

RESEARCH ARTICLE

Strain transformation: enhancement of invertebrate memory in a new rearing environment

Cailin M. Rothwell* and Ken Lukowiak

ABSTRACT

Memory formation is influenced by a variety of factors, including the environmental conditions in which an organism is reared. Here, we studied the memory-forming ability of the lab-bred B-strain of *Lymnaea stagnalis* following a change in their rearing environment from Brock University to the University of Calgary. We have previously demonstrated that this move enhances memory-forming ability and here we studied the magnitude of this phenotypic change. Once reared to adulthood at the University of Calgary, the B-strain animals were first tested to determine how many training sessions were required for the formation of long-term memory (LTM) to occur. Following the change in environment, the B-strain transformed into a 'smart' lab-bred strain requiring only a single 0.5 h session to form LTM. Next, we tested whether exposure to physiologically relevant stressors would block the formation of LTM in this 'transformed' B-strain, as this obstruction has previously been observed in 'smart' snails collected from the wild. Interestingly, neither stressor tested in this study perturbed memory formation in this transformed lab-bred strain. Additionally, both the smart memory phenotype and increased stress resilience were observed in the second generation of transformed B-strain animals at both juvenile and adult stages. This suggests that a change in rearing environment can contribute to the memory-forming ability of lab-bred *L. stagnalis*.

KEY WORDS: Aerial respiration, Associative learning, Operant conditioning, Stress

INTRODUCTION

In order to not only survive but also thrive, an organism must detect and respond to changes in its surroundings, such as the presence of a predator or the restriction of essential resources. This adaptation to an ever-changing environment reflects an animal's ability to learn and remember, with specific conditions being shown to influence memory-forming ability. For instance, species ranging from *Sepia officinalis* (cuttlefish; Dickel et al., 2000), to *Acheta domesticus* (cricket; Mallory et al., 2016), to rodents (Hullinger et al., 2015; Sparling et al., 2018) demonstrate enhanced memory formation following exposure to enriched environments.

The mollusc *Lymnaea stagnalis* is a well-established model for studying the impact of environmental changes and stress on memory-forming ability. Specifically, associative learning and memory can be examined following the operant conditioning of

L. stagnalis's aerial respiratory behaviour (Lukowiak et al., 1996, 1998, 2000). *Lymnaea stagnalis* is capable of forming both intermediate-term memory (ITM) and long-term memory (LTM), depending on the number and duration of training sessions administered (Lukowiak et al., 2000). Additionally, memory formation in this mollusc can be enhanced or obstructed by various environmental perturbations and/or physiological stressors, including exposure to a thermal stress (Teskey et al., 2012), detection of a predator (Orr and Lukowiak, 2008), overcrowding (De Caigny and Lukowiak, 2008), damage to their shell (Hughes et al., 2017) and low levels of aquatic calcium (Dalesman et al., 2011a).

Interestingly, the location from which a strain of *L. stagnalis* originates and is, thus, reared also influences memory-forming ability. For instance, strains collected from different geographic locations in the wild demonstrate varying strengths of memory-forming ability, even when the geographic separation is minimal (Orr et al., 2008, 2009a; Dalesman et al., 2011b; Braun et al., 2012). Additionally, differences are observed between in-bred laboratory strains (with the same Dutch origin) maintained in separate environments for many years (Rothwell and Spencer, 2014; Rothwell et al., 2018). These observed differences have led to the classification of *Lymnaea* strains as 'smart', 'average' or 'below average'. A 'smart' strain forms LTM with a single 0.5 h training session (Orr et al., 2009a), an 'average' strain requires two 0.5 h sessions (Braun and Lukowiak, 2011; Hughes et al., 2016) and a 'below average' strain requires four 45 min sessions to produce LTM (Rothwell and Spencer, 2014). The smart phenotype has previously only been observed in juvenile and adult animals from strains collected in the wild (Shymansky et al., 2017), while the average ability has been reported in both wild and lab-bred populations. Interestingly, juveniles belonging to an average strain are unable to form LTM, but this inability is overcome by predator detection as well as development into adulthood (McComb et al., 2005; Orr et al., 2010; Forest et al., 2016; Shymansky et al., 2017).

The response of a *L. stagnalis* strain when faced with stress varies based on their memory-forming ability. Specifically, stressors that enhance LTM formation in average strains (such as a thermal stressor, predator detection or tissue damage) obstruct LTM formation in smart wild strains (Hughes et al., 2017). This suggests that smarter *L. stagnalis* strains may be more susceptible to the negative effects of stress exposure than strains possessing a weaker memory-forming ability.

We previously demonstrated that a change in rearing environment leads to an enhanced memory-forming ability in the below average lab-bred B-strain (Rothwell et al., 2018). In this study, we aimed to explore the magnitude of this phenotypic change and our results indicate that the B-strain transformed into a smart strain when their rearing environment was changed to the University of Calgary. Despite possessing the smart phenotype, this 'transformed' B-strain demonstrated stress resilience when challenged with physiologically relevant stressors. The smart phenotype and stress resiliency were

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also observed in the second generation of this population at the juvenile and adult stages.

MATERIALS AND METHODS

Animals

The strains of *Lymnaea stagnalis* (Linnaeus 1758) used in this study were obtained from separate lab-bred populations maintained in two locations: (i) Brock University (St Catharines, ON, Canada; termed the B-strain) and (ii) the University of Calgary [Calgary, AB, Canada; termed the C-strain (Rothwell et al., 2018); note that this population is referred to as the W-strain in other publications from the Lukowiak laboratory (e.g. Forest et al., 2016; Dodd et al., 2018)]. The two strains are derived from the same lab-bred population maintained at the Vrije University (Amsterdam, The Netherlands) which was originally cultivated via the collection of animals from polders in The Netherlands in the 1960s. However, the physical separation of the B- and C-strains for over a decade has resulted in the emergence of different memory-forming abilities between these populations (Rothwell et al., 2018).

