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RESEARCH ARTICLE

The effect of rearing environment on memory formation

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ABSTRACT

Lymnaea stagnalis is a well-studied model system for determining how changes in the environment influence associative learning and memory formation. For example, some wild strains of *L. stagnalis*, collected from separate geographic locations, show superior memory-forming abilities compared with others. Here, we studied memory formation in two laboratory-bred *L. stagnalis* strains, derived from the same original population in The Netherlands. The two strains were reared in two different laboratories at the University of Calgary (C-strain) and at Brock University (B-strain) for many years and we found that they differed in their memory-forming ability. Specifically, the C-strain required only two training sessions to form long-term memory (LTM) whereas the B-strain required four sessions to form LTM. Additionally, the LTM formed by the B-strain persisted for a shorter amount of time than the memory formed by the C-strain. Thus, despite being derived from the same original population, the C- and B-strains have developed different memory-forming abilities. Next, we raised the two strains from embryos away from home (i.e. in the other laboratory) over two generations and assessed their memory-forming abilities. The B-strain reared and maintained at the University of Calgary demonstrated improved memory-forming ability within a single generation, while the C-strain reared at Brock University retained their normal LTM-forming ability across two subsequent generations. This suggests that local environmental factors may contribute to the behavioural divergence observed between these two laboratory-bred strains.

KEY WORDS: Invertebrate, Operant conditioning, Aerial respiration, Learning

INTRODUCTION

Environmental conditions are known to influence learning and memory formation in many species. The observation that environmental enrichment results in behavioural improvements, such as enhanced problem-solving abilities, has been reported for more than 70 years (Hebb, 1947). Additionally, it is well established that environmental enrichment leads to improved spatial memory in rodents (Frick et al., 2003; Hullinger et al., 2015; van Praag et al., 2000). For instance, rodents exposed to an enriched environment demonstrate enhanced spatial learning and memory compared with rats maintained in normal conditions (Hullinger et al., 2015). The memory-enhancing influence of environmental enrichment is also observed in invertebrates such as the cricket *Acheta domesticus* (Mallory et al., 2016) and the mollusc *Sepia officinalis* (cuttlefish; Dickel et al., 2000).

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In addition to the effects of an enriched environment on memory formation, other changes to the external environment have been reported to influence learning and memory. For instance, the memory-forming ability of the mollusc *Lymnaea stagnalis* is susceptible to changes in their surrounding environment (Lukowiak et al., 2014). Specifically, predator detection, thermal stress or prolonged exposure to darkness enhances long-term memory (LTM) formation (Orr and Lukowiak, 2008; Teskey et al., 2012; Carpenter et al., 2016). Conversely, other factors, such as crowding or reduced environmental calcium, obstruct memory formation (de Caigny and Lukowiak, 2008; Dalesman et al., 2011a). Additionally, strains of wild *L. stagnalis* collected from different ponds can show different memory-forming abilities (Braun et al., 2012; Lukowiak et al., 2014) and this effect persists even if the geographical separation is less than 1 km (Dalesman et al., 2011c).

Lymnaea stagnalis has been used as a model to study associative learning and memory formation for at least 35 years (Audesirk et al., 1982). For more than two decades, operant conditioning of the aerial respiratory behaviour has been studied (Lukowiak et al., 1996), along with the underlying neuronal mechanisms (Spencer et al., 1999; Scheibenstock et al., 2002; Lowe and Spencer, 2006; Braun and Lukowiak, 2011). Depending on the training procedure employed, *L. stagnalis* can form both intermediate-term memory (ITM) and LTM (Lukowiak et al., 2000; Sangha et al., 2003a). ITM, dependent on *de novo* protein synthesis, is defined as lasting 2–3 h, whereas LTM, dependent on both *de novo* protein synthesis and altered gene activity, lasts at least 24 h (Sangha et al., 2003c). The most common training procedure used to produce LTM in *L. stagnalis* consists of two training sessions separated by 1 h (Lukowiak et al., 2014). However, some strains demonstrate an enhanced memory-forming ability and only require a single training session to form LTM (Orr et al., 2008, 2009a; Dalesman et al., 2011c), whilst other strains require up to four training sessions (Rothwell and Spencer, 2014; Carpenter et al., 2016).

There is a heritable component to the different memory-forming abilities in *L. stagnalis*, as the differences observed between freshly collected 'wild' strains are maintained in their lab-bred offspring (Orr et al., 2009a; Dalesman et al., 2011c; Shymansky et al., 2017). Moreover, memory enhancement, triggered, for example, by the detection of a predator, is obstructed by application of a DNA methylation inhibitor. This obstruction persists for weeks, but this change is not inherited by their offspring (Forest et al., 2016).

Previous studies in *L. stagnalis* have largely focused on differences in memory-forming ability across wild strains and a University of Calgary laboratory-raised strain (the C-strain; Forest et al., 2016; Shymansky et al., 2017). Other studies have used a lab-bred strain reared at Brock University (B-strain; Lowe and Spencer, 2006; Rothwell and Spencer, 2014). While the C- and B-strains are derived from the same laboratory-bred population (in The Netherlands), they have been reared in separate environments for many years. In this study, we sought to examine whether these two strains would have different memory-forming abilities. We also aimed to determine whether any potential differences in memory-forming ability were the

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result of differences in the local lab conditions. Our results showed a difference between the strains in their memory-forming ability when trained in their 'home' environment (i.e. B-strain animals trained at Brock University and C-strain animals trained at the University of Calgary). However, changing the lab environment in which these two strains were reared (while keeping the 'trainer' constant) resulted in a change in memory-forming ability. This suggests that the differences in memory-forming ability between these lab-bred strains may be influenced by environmental factors encountered during their development.

MATERIALS AND METHODS

Animals

Two different strains of laboratory-bred *Lymnaea stagnalis* (Linnaeus 1758) were used. These two strains, the Calgary strain (C-strain) and the Brock strain (B-strain) represent two separate populations which have been reared in separate laboratory environments (at the University of Calgary and Brock University, respectively) for more than 15 years.

The C-strain from the University of Calgary

The C-strain (maintained at the University of Calgary, AB, Canada; referred to as the W-strain in previous publications, e.g. Forest et al., 2016) was derived from a lab-bred strain originating at Vrije University in Amsterdam, The Netherlands. Animals were originally collected from polders in Utrecht, The Netherlands, in the 1950s and were subsequently bred at Vrije University for many generations (Lever et al., 1961; i.e. the original Dutch population). Animals from this original Dutch population were brought to the University of Calgary in the 1980s, and have since been continually reared and maintained in this laboratory setting.

