RESEARCH ARTICLE
Evolution of alarm cues: a role for kin selection? [version 1; peer review: 2 approved with reservations]

Denis Meuthen, Sebastian A Baldauf, Timo Thünken
Institute for Evolutionary Biology and Ecology, University of Bonn, An der Immenburg, Bonn, D-53121, Germany

Abstract
The evolution of alarm signals has puzzled evolutionary ecologists for decades. This is particularly true for alarm cues (‘Schreckstoff’) which are present in many fishes. They are passively released through injuries and signal the presence of a predator. Although the benefits for conspecific receivers are obvious (they can adjust their behavior to avoid predation), those for the senders are not which is, however, a necessary requirement for the evolution of alarm signals. Several hypotheses relying on potential direct benefits for the senders have been suggested. Alarm cues might attract secondary predators which in turn might increase the escape probability of the sender. A primary immune enhancing role was suggested as well. An alternative explanation is based on Hamilton’s inclusive fitness theory stating that individuals can indirectly increase their fitness by increasing the survival of genetically related individuals (‘kin selection theory’). If related individuals preferentially benefit from alarm signals, for instance by being more receptive to kin-alarm cues, senders could increase their inclusive fitness. Here, we investigate whether individuals of the cichlid fish *Pelvicachromis taeniatus* respond differentially to alarm cues derived from kin and non-kin. *P. taeniatus* possesses alarm cues and is known to adjust its behavior when exposed to alarm cues. We measured the change in activity after the addition of alarm cues (derived from kin- and non-kin) relative to a control treatment. Reduced activity is a widespread behavioral adaptation to reduce predation risk in prey organisms. Fish of the alarm cue treatments significantly reduced their activity relative to control fish. However, fish did not respond differentially to alarm cues derived from kin and non-kin suggesting that potential inclusive fitness benefits are not mediated by responses specific to individual alarm cues. We discuss alternative mechanisms such as kin-related grouping and mating preferences potentially leading to kin-biased alarm cue perception.
Corresponding author: Denis Meuthen (dmeuthen@evolution.uni-bonn.de)

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Introduction

Alarm signals are signals that are emitted by individuals in the presence of predators. These signals may not only divert predator attention or discourage it to attack but also alert conspecifics. Alarm signals can be transmitted via different communication pathways: alarm calls are widespread among social terrestrial animals (rodents, birds, and primates). Other animal species use alarm pheromones for the same purpose (insects or mice). In aquatic environments, chemical alarm signals are widespread especially due to the large number of water-soluble compounds (insects, crustaceans, asteroids, gastropods, amphibians, and fishes). Signaling is usually costly for the individual sender and often appears to primarily benefit the receivers. However, to evolve and be maintained by natural selection, the fitness benefits for the signaling individual must override its costs.

The evolution of alarm cues in fishes is a particularly puzzling example of alarm signaling whose evolution has intrigued evolutionary ecologists since the 1960’s until today. Alarm cues, also termed ‘Schreckstoffes’ are located in specialized epidermal ‘club cells’ and are released following injuries by predators, and widespread among different fish taxa including cichlids. Signal-receiving conspecifics benefit because the presence of alarm cues reliably indicates high predation risk, thus allowing them to respond to predator presence in order to increase survival. However, the benefits to the signaler are unclear because it often may not survive a predatory attack. Furthermore, alarm cue production is energetically demanding.

Several hypotheses have been proposed to explain the phenomenon of alarm cues in fishes. First, alarm cues might benefit the survival of the producing individual. In this context, it has been proposed that alarm pheromones may function to attract secondary predators. During the following interference between competing predators prey might be able to escape. Mathis et al. showed that pike Esox lucius and predatory diving beetles were indeed attracted by minnow alarm cues. In the presence of such secondary predators (pike), escape probabilities of minnows were actually increased. Accordingly, the signaling individual can increase its own fitness by producing alarm cues, provided that it survives an initial predator attack.

Other authors proposed that the alarm function is a mere by-product and instead suggested the primary function of alarm cues to be anti-pathogenic agents or promote the healing of injuries. A recent study supported this hypothesis; club cell production was unrelated to predation risk but stimulated by skin penetrating pathogens and parasites. Moreover they found that UV radiation also affected club cell production, providing evidence for a general immune function of alarm cues.

