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Article *in* Animal Behaviour · August 2011

DOI: 10.1016/j.anbehav.2011.05.005

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Animal Behaviour xxx (2011) 1–9

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Microgeographical variability in long-term memory formation in the pond snail, *Lymnaea stagnalis*

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ARTICLE INFO

Article history:
Received 7 December 2010
Initial acceptance 11 January 2011
Final acceptance 2 May 2011
Available online xxx
MS. number: 10-00846R

Keywords:
behavioural plasticity
kairomone
long-term memory
Lymnaea stagnalis
pond snail
population ecology
predator
stress

The ability to learn and form long-term memory (LTM) can enhance an animal's fitness, for example by allowing it to remember predators, food sources or conspecific interactions. Here we used the great pond snail, *Lymnaea stagnalis*, to assess whether variability among natural populations in memory-forming capabilities occurs on a microgeographical scale. We used four populations from two different habitat types separated by 1–20 km: two from large, permanent canals and two from small, fluctuating drainage ditches. Of the four populations tested, only one, from a small drainage ditch, formed LTM lasting 24 h after a 0.5 h operant training session to reduce aerial respiration in hypoxic conditions when trained in pond water alone. Each of the four populations demonstrated the same memory retention capability over 2 consecutive years, indicating temporal stability within each population tested. Despite this lack of a consistent ability for LTM formation among populations in pond water, all populations tested demonstrated LTM formation in the presence of predator kairomones, from both tench, *Tinca tinca*, a predatory fish present at the large canal sites, and crayfish, *Pacifastacus leniusculus*, known to extend memory in a Dutch *L. stagnalis* population. Therefore, while we found differences between populations in LTM retention after training in pond water, the response to predator kairomones during training, an ecologically relevant stressor, appears highly conserved in this species, enabling all populations to form LTM.

The ability of animals to learn and remember during their lifetime enables them to adapt to changes in predator threat (e.g. Kelley & Magurran 2003; Dalesman et al. 2006), food availability (e.g. Healy et al. 2009) or food quality (e.g. Skow & Jakob 2006; Munoz & Bonal 2008), as well as to remember conspecific interactions that may alter social status or mate preference (e.g. Griffiths 2003; Ward et al. 2009), all of which may directly affect an animal's fitness. Hence, it seems logical that enhanced memory-forming capabilities should always be selected for. However, the ability to demonstrate behavioural plasticity, including memory formation, can also carry associated costs (reviewed in Auld et al. 2010; Mery & Burns 2010). Therefore, these costs may limit the potential for the evolution of memory capability if they outweigh benefits gained by the animal remembering aspects of its environment.

Variation in the ability to learn and form long-term memory (LTM) has been found in both closely related species and natural populations. For example, intersubspecies and intercolony variability has been found in bumblebees (in the *Bombus terrestris* species complex) in the rate of learning to associate a particular coloured flower with a food resource; however, memory retention was not assessed (Raine & Chittka 2008; Ings et al. 2009). Closely related parasitic wasp species differ in both acquisition and retention of LTM (Smid et al. 2007), and invasive species of Crustacea demonstrate enhanced memory retention relative to native species (Hazlett et al. 2002). A comparison among populations of tortoise beetles, *Deloyala guttata*, that aimed to assess habitat effects on acquired oviposition site preference found no population differences in memory formation (Rausher 1983). In vertebrates, however, learning and memory have been found to vary consistently, in some cases with differences in the environment experienced by each population. For example, cache retrieval in black-capped chickadees, *Poecile atricapillus*, was found to relate to habitat quality where spatial memory was enhanced in a population experiencing harsher conditions (Pravosudov & Clayton 2002). Three-spined sticklebacks, *Gasterosteus aculeatus*, demonstrate differences in spatial orientation learning and memory that relate to habitat stability (Brydges et al. 2008). In addition, both

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0003-3472/$38.00
doi:10.1016/j.anbehav.2011.05.005

three-spined stickleback and Trinidadian guppy, *Poecilia reticulata*, populations that naturally experience high predation risk showed a greater tendency to learn about predator threat than those from low-risk populations (reviewed in Kelley & Magurran 2003).

There is now evidence that interpopulation variability in learning and memory retention has a genetic basis in some species, suggesting that these traits may be subject to selection. For example, a population of the great pond snail, *Lymnaea stagnalis*, a freshwater gastropod, that demonstrated enhanced memory formation in wild-collected animals also showed similar memory enhancement in F1 generation individuals reared in the laboratory (Orr et al. 2008). Similarly, the ability to learn avoidance behaviour differed between F1 generation laboratory-reared three-spined stickleback populations (Huntingford & Wright 1992), and families of the cabbage white butterfly, *Pieris rapae*, demonstrate significant differences in learning which correspond with differences in mushroom body size (Snell-Rood et al. 2009). Learning and memory retention can be enhanced in *Drosophila melanogaster* (Mery & Kawecki 2002) and diminished in a parasitic wasp, *Cotesia glomerata* (van den Berg et al. 2011) using artificial selection, indicating that memory may also be subject to natural selection in these species. An alternative mechanism leading to natural variability in learning and memory populations is the founder effect, where by chance certain populations are founded by individuals with particularly good or poor memory retention. For example, a founder effect appears to account for nonadaptive divergence in memory retention between Trinidadian guppy populations, where population differences in memory retention do not relate to habitat characteristics, but instead to the river system from which they were sampled (Burns & Rodd 2008).

In addition to heritable memory retention, the probability of forming memory in response to experience can also be altered by the environment encountered before and during the learning period. For example, environmental enrichment has been shown to enhance social learning in cod, *Gadus morhua* (Strand et al. 2010) and rats, *Rattus norvegicus* (Harris et al. 2009); in both cases a reduction in stress appears to be related to their enhanced learning abilities. Experience of a stable foraging environment was also found to enhance the probability of long-term spatial memory in bumblebees, *Bombus impatiens* (Saleh & Chittka 2007). Environmental stressors can also directly alter LTM formation, the effect of which is highly dependent on both the type of stress experienced and the timing of the stressful event relative to the learning period (Shors 2004).

