

Epidermal ‘alarm substance’ cells of fishes maintained by non-alarm functions: possible defence against pathogens, parasites and UVB radiation

Douglas P. Chivers^{1,*}, Brian D. Wisenden², Carrie J. Hindman¹, Tracy A. Michalak¹, Robin C. Kusch¹, Susan G. W. Kaminsky¹, Kristin L. Jack¹, Maud C. O. Ferrari¹, Robyn J. Pollock¹, Colin F. Halbgewachs¹, Michael S. Pollock¹, Shireen Alemadi², Clayton T. James³, Rachel K. Savaloja², Cameron P. Goater³, Amber Corwin², Reehan S. Mirza⁴, Joseph M. Kiesecker⁵, Grant E. Brown⁶, James C. Adrian Jr⁷, Patrick H. Krone⁸, Andrew R. Blaustein⁹ and Alicia Mathis¹⁰

¹Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, Saskatchewan, Canada S7N 5E2

²Department of Biosciences, Minnesota State University Moorhead, Moorhead, MN 56563, USA

³Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, Canada T1K 3M4

⁴Department of Biology, Pennsylvania State University, University Park, PA 16802-5301, USA

⁵The Nature Conservancy, Wyoming Field Office, 258 Main Street, Suite 200, Lander, WY 82520, USA

⁶Department of Biology, Concordia University, 7141 Sherbrooke West, Montreal, Quebec, Canada H4B 1R6

⁷Department of Chemistry, Union College, 807 Union Street, Schenectady, NY 12308-3107, USA

⁸Department of Anatomy and Cell Biology, University of Saskatchewan, 107 Wiggins Road, Saskatoon, Saskatchewan, Canada S7N 5E5

⁹Department of Zoology, Oregon State University, Corvallis, OR 97331, USA

¹⁰Department of Biology, Missouri State University, 901 South National Avenue, Springfield, MO 65897, USA

Many fishes possess specialized epidermal cells that are ruptured by the teeth of predators, thus reliably indicating the presence of an actively foraging predator. Understanding the evolution of these cells has intrigued evolutionary ecologists because the release of these alarm chemicals is not voluntary. Here, we show that predation pressure does not influence alarm cell production in fishes. Alarm cell production is stimulated by exposure to skin-penetrating pathogens (water moulds: *Saprolegnia ferax* and *Saprolegnia parasitica*), skin-penetrating parasites (larval trematodes: *Teleorchis* sp. and *Uvulifer* sp.) and correlated with exposure to UV radiation. Suppression of the immune system with environmentally relevant levels of Cd inhibits alarm cell production of fishes challenged with *Saprolegnia*. These data are the first evidence that alarm substance cells have an immune function against ubiquitous environmental challenges to epidermal integrity. Our results indicate that these specialized cells arose and are maintained by natural selection owing to selfish benefits unrelated to predator–prey interactions. Cell contents released when these cells are damaged in predator attacks have secondarily acquired an ecological role as alarm cues because selection favours receivers to detect and respond adaptively to public information about predation.

Keywords: club cells; alarm substance; *Schreckstoff*; immune function; Ostariophysii; Percidae

1. INTRODUCTION

There is strong selection on animals to attend to public information about predation risk (Danchin *et al.* 2004; Wisenden & Chivers 2006). In aquatic habitats, public information about risk often takes the form of chemical cues that are passively released during the process of predation (Chivers & Smith 1998; Wisenden & Stacey 2005; Wisenden & Chivers 2006). This is not surprising because water is the universal solvent and ideal for the dispersal of a wide range of chemical compounds. Moreover, the aquatic

milieu is the oldest of all environments, leaving aquatic life forms long evolutionary opportunity to innovate and elaborate receptors and behavioural patterns to exploit this information (Wisenden 2003).

An intriguing problem for evolutionary theory is the presence of ‘alarm substance’ cells (figure 1) of fishes in the superorder Ostariophysii (e.g. minnows, characins, catfishes, etc.) that comprise some 64% of all freshwater fish species and 27% of all fish species in the world (Nelson 1994). Some non-ostariophysians (e.g. Percidae: perch, walleye, sauger and darters) also have specialized epidermal club cells with similar histological characteristics (see Smith (1992) for

* Author for correspondence (doug.chivers@usask.ca).

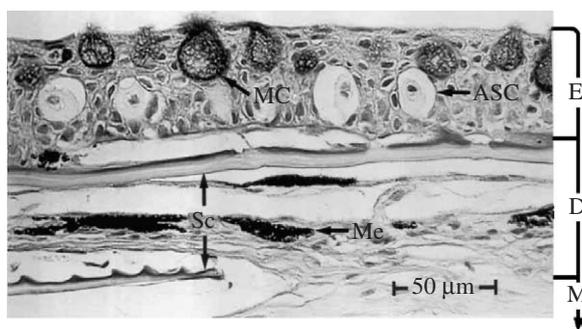


Figure 1. Cross section (4–5 μm thick) of minnow skin showing alarm substance cells (ASC) and mucous cells (MCs) in the epidermal layer (E). Fish scales (Sc) and melanophores (Me) are dermal in origin and lie above the muscle layer (M).

review). Club cells lie in the surface layers of the epidermis and are among the first cells to be damaged in an attack by a predator. The contents of club cells cannot be released voluntarily. Release of chemical alarm cues is a reliable form of public information about the presence of an actively foraging predator. This substance was originally termed *Schreckstoff* (fear substance) by Von Frisch (1941). There are abundant examples of the effect of chemical alarm cues on receiver behaviour, life history and morphology (Chivers & Smith 1998; Ferrari *et al.* 2007; Chivers *et al.* in press).

The presence of these cells, apparently specialized for synthesis and release of olfactorily conspicuous chemicals, is an evolutionary enigma. Club cells are energetically costly to produce (Wisenden & Smith 1997, 1998) but fitness benefits that might reconcile these costs, and maintain production of these cells over evolutionary time, are not obvious (Williams 1992). Smith (1992) summarized 16 hypotheses on mechanisms by which individual fitness might accrue to the sender of the alarm cue; only one among them, attraction of secondary predators, has thus far received empirical support. Laboratory and field experiments demonstrate that predators are differentially attracted to skin extract with club cells (Mathis *et al.* 1995; Wisenden & Thiel 2002). Once a predator has captured a prey, attempts by a second predator to disrupt the primary predator and pirate the prey can lead to an escape opportunity for the captured prey (Chivers *et al.* 1996). This hypothesis argues that club cells are a feeding signal to secondary predators, and that their function as an alarm cue occurred secondarily. This explanation provides some satisfaction, but the frequency with which secondary predators exploit damage-released chemical cues is unknown and possibly not common.