A further hypothesis relying on Hamilton’s inclusive fitness theory predicts that individuals can increase their fitness indirectly by supporting the survival of genetically related individuals, coined ‘kin selection’ by Maynard-Smith. Generally, costly communication is facilitated when interacting individuals are related. While kin selection has been suggested to play an important role in the evolution of mammalian alarm calls (but see), its significance for the evolution of alarm cues in fishes has received little attention. Indirect benefits can be gained when kin particularly benefit from alarm cue production. This might be reached when individuals are able to discriminate between the alarm signals of different senders. Higher sensitivity to kin-alarm cues may result in an improved response to predation, and thus higher survival of individuals related to the sender which in turn may increase the indirect fitness of the sender.

The aim of the present experiment was to test whether the cichlid fish Pelvicachromis taeniatus discriminates between alarm cues produced by kin and non-kin. P. taeniatus is a socially monogamous small cave-breeder with biparental brood care which inhabits streams in Western Africa. Previous studies revealed that this species possesses alarm cues, recognizes conspecific alarm cues and adjusts its behavior in the presence of alarm cues (Meuthen et al., submitted, unpublished data). Furthermore, P. taeniatus is capable of kin recognition which is most likely based on chemical cues. In the experiment we measured the change in activity in individual P. taeniatus after the addition of alarm cues derived from kin and non-kin, respectively.

Material and methods

Ethics statement

This study conforms to the Association for the Study of Animal Behaviour’s Guidelines for the Use of Animals in Research and was carried out according to the German laws for experimentation with animals (§ 8 Abs. 1 TierSchG, V.m. § 2 Abs. 1.1 TierSchZustV NW 26.9.1989). No additional licences were required.

Animal collection and maintenance

We conducted an experiment using female F2 progeny of the cichlid species Pelvicachromis taeniatus, whose ancestors (F0) were collected from the Moliwe river near Limbe, Cameroon (04°04’ N, 09°16’ E). Female P. taeniatus were used exclusively due to their consistent activity levels. Prior to experiments, fish were kept in mixed-sex 50 x 30 x 30 cm (L x W x H) stock tanks at densities up.
to 20 individuals and were fed daily with frozen invertebrate larvae ad libitum. These tanks were illuminated in a 12:12 h light:dark cycle; water temperature was kept at 25 ± 1°C.

**Experimental setup**

During experiments, we manipulated predation risk in three treatments by exposing individual fish to one of the following extracts: (1) Alarm cues derived from kin (from familiar and unfamiliar siblings); (2) Alarm cues derived from unfamiliar, unrelated conspecifics (non-kin); (3) Distilled water to control for disturbance effects upon introduction. We produced alarm cues from 26 donor cichlids which were previously starved for two days to exclude any effects caused by the individual’s selective diet. Each alarm cue consisted of a male and a female cichlid to control for sex effects. Fish were anaesthetized with a blow to the head and afterwards euthanized by cervical dislocation in accordance to § 4 of the German animal welfare act (BGB I S. 1207, 1313). They were then placed into a mortar and grinded with a pestle. This procedure, imitating a lethal predation event, ruptured cells and thus allowed alarm pheromones to be released. The homogenate was diluted with distilled water, passed through filter floss and frozen in 1 ml aliquots at -20°C until use. The final concentration each fish was exposed to during trials was 3.6 mg/l donor wet fish weight. Likewise, we prepared 1 ml aliquots of pure distilled water for control experiments.

Trials were run in 30 × 20 × 20 cm tanks which were supplemented with a 0.5 × 0.5 cm grey plastic tube leading below the water level at the middle of their short side. This duct allowed the direct addition of chemical cues into the tanks while minimizing fish disturbance. Furthermore, experimental tanks were surrounded on all sides (except the top) with white polystyrene to prevent fish agitation by neighboring fish or the experimenter. A video camera (QuickCam 9000, Logitech, China) viewing the tanks from the top enabled recording of fish behavior for evaluation. Tanks were filled with substrate-treated water: individual fish were then introduced and acclimatized for 1 h, this period is referred to as the pre-stimulus phase from now on. Experimental stimuli were thereafter temperature-adjusted to tank conditions and introduced at the point of 1 h 15min. Subsequently, fish behavior was recorded for another hour. Afterwards, the experimental subjects were sized accurate to the nearest millimeter and weighed accurate to one milligram on an electrical precision scale (LC 2215, Sartorius, Germany). Between trials, tanks were cleaned with 3% hydrogen peroxide and then rinsed with tap water to remove remaining olfactory traces. Furthermore, experimental stimuli assigned to individual tanks were alternated between trials.