(i) The B-strain at Brock University

The B-strain was established at Brock University between the years 2001 and 2002 by combining *L. stagnalis* populations bred at the University of Calgary and the Vrije University (Amsterdam, The Netherlands). At Brock University, the B-strain was reared and housed in aerated artificial pond water (PW; filtered, dechlorinated tap water containing 0.25 g l⁻¹ Instant Ocean salts; Aquarium Systems, Mentor, OH, USA) at room temperature on a fixed light–dark cycle. Animals were fed a combination of romaine lettuce and NutraFin Max Spirulina fish food (Hagen) and permitted to freely perform aerial respiration in their home tanks prior to all experiments.

Transfer of the B-strain from Brock University to the University of Calgary

In order to rear the B-strain at the University of Calgary, B-strain egg masses were transferred from Brock University to the University of Calgary, where they hatched and the embryos developed into adults in the laboratory environment. These animals were reared in different PW from that used at Brock University (i.e. they were reared under the standard Calgary conditions; see below). This permitted examination of the memory-forming ability of a new, separate *L. stagnalis* population, which we have termed the ‘transformed’ B-strain.

(ii) The C-strain at the University of Calgary

The C-strain was established at the University of Calgary in the 1980s from the lab-bred population at the Vrije University in Amsterdam. This strain was reared and raised at room temperature in artificial PW (deionized water containing 80 mg l⁻¹ CaSO₄ and 0.25 g l⁻¹ Instant Ocean salt). Animals were maintained on a regular light–dark cycle and fed a combination of romaine lettuce and trout pellets. Thus, conditions (the PW used and the addition of trout pellets to the diet) at the University of Calgary differ from those at Brock University.

Operant conditioning of aerial respiration

The operant conditioning of aerial respiration was conducted under hypoxic conditions as previously described (Lukowiak et al., 1996, 2000). Prior to each training session (TS) and memory test (MT), 100% N₂ gas was vigorously bubbled into 500 ml of PW in order to create a hypoxic environment. This bubbling was maintained at a lower rate during all TSs and the following MT. A period of 10 min was provided prior to the initiation of each session to allow the animals to acclimate to this new hypoxic environment. Immediately prior to

the initiation of each TS and MT, all animals were gently propelled to the bottom of the test beaker.

In this study, two different training protocols were employed to operantly condition aerial respiratory behaviour. The first consisted of two 0.5 h TSs spaced 1 h apart with a MT administered 24 h after the second TS. This procedure is sufficient to produce LTM lasting 24 h in *L. stagnalis* strains possessing the average memory-forming phenotype (e.g. C-strain; Braun and Lukowiak, 2011; Hughes et al., 2016). The second training procedure consisted of a single 0.5 h TS followed by a MT 24 h later. This procedure produces LTM in smart but not average strains of *L. stagnalis* (Orr et al., 2008, 2009a; Dalesman et al., 2011b).

A tactile stimulus was applied to the pneumostome each time it began to open (i.e. at the start of an attempted pneumostome opening) during each TS and subsequent MT. This stimulus induced the immediate closure of the pneumostome without causing the animal to fully withdraw into its shell.

Both juvenile (average shell length of approximately 15 mm; approximately 2–3 months old; McComb et al., 2003, 2005) and adult (average shell length of approximately 25 mm; approximately 4–5 months old; McComb et al., 2003, 2005) animals were used in these experiments. For identification purposes, a coloured mark was applied to the shell of each individual animal approximately 24 h before the initiation of a training procedure.

The number of attempted pneumostome openings performed by each animal was recorded during each TS and subsequent MT. Animals were always returned to aerated home tanks and permitted to freely perform aerial respiration between TSs. The same individual (C.M.R.) administered all training procedures to ensure consistency across experiments.

Operational definition of memory

Memory formation was operationally defined as in previous studies (Lukowiak et al., 1996, 1998; Rothwell et al., 2018). Specifically, when two sessions were administered, LTM was deemed to be present when (i) the number of attempted pneumostome openings performed during the MT was significantly lower than the number observed during the first TS (i.e. TS1) and (ii) the number of attempted pneumostome openings during the MT was not significantly different from that seen in the final TS (i.e. TS2). When only a single TS was administered, the number of attempted pneumostome openings during the MT had to be significantly lower than the number observed during the TS for LTM formation to be observed. All values are presented as the mean±s.e.m.

Exposure to stressors

The application of various stressors has been demonstrated to either enhance or obstruct LTM formation in *L. stagnalis* (Lukowiak et al., 2014b). Following exposure to a stressor, memory enhancement is deemed to have occurred when a single 0.5 h TS produces LTM in a strain that is known to possess an average memory-forming ability under normal laboratory conditions (Orr and Lukowiak, 2008; Teskey et al., 2012; Hughes et al., 2017). Conversely, the application of a stressor is said to obstruct LTM formation when memory is no longer observed following training with a standard conditioning procedure for a given strain of *L. stagnalis* (Hughes et al., 2017).

Here, we examined the potential effect of two different stressors on the transformed B-strain: (i) exposure to a thermal stress and (ii) shell injury via clipping of the animal’s shell.

(i) Thermal stressor

Lymnaea stagnalis were exposed to a thermal stressor as previously described (Teskey et al., 2012; Hughes et al., 2017). Specifically,

500 ml of artificial PW was heated to 30°C using a water bath. Animals were placed in this 30°C environment for 1 h, after which they were permitted to recover for 1 h in their home tank containing room temperature PW. Following this recovery period, animals were operantly conditioned with a single 0.5 h TS and memory was assessed 24 h later.

(ii) Shell injury

An injury was induced by clipping the animal's shell, as previously described (Hughes et al., 2017). Briefly, forceps were used to remove a small strip of shell close to the animal's pneumostome. Following this procedure, *L. stagnalis* were returned to their home tank and permitted to recover for 24 h before the initiation of the operant conditioning procedure.

Statistical analysis

Comparisons of the memory-forming ability of the B- and C-strains were conducted using two-way RM ANOVA and a Šidák's multiple comparisons test for *post hoc* analyses. The memory-forming ability of the transformed B-strain was assessed at 24, 72 and 120 h using paired *t*-tests. Separate paired *t*-tests were used to assess the influence of either thermal stress or shell clipping on adult and juvenile animals from the transformed B-strain. Differences were considered significant when $P < 0.05$. All figures display data for each individual animal as well as the overall group means and s.e.m.