We sought other benefits in terms of individual fitness that could provide a selection gradient sufficiently steep to maintain club cells over evolutionary time across diverse taxa. We explored the possibility that the primary function of club cells may be to protect the skin against sources of injury and/or promote healing of damaged skin (Smith 1992). Epidermal cells are in a key location to provide a first line of defence against parasites or pathogens that penetrate through the skin, or promote healing of tissue damaged by bite wounds or UV radiation (Al-Hassen *et al.* 1985; Smith 1992; Blazer *et al.* 1997). The cellular contents could act directly on infecting organisms and/or act indirectly by mediating an immune response in the host.

Here, we report an accumulation of data from investigations conducted independently in several laboratories that

test this hypothesis. Our goal was to distinguish between the following predictions: (i) if the primary function of club cells is to warn conspecifics (or kin) of danger, then the density of club cells should vary with predation risk, and (ii) if the primary function of club cells is to promote skin healing, then the density of club cells should vary with the risk of attack by parasites/pathogens/UV exposure/general injury. We gathered data from species in the superorder Ostariophysi (Cyprinidae: fathead minnows (FHM), *Pimephales promelas*) and Acanthopterygii (percid: yellow perch, *Perca flavescens*; Johnny darters, *Etheostoma nigrum*) as model species in our assays.

2. MATERIAL AND METHODS

(a) Effect of predation risk on club cells

Our three predation experiments followed the basic protocols of Wisenden & Smith (1997, 1998). In each experiment, we raised FHM in individual tanks for 16 days under different treatments and then euthanized the fishes and quantified the number of club cells in a 0.36 mm length of skin centred over the dorsal intermyal septum. Minnows were raised in containers holding 2.0 l of water at approximately 17.5°C on a 14 L : 10 D photoperiod where they were fed ad libitum. In the first experiment, we manipulated predation risk by exposing FHM to the following extracts. (i) Conspecific skin extracts (containing club cells)—high risk. (ii) Skin extracts from brook stickleback (SB; *Culaea inconstans*)—medium/high risk; minnows co-occur with stickleback, share common predators with them and respond to stickleback alarm cues with an anti-predator response (Mathis & Smith 1993). (iii) Skin extracts from swordtails (SWTs; *Xiphophorus helleri*)—low/no risk; SWTs and minnows are phylogenetically distant, do not co-occur in the wild, and SWT extracts do not elicit an alarm response from minnows (Chivers & Smith 1994; Pollock *et al.* 2003). SWT cues controlled for general responses to cues from injured fish tissue. (iv) Distilled water—low/no risk; controlled for the effect of disturbance from cue introduction. Minnows were exposed twice weekly to approximately 0.02 cm² of homogenized skin from minnows, stickleback or SWTs, or to a control of distilled water (n ranged from 35 to 42 per treatment). At the end of the experiment, the fishes were euthanized with an overdose of tricaine methane sulphonate, preserved in 10% buffered formalin and histological sections were prepared with Schiff's reagent (periodic acid) and then counterstained with haematoxylin (PAS-H). Club cells are PAS-H negative and appear white with dark central nuclei. The person scoring the slides counted the number of cells per millimetre along the skin cross section; they were blind to the treatments.

In the second predation experiment, we varied predation risk by exposing minnows to cues from either a familiar predator (northern pike, *Esox lucius*) or an unfamiliar predator (oscar cichlid, *Astronotus ocellatus*), each of which had been fed either FHM or SWTs. Anti-predator responses to diet-based predatory cues are widespread in predator-prey systems (review Chivers & Mirza 2001). A fifth treatment of distilled water was also included (n ranged from 37 to 47 per treatment). Predator odours were collected from predators (two of each species) held in 37 l aquaria for 24 hours. The pike and oscars ranged from 18.0 to 20.0 cm standard length.

The third predation experiment exposed fishes to a 0.4 nM concentration of hypoxanthine-3-*N*-oxide (H3NO, the putative ostariophysan fear substance; Brown *et al.* 2000),

the odour of pike fed minnows (5 ml) and a control of distilled water ($n=32-39$ per treatment). The H3NO was synthesized at Union College using the methods described by Brown *et al.* (2000). In this experiment, fishes were exposed to the cues daily not twice per week.

(b) Effect of body region and trematode infestation on distribution of percid club cells

Johnny darters (*E. nigrum*) were collected from the Otter Tail River, MN (46°22'44.85" N, 96°07'32.02" W) on 19 October 2001. They were maintained in 185 l aquaria at 18°C on a 12 L : 12 D light cycle and fed a diet of thawed adult brine shrimp. Fishes were overdosed with tricaine methane sulphate, weighed, measured and epidermal samples were taken from three regions of the body: nape, the dorsal surface between the head and the dorsal fin; flank, the region posterior to the dorsal fin centred about the lateral line; and bottom, the ventral surface of the caudal peduncle. Tissue sections were fixed in 10% formalin and histological sections were processed with PAS-H. For each skin sample ($n=32$), we scored epidermal thickness and the number of mucous and percid club cells per ocular area at 400× magnification. If club cells confer protection against UV exposure, which is highest on the dorsal surface, then we predicted that club cell density on different regions of the body would rank as nape > flank > bottom.

Uvulifer ambloplites (black spot disease) is a trematode parasite of fish-eating birds (kingfishers and herons) that use littoral fishes as second intermediate host (Lemly & Esch 1984). Cercaria released from snails penetrates the epidermis of a fish and encyst as metacercariae in the epidermis to await ingestion of the fish by a final host. Black spot disease is a convenient system for quantifying infestation because the metacercariae encysted in the epidermis are enveloped in melanin (hence 'black spot'), allowing parasite abundance (numbers of parasites per individual host) to be evaluated without dissection. We used a beach seine to collect 44 perch at the Itasca Biological Field Station (University of Minnesota) in June 2003 (47°13'34.65" N, 95°11'48.84" W). We sorted catch by parasite abundance: fewer than 25 black spots per side were classified as low mean abundance ($n=20$); 25–50 black spots per side as intermediate mean abundance ($n=14$); and perch with more than 50 black spots per side as high parasite abundance ($n=10$). Tissue was removed from the nape, flank and ventral side of the caudal peduncle. Tissue processing for histological examination was identical to that of darter tissue.