Animals were raised and maintained in artificial pond water [deionized water containing 80 mg l^{-1} $CaSO_4$ and 0.25 g l^{-1} Instant Ocean (Aquarium Systems, Mentor, OH, USA)] at room temperature on a regular light–dark cycle. Both romaine lettuce and trout pellets were provided *ad libitum*.

The B-strain from Brock University

The B-strain was maintained at Brock University (St Catharines, ON, Canada). This strain is derived from a combination of *L. stagnalis* bred at Vrije University (i.e. the original Dutch population) and *L. stagnalis* bred at the University of Calgary (i.e. the C-strain) which were transferred to Brock University between the years 2001 and 2002. At Brock University, animals were reared and raised at room temperature in artificial pond water [dechlorinated and filtered city tap water containing 0.25 g l^{-1} Instant Ocean salts (Aquarium Systems)]. Animals were maintained on a fixed light–dark cycle and fed a diet consisting of romaine lettuce and NutraFin Max Spirulina fish food (Hagen).

Transfer of strains between the University of Calgary and Brock University

Egg masses containing *L. stagnalis* embryos were transferred between the lab at the University of Calgary and the lab at Brock University, allowing us to subsequently rear each strain in a new environment. Embryos raised in their strain-specific environment are referred to as 'home', while embryos transported to a new environment are referred to as 'away'. That is, egg masses containing C-strain embryos from University of Calgary were transferred to Brock University where they hatched and developed into adults (away); similarly, egg masses containing B-strain embryos were transferred to University of Calgary, where they subsequently hatched and developed into adults (away). Upon reaching adulthood, animals were randomly selected for inclusion in the experimental procedures.

Lymnaea stagnalis transported as embryos, which hatched and were raised in a new environment are referred to as the first generation of that strain. Eggs laid by the first generation snails were collected and these offspring are referred to as the second generation of that strain.

Operant conditioning of aerial respiratory behaviour

Aerial respiratory behaviour of *L. stagnalis* was operantly conditioned as described previously (Lukowiak et al., 1996, 2000; Sangha et al., 2003a; Rothwell and Spencer, 2014; Rothwell et al., 2014). All training and testing sessions were conducted in hypoxic pond water as this drives aerial respiration via the opening of the pneumostome at the water's surface (Lukowiak et al., 1996). Hypoxic pond water was created by bubbling 100% N_2 gas into 800 ml of pond water in the 'test' beaker for 20 min before each training session and memory test. This bubbling was continued at a reduced rate during all sessions. Animals were permitted to acclimate to this hypoxic environment for 10 min before a training session or memory test was initiated. Following this period of acclimation, all animals were gently propelled to the bottom of the 'test' beaker to signify the start of the experimental session.

In our initial experiments, we employed the protocol normally used to train *L. stagnalis* at Brock University (Rothwell and Spencer, 2014; Rothwell et al., 2014; Carpenter et al., 2016), which consists of four 45 min training sessions (S1 to S4), spaced 1 h apart. Memory duration was assessed by conducting a memory test (MT) between 24 and 96 h after the last training session (S4). During each training session, as well as the subsequent MT, a tactile stimulus was immediately applied to the open pneumostome each and every time the animal attempted to perform aerial respiration. This stimulus was sufficient to cause the immediate closure of the pneumostome without inducing the whole-body withdrawal response. Learning and memory were statistically analysed using a one-way repeated measures ANOVA and a Tukey test was used for *post hoc* comparisons. Differences were deemed significant when $P<0.05$. Effect size was measured as the partial η^2 and all values are presented as means±s.e.m. Detailed statistics can be found in Fig. 1 legend.

In order to carry out additional comparisons between the C- and B-strains, we also used a training procedure consisting of two 45 min training sessions (S1, S2) separated by a 1 h consolidation period. In these experiments, LTM was assessed with a MT 24 h after the completion of the second training session (S2). Learning and memory were statistically analysed across different strains using a two-way repeated measures ANOVA. *Post hoc* comparisons were made using a Tukey test. All data are presented as means±s.e.m. and differences were deemed significant when $P<0.05$. The partial η^2 was used to measure effect size. Detailed statistics are located in Fig. 3 legend.

Some animals served as yoked controls and received non-contingent tactile stimulation during the training sessions and memory test. As previously published studies have demonstrated that the behavioural change resulting from operant conditioning is due to the association between pneumostome opening and contingent tactile stimulation both at University of Calgary (Lukowiak et al., 1996, 1998, 2000) and at Brock University (Rothwell and Spencer, 2014; Carpenter et al., 2016), this control was not repeated with every experimental group in this study.

Adult *L. stagnalis*, ranging in shell length from 20 mm to 28 mm, were used for all behavioural experiments. The shell of each individual snail was labelled with a coloured mark 24 h before training for identification purposes.

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The number of attempted pneumostome openings performed by each animal was recorded during each training session, as well as during the MT. All snails were returned to their eumoxic home tanks between training sessions, as well as between the final training session and the MT. To ensure consistency of the training, the same person (C.R.) administered all operant conditioning procedures at Brock University and University of Calgary. Sample sizes for all experiments were selected based on previous studies conducted at University of Calgary and Brock University (Lukowiak et al., 1998; Forest et al., 2016; Rothwell and Spencer, 2014; Rothwell et al., 2014; Carpenter et al., 2016).

Operational definition of learning and memory

Learning was operationally defined as a significant reduction in the number of attempted pneumostome openings from the initial training session (S1) to the final training session (either S2 or S4 depending on the experiment; Lukowiak et al., 1996; Rothwell and Spencer, 2014). LTM was deemed to have formed when the number of attempted pneumostome openings during the memory test (MT) was (i) significantly less than that observed in S1 and (ii) not significantly greater than that observed during the final training session (Lukowiak et al., 1996, 1998; Rothwell and Spencer, 2014). The proportion of 'good' and 'poor' learners was also compared both at home and away. To be considered a good learner, an animal needed to show more than a 30% reduction in attempted aerial respiratory behaviour from S1 to the MT. This criterion was chosen as it is approximately twice the value of the s.e.m. for yoked controls (which do not show a significant change in pneumostome opening).

Observation of baseline behaviours

Homeostatic breathing observations

The oxygen requirements of naive C- and B-strain snails were compared by observing their homeostatic aerial respiration. As with the operant conditioning procedure, 100% N_2 gas was bubbled into 800 ml of artificial pond water for 20 min to create a hypoxic environment. *Lymnaea stagnalis* were then permitted to acclimate for 10 min, after which a 30 min period of observation commenced. During this observation period, *L. stagnalis* were permitted to freely perform aerial respiration as needed (i.e. no tactile stimulus was applied to the open pneumostome). The total time spent performing aerial respiration during the 30 min observation period was recorded for each animal. A two-way ANOVA with a Tukey *post hoc* test was used to compare total breathing time between the C- and B-strains reared both at home and away. Differences were deemed significant when $P<0.05$ and data are presented as means±s.e.m. Fig. 2 legend contains the detailed statistical analysis.

