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ORIGINAL PAPER

Socializing makes thick-skinned individuals: on the density of epidermal alarm substance cells in cyprinid fish, the crucian carp (*Carassius carassius*)

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Abstract In cyprinid fish, density of epidermal club cells (i.e. alarm substance cells) has been found to vary between lakes with different predator fauna. Because predators can be labelled with chemical cues from prey, we questioned if club cell density could be controlled indirectly by predators releasing prey cues. In particular, we suspected a possible feedback mechanism between chemical alarm signals and their cellular source. We raised crucian carp singly and in groups of four. For both rearing types, fish were exposed to skin extracts of either conspecifics or brown trout (without club cells), and provided either low or high food rations. Independent of rearing type, condition factor and club cell density increased with food ration size, but no change was found in club cell density following exposure to conspecific alarm signals. However, the density of club cells was found significantly higher for fish raised in groups than for fish raised alone. We conclude that an increased condition factor results in more club cells, but crucian carp may also possess an awareness of conspecific presence, given by higher club cell densities when raised in groups. This increase in club cell density may be induced by unknown chemical factors released by conspecifics.

Keywords Chemical alarm signals · Club cells · Inducible defences · Growth · Predation

Introduction

Cyprinid fishes display fright behaviour when smelling alarm signals released by injured conspecifics (von Frisch 1938, 1941). The chemical alarm signals are assumed to be present in special club cells of the epidermis, commonly denoted as 'alarm substance' cells, and are released involuntarily when cells are being ruptured (Pfeiffer 1960, 1962). Club cells are found in most fishes of the superorder Ostariophysi, and those species possessing club cells also display a behavioural fright reaction (Pfeiffer 1977). The structure of club cells has been studied in detail (Pfeiffer et al. 1971; Whitear and Mittal 1983), and comparison of autofluorescence emission spectra of club cells with spectra of isolated alarm substance has provided circumstantial evidence for club cells being the source of alarm substance (Reutter and Pfeiffer 1973).

Club cells are subjected to seasonal variation through mechanisms of endocrine regulation. For instance, it has been reported that skin extract of male North-American fathead minnows (*Pimephales promelas*) loses its fright-inducing properties at the onset of spring breeding season and regains it in the fall. Loss of fright-inducing properties was also obtained when males were treated with testosterone outside the breeding season, and a significant reduction in club cell density was found in testosterone-treated fish (Smith 1973). The seasonal decline in club cell density has been attributed to abrasive spawning behaviour because males which try to clean a spawning site by rubbing against it may unintendedly release alarm signals (Smith 1976). However, males of the fathead minnow retain their fright reaction to alarm substance during the breeding season despite their own seasonal loss of club cells (Smith 1976).

Chemical alarm signals are by definition pheromones (Karlson and Lüscher 1959), and pheromones are known to express both direct (releaser) and long time (primer) effects (Wilson and Bossert 1963). In nature, prey fish possessing club cells may detect predator presence by the use of

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conspecific alarm signals because predators are being chemically labelled with alarm signals when ingesting prey (Mathis and Smith 1993a). However, predators labelled with prey chemical cues may also affect prey over time by inducing morphological changes in prey conspecifics. In aquatic organisms, such predator-induced changes (i.e. inducible defences) may be expressed in many ways (Harvell 1990; Tollrian and Harvell 1999), and pheromones known to induce releaser effects should therefore be regarded as potential candidates for expressing primer effects.

In crucian carp (*Carassius carassius*), predator-induced morphology changes (i.e. a body depth increase) have been demonstrated when predator fishes, like pike (*Esox lucius*) or perch (*Perca fluviatilis*), have ingested the prey of study (Brönmark and Miner 1992; Brönmark and Pettersson 1994). Similar changes in body depth have been found in crucian carp as well as goldfish (*Carassius auratus*) when exposed to filtered extracts of conspecific skin (Stabell and Lwin 1997; Chivers et al. 2008). For crucian carp in particular, the classical alarm signals were initially suspected as the active inducers (Brönmark and Pettersson 1994; Stabell and Lwin 1997, but recent studies have revealed that those components in fish skin do not produce such changes (Stabell et al., unpublished data).