(c) Effect of trematodes on minnow club cells

In a laboratory experiment, we tested whether investment in club cells of FHM was influenced by the presence of metacercariae of the trematode *Teleorchis* sp. This species has been shown to penetrate the epidermis of minnows (Michalak 2006). The snails (*Physa acuta*) and minnows used in this study were collected from State Gamelands, Centre County, PA. The experiment followed the general methodology of the predation experiments. Methods used to expose individual FHM to known numbers of cercariae follow Sandland & Goater (2000). For a period of 16 days, 0, 10 or 70 cercariae originating from field-collected *P. acuta* were added to tanks with individual FHM every 4 days (n varied from 39 to 47 per treatment). At the end of the experiment, the minnows were euthanized and the number of alarm cells was scored as in the predation experiment in §2a.

(d) Effects of parasitic oomycetes (*Saprolegnia ferax* and *Saprolegnia parasitica*) on club cells

In laboratory experiments, we tested whether investment in club cells was influenced by the presence of water moulds (oomycetes of *S. ferax* and *S. parasitica*), which are common fish pathogens (Bruno & Wood 1994). FHM were the test species for these experiments. We used the same general methodology as in the predation experiments in §2a. Minnows were raised in 9.5 l aquaria and exposed to a control solution of dilute salts (DSA and DSB, recipe by Dill & Fuller (1971)) or various concentrations of infective cysts of *S. ferax* (ATCC 36051) or *S. parasitica* (ATCC 200013) for the 11 day duration of the experiments. The dilute salt solution was the carrier solution used to induce zoosporogenesis. The first *Saprolegnia* experiment employed a high dose of both *S. parasitica* and *S. ferax* (20 000 cysts l^{-1}) and a lower concentration of *S. parasitica* (2000 cysts l^{-1}). *Saprolegnia parasitica* is considered more infective than *S. ferax* (Beakes *et al.* 1994). The second experiment followed the same protocol as the first but used much lower concentrations of zoospores of *S. ferax* (20, 200 and 2000 cysts l^{-1}).

If alarm substance has an anti-pathogenic role, this function might be inhibited by immunosuppression. Certainly, fishes reared in crowded hatcheries and those living in polluted waters have relatively high disease incidence (Richards & Pickering 1978). To test this hypothesis, we stressed fishes for 11 days with environmentally relevant levels of Cd (0.564 and 5.64 $\mu g l^{-1}$; Jensen & Bro-Rasmussen 1992), a heavy metal that acts as an immunosuppressant in vertebrates (Sanchez-Dardon *et al.* 1999), simultaneously with a challenge of *S. ferax* (and controls without Cd). The experimental design followed the same protocol as the other *Saprolegnia* experiments using a concentration of 1900 zoospores per litre of *S. ferax*.

(e) Effects of fish skin extracts on growth of *S. ferax*

We determined whether growth of water mould was inhibited by skin extracts from FHM. We cultured *S. ferax* in the presence of skin extracts of minnows (with club cells), SWTs (without club cells) and a control of distilled water. Skin extracts were used at two concentrations: (i) high, 0.205 cm² of skin homogenized in 1.0 ml of distilled water and (ii) low, 0.0041 cm² of skin in 1.0 ml of water. *Saprolegnia ferax* was cultured on GY agar plates by inoculating the plates with a 2 mm³ of *S. ferax* taken from a master culture. Cultures were maintained at 18°C for 36 hours after which we measured the maximum linear dimension of the cultures to the nearest millimetre with a plastic ruler. All measurements were done blind.

(f) Effect of general injury to epidermis on proliferation of club cells

The purpose of this experiment was to evaluate whether general mechanical injury to the epidermis caused a proliferation of club cells. Our intention was to approximately simulate the penetration of free-swimming trematode cercariae. Yellow perch were collected by seine from Silver Lake, MN (46°49'56.71" N, 96°19'35.67" W) and transferred to two, 555 l holding tanks. Black spot disease occurs in this population of perch, but there was no significant difference in infestation rate between the treatment groups (mean \pm s.e. number of black spots per side: poked group = 11.1 \pm 3.6, control group = 13.2 \pm 3.6, $t_{29} = 0.41$, $p = 0.68$). Water temperature was approximately 20°C, photoperiod was 12 L : 12 D. Fishes were fed thawed adult brine shrimp ad libitum twice daily throughout the experiment. Fishes were

anaesthetized with tricaine methane sulphonate. Approximately half ($n=19$) of the fishes had their epidermal tissue lightly poked superficially with a sterile needle (29 gauge) 25 times on each side in the epaxial region of the flank below the spiny dorsal fin. The remaining fishes ($n=15$) were anaesthetized and handled in the same manner as the fishes in the experimental treatment, but not poked. Fishes were returned to separate holding tanks (one for each treatment group) to recover from the anaesthetic. This process was repeated twice more at 4 day intervals. Each time fishes were allowed to recover from the anaesthetic in their holding tanks and feed normally. On the 16th day, all fishes were killed with an overdose of tricaine methane sulphonate and epidermal samples of the epaxial flank region were taken for histological preparation as described for Johnny darters (§2*b*). Histological tissues were scored by an observer blind to the treatments and experimental question.

3. RESULTS

(a) Effect of predation risk on club cells

Despite the large sampling effort ($n=35$ – 47 per treatment in each of the three experiments), there were no significant differences in club cell number for minnows exposed to any of the different treatments in any of the three predation experiments (experiment 1: ANOVA: $F_{3,155}=0.818$, $p=0.486$; experiment 2: ANOVA: $F_{4,208}=1.559$, $p=0.187$; and experiment 3: ANOVA: $F_{2,104}=1.874$, $p=0.159$; figure 2).

(b) Effect of body region and trematode infestation on distribution of percid club cells

There was a significant difference among nape (N), flank (F) and bottom (B) body regions in the concentration of percid club cells in Johnny darters (ANOVA: $F_{2,71}=30.39$, $p<0.001$; figure 3). A similar distribution pattern among body regions was observed in yellow perch (figure 4*a–c*). These effects were independent of a positive correlation between percid club cell number and degree of parasitism (ANOVA: parasite $F_{2,114}=3.72$, $p=0.027$; NFB $F_{2,114}=38.49$, $p<0.001$; interaction $F_{4,114}=0.40$, $p=0.812$). Parasite load had no significant effect on number of mucous cells (ANOVA: parasite $F_{2,114}=1.04$, $p=0.357$; NFB $F_{2,114}=19.56$, $p<0.001$; interaction $F_{4,114}=0.43$, $p=0.785$) or epidermal thickness (ANOVA: parasite $F_{2,114}=2.06$, $p=0.131$; NFB $F_{2,114}=35.14$, $p<0.001$; interaction $F_{4,114}=0.10$, $p=0.983$).

