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RESEARCH ARTICLE

Juveniles of *Lymnaea* 'smart' snails do not perseverate and have the capacity to form LTM

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ABSTRACT

Previously, it was concluded that the nervous systems of juvenile snails were not capable of mediating long-term memory (LTM). However, exposure and training of those juvenile snails in the presence of a predator cue significantly altered their ability to learn and form LTM. In addition, there are some strains of *Lymnaea* which have been identified as 'smart'. These snails form LTM significantly better than the lab-bred strain. Here, we show that juveniles of two smart snail strains not only are capable of associative learning but also have the capacity to form LTM following a single 0.5 h training session. We also show that freshly collected 'wild' 'average' juveniles are also not able to form LTM. Thus, the smart snail phenotype in these strains is expressed in juveniles.

KEY WORDS: Long-term memory, Learning and memory, Smart juveniles, Perseveration, Strain-specific learning abilities

INTRODUCTION

In the operant conditioning procedure used in the Lukowiak laboratory, the pond snail, *Lymnaea*, is trained to decrease aerial respiratory behaviour in a hypoxic environment where this behaviour predominates (Lukowiak et al., 1996). Aerial respiration consists of the snail coming to the surface and opening its breathing tube (pneumostome) to exchange lung air with the atmosphere. Adult *Lymnaea* used initially by the Lukowiak lab, referred to here as the Dutch strain, are capable of learning to decrease aerial respiratory behaviour and to form long-term memory (LTM). That is, they learn and remember not to perform aerial respiration. Subsequently, it was shown that Dutch juvenile snails were incapable of forming LTM (McComb et al., 2005). These snails were said to perseverate (defined as an inappropriate repetition of a behaviour). Typically, younger animals perseverate in situations where they are required to withhold a behavioural response (Peretz and Lukowiak, 1975; Peretz et al., 1976). However, Dutch juveniles are capable of both associative learning and LTM for tasks where withholding a behaviour is not required. For example, juveniles can learn and form LTM for appetitive food conditioning (Yamanaka et al., 2000).

The Dutch strain has been in the laboratory environment since the 1950s and originated from watercourses in a polder near Utrecht in The Netherlands (Orr et al., 2009; Lukowiak et al., 2014). This strain is now used in many universities world-wide. As the Dutch strain has been in the laboratory for some 60 years, it is safe to assume that certain traits have been selected for (some on purpose) to cause differences between this inbred laboratory strain and strains in the wild. For example, the Dutch strain lays eggs throughout the year, whereas freshly collected snails (e.g. TC1 snails, Braun et al., 2012) in our laboratory environment lay eggs primarily during the summer months. There are data suggesting that inbred juveniles reared in laboratory environments behave differently from those reared in enriched laboratory environments or from outbred strains (Campbell et al., 2013). We therefore asked whether juvenile offspring from freshly collected snails would form LTM following operant conditioning of aerial respiration.

Other evidence suggested a reason for developing the hypothesis that freshly collected juveniles might differ from the Dutch strain juveniles in regard to LTM formation following operant conditioning of aerial respiratory behaviour. Dutch juvenile snails have the capability of both learning and LTM if they are trained while detecting the scent of a predator (Orr et al., 2010; Sunada et al., 2010a). This enhancement of memory-forming ability was thought to be an 'inducible defence' caused by predator detection (Orr et al., 2007; Orr and Lukowiak, 2008). That is, training in the presence of crayfish effluent (CE) causes changes in the neural circuit mediating learning and memory formation, such that juveniles gain the capability of exhibiting both learning and LTM. More recently, Forest et al. (2016) have not only confirmed the earlier finding that Dutch strain juveniles trained in CE show the ability to form LTM but also showed that training in CE as a juvenile produced the 'smart' phenotype when snails matured into adults. That is, in pond water, the now adult snails (i.e. 4 weeks later) form LTM following a single 0.5 h training session. The Forest et al. (2016) study as well as a study by Sunada et al. (2016) showed that the long-lasting changes in the nervous system brought about by predator detection or other stressors are the result of epigenetic changes.

