

Costly carotenoids: a trade-off between predation and infection risk?

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Keywords:

carotenoids;
Macrocyclops albidus;
multiple enemies;
parasite infection;
predation;
Schistocephalus solidus.

Abstract

Carotenoid reserves in copepods seem costly in terms of predation risk because they make individuals conspicuous. However, carotenoids also seem to play an important role in immune defence as free radical scavengers. To test whether predation risk influences carotenoid levels and whether changes in carotenoid levels are related to changes in immune defence, I examined individual changes in large carotenoid and other lipid droplets upon exposure to predation risk and subsequent exposure to parasites in the copepod *Macrocyclops albidus*. Copepods reduced carotenoid reserves upon exposure to predators, through which they potentially avoided the costs of being conspicuous under predation risk. Thus, the size of carotenoid reserves is a plastic trait. Such a decrease in carotenoid reserves may also have a negative impact on the copepods' immune system as individuals that decreased their reserves suffered higher parasite prevalence upon exposure to the cestode *Schistocephalus solidus*. These results suggest that carotenoid reserves may be individually optimized to trade-off each individual's unique costs (predation risk) and benefits (immune defence) of having these reserves.

Introduction

In recent years carotenoids have started to play an important role in studies of evolutionary biology. Since animals cannot produce carotenoids *de novo* (Young & Britton, 1993) and carotenoids may be rare in nature (Endler, 1980), it has been suggested that carotenoid reserves or intensity of carotenoid based colour expression may be an indicator of individual quality (Olson & Owens, 1998). Variation between individuals in carotenoids has been suggested to be due to genetic (Hill, 1991) or environmental effects (Bortolotti *et al.*, 2000; Tschirren *et al.*, 2003). Genetic effects may play a role when individuals differ in their physiological need of carotenoids or their ability to absorb or consume carotenoids (Hill, 1991; Hill *et al.*, 1994; Hudon, 1994; Tschirren *et al.*, 2003). Environmental effects may play a role when carotenoids are rare, which may, for example be the case when an addition of carotenoids in the diet leads to an increased expression in carotenoid based colours

(Grether *et al.*, 1999; Blount *et al.*, 2003; McGraw & Ardia, 2003; Navara & Hill, 2003; Ohlsson *et al.*, 2003).

Another reason for increased recent interest of evolutionary biologists in carotenoids is that carotenoids are related to various components of immune defence (Lozano, 1994). Besides playing a role as general immunostimulants (Bendich, 1989; Bendich & Olson, 1989), carotenoids also play an important role during activation of the immune system because of their free radical scavenging capacity. During immune defence highly reactive free radicals are produced and without free radical scavengers, like carotenoids, these free radicals may damage important molecules and proteins in the body (Bendich, 1989; Bendich & Olson, 1989; Bendich, 1993; von Schantz *et al.*, 1999). Several recent studies have focused on the role of carotenoids in the immune system of birds and fish, most often in relation to carotenoid based expression of secondary sexual characters. Some of these studies have shown correlational evidence for an association between plasma carotenoid levels or intensity of carotenoid based colouration on one hand and infection status or immunocompetence on the other (Folstad *et al.*, 1994; Dufva & Allander, 1995; Skarstein & Folstad, 1996; Horak *et al.*, 2001; Saks *et al.*, 2003; Horak *et al.*, 2004). Experimental studies have

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followed two lines of experimental manipulation. First, some of them aimed to prove the importance of carotenoids for the immune system by showing that either plasma levels of carotenoids or carotenoid based expression of secondary sexual characters decreased after immune activation (Milinski & Bakker, 1990; McGraw & Hill, 2000; Saino *et al.*, 2000; Faivre *et al.*, 2003; Alonso-Alvarez *et al.*, 2004; Peters *et al.*, 2004, but see Navara & Hill, 2003). Second, others aimed to show the importance of carotenoids for the immune system by showing that immunocompetence increases after supplementing the diet with carotenoids. However, these studies give inconsistent results, sometimes depending on sex or type of immune response (significant positive effect Amar *et al.*, 2001; Fenoglio *et al.*, 2002; Blount *et al.*, 2003; McGraw & Ardia, 2003; Saino *et al.*, 2003; Alonso-Alvarez *et al.*, 2004; Amar *et al.*, 2004; Grether *et al.*, 2004; McGraw & Ardia, 2004, no significant positive effect Navara & Hill, 2003; Ohlsson *et al.*, 2003; Saino *et al.*, 2003; Grether *et al.*, 2004).

