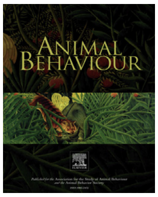
Animal Behaviour 219 (2025) 123017

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav



Social influences on embryonic behaviour and the developmental onset of embryonic acquired predator recognition in minnows

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A R T I C L E I N F O

Article history:
Received 4 July 2024
Initial acceptance 22 July 2024
Final acceptance 20 August 2024
Available online 24 December 2024
MS. number: A24-00427

Keywords:
associative learning
embryonic learning
olfactory cue
ontogeny
predator recognition

For oviparous species, the external environment is replete with cues that contain diverse information relevant to developing embryos, including potential predation threats. Information about predators obtained during the egg stage is known to improve survival after hatching by allowing individuals to recognize and respond to potential threats more effectively, but the development and mechanisms by which embryos learn are not well studied. Here, we sought to identify the developmental onset of embryonic learning in a freshwater fish, the fathead minnow, *Pimephales promelas,* by conditioning embryos to identify a piscivorous predator, the bluegill sunfish, *Lepomis macrochirus*, and evaluating their behaviour in response to predatory cues each day before hatching. In addition, we manipulated egg number (small or large clutch) and configuration (clustered, isolated) in experimental clutches to determine whether social attributes of the clutch, specifically, group size and embryo proximity, influence the acquisition of learned predator information or the expression of antipredator behaviour. Behavioural evidence of learned predator recognition first emerged among predator-conditioned embryos at 4 days postfertilization, expressed as a reduction in locomotor activity in the presence of predator cues. In addition, we found general effects of both the number and proximity of neighbouring eggs on embryonic activity levels but not on the developmental onset of predator recognition in predator-conditioned embryos or the expression of antipredator behaviour. These findings contribute to a growing body of knowledge on embryonic learning in oviparous aquatic vertebrate species and suggest that aquatic vertebrate embryos may be more sensitive to the social environment of the clutch than is commonly considered.

Evidence from diverse taxa with oviparous reproduction indicates that experience during embryogenesis can influence the behaviour and fitness of organisms after hatching (e.g. cephalopods: Darmaillacq et al., 2008; Guibé & Dickel, 2011; Lee et al., 2020; birds: Colombelli-Negrel et al., 2014; Noguera & Velando, 2019; Turatto et al., 2019; amphibians: Garcia et al., 2017; Mathis et al., 2008; arthropods: Peralta Quesada & Schausberger, 2012; Shannon et al., 2022; fish: Horn et al., 2019; Nelson et al., 2013; Poisson et al., 2017). For these organisms, the ability to learn about the postnatal environment prior to directly encountering it is thought to be favoured by selection because it enables individuals to recognize and exploit locally available resources (Guibé & Dickel, 2011; Guibé et al., 2012; O'Brien et al., 2017) and/or reduce risk (e.g. Garcia et al., 2019; Horn & Chivers, 2021). Such improved responsiveness is likely to be especially advantageous during the vulnerable early stages of life, when mortality rates are high (May, 1974). For example, there is excellent evidence that embryonic fish and amphibians can learn to recognize a predator before hatching via a conditioned association between predator chemical cues and alarm cue, as evidenced by enhanced antipredator responses to predatory cues after hatching (e.g. Ferrari & Chivers, 2009, 2010; Garcia et al., 2017, 2019; Horn et al., 2019; Mathis et al., 2008; Nelson et al., 2013; Poisson et al., 2017), and that separately, learned recognition of chemical cues indicative of predation risk significantly improves survival (Chivers et al., 2002; Gazdewich & Chivers, 2002; Mathis & Smith, 1993a). However, whereas the posthatch adaptive benefits of developmental plasticity resulting from embryonic experience are well established (Jonsson et al., 2022; Nettle & Bateson, 2015), few studies have explicitly tested hypotheses related to learning in aquatic embryos prior to hatching (Atherton & McCormick, 2015;

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https://doi.org/10.1016/j.anbehav.2024.10.031

Sehr et al., 2016). Consequently, we know comparatively little about the proximate mechanisms of predator learning at this key stage of ontogeny.

One area of research that has received relatively little attention concerns the timing and developmental onset of embryonic learning and behaviour. Predation poses a risk to individuals at all stages of ontogeny, including the egg stage, and embryos of many oviparous aquatic species can detect predatory cues in the environment using a variety of sensory modalities and respond to those cues in ways that minimize this risk (Du & Shine, 2022). For example, individuals may adjust timing or behavioural decisions related to hatching (Chivers et al., 2001; Gomez et al., 2023; Güell et al., 2022; Warkentin, 2011) or suppress conspicuous activity within the egg to minimize the probability of detection (Gervais et al., 2021; Kempster et al., 2013; Mezrai et al., 2020). However, the earliest ability of an embryo to adjust its behaviour in response to environmental cues is constrained by the development of sensory and locomotor systems responsible for perceiving, processing and responding to such stimuli (Fuiman & Magurran, 1994; Pereira & Moita, 2016; Romagny et al., 2012; Warkentin et al., 2017; Wiedenmayer, 2009). Conditioned context-specific behaviours, such as acquired predator recognition, also require further establishment of functional connections and mechanisms for the formation of associative memories (Eschbach et al., 2020; Fan et al., 2022; Gross & Canteras, 2012). Because most studies of embryonic learning and acquired predator recognition in oviparous aquatic vertebrates conducted to date have inferred learning via the behaviour of individuals after birth, it is often unknown whether embryos themselves show behavioural evidence of learned predator recognition and, if so, when during development the capacity to learn emerges (Sehr et al., 2016).

Moreover, for many aquatic species the physical organization of eggs within the nest presents an opportunity for learning through social mechanisms, in addition to the direct evaluation of environmental cues. Thus, a second understudied area of research concerns the extent to which the social environment of the nest influences learning performance and/or the expression of behaviour before hatching (Colombelli-Negrel et al., 2014; Du & Shine, 2022; Noguera & Velando, 2019). In aquatic vertebrates, conspecific social interactions are known to contribute substantially to the development of behaviour after hatching (Brown & Laland, 2003; Crane et al., 2018), including the development of predator defences (Chapman et al., 2008; Kelley et al., 2003; Kelley & Magurran, 2003; Watve & Taborsky, 2019). For example, juvenile reef fish, *Dascyllus marginatus*, show greater predator avoidance in the presence of an adult conspecific compared to when alone or paired with another juvenile fish (Karplus et al., 2006). However, even in the absence of opportunities for social transmission of previously learned information from experienced individuals (i.e. cases wherein all individuals in a group are naïve), the social context can affect how individuals evaluate and respond to environmental information and influence independent learning processes. For example, individuals frequently show evidence of improved learning performance in the presence of conspecifics versus in isolation (e.g. Brandão et al., 2015; Stanbrook et al., 2020), and both cognitive flexibility and learning rate have been shown to be correlated with group size in some species (Gleason et al., 1977; Triki et al., 2024; but see Manassa et al., 2014; Mathiron et al., 2015). Group size can also influence the intensity of antipredator responses, with individuals in larger groups of some species showing enhanced antipredator behaviour (Mathiron et al., 2015; but see Chivers & Ferrari, 2015).