In cyprinids, further, it has been found that fathead minnows from regions with different predator fauna display a lower club cell density with increasing predator presence (Hugie 1989; referred by Smith 1992), but no explanation for this inverse relationship was given. In any case, the mentioned finding indicates that club cell density is plastic with regard to predator presence, and led us to hypothesize that club cell density could in some way be modified by chemical alarm signals. Because predators may be labelled with chemical alarm signals from prey (e.g. Mathis and Smith 1993a), the indirect presence of alarm signals from ingested prey may therefore theoretically alter club cell density in conspecifics.

The condition factor of fish increases with increased availability of food, and in fathead minnows epidermal thickness and club cell density are found to increase in fish on a high compared to a low food ration (Wisenden and Smith 1997). Accordingly, a variation in food ration should be included in the experimental design when testing for possible effects of alarm signals on club cell density.

We wanted to explore if club cell density in the epidermis of a cyprinid fish will change during long-time exposure to conspecific alarm signals. In accordance with current knowledge, we carried out an experiment where the amount of food and the presence of alarm signals were expressed in a two-factorial design. We raised fish four by four in triplicate aquaria, but also carried out a parallel series of experiments in individual containers for comparison.

Materials and methods

Origin and capture of fish

Crucian carp used in the experiments were captured in the Springvannsdammen pond, sited within the city borders of Arendal, East-Agder County, Norway (58°31′N; 8°46′E). The pond has a surface area of 0.08 ha, and contains crucian carp as the only fish species. Fish were captured with traps that were made from large polycarbonate water bottles (18.9 l) with canned corn as bait, and subsequently transported to the laboratory.

Fish used in the growth experiments were anaesthetized (50 mg/l benzocaine in tap water), weighed to the nearest 0.1 g, photographed with a digital camera against a background millimetre grid, and distributed to their rearing tanks. Photos and weight were later used for length measurements and morphometric calculations, as well as individual identification of fish reared in groups by the use of body length and scale patterns.

The fish used to obtain the number of epidermal cells at the start of the experiment were killed by an overdose of anaesthesia and preserved in 10% buffered formalin. Surplus fish to be used as donors for preparing skin extracts were killed by a blow to the head, and frozen at −20°C until use. Brown trout (*Salmo trutta*) from a commercial hatchery were brought live to the laboratory, killed by a blow to the head, and frozen at −20°C until use as skin donors for control skin extracts. Fish of the family Salmonidae have been found not to possess epidermal club cells or display fright behaviour (Pfeiffer 1977), and skin extracts of brown trout do not release fright behaviour in crucian carp (M.M. Durajczyk and O.B. Stabell, unpublished).

Preparation of skin extracts

The procedure used for making skin extract was improved from an earlier method (Pfeiffer 1962; Stabell and Lwin 1997; Chivers et al. 2008) by using centrifugation instead of filtering of homogenates. This improved method was applied to both crucian carp and brown trout skin. Lightly thawed fish were used to obtain a skin layer devoid of flesh. Incision was made behind the gills and along the dorsal and ventral edges on each side, and approximately 2.8 g of skin was peeled off from each species using forceps. The skin from each species was homogenized in 100 ml of tap water in a Waring blender, centrifuged at 2,500 rpm, and two-third of the supernatant was diluted to 3.15 l in tap water.

The diluted supernatants were then transferred to ice-cube bags and frozen at −20°C. Freezing has previously been found not to affect the fright-releasing properties of chemical alarm signals in fish (Lawrence and Smith 1989; Mathis and Smith 1993a, b.

Experimental design and rearing

In the rearing experiments, crucian carp were raised either alone or in groups of four for 50 days at 18–20°C and at a 16 L:8 D photoperiod. Fish reared alone were raised in 5 l plastic buckets (polypropylene), containing water treated with 0.5 ml/l Aquasafe (TetraWerke, Melle, Germany) and aerated by air-stones. Six crucian carp were used in each series of single-raised fish. In the group rearing, the fish were raised in glass aquaria (20 l), with similar Aquasafe and air-stone treatment. Triplicate aquaria were used in each series for the group rearing. A substrate of gravel (3–5 mm size) was used in both type of rearing compartments.

For both rearing methods, two parallel exposure series were used. In one series, conspecific skin extract was added as frozen ice cubes three times a week, while frozen skin extract of brown trout was used for the control series. Each time, one ice-cube of approximately 13 ml was added per fish, i.e. per 5 l of rearing water. Once a week, bottom detritus in buckets and aquaria was removed by siphoning one-third of the water volume, and replacing it with fresh Aquasafe-treated water before adding new ice-cubes.