Lymnaea stagnalis is a temperate freshwater snail species, with an average life span of 2 years (Dillon 2000). It is frequently used as a model organism to study learning and memory because of its relatively simple neuronal network, its simple set of behaviours that are easily identified and recorded, and the ease with which this animal can be maintained in the laboratory. *Lymnaea stagnalis* is a pulmonate snail, respiring both via direct absorption of oxygen across its skin and, in low oxygen conditions, using a basic lung, which is opened to the atmosphere via the pneumostome to perform aerial respiration. Aerial respiration behaviour in this species is driven by a three-neuron central pattern generator (Syed et al. 1990, 1992), and individuals can be operantly conditioned to reduce aerial respiration in an hypoxic environment (Lukowiak et al. 1996). Intermediate-term memory (ITM) to reduce aerial respiration (i.e. memory lasting up to about 6 h) in this species is dependent on protein synthesis alone; however, for LTM to be formed (i.e. memory lasting from 24 h to several weeks) both altered gene activity and protein synthesis are required (Sangha et al. 2003). This memory formation is flexible and can be both enhanced and blocked using environmentally relevant stressors, the timing of which relative to the learning procedure is critical (reviewed in Lukowiak et al. 2010). For example, predator kairomones experienced during the training procedure enhance the duration of memory retention, but only when the predator is sympatric to the snail population (Orr & Lukowiak 2010), and low environmental calcium experienced immediately (1 h) prior to training can block LTM formation (Dalesman et al. 2011). Most laboratory work with this species is carried out using a population originating from wild animals collected in the 1950s from canals in a polder located near Utrecht, Holland, and subsequently maintained at Vrije Universiteit in Amsterdam. However, previous work has shown that wild snails from a single population in the Belly River catchment area, Canada, had an enhanced ability to form LTM after training relative to the standard laboratory population, and also to another Canadian population 200 km away (Orr et al. 2009a).

Here, we used information from work on *L. stagnalis* populations on the Somerset Levels, U.K., to address the following questions. (1) Do U.K. populations of *L. stagnalis* differ in their ability to form LTM? (2) Is variability between populations in memory formation consistent over time? (3) Does the ability to form memory vary consistently between populations dependent on habitat type, as found in some vertebrate populations? (4) Can we enhance memory retention in U.K. populations using predator kairomones? The populations were selected on the basis that they occupy two distinct habitat types, and also demonstrate local adaptation in their innate response to predatory fish kairomones (Dalesman et al. 2007b), developmental temperature (Dalesman & Rundle 2010) and recognition of conspecific alarm cues (Dalesman et al. 2007a), despite being separated by only 1–20 km.

METHODS

Site Characteristics

Adult snails were collected from four sites on the Somerset Levels, an area of the U.K. with a large number of small drainage ditches, canals and rivers forming an interlocking matrix on the landscape. Two of these sites are large stable drainage canals (South Drain: 51.18°N, 2.88°W; Sowy River: 51.07°N, 2.88°W) containing predatory fish including tench, *Tinca tinca*, and two are small fluctuating drainage ditches (Chilton Moor: 51.19°N, 2.88°W; Little Hook: 51.06°N, 2.87°W) lacking predatory fish but containing a number of invertebrate predators including bugs, beetles and leeches. Sites with and without predatory fish are spaced in a pairwise fashion, such that South Drain and Chilton Moor are less than 1 km apart, as are Sowy River and Little Hook; however, the distance between these paired sites is approximately 20 km. The abiotic environment may also have a significant effect on learning and memory in *L. stagnalis*. We therefore took measurements for factors known to alter memory formation: environmental calcium concentration (Dalesman et al. 2011), temperature (K. Lukowiak, unpublished data) and oxygen concentration, which may relate to the response to hypoxia during training.

Collection and Maintenance

We collected adult snails of ca. 25 mm spire height (maximum distance from the outer edge of the aperture to the apex of the shell) in July 2009 and 2010 by randomly sampling from aquatic vegetation over a 40 m stretch of bank at each site. Snail spire height did not differ significantly between any of the populations or treatment groups within a population. Only adult snails were used, as juveniles do not show LTM when trained in pond water (McComb et al. 2005).Wild, freshly collected snails were returned to the laboratory to assess memory-forming capabilities. In the

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laboratory, adult snails were maintained in 6 litres of aerated artificial pond water (ASTM 1980) with 90 mg/litre [Ca^{2+}] at 20 °C under 12:12 h light:dark conditions at a density of 12 adults per aquarium and fed romaine lettuce ad libitum. The same climate-controlled room was used in both 2009 and 2010 to maintain the snails and for behavioural trials to ensure that laboratory conditions were identical between years. Snails were allowed to acclimate to laboratory conditions for a minimum of 48 h before being used for training. Wild adults were maintained in the laboratory after the experiments as breeding stock.

Labelling Individuals

Snails were tested for breathing rate and memory formation in cohorts of five or six individuals. To allow individual breathing rate and number of pneumostome opening attempts during training and test sessions to be measured, we labelled each individual, using a number printed on waterproof paper fixed onto the shell immediately above the pneumostome opening with Instant Krazy Glue (Columbus, Ohio, U.S.A.). Labelling was carried out a minimum of 24 h prior to experiments to prevent labelling stress from affecting results. Snails were labelled while sat on damp paper towel, and the glue was allowed to cure fully for a minimum of 10 min before snails were returned to their home aquaria. This method of labelling has been found not to affect snail behaviour, growth or survival in the past (S. Dalesman, personal observation). Snails were trained in groups rather than individually, following a protocol developed in previous studies, to ensure that the current study is directly comparable to this previous work (Lukowiak et al. 1996, 1998; Orr et al. 2008, 2009a). Group training does not alter the behaviour of individuals within the group in this species. For example, in a previous study, in which yoked controls (where the snail is poked at the same time as the snail to which it is yoked, i.e. not contingent with its pneumostome opening) and trained snails were trained and tested in the same container, only trained snails demonstrated learning and memory (Lukowiak et al. 1996). In addition, snails receiving partial reinforcement of training in the same beaker as fully trained snails did not demonstrate learning or memory, whereas the trained snails did (Sangha et al. 2002), and snails trained in isolation do not learn and form memory differently from those trained in groups (S. Dalesman, unpublished data). These results provide evidence that the response one snail demonstrates in response to training does not significantly alter the response of other animals trained in the same container. No individual snail was used more than once, either for breathing observations or for training, throughout this study.

