17 °C, 18 h light	2
5	Aug. 2006	55 (29)	Two weeks observation at 17 °C, 18 h light	2
6	Oct. 2006	68 (31)	Two weeks observation at 17 °C, 18 h light	2
7	Aug. 2006	52 (26)	Four weeks acclimation to 17 °C, 18 h light, then 2 weeks observation under same conditions	2
8	Aug. 2006	55 (28)	Four weeks acclimation to 11 °C, 12 h light, then 2 weeks observation under same conditions	2
9	Aug. 2006	47 (21)	Four weeks acclimation to 5 °C, no light, then 2 weeks observation at 17 °C, 18 h light	2

* 1, Benesh *et al.* (2008); 2, Benesh *et al.* (2009).

A. lucii; among female cystacanths there can be more than a 2-fold difference between the smallest and largest individuals (Benesh and Valtonen, 2007c). Much of this variation is explained by isopod size; larger hosts harbour larger worms (Benesh and Valtonen, 2007b,c). Therefore, parasites in small isopods may have a lot to gain by remaining in the host and continuing to grow mutually with it (Benesh and Valtonen, 2007a,b). The potential payoffs of remaining in isopods likely differ between parasite sexes, though. Female cystacanths are larger than males and their size increases faster as a function of isopod size, indicating that females allocate more of the available resources to growth (Benesh and Valtonen, 2007c). This suggests that a large transitional size is more important for females than males, possibly because fecundity increases with female size, whereas male reproductive success may only increase with body size under specific forms of competition (Stearns, 1992). Thus, delaying the expression of transmission-relevant traits until a larger size is reached may be particularly profitable for female worms.

As *A. lucii* reaches the cystacanth stage, the respiratory operculae of their hosts become darkly pigmented (Bratney, 1983), and this renders the overall abdominal pigmentation of infected isopods darker than that of uninfected isopods (Benesh *et al.* 2008). Conspicuous isopods are likely to be eaten by fish (Hargeby *et al.* 2004, 2005), so the modified colouration of infected isopods presumably increases their predation risk (Bratney, 1983; Seppälä *et al.* 2008). Although altered host colouration appears related to parasite transmission, a direct link has not been established. This caveat deserves mention because a recent study found no relationship between intermediate host appearance and predation risk in a different acanthocephalan (Kaldonski *et al.* 2009). Here we test (1) if there is a relationship between parasite

size and host colouration and (2) whether this pattern differs between male and female parasites. The main analysis was conducted with naturally infected isopods. However, natural infections can be problematic, as infection is not a randomly assigned treatment. Thus, the relationship between colouration and parasite size was also investigated in a group of experimentally infected isopods.

MATERIALS AND METHODS

Naturally infected isopods

Isopods were collected in 2005 and 2006 from Lake Jyväsjärvi, Central Finland (62°14'N 25°44'E) for use in various other experiments (Table 1). At the end of all experiments, live isopods were frozen in lake water at −20 °C. At a later date, isopods were thawed and individually photographed with a Nikon Coolpix 4500 digital camera (light conditions and camera settings were described by Benesh *et al.* (2008)). After photographs were taken, isopods were measured to the nearest 0.5 mm and then dissected to determine infection status (presence/absence of *A. lucii*; number and sex of cystacanths). Worms reach an advanced state of development in isopods, so their sex can be easily established based on whether there are testes or ovarian balls in the body cavity. Cystacanths were placed in refrigerated tap water to relax and extend. The length and width of all cystacanths were measured to the nearest 0.01 mm using an ocular micrometer on a light microscope. Worms were considered cylindrical in shape, so cystacanth volume (mm³) was calculated with the equation $(\pi lw^2)/4$ where l is worm length and w is worm width. Additionally, a subsample of worms ($n=116$) were dried at 60 °C for 3–4 h and then weighed to the nearest μ g on a microbalance (Sartorius, SE MA 2.1 g).