(c) Effect of trematodes on minnow club cells

In the laboratory experiment, we observed that *Teleorchis* sp. cercariae penetrated the skin of fishes in both the low and high trematode treatments, but there were no trematodes in fishes from the control treatment. An ANOVA revealed that at the end of the 16 day experiment, the number of club cells in the epidermis of the minnows was significantly greater among fishes in the high trematode treatment than the low trematode and control treatments ($F_{2,118}=7.473$, $p<0.001$; figure 5).

(d) Effects of parasitic oomycetes (*S. ferax* and *S. parasitica*) on minnow club cells

Club cell number was greater in minnows challenged with the infective stage of water mould than those given the control solution (ANOVA: $F_{3,154}=9.053$, $p<0.001$).

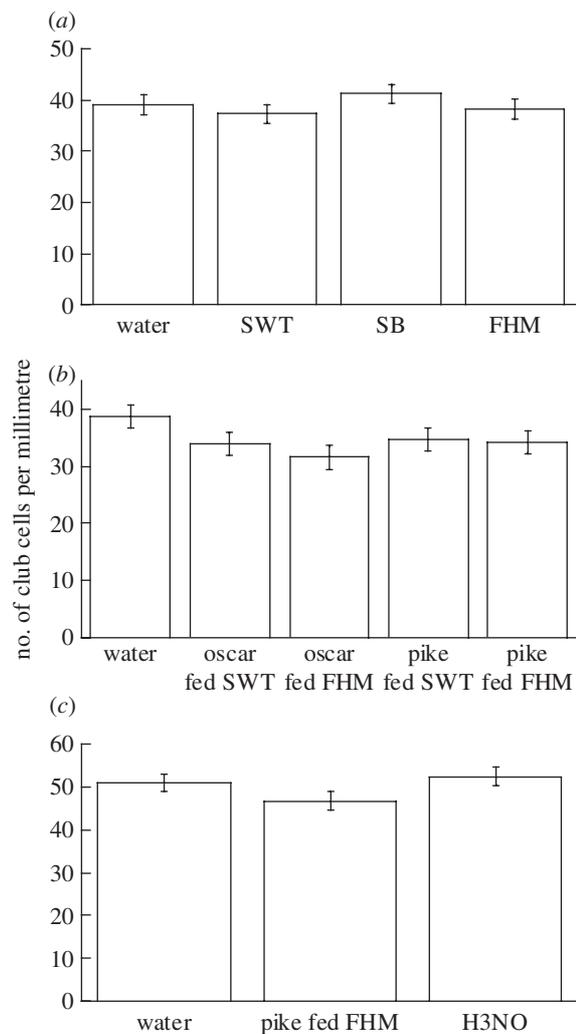


Figure 2. Mean \pm s.e. number of club cells per millimetre section for minnows raised under different risks of predation. (a) Fishes were exposed to a control of distilled water or skin extracts of FHM, SB or SWT. (b) Fishes were exposed to distilled water or cues of oscars or pike fed FHM or SB. (c) Fishes were exposed to distilled water, cues of pike fed FHM or a solution of hypoxanthine-3(*N*)-oxide (H3NO). See text for details of experiments.

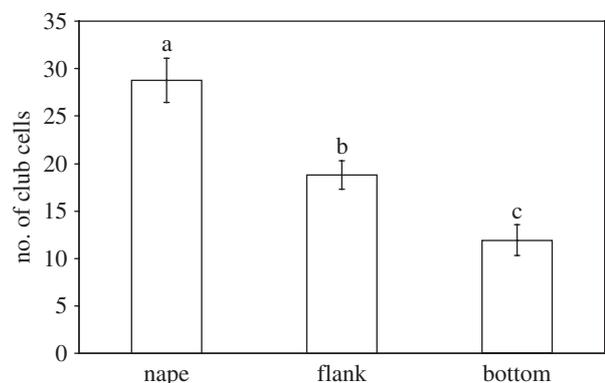


Figure 3. Mean \pm s.e. number of percid club cells in the epidermis of Johnny darters per ocular diameter ($400\times$) for tissues sampled from three regions of the body: nape, flank and bottom. Different letters over bars indicate significant differences based on Tukey *post hoc* comparisons ($p<0.05$).

Interestingly, there was no difference in the number of cells for fishes exposed to *S. ferax* or *S. parasitica* or those exposed to the low and high concentration treatments (figure 6*a*).

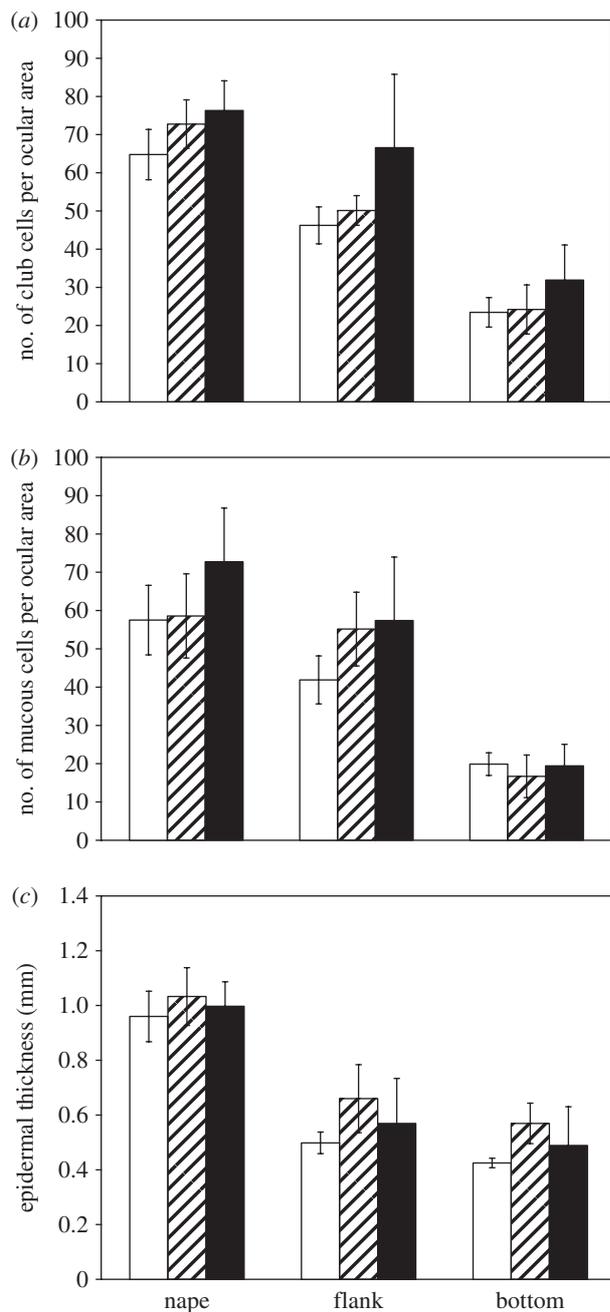


Figure 4. Epidermal quality of yellow perch for tissues samples from three regions of the body: nape, flank and bottom. Bar fill indicates level of infestation by black spot metacercaria: open bars, low parasitism; hatched bars, intermediate; filled bars, heavy infestation. (a) Mean \pm s.e. number of percid club cells in skin of yellow perch per ocular area ($400\times$). (b) Mean \pm s.e. number of mucous cells in skin of yellow perch per ocular area ($400\times$). (c) Mean \pm s.e. epidermal thickness (mm) of skin of yellow perch ($400\times$).

