Alarm substance from adult zebrafish alters early embryonic development in offspring

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Alarm substances elicit behavioural responses in a wide range of animals but effects on early embryonic development are virtually unknown. Here we investigated whether skin injury-induced alarm substances caused physiological responses in embryos produced by two *Danio* species (*Danio rerio* and *Danio albolineatus*). Both species showed more rapid physiological development in the presence of alarm substance, although there were subtle differences between them: *D. rerio* had advanced muscle contraction and heart function, whereas *D. albolineatus* had advanced heart function only. Hence, alarm cues from injured or dying fish may be of benefit to their offspring, inducing physiological responses and potentially increasing their inclusive fitness.

Keywords: alarm substance; *Danio*; development; inclusive fitness

1. INTRODUCTION

Chemical alarm cues exist in a wide range of taxa where they elicit anti-predator behaviours (e.g. Chivers & Smith 1998; Ferrari *et al.* 2007; Shabani *et al.* 2008) and induced defences (Schoeppner & Relyea 2005; Laforsch & Beccara 2006; Hagman *et al.* 2009). Most studies on effects of alarm cues are restricted to juveniles or adults, with effects on early embryonic development virtually unknown. Yet, recent evidence suggests that embryos in the early stages of development may be just as responsive to biotic environmental influences as later developmental stages (Laforsch & Tollrian 2004; Mathis *et al.* 2008).

Ostariophysan fishes (such as minnows, catfish, characins and suckers) produce alarm substance in specialized epidermal cells (Smith 1992; Mathis & Smith 1993a; Brown & Godin 1997; Chivers & Smith 1998; Brown *et al.* 2000). As these cells lack any external duct, their contents are only released into the external environment when skin damage occurs (Wisenden & Thiel 2002), e.g. during a predator attack, thereby alerting nearby conspecifics of a predation risk (Mathis & Smith 1993b; Wisenden & Thiel 2002).

The cost of alarm substance production and release is high (Wisenden & Smith 1997). While alarm

substance may attract other predators to fight over the injured individual allowing it to escape, the outlook for an injured individual is poor. Consequently, many hypotheses on how alarm substance might benefit the inclusive fitness of the sender have been proposed (Smith 1992). The 'alarm' role of alarm substance may have evolved secondarily to functions such as defence against pathogens (Chivers *et al.* 2007); subsequent selection has resulted in an additional alarm benefit to conspecifics. Whether alarm substance could alter the early embryonic development of offspring, which would be of direct benefit to the inclusive fitness of the injured individual has not been considered before.

Here, we investigated whether such communication between adults and developing offspring might be occurring by observing whether the early development of embryos of two species of danio fishes, *Danio rerio* and *Danio albolineatus*, were influenced by alarm substance. In particular we focused on whether the timing of key physiological and morphological events were brought forward in developmental time as a strategy for coping with increased environmental stress (Spicer & Burggren 2003; Spicer & Rundle 2007).

2. MATERIAL AND METHODS

(a) Experimental animals

Adult *D. rerio* were from an existing stock at the University of Plymouth. *Danio albolineatus* were obtained from local aquarists. Single species groups of males and females were kept separate in 10 l glass aquaria ($26 \pm 1^\circ\text{C}$; 12 h light: 12 h dark) and fed three times daily ad libitum on flake and *Artemia*. For embryo collection, males and females of a single species were placed together in a breeding tank and embryos collected immediately post-fertilization. A mixture of individuals from several different breeding groups was used during the course of the experiment to maximize genetic variation among embryos. As the precise time of fertilization was difficult to determine, the two-cell stage of development was used as our initial time point.

(b) Experimental setup

Individual embryos were filmed from the two-cell stage for a period of 24 h using a camera (WATEC 202B/WATEC 221S, WATEC Co. Japan), connected to a VCR (Time-Lapse VCR AG-6730, Panasonic Corp. Japan/Computar 24 h Time-Lapse CTR-3024, CBC Corp., USA). Images were captured at $75\times$ magnification, and were viewed on a monitor (WV-CM1470, Panasonic Corp., Japan) connected to the recorder.

To maintain embryos within the camera frame during filming, they were placed into a well made out of a 5 per cent agarose gel held in a 6.5 cm Ø Petri dish. These wells mimicked a structurally complex substrate where zebrafish would oviposit. The diameter of each well was approximately twice the length of an embryo to avoid growth constraints. The Petri dish was maintained at $28.5 \pm 1^\circ\text{C}$. A continuous flow of water (0.3 ml min^{-1}) to the dish was provided via a peristaltic pump from stock tanks. To minimize disturbance, the water input was >5 cm from the well where the embryo was located.

(c) Experimental protocol

Alarm substance was obtained using the method of Sloman *et al.* (2008). *Danio rerio* embryos were exposed to alarm substance obtained from adult *D. rerio* and *D. albolineatus* embryos to alarm substance from adult *D. albolineatus*. The concentrations of alarm substance used here did not negatively affect survival to first feeding.