In total, we tested 51 individuals from 8 families; extracts from the same donor fish were used throughout different treatments and thus represented – based on the family identity of the focal fish – as either related or unrelated conspecifics. Also, individuals from the same family were evenly distributed among the three extracts. Because in two cases the fish exhibited no activity during the pre-stimulus phase, we excluded them from analysis. Hence, the final sample size consisted of 49 individuals; 12 received alarm cues from familiar siblings, 9 from unfamiliar siblings, 14 from unrelated fish and 14 individuals were exposed to the control stimulus. Because sibling familiarity did not significantly affect focal fish activity (familiar vs. unfamiliar kin: \( \chi^2 = 0.090, p = 0.764 \)), their activity scores were pooled to represent 21 fish receiving alarm cues derived from kin.

**Statistical analysis**

We evaluated fish activity by tracking its movement during 1 h pre-stimulus phase and the following 1 h post-stimulus phase with animal tracking software (Biobverse Viewer, St. Augustin, Germany). Subsequently, we assigned an activity index to each fish by calculating the difference between the distances covered during the two experimental phases. Activity indices did not deviate significantly from normal distribution according to the Kolmogorov-Smirnov test (Lillie.test, R library “nortest”), thus we applied linear-mixed effect models (LME, R library “nlme”) for analysis. All test fish were only used once but to account for the repeated use families, we entered “family identity” as a random factor. All results were based on likelihood ratio tests (LRT); hence degrees of freedom always differed by one.

**Results**

The change in activity of female *P. taeniatus* was significantly affected by the treatment (\( \chi^2 = 10.057, p = 0.007 \), Figure 1). Activity indices of both alarm-cue treatment groups (kin/non-kin) were significantly different from those of the water-control group (Kin vs. water: \( \chi^2 = 8.346, p = 0.004 \); Non-kin vs. water: \( \chi^2 = 8.693, p = 0.003 \), Figure 1). Whereas fish of the control group showed on average an increase of 0.67 m in covered distance during the post-stimulus period, fish of both alarm cue treatments showed reduced activity in the post-stimulus phase (on average 0.31 m less

![Figure 1. Activity indices of female *P. taeniatus* (mean±SD) exposed to distilled water (gray open bar) and alarm cues derived from related conspecifics (kin, black hatched bar) or unrelated conspecifics (non-kin, black open bar). Activity indices were calculated by subtracting the distance covered during the 1 h pre-stimulus phase from the following 1 h post-stimulus period. Asterisks above the bars indicate ** p < 0.01; ns p > 0.6.](image-url)
compared to the pre-stimulus phase). However, fish did not respond differentially to alarm cues derived from related and unrelated individuals ($\chi^2 = 0.233, p = 0.630$).

**Fish activity data**

1 Data File

http://dx.doi.org/10.6084/m9.figshare.96320

**Discussion**

Generally, activity of female *P. taeniatus* was affected by the presence of conspecific alarm cues. Fish of the alarm cue treatment significantly decreased their activity relative to control fish. However, *P. taeniatus* did not discriminate between alarm cues derived from kin or non-kin in terms of activity changes.

These results are in accordance to numerous studies showing that the presence of conspecific alarm cues decreases prey activity in general. Reduced activity concurrently decreases prey conspicuousness, which is an effective strategy against visual predators and enhances prey survival. Furthermore, our results add to an earlier study, showing that males of *P. taeniatus* reduce territorial aggression in the presence of conspecific alarm cues (Meuthen et al., submitted, unpublished data). Thus both sexes of *P. taeniatus* are capable of recognizing alarm cues and adjust their behavior accordingly.

Against the expectations of the kin selection hypothesis, fish did not discriminate between kin and non-kin alarm cues. This is also surprising because theoretical models proposed that the evolution of communication in general is driven by the interaction between siblings and kin selection has been suggested to play an important role in the evolution of different alarm signals such as alarm calls but see. Furthermore, individual discrimination of alarm signals is predicted to be highly beneficial. However, lack of discrimination does not necessarily mean lack of recognition. Thus, further studies in different contexts are required to confirm the present results. The results are in accordance with the "direct benefits" hypotheses, including those hypotheses highlighting that alarm cues have primarily evolved as an immune enhancing mechanism and that the alarm signaling function is a by-product. They are also in accordance with the theory that chemical signals have originally evolved from compounds without a communicative function.