Minnows challenged with the infective stage of water mould had a greater number of alarm cells than fishes in the control treatments (ANOVA: $F_{3,122}=11.75$, $p<0.001$). Again, there was no difference in the number of cells among the various concentration treatments, indicating that investment in club cells is not graded to the level of cysts, but rather is a threshold response (figure 6b).

If the presence of *Saprolegnia* cysts induced club cell investment by minnows, then this could be an immune-mediated response. Consequently, we treated fishes with a

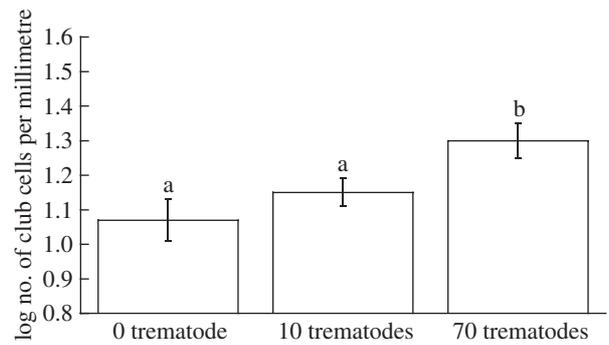


Figure 5. Mean (\pm s.e.) of log[club cell number] per millimetre section for minnows raised in the presence of larval trematodes. The experiment was 16 days in length and 0, 10 or 70 cercariae (family Teleorchidae) were added to the containers every 4 days (sample sizes varied from 35 to 46 per treatment). Different letters over bars indicate significant differences based on Tukey *post hoc* comparisons ($p<0.05$).

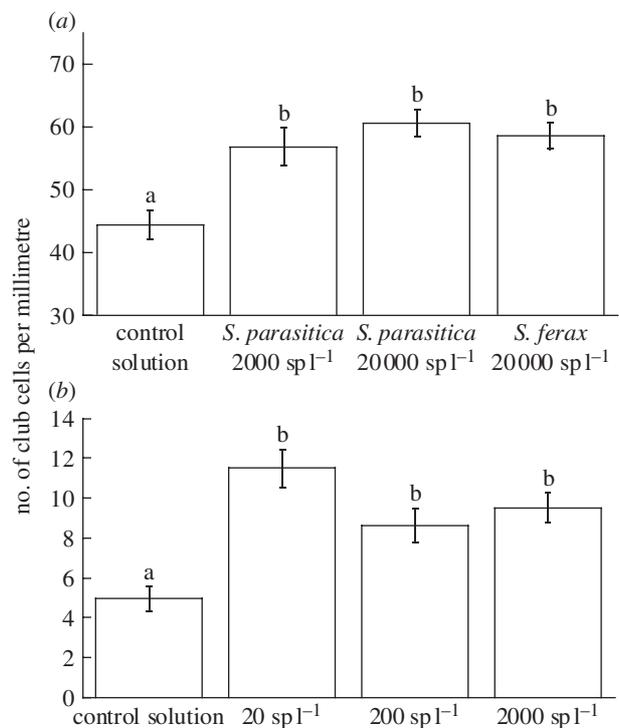


Figure 6. Mean (\pm s.e.) number of club cells per millimetre section for minnows raised in the presence of water moulds. (a) Minnows raised for 16 days in the presence of water moulds (*S. ferax* and *S. parasitica*) or a solution of dilute salts (DSA and DSB). (b) Minnows raised for 14 days in the presence of 20, 200 or 2000 *S. ferax* zoospores or a solution of dilute salts (control). Different letters over bars indicate significant differences based on Tukey *post hoc* comparisons ($p<0.05$).

known immunosuppressant, Cd (Sanchez-Dardon *et al.* 1999). The final experiment examining the effects of *Saprolegnia* on club cell investment provides evidence of a link with immune function (figure 7). Results of the 2×3 ANOVA indicated a significant interaction between the presence of cysts and Cd level ($F_{2,202}=15.104$, $p<0.001$). At high levels of Cd, test fishes were no longer able to increase production of club cells in response to the zoospore challenge.

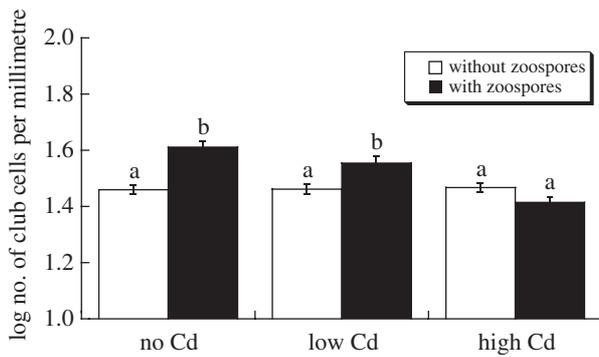


Figure 7. Mean (\pm s.e.) of log[club cell number] per millimetre section for minnows raised for 14 days under two concentrations of Cd (0.564 and $5.64 \mu\text{g l}^{-1}$) in the presence of *S. ferax* or a distilled water control. Different letters over bars indicate significant differences at $p < 0.01$.

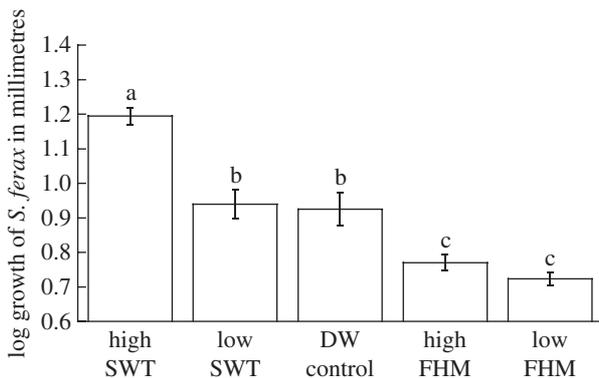


Figure 8. Mean (\pm s.e.) of the log size (mm) of *S. ferax* cultures grown on GY agar plates in the presence of skin extracts of FHM (with club cells), SWT (without club cells) and distilled water (DW, $n = 10$ – 14 per treatment). Different letters over bars indicate significant differences based on Tukey *post hoc* comparisons ($p < 0.05$).

