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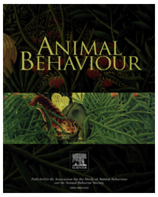

Learning to find food: evidence for embryonic sensitization and juvenile social learning in a salamander

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For many species, learning is an essential mechanism for dealing with the environment correctly and efficiently. Animals that quickly learn important information, and learn at a young age, can gain a competitive advantage in exploiting resources. Moreover, animals that learn indirectly through social observations can avoid the fitness costs of directly learning about potential dangers. Here we tested such learning capabilities in ringed salamanders, *Ambystoma annulatum*, a species where adults are primarily solitary and do not provide parental care. Adults lay eggs in ponds where embryos have the opportunity to learn from chemical cues in their environment before hatching, whereupon the high density of larvae provides an opportunity to learn from social information. In this study, we found that these salamanders can learn an attraction to novel food stimuli as embryos and that naïve observer larvae can learn from conspecifics that show attraction to stimuli. Embryonic exposure to a novel food stimulus (shrimp odour) caused attraction to that stimulus posthatching, and this response appeared to be generalized to another potential prey stimulus (mussel odour) but not to a novel plant stimulus. In a test of social learning, only observers that were paired with models corralled near a novel food stimulus were subsequently attracted to the stimulus. This study is the first to report embryonic learning of food or social learning by salamanders, providing more evidence for generalized learning by embryos and social learning by species lacking more complex social behaviours.

Correctly responding to environmental stimuli is essential to maximizing fitness, but fluctuations in the environment can pose challenges to decision making (Dall, 2010; Kacelnik & Bateson, 1996; Lima & Dill, 1990). For instance, changes in food availability or predation pressure may lead to a failure to accurately assess such information. Learning is generally viewed as the act of acquiring new information or the modification or reinforcement of existing information based on experience, resulting in behavioural changes (Brown & Chivers, 2005; Papaj & Prokopy, 1989; Stephens, 1991). According to learning theory, stable environments facilitate the evolution of innate responses to stimuli, whereas variable and complex environments can promote learned responses (Stephens, 1991, 1993). In a changing environment, individuals that learn can better exploit resources and have an increased probability of survival and reproductive success (Brown & Chivers, 2005). Such learning capabilities may be particularly important during early life periods where mortality rates are generally high (Pianka, 1970; Sogard, 1997), and indeed, young animals often show a high capacity for learning (Bornstein, 1989; Fawcett & Frankenhuis, 2015). In classic research by Lorenz (1935), geese, *Anser anser*, during a sensitive period in early development would instinctively bond with other moving stimuli in place of their parents. This phenomenon, known as imprinting, allows goslings to quickly learn to recognize their parents. Imprinting (or a learning process akin to imprinting) is not restricted to social stimuli however (Immelmann, 1975). For instance, several studies have explored imprinting of food stimuli during sensitive learning periods (e.g. Burghardt & Hess, 1966; Guibé, Poirel, Houdé, & Dickel, 2012; Punzo, 2002).

Even as embryos, animals are capable of learning how to maximize their probability of survival in their postnatal environment via a variety of sensory modalities (e.g. Darmaillacq, Lesimple, & Dickel, 2008; Hepper & Waldman, 1992; Lickliter & Hellewell, 1992). Chemosensory cues, for instance, are relatively long lasting, can move around barriers, and are available when visibility is

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low (Alcock, 2005; Mathis & Crane, 2017). Animals can use chemical cues to become familiar with the activity patterns of other species, such as those that are predators or prey. Although relatively few species have been studied, embryonic learning of chemical information occurs across a wide range of animal taxa (e.g. in dogs: Wells & Hepper, 2006; chickens: Sneddon, Hadden, & Hepper, 1998; crocodiles: Sneddon, Hepper, & Manolis, 2000; fish: Nelson, Alemadi, & Wisenden, 2013; cuttlefish: Guibé et al., 2012; mites: Quesada & Schausberger, 2012). For species with aquatic eggs, such chemical information about the environment is widely available. In this context, perhaps amphibians are the most studied group, usually in the context of learning about predation risk (e.g. Ferrari & Chivers, 2009b; Ferrari, Manek, & Chivers, 2010; Garcia, Urbina, Bredeweg, & Ferrari, 2017; Mathis, Ferrari, Windel, Messier, & Chivers, 2008). Hepper and Waldman (1992) were the first to document embryonic learning in amphibians, where exposure to a novel odour (pure orange essence) caused embryonic frogs, *Rana temporaria* and *Lithobates sylvaticus*, to prefer that odour after hatching. Such learning could better prepare individuals for locating available food types in their home pond.