Breathing Rate

First, we wanted to assess whether there were any population differences in the basic aerial respiratory behaviour. *Lymnaea stagnalis* did not show significant differences in aerial respiration between widely separated populations (Orr et al. 2009a); however, these populations did not differ consistently in habitat type. We measured total breathing time in both ca. 100% [O_2] and hypoxic (<5% [O_2]) conditions using a randomly chosen group from each field population collected in 2009. To measure breathing rate in ca. 100% [O_2] conditions, we vigorously aerated 500 ml of pond water in a 1-litre beaker for 20 min; air pressure was approximately 0.5 PSI delivered through a 6 mm diameter air tube with an air stone attached. Aeration was reduced (to approximately 0.01 PSI) prior to placing the snails in the beaker to minimize disturbance to the snails, and maintained at this level throughout the observation period. Snails were randomly selected from each population, placed into the beaker in cohorts of five or six individuals (two cohorts per population) and allowed to acclimate for 10 min prior to observation. The total time each individual snail spent at the surface respiring aerially was recorded over 0.5 h; snails were then returned to their home aquaria. To assess breathing rate in hypoxia a further group of snails was randomly selected from each population. To make water hypoxic, nitrogen was vigorously bubbled through 500 ml of pond water in a 1-litre beaker for 20 min before the observation period; nitrogen pressure was approximately 0.5 PSI delivered through a 6 mm diameter air tube with an air stone attached. Bubbling was continued at a reduced level (approximately 0.01 PSI) throughout the observation period to maintain hypoxic conditions. Again, snails were placed into the beaker in groups of five or six individuals (two cohorts per population) and allowed to acclimate for 10 min prior to observation. The total time each individual snail spent at the surface respiring aerially was recorded over 0.5 h; snails were then returned to their home aquaria.

LTM in Pond Water

While in ca. 100% [O_2] conditions *L. stagnalis* absorb oxygen from the water directly though their skin, in hypoxic conditions they switch to aerial breathing using the pneumostome (see Introduction). They can be trained to reduce aerial breathing rate under hypoxic conditions by gently prodding the pneumostome each time the snail attempts to open it (Lukowiak et al. 1996, 1998, 2000). This physical contact results in the snail immediately closing the pneumostome but not in full body withdrawal. To increase snail aerial breathing rate, artificial pond water was made hypoxic by vigorously bubbling nitrogen through 500 ml of water in a 1-litre beaker for 20 min before training commenced; bubbling was continued at a reduced rate throughout the training session to maintain hypoxic conditions (as outlined in breathing rate methods above). Snails were introduced in cohorts of five or six individuals into the beaker, two cohorts per treatment group giving 10–12 snails per treatment group, and allowed to acclimate for 10 min before the training session. The acclimation period was then followed by a 0.5 h training period, such that each time a snail attempted to open its pneumostome at the water's surface the pneumostome was gently poked using a sharpened wooden stick (Lukowiak et al. 1996, 2000). Snails were then returned to their home aquaria in ca. 100% [O_2] conditions between training and testing. Testing for LTM at 24 h was carried out using the identical protocol to the training session. Memory is considered to have formed if the number of attempted pneumostome openings by an individual decreases significantly between the training session and the test session. Occasionally snails did not attempt to breath during the training session and were therefore excluded from the analysis. This procedure was repeated with snails collected in 2009 and 2010 from all four sites to assess the stability in memory retention in pond water alone over time within each population.

ITM in Pond Water

To assess whether populations were not demonstrating LTM at 24 h owing to an inability to learn, or alternatively an inability to form or retain memory of the training procedure, we wanted to test for ITM 1 h after the training procedure. This was carried out using snails collected in 2010. Unfortunately we were unable to collect enough wild snails from the Sowy River site to test both the effects of predator kairomones and stability in LTM and ITM formation, and so this assessment was for the other three sites only (Chilton Moor, South Drain and Little Hook). Training was carried out in pond water, as outlined above, using two cohorts of individually labelled snails from each population. After training, snails were returned to ca. 100% [O_2] conditions in their home aquaria, and then tested for memory following the protocol for memory testing at

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24 h, except this time they were tested only 1 h after the training session. Snails were not retested at 24 h, as the test session at 1 h would reinforce the training procedure. The data for the groups tested at 1 h were compared with the data for memory retention at 24 h, after training in pond water, obtained in 2010.

LTM in Predator Kairomones

The presence of sympatric predator kairomones has been found to enhance memory formation in *L. stagnalis* (Orr et al. 2009a). To assess whether this is the case with the Somerset Levels populations tested, we exposed snails to kairomones from known molluscivorous predators during the training phase. The predator species we chose were crayfish, *Pacifastacus leniusculus*, which have been found to enhance LTM formation in Dutch laboratory snails (Orr & Lukowiak 2008), and tench, as this species has previously been found to elicit antipredator behaviour in juvenile *L. stagnalis* from the Somerset Levels (Dalesman et al. 2007b). Two different predator species were used to assess whether the response to kairomones during training would be generalized or whether different predator species would differentially alter the response to training. Snails collected in 2009 were exposed to crayfish kairomones during training, while those collected in 2010 were exposed to tench kairomones during training.