Freshly collected juvenile snails develop in a more 'enriched environment' and presumably have detected a predator of some sort following hatching and development. Thus, an 'inducible defence', such as that seen in the Forest et al. (2016) study, may have been initiated. It therefore seemed reasonable to hypothesize that the juvenile offspring of freshly collected snails would have the ability to form LTM following operant conditioning of aerial respiratory behaviour. If our just-mentioned hypothesis turned out to be the case, then the perseveration seen first in the McComb et al. (2005) study may be a behavioural trait that was 'selected' for in the development of the Dutch strain.

We have defined the smart snail phenotype as the ability to form LTM after a single 0.5 h training session (Braun et al., 2012). In the Dutch strain, which can be considered as the world-wide standard (Lukowiak et al., 2014), LTM typically only forms following two

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0.5 h training sessions that are separated by a 1 h interval (Lukowiak et al., 2000; Sangha et al., 2003; Parvez et al., 2005). This strain has been designated as an 'average' snail in regards to how easily snails form LTM. An exception to this is that certain stressors when experienced by the Dutch snails can cause an enhancement of memory formation (Lukowiak et al., 2014). The smart snail phenotype, in contrast, is one where a single 0.5 h training session is sufficient to cause LTM formation (Orr et al., 2009; Dalesman et al., 2011; Braun et al., 2012; Dalesman and Lukowiak, 2012). With the exception of lab-reared snails in the Forest et al. (2016) study that were exposed and trained in CE as juveniles and then tested as adults, all smart snails have come from naturally occurring populations of *Lymnaea stagnalis* in specific ponds in the UK, Alberta and, as shown here, Saskatchewan. However, it must be kept in mind that there are not only quantifiable differences in memory in *Lymnaea* (e.g. duration of memory) but also qualitative differences (Hughes et al., 2016). Whether the memory seen in smart juveniles is qualitatively different from that seen in the adults remains to be determined.

Strain-specific differences in the ability to learn and remember are not unique to *Lymnaea* as they have been observed for many years across phyla (Ings et al., 2009; Healy et al., 2009; van den Berg et al., 2011). Why some *Lymnaea* strains are smarter than others is not clear. It is also unclear whether the smart snail phenotype confers any selective advantage to the strain. The smart snail phenotype is heritable and has been observed across many years in freshly collected snails from known ponds (Dalesman et al., 2011; Lukowiak et al., 2014). As well, offspring from freshly collected snails (the F1 generation) and subsequent generations (i.e. F2) continue to exhibit the smart snail phenotype. We have also hypothesized that smart snails are more easily stressed than are average snails (Hughes et al., 2017). Whether this will also be seen in how smart versus average juveniles respond to various stressors also remains to be demonstrated.

Here, we tested the hypothesis that the juvenile offspring from all freshly collected *Lymnaea* have the ability to form LTM following operant conditioning of aerial respiratory behaviour in contrast to the lab-reared Dutch strain. Further, we tested the hypothesis that juveniles of the smart snail strain are also smart.

MATERIALS AND METHODS

Lymnaea

Snails (*Lymnaea stagnalis* L.) used in these studies were freshly collected in spring and summer from known ponds in Alberta and Saskatchewan and were maintained in the laboratory at room temperature (20–22°C) on an approximately 16 h:8 h light:dark (LD) cycle in aerated artificial pond water (0.26 g l^{-1} Instant Ocean, Spectrum Brands Inc., USA) in our standard calcium conditions with 80 mg l^{-1} Ca^{2+} (Dalesman and Lukowiak, 2010; Dalesman et al., 2011) and fed romaine lettuce *ad libitum*. We used this particular LD cycle primarily because it approximates the LD cycle encountered in Alberta/Saskatchewan during the summer time when snails are collected and when they typically lay their eggs. Snails were typically trained in the morning and tested for LTM at approximately the same time the following day (i.e. 24 h later).

