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Predator avoidance and immune defence: costs and trade-offs in snails

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Organisms are often confronted by both predators and pathogens. Defending against such widely divergent enemies requires more than one type of defence. Multiple defences, however, raise the possibility of trade-offs among defences. We tested for such trade-offs by manipulating the level of predator-avoidance behaviour and immune function in the freshwater snail *Lymnaea stagnalis* (Gastropoda: Pulmonata). Our results show that predator avoidance and immune function had clear costs in terms of reproduction and survival. Further, we show that increased levels of predator-avoidance behaviour reduced the snails' ability to defend against potential pathogens. Predator-avoidance behaviour may thus have the additional indirect cost of reduced immunocompetence and increased susceptibility to pathogens. Our results suggest that ecological factors (e.g. predator density) may considerably modify the expression and costs of immune defences.

Keywords: cost of defence; predator avoidance; immune defence; immunocompetence; *Lymnaea stagnalis*

1. INTRODUCTION

Optimal defence theory (Fagerström *et al*. 1987; Feeny 1976; Rhoades 1979; Simms & Rausher 1987) suggests that the resource allocation to defence is flexible. The resources allocated to defence should be derived from a common pool of limited resources used by all fitness-associated traits. At optima the total amount of resources allocated to defence is predicted to maximize the benefit of defence versus its cost in terms of other fitness-associated traits (Demeester *et al*. 1995; Fineblum & Rausher 1995; Simms & Fritz 1990). This approach, however, does not attempt to investigate the optimal use of the allocation to defence. As organisms are often confronted by multiple enemies (Thompson 1998), the allocation to defence will rarely achieve any large degree of success if it is channelled into only one defence. This suggests that, in the presence of multiple enemies, the total allocation to defence may be partitioned among different defences (Thompson 1994). For example, the fruit fly *Drosophila melanogaster* uses at least seven different antimicrobial peptides to defend against micro-organisms alone (Lemaitre *et al*. 1997). Given that many organisms have both multiple predators and pathogens, partitioning the allocation to defence among multiple defences should be common.

If optimal defence theory is applied to the use of multiple defences, we may predict trade-offs both between defence and other fitness components, and among defences. These trade-offs are similar to those predicted by optimal allocation theory (Cody 1966; Sibly & Calow 1986), and are a consequence of competition for limited resources and of the variance in benefits and costs among defences. In the above example, antimicrobial peptides are made of amino-acid chains of varying length (i.e. they use the same resources but have differing costs) and each peptide defends against a different group of pathogens (e.g. Hetru *et al*. 1998; Lemaitre *et al*. 1997). Additionally, defences may compete for limited machinery. For example, in insects, the fat body synthesizes antimicrobial peptides (e.g. Hetru *et al*. 1998) and may limit the rate of synthesis. Lastly, defences may have conflicting actions. For example, a defence may repel one enemy but attract another (e.g. DaCosta & Jones 1971), requiring increased levels of other defences. Defences may thus be expected to exhibit trade-offs based on resources, machinery and conflicting actions, potentially constraining the optimal level of each defence.

We used the freshwater pond snail *Lymnaea stagnalis* (Gastropoda: Pulmonata) to study trade-offs between predator-avoidance behaviour and immune defence. Many gastropods retreat into the relative safety of their shells when attacked by predators (reviewed in Vermeij 1987). This behaviour permits *L. stagnalis* to escape from predatory crayfish, e.g. *Astacus leptodactylus* and *A. astacus* (M. C. Rigby, unpublished data). In order to retreat deeply within its shell, *L. stagnalis* must expel its blood (Luchtel *et al*. 1997), which should have obvious energetic costs. Soon after the predator's attack, the expelled blood is replaced with water from the surrounding environment (Wood *et al*. 1981). This water may contain micro-organisms, which are then killed and removed by immune defences. Hence, predator avoidance imposes the double costs of increased immune function and replacing lost blood. Expelling blood during predator avoidance may conflict with proper immune function as blood contains many immune defences. Thus, predator-avoidance behaviour may have the indirect cost of reduced immunocompetence.

