

## SHORT COMMUNICATION

Configural learning in freshly collected, smart, wild *Lymnaea*

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**ABSTRACT**

An inbred laboratory strain (W-strain) of *Lymnaea stagnalis* is capable of configural learning. Configural learning, a higher form of learning, is an association between two stimuli experienced together that is different from the simple sum of their components. In our configural learning procedure, a food odour (carrot odour, CO) is experienced together with crayfish effluent (CE) (i.e. CO+CE). Following configural learning, CO now elicits a fear state rather than increased feeding. We hypothesized that freshly collected wild strains of predator-experienced *L. stagnalis* also possess the ability to form configural learning, even though they experience crayfish daily in their environment. We therefore subjected freshly collected wild strain *L. stagnalis* to the configural learning procedure. Following the configural learning procedure, CO became a risk signal and evoked anti-predator behaviours. Thus, configural learning was demonstrated in wild, freshly collected snails. We believe that configural learning occurs in the snail's natural environment and is important for their survival.

**KEY WORDS:** Memory, Predator experience, Cognitive ability**INTRODUCTION**

Learning and memory are necessary if organisms are to survive in a changing environment. An organism's ability to contextualize important environmental information allows for cognitive and behavioural flexibility (Maren et al., 2013). The ability to assign importance to specific stimuli over others is a prime cognitive event essential for survival. Previously, we found that *Lymnaea stagnalis* have the cognitive capability for configural learning; thus, they are competent to form higher-order learning (Swinton et al., 2019a). Configural learning is an association between two stimuli experienced together that is different from the simple sum of their components (Giurfa, 2003). We hypothesized that this learning occurred in the snail's natural environment and was important for their survival (Swinton et al., 2019a). In that study, a laboratory-reared inbred strain of *L. stagnalis* (the W-strain) was used and the snails had never experienced a crayfish predator. It is uncertain whether freshly collected wild snails that experience crayfish on a daily basis are capable of configural learning.

*Lymnaea stagnalis* are strain specific (Dodd et al., 2018) and can be categorized by their behavioural and memory-forming abilities (Rothwell et al., 2018; Swinton et al., 2019b). With regard to the crayfish predator, in *L. stagnalis* there are both predator-naïve and predator-experienced strains (Orr et al., 2009; Swinton et al., 2019b).

A strain is defined as predator naïve if the snails do not innately respond to chemical cues transmitted by the predator with anti-predator behaviours (Orr et al., 2009). Predator-experienced *L. stagnalis* strains are those that instinctively respond with anti-predator behaviours following predator detection (Alexander and Covich, 1991). The laboratory inbred W-strain snails used earlier are predator experienced. Sensing crayfish effluent (CE), W-strain snails show anti-predator behaviours such as decreased feeding, enhancement of long-term memory (LTM) formation and configural learning (Swinton et al., 2019a; Orr and Lukowiak, 2008).

The W-strain snails form configural learning when they experience food (carrot odour, CO) together with CE (Swinton et al., 2019a). Following configural learning, CO comes to elicit a fear state and no longer elicits a feeding response. It was speculated that configural learning in the snails' natural environment would have positive survival value. However, snails in a pond populated with crayfish would be subjected to almost continual exposure to crayfish kairomones (i.e. CE), along with food odours. Thus, the configural learning exhibited by laboratory-bred snails that do not have a history of exposure to CE will possibly not occur in freshly collected wild *L. stagnalis*.

**MATERIALS AND METHODS****Animal collection and maintenance**

*Lymnaea stagnalis* (Linnaeus 1758) used were freshly collected from White Sand Lake (WSL; 51°46'12.45"N, 103°21'14.16"W) and Stony Lake (51°47'01.37"N, 103°21'51.85"W). Both lakes are approximately 250 km east of Saskatoon, SK, Canada, and are separated by less than 0.5 km. Northern crayfish, *Orconectes virilis*, are resident in large numbers in both lakes. We have been collecting snails from these two lakes since 2014. Interestingly, pre-2016, crayfish were not found in Stony Lake (K.L., personal observation). However, they invaded Stony Lake from White Sand Lake in the late summer of 2016. Since the summer of 2017, we have been unable to collect as many Stony Lake *L. stagnalis* as pre-2016. We are uncertain whether this is the result of crayfish predation. The Stony Lake snails used in this study were collected in the summer/autumn of 2018 and 2019. Both White Sand Lake and Stony Lake snails are classified as 'smart' and are predator experienced (Swinton et al., 2019a,b).