Rate of locomotion

Rates of locomotion (in mm s^{-1}) were compared across naive snails from both the C- and B-strains. Individual snails were placed in the middle of a Petri dish (14 cm diameter with a 2 cm×2 cm grid) containing pond water and the 10 min period of observation commenced once the snail emerged from its shell and fully extended its tentacles. The rate of locomotion (mm s^{-1}) was then calculated for each individual animal. The rate of locomotion was compared for the C- and B-strains both at home and away using a two-way ANOVA and a Tukey test was used for *post hoc* comparisons. Differences were considered significant when $P<0.05$. All values are presented as means±s.e.m. Detailed statistics are given in Fig. 2 legend.

RESULTS

The C- and B-strains possess different memory-forming abilities at home

Previous findings (at the home institutions) have suggested that LTM lasting 48 h requires four 45 min training sessions for the B-strain (Carpenter et al., 2016), whereas the C-strain can produce 48 h LTM from only two 45 min training sessions (Scheibenstock et al., 2002). These previous studies suggest potential differences between these lab-bred strains with respect to memory-forming ability. However, the same laboratory personnel did not conduct all of the training in these previous studies. In this study, we used the same trainer at each location to determine whether strain differences do indeed exist, and whether these depend on the rearing conditions present during development.

Our first aim was to use four 45 min training sessions to test the duration of LTM for each strain in their home environment. As shown previously, the B-strain trained at home (i.e. at Brock University) demonstrated both learning and LTM persisting for 24 h ($F_{2,36}=15.52$, $P<0.0001$; $\eta^2=0.463$; $n=19$). Specifically, these animals demonstrated a significant reduction in behaviour from S1 to S4, indicating learning [$P<0.0001$; 95% confidence interval (CI) 5.080, 1.762] and this reduction was maintained for 24 h, indicating LTM formation ($P=0.0002$; 95% CI 4.764, 1.446; data not shown). Memory was also assessed at both 48 and 72 h after the final training session. The B-strain demonstrated both learning ($P<0.0001$) and LTM ($P<0.0001$) when tested 48 h after S4 ($n=18$; Fig. 1A). However, when memory was assessed 72 h after training, the B-strain no longer demonstrated LTM ($P=0.2956$; $n=17$; Fig. 1B; more detailed statistics are contained in the figure legend). Thus, using four 45 min training sessions, the B-strain animals at home form LTM that persists for 48 h, but not 72 h.

The C-strain trained at home (i.e. at University of Calgary; $n=15$) with four 45 min training sessions showed LTM that lasted for a longer duration than the B-strain. Following four training sessions, the C-strain demonstrated a significant reduction in aerial respiratory behaviour from S1 to S4, indicative of learning ($P<0.0001$). The LTM formed by the C-strain animals following training persisted for at least 96 h ($P=0.0002$; Fig. 1C).

In summary, in their home environments, the B-strain demonstrated memory that lasted for 48 h (but not 72 h), whereas the C-strain demonstrated memory that persisted for at least 96 h. We next determined whether the environment in which the animals were reared was responsible for these observed differences. To address this, we switched the laboratory setting in which the two strains were reared. Egg masses containing C-strain embryos were moved to Brock University, subsequently allowed to hatch and then raised to adulthood in this new away environment (referred to as the first generation C-strain). Similarly, egg masses containing B-strain embryos were transferred to University of Calgary (away environment) where they hatched and were raised to adulthood (first generation B-strain).

Baseline locomotor and respiratory behaviours do not differ between different strains reared in the same environment

Before assessing learning and memory in strains reared away from home, we first needed to ensure that the two environments did not differentially influence various baseline behaviours. Differences in the performance of baseline behaviours in the two environments could influence aerial respiratory activity and, consequently, memory-forming ability. We thus asked whether there was significant variation in either (i) homeostatic aerial respiratory behaviour or (ii) rate of locomotion between the C- and B-strains,

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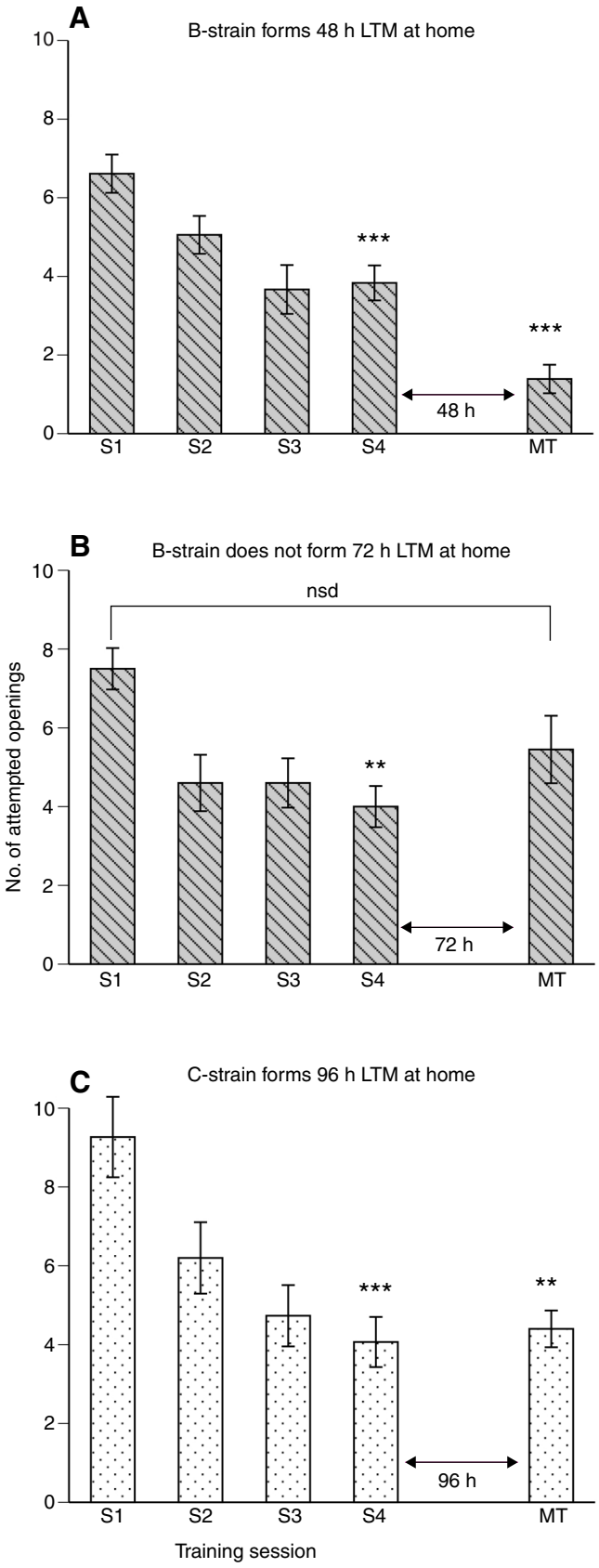