Many crustaceans are known to contain large amounts of carotenoids. In crustaceans these carotenoids seem to play an important role in protecting against UV exposure and repairing DNA damage after exposure to UV light (Hairston, 1976, 1979a,b, 1980; Rhode *et al.*, 2001). It has been shown that in lakes where UV exposure is high, copepods generally contain more carotenoids and are thus redder (Hansson, 2000). However, being red seems to be costly for copepods. Red prey are more easily detected by predatory fish and thus preferentially preyed upon (Hairston, 1979a; Endler, 1980; Ohguchi, 1981; Bakker *et al.*, 1997), and several studies have shown that in lakes where visual predators occur, copepods are generally less red and contain fewer carotenoids (e.g. Hansson, 2000, 2004). Such a difference could be due to selection, when red individuals are removed from the lakes by predators, or to plasticity, when individuals adjust their carotenoid reserves to the prevailing risk. Recently, Hansson (2000, 2004) suggested that the carotenoid reserves of copepods might actually be a plastic trait. He showed by taking samples from the same aquarium before and after exposure to predation risk that carotenoid reserves were lower after exposure to predation risk than before. He argued that, since the copepods were only exposed to predation risk and not to actual predators, this cannot be due to differential selection by predators, but is due to individuals adjusting their carotenoid content to the prevailing predation risk. The problem with these studies is that such an effect could also appear when copepods that differ in carotenoid content show differential mortality, which could, for example, happen when copepods that have more carotenoids are more sensitive to predation stress. In this study I aim to show that carotenoid reserves of copepods are plastic by tracking individual changes in carotenoid content of copepods from before to after exposure to predation risk. In this

way differential mortality can be ruled out as an alternative explanation.

Not only in vertebrates, but also in invertebrates free radical scavenging plays an important role during immune activation. In vertebrates free radicals are produced during the respiratory burst reaction and the NO reaction (Janeway *et al.*, 1999). In invertebrates, in addition to those two reactions, free radicals are also produced during the phenoloxidase cascade (Nappi & Vass, 1993; Söderhäll & Cerenius, 1998; Sugumaran *et al.*, 2000). In my study system the resistance of the copepod *Macrocyclops albidus* Jurine against the cestode *Schistocephalus solidus* Muller seems to depend on the stage during which the parasite penetrates through the gut wall and enters the haemocoel (van der Veen & Kurtz, 2002; van der Veen, 2003). At this stage of the immune defence it is likely that free radicals are produced (e.g. Kumar *et al.*, 2003) and thus it is likely that carotenoids play a role as free radical scavengers in the immune defence of copepods against this parasite. Therefore, *M. albidus'* immune defence may be down regulated and their resistance may deteriorate when they reduce carotenoid reserves upon exposure to predation risk (Bendich, 1989; Bendich & Olson, 1989). I will therefore study if an adjustment of carotenoid reserves to the prevailing predation risk has consequences for parasite resistance. This can be done by showing that those copepods that decreased their carotenoid reserves upon predation risk suffer higher parasite prevalence after experimental exposure to parasites. This would then indicate that in copepods avoidance of predation risk by means of reducing carotenoid reserves is costly in terms of infection risk, and thus, that copepods face a trade-off between predation risk and infection risk mediated by carotenoids.

Material and methods

Copepods

M. albidus are predatory copepods (Einsle, 1993). *M. albidus* copepods originating from the Kremper Au, which connects to the Neustadter Binnensee, were cultured as described in van der Veen & Kurtz (2002). In the culture the copepods were kept in water in which hay had been boiled and were fed with *Paramecium caudatum* Ehrenberg. While being fed with *P. caudatum*, the copepods did not build up visible carotenoid reserves.

Start of the experiment

The experiment started on the 5th and the 6th of February 2002 with 148 c5 copepodites (the last stage before becoming adult) on each day. On day 1 of the experiment the copepodites were individually isolated in 1 mL hay-water in wells of 24-well ELISA plates. Water was always filtered through a 0.45 µm filter to remove ciliates and bacteria before adding it to the wells.