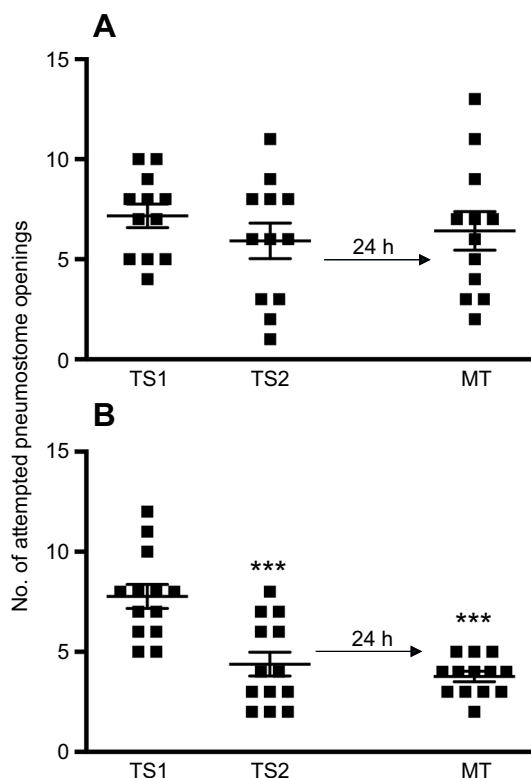


Fig. 1. The C-strain of *Lymnaea stagnalis* forms long-term memory (LTM) following two training sessions, but the B-strain does not. Adult *L. stagnalis* from the B-strain (A; $n=12$) and C-strain (B; $n=13$) were conditioned with two 0.5 h training sessions (TS) and memory was assessed 24 h later (MT, memory test). (A) The B-strain showed no significant reduction in aerial respiratory activity across sessions. (B) The C-strain learned to reduce the number of attempted pneumostome openings from TS1 to TS2 and maintained this reduction for 24 h, demonstrating LTM (** $P < 0.0001$ relative to TS1).

RESULTS

Neither the B-strain nor the C-strain forms LTM following a single 0.5 h TS

It has previously been demonstrated that a *L. stagnalis* strain possessing an average memory-forming ability requires two 0.5 h TSs to form LTM, while smart strains form LTM with only a single TS (Orr et al., 2008, 2009a; Braun and Lukowiak, 2011; Dalesman et al., 2011b; Hughes et al., 2016). In this study, as a control, we first needed to verify the memory-forming ability of both the B- and C-strains in their home environment (at Brock University and the University of Calgary, respectively). First, we tested whether the B- or C-strain exhibit the average phenotype by administering two 0.5 h TSs spaced 1 h apart and testing for LTM formation 24 h later (Fig. 1). A two-way RM ANOVA revealed a significant interaction ($F_{2,46}=4.917$, $P=0.0116$) and a Šidák's multiple comparisons test was used for *post hoc* comparisons. As expected, the B-strain did not show a significant reduction in aerial respiration (TS1 versus TS2: $P=0.2870$; TS1 versus MT: $P=0.6973$; $n=12$; Fig. 1A), while the C-strain demonstrated both learning and LTM formation (TS1 versus TS2: $P < 0.0001$; TS1 versus MT: $P < 0.0001$; $n=13$; Fig. 1B), verifying that in their home environment, C-strain snails possess an average memory-forming ability, while B-strain snails do not.

Previous studies indicated that neither the B-strain nor the C-strain are classified as smart in their home environments (Rothwell et al., 2018; Sunada et al., 2017). We next aimed to verify this by administering a single 0.5 h TS to both strains and assessing memory 24 h later in their home environment (Fig. 2).

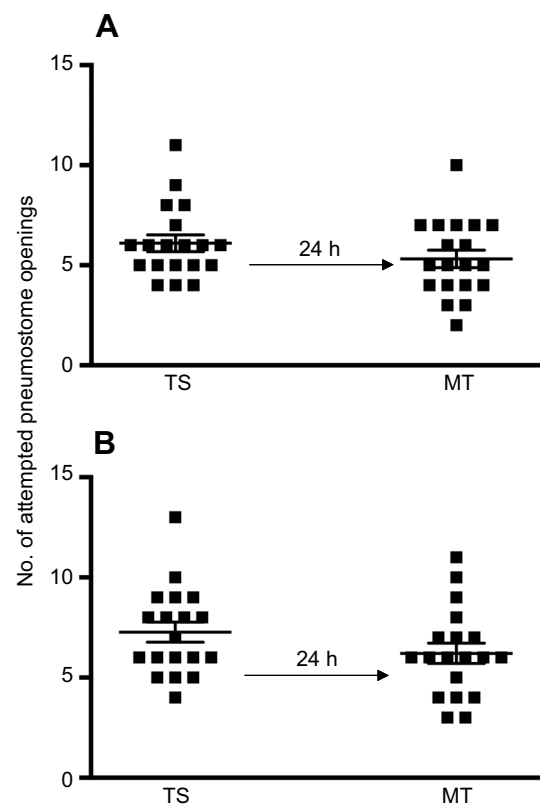


Fig. 2. A single 0.5 h TS does not facilitate LTM in either the B- or the C-strain. Adult *L. stagnalis* of the B-strain (A; $n=19$) and the C-strain (B; $n=19$) were given one 0.5 h TS in their home environment. A MT was administered 24 h later to test for LTM. Neither the B-strain (A) nor the C-strain (B) showed a significant reduction in aerial respiratory behaviour and, thus, neither strain was able to form LTM following a single TS.

A two-way RM ANOVA revealed a significant effect of session ($F_{1,36}=5.178$, $P=0.0289$) and a Šidák's multiple comparison test was used to compare the number of attempted pneumostome openings during the TS with that observed during the MT for each strain. Following a single TS, neither the B-strain ($P=0.3216$, $n=19$; Fig. 2A) nor the C-strain ($P=0.1429$, $n=19$; Fig. 2B) demonstrated a significant reduction in attempts at aerial respiration 24 h later. Thus, neither the B- nor the C-strain possessed the smart memory-forming phenotype in their home environment.