To gain insight into the mechanisms of embryonic learning and behaviour in aquatic vertebrates, we evaluated the development and expression of acquired predator recognition in embryonic fathead minnows, *Pimephales promelas*. Our goals were to (1) determine when learned predator recognition via direct evaluation of environmental cues is first behaviourally expressed in embryos and (2) test the hypothesis that embryonic antipredator behaviour is influenced by two physical attributes of clutch structure: group size and embryo proximity. During the reproductive season (May—September), female minnows lay clutches of up to 400 eggs in a single layer on the underside of wood, rocks and floating aquatic substrates (Andrews & Flickinger, 1973; Page & Burr, 2011; Wisenden et al., 2009). The eggs are typically densely organized, and most individuals develop in close physical contact with siblings. However, both the physical structure and social composition of a nest may vary according to the natural ecology of the species. Fathead minnows are fractional spawners (Gale & Buynak, 1982), and eggs in smaller clutches may be more sparsely distributed on the spawning substrate (B. Karasch & J. Ward, personal observations). In nature, female minnows also frequently lay their eggs in the nests of males that are already guarding eggs, resulting in nests that contain embryos of varying age and genetic relatedness (Bessert et al., 2007; Stone et al., 2019; Unger & Sargent, 1988). Embryonic development in *P. promelas* is well characterized (Bohler, 2012; Devlin et al., 1996), with individuals passing through several distinct stages before hatching at approximately 5 days postfertilization at room temperature, under laboratory conditions (Crowder & Ward, 2022). Fathead minnows are an ideal species for investigations of embryonic behaviour and learning because they exhibit well-developed, unconditioned antipredator responses to chemical cues from injured conspecifics (i.e. conspecific alarm cue; Chivers & Smith, 1994, 1998; Crane et al., 2020; Mathis & Smith, 1993a), including at the embryonic stage (Crowder & Ward, 2022). Embryonic *P. promelas* reared in the presence of olfactory cues indicative of predatory threat exhibit reduced spontaneous, conspicuous movement (e.g. embryonic ‘flexing’) inside the egg under conditions of perceived predation risk (Crowder & Ward, 2022), which is thought to minimize detection by a predator (Gervais et al., 2021; Kempster et al., 2013; Mezrai et al., 2020). This research also showed that embryos conditioned to associate alarm cue with the odour of a predator display enhanced antipredator behaviour in the presence of predator odour alone after hatching, which is evidence for embryonic learning in this species.

Our first objective was to determine whether embryos show behavioural evidence of learned predator recognition prior to hatching and, if so, to identify the developmental window in which such behaviour is first expressed. In minnows, olfactory canals and supporting structures develop between 24 and 50 hours post fertilization (Devlin et al., 1996). Thus, we predicted that embryos would not show behavioural evidence for learned predator recognition until after the olfactory and neural structures responsible for conspecific alarm cue recognition and learning were established, as evidenced by reduced embryonic activity in the presence of predator cues. Our second objective was to evaluate the effect of social features of the clutch (e.g. group size and embryo proximity) on embryonic behaviour and predator learning (Manassa et al., 2014; Mathiron et al., 2015). To assess the potential for clutch-specific influences, we conditioned and tested embryos in either small groups (2—5 embryos) or large groups (10—15 embryos) and in direct contact with, or physically isolated from, neighbouring siblings. If embryos are sensitive to the presence of conspecifics in the nest, we predicted that those that were in physical contact with one another and those in larger groups would show more pronounced responses to predator cues than those that were not, given the potential to obtain environmental information though both direct sampling and indirect (social) means.

METHODS

Animals, Maintenance and Housing

Reproductively mature minnows (6+ months) were purchased from a laboratory culture facility (Aquatic Biosystems, Fort Collins, CO, U.S.A.) and bred to generate minnow eggs for the study. The hatchery population from which the parental generation was sourced was raised in a single-species, indoor recirculating hatchery, with no prior predator experience for at least 10 generations. Upon arrival at the laboratory, breeding groups consisting of one male and two females were introduced into 6-litre tanks breeding tanks containing a PVC spawning tile in a recirculating flow-through aquatic housing system (Aquaneering Inc., San Diego, CA, U.S.A.). The fish were fed an ad libitum diet of live brine shrimp (*Artemia* spp.) supplemented with bloodworms (*Glycera* spp.) twice daily and were maintained under a 16:8 hour light:dark cycle at 23 °C for the duration of the experiment. Spawning tiles were checked for the presence of eggs twice each day. Eggs were immediately removed from the tank when found.

Ethical Note

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Ball State University (protocol number 1220881-24).

Developmental Treatments and Rearing

Newly laid clutches were assigned to one of two developmental water treatments, control water or water containing olfactory cues from a piscivorous predator (bluegill sunfish, *Lepomis macrochirus*) combined with conspecific alarm cue, following previously described methods (Crowder & Ward, 2022). A stock solution of conspecific alarm cue was created before the start of the experiment from naturally deceased or humanely euthanized (via a lethal dose of MS-222) frozen adult female minnows (Cashner, 2004; Crowder & Ward, 2022; Mathis & Smith, 1993b). To generate the alarm cue, 60 minnows were thawed at room temperature and rinsed thoroughly in tap water. The skins were removed with a scalpel, homogenized for 10 min in 20 ml of deionized water and then diluted with 350 ml of deionized water. The homogenate was mechanically filtered to remove any tissue debris, and 3 ml quantities of the filtered cue solution were aliquoted into vials and frozen at -20 °C until use. Water containing olfactory predator cues was obtained from a 140-gallon (530-litre) tank (model LS-700, Frigid Units Inc., Toledo, OH, U.S.A.) containing four adult bluegill sunfish. The sunfish were fed the same diet as the minnow population to eliminate confounding novel dietary cues. Fresh predator + alarm cue treatment water was made each day by mixing 800 ml of the aged, aerated water containing predator cue with 3 ml of conspecific alarm cue. Fresh control treatments were also made each day, consisting only of aged and aerated dechlorinated water that was free of predator or alarm cues.

Eggs were selected at random from the spawning tile on the day that they were laid and divided among four, 3.5 cm diameter dishes containing 6 ml of the appropriate water treatment. The number and arrangement of embryos in each of the dishes was experimentally manipulated such that eggs were placed in either a small group (5 embryos) or a large group (15 embryos). For each group size treatment, half of the embryos were clustered, meaning they were placed in physical contact with at least one other embryo, and half were dispersed, meaning that they were not in physical contact with any other embryos. We maintained the embryos under these conditions for 5 days at 21–23 °C (~12–24 hour before hatching). Each day, half (~3 ml) of the water was gently removed from each dish using a fine pipette and replaced with an equivalent volume of water containing freshly prepared olfactory cues or fresh control water. Embryos were assessed three times daily for mortality and dead embryos were gently removed with forceps. Mean final group sizes were 3.8 (range 2–5 embryos) and 14.9 (range 10–15 embryos) for the small and large groups, respectively.

Behavioural Tests

To determine whether embryos that develop in different clutch configurations differ in their behaviour, embryos in large or small groups and those physically clustered or dispersed were presented with water containing either predator olfactory cues alone or control water on days 3, 4 and 5 of development (Fig. 1). Embryos remained in their dishes of residence during tests.

To begin a test, the dish was placed on the stage of a dissecting microscope (Zeiss Stemi 508) outfitted with a camera (Axiocam 208 Color) and the embryos were permitted 2 min to acclimate. Thirty to 60 s before the start of a test, 3 ml of either control water or predator cue was added to the dish. Half of the clutches were presented with predator cue on days 3–5 of development, and the other half were tested with control water. Embryonic behaviour was recorded for 5 min after administration of the stimulus, and an automated behavioural detection program (DanioScope, EthoVision, Noldus, Leesburg, VA, U.S.A.) was used to extract embryonic activity data from the tapes. For each embryo, we recorded the proportion of trial time spent engaged in locomotor activity. Within the egg, embryos exhibit frequent spontaneous locomotion in the form of twitching or rolling. However, embryos are less conspicuously active under perceived conditions of predation risk (Crowder & Ward, 2022).

Statistical Analysis

General linear mixed-effects models (GLMMs) were used to determine when embryonic learning was first expressed during development and to assess the influence of group size and neighbour proximity on embryo behaviour. In initial analyses, we included developmental treatment (predator-conditioned or naïve), test stimulus (predator cue or control water), neighbour proximity (clustered or dispersed), group size (small or large) and developmental day (3, 4 or 5) as main factors in the model, as well as all two-way interactions between these factors. In addition, we included the three-way interaction between embryo developmental treatment, test stimulus and developmental day to determine when embryonic antipredator behaviours are first expressed. We also included three-way interactions between embryo developmental treatment, test stimulus and group size and between embryo developmental treatment, test stimulus and neighbour proximity, respectively, to evaluate whether the responses of conditioned and nonconditioned embryos to a predatory stimulus varied across social contexts. Clutch identity was included as a random effect in all models. For significant interaction terms of interest, we conducted additional GLMM analyses followed by Tukey post hoc tests to determine the direction and magnitude of the effect. All analyses were conducted using the package ‘nlme’ in R (version 4.0.3; R Core Team, 2021).

