



# Coping with continual danger: Assessing alertness to visual disturbances in crucian carp following long-term exposure to chemical alarm signals



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## HIGHLIGHTS

- Supernatants of centrifuged fish-skin homogenates contain chemical alarm signals.
- Crucians display the strongest instant alarm reaction to extract of conspecific skin.
- Alertness-effects from long-term exposure to chemical alarm signals were questioned.
- Fright reaction to visual disturbances was not found after 7 weeks of exposure.
- Instant acting alarm signals are not the causative agents for inducing alertness.

## ARTICLE INFO

### Article history:

Received 3 April 2013

Received in revised form 26 August 2013

Accepted 23 December 2013

### Keywords:

Alarm substance

Predation

Cross-species responses

Fright reaction

Skin extracts

## ABSTRACT

Chemical alarm signals in fish are passively released into the water from ruptured epidermal cells, and induce instant fright responses in conspecifics. Fish also display alarm responses to injured heterospecific skin, as well as to scent of piscivorous predators that have ingested prey. A conspicuous alertness to visual disturbances has also been observed in fish following long-term exposure to extracts of filtered, homogenized skin, but the chemical inducers of such vigilance are actually unknown. We tested if a continual exposure to water-soluble alarm signals, from either conspecifics or heterospecifics, affects alertness of fish. Based on previous experience, it was assumed that alertness could be detected following visual disturbances. Naïve crucian carp were initially exposed to the aqueous extracts of centrifuged skin homogenates, from either conspecifics, or from one out of four heterospecific species (tench, perch, Arctic charr, and brown trout). Darting movements, inter-individual distances, and vertical distribution were used to measure behavioral fright responses released by the test stimuli. After seven weeks of continual exposure to the same extracts, behavioral observations were repeated during visual disturbance. Compared with fish that were long-term exposed to skin extracts of tench or charr, crucian carp exposed to extracts of conspecifics, or to extracts of trout or perch, displayed lower inter-individual distances before being visually disturbed. However, no apparent fright responses were observed following such disturbances, and fish that had been continually exposed to conspecific chemical alarm signals displayed feeding behavior. Our results revealed that fish under assumed continual stress, induced by long-term presence of water-soluble alarm cues, only moderately changed their behavioral pattern. This further demonstrates that the aqueous part of extracts from homogenized skin does not contain any causative agents for inducing any conspicuous alertness.

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## 1. Introduction

Chemical alarm signals were discovered accidentally by Karl von Frisch, who observed that injury to a minnow (*Phoxinus phoxinus*) caused nearby conspecifics to alter their behavior. He also noticed that the alarm signals came from damaged skin [1]. Several prey fish species respond with fright reaction to chemical alarm signals from injured skin of conspecifics [2,3]. In crucian carp (*Carassius carassius*), for instance, a fully expressed fright reaction appears with initial darting, followed by

rapid tail movements combined with typical head-down dashing against the bottom of the aquarium. If present in shoals, fish may also display vigilant behavior when detecting alarm signals, like for instance increased aggregation, hiding, or decreased motion, which reduces the chances of being captured by potential predators [2,4].

Chemical alarm signals were first described in the carp family (Cyprinidae), but have later been found in a wide diversity of other fish species [5–14]. Fish do not only respond with fright reaction to skin extracts of conspecifics, but to heterospecific skin extracts as well [15–18]. Studies on cross-species responses to chemical alarm signals have also confirmed that fish of several super-orders do distinguish between chemical signals of conspecifics versus heterospecifics, and that the response is stronger to fish of the same taxonomic group [16,19,20]. The

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olfactory system of crucian carp discriminates well between odors in conspecific skin extract and similar extracts from other species of fish. With respect to fright responses, different groups of sensory neurons in the alarm region of the olfactory bulb are activated by odorants from conspecifics compared to odorants from other species. It has therefore been suggested that such differences are connected to affinity and properties of the olfactory receptors [21].

The behavior of crucian carp that co-occur with predators has been widely investigated, and several studies have particularly focused on their reactions to waterborne chemical cues from piscivorous and non-piscivorous fish. For instance, it has been shown that the anti-predator response is not directed towards all new stimuli, and that waterborne cues are sources of important information such as, for example, predator diet [22]. It has also been found that predation influences structure of crucian carp populations in small lakes, and that young crucian carp alter their pattern of habitat use in the presence of perch (*Perca fluviatilis*) [23]. When coexisting with predators, fish prefer the highly structured, shallow-water inshore habitat as refuge area rather than deep, unpredictable terrains. Another study which investigated long-term effects of predator chemical cues has shown that crucian carp reduced their overall activity, increased usage of refuge, and changed common activity pattern from nocturnal to aperiodic during the exposed period [24]. However, most of the research carried out has focused on the effects of chemical cues from predators, and little is known about the effects on fish from long-term exposure to chemical alarm signals, derived from skin of conspecifics or heterospecifics.

Working with predator-induced morphology in crucian carp, one of us (O.B. Stabell) observed a conspicuous alertness of fish in aquaria to which filtered homogenates of conspecific skin were added every second day over long time-periods (i.e. up to 50 days) [25]. When being observed from behind the door of the aquarium room, fish in aquaria that had been added skin homogenates swam around calmly, using the water column in a similar way to fish in control aquaria. When the door to the room was opened, although slowly, and the observer appeared into the view of the fish, two striking behaviors could be seen. In the aquaria where water had been added as a control stimulus, the fish rapidly approached the glass walls, presumably waiting to be fed; while in the aquaria treated with skin homogenates, the fish displayed a sudden change in swimming behavior, searched for the innermost corners, and tried to hide in the bottom substrate similar to the classical fright reactions released by chemical alarm signals. Accordingly, we suspected that the chemical alarm signals, when permanently present, would increase the alertness of fish to visual disturbances. In nature, chemical alarm signals may potentially be permanently present over long time periods because predators may be 'labeled' by such cues following ingestion of prey [26].