A change in rearing environment leads to the transformation of the B-strain

We have previously demonstrated that rearing the B-strain at the University of Calgary results in the development of an improved memory-forming ability (Rothwell et al., 2018), which led us to term this new population the 'transformed' B-strain. In this study, we aimed to further characterize the enhancement of memory-forming ability which occurs following the change in rearing environment. First, we aimed to examine whether the transformed B-strain population had become smart as a result of being reared at the University of Calgary by administering a single 0.5 h TS and assessing LTM formation 24 h later (Fig. 3A). A paired t -test indicated that the behaviour observed during the MT was significantly reduced compared with that seen during the TS ($t=7.842$, d.f.=25, $P<0.0001$, $n=26$; Fig. 3A), indicating that LTM had formed.

Having demonstrated that this transformed B-strain is capable of forming LTM lasting 24 h following a single TS, we next examined how long this memory persists following the single TS protocol. Thus, a separate group of naive adult transformed B-strain animals was again conditioned with a single 0.5 h TS, but this time memory was tested 72 h later. Again, a paired t -test indicated that there was a significant reduction in attempts at aerial respiration during the MT compared with the TS ($t=6.009$, d.f.=25, $P<0.0001$, $n=26$; Fig. 3B), indicating that LTM was present 72 h after training. In order to further test the limits of the transformed B-strain's LTM-forming ability, we again repeated this training protocol with a third group of naive animals and tested for memory 120 h later. Interestingly, LTM formation was also observed at this time point, as indicated by a significant reduction in attempted pneumostome openings performed during the MT compared with the TS (paired t -test; $t=4.002$, d.f.=17, $P=0.0009$, $n=18$; Fig. 3C). Thus, following the administration of a single TS, the transformed lab-bred B-strain can form LTM persisting for at least 120 h at the University of Calgary.

The adult transformed B-strain still forms LTM when challenged with stressors

Lymnaea stagnalis respond to a number of different environmental and/or physiological factors including, but not limited to, an increase in water temperature (Teskey et al., 2012), detection of a predator (Orr and Lukowiak, 2008) and tissue (shell) damage (Hughes et al., 2017). Interestingly, some stressors that enhance the memory-forming ability of an average strain instead impair memory formation in smart wild strains (Hughes et al., 2017), which indicates that memory-forming ability influences stress responses in *L. stagnalis*.

Having demonstrated that the transformed B-strain displays a smart memory-forming phenotype, we next aimed to examine whether this strain shows the same response to physiological stressors as smart strains collected from the wild [e.g. the TC1 (Trans-Canada 1) strain; Braun et al., 2012]. Specifically, two separate cohorts of adult transformed B-strain animals were presented with two different stressors, both of which have

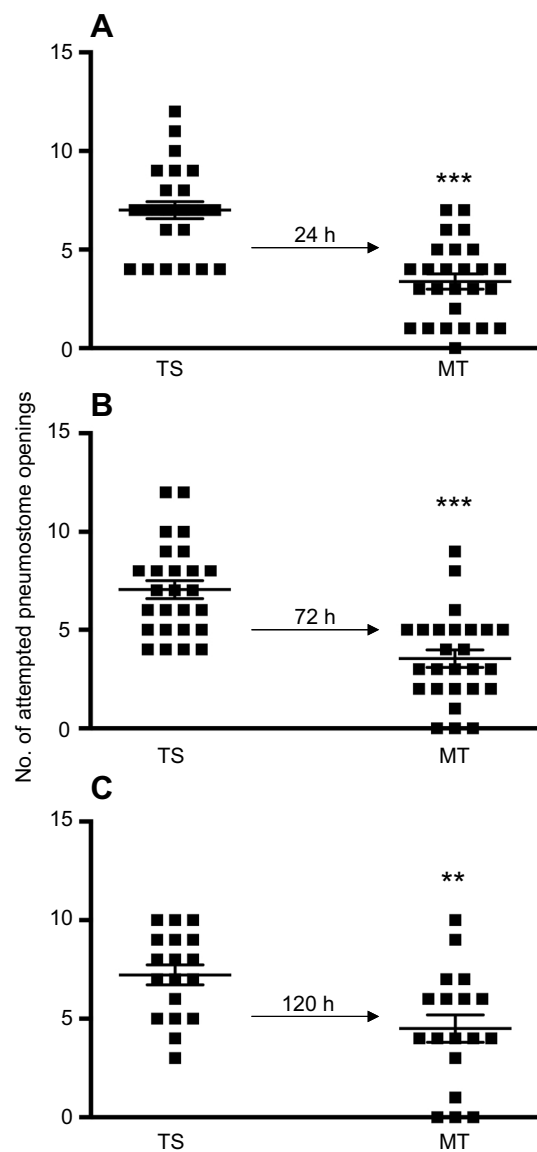


Fig. 3. The B-strain 'transforms' to a 'smart' strain when reared in a new laboratory environment. Eggs from the B-strain were transported from Brock University to the University of Calgary, where they hatched and the animals developed into adults. Following the administration of a single TS, LTM was observed at 24 h (A; $n=26$, $***P<0.0001$), 72 h (B; $n=26$, $***P<0.0001$) and 120 h (C; $n=18$, $**P<0.001$).

previously been demonstrated to obstruct memory formation in a 'smart' strain collected from the wild (Hughes et al., 2017): (i) a thermal stress and (ii) shell tissue damage (injury to the animal's shell).

First, we exposed a naive cohort of transformed B-strain animals to a thermal stress (PW heated to 30°C) for 1 h prior to the administration of a single 0.5 h TS (Fig. 4A). Memory was then assessed 24 h later. Interestingly, unlike the previously studied smart snails collected from the wild, the transformed B-strain did not show memory impairment when faced with this stressor. That is, a significant reduction in attempted pneumostome openings was still observed during the MT relative to the TS (paired t -test; $t=7.251$, d.f.=21, $P<0.0001$, $n=22$; Fig. 4A). Thus, exposure to thermal stress did not impair the memory-forming ability of this transformed lab-bred strain.