On a molecular level, the missing discrimination may result from a lack of kin-related information within alarm cues. Although to date, some studies were able to identify single chemical components of alarm cues such as Hypoxanthine-3-N-oxide or the glycaminoglycan chondroitin, the full suite of alarm cue components still remains to be identified. The results of behavioral experiments have suggested that substantial individual variation in alarm cues exists. Sender body condition was determined to be an important factor affecting alarm cue response. These results were, however, attributed to differences in club cell quantity because fish display graded responses based on alarm cue concentration. Unidentified qualitative effects could, however, also be responsible for the altered response, especially because not all behaviors were specific to sender condition – whereas changes in activity and shoaling density were specific to sender condition, aggression remained unaffected.

Kin discrimination in alarm cues might simply not have evolved in *P. taeniatus* due to the lack of necessity. The increase in fitness due to kin discrimination in alarm cues might not be sufficient to cause the necessary impact on natural selection, causing kin-related information to evolve in alarm cues. Many fishes are capable of identifying kin and live in kin-shaped groups. This is also true for *P. taeniatus* which lives the first weeks of hatching in family group guarded by their parents. Afterwards they live in shoals without defined territories throughout the juvenile stage. Laboratory experiments showed that they prefer to shoal with familiar kin (Thünken et al., unpublished data). Furthermore, *P. taeniatus* preferentially mate with kin which is most likely also true for the natural population. Accordingly, throughout their life the social environment of *P. taeniatus* is probably largely kin-structured in nature. In this case, individuals receiving the information transmitted by alarm cues are most likely kin. As a consequence, signaling individuals may increase their inclusive fitness.

In conclusion, our study found no evidence for differential response to kin derived alarm cues. However, under natural conditions behavioral mechanisms may lead to kin-biased alarm cue perception. Thus, kin selection potentially plays a role in alarm signaling in our model system. Still, further research is required determining the direct fitness benefits and costs for the signaling individual as well as the benefits for the receivers which are fundamental parameters to understand the evolution and maintenance of alarm cues.

**Author contributions**

TT and DM conceived the study. DM, SAB and TT designed the experiments. DM carried out the research. DM, SAB and TT analysed the data. DM and TT wrote the paper. All authors had read and improved the manuscript and agreed to the final content.

**Competing interests**

No competing interests were disclosed.

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Maurice W Sabelis
Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Amsterdam, The Netherlands

Paulien JA de Bruijn
Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Amsterdam, The Netherlands

Martijn Egas
Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Amsterdam, The Netherlands

Meuthen et al. describe the results of experiments to test whether the cichlid fish *Pelvicachromis taeniatus* discriminates between alarm cues produced by kin and non-kin upon being injured by a predator.

They manipulated alarm cues (as a proxy of predation risk) in three treatments by exposing individual fish to one of the following: (1) Alarm cues derived from kin (from familiar and unfamiliar siblings); (2) Alarm cues derived from unfamiliar, unrelated conspecifics (non-kin); (3) Distilled water to control for disturbance effects upon introduction. They showed that activity of fish receiving alarm cues from treatment 1 and 2 was reduced compared to the control (treatment 3), but familiarity of siblings (treatment 1) and kinship (treatment 1 vs 2) had no effect on activity. Meuthen et al. conclude that *P. taeniatus* fish do not discriminate between alarm cues from kin and non-kin. Finally, they argue that kin selection can only play a role if this fish usually lives in groups of kin and that alarm cues are more likely to have evolved as a by-product of wound-healing or immune responses to pathogens colonizing cells wounded by predators.

While their experiments are clearly described and carefully designed, we question whether the question in the title of the paper by Meuthen et al. is well posed: Evolution of alarm cues: a role for kin selection? First, alarm cues represent public information and we see no reason why kin and non-kin should respond differently to these cues (except in the very special case where predation risk differs between kin and non-kin). Hence, we disagree where the authors state: “Higher sensitivity to kin-alarm cues may result in an improved response to predation, and thus higher survival of individuals related to the sender which in turn may increase the indirect fitness of the sender”. Why would a higher sensitivity to kin alarm be
expected in the first place? Second, the experiments by Meuten et al. were designed such that they exclude the possibility for wounded fish to decide on what and how much to send depending on whether it is surrounded by kin or non-kin. This presupposes that the wounded fish has some form of control over the amount of alarm cues that are released. In our view this extent of this sender control is an essential target for kin selection. Thus, to answer the question posed in the title of the paper the experiments should have been designed so as to allow the potential sender to perceive who are the potential receivers and so as to measure the amount of alarm cues released depending on their environment.