Fig. 1. Long-term memory (LTM) persists longer in the *Lymnaea stagnalis* C-strain than in the B-strain following four training sessions at 'home'. (A) Following four training sessions in their home environment, B-strain animals demonstrated learning and LTM that persisted for 48 h [$F_{2,34}$=44.66, P<0.0001; η^2=0.724; S1 versus S4: P<0.0001; 95% confidence interval (CI) 4.133, 1.423; S1 versus memory test (MT): P<0.0001; 95% CI 6.577, 3.867]. (B) However, when memory was assessed 72 h after training, the B-strain still demonstrated learning, but no longer demonstrated LTM ($F_{2,32}$=8.867, P=0.0009; η^2=0.357), i.e. these animals showed learning (S1 versus S4: P=0.0006; 95% CI 5.895, 1.517), but behaviour during the MT was not significantly reduced from that in S1 (P=0.2956; 95% CI 3.542, −0.8357). (C) Following this same training procedure, the C-strain animals demonstrated learning and LTM at home ($F_{2,28}$=16.04, P<0.0001; η^2=0.534). After four training sessions, a significant reduction in behaviour indicating learning was observed (S1 to S4: P<0.0001; 95% CI 7.743, 2.657). These animals formed LTM that persisted for at least 96 h (S1 versus MT: P=0.0002; 95% CI 7.410, 2.324). ***P<0.0001, **P<0.001 relative to S1; nsd, no significant difference. All values are means±s.e.m.

when raised in the same laboratory environments. Observations were made for both strains in their home environment, as well as for the first generation of each strain in the away environment.

When tested at Brock University, the B-strain (n=28) and the first generation C-strain animals (n=27) did not demonstrate any significant difference in total breathing time (P=0.9367; Fig. 2Ai). Similarly, when tested at University of Calgary, the C-strain (n=25) and first generation B-strain animals (n=37) showed no significant differences in their total breathing time (P=0.3564; Fig. 2Bi). Thus, homeostatic aerial respiratory behaviour did not differ between these two strains when reared under the same environmental conditions.

Because *L. stagnalis* must travel to the surface of the water to perform aerial respiration, we also examined whether locomotor activity was different between the strains. When tested at Brock University, the B-strain (n=20) and the first generation C-strain animals (n=18) showed no significant difference in their rate of locomotion (P=0.8955; Fig. 2Aii). Similarly, when tested at University of Calgary, the C-strain (n=30) and the first generation B-strain animals (n=19) also showed no significant difference in locomotion (P=0.9974; Fig. 2Bii). Therefore, we found no significant differences between the C- and B-strains either at home or away.

The B-strain exhibits improved memory-forming ability when reared away at University of Calgary

Having determined that the two strains do not demonstrate differences in either respiratory or locomotor behaviours either at home or away, we next aimed to compare the memory-forming ability of animals in the two environments. The memory-forming ability of both strains was compared following only two training sessions both at home and away. This procedure can usually produce 24 h LTM in the C-strain (Scheibenstock et al., 2002; Sangha et al., 2003a; Hughes et al., 2016) but only ITM in the B-strain (Rothwell and Spencer, 2014). Both strains were trained in their home environment. First generation snails in their away environment, as well as their offspring (second generation away snails), were also trained. A two-way repeated measures ANOVA, used to compare all six groups, detected a significant interaction ($F_{10,318}$=8.267, P<0.0001; η^2=0.206) and a Tukey test was used for *post hoc* comparisons.

As expected, when the C-strain was trained at home, LTM was observed 24 h after training (n=19). Following two 45 min training sessions, a significant reduction in the number of attempted pneumostome openings was observed from S1 to S2 (P<0.0001), indicative of learning, and this reduction was maintained for 24 h (P<0.0001), indicating the presence of LTM (Fig. 3A).

When the B-strain was tested with two training sessions at home, the snails also demonstrated learning, as the number of attempted pneumostome openings observed during S2 was significantly

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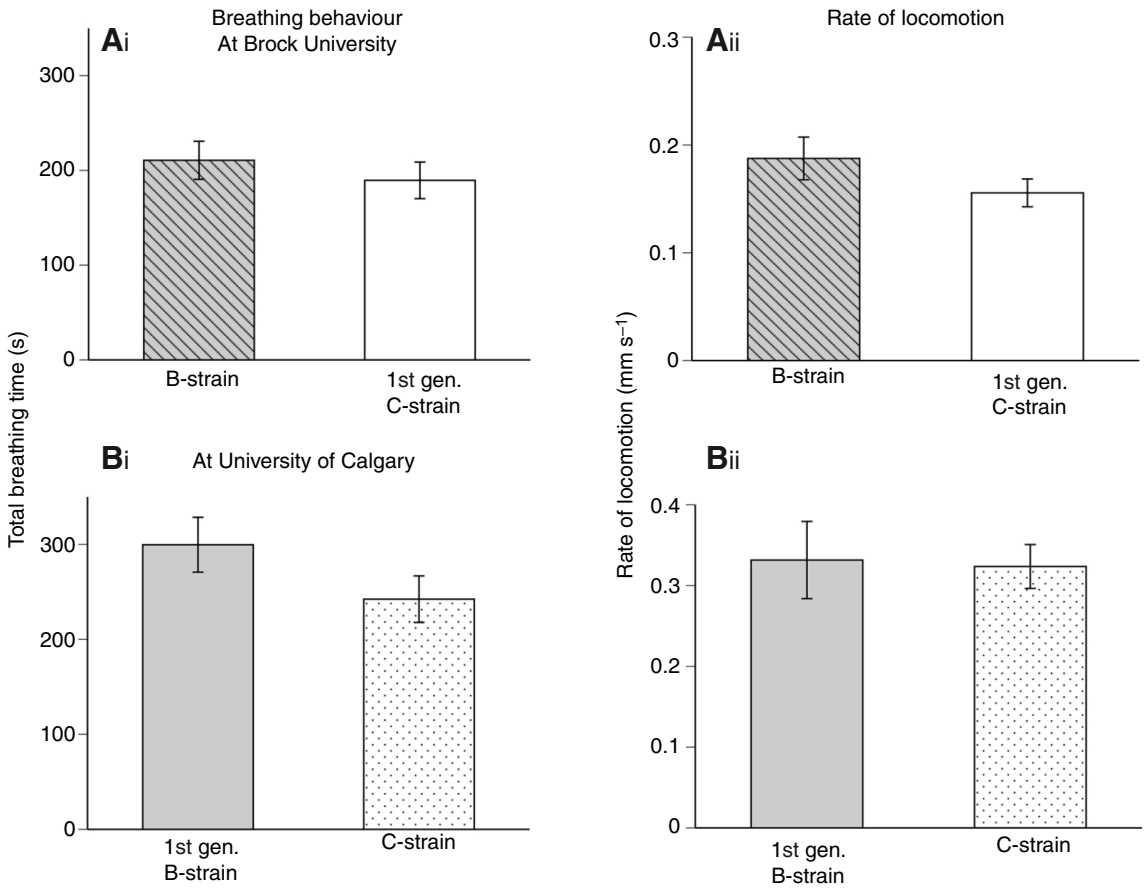