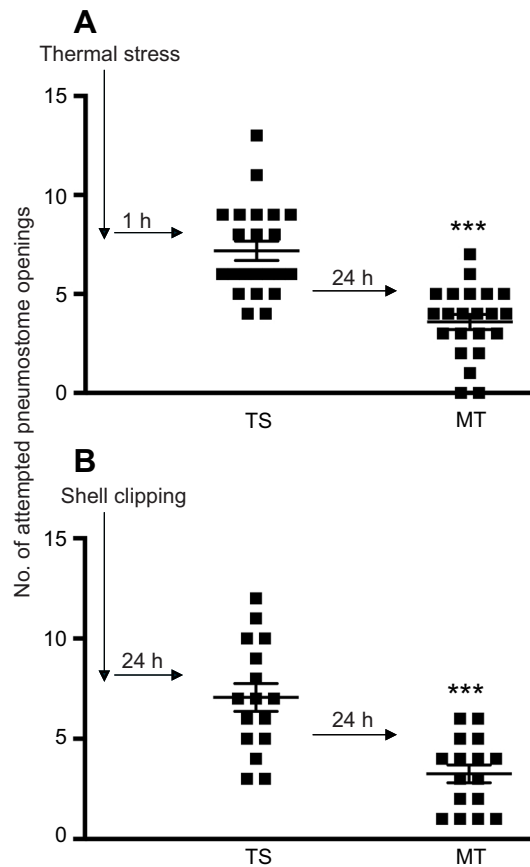


Fig. 4. Stressors do not block LTM formation in adults from the transformed B-strain. (A) Adult, transformed B-strain animals were exposed to a thermal stressor 1 h before being conditioned with a single 0.5 h TS. These animals formed LTM lasting 24 h, as the number of attempted pneumostome openings observed during the MT was significantly reduced compared with that in the TS ($n=22$; $***P<0.0001$). (B) Damaging the shells of adult transformed B-strain animals 24 h before the administration of a single TS did not prevent the formation of LTM lasting at least 24 h ($n=16$; $***P<0.0001$).

Second, we tested whether inducing an injury to the animal's shell would influence the memory-forming ability of this transformed lab-bred strain. Thus, 24 h before the initiation of training, a separate cohort of naive transformed B-strain animals had their shells clipped, after which all animals received a single 0.5 h TS (Fig. 4B). Interestingly, animals from the transformed lab-bred strain retained their ability to form LTM when challenged with this tissue damage, unlike what was previously observed in a smart wild strain (Hughes et al., 2017). Specifically, a significant reduction in attempted pneumostome openings was observed in the MT compared with that seen in the TS (paired t -test, $t=5.62$, $d.f.=15$, $P<0.0001$, $n=16$; Fig. 4B). Thus, the memory-forming ability of the transformed lab-bred B-strain was maintained following exposure to two different physiologically relevant stressors prior to operant conditioning.

The enhanced memory-forming phenotype is also observed in the second generation of the transformed B-strain

Having demonstrated that the first generation of the transformed B-strain shows a phenotypic change and becomes smart when reared at the University of Calgary, we next asked whether this change was maintained in their offspring. Eggs laid by adult transformed B-strain animals hatched and developed at the University of Calgary, allowing us to establish a second

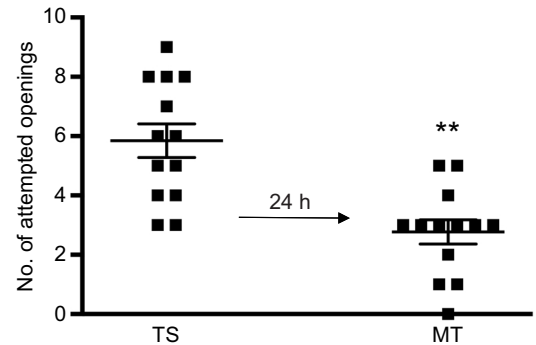


Fig. 5. Adults from the second generation of the transformed B-strain are also smart. Offspring of the first generation of the transformed B-strain were raised to adulthood at the University of Calgary and subsequently received a single 0.5 h TS ($n=13$). A significant reduction in the number of attempted pneumostome openings was observed during the MT compared with that during the TS ($**P<0.001$), indicating the presence of LTM.

generation of this strain. Upon reaching adulthood, naive animals from this second generation were trained with a single 0.5 h TS and memory was assessed 24 h later (Fig. 5). A paired t -test indicated that there was a significant reduction in attempts at aerial respiration during the MT compared with the TS ($t=4.5$, $d.f.=12$, $P=0.0007$, $n=13$; Fig. 5) and, thus, adult members of the second generation of the transformed B-strain also possess the smart phenotype.

The smart phenotype is also observed in juvenile transformed B-strain animals

The memory-forming ability of juvenile *L. stagnalis* has previously been examined in both average strains (the lab-bred C-strain as well as wild strains; McComb et al., 2005; Shymansky et al., 2017) and smart wild *L. stagnalis* strains (Shymansky et al., 2017). Juvenile *L. stagnalis* from average strains demonstrate an inability to form LTM, which is overcome by development into adulthood (McComb et al., 2005; Shymansky et al., 2017). However, juveniles from two different smart wild strains have been shown to possess the same memory-forming ability as adults from these populations. Thus, it appears that the physiological basis of the smart phenotype develops early in the lifespan, at least in wild *L. stagnalis* (Shymansky et al., 2017). In the current study, we asked whether the smart phenotype also emerges early in the lifespan of the transformed lab-bred B-strain (Fig. 6).