Freshly collected adult snails of each strain were maintained in separate aquaria in the lab for at least 1 week before training. Following training, each strain of adult snail was kept separate so that they could lay eggs from which we obtained F1 juvenile snails. We did not perform any crossbreeding experiments with these strains.

Snails from four different ponds were used. Two of the ponds, termed Trans Canada (TC)1 and TC2, have previously been described (Braun et al., 2012; Lukowiak et al., 2014). These ponds are situated alongside the Trans Canada Highway approximately 50 km west of the city of Calgary. Sadly, and importantly for the present study, the TC1 pond suffered a catastrophic accident when the beaver dam that caused the pond to form suffered a breakage during a heavy rainstorm. Thus, we were not able to collect TC1 snails in the 2016 collecting season, and instead used previously unpublished data from adult snails collected in a previous year (2012) and their F1 offspring from that same year. Adult snails from TC2 were collected in 2016. We were able to collect both juveniles and adults in 2016 from a third pond, termed TC3, located on a gravel road (Township Road 252; 51°07′28.7″N, 114°40′32.8″W) that runs parallel to the Trans Canada Highway due north and slightly west from the TC1 pond. Finally, adult snails were freshly collected in 2016 from a pond in Saskatchewan termed Whitesand Lake (WSL; 51°46′12.45″N, 103°21′14.16″W), approximately 3 h east of the city of Saskatoon. This pond was chosen as it provided *Lymnaea* for use in a future study of the effects of a natural invasion of the northern crayfish (*Orconectes virilis*) up the Assiniboine River drainage on populations of *L. stagnalis* and their response to this new naturally occurring predator.

Juvenile snails

As has been the case previously (e.g. McComb et al., 2005), snails were considered to be juveniles if their shell length was 1.5 cm or less, while adults had a shell length of at least 2.5 cm. Juvenile snails were never observed to copulate nor were egg masses observed in their aquaria. All the TC1, TC2 and WSL juveniles used were F1 individuals reared under laboratory conditions from eggs laid by freshly collected snails. Freshly collected TC3 juveniles were also used.

Operant conditioning

Snails were removed from their home aquaria and placed into a 1 l beaker containing 500 ml of hypoxic pond water at room temperature. The water was made hypoxic by bubbling N_2 gas through the water for 20 min prior to introducing the snails. Snails were given a 10 min acclimatization period prior to the 0.5 h training session. To determine whether a specific strain was smart, animals were operantly conditioned with a single 0.5 h training session (TS1) by applying a tactile stimulus with a sharpened wooden applicator to the pneumostome as it began to open (i.e. an attempted pneumostome opening). The stimulus was strong enough to cause the snails to close the pneumostome yet gentle enough that the snails did not perform the full-body withdrawal response. Following the single 0.5 h training session, snails were returned to their home aquaria and then underwent a memory test (MT) 24 h later using a similar procedure to that in TS1 (i.e. a 'savings-test'). Two strains of juveniles, TC2 and TC3, which were found not to be smart, received two 0.5 h training sessions separated by a 1 h interval. Memory was then tested 24 h later. This procedure was used to determine whether these average juvenile snails could form LTM.

In previous studies employing juvenile and adult *Lymnaea*, various control procedures (e.g. yoked controls) were performed to demonstrate that the observed changes in behaviour following training were the result of associative learning. Because of a limited stock of freshly collected juveniles, we did not repeat these control procedures as we are quite certain that *Lymnaea* are capable of associative learning when the training procedures used since 1996 are employed.

Operational definition of learning and memory

As previously described (Lukowiak et al., 2000; Martens et al., 2007), for one-session training, memory is defined as a significant

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reduction in the number of attempted pneumostome openings after training. That is, for LTM to be present, the number of attempted pneumostome openings in the MT session must be significantly less than in TS1.