Tench used to produce kairomones were maintained as laboratory stock, and were fed Salmon Diet P20 (EWOS Ltd, Bathgate, U.K.), which contains no mollusc extract, and hence avoids the potential for dietary cues to affect the trials. Tench were maintained in a darkened aquarium, with a gravel base and structures to provide shelter to minimize fish stress. To minimize disturbance to the tench, and also to avoid any predator size effects as our laboratory tench population varied in size from 10 to 20 cm total length, we used water obtained directly from the tench-holding aquaria. Separate batches were collected for each training session; water was obtained from a 120-litre aquarium holding six tench, then diluted to a 10% concentration using artificial pond water to produce the final concentration in which snails were trained. Crayfish were maintained as laboratory stock and fed pollock, potato and carrot to avoid dietary cues containing mollusc. Kairomones used during training were produced by taking water from a 60-litre aquarium containing three crayfish, 5–7 cm mantle length, again diluted to a 10% concentration using artificial pond water to produce the final concentration in which snails were trained. Once diluted to 10% concentration, 500 ml of predator kairomone water was transferred into 1-litre beakers and nitrogen was bubbled through for 20 min to make the water hypoxic to train the snails as outlined above. Training was identical to the methods carried out in pond water alone except being carried out in the presence of tench or crayfish kairomones. Memory was then tested at 24 h in pond water alone.

The order in which snail groups from each population (and cohort within population) were trained in either pond water or predator kairomones was fully randomized within each year. The response of the snails exposed to kairomones during the training session was compared to the response of those trained in pond water alone and tested at 24 h within each year (e.g. the response to crayfish kairomones during training was compared to the response to training in pond water alone in 2009).

Statistical Analysis

Abiotic factors at the field sites

Data for abiotic factors were analysed using ANOVA in SPSS 17.0 (SPSS Inc., Chicago, IL, U.S.A.) with habitat type and individual population nested within habitat as factors.

Analysis of breathing rate

Data for breathing rate were initially analysed within population, using ANOVA in SPSS 17.0, using oxygen conditions (hypoxic versus ca. 100% [O_2]) and cohort nested within oxygen condition (four cohorts per population, two in each oxygen concentration) as factors in the analysis. No significant effect of cohort was found within any of the four populations, and so within-population data were grouped for each oxygen level to test between-population effects. Between-population differences were then tested using a two-way ANOVA in SPSS with population (four levels: South Drain, Chilton Moor, Sowy River and Little Hook) and oxygen concentration (hypoxic versus ca. 100% [O_2]) as factors.

For all repeated measures analyses used to analyse the response to training, homogeneity of variance was confirmed using Mauchly's test for sphericity prior to analysis; where sphericity could not be assumed we used the more conservative Greenhouse–Geisser *P* values. Tukey's post hoc pairwise tests were used to assess where significant differences lay between subjects, and post hoc pairwise *t* tests were used to assess significant within-subject differences. An initial analysis was carried out using repeated measures ANOVA in SPSS 17.0 for each treatment group within population, including cohort as a factor in the analysis. As no significant effect of cohort was found in any of the treatment groups, cohorts were grouped to compare the effect of treatment within each population.

LTM in pond water

To test whether LTM retention after training in pond water differed within each population between sample years we used repeated measures ANOVA, with training session versus test session as the within-subject factor, and year (two levels: 2009 versus 2010) as the between-subject factor. Final *N* values are given in the figure legend.

ITM in pond water

Repeated measures ANOVA was used to test whether ITM at 1 h and LTM at 24 h differed after training in pond water in each of the three populations tested (South Drain, Chilton Moor and Little Hook). Training versus test session was used as the within-subject factor, with time at which memory was tested (1 h versus 24 h) as the between-subject factor as testing at each time period was carried out on separate groups of snails. Final *N* values are given in the figure legend.

LTM in predator kairomones

Data were analysed separately for each year, comparing the response in pond water alone to the response when exposed to predator kairomones (crayfish: 2009; tench: 2010). Data were analysed for each population using repeated measures ANOVA, with training versus test session as the within-subject factor and presence of predator kairomones (either crayfish or tench) during training (present versus absent) as the between-subject factor. Final *N* values are given in the figure legend.

RESULTS

Abiotic Field Conditions

No significant effect of population site, nested within habitat type, was found in any of the analyses of abiotic conditions. Oxygen saturation was significantly higher at the large canal sites than in the small drainage ditches (ANOVA: $F_{1,12} = 6.05$, $P = 0.030$; South Drain: $53.6 \pm 14.7\%$; Sowy River: $57.3 \pm 14.8\%$; Chilton Moor: $30.2 \pm 10.5\%$; Little Hook: $19.1 \pm 9.0\%$). There was no significant variation between habitat types in either temperature (16 ± 1.5 °C) or calcium concentration (89 ± 8 mg/litre).

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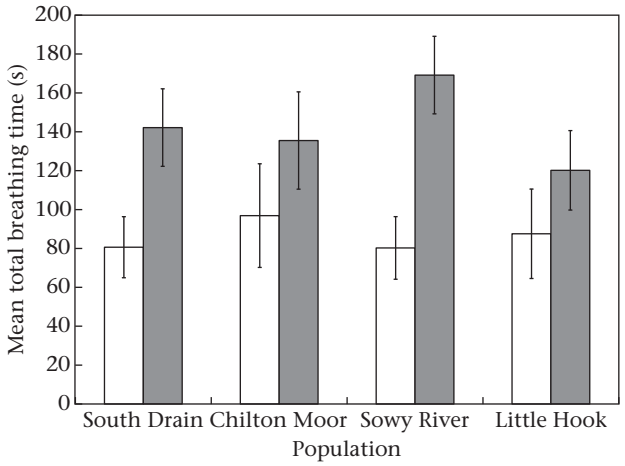

Figure 1. Mean total breathing time (s) ± SE over 0.5 h in ca. 100% [O_2] (white bars) and hypoxic (grey bars) artificial pond water. $N = 11$ for each treatment group (five or six individuals per cohort).