After collection, snails were brought back to Calgary and housed in artificial pond water (PW, 0.25 g l<sup>-1</sup> of Instant Ocean in deionized water, Spectrum Brands, Madison, WI, USA) supplemented with CaCO<sub>3</sub>, ensuring calcium concentrations remained above 50 mg (Dalesman and Lukowiak, 2010). Snails were fed romaine lettuce *ad libitum* and maintained at 20±1°C on a 16 h:8 h light:dark cycle. A total of 39 snails were used. Snails maintained in Calgary do not experience crayfish until a specific experiment.

**Crayfish effluent (CE)**

Crayfish are natural predators of *Lymnaea* (Orr et al., 2007). Snails were considered to be predator experienced if they innately detect and respond to CE with anti-predator behaviours (Alexander and

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Covich, 1991), as noted above. The *O. virilis* crayfish used in the studies reported here was collected in White Sand Lake in the summer of 2018 and was housed in a 70 l aquarium of artificial pond water and fed fish pellets, lettuce and snails. The water in the crayfish tank is termed crayfish effluent (CE) (Orr and Lukowiak, 2008). We have found that the effect of CE is not dependent on crayfish eating *Lymnaea*, as CE obtained from crayfish fed only lettuce had a similar effect on anti-predator behaviours in *L. stagnalis* (Orr et al., 2007; Orr and Lukowiak, 2008).

### Carrot slurry

The carrot slurry (CO) was made by combining two medium-sized commercially obtained organic carrots (purchased from multiple grocery stores) in a blender along with approximately 475 ml of PW. Carrots were peeled before blending. Following blending and repeated straining of the mixture, a liquid carrot–PW slurry was obtained without any visible carrot pieces (i.e. CO).

### Rasping behaviour

In *Lymnaea*, a rasp is a rhythmic motor behaviour in which repeated movements of the radulae scrape the surface of a substrate, leading to the ingestion of food (Ito et al., 2013). Snails were placed in a 14 cm Petri dish with enough PW or enough CO for them to be partially submerged. The snails were given a 2–5 min acclimation period in each session. Each snail was then observed for 2 min and the number of rasps counted; the average number of rasps per minute was then calculated and graphed.

### Configural learning procedure

Snails were first placed in PW and the number of rasps counted. They were then returned to their home tank; 2 h later, the snails were again exposed to CO and their rasping behaviour was recorded. The snails were then returned to their home aquaria. The following day (i.e. 18 h later), they were exposed simultaneously to CO+CE for 45 min; 2 h later, they were exposed to CO only and rasps were counted. Snails were food deprived starting from the initial observation of rasping behaviour in PW.