(e) Effects of fish skin extracts on growth of *S. ferax*

When compared with the distilled water control treatment, the presence of minnow skin extract reduced *Saprolegnia* growth, whereas SWT skin extract increased *Saprolegnia* growth in comparison to the control treatments (ANOVA: $F_{4,58} = 85.461$, $p = 0.001$; figure 8).

(f) Effect of general injury to epidermis on proliferation of club cells

There was no significant response of perch epidermis to the experimental penetration injury (mucous cell count: $t_{32} = 1.21$, $p = 0.235$; percid club cell count: $t_{32} = 1.44$, $p = 0.161$; epidermal thickness: $t_{32} = 0.75$, $p = 0.460$; table 1).

4. DISCUSSION

Our data suggest that epidermal club cells in fishes of the Ostariophysi and Acanthopterygii may be maintained by natural selection owing to protection they confer against pathogens, parasites and UV radiation as agents that compromise the integrity of the epidermal layer. A significant element of this argument is the apparent parallel evolution of similar cells within two major and independent lineages of fishes. Cells of similar histological characteristics have also been reported in other fish groups

Table 1. Mean (s.e.) number of mucous cells, club cells and mean epidermal thickness (mm) in skin samples of yellow perch collected from the nape region. (Half of the perch received needle pokes (under anaesthesia) to injure the epidermis. Control fishes received no pokes. *t*-test showed no significant difference in any measure of epidermal quality ($p > 0.05$).

	mucous cells	club cells	epidermal thickness
injured	138.05 (6.3)	6.63 (0.8)	1.78 (0.1)
control	148.36 (5.2)	5.27 (0.4)	1.67 (0.1)

such as the Poeciliidae, Eleotridae, Centrarchidae and Cottidae (Wisenden & Chivers 2006 and references therein). Studies directly testing for a protective function of club cells are needed.

Club cells are in the surface layers of the epidermis where they may serve as a first line of defence to protect underlying tissues. Owing to this positioning, they are preadapted to be also the first cells ruptured by predatory attack. There is strong selection on receivers to respond adaptively to chemical compounds released from injured conspecifics (Chivers & Smith 1998; Wisenden & Chivers 2006). However, responses of receivers do not probably result in a fitness benefit to alarm cue senders with a frequency sufficient to maintain club cells over evolutionary time and across such an ecologically diverse array of freshwater fish taxa.

Taken together, these data suggest that the alarm function of club cells evolved secondarily. We submit that epidermal injury from pathogens and parasites is ubiquitous and universal. Moreover, exposure to UVB radiation is of major concern for fishes living near the surface. These conditions set the stage for evolutionary innovation of counter measures in the form of club cells to ameliorate fitness costs associated with injury. This evolutionary scenario presents a much steeper selection gradient than fitness benefits associated with the attraction of secondary predators (Mathis *et al.* 1995; Chivers *et al.* 1996) and other hypotheses premised on an anti-predator evolutionary origin for club cells (Smith 1992). Once present, the contents of club cells secondarily assumed the role of an alarm cue (in the context of public information; Wisenden & Stacey 2005; Wisenden & Chivers 2006). Abundant laboratory and field evidence demonstrates the results of past selection on nearby receivers, which has led to an adaptive response to the presence of these public chemical cues. Prey species perceive predation risk (Chivers & Smith 1998), while predators perceive an opportunity for piracy (Mathis *et al.* 1995; Chivers *et al.* 1996).

We were not able (§2f) to experimentally demonstrate a causal link between general epidermal injury and club cell production. We predicted that if club cells respond to general epidermal injury, then perch with a recent history of injury would have more club cells than those without that history. The results were in the predicted direction, indicating that we should be cautious about strongly concluding that general injury plays no role in triggering production of club cells. It may be the case that injury *per se* is only part of the stimulus required to stimulate club cell production. Additional chemical cues from the causative agent (pathogen, parasite, etc.) may be required

to stimulate adaptive shifts in the chemistry within the club cells.

Club cells of some fish species contain chondroitin and keratins (Ralphs & Benjamin 1992; Zaccone *et al.* 1999), which could help in repair of damaged tissue (but see Iger & Abraham (1990)). Hypoxanthine-3(*N*)-oxide was named as the putative active ingredient of ostariophysan alarm cells by Argentini (1976; after Pfeiffer 1982) and subsequent work confirmed its ability to induce alarm behaviour (Pfeiffer *et al.* 1985; Brown *et al.* 2000). In contrast, the most biologically active fractions from gel chromatography of minnow skin extracts show that components that elicit fright reactions are large molecules with molecular weights of approximately 1100 Da and with weights greater than 1500 Da (Lebedeva *et al.* 1975; Kasumyan & Ponomarev 1987). Compounds with smaller molecular weights (350–550 Da), where free hypoxanthine-3(*N*)-oxide would resolve, did not induce alarm behaviour in experimental trials (Lebedeva *et al.* 1975; Kasumyan & Ponomarev 1987). The evolution of cellular chemistry that best defends skin against pathogens, parasites and UV radiation may well occur independently of selection (albeit relatively weaker) for cellular chemistry that is most effectively detected by nearby conspecifics and predators. Basic chemical characterization of the contents of club cells is currently lacking. This knowledge would shed light on club cell functions and may potentially open new research avenues for pharmaceutical applications against disease agents or UV radiation.

The mechanism(s) by which club cells inhibit pathogens and parasites remains to be determined. However, given the immunosuppressive nature of heavy metals, there is a clear link with the immune system. Susceptibility to skin infections such as *Saprolegnia* is increased for fishes that have been subjected to environmental stress (Bruno & Wood 1994). Likewise, the interaction between FHM and cercariae of one of their common trematodes is strongly affected by low concentrations of cadmium (Pietroock & Goater 2005). There is little doubt that stressors (UVB, pollution and parasites) are increasing due to global environmental changes. For example, decreases in stratospheric ozone increase exposure to UVB radiation (Blaustein *et al.* 1997; Rex *et al.* 2004), emerging diseases are of increasing local and global importance (Kiesecker *et al.* 2004), and environmental contamination increases exposure to pollutants, including heavy metals (Jensen & Bro-Rasmussen 1992). Our data demonstrate that the club cells of fishes may provide a defence against epidermal damage, but that this defence may be compromised by the presence of environmental stressors such as cadmium. The nature and extent of the protection provided by the cells should become a fascinating area of future research.