Breathing Rate

Population of origin did not affect the total breathing time over 0.5 h in either ca. 100% [O_2] or hypoxic conditions; however, the mean total breathing time was significantly greater in hypoxic (143 ± 10.5 s) than in ca. 100% [O_2] (85 ± 9.5 s) conditions (two-way ANOVA: main effect of oxygen concentration: $F_{1,81} = 14.21$, $P < 0.001$; Fig. 1).

LTM in Pond Water

Three of the populations, two from the large canal sites (South Drain and Sowy River) and one from a small ditch site (Little Hook), did not demonstrate memory at 24 h after training in pond water alone (i.e. there was no significant difference between the number of pneumostome openings in the training session compared with the test session 24 h later). However, the Chilton Moor population from a small drainage ditch did demonstrate LTM at 24 h after training in pond water (repeated measures ANOVA: $F_{1,20} = 34.59$, $P < 0.001$; Fig. 2). This response to training in pond water alone was consistent between years for all the populations tested; there was no significant main effect of year, nor any interaction between year and the response to training (Fig. 2).

ITM in Pond Water

After training in pond water alone, all three populations tested demonstrated ITM 1 h later (Fig. 3). The two populations, South Drain (large canal, <1 km from Chilton Moor) and Little Hook (small ditch 20 km from Chilton Moor), that had not shown LTM when tested at 24 h, demonstrated a significant reduction in the number of pneumostome opening attempts when tested 1 h after training (repeated measures ANOVA: interaction between training versus

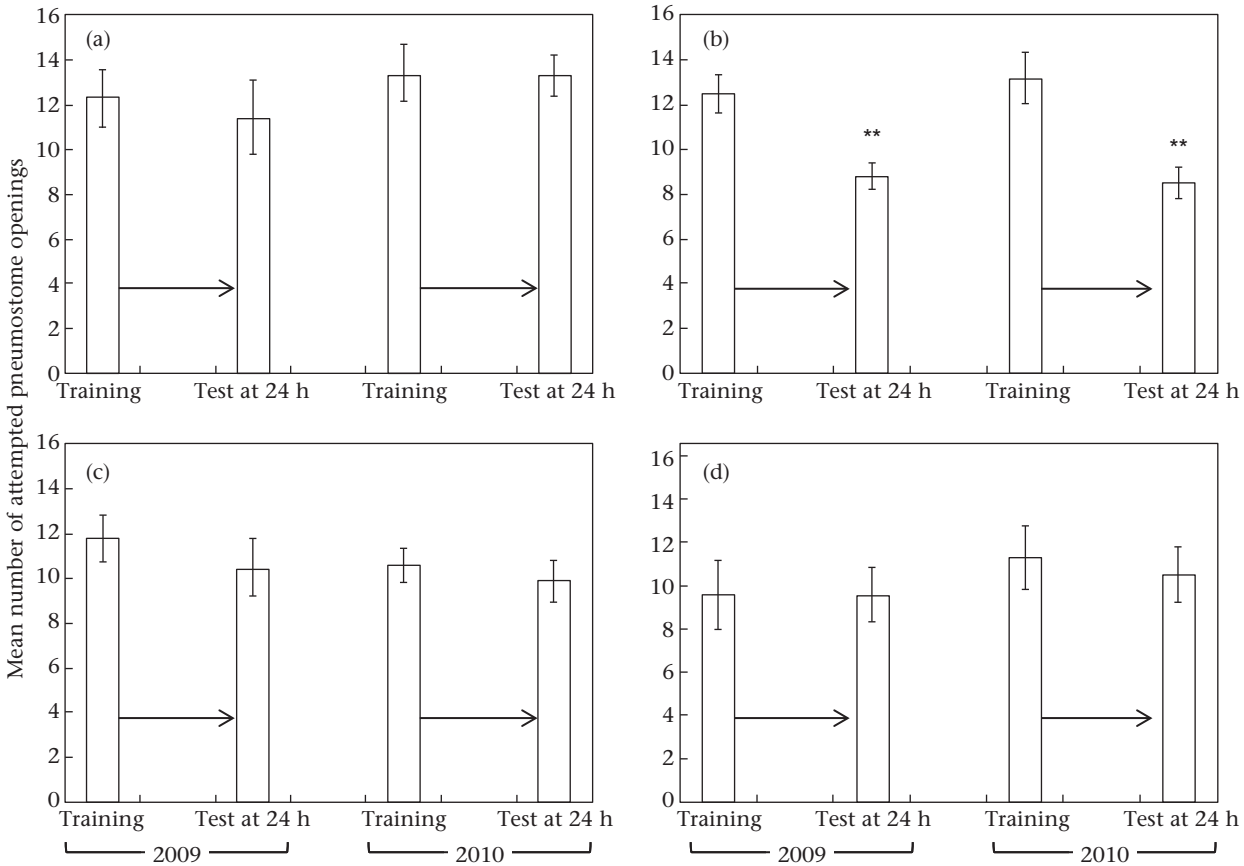

Figure 2. Mean ± SE number of pneumostome opening attempts over 0.5 h during the training session in pond water and test session for long-term memory (24 h after training) in 2009 and 2010 for (a) South Drain, (b) Chilton Moor, (c) Sowy River and (d) Little Hook populations. In 2009: South Drain $N = 12$; Chilton Moor $N = 12$; Sowy River $N = 10$; Little Hook $N = 10$. In 2010: South Drain $N = 10$; Chilton Moor $N = 10$; Sowy River $N = 11$; Little Hook $N = 10$. **Test session significantly different from the training session (paired t test: $P < 0.01$).