In this study, we examined the costs of predator-avoidance behaviour and immune function and the potential for a trade-off between them. First, we simulated a predator's attack without harming the snails. Following the expulsion of blood, we increased immune function in half of the snails by placing them in a stock solution of non-pathogenic micro-organism-enriched water. We found that predator-avoidance behaviour and immune function had substantial costs in terms of reproduction, survival and fat reserves. Additionally, increased predator-avoidance behaviour reduced the ability to remove potential pathogens. This

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suggests that trade-offs between defence types, due to ecological interactions, may limit the effectiveness of immune defences.

2. MATERIAL AND METHODS

(a) *Snails*

In this experiment, we used the laboratory reared F_1 offspring from 13 wild snails caught in a pond near Einsiedeln, Switzerland (47°6′30″ N, 8°43′40″ E). The F_1s were raised together in aquaria at 20 °C with a 16 L:8 D photoperiod, and fed iceberg lettuce *ad libitum* until they were mature, at approximately two months of age.

(b) *Experimental design*

For the experiment, we maintained each snail individually in a 600 ml plastic container, supplied iceberg lettuce *ad libitum*, and changed both the water and container every second day under the above light and temperature conditions. To acclimatize the snails to these conditions before the experiment began, we waited one week after isolating the snails before starting the experiment. We then manipulated the frequency of predator-avoidance behaviour and the level of immune function in a factorial design for 12 days. To stimulate predator-avoidance behaviour, we gently tapped the snail's foot with a micropipette tip until the snail stopped expelling blood. We gave snails this predator-avoidance treatment with one of three frequencies: once every six days ($n = 30$), once every second day ($n = 30$), or once every day ($n = 40$). As the cost of predator avoidance should be determined by the amount of blood expelled, we also measured the volume of blood expelled. We manipulated the level of immune function by placing half of the snails from each predator avoidance frequency in either clean water or micro-organism-enriched water (see below) immediately following the expulsion of blood. This permits snails to replace expelled blood with either micro-organism-enriched or clean water. Snails in micro-organism-enriched water should have a higher level of immune function as immune defences will be used to kill and remove the greater amount of micro-organisms taken in with the water. There were thus six treatment combinations in this experiment (three predator avoidance, two water).

We maintained the snails in their respective water treatments throughout the experiment. To make micro-organism-enriched water, we aged a solution of LB medium (2 ml l^{-1}) (10 g l^{-1} tryptone, 10 g l^{-1} NaCl, 5 g l^{-1} yeast extract), algae medium (1 ml l^{-1}) (0.1 M KNO_3, 0.007 M KH_2PO_4, 0.004 M $MgSO_4 \cdot 7H_2O$), and Apiinvert sugar water (0.3 ml l^{-1}) (Sudzücker AG, Ochsenfurt, Germany) for five days. Before use, we made a 1:3 dilution in aged tap water. We added calcium to both clean and micro-organism-enriched water.

To determine whether the effects of micro-organism-enriched water were due to pathogens or the use of immune defences to remove micro-organisms, we maintained approximately half of the surviving snails (the other half were used to determine fat reserves at the end of the experiment) without either experimental treatment for three weeks following the experiment. We then measured reproductive output, fat reserves and survival. Had micro-organism-enriched water included pathogens, we would expect lower survival and a slower recovery of reproduction and fat reserves in the snails that had been maintained in micro-organism-enriched water during the experiment. Examining the snails that died during the experiment for pathogenic micro-organisms would have yielded little meaningful information as pathogenic micro-organisms cannot readily be distinguished from opportunistic micro-organisms that rapidly colonize snails after death.

(c) *Response variables*

Snails were removed from their containers, blot dried, bled as described above, then returned to their containers. To measure the volume of the resulting blood, we used a volumetric micropipette (Socorex, Isba SA, Ecubleus, Switzerland). We measured shell length using manual callipers. To calculate fat reserves, we measured the difference between dry body weight and dry body weight following removal of the fat reserves with ether (Reznick 1983). This quantity was then converted into the percentage of fat out of the total body mass (% fat) for statistical analysis. As *L. stagnalis* egg masses are laid in a single column consisting of three eggs per row, we counted the number of egg rows in an egg mass and multiplied that by three to estimate reproductive output. We measured the concentration of haemocytes in the expelled blood using a Neubauer improved haemocytometer (Merck AG, Dietikon, Switzerland). Lastly, we assayed the ability of the haemocytes to remove a potential pathogen via phagocytosis by exposing the haemocytes to Baker's yeast (*Saccharomyces cerevisiae*). To perform this assay, we allowed the haemocytes in 20 µl of snail blood (diluted to 40 µl in snail saline (Chernin 1963), with 11 mM EDTA dihydrate and 17 mM TRIS, pH 7.4)), to attach to glass immunofluorescence slides for 15 min. After removing the overlying solution, we added 40 µl of yeast solution (0.13 g yeast ml^{-1} snail saline as above) and let it stand for 5 min. We then fixed the attached haemocytes in methanol and Gram-stained them. Lastly, we examined 100 haemocytes under ×1000 magnification oil immersion and scored them as either having phagocytosed yeast or not. We converted this to the percentage of cells capable of phagocytosing (% phagocytosis) for statistical analysis.