We wanted to compare the initial responses of crucian carp to the aqueous part of skin extracts from conspecific and heterospecific fish, i.e. to the water-soluble fractions where the classical alarm signals are to be found, in order to subsequently explore how long-term exposure to such extracts affects fish behavior. In particular, we speculated that crucian carp, following long-term exposure to alarm cues, would react differently to visual disturbances compared with unexposed fish. Our hypothesis was that fish, when long-term exposed to conspecific skin extracts, would display higher alertness (i.e. increased vigilance) than unexposed fish, and that fish exposed to extracts of heterospecifics would display intermediate levels of alertness.

In the current study, crucian carp were exposed to skin extracts made from conspecifics and four other species of fish: tench (*Tinca tinca*), Arctic charr (*Salvelinus alpinus*), brown trout (*Salmo trutta*), and perch (*P. fluviatilis*). Tench is a cyprinid, and possess alarm signals that should be expected to elicit a partial response in crucian carp [15]. Arctic charr and brown trout are not common sympatric species with crucian carp, but alarm cues from skin have been reported in brown trout as well as in the related brook charr (*Salvelinus fontinalis*) [20]. Perch is a

common predator to crucian carp, containing skin odors that have been found to release behavioral as well as neural responses in its prey [23,27].

## 2. Materials and methods

### 2.1. Fish and experimental conditions

Crucian carp were caught by baited traps in the Springvanndammen pond, Arendal, Norway, (58°31'N; 8°46'E) and brought to the campus at the University of Agder, Kristiansand. Springvannsdammen is a small monospecific fish pond (0.08 ha), with common newt (*Triturus vulgaris*) and common toad (*Bufo bufo*) as the only two cohabiting but non-predatory vertebrate species [28,29].

Tench were captured by baited traps in a small lake at Nes Verk, Tvedestrand Community, Øst-Agder County, while perch were captured by similar traps in the Kvernhusvannet Lake, Søgne Community, Vest-Agder County. Arctic charr were brought by air from Kårvik Research Station outside Tromsø, North Norway, while brown trout were purchased from the Syrtveit Hatchery at Evje in the Setesdal Valley, Øst-Agder County. All fish were transported live to the laboratory. Fish used as skin donors were killed by a blow to the head, and frozen fresh at  $-20^{\circ}\text{C}$  until preparation of skin extracts. Crucian carp to be used in the long-term exposure experiments were anesthetized (50 mg/l benzocaine in tap water), weighed to the nearest 0.1 g, and randomly distributed with four fish in each aquarium.

During the experiment, crucian carp were reared in 25-l aquaria (dimensions: 48 × 25 × 25 cm, length × height × width), containing tap water treated with 0.5 ml/l AquaSafe (TetraWerke, Melle, Germany) and oxygenated by air stones. The water temperature was kept at 18–20 °C, and half of the water volume in each tank was changed weekly after removal of bottom detritus by siphoning. The walls of the aquaria were covered with brown wrapping paper at both ends and at the back, to avoid visual contact between fish subjected to different treatments. To minimize distortions in the measurements of inter-individual distances, resulting from the sidewise view of a single camera on the horizontal projection of the fish (i.e. a missing z-axis), a plastic grid was mounted vertically and lengthwise in every aquarium, restricting fish to the frontal 1/3 of the volume. The fish were fed once a day with rolled oats mixed 1:1 with commercially fish feed (Tetra Goldfish Pellets, Melle, Germany) by weight, at an average level of approx. 3% of fish biomass per day.

### 2.2. Preparation of skin extract

To prepare skin extracts we used 2.7 g skin of crucian carp, 3.3 g skin of tench, 3.3 g skin of perch, 3.4 g skin of trout and 3.6 g skin of charr. Skin came from three crucian carp (weight:  $10.23 \pm 2.36$  g; length:  $8.2 \pm 0.3$  cm), two tench (weight: approx. 350 g; length: approx. 25 cm), three perch (weight:  $42.7 \pm 49.7$  g; length:  $14.0 \pm 5.2$  cm), five trout (weight:  $8.1 \pm 1.1$  g; length:  $8.5 \pm 0.4$  cm), and three charr (weight:  $32.2 \pm 2.3$  g; length:  $5.8 \pm 0.3$  cm).

Non-turbid skin extracts, i.e. without minute tissue particles, were made according to standard procedures [28,29]. Frozen donor fish were lightly thawed, and incisions were made behind the gills and along the dorsal and ventral edges on each side. The skin was gently peeled off with forceps and immediately placed into 50 ml chilled, distilled water, and homogenized in a blender. The homogenate was subsequently centrifuged at 5000 rpm for 5 min to remove tissue particles, and the supernatant was diluted to 800 ml, transferred to plastic ice cube bags, and frozen at  $-20^{\circ}\text{C}$  until use. Tap water treated with AquaSafe was frozen in ice cube bags to be used as control stimulus. Freezing has previously been found not to affect the fright-releasing properties of chemical alarm signals in fish [30].

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