For all rearing methods and exposure types, double sets were run, supplying fish with either a low (0.03 g/fish/day) or a high (0.1 g/fish/day) food ration per day. A mixture of commercial fish food (Tetra AniMin Goldfish Colour, TetraWerke) and rolled oats in the ratio 1:1 by weight was used, representing approximately 1 and 3% of average fish biomass per day. A randomized block-design was used between treatments in the distribution of rearing compartments both for the single and the group rearing. At the end of the experiment, all fish were killed by an overdose of anaesthesia, weighed, photographed and conserved in 10% buffered formalin for subsequent skin histology studies. Two fish from each of the group rearing aquaria were randomly chosen for further histology work to equalize the number of fish from each treatment in single rearing (i.e. $n = 6$ for all treatment groups).

Histology preparations and morphometric analysis

Epidermal samples, including a portion of the underlying adherent muscle tissue, were dissected from the dorsal surface of formalin-fixed fish. Each piece of tissue was taken from between the dorsal fin and the head, from the dorsal midline and laterally on both sides according to established procedures (Wisenden and Smith 1997; Chivers et al. 2007). Depending on the fish size, the pieces were ranging between 3 and 6 mm in length and depth. The formalin-fixed tissues were subsequently dehydrated and embedded in paraffin wax following standard procedure.

Histological transverse sections of approximately 3 μm were cut, and every tenth section was placed onto slides to avoid overlapping histological areas. Totally three sections from each sample were prepared with 1% periodic acid for 10 min, thoroughly rinsed with running tap water followed by incubation in Schiff ‘s reagent for 20 min. After rinsing in tap water, the sections were counterstained with Mayer’s haematoxylin.

All sections were observed using an Axioskop MOT light microscope (Zeiss, Germany). A Delta Pix 450 camera integrated with the microscope was used to capture digitized images. Images for quantification of the number of club cells were captured from a 3–6-mm length of skin, depending on the size of the fish. The portion of epidermis covering the area centred above the thick fat layer in the dorsal intermyal septum contained very few club cells (between arrows, Fig. 1a). This part of the epidermis also coincided with an area of especially high concentration of mucus cells. Accordingly, this area was not included in the analysis of club cell density. The club cells were not uniformly dispersed along the sides of the dorsal intermyal septum, but showed minor variations in distribution over the epidermis (below arrows, Fig. 1a). Similarly, thickness of the epidermis displays minor variations over the scales (e.g. Strüssmann et al. 1994). Therefore, 6–10 images were taken from each section, depending on the size of the fish. The number of club cells was scored, and epidermal thickness was measured on all image acquisitions within each of the three sections. Average values for the three sections were then calculated for both parameters. Length and thickness of the epidermis was measured using ImageJ image analysis software (National Institutes of Health, USA). The image analysis was carried out double blind.

Measurements and data treatment

Measurement of fish length was carried out on the photos using ImageJ image analysis software. Fulton’s condition factor (CF) was calculated from the formula: $CF = 100W/L^3$, where W weight in grams and L fork length in cm (Ricker 1975; Nash et al. 2006).

For testing differences between treatment groups, the Wilcoxon rank sum test was used with two-tailed P value for normal approximation (Siegel and Castellan 1988). Comparison of scattered plots was carried out by linear regression analysis (Sokal and Rohlf 1987), followed by comparison of regression lines by the linear regression procedure (Snedecor and Cochran 1980), using the