Learning about novel foods has clear benefits, but sampling different foods can be time consuming and potentially dangerous (e.g. consuming something toxic). However, learning by observing experienced companions (i.e. social learning), allows animals to minimize such costs (Galef, 1993; Galef & Laland, 2005). One social learning mechanism is ‘stimulus enhancement’ where an animal learns to approach certain stimuli after observing the attraction of another individual to the stimuli (Heyes, 1994), but for many species, the opportunities for such learning are limited due to their solitary life history. Most studies on social learning have involved species that provide parental care (e.g. mammals: Whiten, 2000; birds: Lefebvre & Bouchard, 2003) or are highly gregarious throughout their lives (e.g. fishes: reviewed in Brown & Laland, 2003). However, there is a growing body of literature revealing that species that are primarily solitary and are not socially complex can be influenced by the behaviour of conspecifics (Coolen, Dangles, & Casas, 2005; Crane, Mathis, & McGrane, 2012; Wilkinson, Mandl, Bugnyar, & Huber, 2010). A behavioural response in the presence of other individuals that are performing that same behaviour is referred to as ‘social facilitation’ (Clayton, 1978), but to demonstrate that social learning has occurred, observer individuals must display the behavioural change in the absence of others. A few studies have documented social learning in species lacking more complex social behaviours, usually in the context of learning the locations of food (e.g. Brown, Markula, & Laland, 2003; Guttridge et al., 2013; Kis, Huber, & Wilkinson, 2015; Noble, Byrne, & Whiting, 2014; Wilkinson, Kuenstner, Mueller, & Huber, 2010).

Here we tested whether ringed salamanders, *Ambystoma annulatum*, can learn about prey stimuli using two different mechanisms (imprinting and social learning) during two different life stages (embryonic and larval). Ringed salamander embryos are surrounded by a vitelline membrane and outer jelly capsules (Petranka, 1998) that allow environmental cues to diffuse into the immediate vicinity of the embryos, providing an opportunity to become familiar with their future environment prior to hatching (Mathis et al., 2008). These salamanders do not appear to be socially complex; adults spend time alone underground, except when gathering at ponds to breed in the fall (Spotila & Beumer, 1970). Larvae are initially found in close proximity upon hatching (>500 individuals per 1 m^2 in one study) (Peterson, Wilkinson, Moll, & Holder, 1991). As is typical for salamander larvae, ringed salamander larvae do not show schooling or shoaling behaviour (A. L. Crane, personal observations), and actively avoid contact with conspecifics, unlike anuran larvae (Wells, 2010). Over the winter, densities of larval ringed salamanders drop to ~70/m^2, and many become cannibalistic (Jefferson et al., 2014). Densities drop to zero as the larvae metamorphosize and leave the pond in May (Peterson et al., 1991). During this period, larval ringed salamanders should have ample opportunities to acquire information from nearby conspecifics (i.e. social information) while foraging on small aquatic invertebrates and being exposed to risk from a diversity of predators (Crane et al., 2012; Mathis, Murray, & Hickman, 2003). We expect that such opportunities can be used to learn about food locations. Here, we exposed salamander embryos to a novel prey stimulus during a conditioning period, predicting increased preference and foraging behaviour towards the stimulus posthatching. In a separate experiment, we tested the prediction that larval ringed salamanders would learn to approach a novel prey stimulus via the social learning mechanism of stimulus enhancement. In this experiment, we expected larvae to first show attraction to a conspecific individual (a ‘model’) that was corralled near a novel stimulus, and then subsequently show attraction to that stimulus in the absence of the model.

METHODS

Ethical Statement

This research was approved by the Institutional Animal Care and Use Committee at Missouri State University (protocol no. 10030). The Missouri Department of Conservation granted permission (permit no. 15193) to collect the salamanders used in this study, and also for their release at their collection site after the completion of this study. The salamanders were collected as eggs (details below) and were transported to the laboratory (a 1 h drive) in buckets with pond water and battery-powered aeration. A total of 316 individuals (sex undetermined) were used in these experiments when larvae were <5 months of age. Throughout the experiments, larvae were maintained in groups (1–30 individuals, depending on their body size to avoid cannibalism) in plastic holding containers (34.5 × 20 × 12.5 cm) filled with filtered water (2 litres) and with an airstone attached to an air pump for aeration. A few rocks and artificial plants were also added for enrichment. Larvae were fed daily with *Daphnia*, and over development were transitioned to a diet of aquatic worms (*Lumbriculus variegatus*). The experiments involved no potentially harmful, painful or distressful manipulations.

Collection, Housing and Maintenance

We collected what appeared to be ~40 clutches of eggs (2–31 per clutch at stage 28–31) (Harrison, 1969) in October of two consecutive years (2011–2012) from a pond at Bull Shoals Field Station in southwestern Missouri, U.S.A. Each year, eggs were housed in 24 plastic containers (10 cm^3) with approximately 10 eggs per container, sometimes together for large clutches while being combined for smaller clutches. Each container was filled with an equal mixture of pond-water and dechlorinated municipal filtered and dechlorinated water (hereafter, water) and was kept inside an environmental chamber at 14 °C and on a 12:12 h light:dark cycle, with weekly water changes. After hatching, larvae from different clutches were mixed and moved into larger plastic holding containers (34.5 × 20 × 12.5 cm) with aeration. Larvae were separated by size to prevent cannibalism. Because larvae were mixed across clutches and separated by size, we did not account for clutch variation in our experiments. Each experiment involved 12 or more clutches, with individuals being randomly assigned to treatments (i.e. approximately equal numbers from each clutch in each treatment).