RESULTS

A total of 58 clutches were collected during the experimental period. Of these, 20 were reared under control conditions (11

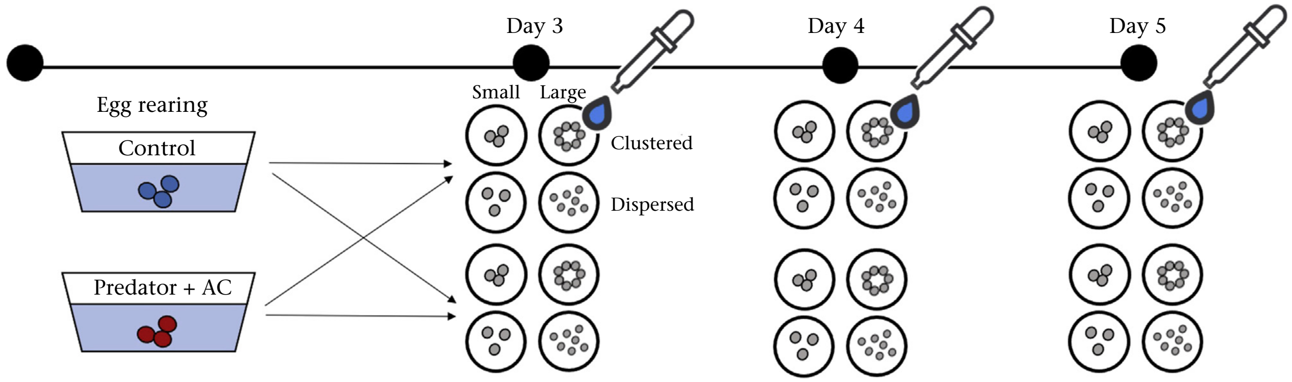

Figure 1. Testing conditions and experimental design. Predator-conditioned embryos (daily exposure to bluegill sunfish odour combined with minnow alarm cue, AC) and predator-naïve embryos (control water exposure) were reared to hatching in one of four social configurations (small or large group, with or without direct contact with other embryos). Embryonic behaviour in response to predator cue or control water stimuli was tested on days 3, 4 and 5 of development.

clutches tested on day 5 of development for response to a control stimulus and 9 tested in response to predator olfactory cues). Thirty-eight clutches underwent daily exposure to predator cue combined with conspecific alarm cue (14 of which were subsequently tested in response to control stimulus and 24 of which were tested in response to predator olfactory cues).

Effects of Developmental Age and Clutch Structure on Embryonic Behaviour

The proportion of trial time that embryos spent engaged in locomotor activity was variable across trial scenarios (range 2.5–22.4% across all treatments and days). However, mean activity levels showed consistent decreases with increased developmental age. Across groups, the mean (± SD) proportion of embryonic activity was 13.51 ± 0.69% on day 3 of development, 12.63 ± 0.69% on day 4 of development and 5.23 ± 0.69% on day 5 of development. Accordingly, there was a significant main effect of day of embryonic development on activity level (Table 1). Pairwise Tukey post hoc tests indicated that activity significantly and linearly decreased over the developmental period (day 3 versus day 4: $P = 0.02$; day 3 versus day 5: $P < 0.0001$; day 4 versus day 5: $P < 0.0001$; Fig. 2a).

Both the number of embryos in the clutch and the proximity of embryos to one another had significant effects on behaviour (Table 1). Specifically, mean (± SD) trial embryonic activity levels were moderately higher (18% increase) in larger experimental clutches (11.32 ± 0.67% versus 9.59 ± 0.7%; Fig. 2b) and substantially lower (52% decrease) in trials in which embryos were in physical contact with one another compared to trials in which they were dispersed (12.62 ± 0.68% versus 8.29 ± 0.68%; Fig. 2c).

Embryonic Learning

We did not observe significant main effects of either the developmental environment (predator + alarm cue or control) or test stimulus (predator cue or control) on the percentage of time spent active during trials (Table 1). However, there was a significant interaction between embryonic rearing environment and test stimulus, indicating that embryos that were conditioned to learn the identity of a predator responded differently to olfactory stimuli than those that were not (Table 1). Subsequent analyses conducted separately for embryos reared under control conditions and those exposed to predator cues combined with alarm cue revealed that whereas embryos reared under control conditions showed similar levels of activity in the presence of control water or predator cues ($F_{1,1889} = 2.07$, $P = 0.15$; Fig. 3), embryos reared in the presence of predator cue + alarm cue showed significantly reduced activity in response to alarm cue alone compared to control water ($F_{1,36} = 12.5$, $P = 0.001$; Fig. 3).

Emergence of Learned Predator Recognition during Development

The three-way interaction between developmental treatment, test stimulus and day of development was significant (Table 1), indicating that how predator-conditioned and nonconditioned embryos responded to predator cues diverged over the developmental period. Subsequent analyses conducted separately for predator-conditioned and naïve embryos on each day of the experiment showed that changes in behaviour indicative of learned predator recognition were first evident on day 4 of development (Fig. 4). Neither predator-conditioned embryos ($F_{1,29} = 2.85$,

Table 1
Results of a linear mixed-effects model examining the effects of developmental environment (control, predator cue + alarm cue), test stimulus (control, predator cue) and clutch features (group size, neighbour proximity) on the development and expression of learned predator recognition prior to hatching (3, 4 or 5 days postfertilization)

	F	*df*	*P*
Developmental environment	0.257	1, 4769	0.612
Test stimulus	0.0642	1, 4769	0.800
Day	417.520	2, 4769	<0.0001
Group size	26.637	1, 4769	<0.0001
Neighbour proximity	246.640	1, 4769	<0.0001
Developmental environment∗test stimulus	6.742	1, 54	0.012
Developmental environment∗day of development	36.250	2, 4769	<0.0001
Test stimulus∗day of development	6.287	2, 4769	0.002
Developmental environment∗test stimulus∗neighbour proximity	12.088	1, 4763	0.0005
Developmental environment∗test stimulus∗group size	0.420	1, 4763	0.517
Developmental environment∗test stimulus∗day of development	15.239	2, 4769	<0.0001

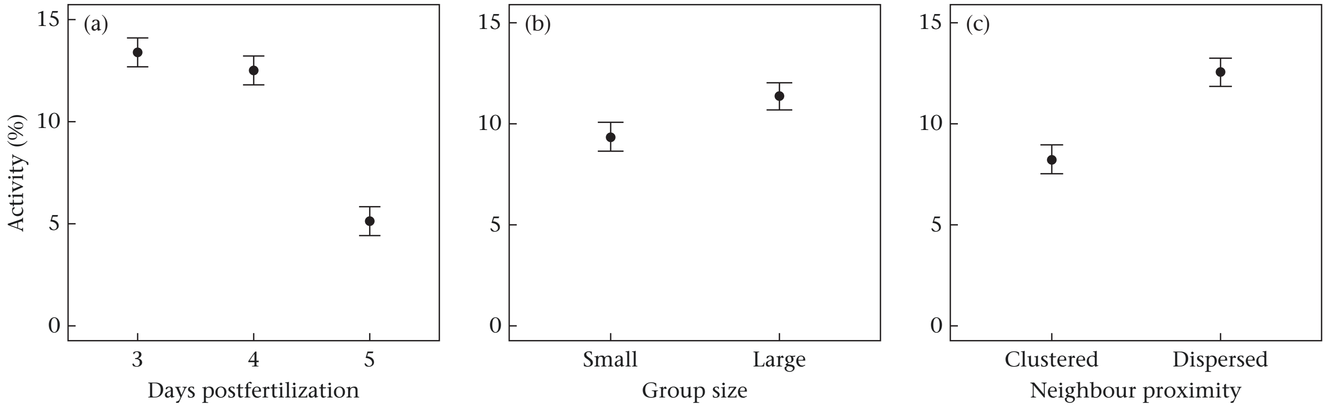

Figure 2. Marginal means from a linear mixed model showing main effects on embryonic locomotor activity (percentage of trial time spent active) of (a) embryonic development on day 3 ($N = 1723$), day 4 ($N = 1676$) and day 5 ($N = 1586$) postfertilization, (b) embryo group size (small, 2–5 embryos, $N = 1163$; large, 10–15 embryos, $N = 3674$) and (c) proximity of neighbouring embryos (clustered, in physical contact, $N = 2652$; dispersed, no physical contact, $N = 2185$). Points and whiskers represent means and standard errors.