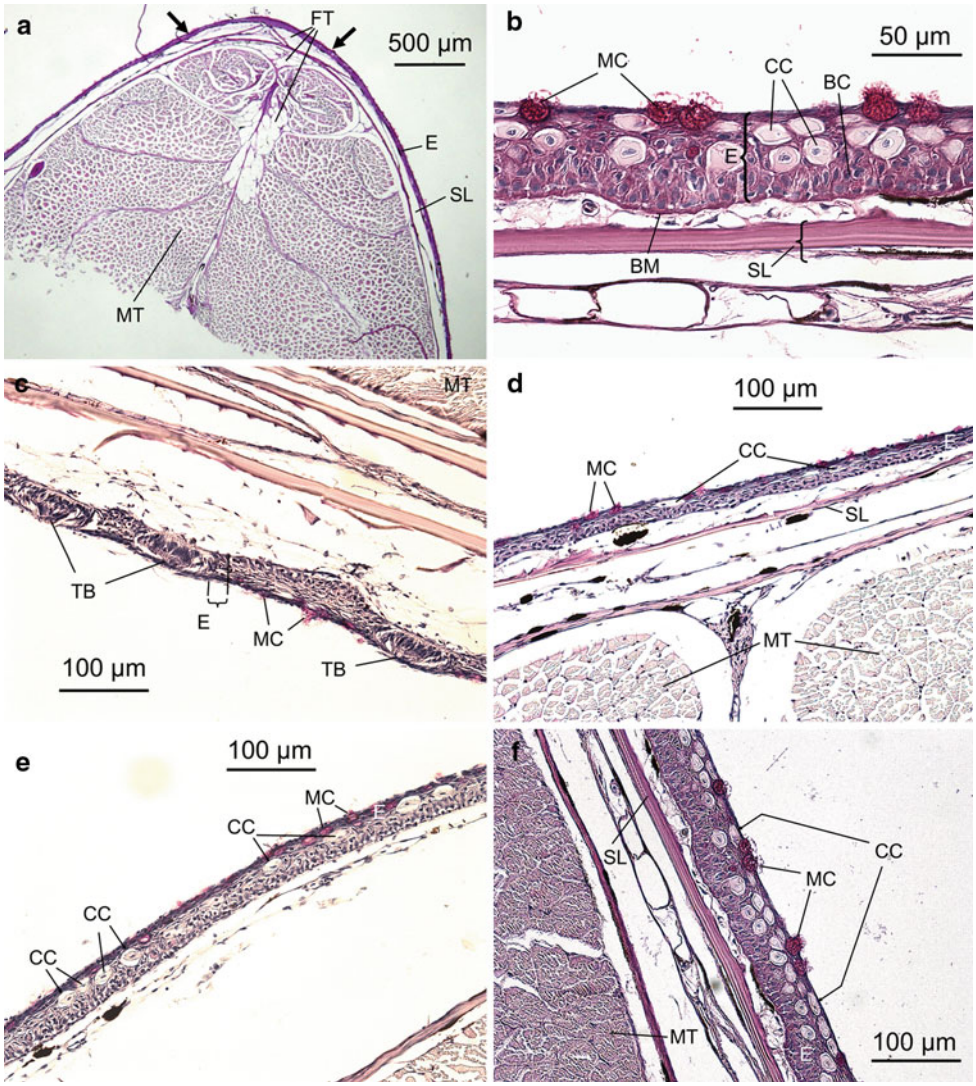

Fig. 1 **a** Micrograph showing a histological transverse section of the epidermal layer and the underlying muscle tissue, dissected from the region between the dorsal fin and the head of crucian carp. Images for quantifying number of club cells were captured from a 3–6-mm length downward from *arrows*. **b** Morphology of club cells and mucus cells. Club cells (*CC*) are PAS negative and appear *white* with *dark* central nuclei, while mucus cells are PAS positive and appear *dark red* (*MC*). **c** Taste buds (*TB*) were often observed to oust club cells in some areas of the epidermis. **d** Epidermis from fish at start of the experiment was thin (20–30 µm) and contained few club cells. **e** Fish reared alone developed an epidermis thickness of approximately 35 µm during the experiment, and contained more club cells than observed at start. **f** Fish raised in groups developed the thickest epidermis (max 70 µm) and the highest density of club cells. **e**, **f** Fish kept at a high feed ratio. *BC* basal cells, *BM* basal membrane, *E* epidermis, *FT* fat tissue, *MT* muscle tissue, *SL* scale layer

computer programme Statistix7 (Analytical Software, Tallahassee, FL, USA).

Results

Morphology and distribution of club cells

The club cells were identified based on their cytological properties. They are prominent and easily recognized as large periodic acid-Schiff reagent (PAS)-negative cells, and appear white with dark central nuclei. In contrast, the mucus cells are PAS positive and appear red (Fig. 1b). The club cells were embedded in the epithelium and never reached the epidermal surface, as could be frequently observed for the mucus cells. Occasionally, areas occupied by taste buds were found to oust club cells in the epithelium (Fig. 1c). These areas were not included in calculations of club cell density. In general, club cells were distributed in a single layer in the epidermis (Fig. 1d), often appearing like "pearls on a string" when density increased (Fig. 1e). In more thick-skinned individuals, however, club cells were often distributed in two or three "layers" in the epidermis (Fig. 1f).