Statistics

A paired sample *t*-test was used to determine whether LTM was present in the cohorts where we trained snails with a single 0.5 h training session and tested for memory 24 h later. For LTM to be present, the number of attempted openings in MT had to be significantly less than in TS1 for each cohort of snails used. In the cohorts receiving the two 0.5 h training sessions (TS1 and TS2) with a 1 h interval between the sessions, a one-way ANOVA was used. If a significant difference was found, *post hoc* paired *t*-tests were run to compare training session versus MT, with Bonferroni corrections to assess which group learned and formed memory. In all cases, significance was at least at the $P<0.05$ level. All statistics were performed using PRISM 6 for Macintosh.

As mentioned above, experiments using the TC1 adult and juvenile cohorts were not performed in 2016.

RESULTS

While data on adult snails have previously been published using TC1 and TC2 snails (e.g. Braun et al., 2012), we thought that it would be best to test these two strains again and additionally test two new strains: TC3 and WSL. Thus, separate naive cohorts of each adult strain were trained with a single 0.5 h training session (TS1) and LTM was tested (MT) 24 h after TS1.

As can be seen in Fig. 1A, the TC1 adult snails, obtained before the destruction of the beaver dam, given the single 0.5 h training session ($N=64$), were able to form LTM. That is, the number of attempted pneumostome openings in MT was significantly less than the number of attempted openings in TS1 ($t=10.307$; $P<0.01$). Thus, these snails have been classified as smart snails, consistent with previous data (Braun and Lukowiak, 2011; Braun et al., 2012). In contrast (Fig. 1B), the TC2 snails collected in 2016 ($N=61$) that received the single 0.5 h TS1 did not form LTM. That is, the number of attempted pneumostome openings in MT was not significantly different from the number of attempted openings in TS1 ($t=0.1653$; $P=0.87$). Thus, the TC2 snails were classified as average snails.

We next determined whether the WSL snails were smart or average. As can be seen (Fig. 1C), the WSL snails ($N=28$) obtained in the 2016 collection season exhibited LTM following a single 0.5 h training procedure. That is, the number of attempted openings in MT was significantly less than that in TS1 ($t=5.22$; $P<0.01$) and thus these snails earned the smart snail designation. Finally (Fig. 1D), when the TC3 snails collected in 2016 ($N=11$) received the single 0.5 h training session, they did not exhibit LTM when tested 24 h later. The number of attempted pneumostome openings in MT was not significantly different from the number of attempted openings in TS1 ($t=0.2802$; $P=0.78$). Thus, the TC3 snails are not considered smart. Therefore, both the TC1 and WSL strains of *Lymnaea* were classified as smart snails while the TC2 and TC3 strains were classified as average snails.

In all previous studies examining the ability of juvenile *Lymnaea* to form LTM, researchers have used laboratory-reared (over 250 generations) Dutch snails. Here, we examined whether the so-called average freshly collected snails (i.e. TC2 and TC3) are able to form LTM as juveniles. We first tested the ability of the TC2 juveniles to form LTM following either a single 0.5 h training session or two 0.5 h training sessions with a 1 h interval between (Fig. 2A,B). The TC2 juvenile snails were not able to form LTM with only a single 0.5 h training session (Fig. 2A). That is, the number of attempted pneumostome openings in MT was not significantly different from the number of attempted openings in TS1 ($N=19$; $t=0.1664$; $P=0.87$). In a similar manner (Fig. 2B), LTM was not observed when the TC2 juvenile snails received the two-session training procedure ($N=14$; $F_{2,36}=0.8918$; $P=0.42$). Thus, the TC2 juvenile snails behave in a similar manner to the Dutch juvenile snails. We next tested the TC3 juvenile snails in the same manner (Fig. 2C,D) and found comparable results. That is, LTM was not formed following the single 0.5 h training session ($N=10$; $t=0.8762$; $P=0.40$), nor did it form following the two-training session procedure ($N=13$; $F_{2,36}=0.7805$; $P=0.4658$). Thus, the TC2 and TC3 juvenile snails behave in a similar manner to the Dutch strain in regards to the inability of juveniles to form LTM.