At the two-cell developmental stage, for embryos exposed to alarm substance ($n = 11$, *D. rerio*; $n = 10$, *D. albolineatus*), 2 ml of alarm substance (at 28.5°C) was pipetted continuously into a well containing an embryo. The alarm substance was removed from the tank after 30 min by flushing the system using a peristaltic pump (75 ml min^{-1}). In control experiments, 2 ml of system water (at 28.5°C) replaced alarm substance ($n = 11$, *D. rerio*; $n = 11$, *D. albolineatus*).

The time of appearance of three developmental features were recorded for each embryo: eye primordium (i.e. the first appearance of a clear oval shape of the eye contour in the head region of the developing embryo), initiation of the first muscular contraction and first heartbeat.

Figure 1. (a) Time taken by *D. rerio* embryos to reach a designated stage of development. Asterisks indicate a significant difference between treatments ($p < 0.05$). Mean $\pm$ s.e. Filled bars, control; open bars, alarm substance. (b) Developmental timeline where each bar and corresponding p -value indicates if alarm substance causes a significant difference in the developmental time between stages indicated.

(d) Statistical analyses

Data (decimalized timings expressed in minutes) were normally distributed and analysed parametrically using SPSS 15.0 for Windows (SPSS Inc. Chicago, IL, USA). The overall difference in the time it took from the two-cell stage to reach the onset of the eye primordium, the first muscular contraction and the first heartbeat was compared between control embryos and those exposed to alarm substance using a repeated-measures analysis of variance (ANOVA). One-way ANOVAs were then used to test for differences in specific timings between stages (i.e. using each stage as an initial time point and testing for differences in the time taken to reach further stages between control and alarm substance samples).

3. RESULTS

There was a significant effect of alarm substance on the time it took *D. rerio* embryos exposed to alarm substance to reach the different developmental features recorded (repeated-measures: $F_{1,20} = 6.435$, $p < 0.05$). Post hoc testing showed that embryos reached the first muscular contraction ($p < 0.05$) and the first heartbeat earlier ($p < 0.05$; figure 1a), with no significant effect on time to the eye primordium stage ($p = 0.05$). There were also differences in the time it took for *D. rerio* embryos to develop between specific developmental stages (figure 1b). The time required for embryos exposed to alarm substance to develop to

the heartbeat stage from the eye primordium stage was significantly smaller ($F_{1,20} = 5.180$, $p < 0.05$) when compared with controls, and the developmental time between the first muscular contraction and the heartbeat was also significantly smaller in alarm substance embryos ($F_{1,20} = 6.445$, $p < 0.05$).

Overall, the developmental rate of *D. albolineatus* embryos was not significantly altered when exposed to alarm substance ($F_{1,19} = 1.983$, $p = 0.175$; figure 2a), although post hoc analyses revealed that the time between the two-cell stage and onset of heartbeat was significantly shorter in embryos exposed to alarm substance. There was also a significant decrease in the development time between eye primordium and heartbeat ($F_{1,19} = 4.591$, $p < 0.05$), and between first muscular contraction and heartbeat for alarm substance samples compared with controls ($F_{1,19} = 6.107$, $p < 0.05$; figure 2b).

4. DISCUSSION

This is, to our knowledge, the first report of an alarm substance affecting embryonic development. For both species, the development of the heartbeat was significantly advanced relative to other developmental

Figure 2. (a) Time taken by *D. albolineatus* embryos to reach a designated stage of development. Mean $\pm$ s.e. Filled bars, control; open bars, alarm substance. (b) Developmental timeline where each bar and corresponding *p*-value indicates if alarm substance causes a significant difference in the developmental time between stages indicated.

events, with *D. rerio* also showing an advance in muscle contraction. Such advances in developmental events might signal an increase in the overall development rate, with animals rushing through early development in a risky environment. Such increased development rates in response to predation threat are common place (e.g. Tollrian 1995) but may have associated costs (Peckarsky *et al.* 2002). Alternatively, the early occurrence of developmental events could relate to later physiological or behavioural traits, as has been demonstrated in tadpoles, which learnt to respond to novel predator cues following exposure as embryos (Mathis *et al.* 2008).

The mechanism by which embryos are able to detect alarm substance at such an early stage of development remains unknown. The clear difference in response between species, however, suggests that there may be variation between species in terms of when they are able to detect cues in their environment, or when they are able to initiate developmental responses to such cues. If such responses do confer advantages in terms of the inclusive fitness, there could also be differences between species in terms of their abilities to respond developmentally to the biotic environment.

In conclusion, alarm substance induces an acceleration of the overall developmental rate in *D. rerio*;

D. albolineatus did not respond as strongly, although some changes between developmental stages were seen. Further studies should investigate the mechanisms by which alarm substance is affecting these embryos. Phenotypic effects of these changes in developmental rate in larval and adult stages also make an exciting area for future research.

The study was carried out in accordance with the University of Plymouth ethical guidelines.

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