Morphometry

Mean length and weight at start of the experimental series were 59.1 mm (SD 5.9) and 3.1 g (SD 0.8) for the single-reared fish, and 61.0 mm (SD 3.4) and 3.2 g (SD 0.6) for the group-reared ones. At the end of the experiment, these figures had increased to 63.4 mm (SD 5.4) and 4.8 g (SD 1.3) for the single-reared fish, and 65.3 mm (SD 4.5) and 5.3 g (SD 1.5) for the group-reared. Details on length and weight for the different rearing groups are given in Table 1.

The condition factor at start of the experiment ranged between 1.20 and 1.80 in mean values of treatment groups. For fish given a low feed ration a minor increase was found

Table 1 Average length and weight of crucian carp at the start and the end of the experimental series

	Start		End	
	Length ± SD (mm)	Weight ± SD (g)	Length ± SD (mm)	Weight ± SD (g)
Single (low)				
Trout	59.0 ± 3.2	3.2 ± 0.6	61.1 ± 2.3	4.0 ± 0.4
Crucian	57.4 ± 8.2	2.8 ± 1.0	60.4 ± 6.9	3.7 ± 1.1
Single (high)				
Trout	58.5 ± 5.8	2.9 ± 0.9	64.0 ± 4.6	5.0 ± 0.9
Crucian	61.3 ± 6.2	3.4 ± 0.9	68.2 ± 4.0	6.3 ± 1.0
Group (low)				
Trout	61.1 ± 4.1	3.3 ± 0.7	62.9 ± 4.7	4.2 ± 1.0
Crucian	60.3 ± 3.2	3.2 ± 0.6	62.7 ± 2.9	4.2 ± 0.6
Group (high)				
Trout	59.1 ± 2.5	3.0 ± 0.4	67.2 ± 3.1	6.3 ± 1.0
Crucian	62.0 ± 3.5	3.6 ± 0.6	68.4 ± 4.1	6.4 ± 1.2

All fish were exposed to the supernatants obtained from centrifugation of homogenized skin of either crucian carp (crucian) or brown trout (trout). The fish were reared either singly (single) or in groups of four (group), and within both rearing types they were provided a food ration either of 0.03 g/fish/day (low) or 0.1 g/fish/day (high). Only data of fish used in histological examination are given for group-raised fish, i.e. $n = 6$ for all treatment groups within both single and group rearing

during the experiment, final mean values ranging between 1.64 and 1.75 for those groups. Fish given a high feed ration showed a somewhat higher increase in condition factor, reaching mean values between 1.88 and 2.08. The changes in condition factor from start to end of the experiment are given in Fig. 2a. Fish in all groups significantly increased their condition factor during the experiment (Wilcoxon, $P < 0.05$), but there was no significant difference in condition factor between alarm-signal exposed fish and control fish within any treatment groups. When data from the two exposure types were pooled, significant differences were not found in final condition factor between fish raised singly and fish raised in groups.

Fish kept on a high feed ration showed the highest increase in condition factor during the experiment, ranging from 0.26 to 0.89 in mean values, whereas fish kept on a low feed ration showed a minor increase in mean values, ranging from 0.15 to 0.29 (Fig. 2a). In general, the fish given a high feed ration increased their condition factor significantly more than fish kept on a low feed ration for both single and group rearing (Wilcoxon, $P < 0.01$), the exception being control fish raised singly. It should also be noted that the fish on a high feed ration, raised in groups, tended to increase their condition factor even more than fish on a high feed ration raised singly.

Club cell number

The number of club cells was very low in fish at the start of the experiment, ranging from approximately 1 to 5 cells per millimetre of epidermis with a mean value of 2.5 (Fig. 2b). The cell density increased during the experimental period for all fish. Within both feeding regimes, the increase in cell density was significantly higher for fish reared in groups, compared with fish reared alone (Wilcoxon, $P < 0.05$). The increase was especially prominent for fish kept in groups at a high feed ration, reaching a mean number of 20–22 cells per millimetre epidermis, whereas fish in groups with a low feed ration reached a level of 13–15 cells per millimetre.

Compared with fish reared in groups, fish raised singly developed a much lower density of club cells (7–9 cells/mm). For single fish kept at a low feed ration, the increase was not significantly different from values obtained at start. For fish raised singly on a high feed ration, however, a significant increase in cell number was found (Wilcoxon, $P < 0.05$). For fish reared alone, there were no significant differences in cell density between feeding regimes. Neither did exposure to alarm substance induce any significant differences in cell density compared with control fish within any treatment groups.