Offspring of the first generation of the transformed B-strain (i.e. the second generation) were randomly selected for inclusion in this experiment when they reached the juvenile stage of development (determined by having a shell length of approximately 15 mm; McComb et al., 2003, 2005). As a control, juvenile C-strain *L. stagnalis* were also raised and trained under the same laboratory conditions, as it has previously been shown that juveniles of this lab-bred strain are not capable of forming LTM (McComb et al., 2005). All animals received a single 0.5 h TS and LTM formation was assessed 24 h later. A two-way RM ANOVA revealed a significant interaction ($F_{1,27}=5.7$, $P=0.0242$). Šidák's *post hoc* test revealed the presence of LTM in the juveniles of the transformed B-strain ($P=0.0003$, $n=15$; Fig. 6A), but not the C-strain ($P=0.5528$, $n=14$; Fig. 6B). Thus, the smart phenotype emerges at the juvenile stage in the transformed B-strain.

Juvenile transformed B-strain animals demonstrate stress resilience

Having demonstrated that juveniles of the transformed B-strain possess the same smart phenotype as adults from this population, we

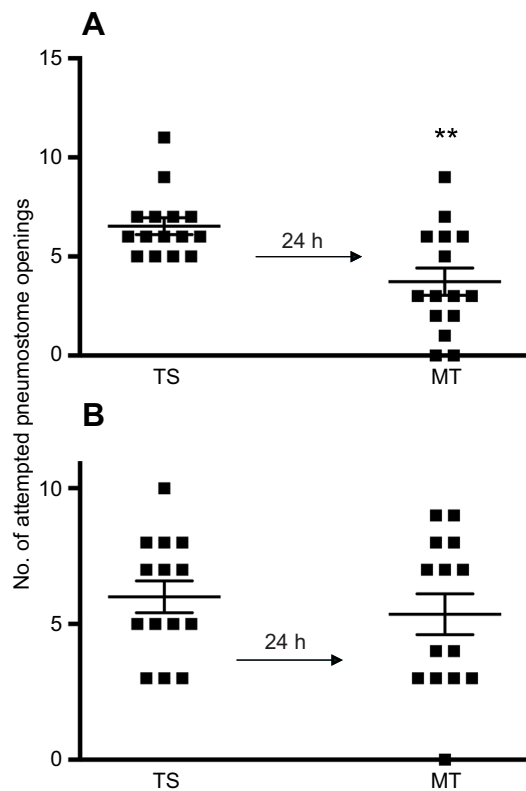


Fig. 6. Juvenile 'transformed' B-strain animals demonstrate the smart phenotype. (A) Juvenile B-strain animals received a single 0.5 h TS and memory was assessed 24 h later ($n=15$). These animals demonstrated LTM formation, as there was a significant reduction in the number of attempted pneumostome openings during the MT versus the TS (** $P<0.001$). (B) When C-strain juvenile animals were subjected to the same training procedure, LTM was not observed ($n=14$; $P=0.5528$).

next examined their response to physiologically relevant stressors. Although we demonstrated that adults of the transformed B-strain do not show a change in memory-forming ability when challenged with stress, we hypothesized that perhaps the juveniles of this strain, with the ongoing development of their nervous systems, may be more vulnerable to the potential effects of stress.

Thus, naive juveniles from the transformed B-strain were exposed to the same two stressors that we previously used with the adults: (i) thermal stress and (ii) tissue damage (shell clipping; Fig. 7). Exposure to a thermal stressor 1 h before training did not result in the obstruction of LTM formation, as a significant reduction in the number of attempted pneumostome openings was observed during the MT compared with the TS (paired t -test; $t=4.234$, d.f.=16, $P=0.0006$, $n=17$; Fig. 7A). A second cohort of naive juvenile transformed B-strain *L. stagnalis* received damage to their shells 24 h before the initiation of the operant conditioning procedure. These animals also demonstrated LTM formation, as there was a significant reduction in attempted aerial respiratory activity during the MT compared with the TS ($t=2.293$, d.f.=12, $P=0.0407$, $n=13$; Fig. 7B), indicating that the shell damage did not obstruct LTM formation in these animals. Thus, the juvenile members of the transformed B-strain demonstrate a level of stress resilience similar to that seen in adults of this population.

DISCUSSION

Lymnaea stagnalis is a holarctic mollusc and thus can be collected from a wide array of geographic locations throughout North

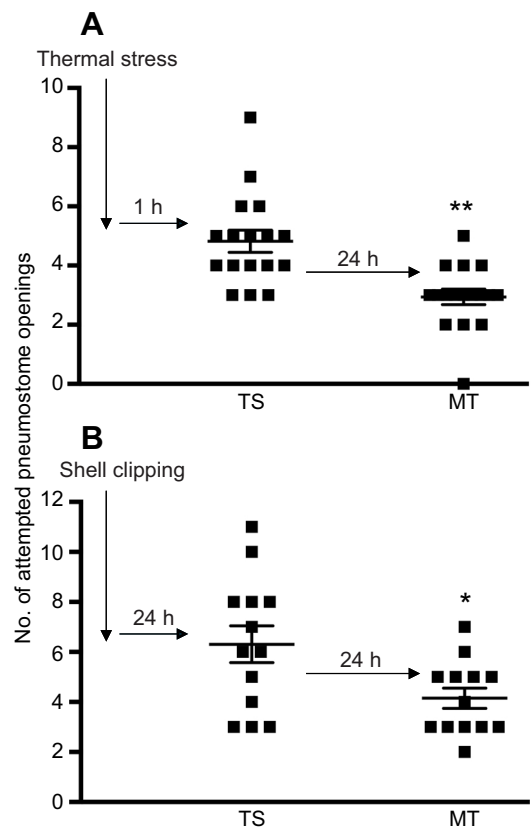


Fig. 7. Stress does not obstruct memory formation in juvenile transformed B-strain animals. (A) Juvenile transformed B-strain animals were exposed to a thermal stressor 1 h before training. The presence of this physiologically relevant stressor did not impair LTM formation, as a significant reduction in the number of attempted pneumostome openings was still observed in the MT compared with the TS ($n=17$; ** $P<0.001$). (B) Damage to the juvenile animals' shell prior to training was also insufficient to obstruct LTM formation, as the number of attempts at aerial respiration during the MT was significantly lower than that observed during the TS ($n=13$; * $P<0.05$).