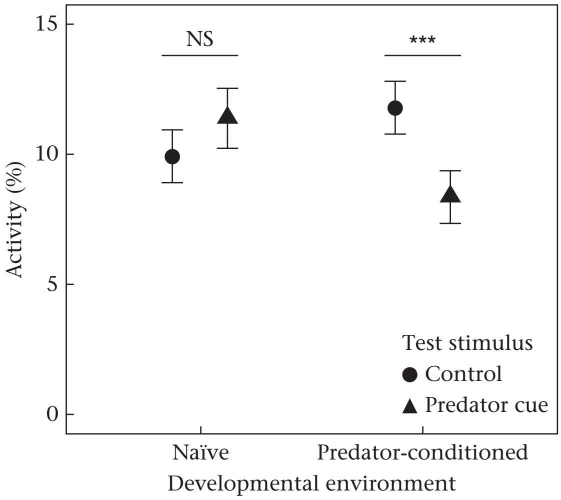

Figure 3. Marginal means from a linear mixed model showing mean locomotor activity (percentage of trial time spent active) of embryos as a combination of embryo developmental environment and olfactory test stimuli. Control stimulus: $N = 1067$ naïve fish, $N = 1067$ predator-conditioned fish; predator cue stimulus: $N = 889$ naïve fish, $N = 889$ predator-conditioned fish. Points and whiskers represent means and standard errors. ***$P \leq 0.001$ compared with control.

$P = 0.102$) nor naïve embryos ($F_{1,626} = 0.67$, $P = 0.411$) differed in their response to predator cues versus control water on day 3. However, on day 4, predator-conditioned embryos showed a ~5% decrease in activity in the presence of predator cues compared to their behaviour in control water ($F_{1,33} = 7.76$, $P = 0.009$), which persisted through day 5 ($F_{1,31} = 6.78$, $P = 0.014$). By contrast, naïve embryos showed higher levels of activity in the presence of predator cues compared to control water on both days 4 and 5 (day 4: $F_{1,617} = 5.56$, $P = 0.012$; day 5: $F_{1,607} = 7.76$, $P = 0.006$; Fig. 4).

Effects of Clutch Size and Neighbour Proximity on Embryonic Responses to Test Stimuli

Our initial results indicated that differences between naïve versus predator-conditioned embryos in their responses to the test stimuli depended on aspects of clutch structure (group size and embryo proximity; Table 1). Because we found that learned behavioural responses to the predator did not emerge until day 4 of development, we restricted follow-up analyses conducted separately for naïve and predator-conditioned embryos to data collected on days 4 and 5 of development. Predator-conditioned embryos showed consistent, parallel decreases in activity in the presence of predator cues compared to control water irrespective of group size ($F_{2,1951.16} = 0.69$, $P = 0.50$; Fig. 5a) or neighbour proximity ($F_{2,1948.88} = 1.28$, $P = 0.28$; Fig. 5b). By contrast, embryos reared in control water showed no difference in activity levels in the presence of predator cues in larger or smaller groups ($F_{2,1272.28} = 0.63$, $P = 0.53$; Fig. 5a) but were more active when not in physical contact with any other embryos ($F_{2,1277.54} = 9.23$, $P < 0.001$; Fig. 5b).

DISCUSSION

In this study, we investigated (1) whether and at what stage of development fathead minnow embryos express behavioural evidence of acquired predator recognition and (2) whether social features of the clutch have an observable influence on embryo behaviour. We found that embryos conditioned to identify a predator showed changes in locomotor activity in the presence versus absence of predator cues consistent with an antipredator interpretation and that behavioural expression of this response emerged at approximately 4 days postfertilization. We also found that two features of clutch structure and organization (group size and embryo proximity) had a detectable influence on behaviour; specifically, embryos in physical contact with other embryos and those in smaller groups showed lower levels of activity than those in isolation. However, this finding was observed in both naïve and predator-conditioned embryos, suggesting that embryos may generally attend to the presence of neighbours regardless of prior experience.

The finding that embryos conditioned to identify a predator based on olfactory cues exhibited differences in spontaneous activity in the presence versus absence of those olfactory cues adds to a growing body of research demonstrating that not only do embryos possess the capacity to acquire information from the environment (Garcia et al., 2017; Godoy et al., 2021; Horn & Chivers, 2021; Poisson et al., 2017), but that they also respond to those environmental cues before hatching (Horn et al., 2019; Mezrai et al., 2020; Noguera & Velando, 2019). In the context of predation, the ability to learn and respond to predator cues at the egg stage has been shown to improve survival in one of two ways: by allowing for escape or by decreasing the probability of detection (Fuiman &

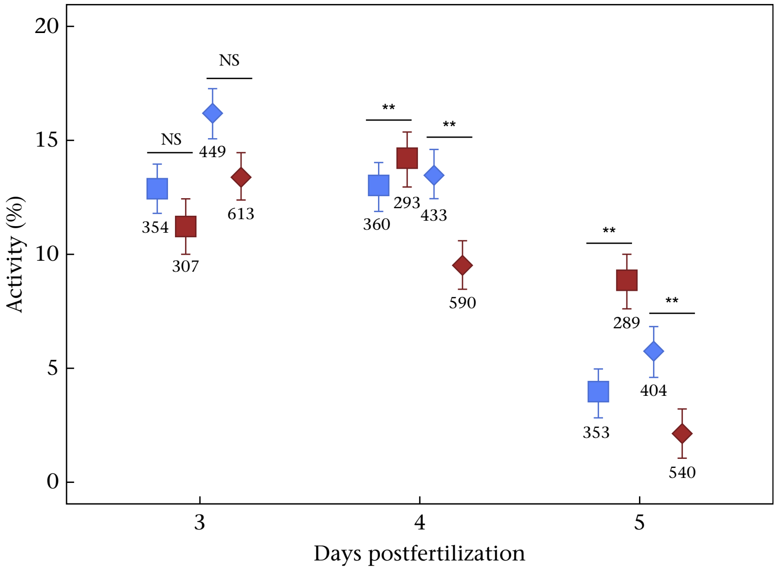

Figure 4. Marginal means from a linear mixed model showing mean locomotor activity (percentage of trial time spent active) of embryos over time (days postfertilization) in relation to developmental environment (naïve (control): square symbols; predator-conditioned (predator + alarm): diamonds) and olfactory test stimulus (control stimulus: blue symbols; predator stimulus: red symbols). Points and whiskers represent means and standard errors. Numbers below the symbols are the number of embryos in each test combination. $^{**}P \leq 0.01$ compared with control.

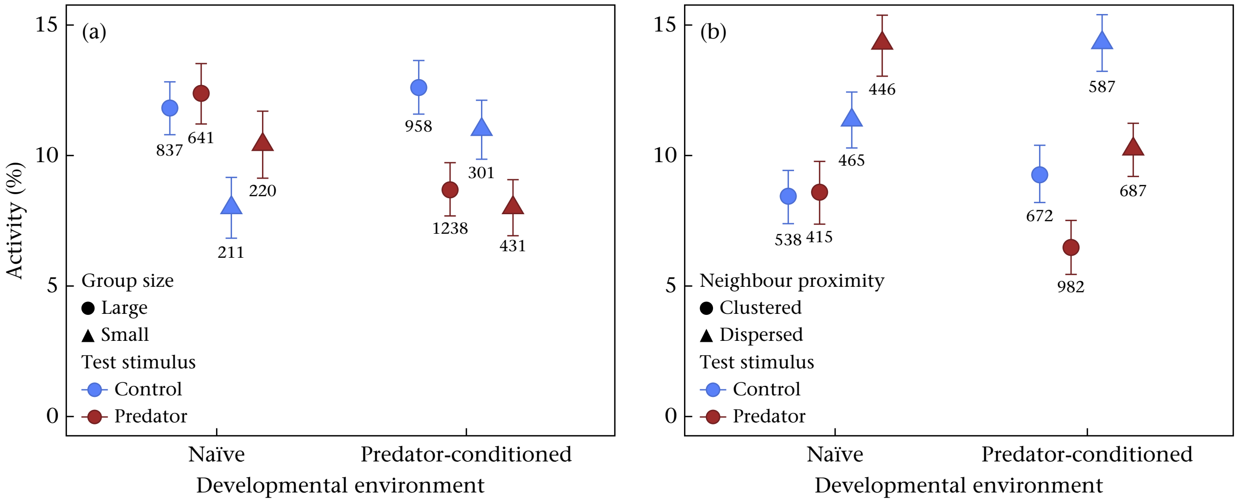

Figure 5. Marginal means from a linear mixed model showing mean response (percentage of trial time spent active) of naïve (control) and predator-conditioned (predator + alarm) embryos in response to control versus predator cues as a function of (a) group size (small, 2–5 embryos; large 10–15 embryos) and (b) neighbour proximity (clustered, in physical contact; dispersed, no contact). Points and whiskers represent means and standard errors. Numbers below the symbols are the number of embryos in each test combination.