Epidermis thickness versus condition factor

Analysis of epidermal thickness revealed that fish with a high condition factor developed a thicker epidermis compared to fish with a lower condition factor. Exposure to chemical alarm cues had no effect on epidermal thickness compared with control fish. Therefore, data from alarm signal exposed and control fish were pooled in

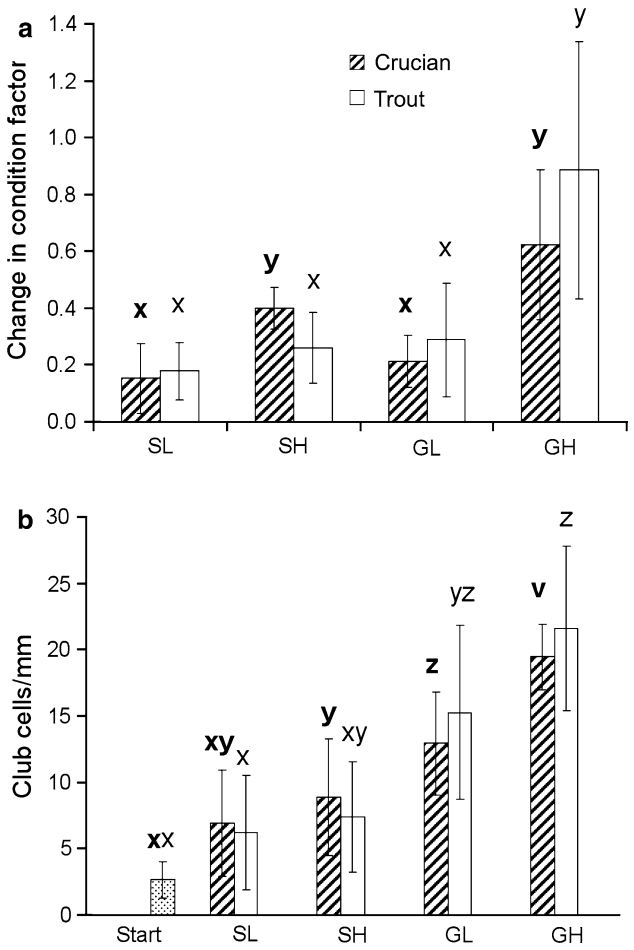

Fig. 2 **a** Change in condition factor and **b** epidermal club cell density (club cells/mm) in crucian carp raised either in groups of four (*G*) or as single fish (*S*), and provided either high (*H*) or low (*L*) feed ratios. The fish were exposed to either skin extract of crucian carp (Crucian) or brown trout (Trout) during an experimental period of 7 weeks. *Start* represents data from fish killed at the onset of the experiment. Each *bar* gives mean values (±SD) of six fishes; two fish were randomly selected from each aquarium of group rearing. Different *letters* indicate significant differences at $P < 0.01$ (**a**) or $P < 0.05$ (**b**) between experimental groups within exposure series (Crucian or Trout); the differences between groups exposed to Crucian skin extract given in *bold*. No significant differences were found between fish exposed to Crucian and Trout extracts within any rearing regimes