Behavioural ecologists have often reported considerable variation in the intensity of anti-predator responses of fishes to alarm cues. Under some conditions, fishes do not appear to exhibit anti-predator responses at all (Magurran *et al.* 1996), while in other cases, they show stronger intensity anti-predator responses to increasing concentrations of alarm cues (Ferrari *et al.* 2005; Ferrari & Chivers 2006). The increase in response intensity has always been interpreted in an anti-predator context. Our data lead us to question whether the intensity of response to increasing concentrations of cues could also be

interpreted in a disease context. A high concentration of cues may indicate damage from a conspecific in close proximity. Such damaged prey may pose a significantly greater risk of disease than those at a distance. Perhaps the intensity of responses to cues of injured conspecifics reflects selection on receivers to avoid both predators and disease.

In summary, these data resolve a long-standing problem in evolutionary ecology (Williams 1992). Individual ostariophysan and percid fishes benefit from investing in club cells owing to the protective function the cells provide. These cells are ruptured as a natural by-product of predation and subsequent selection on nearby receivers has driven the development of receptors and responses to these 'alarm cues' that reduce the probability of predation.

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REFERENCES

- Al-Hassen, J. M., Thompson, M., Criddle, K. R., Summers, B. & Criddle, R. S. 1985 Catfish epidermal secretions in response to threat or injury: a novel defense response. *Mar. Biol.* **88**, 117–123. (doi:10.1007/BF00397158)
- Beakes, G. W., Wood, S. E. & Burr, A. W. 1994 Features which characterize *Saprolegnia* isolates from salmon fish lesions—a review. In *Salmon saprolegniasis* (ed. G. J. Mueller), pp. 33–66. Portland, OR: U.S. Department of Energy, Bonneville Power Administration.
- Blaustein, A. R., Kiesecker, J. M., Chivers, D. P. & Anthony, R. G. 1997 Ambient UV-B radiation causes deformities in amphibian embryos. *Proc. Natl Acad. Sci. USA* **94**, 13 735–13 737. (doi:10.1073/pnas.94.25.13735)
- Blazer, V. S., Fabacher, D. L., Little, E. E., Ewing, M. S. & Kocan, K. M. 1997 Effects of ultraviolet-B radiation on fish: histologic comparison of a UVB-sensitive and a UVB-tolerant species. *J. Aquat. Anim. Health* **9**, 132–143. (doi:10.1577/1548-8667(1997)009<0132:EOUBRO>2.3.CO;2)
- Brown, G. E., Adrian Jr, J. C., Smyth, E., Leet, H. & Brennan, S. 2000 Ostariophysan alarm pheromones: laboratory and field tests of the functional significance of nitrogen oxides. *J. Chem. Ecol.* **26**, 139–154. (doi:10.1023/A:1005445629144)
- Bruno, D. W. & Wood, B. P. 1994 *Saprolegnia* and other oomycetes. In *Fish diseases and disorders*, vol. 3 (eds P. T. K. Woo & D. W. Bruno). Viral, bacterial and fungal infections, pp. 599–659. Wallingford, UK: CABI Publishing.
- Chivers, D. P. & Mirza, R. S. 2001 Predator diet cues and the assessment of predation risk by aquatic vertebrates: a review and prospectus. In *Chemical signals in vertebrates*, vol. 9 (eds A. Marchlewska-Koj, J. J. Lepri & D. Müller-Schwarze), pp. 277–284. New York, NY: Plenum Press.

