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Odour recognition learning of multiple predators by amphibian larvae

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Many aquatic animals learn to recognize novel predators when they simultaneously perceive the odours of these novel threats paired with alarm cues released by injured conspecifics. Since the odours of several organisms may be present simultaneously in the environment during this process, selection is expected to favour learning mechanisms that allow prey to respond independently to the odour of each species in a mixture of odours. We tested this hypothesis by exposing tadpoles of the edible frog, *Pelophylax esculentus*, to injured conspecific cues paired with either the odour of two fish species (experiment 1) or one fish and one crayfish species (experiment 2). We subsequently tested the ability of tadpoles to respond to each odour separately. We found clear evidence that tadpoles learned to recognize the odour of individual species in the mixture and that the response to each odour of a mixture was equally strong. However, the learned response was weaker overall in tadpoles conditioned with the mixture of fish and crayfish compared to those with the two fish species. Our study reveals that tadpoles can adaptively handle the presence of multiple predator odours in their environment during conditioned learning, but highlights some constraints that may due to the diversity of predators in the mix.

It is not uncommon for prey individuals to be vulnerable to many potential predators across their life span (Brilot, Bateson, Nettle, Whittingham, & Read, 2012; Polis, 1991). Multiple predator species may simultaneously live in the same environment or they may occupy different but adjacent habitats; furthermore, predator abundance often varies between seasons, and some predators feed on specific prey age classes (Ferrari, Sih, & Chivers, 2009; Hammond, Luttbeg, & Sih, 2007; Schoener, 1989; Sih, Englund, & Wooster, 1998). It has been estimated that each prey taxon is exposed, on average, to two to three predator taxa per food web (Schoener, 1989). For individuals of many species, it is therefore paramount to gather information about which species represent a threat and natural selection has equipped these species with sophisticated cognitive mechanisms for predator recognition learning (Brown, 2003; Caro, 2005; Kelley & Magurran, 2003). In aquatic environments, amphibians, fish and invertebrates exploit a learning mechanism based on chemical cues to recognize predators: when an individual perceives a novel odour paired with the chemicals released by an injured conspecific (hereafter 'alarm cues'), it associates the novel odour with danger and will thereafter respond to that odour by displaying antipredator behaviours (reviewed in Brown, 2003; Ferrari, Wisenden, & Chivers, 2010; Kelley & Magurran, 2003). Indeed, these alarm cues are only released through mechanical damage to the skin, as would occur during a predation event, and, hence, represent a reliable indicator of risk for nearby conspecifics (reviewed in Ferrari et al., 2010).

Alarm cue-mediated learning is usually studied in controlled settings in which prey are exposed to a single predator cue in clean water, and therefore no (or limited) potential exists for interference from other unknown odours (reviewed in Brown, 2003; Ferrari et al., 2010). Although useful to understand the basic mechanisms of predator recognition learning, the use of such controlled settings might not reflect the complexity of chemical communication in natural environments because several organisms are often present simultaneously in the same microhabitat (Sih et al., 1998). As a consequence, aquatic prey are likely to be exposed to alarm cues along with a mixture of olfactory cues of different species simultaneously (Darwish, Mirza, Leduc, & Brown, 2005), with all or a portion of the odours belonging to the predator species. Selection is expected to favour the evolution of learning mechanisms allowing prey to recognize each odour in the mixture and independently

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respond with an antipredator behaviour to each odour encountered alone because each of them can belong to the predator. This hypothesis has found support in two tropical fish species. Darwish et al. (2005) exposed glowlight tetras, *Hemigrammus erythrozonus*, to conspecific alarm cues paired with three novel fish odours; in a following test phase, tetras displayed antipredator responses to each fish odour individually. Similarly, Mitchell, McCormick, Ferrari, and Chivers (2011) showed that lemon damselfish, *Pomacentrus moluccensis*, can learn to recognize each of four novel predator odours that were simultaneously paired with alarm cues.

Larval amphibians show predator recognition learning abilities and mechanisms that are often similar to those of fish: for example, both groups display generalization of learned predator odours (Chivers, Mitchell, Lucon-Xiccato, Brown, & Ferrari, 2016; Ferrari, Brown, Messier, & Chivers, 2009), embryonic learning (Atherton & McCormick, 2015; Mathis, Ferrari, Windel, Messier, & Chivers, 2008) and latent inhibition (Ferrari & Chivers, 2006; Ferrari & Chivers, 2009). This might be indicative of convergent evolution of alarm cue-mediated predator recognition in aquatic environments. Yet, it is unknown whether larval amphibians conditioned with a mixture of the odour of different species can learn to respond to each individual odour. The main aim of this study was to address this question. To do so, in our two experiments, we conditioned tadpoles of the edible frog, *Pelophylax esculentus*, to alarm cues (or a water control) paired with a mixture of odours from two different species. In experiment 1, we used the odour of two fish species, while in experiment 2, we used the odour of two species with greater phylogenetic distance, one fish and one crayfish. We then measured the antipredator response of tadpoles when exposed to each predator odour of the mixture individually. If tadpoles can learn multiple predator odours in a mixture, we expected that subjects conditioned with alarm cues would respond to each individual odour more than subjects conditioned with the water control. Based on research on odour mixture discrimination in other species (Laska & Hudson, 1993; Livermore & Laing, 1998; Mandairon, Stack, & Linster, 2006; Rabin, 1988), we also expected that tadpoles might learn to recognize the two odours in the same mixture with different accuracy, failing more often to recognize one of the two odours.