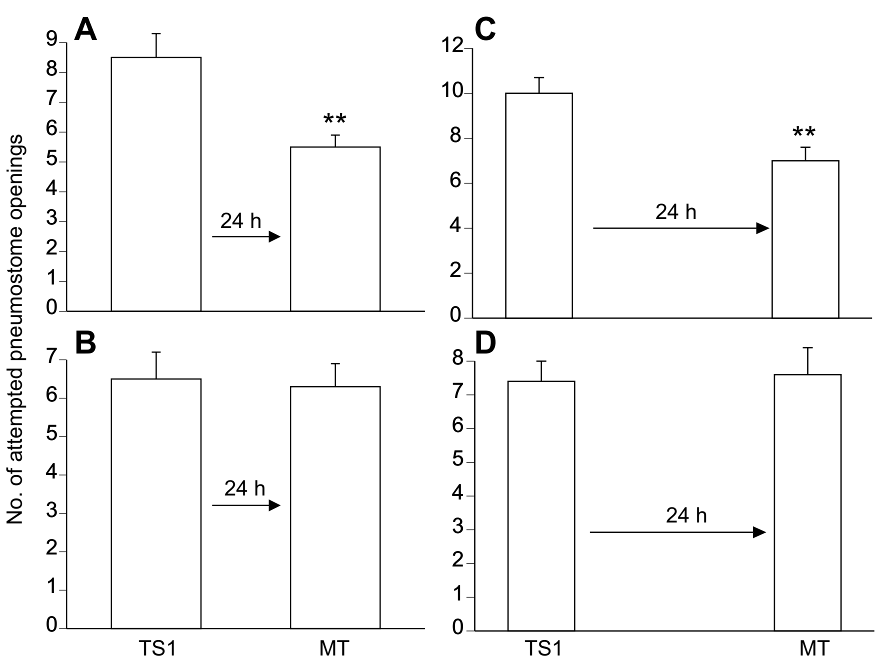

Fig. 1. TC1 and Whitesand Lake (WSL) adult snails are classified as 'smart' and TC2 and TC3 adults as 'average'. (A) Adult snails from the TC1 pond received a single 0.5 h training session (TS1) and memory was tested 24 h later (MT). In these snails, the number of attempted pneumostome openings in MT was significantly less than that in TS1, showing that long-term memory (LTM) was present. (B) As in A except snails from TC2 pond were used. In these snails, LTM was not formed and thus these snails were classified as average. (C) WSL snails are smart. Adult snails receiving a single 0.5 h TS1 showed LTM when tested (MT). (D) TC3 snails are average. LTM did not form (MT) following the single 0.5 h TS1. $^{**}P<0.01$.

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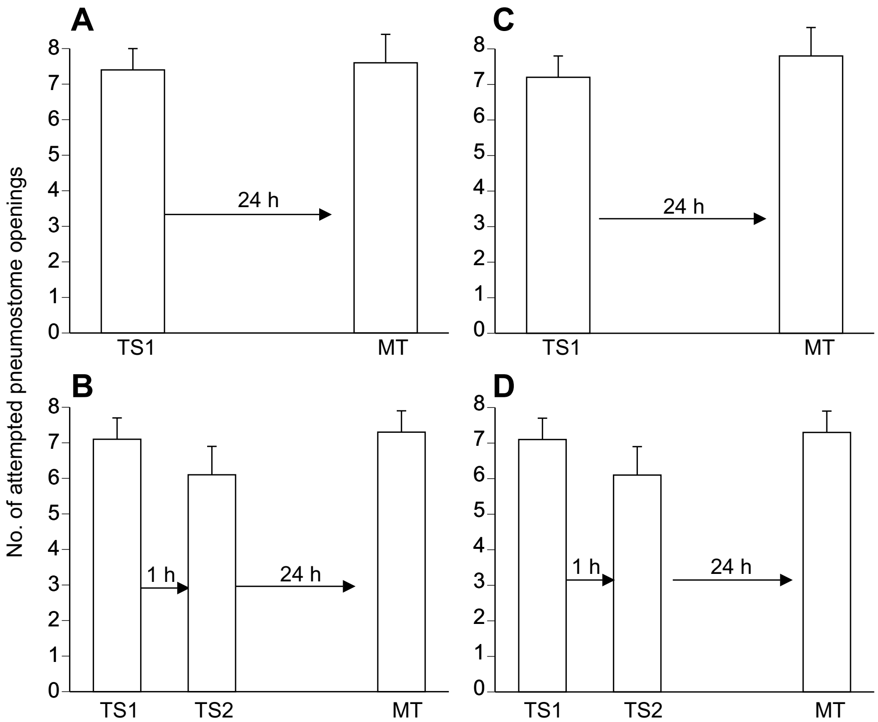