Throughout the whole experiment the copepods were moved to a well with freshly filtered water every day. All copepods were fed with two one-day-old *Artemia salina* L. nauplii three times a week. The food level was set at a level at which all copepods would always eat all their food. As a consequence the food level was rather restricted. Each day survival and moulting of the copepods was checked. Upon moulting, adult body size was measured as the length between the central posterior side of the eye and the dorsal anterior end of the cephalothorax by means of image analysis (Scion Image, a modification of NIH Image <http://rsb.info.nih.gov/nih-image>). From each copepod three size measurements were taken (repeatability 99.0%) and for analyses the average was used. Since during moulting large changes in carotenoid and other lipid reserves occur (pers. obs.), all copepods that had not moulted during the acclimatisation period (days 1–7) were removed from the experiment ($n = 125$).

Counting of droplets

A. salina nauplii contain high amounts of the carotenoid canthaxanthin (Czygan, 1968; Hsu *et al.*, 1970). After feeding copepods with *Artemia* nauplii, brightly orange coloured lipid droplets started to appear in the copepods' haemocoel. It is likely that these droplets contain the stored form of a derivative of canthaxanthin (Olson & Owens, 1998). On day 8 of the experiment (after 1 week of acclimatisation) I counted lipid droplets in the haemocoel (see also Franz & Kurtz, 2002). I distinguished between carotenoid containing lipid droplets, which are brightly orange, and other lipid droplets, which are similarly coloured as the rest of the copepod haemocoel. Franz & Kurtz (2002) estimated the volume of all droplets in each copepod by measuring the area of each droplet by means of image analyses. However, since this implies long screening time (up to 39 min per copepod, Franz, 2001), which can be detrimental for copepods, such a method was not suitable for the current experiment. However, since in that study total area related rather well with number of droplets (carotenoids: $F_{1,135} = 459.75$, $P < 0.0001$, $r^2 = 0.77$, other: $F_{1,135} = 633.19$, $P < 0.0001$, $r^2 = 0.83$, data from Karoline Franz), I counted the number of lipid droplets instead of measuring the area. I counted the droplets in three estimated size classes (min-max diameter (μm): carotenoid droplets: small 12.88–22.81, $n = 14$, medium 22.81–36.68, $n = 14$, large 34.94–67.22, $n = 11$, other droplets: small 10.91–19.10, $n = 11$, medium 20.84–29.79, $n = 9$, large 34.64–67.09, $n = 8$). Counting of lipid droplets was always done one day after feeding (cf. Franz & Kurtz, 2002).

Predation treatments

In each plate two of the four rows were randomly assigned to be controls and the other two were assigned

to be exposed to predation risk (fish exudates). From day 8 to 25, in the predation risk treatment, copepods were moved every day to wells with a concentration of 0.1 fish/l (0.9 mL hay-water with 0.1 mL water from a 5 L aquarium in which five sticklebacks *Gasterosteus aculeatus* L. were kept), whereas in the control treatment copepods were moved to wells without fish exudates (0.9 mL hay-water with 0.1 mL water from a 5 L aquarium in which no sticklebacks were kept). Every morning, after moving the copepods to fresh water, the control as well as the fish aquarium were refreshed. After this, the fish were fed *ad libitum* with frozen chironomid larvae and 20–30 living *M. albidus* copepods. Throughout the whole experiment the observer was blind concerning treatment, because the daily water changing was performed by somebody else. On day 15 (after 1 week of treatment) lipid droplets were counted again.

Infections

S. solidus eggs, obtained from matings of four pairs of parasites dissected from sticklebacks caught in the Neustadter Binnensee in September and October 2001, were put from 4 to 20 °C 3 weeks before the infection day and were kept in the dark. When they were put in light on the afternoon before the infection day, the light stimulus caused the eggs to hatch (Dubinina, 1966). On day 18 each copepod was exposed to four larvae of the parasite. The parasite clutches were randomly assigned to the six columns of a plate, so that in every two plates each of the four clutches was used three times. On day 25 (1 week after exposure) lipid droplets were counted again and copepods were screened under the microscope for parasite prevalence.

Statistical analyses

For each counting of lipid droplets I estimated the carotenoid and other lipid volume with the size class distribution of droplets mentioned above. However, even after transformation, the distribution of lipid reserves was not normal and therefore I chose to analyse the number of droplets in each size category by means of linear logistic models. For this I used Proc Catmod in SAS 612 (SAS, 1989), with a logit link function.