Host size – parasite size relationship

Using a subset of the isopods listed in Table 1, Benesh and Valtonen (2007c) showed that, in single-cystacanth infections, the slope of the correlation between cystacanth size and isopod size was higher for female worms than for males. Because this growth pattern is a major assumption for our hypothesis of sexually-divergent manipulation strategies, the relationship between host size and parasite size was checked using all the collected isopods that harboured a single cystacanth. An analysis of covariance (ANCOVA) was performed with parasite volume as dependent variable, worm sex as a fixed factor, and isopod size as a covariate. A second ANCOVA using parasite dry mass instead of volume was also performed. Mass and volume measurements need not give identical results, e.g. if sexual organs (testes *vs* ovarian balls) have different weights.

Analysis of isopod colouration

Photographs of whole isopods were analysed using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, USA). The analysis of whole-isopod photographs was described previously (Benesh *et al.* 2008). Briefly, all pictures were converted to grey-scale and reflectance values for the first, fourth, and seventh segments were averaged to give a mean value for body pigmentation. A reflectance value for the abdomen was also calculated. The scale of reflectance in the software ranged between 0 (black, 100% saturation) and 255 (white, 100% reflectance). Histograms of reflectance of individual pixels within the analysed areas resembled a normal distribution, so the mean value of reflectance from each area was taken as a measure of colouration. Reflectance values ranged from 41.2 to 126.2 for body colouration and 32.5 to 142.3 for abdominal colouration. This method was highly repeatable (Benesh *et al.* 2008).

Isopods were either uninfected ($n=340$), infected with a single male cystacanth ($n=135$), or infected with a single female cystacanth ($n=143$). A number of isopods harboured 2 or more cystacanths ($n=44$). These multiply infected isopods tended to be larger than average (one-sample *t*-test against overall mean isopod size, $t_{43}=4.54$, $P<0.0001$). As the upper portion of the host size distribution was over-represented, size by colouration correlations for multiply infected isopods may not be comparable to singly infected and uninfected isopods, so they were excluded from the analysis. Also, the few isopods harbouring small, uninfected parasites ($n=11$) were excluded from the data.

The first and main statistical analysis compared the colouration of uninfected and infected isopods (split by worm sex). Both isopod body and abdominal colouration were evaluated with ANCOVA. Infection status and isopod sex were fixed factors and

isopod size was used as a covariate. Because isopods were collected at different times and kept under different lab conditions, an experimental 'block' factor was also included in the model (see Table 1). Interactions between 'block' and other factors were not assessed because of the low sample sizes for some subgroups in some blocks (i.e. there were too few female isopods, too few isopods with a male cystacanth, etc.). However, Benesh *et al.* (2009) found that the effect of infection on colouration did not vary with season or light/temperature treatment, suggesting that any interaction effects are weak. All other interactions were initially included in the model. Non-significant interactions were sequentially removed to reduce model complexity.

A second, subanalysis assessed whether parasite size affects host colouration, and thus involved only infected isopods. The most parsimonious ANCOVA models (i.e. with non-significant interactions removed) from the first analysis were taken as a basis for the second analysis. Parasite size was added as a covariate to these base models and its main effect and interaction effects were checked. This analysis, therefore, assessed whether parasite size explains any additional variation in isopod colouration not covered by the main model.

Experimental infection and opercular colouration

To check the validity of the results obtained with naturally infected isopods, colouration changes were also observed in experimentally infected isopods. Isopods were collected in August 2005 with a dipnet from Niemijärvi, a small pond in central Finland (62°12'N 25°45'E) in which the only fish species present is *Carassius carassius*, the crucian carp. Thus, all isopods were uninfected because the definitive host of the parasite is not present in the pond. Isopods were either exposed to fish faeces containing parasite eggs or sham-exposed with distilled water (the details of this infection have been reported by Benesh and Valtonen (2007a)). Isopods were observed for 101 days before any remaining animals were killed and dissected. Nearly all exposed isopods were infected with multiple parasites, and the number and size of all parasites were recorded from each isopod. The average infection intensity for the isopods used in this analysis was 17.24 (S.D. ± 8.35). For most of the isopods that died 75 days or more post-exposure, the respiratory operculae were collected and stored in 70% EtOH. Opercula were dehydrated through an EtOH series, and then mounted, ventral side up, on microscope slides in Euparal medium. The opercula were photographed at 40 $\times$ magnification using a Nikon Coolpix 4500 camera (scene mode: close up, focal length: 96 mm, aperture: F5.4, shutter speed: 1/500, sensitivity: ISO100, image size: 1600 $\times$ 1200 pixels, image quality: fine, focus mode: auto) attached to a light microscope with an

M28×0.75 digital coupler (Thales Optem Inc., Fairport, NY, USA). Opercular colouration was analysed in a similar manner as described above for whole isopod photographs. Reflectance was measured from a circular area (400-pixel diameter) in the middle of both the left and right operculae, and these values were averaged to give a mean reflectance value for each isopod. A subsample of operculae was photographed a second time to establish that the method was repeatable ($n=29$, $R=0.99$, $F_{28,29}=271$, $P<0.001$).