Fig. 2. Baseline behaviours do not differ between the different strains reared in the same environment. The breathing and locomotor behaviour of both strains were observed at Brock University (A) and University of Calgary (B). (A) When both strains were raised at Brock University, there were no significant differences in breathing behaviour (Ai: P=0.9367; 95% CI 115.6, −73.32) or rate of locomotion (Aii: P=0.8955; 95% CI 0.1510, −0.08714) between the two strains. (B) Similarly, there were no significant differences in total breathing time (Bi: P=0.3564; 95% CI 33.39, −148.0) or rate of locomotion (Bii: P=0.9974; 95% CI 0.09949, −0.1154) when both strains were reared at University of Calgary. All values are means±s.e.m.

reduced compared with the initial behaviour observed in S1 (P<0.0001; n=41). However, LTM was not present 24 h after training, as the behaviour observed during the MT was not significantly different from that observed in S1 (P=0.1609; Fig. 3B).

In summary, these results show that when trained at home, C-strain animals are capable of forming 24 h LTM with only two training sessions, but the B-strain animals are not. This further indicates that the memory-forming capabilities of these two lab-bred strains are different.

We hypothesized that the differences in memory-forming ability between the B- and C-strains were due to differences in the environmental conditions used to rear and house the animals. Having switched the laboratory setting in which the two strains were reared, we next examined the memory-forming ability of both strains away from home.

The first generation C-strain animals, raised away, demonstrated both learning (P<0.0001) and 24 h LTM (P<0.0001), when trained with two sessions (n=28; Fig. 3C). Thus, these animals performed as if at home and retained their ability to form LTM (with two training sessions) when raised and tested away at Brock University.

The B-strain, which did not previously form LTM following two training sessions at home, did, however, show LTM when raised away (at University of Calgary; n=26). These first generation B-strain animals trained with two sessions showed both learning (P<0.0001) and LTM at 24 h (P<0.0001; Fig. 3D). Thus, the first generation B-strain reared away gained the capability to form LTM with the two-session training procedure. To ensure that this dramatic change of behaviour leading to LTM was indeed the result of contingent learning following the operant conditioning procedure, first generation B-strain animals were also subjected to a yoked control procedure (Rothwell and Spencer, 2014). As expected, animals subjected to the control procedure showed no significant change in aerial respiratory behaviour (n=16; S1: 5.63±0.55, S2: 5.63±0.58, MT: 4.63±0.38; one-way RM ANOVA; P=0.3219; data not shown).

Second generation animals maintain their memory-forming ability away from home

When reared away from home, the first generation B-strain animals gained the capability to form LTM with only two training sessions while the first generation C-strain snails showed no phenotypic change and maintained their memory-forming ability (Fig. 3C,D). We next examined whether the offspring of these first generation animals reared away (i.e. the second generation) would demonstrate LTM following two training sessions.

Second generation C-strain animals reared away (i.e. at Brock University) maintained their ability to form LTM and demonstrated both learning (P<0.0001) and memory 24 h after training (P<0.0001; n=35; Fig. 3E). The second generation B-strain animals raised away (i.e. at University of Calgary) also continued to demonstrate LTM formation with two training sessions (as exhibited by the first generation raised away; n=16). Specifically,

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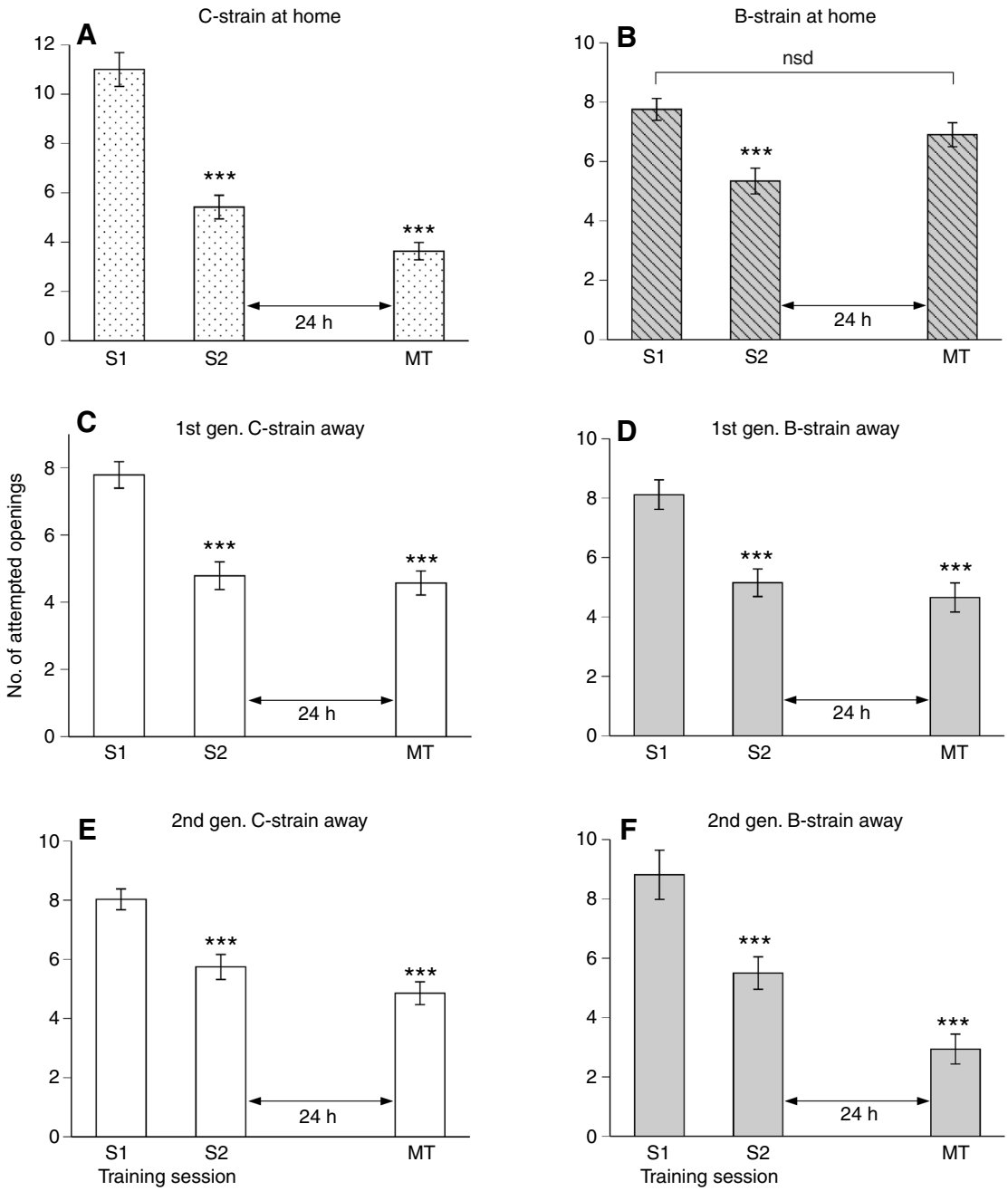