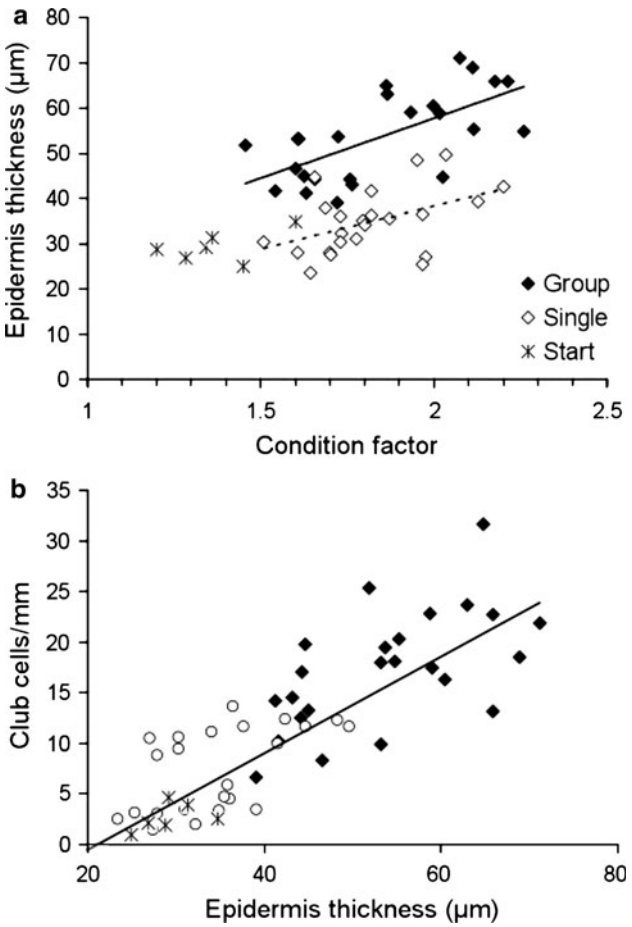

Fig. 3 **a** The relationship between epidermis thickness and condition factor and **b** the relationship between club cell density (club cells/mm) and epidermis thickness in crucian carp raised either in groups of four (*Group*) or as single fish (*Single*) for 7 weeks. Measurements from fish that were exposed to two types of skin extract (crucian carp or brown trout) and provided two different feeding regimes (high or low) for each exposure type have been pooled within the *Group* and the *Single* categories. *Start* represents data from fish killed at the onset of the experiment. Regarding epidermis thickness (*A*), the regression line for fishes raised in groups differs from that of fishes raised singly in elevation ($P < 0.001$), but not in slope ($P > 0.05$)

the analysis further on. On the other hand, conspecific presence had a significant impact on epidermis thickness (Fig. 3a). The mean epidermis thickness of fish was approximately 29 µm at start of the experiment, increasing to 40–70 µm (mean value 54 µm) for fish reared in groups, regardless of feeding regime. Fish kept singly developed an epidermal thickness between 24 and 50 µm (mean value 35 µm), with minor variations between dietary levels. The correlation between epidermal thickness (ET) and condition factor (CF) of fish raised in groups is given by the equation:

$$\mathrm{ET} = 26.89\mathrm{CF} + 4.05 \quad \left(R^2 = 0.43\right),$$

and the correlation of fish raised singly is given by:

$$\mathrm{ET} = 18.80\mathrm{CF} + 0.65 \quad \left(R^2 = 0.20\right).$$

Comparison of regression lines revealed significant differences between elevations ($P < 0.001$), but slopes did not differ.

Club cell density versus epidermis thickness

A thin epidermis with a low density of club cells was found in fish at start of the experiment. At the end of the experiment, the number of cells per millimetre epidermis was found higher in fish with a thick epidermis compared to fish

with a thin epidermis (Fig. 3b). When plotting the number of cells per millimetre epidermis as a function of epidermis thickness, a positive relationship appears. The correlation is given by the equation:

$$\mathrm{CD} = 0.47\mathrm{ET} - 8.93 \quad \left(R^2 = 0.69\right),$$

where CD is club cell density (cells/mm) and ET is epidermis thickness (mm). Figure 3b reveals that fish kept in groups developed a significantly thicker epidermis and a higher density of club cell than fish kept singly (Wilcoxon, $P < 0.001$, for both parameters). The club cell density of single-reared fish also differed significantly from that obtained in fish at start ($P < 0.05$).

Discussion

Three findings are revealed from the present study. First, permanent presence of chemical alarm signals, i.e. the signals releasing behavioural fright reactions, seems unimportant for regulating club cell density in the epidermis of crucian carp. Second, epidermis thickness and the club cell density of the epidermis were found to be plastic with regard to nutritional status (i.e. Fulton's condition factor). Third, conspecific presence seems important for regulating density of club cells. This is given by the fact that significantly more club cells were found in fish that are living in close association with conspecifics compared with fish living alone.

Extra-oral taste buds were occasionally found to interrupt a regular distribution of club cells in the surface area studied. An external distribution of taste buds is well known (Atema 1971), and has previously been reported also in crucian carp (Gomahr et al. 1992). It is important to note that areas with taste buds were not accounted for in the calculation of club cell density.

The initial idea of this work was based on a report proposing that fathead minnows from regions with different predator fauna displayed a lower density of club cells with increasing predator presence (Hugie 1989). Accordingly, we postulated that the density of club cells could in some way be affected through the action of conspecific alarm signals, released over time by predators labelled with such chemical cues from prey. In the current study, we used the supernatant obtained by centrifuging homogenates made of crucian carp skin. This procedure removes the minute particles that make filtered homogenates get turbid. The supernatant contains the water-soluble alarm signals that are releasing behavioural fright reactions, but it does not induce any morphological changes in crucian carp (Stabell et al., unpublished data). The results presented here revealed neither negative nor positive effects from the action of chemical alarm signals on the density of club cells following 50 days of exposure, and we therefore conclude that club cell density in crucian carp seems unaffected by that stimulus.