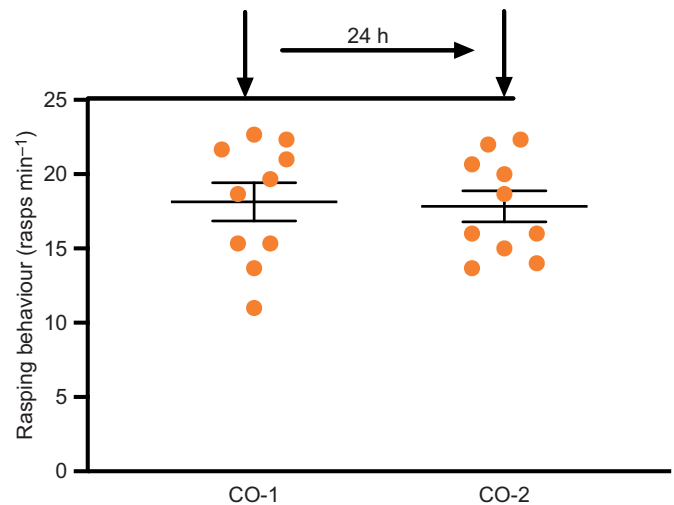
### Statistical analysis

We used Prism 8 for Mac. We first determined whether the data were normally distributed. The complete dataset in Fig. 1 was normally distributed whilst the complete datasets in Figs 2 and 3 were not. Thus, we applied a non-parametric Friedman's ANOVA to those datasets. A Dunn's multiple comparisons test was then performed to compare the feeding responses before and after the configural learning procedure. A paired *t*-test was performed for the data presented in Fig. 1. Differences were considered significant if  $P < 0.05$ .

## RESULTS AND DISCUSSION

We hypothesized that freshly collected predator-experienced, smart White Sand Lake and Stony Lake snails were capable of configural learning.

We first determined whether the feeding response (i.e. the number of rasps per minute) to CO in White Sand Lake snails, which had not previously to our knowledge experienced carrot as a food substance, was altered the second time they were tested in CO (Fig. 1). That is, we compared their initial feeding response to carrot odour (CO-1) with that to a second presentation of carrot odour 24 h later (CO-2). There was no significant difference in the feeding responses to CO (paired *t*-test;  $t = 0.2873$ , d.f. = 9,  $P = 0.7804$ ). These data are similar to those obtained previously in our lab (fig. 7A,B in Swinton et al., 2019a).