- Chivers, D. P. & Smith, R. J. F. 1994 The role of experience and chemical alarm signalling in predator recognition by fathead minnows *Pimephales promelas*. *J. Fish Biol.* **44**, 273–285. (doi:10.1111/j.1095-8649.1994.tb01205.x)
- Chivers, D. P. & Smith, R. J. F. 1998 Chemical alarm signalling in aquatic predator–prey systems: a review and prospectus. *Ecoscience* **5**, 338–352.
- Chivers, D. P., Brown, G. E. & Smith, R. J. F. 1996 Evolution of chemical alarm signals: attracting predators benefits alarm signal senders. *Am. Nat.* **148**, 649–659. (doi:10.1086/285945)
- Chivers, D. P., Zhao, X., Brown, G. E., Marchant, T. A. & Ferrari, M. C. O. In press. Predator-induced changes in morphology of a prey fish: the effects of food level and temporal frequency of predation risk. *Evol. Ecol.* (doi:10.1007/S10682-007-9182-8)
- Danchin, É., Giraldeau, L.-A., Valone, T. J. & Wagner, R. H. 2004 Public information: from nosy neighbors to cultural evolution. *Science* **305**, 487–491. (doi:10.1126/science.1098254)
- Dill, B. C. & Fuller, M. S. 1971 Amino acid immobilization of fungal motile cells. *Arch. Mikrobiol.* **78**, 92–98. (doi:10.1007/BF00409091)
- Ferrari, M. C. O. & Chivers, D. P. 2006 Learning threat-sensitive predator avoidance: how do fathead minnows incorporate conflicting information? *Anim. Behav.* **71**, 19–26. (doi:10.1016/j.anbehav.2005.02.016)
- Ferrari, M. C. O., Trowell, J. J., Brown, G. E. & Chivers, D. P. 2005 The role of learning in the development of threat-sensitive predator avoidance in fathead minnows. *Anim. Behav.* **70**, 777–784. (doi:10.1016/j.anbehav.2005.01.009)
- Ferrari, M. C. O., Gonzalo, A., Messier, F. & Chivers, D. P. 2007 Generalization of learned predator recognition: an experimental test and framework for future studies. *Proc. R. Soc. B* **274**, 1853–1859. (doi:10.1098/rspb.2007.0297)
- Iger, Y. & Abraham, M. 1990 The process of skin healing in experimentally wounded carp. *J. Fish Biol.* **36**, 421–437. (doi:10.1111/j.1095-8649.1990.tb05622.x)
- Jensen, A. & Bro-Rasmussen, F. 1992 Environmental cadmium in Europe. *Rev. Environ. Contam. Toxicol.* **125**, 101–181.
- Kasumyan, A. O. & Ponomarev, V. Y. 1987 Biochemical features of alarm pheromone in fish of the order Cypriniformes. *J. Evol. Biochem. Physiol.* **23**, 20–24.
- Kiesecker, J. M., Belden, L. K., Shea, K. & Rubbo, M. J. 2004 Amphibian decline and emerging diseases. *Am. Sci.* **92**, 138–147. (doi:10.1511/2004.2.138)
- Lebedeva, N. E., Malyukina, G. A. & Kasumyan, A. O. 1975 The natural repellent in the skin of cyprinids. *J. Ichthyol.* **15**, 472–480.
- Lemly, A. D. & Esch, G. W. 1984 Effects of the trematode *Uvulifer ambloplites* on juvenile bluegill sunfish, *Lepomis macrochirus*: ecological implications. *J. Parasitol.* **70**, 475–492. (doi:10.2307/3281395)
- Magurran, A. E., Irving, P. W. & Henderson, P. A. 1996 Is there a fish alarm pheromone? A wild study and critique. *Proc. R. Soc. B* **263**, 1551–1556. (doi:10.1098/rspb.1996.0227)
- Mathis, A. & Smith, R. J. F. 1993 Chemical labeling of northern pike (*Esox lucius*) by the alarm pheromone of fathead minnows (*Pimephales promelas*). *J. Chem. Ecol.* **19**, 1967–1979. (doi:10.1007/BF00983800)
- Mathis, A., Chivers, D. P. & Smith, R. J. F. 1995 Chemical alarm signals: predator-deterrents or predator attractants? *Am. Nat.* **146**, 994–1005. (doi:10.1086/285780)
- Michalak, T. 2006 The effects of pathogens, parasites and familiarity on alarm cell investment in fathead minnows, *Pimephales promelas*. MSc thesis, University of Saskatchewan.
- Nelson, J. S. 1994 *Fishes of the world*, 4th edn. New York, NY: Wiley-Interscience.
- Pfeiffer, W. 1982 Chemical signals in communication. In *Chemoreception in fishes*, vol. 8 (ed. T. J. Hara). Developments in aquaculture and fisheries science, pp. 307–326. New York, NY: Elsevier.
- Pfeiffer, W., Riegelbauer, G., Meir, G. & Scheibler, B. 1985 Effect of hypoxanthine-3(*N*)-oxide and hypoxanthine-1(*N*)-oxide on central nervous excitation of the black tetra *Gymnocorymbus ternetzi* (Characidae, Ostariophysi, Pisces) indicated by dorsal light response. *J. Chem. Ecol.* **11**, 507–524. (doi:10.1007/BF00989562)
- Pietroock, M. & Goater, C. P. 2005 Infectivity of *Ornithodiplostomum ptychocheilus* and *Posthodiplostomum minimum* (Trematoda: Diplostomidae) cercariae following exposure to cadmium. *J. Parasitol.* **91**, 854–856. (doi:10.1645/GE-473R.1)
- Pollock, M. S., Chivers, D. P., Mirza, R. S. & Wisenden, B. D. 2003 Fathead minnows learn to recognize chemical alarm cues of introduced brook stickleback. *Environ. Biol. Fish.* **66**, 313–319. (doi:10.1023/A:1023905824660)
- Ralphs, J. R. & Benjamin, M. 1992 Chondroitin and keratan sulfate in the epidermal club cells of teleosts. *J. Fish Biol.* **40**, 473–475. (doi:10.1111/j.1095-8649.1992.tb02594.x)
- Rex, M., Salawitch, R. J., von der Gathen, P., Harris, N. R. P., Chipperfield, M. P. & Naujokat, B. 2004 Arctic ozone loss and climate change. *Geophys. Res. Lett.* **31**, L04116. (doi:10.1029/2003GL018844)
- Richards, R. H. & Pickering, A. D. 1978 Frequency and distribution patterns of *Saprolegnia* infection in wild and hatchery-reared brown trout *Salmo trutta* L. and char *Salvelinus alpinus* (L.). *J. Fish Dis.* **1**, 69–82. (doi:10.1111/j.1365-2761.1978.tb00006.x)
- Sanchez-Dardon, J., Voccia, I., Hontela, A., Chilmonczyk, S., Dunier, M., Boermans, H., Blakley, B. & Fournier, M. 1999 Immunomodulation by heavy metals tested individually or in mixtures in rainbow trout (*Oncorhynchus mykiss*) exposed *in vivo*. *Environ. Toxicol. Chem.* **18**, 1492–1497. (doi:10.1897/1551-5028(1999)018<1492:IBHMTI>2.3.CO;2)
- Sandland, G. T. & Goater, C. P. 2000 Development and intensity-dependence of *Ornithodiplostomum ptychocheilus* metacercariae in fathead minnows (*Pimephales promelas*). *J. Parasitol.* **86**, 1056–1060.
- Smith, R. J. F. 1992 Alarm signals in fishes. *Rev. Fish Biol. Fish.* **2**, 33–63. (doi:10.1007/BF00042916)
- Von Frisch, K. 1941 Über einen schreckstoff der fischhaut und seine biologische bedeutung. *Z. Vergl. Physiol.* **29**, 46–149. (doi:10.1007/BF00304445)
- Williams, G. C. 1992 *Natural selection: domains, levels, and challenges*. Oxford, UK: Oxford University Press.
- Wisenden, B. D. 2003 Chemically-mediated strategies to counter predation. In *Sensory processing in the aquatic environment* (eds S. P. Collin & N. J. Marshall), pp. 236–251. New York, NY: Springer.
- Wisenden, B. D. & Chivers, D. P. 2006 The role of public chemical information in antipredator behaviour. In *Fish communication* (eds F. Ladich, S. P. Collins, P. Moller & B. G. Kapoor), pp. 259–278. Enfield, NH: Science Publishers.
- Wisenden, B. D. & Smith, R. J. F. 1997 The effect of physical condition and shoal-mate familiarity on proliferation of alarm substance cells in the epidermis of fathead minnows. *J. Fish Biol.* **50**, 799–808. (doi:10.1111/j.1095-8649.1997.tb01973.x)

- Wisenden, B. D. & Smith, R. J. F. 1998 A re-evaluation of the effect of shoalmate familiarity on the proliferation of alarm substance cells in fathead minnows. *J. Fish Biol.* **53**, 841–846. (doi:10.1111/j.1095-8649.1998.tb01837.x)
- Wisenden, B. D. & Stacey, N. E. 2005 Fish semiochemicals and the evolution of communication networks. In *Animal communication networks* (ed. P. K. McGregor), pp. 540–567. Cambridge, UK: Cambridge University Press.
- Wisenden, B. D. & Thiel, T. A. 2002 Field verification of predator attraction to minnow alarm substance. *J. Chem. Ecol.* **28**, 433–438. (doi:10.1023/A:1017950628284)
- Zaccone, G., Ainis, L., Fasulo, S., Mauceri, A., Lauriano, E. R., Licata, A. & Cascher, P. L. 1999 Paraneurons in the skin epithelium of the fishes. In *Water/air transition in biology* (eds A. K. Mittal, F. B. Eddy & J. S. Datta Munshi), pp. 188–213. New Delhi, India: Oxford and IBH Publishing.