Magurran, 1994; Kelley & Magurran, 2003; Weihs & Webb, 1984). The former phenomenon is well documented in multiple frog and fish species, with individuals exposed to predator cues during embryogenesis showing accelerated developmental rates and earlier hatching times (e.g. Doody & Paull, 2013; Smith & Fortune, 2009; Wisenden et al., 2022) and/or rapid escape-hatching behaviour in response to an immediate threat (Güell et al., 2022; Jung et al., 2022; Warkentin, 2005). For example, fathead minnow embryos are sometimes vulnerable to cannibalism by parental and alloparental males (Green et al., 2008; Stone et al., 2019). In such cases, escape may be the only survival strategy available. However, the immediate antipredator benefits associated with early hatching may trade off with longer-term developmental fitness costs. For example, individuals that hatch early are commonly smaller (Musa et al., 2020) and have underdeveloped locomotor and/or sensory systems (Delia et al., 2019) resulting in reduced posthatch individual performance and higher posthatch mortality (Colbert et al., 2010; Willink et al., 2014; Wisenden et al., 2022).

Alternatively, embryos may adjust their behaviour inside the egg to reduce the probability of detection by predators (Kempster et al., 2013; Poisson et al., 2017). Such a strategy may be particularly likely if predator olfactory cues are detected by embryos without accompanying visual or tactile stimuli, indicative of an initial phase of the predation sequence (i.e. before the predator has detected the prey; Brönmark & Hansson, 2000; Landeira-Dabarca et al., 2019). Consistent with this idea and previous work on this species (Crowder & Ward, 2022), in this study minnow embryos conditioned to recognize predator olfactory cues showed lower levels of spontaneous activity within the egg in the presence of olfactory predator cues compared with blank water control conditions. Reduced conspicuousness is also thought to explain changes in the behaviour of other embryonic aquatic species in the presence of predator cues, including cessation of movement of embryonic sharks and cuttlefish (Gervais et al., 2021; Kempster et al., 2013; Mezrai et al., 2020).

Behavioural evidence consistent with an interpretation of acquired predator recognition first emerged at 4 days postfertilization, corresponding to stage 30–31 of development at 25 °C (Devlin et al., 1996). In minnows, the olfactory placodes are first visible at stage 21 of embryonic development (approximately 24 hours postfertilization), with axons extending from the olfactory groove to the olfactory lobes of the telencephalon by stage 28 (74 hours postfertilization) and sensory cells bordering the olfactory groove by stage 29 (85 hours postfertilization; Devlin et al., 1996). By contrast, the first evidence of embryonic body movement in minnow embryos occurs at stage 22 (approximately 26 hours postfertilization), preceding the development of the olfactory sensory system (Bohler, 2012; Devlin et al., 1996). Indeed, locomotor movement is well developed by 72 h postfertilization and individuals show clear changes in behaviour in response to other environmental cues (e.g. mechanical disturbance) at this time (K. Steinberg & J. Ward, personal observation), Thus, the timing of the behavioural onset of associatively learned antipredator responses based on olfaction in minnows may more strongly reflect development of the sensory and neural systems involved in perception and/or processing rather than the functionality of the motor system required to execute a response.

We also detected an observable influence of the structural organization of the clutch on embryonic behaviour, providing the first evidence that embryonic *P. promelas* are sensitive to the presence and behaviour of neighbours. Specifically, we found evidence that embryos reared and tested in physical contact with one another and those in smaller groups were less active than those lacking contact with other embryos and those in bigger groups. Broadly, our findings are consistent with those reported for other taxa that found that embryos perceive cues produced by nestmates and adjust their behaviour in response to them (Aubret et al., 2016; Endo et al., 2019; McGlashan et al., 2012; Noguera & Velando, 2019; Riley et al., 2020; Vergne & Mathevon, 2008). For example, younger embryonic water snakes (*Natrix maura*) in mixed-age nests accelerate development to facilitate synchronized hatching with older nestmates (Aubret et al., 2016), which is hypothesized to reduce predation risk (Colbert et al., 2010; Endo & Numata, 2017). However, our specific predictions that the activity levels of individuals within the chorion would be lower in clustered eggs and in larger groups were only partially supported.

Assuming that all eggs in a clutch are equally vulnerable to piscivorous predators, all members of the group are likely to benefit from heightened responsiveness (Mathiron et al., 2015; Ward & Webster, 2016). Indeed, group coordination has been shown to be an important factor in the effective execution of a collective response to predatory threat in fish (Handegard et al., 2012; Zheng et al., 2005). Consistent with this interpretation, in our study, embryos in physical contact with one another, who presumably had access to social cues from neighbouring eggs in addition to personal information, showed lower levels of activity than isolated eggs. However, in contrast with our expectation, activity levels in larger groups were greater than those in smaller groups. Moreover, we did not find evidence that the presence or number of conspecifics influenced the likelihood or onset timing of associative learning in predator-conditioned embryos, which aligns with the results of previous studies conducted after hatching (Davis et al., 2023; Chivers & Ferrari, 2015; Manassa et al., 2014; Mathiron et al., 2015).

This study adds to a growing body of literature on embryonic behaviour (Du & Shine, 2022) by demonstrating evidence for learned predator recognition prior to hatching and identifying the developmental window over which behavioural evidence of the embryonic learning process emerges in fathead minnows, which is becoming a model species for studies of the development and mechanisms of embryonic learning in fish. Moreover, we present evidence that suggests that embryos are sensitive to the presence of adjacent conspecifics, suggesting the potential for social mechanisms of embryonic learning in addition to direct (asocial) evaluation of environmental cues. An intriguing area for future research would be to understand how embryonic interactions change as a function of nest composition. In *P. promelas*, as in other species, females preferentially lay their eggs in nests that already contain one or more (often older) clutches (Jamieson, 1995; Ridley & Rechten, 1981); these new clutches are usually laid in direct contact with older clutches laid hours to days earlier, and the coefficient of relatedness (r) among individuals in a nest may vary from 0 (in the case of sneak fertilizations or after a nest take-over by another male; Bessert et al., 2007; Unger & Sargent, 1988) to 0.5. A second potential area for follow-up concerns the roles played by different sensory modalities in mediating information transfer among nestmates. For example, one potential source of conspecific-derived information is physical disturbance cues generated by the movement of neighbouring embryos. Indeed, in both avian and nonavian reptiles, physical disturbance cues produced by siblings, including changes in cardiac activity (e.g. Aubret et al., 2016) and motility within the egg (Noguera & Velando, 2019), function as sources of social information that alter developmental programming and coordinate behaviour within the clutch (Doody et al., 2012; McGlashan et al., 2012). However, because fathead minnow eggs are transparent, visual cues may provide an additional source of information. Additional research is now underway to determine whether and how embryos perceive and transmit social information in the nest and how conspecific information influences embryonic behaviour and learning in lower aquatic vertebrates.

Author Contributions

Brooke Karasch: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jessica Ward:** Writing – review & editing, Supervision, Software, Resources, Methodology, Funding acquisition, Conceptualization.

Data Availability

Data are available at https://doi.org/10.17632/8djgh8jp4x.1.

Declaration of Interest

The authors have no competing interests to declare.

Acknowledgments

We thank the dedicated undergraduates who assisted with the care and maintenance of the fish, and Dee Hoffman and Susan Calvin for their unwavering support of our research. We also thank two anonymous referees for their thoughtful suggestions to improve the manuscript. This research was supported by the U.S. National Science Foundation under grant number IOS-2152668.

References

Andrews, A., & Flickinger, S. (1973). Spawning requirements and characteristics of the fathead minnow. *Proceedings of Southeastern Association of Fish and Wildlife*

Agencies Conference, 27, 759–766. https://seafwa.org/journal/1973/spawning-requirements-and-characteristics-fathead-minnow.