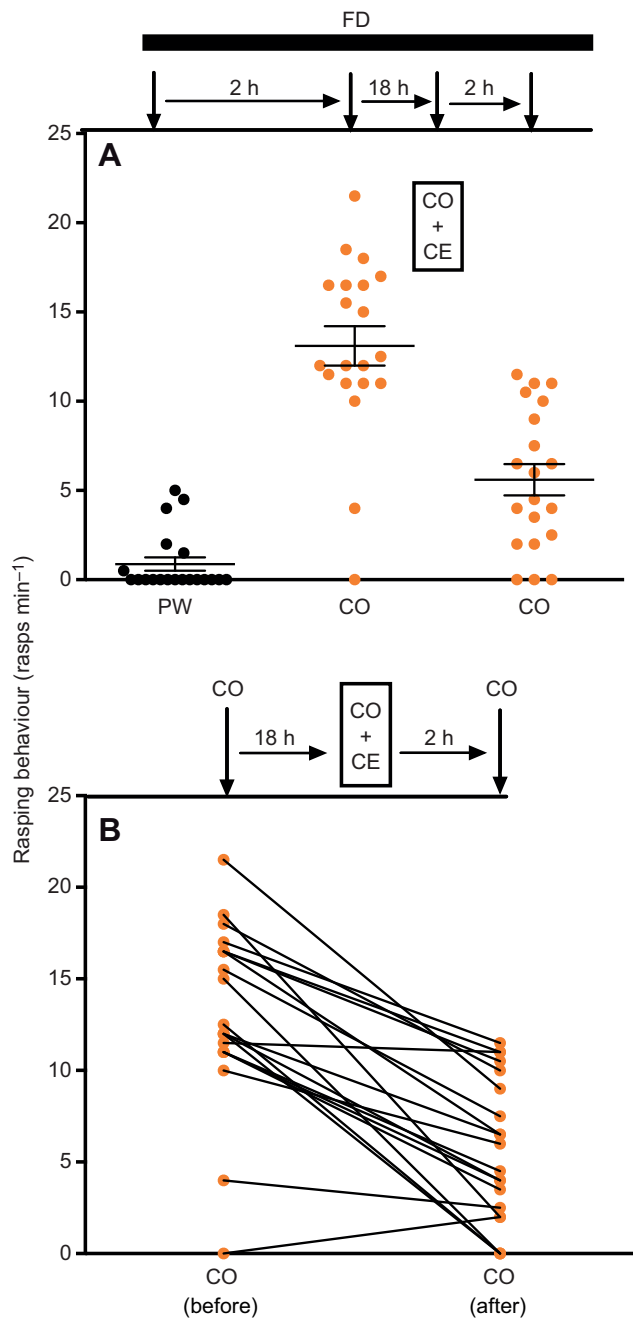


**Fig. 1. Rasping behaviour in wild snails in response to carrot odour does not change over time.** The feeding response in a cohort of freshly collected, previously untested White Sand Lake snails ('wild snails',  $n = 10$ ) was determined in the presence of carrot odour (CO-1) and then tested again 24 h later (CO-2). There was no statistical difference between the two bouts of feeding ( $P = 0.7804$ ). The solid line is the mean and the error bars are the s.e.m.

We then tested whether untrained White Sand Lake snails ( $n = 20$ ) were capable of configural learning (Fig. 2). Snails were first exposed to PW for 2 h and then to CO; 18 h later, they were exposed to CO+CE for 45 min and their response to CO was tested 2 h later (see Materials and Methods). A Friedman test (i.e. non-parametric ANOVA followed by a Dunn's multiple comparisons test) indicated that there were significant differences in the feeding responses in this experiment ( $P < 0.0001$ ). There was a significant increase in the response to the initial CO exposure versus PW ( $P < 0.0001$ ). Importantly, following the configural learning procedure, the response to CO was significantly lower than the initial response to CO ( $P = 0.0027$ ) but was not significantly different from the response to PW ( $P = 0.0984$ ).

Next, we tested the Stony Lake snails ( $n = 19$ ; Fig. 3). The same procedure was used as described above for the White Sand Lake snails. Configural learning also occurred in the Stony Lake snails. A Friedman test (i.e. non-parametric ANOVA followed by a Dunn's multiple comparisons test) indicated that there were significant differences in the feeding response in this experiment ( $P < 0.0001$ ). There was a significant increase in the response to the initial CO compared with PW ( $P = 0.0003$ ). After the configural learning procedure, the response to CO was significantly lower than that to the initial CO test ( $P = 0.0003$ ) and was not different to the response in PW ( $P < 0.999$ ). We concluded that configural learning occurred in both these freshly collected, wild predator-experienced, smart strains.