In this paper I focused on the large droplets, because they are most conspicuous and contain the largest part of the reserves; when I ranked copepods for total lipid volume, copepods with large lipid droplets always ranked higher than copepods without large lipid droplets. Statistical results for the other size categories were either similar, but showed weaker trends, or were nonsignificant. Changes in the number of large droplets were not compensated for by opposite changes in medium sized droplets. Instead, changes in large and medium sized droplets were positively correlated (carotenoids: $r_{\text{spearman}} = 0.24$, $n = 145$, $P < 0.05$, others: $r_{\text{spearman}} =$

0.18, $n = 145$, $P < 0.05$). To increase the power of statistical analyses I categorised the data. Since my aim was to find out if exposure to predation risk caused a decrease in carotenoids and if such a decrease was related to parasite prevalence, I compared those copepods that decreased (≤ -1) in large carotenoid droplets in the first treatment week (days 8–15) with those that either increased or did not change (≥ 0). When analyzing what factor affected whether copepods decreased (≤ -1) or increased (≥ 0) in large lipid droplets, I included body size as a continuous variable and treatment (control or predation) as a class variable in the linear logistic model.

The range of body size distribution differed strongly between infected and noninfected copepods (size (μm) \pm SE: uninfected: 748.27 ± 4.24 , $n = 101$; infected: 725.83 ± 5.33 , $n = 37$, $t_{136} = 2.91$, $P < 0.05$), in the infected group large copepods were lacking. When large copepods respond in a different way than small copepods, including size as a covariate in the analysis on two groups that have such different size distributions may not effectively control for size effects in the data set. To ensure that when testing what factors influence parasite prevalence, this difference in size distribution is not confounding the other factors of interest, I controlled for size differences between the infected and uninfected copepods by matching each infected copepod with an uninfected copepod from the same treatment. During matching I always chose the uninfected copepod that was closest in size to the infected copepod. This resulted in a data set with 37 infected and 37 uninfected copepods with similar size (range of size difference between matched individuals from 0 to 2.7% of body size). It must be noted here that by excluding large copepods from this data set, the analysis done here cannot be taken as representative anymore for the whole size range of copepods. To test what factors influenced parasite prevalence I used a linear logistic model with a logit link function, where treatment (control or predation) and change in large carotenoid droplets from before to after one week of treatment (≤ -1 or ≥ 0) were included as class variables.

In all statistical analyses nonsignificant interactions were removed from the models.

Results

Since carotenoids make copepods conspicuous one can expect copepods to reduce their amount of carotenoid droplets when exposed to predation risk. On the other hand, one should not expect such a change in the other lipid droplets since those are opaque and thus cryptic. Both expectations seem to be fulfilled in this experiment. In the predation treatment copepods more often decreased their number of large carotenoid droplets than copepods in the control treatment (Fig. 1, log-linear regression $\chi^2_1 = 7.57$, $P < 0.05$), and such an effect was not observed for large other lipid droplets (Fig. 1, log-linear regression $\chi^2_1 = 0.37$, n.s.).

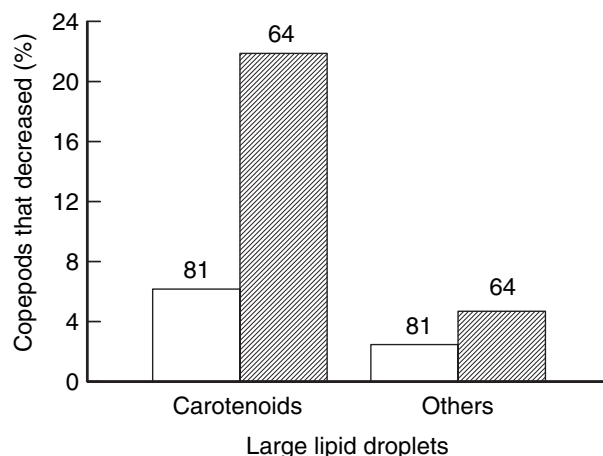


Figure 1 The percentage of copepods that decreased in large carotenoid or other lipid droplets upon 7 days of exposure to predation risk (hatched bars), i.e. water with a concentration of 0.1 fish per litre, or to a control (white bars). Sample sizes are indicated above the bars.

Body size was significantly related to change in large carotenoid droplets; copepods that reduced their amount of carotenoid droplets were significantly smaller than those that did not change or increased in large carotenoid droplets (size (μm) \pm SE: decrease: 723.81 ± 8.48 , $n = 19$, no change or increase: 744.43 ± 3.68 , $n = 126$, log-linear regression $\chi^2_1 = 4.61$, $P < 0.05$). Change in other large lipid droplets did not significantly relate to body size (log-linear regression $\chi^2_1 = 1.00$, n.s.).