Side remarks:
(1) In the Materials and Methods section the authors state: “(experimental stimuli) … were introduced at the point of 1h 15min and then fish behavior was recorded for another hour. Thereafter, all experimental subjects were sized accurate to the nearest millimeter and weighed accurate to one milligram on an electrical precision scale (LC 2215, Sartorius, Germany)”. However, the results of these measurements are not described in the paper.
(2) In the Results section the analysis focuses on activity as the difference in distance covered by the fish before and after application of the treatment. These values may differ (as they do between treatments 1 and 2 vs 3) due to differences before treatment, but the authors seem to assume that any difference in this activity parameter is due to differences after application of the treatment. We strongly suggest the authors to provide a statistical test to show that the distances before treatment were not significantly different among the three groups.

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 27 May 2014

Denis Meuthen, University of Bonn, An der Immenburg, Bonn, Germany

Sabelis et al. critically remarked "whether the question in the title of the paper is well posed". To our opinion the evolution of alarm cues is puzzling as their production is a costly process (individuals with higher condition produce more alarm cues). If individuals benefit from alarm cues while not contributing to the alarm system by producing own alarm cues, they would increase in number and ultimately eliminate this alarm signaling system from evolution. Kin-biased perception could solve this problem. In accordance with this hypothesis a recent paper shows that discrimination between kin and non-kin volatile chemical (alarm) cues occurs in plants (Karban et al. 2013). Moreover, several studies suggest that familiarity with alarm signals (O'Connell-Rodwell et al. 2007) or chemical cues (Coopersmith & Leon 1984, Brown & Smith 1994) leads to improved responses. Along the hypotheses of other researchers concerning the role of kin selection in the evolution of mammalian alarm calls (Sherman 1977, Charnov & Krebs 1975, Sherman 1985, da Silva et al. 2002) and alarm cues of fishes (Smith 1992), we therefore expect that individual fish should respond differently to alarm cues derived from kin and non-kin.

Furthermore, Sabelis et al. suggest that "wounded fish have some form of control over the amount of alarm cues that are released, ultimately providing an essential target for kin selection". If wounded fish had the control, this would be indeed a possibility. However, it is usually assumed that alarm cues are passively released. Alarm cues are putatively located in enclosed subepidermal club cells without external ducts, thus providing little opportunity for short-term
variation in the released alarm cue amount. Accordingly, the only way for alarm cues to be released is the destruction of these cells by predators. Therefore, we focused on the receiver part, i.e. whether individuals are differentially sensitive towards kin and non-kin alarm cues. One possible control by the sender might be differential long-term investment in club cell quantity depending on whether the sender is surrounded by relatives. In the present study all fish grew up in kin groups prior to trials, therefore, we exclude the possibility of differential investment based on the long-term sender environment.

Following Sabelis et al. side remarks we included additional analyses regarding fish sizes and activities before the treatment. These analyses showed that neither fish size nor weight nor prestimulus activity differed significantly among the three treatment groups.

Competing Interests: No competing interests were disclosed.
Rubenstein criticized the reference to the theoretical model by Pollard 2010. We realized that the reference to the Pollard paper was a bit misleading and may have caused confusion. In our revision, we replaced this aspect with a more detailed explanation of why we expect kin selection to play a role in the evolution of alarm cues. We now include other studies previously highlighting the potential role of kin selection in the evolution of alarm signals, including fish alarm cues. A particularly interesting example on plants shows that sagebrush induces strong anti-herbivore protection mechanisms when exposed to the volatile (alarm) cues of wounded close relative plants. In contrast, the anti-herbivore response was weaker when they were exposed to volatiles from distantly related plants.

Second, Rubenstein raised concerns that due to physiological restrictions of alarm cues in fish, dishonest alarm signals are unlikely. We fully agree with the referee. However, it was not our intent to convey that our study is based on the premise of the dishonest or honest alarm signals which are referenced by Pollard 2010. Instead, we focus on the question whether kin are able to respond more quickly towards a predator by being more sensitive towards kin alarm cues. For this purpose, in our revision we present an argument outlining that the recognition of kin alarm cues could be based on an indirect coupling of concurrently present kin-specific cues and alarm cues. Accordingly, fish could be able to discriminate between kin- and non-kin alarm cues based on learned kin recognition. This would not be surprising as kin recognition has been shown to be present in fishes and injuries caused by predators release numerous substances contained in very different areas of the prey body.

**Competing Interests:** No competing interests were disclosed.

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