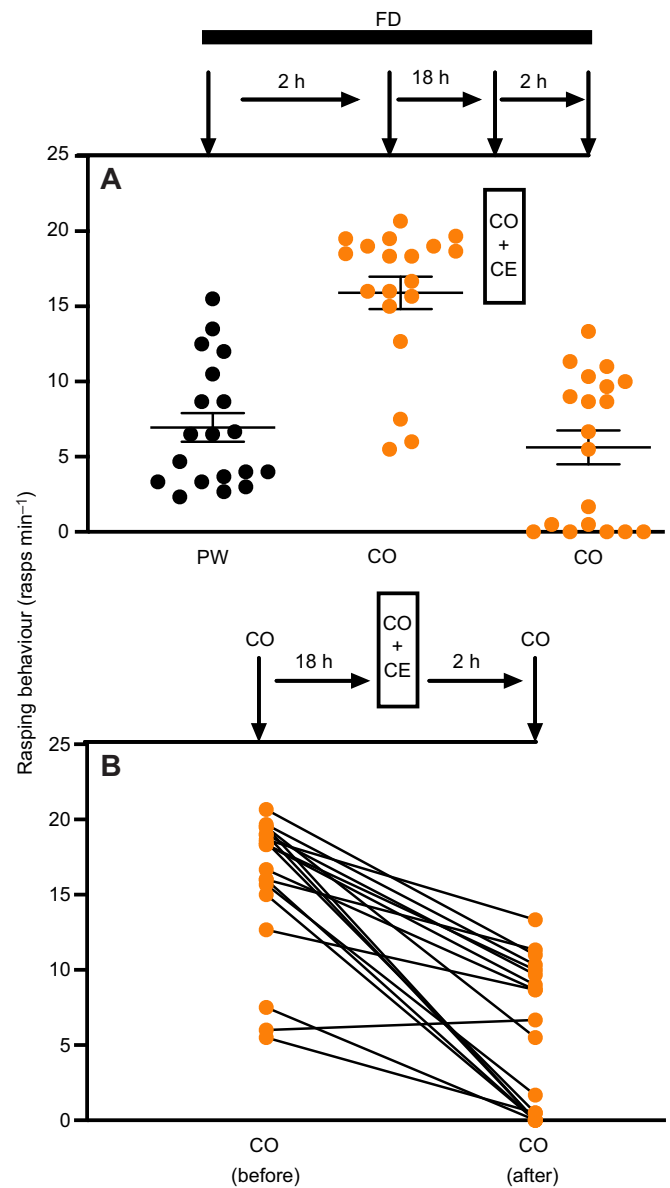
The data obtained here are consistent with our hypothesis that wild, freshly collected, predator-experienced, White Sand and Stony Lake *L. stagnalis* are capable of configural learning. Thus, both freshly collected smart wild and inbred average laboratory W-strain snails (Swinton et al., 2019a) have the capacity for this higher form of learning. Configural learning is an association between two stimuli experienced together that is different from the simple sum of their components (Giurfa, 2003). Following exposure of the snails to the combined stimuli (CO+CE), CO elicits a fear response not a feeding one. This satisfies the definition of configural learning by Sutherland and Rudy (1989). Consequently, snails assign a new meaning to the CO stimulus, i.e. it now elicits a 'fear



**Fig. 2. Rasping behaviour and configural learning in freshly collected White Sand Lake snails.** (A) The feeding response in an untrained cohort ( $n=20$ ) of *L. stagnalis* was determined in pond water (PW, black circles) and 2 h later in CO (orange circles). There was a significant increase ( $P<0.001$ ) in the number of rasps in CO versus PW. Eighteen hours later, the snails were exposed to CO+CE for 45 min. Two hours later, the feeding response in CO was again calculated. The number of rasps in this CO exposure was significantly lower than in the initial exposure to CO ( $P<0.001$ ). Interestingly, it was still significantly greater than the response in PW ( $P<0.01$ ). FD, food deprived. (B) The change in the number of rasps for each snail in response to CO before and after CO+CE exposure.

state', as opposed to its innate motivational state of increased feeding. The induced fear state occurs even though the CO does not contain predator kairomones.

Following the demonstration of configural learning in an inbred laboratory-reared strain of *L. stagnalis*, Swinton et al. (2019a) speculated that this form of learning was of great importance to the



**Fig. 3. Rasping behaviour and configural learning in freshly collected Stony Lake snails.** (A) The feeding response of untrained *L. stagnalis* ( $n=19$ ) was determined in PW (black circles); 2 h later the response to CO was determined. There was a significant increase in rasping behaviour ( $P<0.001$ ). After the configural learning procedure (CO+CE), the number of rasps elicited by CO was significantly lower than that before the configural learning procedure ( $P<0.001$ ) but the number of rasps was not different from that in PW ( $P=0.7448$ ). (B) The change in the number of rasps for each snail in response to CO before and after the CO+CE exposure.

snail, as it may be an essential form of learning in the snails' natural environment. However, while the W-strain laboratory-reared snails satisfy the definition of crayfish predator experienced snails (Orr et al., 2007), the snails used in the Swinton et al. (2019a) study had never previously experienced CE. That is, CE was a novel stimulus for them. The simultaneous exposure to CO+CE in those snails was the first and only time they had encountered CE. Hence, CE was a truly new experience. In contrast, both the White Sand Lake and Stony Lake snails had experienced crayfish kairomones on a daily basis. We were therefore uncertain whether the lack of novelty, etc., would allow freshly collected wild snail strains to undergo configural learning using the Swinton procedure (i.e. CO+CE for

45 min). Because the freshly collected snails experienced CE on a daily basis but had yet not been preyed on by crayfish, combined CO+CE might not have had the same effect as seen in the W-strain snails. As we found here, this is not the case. The two strains of wild, freshly collected, smart snails exhibited configural learning in a similar manner to the laboratory-reared W-strain.