America and Europe. As there are documented strain-specific differences in *L. stagnalis* within a relatively small geographic area, this mollusc is a good model for studying variability in many behaviours, including memory-forming ability (Dodd et al., 2018; Côte et al., 2015; Orr et al., 2009a; Puurtinen et al., 2004a,b). Differences in memory-forming ability have been observed following operant conditioning in strains collected from different locations in the UK and Canada (Orr et al., 2009a; Dalesman et al., 2011b), which has led to the classification of *L. stagnalis* strains based on their ability to form LTM. Specifically, some strains are average and require two TSs to form LTM, while others require only a single session and have, thus, been deemed smart (Orr et al., 2008, 2009a; Dalesman et al., 2011b; Braun et al., 2012). Variability in memory-forming ability is also observed among lab-bred strains of the same Dutch origin, but which have been reared in different laboratory settings for many generations (i.e. the B-strain at Brock University and the C-strain at the University of Calgary; Braun and Lukowiak, 2011; Rothwell and Spencer, 2014; Hughes et al., 2016; Rothwell et al., 2018).

Interestingly, phenotypic differences are also observed between strains with respect to the way in which they respond to physiologically relevant stressors. Specifically, when challenged with a stressor, some *L. stagnalis* strains demonstrate an enhancement of LTM-forming ability, while others show an obstruction of LTM

formation (Hughes et al., 2017). It is currently unclear why these phenotypic differences exist, though differences in neural activity within the central nervous system (CNS) may, at least in part, explain these observations (Braun et al., 2012).

We have previously demonstrated that the lab-bred B-strain develops a different memory-forming ability from that observed at Brock University when their rearing environment is changed to the University of Calgary. Specifically, following rearing at the University of Calgary, the B-strain became capable of forming LTM with half the number of TSs as required in their home environment (i.e. two 45 min sessions compared with four 45 min sessions; Rothwell et al., 2018). This transformation led us to term this new population of B-strain animals reared at the University of Calgary the 'transformed' B-strain in this follow-up study. Here, we aimed to examine the strength of this phenotypic change in memory-forming ability within this new lab-bred strain.

We found that rearing the B-strain snails at the University of Calgary resulted in their transformation to a smart strain, requiring only a single TS to produce LTM. All previously identified smart strains have been collected from populations in the wild across the UK and Canada. However, not all wild strains possess the smart phenotype (Orr et al., 2008, 2009a,b; Dalesman et al., 2011b; Braun et al., 2012). It is currently unknown what natural factors may contribute to this variability in the wild and whether any similar factors contributed to the transformation of the B-strain at the University of Calgary. The laboratory conditions at Brock University and the University of Calgary are reasonably similar, which suggests that the dramatic phenotypic change in memory-forming ability observed here may be the result of a subtle environmental difference. While the specific environmental factors underlying the phenotypic change observed in this study remain to be determined, it can be seen that they rapidly exert their effects, as the enhanced memory-forming ability emerges within a single generation and persists for at least one subsequent generation.

One difference between the environment at Brock University and that at the University of Calgary is the composition of the artificial PW used to raise the animals. At Brock University, artificial PW is composed of dechlorinated tap water (average total calcium concentration of 29.1 mg l⁻¹; Decew Water Treatment Plant, Regional Municipality of Niagara) to which Instant Ocean aquarium salts are added. In Calgary, animals are reared and maintained in deionized water containing the same Instant Ocean salts as at Brock University. However, calcium sulfate (final concentration of 80 mg l⁻¹) is also added to create this artificial PW. It is known that the levels of calcium in the artificial PW have an effect on different behaviours, including learning and memory formation in the C-strain *L. stagnalis* population. For example, in a low-calcium environment (20 mg l⁻¹), cutaneous respiration significantly increases while motility significantly decreases relative to that of animals maintained in a high-calcium environment (Dalesman and Lukowiak, 2010). Further, low levels of environmental calcium block LTM formation following a one-trial operant conditioning procedure (Dalesman et al., 2011a) and also obstruct LTM formation when animals are trained with the same operant conditioning procedure used in this study (Knezevic et al., 2011).

It is unknown what other ionic concentration differences or other molecules may remain in the water obtained from Lake Erie (the source of the water supplied to Brock University) following the water treatment process. That is, there may be other compounds in the tap water used to rear animals at Brock University that are influencing the memory-forming ability of the B-strain. Perhaps the change in the artificial PW used to raise the animals triggered a

physiological response in the B-strain animals and this led to the enhanced memory-forming ability observed here.

Another subtle difference between the rearing environments at the University of Calgary and Brock University is what was used to supplement the animals' standard diet of romaine lettuce. Specifically, at Brock University, spirulina fish food was administered, while the animals at the University of Calgary received trout pellets in addition to romaine lettuce. While these products have similar fat and protein content, the overall combination of ingredients (i.e. vitamins and minerals) differs. Thus, perhaps the combination of ingredients in the trout pellets is more beneficial for memory formation than that in the fish food administered at Brock University. Interestingly, the trout pellets have a higher guaranteed percentage of calcium than the spirulina fish food, which may further highlight the important role that calcium plays in LTM formation, at least in *L. stagnalis*.

A third possible difference that may account for the change observed in this study is the actual geographic location of the rooms where the animals were raised. Specifically, it is possible that an environmental factor other than water (for example, light conditions, air quality, altitude) may be responsible for the phenotypic change observed in this study. Experiments will be conducted to determine whether eggs laid at Brock University and reared in Calgary PW at the Brock location demonstrate the same transformation with respect to memory-forming ability as those raised at the University of Calgary.

One possible explanation for the observed memory enhancement is that the environmental change may bring about a long-term influence on activity within the CNS of the B-strain animals. Studies in wild populations demonstrate that strains with an enhanced memory-forming ability possess reduced excitability of the neuron RPeD1 compared with strains with the average phenotype (Braun et al., 2012). This neuron initiates aerial respiration and has been shown to be essential for the formation of LTM following operant conditioning, with LTM formation being associated with a reduction in RPeD1 activity (Spencer et al., 1999). Thus, it has been hypothesized that the reduced excitability of RPeD1 in the smart wild strains may prime the animals' nervous system, making it easier for LTM formation to occur (Braun et al., 2012). Perhaps RPeD1 within the nervous system of the transformed B-strain snails also demonstrates a similar decrease in excitability, and this may account for the phenotypic change observed in this study. This remains to be investigated in future studies.