Atherton, J. A., & McCormick, M. I. (2015). Active in the sac: Damsel fish embryos use innate recognition of odours to learn predation risk before hatching. *Animal Behaviour, 103*, 1–6. https://doi.org/10.1016/j.anbehav.2015.01.033

Aubret, F., Blanvillain, G., Bignon, F., & Kok, P. J. R. (2016). Heartbeat, embryo communication and hatching synchrony in snake eggs. *Scientific Reports, 6*. https://doi.org/10.1038/srep23519. Article 23519.

Bessert, M. L., Brozek, J., & Ortí, G. (2007). Impact of nest substrate limitations on patterns of illegitimacy in the fathead minnow, *Pimephales promelas* (Cypriniformes: Cyprinidae). *Journal of Heredity, 98*(7), 716–722.

Bohler, S. (2012). The fathead minnow embryo as a model for the development of alternative testing methods in ecotoxicology *(M.S. thesis)*. University of Heidelberg.

Brönmark, C., & Hansson, L.-A. (2000). Chemical communication in aquatic systems: An introduction. *Oikos, 88*(1), 103–109.

Brandão, M. L., Braithwaite, V. A., & Gonçalves-de-Freitas, E. (2015). Isolation impairs cognition in a social fish. *Applied Animal Behaviour Science, 171*, 204–210. https://doi.org/10.1016/j.applanim.2015.08.026

Brown, C., & Laland, K. N. (2003). Social learning in fishes: A review. *Fish and Fisheries, 4*, 280–288.

Cashner, M. (2004). Are spotted bass (*Micropterus punctulatus*) attracted to schreckstoff? A test of the predator attraction hypothesis. *Copeia, 3*, 592–598.

Chapman, B. B., Morrell, L. J., Benton, T. G., & Krause, J. (2008). Early interactions with adults mediate the development of predator defenses in guppies. *Behavioral Ecology, 19*, 87–93.

Chivers, D. P., & Ferrari, M. C. O. (2015). The effect of group size and tutor-to-observer ratio on socially learned antipredator responses in woodfrog tadpoles. *Animal Behaviour, 104*, 25–29.

Chivers, D. P., Kiesecker, J. M., Marco, A., DeVito, J., Anderson, M. T., & Blaustein, A. R. (2001). Predator-induced life history changes in amphibians: Egg predation induces hatching. *Oikos, 92*, 135–142.

Chivers, D. P., Mirza, R. S., & Johnston, J. G. (2002). Learned recognition of heterospecific alarm cues enhances survival during encounters with predators. *Behaviour, 139*, 929–938.

Chivers, D. P., & Smith, R. J. F. (1994). Fathead minnows, *Pimephales promelas*, acquire predator recognition when alarm substance is associated with the sight of unfamiliar fish. *Animal Behaviour, 48*, 597–605.

Chivers, D. P., & Smith, R. J. F. (1998). Chemical alarm signalling in aquatic predator–prey systems: A review and prospectus. *Écoscience, 5*(3), 338–352. https://doi.org/10.1080/11956860.1998.11682471

Colbert, P. L., Spencer, R. J., & Janzen, F. J. (2010). Mechanism and cost of synchronous hatching. *Functional Ecology, 24*(1), 112–121. https://doi.org/10.1111/j.1365-2435.2009.01602.x

Colombelli-Negrel, D., Hauber, M. E., Kleindorfer, S., Colombelli-Négrel, D., Hauber, M. E., & Kleindorfer, S. (2014). Prenatal learning in an Australian songbird: Habituation and individual discrimination in superb fairy-wren embryos. *Proceedings of the Royal Society B: Biological Sciences, 281*(1797), Article 20141154. https://doi.org/10.1098/rspb.2014.1154

Crane, A. L., Ferrari, M. C. O., Rivera-Hernández, I. A. E., & Brown, G. E. (2020). Microhabitat complexity influences fear acquisition in fathead minnows. *Behavioral Ecology, 31*(1), 261–266. https://doi.org/10.1093/beheco/arz187

Crane, A. L., Helton, E. J., Ferrari, M. C. O., & Mathis, A. (2018). Learning to find food: Evidence for embryonic sensitization and juvenile social learning in a salamander. *Animal Behaviour, 142*, 199–206.

Crowder, C., & Ward, J. (2022). Embryonic antipredator defenses and behavioral carryover effects in the fathead minnow (*Pimephales promelas*). *Behavioral Ecology and Sociobiology, 76*, Article 27. https://doi.org/10.1007/s00265-022-03136-2

Darmaillacq, A. S., Lesimple, C., & Dickel, L. (2008). Embryonic visual learning in the cuttlefish, *Sepia officinalis*. *Animal Behaviour, 76*(1), 131–134. https://doi.org/10.1016/j.anbehav.2008.02.006

Davis, O. N., Shah, P., Rahin, O., & Wund, M. A. (2023). Threespine stickleback do not use social cues to learn about predation risk. *Behavioural Processes, 206*, Article 104846. https://doi.org/10.1016/j.beproc.2023.104846

Delia, J., Rivera-Ordonez, J. M., Salazar-Nicholls, M. J., & Warkentin, K. M. (2019). Hatching plasticity and the adaptive benefits of extended embryonic development in glassfrogs. *Evolutionary Ecology, 33*(1), 37–53. https://doi.org/10.1007/s10682-018-9963-2

Devlin, E., Brammer, J., Puyear, R., & McKim, J. (1996). Prehatching development of the fathead minnow *Pimephales promelas* Rafinesque. United States Environmental Protection Agency.

Doody, J. S., & Paull, P. (2013). Hitting the ground running: Environmentally cued hatching in a lizard. *Copeia, 2013*(1), 160–165.

Doody, J. S., Stewart, B., Camacho, C., & Christian, K. (2012). Good vibrations? Sibling embryos expedite hatching in a turtle. *Animal Behaviour, 83*(3), 645–651. https://doi.org/10.1016/j.anbehav.2011.12.006

Du, W. G., & Shine, R. (2022). The behavioural and physiological ecology of embryos: Responding to the challenges of life inside an egg. *Biological Reviews, 97*(4), 1272–1286. https://doi.org/10.1111/brv.12841

Endo, J., & Numata, H. (2017). Effects of embryonic responses to clutch mates on egg hatching patterns of Pentatomidae (Heteroptera). *Physiological Entomology, 42*(4), 412–417. https://doi.org/10.1111/phen.12219

Endo, J., Takanashi, T., Mukai, H., & Numata, H. (2019). Egg-cracking vibration as a cue for stink bug siblings to synchronize hatching. *Current Biology, 29*(1), 143–148. https://doi.org/10.1016/j.cub.2018.11.024

Eschbach, C., Fushiki, A., Winding, M., Schneider-Mizell, C., Shao, M., Arruda, R., Eichler, K., Valdes-Aleman, J., Ohyama, T., Thum, A., Gerber, B., Fetter, D., Truman, J., Litwin-Kumar, A., Cardona, A., & Zlatic, M. (2020). Recurrent architecture for adaptive regulation of learning in the insect brain. *Nature Neuroscience, 23*, 544–555. https://doi.org/10.1038/s41593-020-0607-9

Fan, R., Reader, S. M., & Sakata, J. T. (2022). Alarm cues and alarmed conspecifics: Neural activity during social learning from different cues in Trinidadian guppies. *Proceedings of the Royal Society B: Biological Sciences, 289*(1981), Article 20220829. https://doi.org/10.1098/rspb.2022.0829

Ferrari, M. C. O., & Chivers, D. P. (2009). Latent inhibition of predator recognition by embryonic amphibians. *Biology Letters, 5*(2), 160–162. https://doi.org/10.1098/rsbl.2008.0641

Ferrari, M. C. O., & Chivers, D. P. (2010). The ghost of predation future: Threat-sensitive and temporal assessment of risk by embryonic woodfrogs. *Behavioral Ecology and Sociobiology, 64*(4), 549–555. https://doi.org/10.1007/s00265-009-0870-y

Fuiman, L. A., & Magurran, A. E. (1994). Development of predator defences in fishes. *Reviews in Fish Biology and Fisheries, 4*, 145–183.