Fig. 3. Memory-forming ability of the C- and B-strains at home and away. (A) Following two training sessions at home, the C-strain (trained at University of Calgary) demonstrated both learning (S1 versus S2: $P<0.0001$; 95% CI 7.191, 3.967) and LTM lasting 24 h (S1 versus MT: $P<0.0001$; 95% CI 8.980, 5.756). (B) The B-strain also demonstrated learning in their home environment (S1 versus S2: $P<0.0001$; 95% CI 3.512, 1.317), but did not form LTM 24 h later (S1 versus MT: $P=0.1609$; 95% CI 1.951, −0.2437). (C) The first generation of C-strain snails raised 'away' at Brock University demonstrated both learning (S1 to S2: $P<0.0001$; 95% CI 4.328, 1.672) and LTM 24 h after training (S1 to MT: $P<0.0001$; 95% CI 4.542, 1.886). (D) The first generation of B-strain snails raised away at University of Calgary demonstrated both learning (S1 to S2: $P<0.0001$; 95% CI 4.340, 1.583) and LTM persisting for 24 h (S1 to MT: $P<0.0001$; 95% CI 4.840, 2.083). (E) The second generation of C-strain snails raised away at Brock University demonstrated learning (S1 to S2: $P<0.0001$; 95% CI 3.473, 1.098) and LTM 24 h after training (S1 to MT: $P<0.0001$; 95% CI 4.359, 1.984). (F) The second generation of B-strain snails reared away at University of Calgary demonstrated both learning (S1 to S2: $P<0.0001$; 95% CI 5.069, 1.556) and LTM lasting for 24 h (S1 to MT: $P<0.0001$; 95% CI 7.632, 4.118). ***$P<0.0001$ relative to S1; nsd, no significant difference. All values are means±s.e.m.

the second generation B-strain demonstrated a significant reduction in the number of attempted pneumostome openings from S1 to S2 ($P<0.0001$), indicative of learning. This reduction was maintained for 24 h until the MT ($P<0.0001$; Fig. 3F). Thus, the ability of the B-strain animals to form LTM with the two-session training procedure persisted in the second generation reared away. Overall, memory-forming ability was maintained from the first to second generations for both the C- and B-strain animals raised away from home.

To further examine the difference in memory-forming ability between strains, we also examined the proportion of good and poor learners in each environment. To be considered a good learner, an

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animal needed to demonstrate more than a 30% reduction in behaviour during the MT compared with the first training session (S1). This allowed us to examine whether there were subtle changes in memory-forming ability when animals were reared at home versus away, which may not be obvious when only examining the group means. Overall, the proportion of C-strain animals classified as good learners was reduced when this strain was reared away at Brock University for two generations. Conversely, the B-strain showed an increase in the proportion of good learners over two generations when reared away at University of Calgary (Fig. 4).

The memory-forming ability of both strains at home and away is summarized in Table 1. The change in the behaviour that occurred as a result of learning and memory for both B- and C-strains is also summarized in Fig. 5. While the C-strain formed LTM both at home and away, the change in behaviour during the MT was significantly greater at home (Kruskal–Wallis one-way ANOVA on ranks, P=0.002; Fig. 5A). Conversely, the B-strain did not form LTM at home, but respiratory behaviour during the MT was significantly reduced for two generations away from home (Kruskal–Wallis one-way ANOVA on ranks, P<0.0001; Fig. 5B). Thus, the behavioural response to operant conditioning was significantly changed for both strains when they were reared away compared with at home. This suggests that the environmental conditions in the two laboratory settings are indeed differentially influencing memory-forming ability in *L. stagnalis*.