Interestingly, the smart phenotype was also observed in the second generation of transformed B-strain animals reared at the University of Calgary, suggesting that the mechanism underlying this enhancement may be a genetic (or epigenetic) change. The heritability of memory-forming ability has previously been demonstrated in multiple *L. stagnalis* strains. For example, offspring of freshly collected wild strains that are reared in a laboratory environment in artificial PW demonstrate the same memory-forming ability as their parents reared in a pond (Orr et al., 2008, 2009a,b). Thus, at least in those populations, a change in the composition of the PW was insufficient to induce a phenotypic change and the heritability of the enhanced memory-forming ability was maintained (Dalesman et al., 2011b). Interestingly, the lab-bred B-strain seems more susceptible to an environmental change than the previously studied wild strains, as transport to the University of Calgary triggered memory enhancement that was passed from the first to the second generation. Specifically, transport of freshly collected wild strains (e.g. from the UK) did not alter their memory-forming phenotype (Dalesman et al., 2011b).

We hypothesize that some factor in the new environment combined with the experience of transport to the new environment may have induced epigenetic changes within the nervous system of the B-strain. Epigenetic changes have previously been shown to be triggered by a number of environmental factors, including nutrition and pollution (Alegria-Torres et al., 2011). Additionally, it has been established that DNA methylation is involved in memory enhancement in *L. stagnalis* (Lukowiak et al., 2014a). As methylation has been associated with an enhanced memory response in *L. stagnalis*, perhaps something in the new environment at the University of Calgary (such as a higher calcium content in the artificial PW or the slightly different diet) induces the methylation of genes involved in LTM formation in the transformed B-strain animals. This remains to be elucidated. However, in a previous study showing that the memory enhancement observed following exposure to the scent of a predator involves DNA methylation, the enhanced (smart) phenotype was not observed in the offspring of these animals (Forest et al., 2016). This suggests that, at least in some cases, epigenetic changes involved in memory-forming ability are not heritable in *L. stagnalis*.

Memory-forming ability has also been shown to change across the lifespan of some *L. stagnalis* strains. Specifically, in *L. stagnalis* strains demonstrating an average memory-forming ability (both lab-bred and collected from the wild), juveniles are unable to form LTM, but this is overcome by development into adulthood (McComb et al., 2005; Shymansky et al., 2017). In addition, it has been demonstrated that the ability of the lab-bred C-strain snails to form LTM is compromised with age when they are trained with an appetitive conditioning paradigm (Hermann et al., 2007; Watson et al., 2012). This age-related memory impairment has yet to be demonstrated following training with the same operant conditioning procedure used in this current study. Interestingly, some strains of *L. stagnalis* have been shown to demonstrate different memory-forming abilities following the operant conditioning of breathing behaviour versus the classical conditioning of the feeding behaviour (Sunada et al., 2017). Thus, it is important for memory-forming ability to be tested at different developmental stages using the same training procedure in order to gain insight into how this ability changes across an animal's lifespan.

Interestingly, both juveniles and adults demonstrate LTM-forming ability in wild smart strains following operant conditioning of their aerial respiratory activity (Shymansky et al., 2017). Here, we also observed the smart phenotype in transformed lab-bred B-strain juveniles. Thus, the smart phenotype emerges at an early stage of development, regardless of whether the animals are reared in the wild or in an artificial laboratory setting. It remains to be determined whether the same cellular and molecular mechanisms underlie the smart phenotype in the wild and the laboratory setting. McComb et al. (2003) demonstrated that the observed difference in the memory-forming ability between juvenile and adult average C-strain animals following the conditioning of pneumostome opening is related to activity within the CNS. Specifically, the juveniles show less suppressive input to their CNS than adults. As reduced excitability and activity of RPeD1 is correlated with LTM formation, it has been suggested that the inability of average juveniles to form LTM may be due to the lack of inhibitory CNS input (McComb et al., 2003). Thus, it can be hypothesized that this necessary inhibitory input may develop at an earlier stage in smart strains, regardless of whether the strain is in the wild or an artificial laboratory environment.

We further show that the transformed B-strain shows resilience to stressors that have previously been reported to impair LTM formation in wild smart strains. Specifically, Hughes et al. (2017) demonstrated that various physiologically relevant stressors, including (i) exposure to a thermal stress and (ii) the induction of injury to an animal's shell,

obstruct LTM formation in a smart wild strain. Here, we hypothesized that a similar obstruction would be observed in the transformed B-strain. However, the transformed B-strain instead showed resilience to both of these stressors at the juvenile and adult stages. Thus, the hypothesis that all smart strains are more easily stressed than average strains is not supported. We are uncertain at this time why the transformed B-strain snails show greater resilience than the other wild strains exhibiting the smart phenotype. Experiments are underway to determine the basis of this resilience in the transformed B-strain. It is important to note that the average lab-bred C-strain responds to these stressors with enhanced LTM formation (Teskey et al., 2012; shell-clipping: C.M.R. and K.L., unpublished observations), but the effect of these stressors on memory formation in the B-strain animals reared at Brock University has not yet been investigated.

In summary, we have demonstrated that the lab-bred B-strain can be transformed from below average to smart within a single generation following a change in their rearing environment. This is the first observation of a lab-bred *L. stagnalis* strain showing the smart phenotype without the aid of other memory-enhancing factors. The change in memory-forming ability also corresponded to stress resilience in these animals. While the cellular and molecular mechanisms underlying this dramatic phenotypic change remain to be elucidated, we posit that the environmental conditions at the University of Calgary may be influencing changes within the mollusc's CNS. Whether these involve genetic (or epigenetic) changes or possibly changes in the firing pattern of specific neurons needs to be investigated in future studies. However, these results confirm that a change in rearing environment, even if only subtle, can greatly influence memory formation in this mollusc.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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