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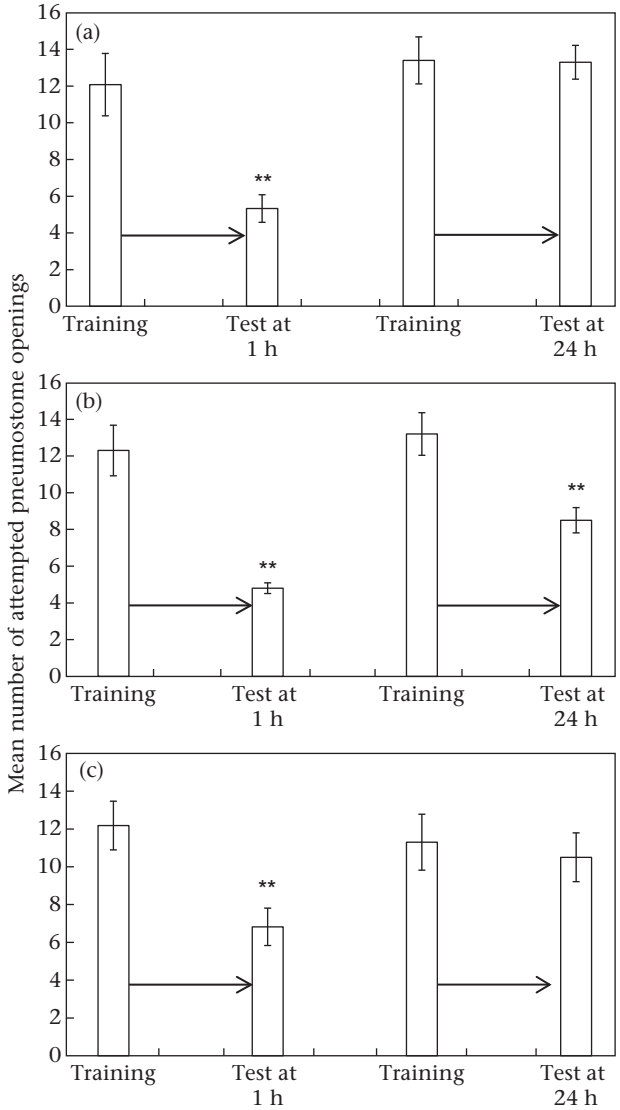

Figure 3. Mean ± SE number of pneumostome opening attempts over 0.5 h during the training session, test session for intermediate-term memory (1 h after training) and long-term memory (24 h after training) for (a) South Drain, (b) Chilton Moor and (c) Little Hook populations. Tested at 1 h post-training: South Drain $N = 12$; Chilton Moor $N = 10$; Little Hook $N = 11$. Tested 24 h post-training: $N = 10$ for each population. **Test session significantly different from the training session (paired t test: $P < 0.01$).

testing and the time of the test: South Drain: $F_{1,20} = 7.69$, $P = 0.012$; Little Hook: $F_{1,19} = 4.63$, $P = 0.045$; Fig. 3a, c). This indicates that both the South Drain and Little Hook populations demonstrate ITM, but not LTM, to reduce aerial respiration after a single 0.5 h training session. In contrast, the Chilton Moor population (small ditch site) demonstrated both ITM and LTM after training in pond water alone (repeated measures ANOVA: main effect of training: $F_{1,18} = 44.86$, $P < 0.001$; Fig. 3b). However, for the Chilton Moor population, the overall number of pneumostome opening attempts was greater during training and testing at 24 h than at 1 h (repeated measures ANOVA: main effect of test period: $F_{1,18} = 4.95$, $P = 0.039$).

LTM in Predator Kairomones

When trained in crayfish kairomones all four populations demonstrated LTM at 24 h (Fig. 4). For three of the populations, those that did not demonstrate LTM after training in pond water alone (Fig. 2: both large canal sites, South Drain and Sowy River, and a small ditch site, Little Hook), there was a significant interaction between the response to training and whether snails were trained in pond water or crayfish kairomones (repeated measures ANOVA: South Drain: $F_{1,21} = 5.09$, $P = 0.035$; Sowy River: $F_{1,18} = 5.26$, $P = 0.034$; Little Hook: $F_{1,19} = 7.32$, $P = 0.014$; Figs 2, 4), whereby snails from all three populations showed a significant reduction in aerial respiration when trained in crayfish kairomones (paired t test: $P < 0.01$ for all comparisons; Fig. 4), but not when trained in pond water alone (Fig. 2). However, the Chilton Moor population demonstrated LTM at 24 h irrespective of whether snails were trained in pond water alone or in the presence of crayfish kairomones, that is, there was no significant effect of the presence of crayfish kairomones on training to reduce aerial respiration, only a significant reduction in aerial respiration between the training and test sessions (repeated measures ANOVA: $F_{1,20} = 35.09$, $P < 0.001$; Figs 2, 4).

We found similar results when assessing the effect of tench kairomones on the ability of the four populations to form LTM. All four populations demonstrated LTM at 24 h after training in the presence of tench kairomones (paired t test: $P < 0.01$ for all comparisons: Fig. 4); however, three of the four populations (South Drain, Sowy River and Little Hook) did not demonstrate LTM after training in pond water alone (Fig. 2), that is, there was a significant interaction between the response to training and whether snails were trained in pond water or tench kairomones (repeated measures ANOVA: South Drain: $F_{1,20} = 18.01$, $P < 0.001$; Sowy River: $F_{1,18} = 5.21$, $P = 0.035$; Little Hook: $F_{1,18} = 5.94$, $P = 0.025$; Figs 2, 4). The Chilton Moor population demonstrated LTM at 24 h irrespective of whether they were trained in tench kairomones or pond water alone (repeated measures ANOVA: main effect of training: $F_{1,19} = 35.44$, $P < 0.001$; Figs 2, 4).

Together, these results indicate that the Chilton Moor population from a small drainage ditch formed LTM to reduce aerial respiration irrespective of whether predator kairomones were present during training. However, the other three populations tested, South Drain (large canal <1 km from Chilton Moor), Sowy River (large canal 20 km from Chilton Moor) and Little Hook (small drainage ditch 20 km from Chilton Moor), showed LTM formation to reduce aerial respiration in the presence of predator kairomones from both crayfish and tench, but not in pond water alone.