Güell, B. A., Jung, J., Almanzar, A., Cuccaro-Díaz, J., & Warkentin, K. M. (2022). Ontogeny of risk assessment and escape-hatching performance by red-eyed treefrog embryos in two threat contexts. *Journal of Experimental Biology, 225*(20), Article jeb244533. https://doi.org/10.1242/jeb.244533

Gale, W. F., & Buynak, G. L. (1982). Fecundity and spawning frequency of the fathead minnow—a fractional spawner. *Transactions of the American Fisheries Society, 111*(1), 35–40. https://doi.org/10.1577/1548-8659(1982)111<35:FASFOT>2.0.CO;2

Garcia, T. S., Bredeweg, E. M., Urbina, J., & Ferrari, M. C. O. (2019). Evaluating adaptive, carry-over, and plastic antipredator responses across a temporal gradient in Pacific chorus frogs. *Ecology, 100*(11), Article e02825. https://doi.org/10.1002/ecy.2825

Garcia, T. S., Urbina, J. C., Bredeweg, E. M., & Ferrari, M. C. O. (2017). Embryonic learning and developmental carry-over effects in an invasive anuran. *Oecologia, 184*(3), 623–631. https://doi.org/10.1007/s00442-017-3905-5

Gazdewich, K. J., & Chivers, D. P. (2002). Acquired predator recognition by fathead minnows: Influence of habitat characteristics on survival. *Journal of Chemical Ecology, 28*(2), 439–445. https://doi.org/10.1023/A:1017902712355

Gervais, C. R., Nay, T., & Brown, C. (2021). Friend or foe? Development of odour detection, differentiation and antipredator response in an embryonic elasmobranch. *Marine and Freshwater Research, 72*(7), 942–949. https://doi.org/10.1071/MF20108

Gleason, P. E., Weber, P. G., & Weber, S. P. (1977). Effect of group size on avoidance learning in zebra fish, *Brachydanio rerio* (Pisces: Cyprinidae). *Animal Learning & Behavior, 5*, 213–216. https://doi.org/10.3758/BF03214081

Godoy, R. S., Weber, V., Lanés, L. E. K., Reichard, M., Gemelli, T., Hohendorff, R. V., & Maltchik, L. (2021). Recognizing the enemy: Do predator cues influence hatching in Neotropical annual killifish? *Journal of Fish Biology, 99*(4), 1476–1484. https://doi.org/10.1111/jfb.14856

Gomez, E. K., Chaiyasarikul, A., Güell, B. A., & Warkentin, K. M. (2023). Developmental changes in red-eyed treefrog embryo behavior increase escape-hatching success in wasp attacks. *Behavioral Ecology and Sociobiology, 77*(5), Article 52. https://doi.org/10.1007/s00265-023-03324-8

Green, W., Mirza, R., & Pyle, G. (2008). Kin recognition and cannibalistic behaviours by adult male fathead minnows (*Pimephales promelas*). *Naturwissenschaften, 95*, 269–272. https://doi.org/10.1007/s00114-007-0319-y

Gross, C. T., & Canteras, N. S. (2012). The many paths to fear. *Nature Reviews Neuroscience, 13*(9), 651–658. https://doi.org/10.1038/nrn3301

Guibé, M., & Dickel, L. (2011). Embryonic visual experience influences post-hatching shelter preference in cuttlefish. *Vie et Milieu, 61*, 243–246.

Guibé, M., Poirel, N., Houdé, O., & Dickel, L. (2012). Food imprinting and visual generalization in embryos and newly hatched cuttlefish, *Sepia officinalis*. *Animal Behaviour, 84*(1), 213–217. https://doi.org/10.1016/j.anbehav.2012.04.035

Handegard, N. O., Boswell, K. M., Ioannou, C. C., Leblanc, S. P., Tjostheim, D. B., & Couzin, I. D. (2012). The dynamics of coordinated group hunting and collective information transfer among schooling prey. *Current Biology, 22*(13), 1213–1217. https://doi.org/10.1016/j.cub.2012.04.050

Horn, M. E., & Chivers, D. P. (2021). Preschool for small frys: Threat-sensitive learning of predators by embryonic fathead minnows. *Animal Behaviour, 178*, 49–55. https://doi.org/10.1016/j.anbehav.2021.05.005

Horn, M. E., Ferrari, M. C. O., & Chivers, D. P. (2019). Retention of learned predator recognition in embryonic and juvenile rainbow trout. *Behavioral Ecology, 30*(6), 1575–1582. https://doi.org/10.1093/beheco/arz116

Jamieson, I. (1995). Do female fish prefer to spawn in nests with eggs for reasons of mate choice copying or egg survival? *American Naturalist, 145*, 824–832.

Jonsson, B., Jonsson, N., & Hansen, M. M. (2022). Knock-on effects of environmental influences during embryonic development of ectothermic vertebrates. *Quarterly Review of Biology, 97*(2), 95–139. https://doi.org/10.1086/720081

Jung, J., Guo, M., Crovella, M. E., McDaniel, J. G., & Warkentin, K. M. (2022). Frog embryos use multiple levels of temporal pattern in risk assessment for vibration-cued escape hatching. *Animal Cognition, 25*(6), 1527–1544. https://doi.org/10.1007/s10071-022-01634-4

Karplus, I., Katzenstein, R., & Goren, M. (2006). Predator recognition and social facilitation of predator avoidance in coral reef fish *Dascyllus marginatus* juveniles. *Marine Ecology Progress Series, 319*, 215–223. https://doi.org/10.3354/meps319215

Kelley, J. L., Evans, J., Indar, R., & Magurran, A. (2003). Back to school: Can antipredator behaviour in guppies be enhanced through social learning? *Animal Behaviour, 65*, 655–662.

Kelley, J. L., & Magurran, A. E. (2003). Learned predator recognition and antipredator responses in fishes. *Fish and Fisheries, 4*, 216–226.

Kempster, R. M., Hart, N. S., & Collin, S. P. (2013). Survival of the stillest: Predator avoidance in shark embryos. *PLoS One, 8*(1), Article e52551. https://doi.org/10.1371/journal.pone.0052551

Landeira-Dabarca, A., Näslund, J., Johnsson, J. I., & Alvarez, M. (2019). Cue recognition and behavioural responses in the three-spined stickleback (*Gasterosteus aculeatus*) under risk of fish predation. *Acta Ethologica, 22*, 209–221.

Lee, Y. C., Darmaillacq, A. S., Dickel, L., & Chiao, C. C. (2020). Effects of embryonic exposure to predators on the postnatal defensive behaviors of cuttlefish. *Journal of Experimental Marine Biology and Ecology, 524*, Article 151288. https://doi.org/10.1016/j.jembe.2019.151288

Manassa, R. P., McCormick, M. I., Dixson, D. L., Ferrari, M. C. O., & Chivers, D. P. (2014). Social learning of predators by coral reef fish: Does observer number influence acquisition of information? *Behavioral Ecology and Sociobiology, 68*(8), 1237–1244. https://doi.org/10.1007/s00265-014-1734-7

Mathiron, A. G. E., Crane, A. L., & Ferrari, M. C. O. (2015). Individual versus. social learning of predator information in fish: Does group size affect learning efficacy? *Behavioral Ecology and Sociobiology, 69*, 939–949.

Mathis, A., Ferrari, M. C. O., Windel, N., Messier, F., & Chivers, D. P. (2008). Learning by embryos and the ghost of predation future. *Proceedings of the Royal Society B: Biological Sciences, 275*(1651), 2603–2607. https://doi.org/10.1098/rspb.2008.0754

Mathis, A., & Smith, R. J. F. (1993a). Chemical labeling of northern pike (*Esox lucius*) by the alarm pheromone of fathead minnows (*Pimephales promelas*). *Journal of Chemical Ecology, 19*(9), 1967–1979. https://doi.org/10.1007/BF00983800

Mathis, A., & Smith, R. J. F. (1993b). Chemical alarm signals increase the survival time of fathead minnows (*Pimephales promelas*) during encounters with northern pike (*Esox lucius*). *Behavioral Ecology, 4*, 260–265. https://doi.org/10.1093/beheco/4.3.260

May, R. C. (1974). Larval mortality in marine fishes and the critical period concept. In J. H. S. Blaxter (Ed.), *The early life history of fish* (pp. 3–19). Springer.