Since size was significantly related to prevalence of parasites and size distributions differed between infected and noninfected copepods, I controlled for size differences between the infected and uninfected copepods by matching each infected copepod with an uninfected copepod (see Methods). If there is a trade-off between predation risk and infection risk, copepods that reduced their amount of carotenoid droplets should suffer higher parasite prevalence. In this data set, prevalence was significantly higher in copepods that reduced their amount of large carotenoid droplets than in those that did not change or increased their amount of large carotenoid droplets (Fig. 2, log-linear regression $\chi^2_1 = 4.75$, $P < 0.05$), whereas treatment (control or predation) did not affect prevalence by itself (log-linear regression $\chi^2_1 = 0.38$, n.s.). Such effects were not visible for the large other lipid droplets, however only two individuals reduced their amount of large other lipid droplets, which limits the power for statistical tests (Fig. 2).

Discussion

This paper is, as far as I know, the first in which within individual changes in carotenoid reserves upon exposure to predation risk are studied. This study shows that

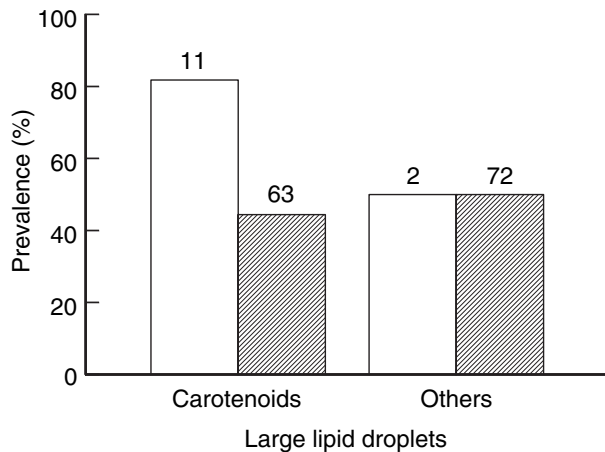


Figure 2 Parasite prevalence 7 days after experimental exposure to four larvae of *S. solidus* among copepods that decreased (white bars) or either increased or did not change (hatched bars) in large carotenoid or other lipid droplets during 7 days preceding the exposure. Sample sizes are indicated above the bars.

carotenoid reserves of copepods are a plastic trait and that copepods adjust their carotenoid reserves to prevailing predation risk; when predation risk increases, carotenoid reserves are reduced. Carotenoid reserves make copepods conspicuous and give them an orange colour. It has been shown that fish preferentially prey on red prey (Hairston, 1979a; Endler, 1980; Ohguchi, 1981; Bakker *et al.*, 1997). Plasticity of carotenoid reserves may therefore be an important trait in copepods and may be under strong natural selection.

It could be argued that the observed reduction in the number of large carotenoid droplets may not indicate a reduction of total carotenoid reserves, but may indicate a redistribution of carotenoids instead. Such redistribution may be indicated by weaker colouration of the carotenoid droplets in the predation treatment than in the control treatment. However, when the colour struck me as weak I usually made a note on this and in these notes this effect was rather the opposite. For significantly more individuals in the control than in the predation treatment I made a note on weak colouration of the carotenoid droplets (control 14.0 %, predation 3.8 % were noted as having a weak colour, $\chi^2_1 = 5.02$, $P < 0.05$). Hansson (2000) did not estimate carotenoid reserves visually, but chemically and his results also indicate reduced carotenoid reserves upon predation risk. Although the possibility of redistribution of carotenoids cannot fully be ruled out in this experiment, the similarities between the current results and the results of Hansson (2000) indicate that redistribution may be of minor importance.

This paper is also, as far as I know, the first that aims to study the consequences of adjustments of carotenoid reserves to prevailing predation risk in terms of infection risk. This study indicates higher parasite prevalence

following a reduction in carotenoid reserves. It could be argued that a significant association between predation risk and parasite prevalence, which was not found in this study, would be more convincing evidence for a trade-off between predation and infection risk than the present result of a significant association between a reduction in carotenoids and parasite prevalence. However, under the hypothesis that carotenoids are the mechanism through which the trade-off between predation and infection risk is mediated, it is to be expected that carotenoid reduction shows a stronger correlation with parasite prevalence than predation risk itself. This is because, even though significantly more copepods reduced their carotenoid reserves in the predation than in the control treatment, not every single copepod actually reduced its carotenoid reserves in the predation treatment, which decreases the strength of a potential statistical association between predation and infection risk.