(d) *Statistical analyses*

The cost of predator avoidance is likely to be determined by the volume of blood expelled. The volume of blood expelled increased with the frequency of predator avoidance (ANOVA, $F_{2,61} = 293$, $p < 0.001$), with snails in the most frequent treatment expelling approximately four times as much blood as snails in the least frequent treatment. However, the volume of blood expelled varied considerably within each predator-avoidance treatment (coefficient of variation: 20–26%). Consequently, predator-avoidance frequency may not accurately reflect the costs of predator avoidance. Therefore, we performed our statistical analyses using the volume of blood that each individual expelled during the experiment as a measure of predator-avoidance activity. Additionally, as reproduction and the volume of blood expelled may depend on the snail's size, we used initial shell length as a covariate in all analyses, excluding it when it was not significant.

We used a logistic regression with backward selection from all possible models to examine the effects of water treatment (categorical covariate), the volume of blood expelled and shell length (continuous covariates) on the probability of reproduction and survival during the experiment. The probability of reproduction was analysed in surviving snails with respect to the total volume of blood expelled (non-transformed) and the probability of survival was analysed with respect to the average volume of blood expelled per day while alive. It was necessary to use daily average amount of blood expelled in the survival analysis because the total volume of blood expelled depends on

survival time. We used an ANCOVA to examine the effects of water treatment, the total volume of blood expelled and length (the last two variables were used as covariates) on total reproductive output, fat reserves, the average number of haemocytes and the average percentage of phagocytic haemocytes maintained throughout the experiment. A significant interaction term between water treatment and the volume of blood expelled indicates that the pattern observed in the response variable differed by water treatment. Using predator-avoidance frequency as a categorical factor, instead of the volume of blood expelled as a covariate, yields largely similar results. We chose to use the volume of blood expelled as a covariate because the variance within each predator avoidance treatment indicated that using the actual volume of blood expelled would allow a more powerful analysis of the costs enforced by the predator-avoidance treatment. Reproductive output for the three weeks following the experiment was analysed using a repeated measures ANOVA with the data pooled for each group of two consecutive days. The total volume of blood expelled, reproductive output, percentage phagocytosis and post-experiment fat reserves were $\log_e$ transformed prior to statistical analysis.

3. RESULTS

Our results showed clear costs (in terms of survival, reproduction and fat reserves) of predator-avoidance behaviour and immune function. Survival decreased sharply as the allocation to predator avoidance increased, independent of water treatment (figure 1*a*). The probability of survival increased with snail size (logistic regression; $b = 0.528$, Wald $= 5.619$, d.f. $= 1$, $p = 0.0178$). Both the probability of reproduction and reproductive output decreased as the allocation to predator avoidance (shown by the volume of blood expelled) increased (figures 1*b* and 2*a*; table 1). These traits were more strongly affected by predator-avoidance behaviour in clean water than in micro-organism-enriched water (figures 1*b* and 2*a*; table 1). Further, micro-organism-enriched water reduced the probability of reproduction for any given allocation to predator avoidance. A similar effect was observed in reproductive output (figures 1*b* and 2*a*; table 1).

Micro-organism-enriched water also reduced fat reserves, especially in snails that were allowed to allocate less to predator avoidance (figure 2*b*; table 1). In the snails with high allocation to predator avoidance, fat content did not differ with respect to water treatment (figure 2*b*; table 1).

As haemocytes should be expelled with the blood and are used in defence against micro-organisms, we would have expected to observe a response in haemocyte numbers to the treatments. Surprisingly, no significant response was observed (table 1). The percentage of phagocytic haemocytes, however, decreased as the allocation to predator avoidance increased (figure 2*c*; table 1). Contrary to the effect observed on reproduction, survival and fat reserves, the decrease in the percentage of phagocytic haemocytes was stronger in micro-organism-enriched water than clean water. Also, micro-organism-enriched water increased the percentage of phagocytic haemocytes (figure 2*c*; table 1) whereas it decreased reproduction, survival and fat reserves.