Lastly, we investigated the effect of the odour mixture on recognition learning by comparing the learned antipredator response of tadpoles from the two experiments. Research on other species has revealed that the type of odours in a mixture has an effect on discrimination performance. For instance, squirrel monkeys, *Saimiri sciureus*, are more efficient in discriminating between odour mixtures in the presence of specific components (Laska & Hudson, 1993). Also, in the case of innate reaction to predator odours that do not require previous learning, it has been found that prey respond more strongly to the cue of a single predator in a mixture (Eklöv, 2000; Hoverman & Relyea, 2007; Smith et al., 2010). Studies on predator recognition learning have suggested that the difference between the odours of two species increases as a function of phylogenetic distance (Ferrari, Gonzalo, Messie, & Chivers, 2007). This leads to two different predictions for the results of our experiments. On one hand, if the two odours are highly different, one might expect that they are more distinguishable, and hence result in a better learning of the two cues separately; according to this prediction, we expected greater learned response to the individual odours in experiment 2 compared to experiment 1. On the other hand, if the mix is learned as a unit, a greater divergence between the two cues may lead to a greater mismatch between the conditioning and the test cue, resulting in a weaker response to each cue separately; this would cause a greater learned response to the individual odours in experiment 1 compared to experiment 2.

METHODS

Subjects

We collected edible frog eggs from 12 egg masses immediately after spawning in a stream in northeast Italy (45°32′30″N, 11°53′40″E). To prevent any exposure to predators, we raised the eggs and the tadpoles in 20-litre pails (50×36 cm, water depth 12 cm) filled with pond water. Water used in the pails was collected from a nearby artificial pond (6×4 m, depth 60 cm), which was filled 4 weeks prior to the start of the experiments. Plants and algae collected from the sampling site were added to the pond to provide natural cues to the water, while ensuring no predator cues were present. The pond was isolated from any water drainage and free from fish and crayfish. The pails were kept outdoors under natural conditions (light, temperature, precipitation, wind) and underwent a 50% water change every other day. After hatching, tadpoles were fed rabbit pellets (alfalfa) daily to complement the algae present in the pails. We used 168 tadpoles randomly selected from the pails for the experiments; these tadpoles were randomly assigned to the two experiments and to the different conditions of each experiment.

Alarm Cue and Predator Odour Preparation

We prepared alarm cues in line with previous studies on amphibian larvae (Ferrari, Vrtělová, Brown, & Chivers, 2012; Lucon-Xiccato, Chivers, Mitchell, & Ferrari, 2016). We randomly collected donor tadpoles ($N = 25$) from the pails with a small hand net and killed them with a blow to the head. The use of this standard physical euthanasia (AVMA, 2013) was necessary because chemical methods have been reported to interfere with alarm cue responses (Losey & Hugie, 1994). Immediately after euthanasia, the donors were emulsified with a mortar and pestle, and the solution suspended in pond water, to obtain approximately one tadpole per 20 ml of water.

In experiment 1, we used odours from two fish species from different families, the catfish, *Pangasius hypophthalmus* (family Pangasiidae), and the common rudd, *Scardinius erythrophthalmus* (family Cyprinidae). In experiment 2, we used odours from the catfish and the red swamp crayfish, *Procambarus clarkia*. These species were not observed in the sampling site; since tadpoles were maintained in pails filled with pond water with no fish and crayfish, the predators were novel for the tadpoles and tadpoles were not exposed to the cues used for conditioning before the experiments. We used four size-matched (approximately 12 cm) individuals for each species. The fishes were laboratory-raised and maintained under standard conditions. Their maintenance aquaria (150 litres) were provided with a gravel bottom, natural plants and water filters, and kept at 26 ± 1 °C. Fish were fed three times per day ad libitum, alternating commercial fish flakes and *Artemia salina* nauplii. Crayfish were collected in a small river 1 month before the experiments, housed individually in 10-litre pails (35×24 cm, water depth 12 cm) and fed ad libitum with rabbit and shrimp pellets. We prepared predator odours by soaking two individuals of each species in a 10-litre tank for 24 h. During these 24 h, the animals were not fed to avoid confounding effects due to diet cues (Chivers & Mirza, 2001; Mitchell, Ferrari, Lucon-Xiccato, & Chivers, 2016). Water from these tanks was used as odour cues in the experiment.

Conditioning with Odour Mixture

To study predator recognition learning in tadpoles, we used a well-established bioassay (Chivers et al., 2016; Ferrari, Brown, et al.,