McGlashan, J. K., Spencer, R. J., & Old, J. M. (2012). Embryonic communication in the nest: Metabolic responses of reptilian embryos to developmental rates of siblings. *Proceedings of the Royal Society B: Biological Sciences, 279*(1734), 1709–1715. https://doi.org/10.1098/rspb.2011.2074

Mezrai, N., Arduini, L., Dickel, L., Chiao, C. C., & Darmaillacq, A. S. (2020). Awareness of danger inside the egg: Evidence of innate and learned predator recognition in cuttlefish embryos. *Learning & Behavior, 48*(4), 401–410. https://doi.org/10.3758/s13420-020-00424-7

Musa, S. M., Ripley, D. M., Moritz, T., & Shiels, H. A. (2020). Ocean warming and hypoxia affect embryonic growth, fitness and survival of small-spotted catsharks, *Scyliorhinus canicula*. *Journal of Fish Biology, 97*(1), 257–264. https://doi.org/10.1111/jfb.14370

Nelson, A. B., Alemadi, S. D., & Wisenden, B. D. (2013). Learned recognition of novel predator odour by convict cichlid embryos. *Behavioral Ecology and Sociobiology, 67*(8), 1269–1273. https://doi.org/10.1007/s00265-013-1554-1

Nettle, D., & Bateson, M. (2015). Adaptive developmental plasticity: What is it, how can we recognize it and when can it evolve? *Proceedings of the Royal Society B: Biological Sciences, 282*(1812), Article 20151005. https://doi.org/10.1098/rspb.2015.1005

Noguera, J. C., & Velando, A. (2019). Bird embryos perceive vibratory cues of predation risk from clutch mates. *Nature Ecology and Evolution, 3*(8), 1225–1232. https://doi.org/10.1038/s41559-019-0929-8

O'Brien, C. E., Mezrai, N., Darmaillacq, A. S., & Dickel, L. (2017). Behavioral development in embryonic and early juvenile cuttlefish (*Sepia officinalis*). *Developmental Psychobiology, 59*(2), 145–160. https://doi.org/10.1002/dev.21476

Page, L. M., & Burr, B. M. (2011). *A field guide to freshwater fishes of North America north of Mexico*. Houghton Mifflin Harcourt.

Peralta Quesada, P. C., & Schausberger, P. (2012). Prenatal chemosensory learning by the predatory mite *Neoseiulus californicus*. *PLoS One, 7*(12), Article e53229. https://doi.org/10.1371/journal.pone.0053229

Pereira, A. G., & Moita, M. A. (2016). Is there anybody out there? Neural circuits of threat detection in vertebrates. *Current Opinion in Neurobiology, 41*, 179–187. https://doi.org/10.1016/j.conb.2016.09.011

Poisson, A., Valotaire, C., Borel, F., Bertin, A., Darmaillacq, A. S., Dickel, L., & Colson, V. (2017). Embryonic exposure to a conspecific alarm cue triggers behavioural plasticity in juvenile rainbow trout. *Animal Behaviour, 133*, 35–45. https://doi.org/10.1016/j.anbehav.2017.09.013

R Core Team. (2021). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/.

Ridley, M., & Rechten, C. (1981). Female sticklebacks prefer to spawn with males whose nests contain eggs. *Behaviour, 76*, 152–161.

Riley, J. L., Hudson, S., Frenette-Ling, C., & Davy, C. M. (2020). All together now! Hatching synchrony in freshwater turtles. *Behavioral Ecology and Sociobiology, 74*(5), Article 58. https://doi.org/10.1007/s00265-020-2800-y

Romagny, S., Darmaillacq, A. S., Guibé, M., Bellanger, C., & Dickel, L. (2012). Feel, smell and see in an egg: Emergence of perception and learning in an immature invertebrate, the cuttlefish embryo. *Journal of Experimental Biology, 215*(23), 4125–4130. https://doi.org/10.1242/jeb.078295

Sehr, E. K., Beasley, L. N., Wilson, K. W., & Gall, B. G. (2016). When can embryos learn? A test of the timing of learning in embryonic amphibians. *Ecology and Evolution, 6*(8), 2486–2493. https://doi.org/10.1002/ece3.2018

Shannon, H., Kutz, D., & Persons, M. (2022). The effects of prenatal predator cue exposure on offspring substrate preferences in the wolf spider *Tigrosa helluo*. *Animal Behaviour, 183*, 41–50.

Smith, G. R., & Fortune, D. T. (2009). Hatching plasticity of wood frog (*Rana sylvatica*) eggs in response to mosquitofish (*Gambusia affinis*) cues. *Herpetological Conservation and Biology, 4*, 43–47.

Stanbrook, E., Jodoin, J., Culbert, B., Shultz, S., & Balshine, S. (2020). Learning performance is influenced by the social environment in cichlid fishes. *Canadian Journal of Experimental Psychology, 74*, 215–227.

Stone, N. M., Roy, L. A., & Park, E. D. (2019). Fathead minnow reproduction: Implications for commercial culture. *Journal of the World Aquaculture Society, 50*, 267–298.

Triki, Z., Zhou, T., Argyriou, E., de Novais, E. S., Servant, O., & Kolm, N. (2024). Social complexity affects cognitive abilities but not brain structure in a poeciliid fish. *Behavioral Ecology, 35*(3), Article arae026. https://doi.org/10.1093/beheco/arae026

Turatto, M., Dissegna, A., & Chiandetti, C. (2019). Context learning before birth: Evidence from the chick embryo. *Biology Letters, 15*(7), Article 20190104. https://doi.org/10.1098/rsbl.2019.0104

Unger, L. M., & Sargent, R. C. (1988). Alloparental care in the fathead minnow, *Pimephales promelas*: Females prefer males with eggs. *Behavioral Ecology and Sociobiology, 23*, 27–32.

Vergne, A. L., & Mathevon, N. (2008). Crocodile egg sounds signal hatching time. *Current Biology, 18*(12), 513–514. https://doi.org/10.1016/j.cub.2008.04.011

Ward, A., & Webster, M. (2016). *Sociality: The behaviour of group-living animals*. Springer.

Warkentin, K. M. (2005). How do embryos assess risk? Vibrational cues in predator-induced hatching of red-eyed treefrogs. *Animal Behaviour, 70*(1), 59–71. https://doi.org/10.1016/j.anbehav.2004.09.019

Warkentin, K. M. (2011). Plasticity of hatching in amphibians: Evolution, trade-offs, cues and mechanisms. *Integrative and Comparative Biology, 51*(1), 111–127. https://doi.org/10.1093/icb/icr046

Warkentin, K. M., Cuccaro Diaz, J., Güell, B. A., Jung, J., Kim, S. J., & Cohen, K. L. (2017). Developmental onset of escape-hatching responses in red-eyed treefrogs depends on cue type. *Animal Behaviour, 129*, 103–112. https://doi.org/10.1016/j.anbehav.2017.05.008

Watve, M., & Taborsky, B. (2019). Presence of parents during early rearing affects offspring responses towards predators. *Animal Behaviour, 158*, 239–247. https://doi.org/10.1016/j.anbehav.2019.09.012

Weihs, D., & Webb, P. W. (1984). Optimal avoidance and evasion tactics in predator–prey interactions. *Journal of Theoretical Biology, 106*, 189–206.

Wiedenmayer, C. P. (2009). Plasticity of defensive behavior and fear in early development. *Neuroscience & Biobehavioral Reviews, 33*, 432–441.

Willink, B., Palmer, M. S., Landberg, T., Vonesh, J. R., & Warkentin, K. M. (2014). Environmental context shapes immediate and cumulative costs of risk-induced early hatching. *Evolutionary Ecology, 28*(1), 103–116. https://doi.org/10.1007/s10682-013-9661-z

Wisenden, B., Dye, T., Geray, K., Hendrickson, J., Rud, C., Jensen, M., Sonstegard, G., Malott, M., & Alemadi, S. (2009). Effects of nest substrate on egg deposition and incubation conditions in a natural population of fathead minnows. *Canadian Journal of Zoology, 87*, 379–387.

Wisenden, B. D., Paulson, D. C., & Orr, M. (2022). Zebrafish embryos hatch early in response to chemical and mechanical indicators of predation risk, resulting in underdeveloped swimming ability of hatchling larvae. *Biology Open, 11*(12), Article bio059229. https://doi.org/10.1242/bio.059229

Zheng, M., Kashimori, Y., Hoshino, O., Fujita, K., & Kambara, T. (2005). Behavior pattern (innate action) of individuals in fish schools generating efficient collective evasion from predation. *Journal of Theoretical Biology, 235*(2), 153–167.