Opercular colouration for unexposed controls ($n=34$) was compared to that of exposed, infected isopods ($n=45$) with a Mann-Whitney U-test. A multiple regression model was then used to examine how the characteristics of the parasite infrapopulation affected opercular colouration. The total worm volume harboured by an infected isopod was included as a predictor. However, the distribution of parasite volume among individuals may also be important, i.e. pigment alteration may differ between a host with a few large worms and one with several small worms. In hosts harbouring a few big parasites, the average volume of worms, relative to the total, should increase. Residuals were taken of a regression of average worm size on total worm size. These residuals represent the variation in average worm size independent of the total parasite volume. For example, positive residuals (a large average volume relative to the total) characterize hosts in which the total parasite volume is concentrated into fewer individuals. Using residuals as a measure of average worm size in the regression model also circumvented the problem of collinearity (Mason and Perreault, 1991). Because *A. lucii* is sexually dimorphic, the sex ratio of the infrapopulation may affect how worm volume is distributed among individual parasites, so log sex ratio was also included in the regression model. The operculae from most infected isopods (77.8%) were collected 94–101 days post-exposure, so parasite age varied little and was not included in the model.

All statistical analyses were performed with SPSS 14.0 (SPSS Inc., Chicago, Illinois) software.

RESULTS

Host size – parasite size relationship

In natural infections, the relationship between parasite volume and isopod size was dependent on worm sex (ANCOVA, isopod size × worm sex, $F_{1,274}=84.0$, $P<0.0001$). In accordance with the results of Benesh and Valtonen (2007c), female cystacanth size increased more steeply with host size than male size (Fig. 1A). The same pattern was observed when parasite dry mass was used (ANCOVA, isopod size × worm sex, $F_{1,112}=34.4$, $P<0.0001$), although the difference between males and females was not as pronounced (Fig. 1B).

Fig. 1. The relationship between isopod size and parasite size measured as (A) volume (mm³) or as (B) dry mass (mg). Male and female cystacanths came from singly infected isopods collected from the field.

Isopod colouration in naturally collected isopods

After removing non-significant interactions, the terms included in the ANCOVAs were identical for body and abdominal coloration (from here BC and AC, respectively; Table 2). For both BC and AC, there was a significant interaction between infection status and isopod size ($F_{2,609}=4.14$, $P=0.016$ and $F_{2,609}=8.21$, $P<0.001$, respectively; Table 2). This is a violation of the ‘homogeneity of regressions’ assumption of ANCOVA, i.e. the relationship between the dependent variable (colouration) and the covariate (isopod size) differs among levels of the factor (infection status). Consequently, the estimated main effect of infection status may be biased. Thus, uninfected, male-infected, and female-infected isopods are only compared within the context of isopod size. The slope of the BC by size relationship was steepest for isopods infected with a female cystacanth, but the between-group differences were small (Fig. 2A). The interaction was clearer for AC. The difference between infected and uninfected isopods increased with isopod size, because the AC of

Table 2. Summary of ANCOVA analyses evaluating isopod colouration

(Isopods were classified as uninfected, infected with a male cystacanth, or infected with a female cystacanth. The block factor refers to the different experimental treatments listed in Table 1. Non-significant interaction terms ($P>0.05$) were sequentially removed from the ANCOVAs to produce more parsimonious models.)