Both the White Sand Lake and Stony Lake snails have been classified as smart snails (Hughes et al., 2017; Shymansky et al., 2017; Dodd et al., 2018; Rothwell and Lukowiak, 2019). Previously, Hughes et al., (2017) found that smart snails respond to combinations of stressors differently from 'average' freshly collected wild snails (the TC2 strain) or laboratory-bred W-strain snails. The smart snails did not exhibit memory formation, whilst the average snails did. Hughes et al. (2017) concluded that the inability to form memory with the combination of stressors used was a 'cost' of being smart. Thus, it was also possible that these two outbred, freshly collected smart snail strains might be overwhelmed by the simultaneous presentation of two stimuli (CO+CE). Here, we show that this was not the case, and that a cost of being smart in these snails is not an inability to form configural learning. We could not test whether average freshly collected snails undergo configural learning for two reasons: (1) freshly collected TC2 snails (from an Alberta pond) are predator naive (i.e. do not respond to CE); and (2) a population of average predator-experienced snails (e.g. South Drain strain) from the Somerset Levels in the UK could not be collected in 2019. Until such time as we can collect and test those snails, we cannot confidently say that an average, freshly collected predator-experienced snail will also undergo configural learning.

Configural learning in an invertebrate has been previously demonstrated in bees (Giurfa, 2003, 2007; Dyer, 2012; Devaud et al., 2015). Bees have well-developed learning and memory capabilities, giving them the ability to undergo non-elemental forms of learning, including categorization, contextual learning, and rule obstruction in the visual and the olfactory domain (Giurfa, 2003, 2007). In both the inbred W-strain and freshly collected wild snails, configural learning also altered the learning and memory caused by an operant conditioning procedure. Following the configural learning procedure, operant conditioning of W-strain snails in CO resulted in enhanced LTM formation (Swinton et al., 2019a). Enhanced LTM formation is a component of anti-predator behaviours seen in *L. stagnalis* (Orr and Lukowiak, 2008; Orr et al., 2009a,b).

We have noted that crayfish were not present in Stony Lake in our 2016 summer collecting season. We were also able then to easily collect large numbers of Stony Lake snails. However, since then (2017–2019), we have found it difficult to collect large numbers of Stony Lake snails. We hypothesized that this was the result of the crayfish invasion. Based on some pilot 2016 experiments, it appeared to us that the majority of Stony Lake *L. stagnalis* were predator naive to CE. However, since 2018, the majority of Stony Lake snails sampled are predator experienced to CE. We are now conducting experiments to determine how a population that is initially predator naive becomes predator experienced. We speculate that in any large population there will be predator-experienced snails; these are the ones that are most likely to undergo configural learning as described here and to better survive the invasion of crayfish and reproduce. The exact opposite would also be expected: in the White Sand Lake snails, which as a population are considered to be predator experienced, there will be snails that do not respond to CE (i.e. are predator naive). This may be why in some experiments configural learning following CO+CE pairing did not occur.

As the ability to form associations (i.e. configural learning) between predator risk factors and the surrounding environment is necessary for predator avoidance strategies, configural learning as shown here in wild populations should be seen as a key adaptation enabling snails to assign new meaning to stimuli in their environment. Our data directly demonstrate that freshly collected predator-experienced wild snails have the capacity and ability to form a relationship between two stimuli and can treat the stimuli as different from the simple sum of the stimuli alone (i.e. are capable of configural learning).

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: D.K., K.L.; Methodology: K.L.; Formal analysis: K.L.; Investigation: D.K., K.L.; Writing - original draft: D.K.; Writing - review & editing: K.L.; Supervision: K.L.; Funding acquisition: K.L.

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