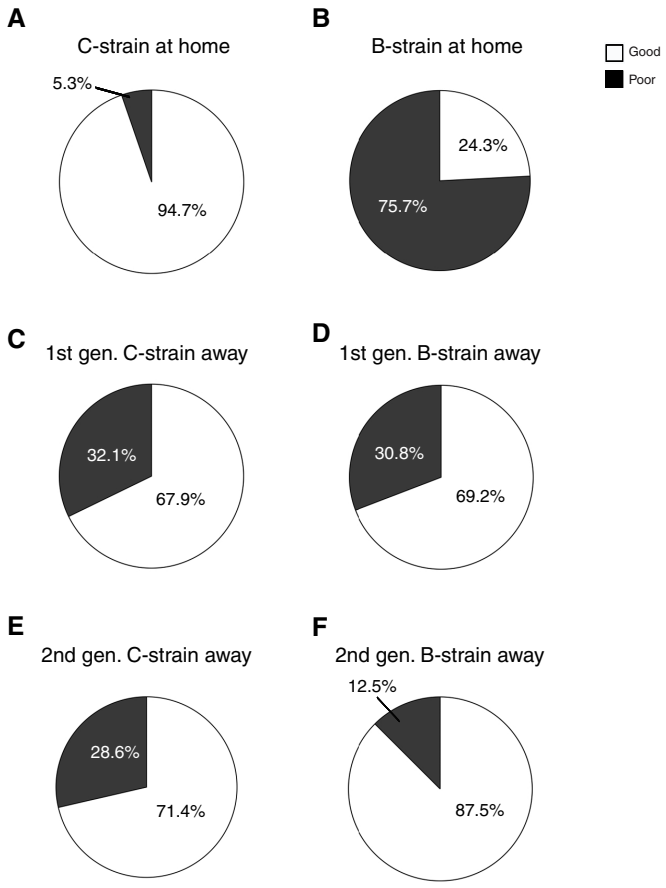

Fig. 4. The proportion of 'good' and 'poor' learners changes when away from home. Each animal was deemed to be either a good or poor learner based on the reduction in aerial respiratory behaviour observed during the memory test. The proportion of C-strain animals classified as good learners was greater at home (A) than when away at Brock University (1st generation, C; 2nd generation, E). Conversely, the proportion of B-strain animals classified as good learners was lower at home (B) than when away from home at University of Calgary (1st generation, D; 2nd generation, F).

Table 1. LTM-forming ability at 'home' versus 'away'

	C-strain	B-strain
Home	LTM	No LTM
First generation away	LTM	LTM
Second generation away	LTM	LTM

C-strain *Lymnaea stagnalis* maintained their LTM-forming ability for two generations when reared away at Brock University. B-strain animals demonstrated enhanced memory-forming ability within a single generation when reared away at University of Calgary. This change was maintained into the second generation away from home.

DISCUSSION

As a Holarctic species, *L. stagnalis* can be collected from ponds across Northern Eurasia and North America. However, despite being the same species, there is evidence of genetic diversity between snails from different geographic locations (Remigio, 2002; Puurtinen et al., 2004a,b, 2007). These genetic differences are associated with phenotypic differences, one example being tolerance to the aquatic levels of environmental copper (Côte et al., 2015). We have previously found that freshly collected *L. stagnalis* from different ponds (only 500 m apart) in England (Somerset Levels of South West England) possess significantly different memory-forming abilities (Dalesman et al., 2011c; Lukowiak et al., 2014). The same is also true of freshly collected *L. stagnalis* from ponds only 9 km apart in Canada (Calgary, AB; Orr et al., 2009a; Dalesman et al., 2011c; Braun et al., 2012; Lukowiak et al., 2014). What underlies this variability in memory-forming ability has not yet been determined, although significant differences have been found in the activity of a key neuron, RPeD1. This neuron drives aerial respiration and is necessary for memory formation, extinction, reconsolidation and forgetting, following conditioning of the behaviour (Syed et al., 1990; Scheibenstock et al., 2002; Sangha et al., 2003b, 2003d, 2005; Braun et al., 2012).

In this study, we found variations in memory-forming ability between two inbred laboratory-reared populations of *L. stagnalis* derived from the same population in The Netherlands (from snails collected in a polder near Utrecht in the 1950s). The two strains in this study have been separated for more than 15 years and reared in different laboratory environments at University of Calgary (C-strain) and Brock University (B-strain). As shown here, this separation has resulted in the divergence of their memory-forming ability. Specifically, the C-strain demonstrates superior memory-forming capability compared with the B-strain. However, despite this difference, we found no difference in either their homeostatic aerial respiratory behaviour or locomotor activity when the two strains were reared in the same laboratory environment.

We also observed a change in the ability of these animals to form memory depending on where an individual strain was reared (University of Calgary versus Brock University). This observation is consistent with the hypothesis that differences in the laboratory conditions at the two locations underlie the divergence in memory-forming ability. Specifically, when the B-strain snails were transferred to University of Calgary, they demonstrated an improved memory-forming ability within a single generation and an approximate 3-fold increase in the proportion of good learners. This enhanced memory-forming ability was further maintained into the second generation away from home. In contrast, the C-strain maintained their ability to form LTM when reared away, but the proportion of good learners was reduced. However, a complete loss

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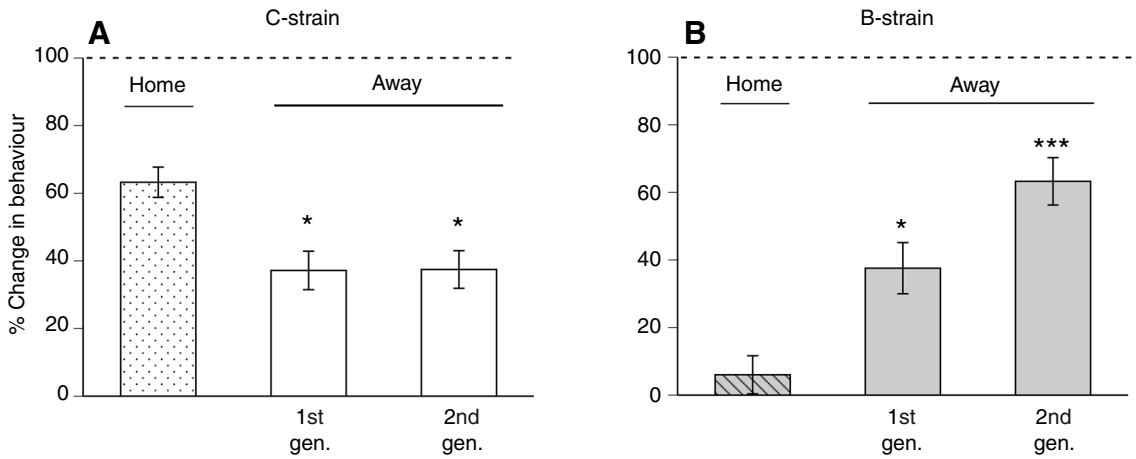

Fig. 5. The change in behaviour is significantly different at home versus away for both strains. The reduction in pneumostome openings during the MT, relative to S1, is expressed as a percentage change in behaviour. (A) The C-strain snails showed significantly less change in behaviour away from home, though they maintained their memory-forming ability at Brock University for two generations. (B) The B-strain demonstrated a significant change in behaviour when reared and maintained away compared with at home; the change in behaviour of the B-strain during the MT was significantly greater away than at home. **P*<0.01, ****P*<0.0001 away versus at home. All values are means±s.e.m.

of LTM-forming ability was not observed for at least two generations away from home. The factors responsible for this divergence in memory-forming ability remain to be elucidated. However, the results from this study suggest there may be factors at University of Calgary which are conducive to memory formation, but factors at Brock University which may hinder memory-forming ability in *L. stagnalis*.