Although this is a promising result, the effect may be correlative rather than causal. It could be that weak or low quality individuals were the ones that had the need to reduce their carotenoid reserves upon predation risk, for example, because they have a worse escape performance than high quality individuals. These weak or low quality individuals may have suffered higher parasite prevalence as a result of their bad condition or bad quality instead of as a result of their reduction in carotenoid reserves. Although this needs further study, I think that the presented results are nonetheless interesting, because of the following reasoning. In the present and earlier studies it was found that body size is an important indicator of individual quality in *M. albidus*. Compared to small copepods, large copepods are better at resisting *S. solidus* (van der Veen & Kurtz, 2002, present study), large females produce more clutches (Sivars Becker & van der Veen, 2004) and more eggs per clutch (Wedekind, 1997). Size also seems to play a role in carotenoid reserves; copepods that decreased their carotenoid reserves were smaller than those that did not. However, in the current study, even when size effects, and thus quality effects, were removed, copepods that reduced carotenoid reserves still suffered higher parasite prevalence. This indicates that individual quality may be of minor importance as an alternative explanation for the higher prevalence in copepods that had reduced their carotenoid reserves.

It seems likely that, since in copepods carotenoids are costly as well as beneficial, each individual carries its own optimal amount of carotenoids (cf. individual optimisation of fat reserves in birds in winter, McNamara & Houston, 1990). Since the extent of the costs and benefits of carotenoid reserves can be expected to vary with the state of the individual, each individual is expected to adjust the size of its carotenoid reserves to the prevailing predation risk according to its individual costs and benefits of having these reserves. If individual optimisation of carotenoid reserves takes place in copepods, this

study suggests that those copepods that need to adjust their optimal reserves to the prevailing predation risk are the ones that suffer because they also face a higher infection risk.

It could be argued that, besides predation cues, toxic compounds may also have been present in the fish water of the predation treatment. However, exposure to toxic compounds would lead to stress, which would, through increased metabolism, lead to a decrease in the other lipids reserves. Since no significant effects of predation risk on the other, not conspicuous, lipid reserves were found, the effect of potential toxic compounds may not be of major importance in this study. Even though, in this study, the other lipid reserves were not significantly related to parasite prevalence, those other lipids seem very important for survival and reproduction in copepods (Klein Breteler & Gonzalez, 1988; Sargent & Falk Petersen, 1988; Santer & Boldt, 1998; Swadling *et al.*, 2000). A previous study on lipid reserves of *M. albidus* showed that total lipid reserves (carotenoid and other lipids) were positively related to survival (Franz & Kurtz, 2002).

This study investigates facing multiple enemies: predators and parasites. A few other studies have concentrated on this issue, although from a slightly different angle. Decaestecker *et al.* (2002) concentrated on habitat selection in water fleas *Daphnia magna* and showed that by avoiding high predation risk in the top layer of the water, where light facilitates hunting for visually hunting fish, water fleas faced higher infection risk in the deeper parts of the water. Rigby & Jokela (2000) focussed on another but also very interesting trade-off. Snails *Lymnaea stagnalis* avoid predation by retreating in their shells. However, to be able to fit in, they have to excrete blood and with this blood they also excrete immune cells. Therefore, upon exposure to a predator these snails suffer a higher infection risk. These two examples together with the present study, where this trade-off seems to be mediated through carotenoid reserves, show how varied and potentially common such trade-offs between facing multiple enemies may be.

Acknowledgments

I would like to thank Joachim Kurtz and Lena Sivars Becker for discussions, Karoline Franz for providing me with data on the correlation between number and total volume of droplets and Kirsten Kessler for discussions on fish concentrations. Joachim Kurtz, Manfred Milinski and Lena Sivars Becker are thanked for commenting on earlier drafts of this paper. I also thank Regina Leipnitz and Lore Janke for taking care of the copepod cultures and for their help during this experiment. Furthermore I thank everybody in the evolutionary ecology group for catching infected sticklebacks and breeding the stock parasites.

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Received 16 November 2004; revised 20 January 2005; accepted 22 January 2005