DISCUSSION

The freshwater gastropod *L. stagnalis* is an excellent model for studying natural variability in learning and memory. A previous study has demonstrated that this species exhibits variation in its memory-forming ability, but these results were from geographically distant populations, and no information was gathered about habitat characteristics (Orr et al. 2009a). Here we have demonstrated that the memory-forming ability in this species can vary at a microgeographical scale, but that interpopulation differences were overridden by the presence of predator kairomones during the training period. This predator effect was generalized in that it occurred in the presence of kairomones from both a predatory fish (tench) and crayfish. Crayfish prey on *L. stagnalis* snails by accessing the soft body parts via the aperture (Nyström & Perez 1998). During aerial respiration the snail must expose these soft body parts, so aerial respiration will increase vulnerability to this predator. Tench consume snails primarily by engulfing the entire snail and crushing the shell using strong pharyngeal plates (Weatherley 1959); therefore whether the soft parts are exposed will have little influence on tench predation success. It seems counterintuitive, therefore, that *L. stagnalis* responds to training to reduce aerial respiration in the presence of tench kairomones to the same extent

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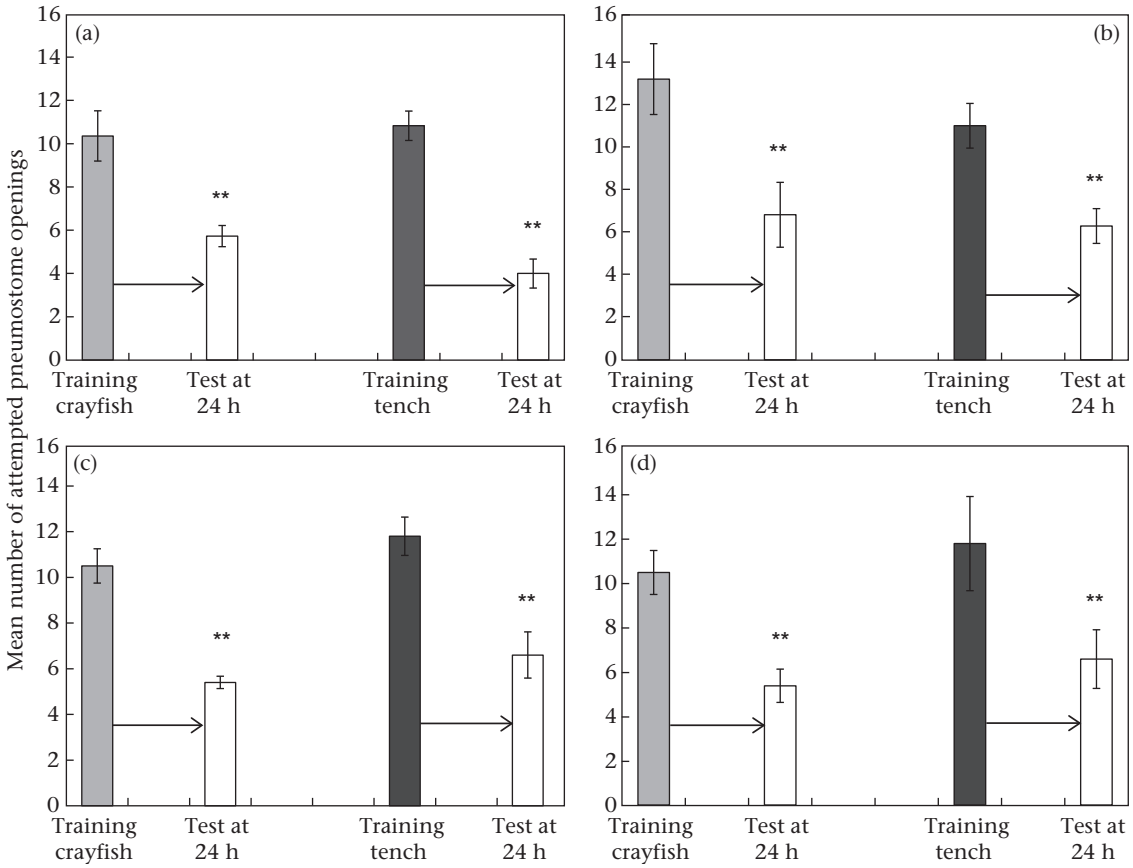

Figure 4. Mean ± SE number of pneumostome opening attempts over 0.5 h during the training session in crayfish kairomones in 2009 (pale grey) or tench kairomones in 2010 (dark grey) and test session for long-term memory 24 h after training for (a) South Drain, (b) Chilton Moor, (c) Sowy River and (d) Little Hook populations. Crayfish kairomones: South Drain $N = 11$; Chilton Moor $N = 10$; Sowy River $N = 11$; Little Hook $N = 11$. Tench kairomones: South Drain $N = 12$; Chilton Moor $N = 11$; Sowy River $N = 12$; Little Hook $N = 10$. **Test session significantly different from the training session (paired t test: $P < 0.01$).

it does in the presence of crayfish kairomones. It appears the presence of predator kairomones has a generalized sensitizing effect on the snail, suggesting that *L. stagnalis* responds by enhancing memory formation in the presence of chemicals released by a predator, independent of the predator species used.

We chose the populations based on their geographical proximity and knowledge of behavioural variability and habitat characteristics at each location. These populations demonstrate adaptation to their local environment, including predator regime (Dalesman et al. 2007b) and temperature (Dalesman & Rundle 2010). Hence, we proposed that snails originating from a particular habitat type might also demonstrate local adaptation in memory retention. For example, those experiencing a high-risk predation environment, as found in the large water bodies that contain predatory fish (South Drain and Sowy River), may have been selected for enhanced memory to enable greater behavioural matching to current predation risk (Kelley & Magurran 2003). Alternatively, the reduced habitat stability in the small drainage ditch sites (Chilton Moor and Little Hook) may select for enhanced memory capabilities to enable the snail to match environmental fluctuations more closely (Brydges et al. 2008). When we tested adult snails in pond water alone (i.e. in the absence of predator kairomones) we found no evidence to support either of these hypotheses.