Our conclusion regarding lack of effect from alarm signals seems supported by other findings. Chivers et al. (2007) tested the idea that the density of club cells should vary with predation risk. Fathead minnows were used, and filtered skin extract of that species as well as predator cues were tested in experiments lasting for 16 days. Predator cues were produced by both common (pike) and an alien predator (oscar cichlids; *Astronotus ocellatus*), and predator fishes were fed fathead minnows as well as control live food, but no effect was found for any of the treatments. On the other hand, it was found that the density of club cells increased following exposure to pathogenic organisms, like water moulds (*Saprolegnia* spp.) and skin-penetrating trematode parasites (*Teleorchis* sp. and *Uluvifer* sp.). The conclusion of that paper was that club cells arose from and are maintained by natural selection owing to selfish benefits, unrelated to predator–prey interactions.

However, some other interesting properties related to growth and epidermal club cells have been revealed by this study. The epidermis of crucian carp demonstrates a linear increase in thickness with an increase in condition factor, i.e. with the nutritional status of the fish. Because the number of club cells was also found as a linear function of epidermal thickness, club cell number and fish condition factor seems interrelated. These findings are in accordance with another report (Wisenden and Smith 1997), which found that epidermal thickness and club cell density were greater in fathead minnows kept on a high feed ration than on a low ration. In this context, it is interesting to note that it has been known for some time that starvation has a marked effect on the content of alarm substance in the skin of minnows (von Frisch 1941; Pfeiffer 1974). Provided that common mechanisms exist among cyprinids for producing alarm substance, this further suggests that it is the number of cells, and not the cellular content of alarm substance, which is compensated for following changes in the nutritional status of fishes.

For the same level of condition factor, fish exposed to the presence of conspecifics develop a thicker epidermis, and thereby a higher density of club cells, than fish reared alone. An increase in club cell density, independent of epidermal thickness, has been reported in fathead minnows when placed into containers with non-familiar shoalmates compared to familiar ones (Wisenden and Smith 1998). In crucian carp, however, an increase in club cell number was only found concurrent with an increase in epidermal thickness, whereas familiarity was not an approach in our study.

Differences in growth between isolated and grouped fish have been reported in immature goldfish that, when raised

singly, grew better if their rearing water were conditioned with water taken from aquaria containing groups of conspecific fish (Allee et al. 1934, 1940). The phenomenon was denoted 'mass physiology', and growth promoting factors released by conspecifics were suspected to be the cause of improved growth. In the current study, and independent of food levels, the fish raised in groups grew slightly better than the fish raised singly. Because the volume of water was adjusted to the number of fish in each container, i.e. with 5 l of water per fish, and feed ratio was adjusted in a similar fashion, a chemical factor similar to the previous one reported (Allee et al. 1934, 1940) may have been acting among the group-raised fish. Such growth factors may have induced better growth and a thicker epidermis in the group-raised fish.

On the other hand, rearing in isolation may be a stress that inhibits growth, and our results could simply mean that thriving, through conspecific presence, may have improved growth. In this respect, sensory factors other than chemical ones cannot be ruled out. However, within a similar range of final condition factor, group-raised fish possessed a higher club cell density than single-raised fish in our study, a result that is difficult to explain by growth alone. Also in fathead minnows, rearing with unfamiliar shoalmates has been found to increase club cell density compared with minnows reared alone, but a decreased club cell density was found in fish reared with familiar shoalmates (Wisenden and Smith 1998). Taken together, we therefore suggest that conspecific chemicals signals may in some way interfere with club cell density, but further experiments are necessary to clarify this hypothesis.

If club cells are the true source of chemical alarm signals causing fright reactions, then our data suggest that group-raised fish are more chemically on guard than those reared singly. Our data also demonstrate that up to a tenfold increase in club cell density can be obtained through an increased feeding regime combined with group rearing. Based on the results of the current study, as well as the literature cited, we conclude that presence and density of club cells in the epidermis of fish is plastic and regulated by factors that are independent of skin injury and pathogenic organisms (e.g. Chivers et al. 2007). The density of club cells seems to be controlled by physiological as well as external regulators, like blood androgen levels, nutritional status and possibly growth-promoting factors working through chemical sensing. However, the sensory mechanisms involved in determining club cell density in cyprinid fish are still not fully understood.

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