It has been shown that the environment in which an animal is raised influences various behaviours, including memory formation, across species. For instance, in rodents, exposure to an enriched environment improves spatial memory in maze-based tests (van Praag et al., 2000). Additionally, young rats exposed to either social or environmental enrichment for 1 month demonstrate enhanced learning and memory (using the Morris water maze) compared with rats maintained in normal, control conditions (Hullinger et al., 2015). Interestingly, after 4 months of exposure, environmentally enriched animals performed better than the socially enriched group, which may suggest that the long-term influence of environmental enrichment is stronger than that of social enrichment, at least in a laboratory setting (Hullinger et al., 2015). Age-related spatial memory impairments are also reduced by environmental enrichment in middle-aged mice (Frick et al., 2003). This suggests that, at least in some instances, environmental enrichment presented later in life can still influence cognitive ability. The influence of environmental enrichment on memory-forming ability is also observed in invertebrates, including the cricket *A. domesticus* (Mallory et al., 2016) and the cuttlefish *S. officinalis* (Dickel et al., 2000). Specifically, crickets exposed to enriched conditions as young adults perform better in memory-based tasks (involving odour preference) than those maintained under impoverished conditions (Mallory et al., 2016). Similarly, cuttlefish reared in an enriched environment demonstrate stronger memory retention following an associative learning protocol than those in impoverished conditions (Dickel et al., 2000).

It has also previously been shown that the local environment can alter a range of animal behaviours. For example, when standardized behavioural testing procedures were used on the same strain of mouse (shipped on the same day from the same company) and tested at the same circadian time, but in different locations, significant differences were noted (Crabbe et al., 1999). Specifically, the environment in which an animal was tested was shown to significantly influence both locomotor activity and anxiety (as measured by the elevated plus maze; Crabbe et al., 1999). In that study, the authors concluded that factors in the local environment caused these changes. One difference noted was that different personnel tested the mice, which highlights the importance of using the same trainer in our current study. More recently, behavioural differences occurred relatively quickly in sub-strains of the inbred C57BL/6 mouse, reared in different laboratories. For example, a sub-strain at the National Institutes of Health (Bethesda, MA, USA) is 'uninterested' in alcohol, whereas a sub-strain bred at The Jackson Laboratory (Bar Harbor, ME, USA) has a preference for alcohol (Reardon, 2017). This indicates that some (unknown) factors which may differ between laboratory settings can lead to phenotypic changes, at least in that inbred mouse strain. Interestingly, *L. stagnalis* collected from ponds in the 'wild' demonstrate the same memory-forming ability whether they are maintained and subsequently trained in the lab or in their natural environment (i.e. their home pond; Orr et al., 2009b). That is, a change from the natural, 'wild' pond to the artificial laboratory environment was not sufficient to alter memory-forming ability in the adult animals in that study (Orr et al., 2009b).

Here, we eliminated one major source of variability, which was that of the experimenter (or trainer). The same researcher, familiar with both laboratories, performed the training and memory-testing experiments in each location. This allowed us to ensure consistency across all populations, as different researchers may apply slightly different stimuli during training. As noted above, the environmental factors, as well as the cellular mechanisms underlying the difference in memory-forming ability between the two strains, remain to be elucidated. The environmental differences between the two laboratories appear to be subtle, as procedures and reagents used in the rearing of snails were similar. One possible explanation is that subtle differences in environmental factors might be inducing epigenetic changes in these snails. Genetic and epigenetic changes have been linked to environmental stressors, diet and even pollution in a number of instances (Alegría-Torres et al., 2011). Additionally, it is known that impairing DNA methylation prevents memory enhancement following operant conditioning in *L. stagnalis* (Lukowiak et al., 2014; Rothwell and Lukowiak, 2017).

Moreover, changes in DNA methylation can persist for many weeks in *L. stagnalis*. For instance, training juvenile C-strain *L. stagnalis* in the presence of a predator scent enhanced their ability to form LTM. More interesting was the fact that these same animals demonstrated the enhanced memory-forming phenotype 4 weeks later when tested in the absence of predator scent. Impairing DNA methylation before exposing *L. stagnalis* to the predator scent prevented this memory enhancement, indicating that epigenetic changes can induce long-term phenotypic changes in *L. stagnalis* (Forest et al., 2016). It is thus possible that the phenotypic changes observed in this study may be a reflection of changes in DNA methylation induced by an environmental factor, which may include the quality of the water used to rear and maintain the snails or the presence of an unknown stressor. However, this remains to be determined.

We have to consider the possibility that the different environmental laboratory conditions may also be directly influencing the activity of the neuronal network underlying aerial respiration. A single neuron, RPeD1, controls the initiation of this behaviour (Syed et al., 1990) and it has been shown to be a necessary site for LTM formation, extinction, reconsolidation and forgetting (Scheibenstock et al., 2002; Sangha et al., 2003b,d, 2005). Moreover, following operant conditioning, this neuron is more likely to become quiescent and its intrinsic excitability is decreased (Spencer et al., 1999, 2002; McComb et al., 2005; Khan and Spencer, 2009; Braun et al., 2012). It is possible that the different environmental conditions at Brock University and University of Calgary influence RPeD1 activity and, in turn, memory-forming ability. For example, it has been shown that C-strain snails detect differing levels of calcium in their aquatic environment and this alters their behaviour: low levels of calcium negatively impact learning and memory, as well as the activity of RPeD1 (Dalesman and Lukowiak, 2010; Dalesman et al., 2011a,b; Karnik et al., 2012; Lukowiak et al., 2014). In addition, we know that different strains of freshly collected *L. stagnalis* show differences in the level of RPeD1 excitability that correlate with differences in memory-forming ability (Braun et al., 2012). Indeed, it is thought that the reduced excitability of RPeD1 in snails with stronger memory-forming ability causes this neuron to be 'primed' for LTM formation (Braun et al., 2012).

In summary, we have demonstrated a divergence in memory-forming ability between two laboratory-reared *L. stagnalis* strains derived from the same original inbred population. At home, the B-strain demonstrates weaker memory-forming ability than the C-strain. However, this weaker memory-forming ability is mitigated within a single generation when B-strain embryos are reared away. The potential mechanisms underlying this divergence between the separate populations remain to be elucidated. However, these results suggest that environmental factors may be influencing the nervous system, resulting in different memory-forming abilities, despite the shared ancestral origin of these strains.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.M.R., G.E.S., K.L.; Methodology: C.M.R., G.E.S., K.L.; Formal analysis: C.M.R.; Investigation: C.M.R.; Resources: G.E.S., K.L.; Writing - original draft: C.M.R., G.E.S., K.L.; Writing - review & editing: C.M.R., G.E.S., K.L.; Supervision: K.L.; Project administration: G.E.S., K.L.; Funding acquisition: G.E.S., K.L.

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