Only one of the four populations, that from a small drainage ditch, Chilton Moor, demonstrated LTM after a 0.5 h training session to reduce aerial respiration in hypoxic pond water alone. This result was stable over 2 consecutive years, indicating that we had not chosen particularly 'bright' snails by chance during the first year from the Chilton Moor site. While we found enhanced memory formation in just one of the populations on the Somerset Levels, the presence of enhanced memory in this population does indicate that this behavioural phenotype is not restricted to a single population on the North American continent (Orr et al. 2009a), and may be a common feature of *L. stagnalis* populations elsewhere. However, the results also indicate that geographically close populations do not necessarily share the same memory-forming capabilities. That is, the other three populations tested (Sowy River, South Drain and Little Hook) showed no LTM formation in pond water, despite the South Drain population from a large canal site being separated by less than 1 km from the Chilton Moor population.

One reason for a lack of LTM when trained in pond water alone may be that particular populations are not responding to the training regime, that is, they are not learning in the first place. While we were unable to test whether this is the case in all the populations, owing to a lack of wild-caught individuals from the Sowy River site (canal 20 km from Chilton Moor), we were able to test it in three of the four populations. The Chilton Moor population (small ditch site), normally showing LTM after training in pond water, also demonstrated ITM 1 h after training. In addition, the other two populations, South Drain (canal <1 km from Chilton Moor) and Little Hook (small ditch 20 km from Chilton Moor) that did not demonstrate LTM did demonstrate ITM after a single 0.5 h training session, showing that the lack of response was not due to a lack of ability to learn. ITM to reduce aerial respiration is dependent on protein synthesis, while LTM relies on both protein

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synthesis and altered gene transcription (Sangha et al. 2003). It seems, therefore, that gene transcription in the Chilton Moor population is more easily turned on after a single 0.5 h training session than in the other three populations, whereas protein synthesis occurs equally in the three populations tested for ITM. How the ability to retain information to reduce aerial respiration after training in pond water alone relates to the ability of this species to form memory after other experiences has yet to be elucidated; however, evidence from selection lines in *Drosophila* suggests that memory capability may at least be generalized across similar tasks (Mery et al. 2007).

Previous work has demonstrated that juvenile *L. stagnalis* use recent experience to adjust their response to a predator (Dalesman et al. 2006); hence the ability to learn and form memory may be an important element in an effective antipredator response in this species. Therefore the question arises, why don't all populations demonstrate the enhanced memory capabilities seen in the Chilton Moor population? Costs associated with phenotypic plasticity in general are thought to be common (reviewed in Relyea 2002; Auld et al. 2010) and memory resulting in behavioural plasticity is also thought to be costly (Mery & Burns 2010). Therefore, at the South Drain, Sowy River and Little Hook sites selection against enhanced memory in the absence of predator threat may be occurring, if benefits derived from remembering their environment are outweighed by these costs. Alternatively, if conditions at these other three sites change very frequently, devaluing recently acquired information rapidly, there may be selection for rapid forgetting in all populations apart from Chilton Moor (Kraemer & Golding 1997). Enhanced memory at the Chilton Moor site might instead be a result of a founder effect similar to that found for memory formation in Trinidadian guppies (Burns & Rodd 2008), that is, the snails that originally populated the Chilton Moor site had, by chance, better memory retention than seen on average. Previous work indicates that in a population demonstrating poor memory there is always a small proportion of individuals that do demonstrate enhanced memory (Orr et al. 2009b). It is therefore possible that, by chance, the Chilton Moor population was founded by such individuals. However, as our current results show no pattern with habitat type when tested in pond water, thus far we can only speculate what processes may be maintaining these differences in memory retention in pond water among *L. stagnalis* populations. Given the small number of populations tested here, further work is required, sampling from a larger number of sites, to elucidate the processes resulting in differences in LTM formation among *L. stagnalis* populations.

Tench kairomones elicited antipredator behaviour in juvenile *L. stagnalis* from all the populations used in this study, although the response was population specific to some degree, with a stronger innate response in the populations from large water bodies (South Drain and Sowy River) that cohabit with tench (Dalesman et al. 2007b). When we tested the effects of tench kairomones on memory of the operant training procedure to reduce aerial respiration, we found that LTM was enhanced in all populations, as might be predicted from previous work showing memory enhancement by sympatric predator kairomones (Orr et al. 2009a). Dutch *L. stagnalis*, commonly used as the source animals to study learning and memory worldwide, demonstrate enhanced memory in the presence of crayfish kairomones (Orr et al. 2007). We also found that the U.K. populations from the Somerset Levels respond to crayfish kairomones with enhanced LTM formation. In addition, previous work has shown that while juvenile *L. stagnalis* were not able to form LTM after operant conditioning in pond water alone (McComb et al. 2005), they were able to do so in the presence of predators (Orr et al. 2010), and were also able to learn about predation risk (Dalesman et al. 2006). Hence, the presence of recognized predator kairomones has a generalized effect in enhancing learning and memory retention in *L. stagnalis*.

The consistency across all populations tested in their ability to form LTM after training in the presence of predator kairomones suggests, along with previous results from North American and Dutch populations (Orr & Lukowiak 2008; Orr et al. 2009a), that the enhancing effect of predator kairomones on the ability to form LTM is highly conserved in this species. There are considerable costs associated with an incorrect response to predator presence, both in mortality and in lost opportunity to feed or reproduce if demonstrating antipredator behaviour when predators are no longer present (Lima & Dill 1990). Hence, there will be strong selection to match behavioural responses to current predation threat correctly. This potentially explains why the ability to retain information is enhanced in the presence of predator kairomones, which may outweigh any costs associated with both potential and operating costs of memory when the animal is under threat of predation.

Acknowledgments

We thank Ann Torr, Roger Haslam and Richard Ticehurst for technical support, and three anonymous referees for their insightful comments that helped to improve this manuscript. This study was funded by an Alberta Heritage Foundation for Medical Research postdoctoral fellowship from Alberta Innovates – Health Solutions awarded to S.D., the Company of Biologists provided financial assistance to S.D. for travel costs and the Canadian Institute for Health Research funds to K.L.

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