	F	D.F.	P
Body colouration			
Infection status	5.77	2	0.003
Isopod sex	19.70	1	<0.001
Block	23.33	8	<0.001
Isopod size	143.54	1	<0.001
Infection status $\times$ Isopod size	4.14	2	0.016
Isopod sex $\times$ Isopod size	32.57	1	<0.001
Error		609	
Abdominal colouration			
Infection status	8.28	2	<0.001
Isopod sex	8.37	1	0.004
Block	20.10	8	<0.001
Isopod size	89.59	1	<0.001
Infection status $\times$ Isopod size	8.21	2	<0.001
Isopod sex $\times$ Isopod size	11.21	1	0.001
Error		609	

infected isopods became conspicuously darker in larger isopods (Fig. 2B). There was also a difference between worm sexes. Small isopods infected with a male cystacanth tended to have darker AC than those infected with a female cystacanth, but in larger isopods AC was similar (Fig. 2B). For BC and AC, there was a significant interaction between isopod sex and size (Table 2), because female isopods were slightly darker than males when large but not when small.

Adding parasite size to the ANCOVAs, either as volume or mass, did not produce any new results. For both AC and BC, worm size and all its interactions were non-significant (all $P>0.05$), indicating that parasite size did not explain any additional variation in host colouration beyond that described in the main analysis.

Opercular colouration in experimentally infected isopods

The operculae of exposed, infected isopods were darker than those of control isopods (Mann-Whitney U-test, $Z = -5.13$, $P < 0.001$). Hosts harbouring a larger total parasite volume tended to have darker operculae (standardized $\beta = -0.39$, $t_{41} = -3.02$, $P = 0.004$; Fig. 3A). Moreover, a large average worm volume, relative to the total, was also associated with darker operculae (standardized $\beta = -0.41$, $t_{41} = -3.20$, $P = 0.003$; Fig. 3B), which suggests that isopods with a few large worms had more severely altered pigmentation than those with many small worms. The infrapopulation sex ratio did not affect

Fig. 2. Body (A) and abdominal (B) colouration as a function of isopod length. The data are separated into uninfected isopods (circles, solid line), isopods infected with a single female cystacanth (open triangles, dashed line), and isopods infected with a single male cystacanth (open squares, broken line). Statistical analyses treated isopod size as a continuous variable, but trends were difficult to discern in a scatter-plot due to the extensive overlap among individual data points. Thus, for clarity, isopod size is plotted as a categorical variable. Colouration is lighter at higher values on the scale. Bars represent the 95 % CI.

opercular colouration (standardized $\beta = -0.05$, $t_{41} = -0.35$, $P = 0.73$).

DISCUSSION

The alteration of isopod colouration only occurs after parasites become cystacanths (Bratney, 1983). However, maximum host alteration was not achieved immediately after attaining the cystacanth stage. In both naturally and experimentally infected isopods, larger parasites more strongly altered host pigmentation. In the naturally infected isopods, abdominal colouration was darkest, relative to uninfected isopods, in large hosts that harboured bigger worms. Likewise, a large total parasite volume was associated

Fig. 3. The relationship between opercular coloration and (A) the total parasite volume (mm^3) harboured by experimentally infected isopods and (B) the residuals of a regression of average parasite volume against total parasite volume. Positive residuals represent hosts with a large average parasite volume relative to the total, i.e. the total parasite volume is concentrated into fewer individuals. The solid lines are the least squares regression lines for the data, while the dashed lines represent the mean opercular colouration of unexposed, control isopods. Colouration is lighter at higher values on the scale.

with darker opercular colouration in experimentally infected isopods, particularly when it was distributed among fewer individual worms. Moreover, the experimental isopods were sampled at about the same time, so the relationship between parasite size and host colouration was not confounded by potential effects of parasite age. Parasite age might also influence host manipulation, but we could not assess this, as there was little variation in parasite age in our experimental data. Evaluating the relative importance of parasite size versus age requires independent variation in each, e.g. parasites could be sampled at different ages after they had been growing at different rates.