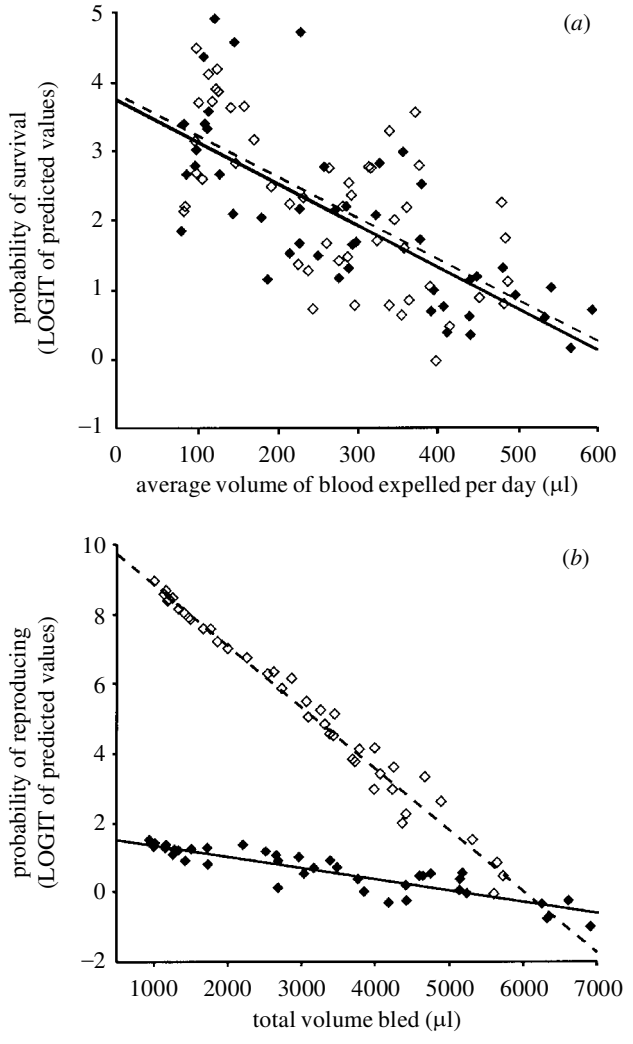

Figure 1. (*a*) The probability of survival with respect to predator-avoidance behaviour (volume of blood expelled) and water treatment (logistic regression; length, $-2\log LR = 7.09$, d.f. $= 1$, $p = 0.008$; volume × length, $-2\log LR = 7.41$, d.f. $= 1$, $p = 0.007$). (*b*) The probability of reproduction with respect to predator-avoidance behaviour and water treatment (volume × length, $-2\log LR = 7.05$, d.f. $= 1$, $p = 0.008$; water, $-2\log LR = 8.67$, d.f. $= 1$, $p = 0.003$; volume × water, $-2\log LR = 3.74$, d.f. $= 1$, $p = 0.053$). Open diamonds represent data points from clean water, with the regression shown by a dashed line. Solid diamonds represent data points from micro-organism-enriched water, with the regression shown by a solid line. (Note: graphs plot logit-transformed predicted values from the best-fitting logistic-regression model.)

During the three weeks following the experiment, only snails that had been kept in clean water died ($n = 7$, 15.6%). Additionally, during the experiment, water quality did not affect survival probability (figure 1*a*). Snails recovered reproductive output following the experiment with no significant effect of water treatment on recovery rate (shown by 'time × water' in table 2) or average reproductive output (shown by 'water' in table 2). Fat reserves also did not differ with respect to the treatments three weeks after the experiment (ANCOVA; water, $F_{1,33} = 1.27$, $p = 0.269$; volume, $F_{1,33} = 1.08$, $p = 0.306$; water × volume, $F_{1,33} = 1.67$, $p = 0.206$). This