Though larger, more modified hosts harboured larger parasites, parasite size was not a significant covariate in the ANCOVA analyses conducted with the field-collected isopods. This was probably because parasite size did not explain any additional variation in colouration beyond that described by host size. That is, parasites that were large (or small) relative to their host's size did not modify host pigmentation more (or less) extensively. Modification of other host traits may also increase as parasites grow, though imperfectly. For example, from late summer to the following spring, the size of isopods and parasites increases, as does the alteration of host hiding behaviour (Benesh *et al.* 2009). However, host behaviour is not clearly modified in late autumn, even though isopods are of a similar size to those collected in spring (Benesh *et al.* 2009). Thus, for both host behaviour and colouration, there is a positive, but imperfect correlation between parasite size and trait alteration. Similar trends have been noted in tape-worms in their fish second intermediate hosts. For example, Brown *et al.* (2001) found that fish infected with larger *Ligula intestinalis* plerocercoids had more altered habitat choice, and Ness and Foster (1999) found larger *Schistocephalus solidus* in demelanized sticklebacks. Size-dependent manipulation may be an adaptive strategy, because, as parasites grow larger, the relative benefits of remaining in the intermediate host decrease while the potential costs increase. At some point, the amount of additional larval growth possible diminishes due to space or resource constraints (Michaud *et al.* 2006; Benesh and Valtonen, 2007b; Shostak *et al.* 2008). Concomitantly, the probability of natural host mortality presumably increases. Therefore, the profitability of transmission, and by association host manipulation, is likely to increase as parasites grow over time.

Many acanthocephalans are sexually dimorphic as cystacanths (e.g. Amin *et al.* 1980; Oetinger and Nickol, 1981; Steinauer and Nickol, 2003), presumably because the benefits of a large larval size are more pronounced for females than males (Benesh and Valtonen, 2007c). This sexual dimorphism might favour divergent manipulation strategies. Oetinger and Nickol (1981), however, did not observe differences in the “pigment dystrophy” exhibited by isopods infected with male or female *Acanthocephalus dirus* cystacanths. The hiding behaviour of isopods infected with a male or female *A. lucii* cystacanth also does not seem to differ (Benesh, unpublished data). In this study, male worms appeared to alter isopod abdominal colouration more extensively than female worms in small hosts, but in larger hosts this difference disappeared. Because female cystacanth size increases rapidly with host size, females in small hosts may have more to gain than males by remaining in and growing mutually with the host (infection does not impair isopod growth, Benesh and Valtonen, 2007a; Hasu

et al. 2007). The more extensive modification of small hosts by male parasites may thus reflect their relatively higher incentive to be transmitted. By contrast, in larger isopods, neither male nor female parasites may profit from staying in the intermediate host, favouring similar, high levels of host modification by both sexes. Sexually divergent manipulation strategies could also arise via differences in resource availability, assuming manipulation entails energetic costs. Because male parasites invest less in growth, they may have more resources available to allocate toward modifying host pigmentation. However, the largest colouration difference between male- and female-cystacanth infections was observed in small isopods when parasite size dimorphism is relatively low and resource pools are presumably similar. This suggests that the sex-specific manipulation strategies stem from different optimal sizes for transmission rather than dissimilarities in resource availability.

Visual-based predation by fish is likely to be a selective force maintaining cryptic colouration in *A. aquaticus* (Hargeby *et al.* 2004, 2005), so the conspicuously darker abdominal pigmentation of naturally infected isopods probably increases their susceptibility to predation by fish definitive hosts (Bratney, 1983; Seppälä *et al.* 2008). If large heavily manipulated hosts harbouring large parasites are taken more easily by predators, then parasite abundance may be higher in hosts of medium size. Consistent with this prediction, natural *A. lucii* abundance peaks in intermediate-sized isopods and is reduced in large isopods (Bratney, 1986). Although a number of processes can produce this pattern (e.g. age-dependent exposure, Duerr *et al.* 2003), it would be interesting to see if other helminths with similar distributions in their intermediate host populations (Thomas *et al.* 1995; Rousset *et al.* 1996; Outreman *et al.* 2007) also increase host manipulation as they grow. Unlike *A. lucii*, however, many parasites exhibit relatively fixed growth strategies, i.e. after developing to an infective stage, growth stops. For these species, there may be no additional benefits, only costs, associated with remaining in the intermediate host after infectivity is reached, so discrete changes in the level of host manipulation might be favoured. Different parasite growth patterns may, thus, lead to different host manipulation strategies.

Jukka Jokela and two reviewers gave helpful comments on an earlier version of this manuscript. D.P.B. was supported by the Biological Interactions Graduate School at the University of Turku and O.S. by the Academy of Finland.

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