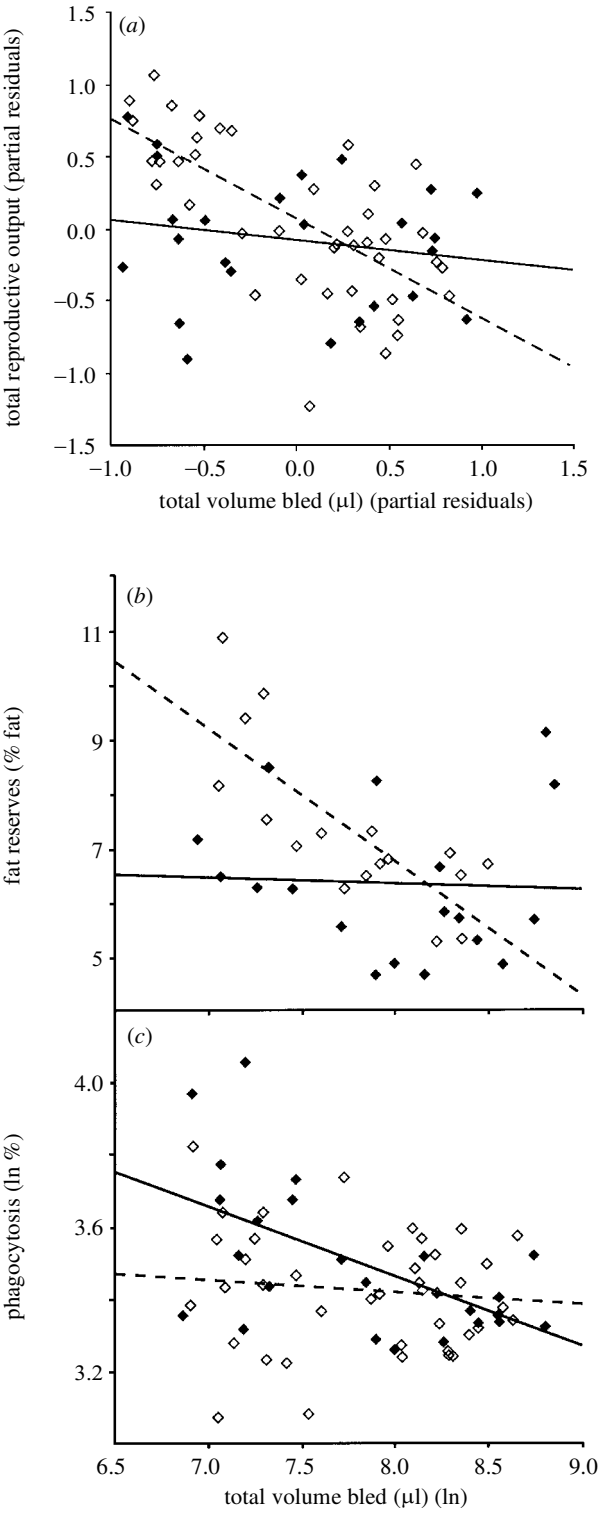

Figure 2. (*a*) Total reproductive output (number of eggs) with respect to predator avoidance and water treatment. Graph shows partial residuals, after the effect of length is removed. (*b*) Fat reserves with respect to predator avoidance and water treatment. Fat reserves are shown here as per cent fat of the total body mass. (*c*) The percentage of haemocytes capable of phagocytosis with respect to predator avoidance and water treatment. Open diamonds represent data points from clean water, with the regression shown by a dashed line. Solid diamonds represent data points from micro-organism-enriched water, with the regression shown by a solid line.

indicates that there were no long-lasting effects of water treatment; e.g. as from infection. We thus assume that micro-organism-enriched water did not contain any pathogens which might have infected the snails.

4. DISCUSSION

In this study, we found significant costs in terms of survival, reproduction and fat reserves for predator avoidance and immune function, and evidence for a trade-off between the two types of defences. Our results show that increased allocation to predator avoidance dramatically reduced the probability of both survival and reproduction (figure 1). Increased allocation to predator avoidance also reduced reproductive output and fat reserves (figure 2*a*,*b*). As fat reserves serve primarily as a form of energetic storage, reduced fat reserves indicate that predator avoidance was energetically costly. Micro-organism-enriched water did not show a cost in terms of survival (figure 1*a*), but did dramatically reduce the probability of reproduction (figure 1*b*). Micro-organism-enriched water also reduced reproductive output (figure 2*a*) and fat reserves (figure 2*b*) in snails with a low-to-medium allocation to predator avoidance. The costs of micro-organism-enriched water seen in reproduction and fat reserves mirror the pattern seen in the percentage of haemocytes capable of removing pathogens, or phagocytosis (figure 2*c*). This suggests that increased immune function in micro-organism-enriched water (as shown by the increased percentage of phagocytic haemocytes), reduced the allocation to reproduction and energetic storage. In other words, there was a trade-off between immune function and reproduction, and immune function was energetically costly. Increased allocation to predator avoidance also depressed the percentage of haemocytes capable of phagocytosis (figure 2*c*). This suggests that there is a trade-off between predator avoidance and immune defence in snails, and that the presence of crustacean predators may have a strong indirect effect on the fitness of snails in the wild.

Predator-avoidance behaviour did not reduce the average number of circulating haemocytes. However, as haemocytes have several other roles in addition to immune defence (Van der Knaap *et al.* 1993), their numbers may be constrained by other demands. While the number of haemocytes may be constrained, the investment per haemocyte may vary with other resource demands (figure 2*c*). Haemocytes capable of phagocytosis may be more costly than non-phagocytic haemocytes as they may require recognition proteins, lytic enzymes and oxidative enzymes. Indeed, predator-avoidance behaviour temporarily reduces the percentage of haemocytes with lytic and oxidative enzymes (Mohandas *et al.* 1992). The reduction in immunocompetence observed here did not carry a significant cost in terms of survival (figure 1*a*) as there were no pathogens in the laboratory. However, in the field, reduced immunocompetence due to predator-avoidance behaviour may have significant costs due to pathogens and parasites. For example, some parasites (digeneans) of snails permanently disable reproduction following infection (e.g. Kuris 1974).

Trade-offs among defences have previously been demonstrated among the chemical defences of plants

Table 1. *ANCOVA for the effect of the water treatment (water) and predator avoidance, expressed as the total volume of blood expelled (volume) on total reproductive output, average number of haemocytes μl^{-1}, average percentage phagocytosis, and fat reserves. Length was used as a covariate only for reproductive output and haemocytes μl^{-1}*

	total reproductive output				average haemocytes (μl^{-1})			average phagocytosis (%)				fat (%)			
effect	MS	d.f.	*F*	*p*	MS	*F*	*p*	MS	d.f.	*F*	*p*	MS	d.f.	*F*	*p*
water	1.64	1	9.09	0.004	78.32	0.11	0.738	0.15	1	5.32	0.024	16.36	1	8.90	0.006
volume	3.53	1	19.53	<0.001	822.79	1.19	0.281	0.27	1	9.70	0.003	18.18	1	9.89	0.004
water × volume	1.54	1	8.51	0.005	156.90	0.23	0.636	0.13	1	4.76	0.033	15.18	1	8.26	0.007
length	2.57	1	14.22	<0.001	4878.75	7.03	0.010	—	—	—	—	—	—	—	—
error	0.29	62	—	—	694.18	—	—	0.03	63	—	—	1.84	31	—	—

Table 2. *Repeated measures ANOVA for the effects of the experimental treatments (see table 1) on reproductive output in snails during the three weeks following the experiment*

	between subjects				within subjects				
effect	MS	d.f.	*F*	*p*	effect	MS	d.f.	*F*	*p*
volume	1513.29	1	8.27	0.007	time	290.66	8	3.63	<0.001
water	227.97	1	1.25	0.272	time × water	86.73	8	1.08	0.374
error	182.96	35	—	—	error	80.01	288	—	—

(Berenbaum & Zangerl 1988). Our results suggest that trade-offs among unrelated defences are possible as well. Here, we have shown a trade-off between predator-avoidance behaviour and immune function. Similarly, other studies have shown trade-offs between reproductive–mating activity and immune defence (e.g. Deerenberg *et al*. 1997; Nordling *et al*. 1998; Siva-Jothy *et al*. 1998; Wiehn & Korpimäki 1998). Together, these observations suggest that some behaviours may have indirect ecological costs in terms of reduced immunocompetence and a greater risk of pathogenic infection. Further, these indirect ecological costs of behaviours may be expected to constrain their expression. In our system, predator-avoidance behaviour may be constrained by the risk of infection. The far-reaching conclusion of our results is that trade-offs among defences and the indirect costs of behavioural interactions with other species, and even within the same species, may considerably modify the effectiveness of immune defence. This factor has largely been overlooked in epidemiological and coevolutionary models of host–pathogen interactions.

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