Fig. 2. TC2 and TC3 juvenile snails are not competent to form LTM. (A) Juvenile TC2 snails received a single 0.5 h training session (TS1) and memory was tested 24 h later (MT). LTM was not formed. (B) TC2 snails received two 0.5 h training sessions (TS1 and TS2) and memory was tested 24 h later. Again, LTM was not formed. (C) Juvenile TC3 snails received a single 0.5 h training session (TS1) and memory was tested 24 h later (MT). LTM was not formed. (D) In juvenile TC3 snails, two 0.5 h training sessions also did not result in LTM formation. Thus, these juvenile snails do not possess the ability to form LTM.

We next turned our attention to the WSL (N=12) and TC1 (N=18) juvenile snails (Fig. 3A,B). In both strains, the single 0.5 h training session was sufficient to cause LTM formation. That is, in both the WSL (t=3.728; P<0.01) and TC1 (t=3.735; P<0.01) cohorts, the number of attempted pneumostome openings in MT was significantly less than that in TS1. Thus, juveniles of smart snails have the ability to form LTM, even when they only receive the single 0.5 h training session procedure.

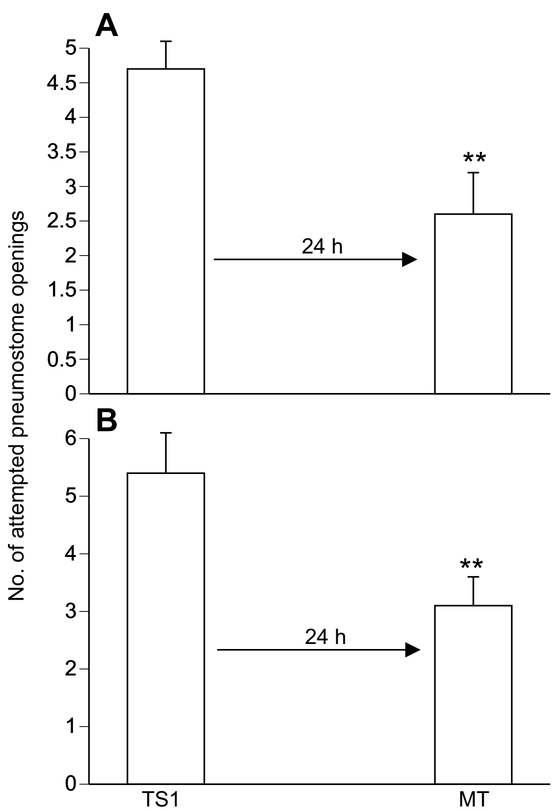

Fig. 3. Juvenile WSL and TC1 snails can form LTM and additionally exhibit the smart phenotype. (A) WSL juveniles received the single 0.5 h training procedure (TS1). When tested 24 h later (MT), these snails exhibited LTM. (B) As in A except TC1 snails were used. TC1 snails also exhibited the smart phenotype as juveniles. **P<0.01.

DISCUSSION

Juvenile Dutch *Lymnaea* were previously found to be incapable of forming LTM following operant conditioning, even if the training procedure consisted of two 45 min training sessions separated by a 1 h interval (McComb et al., 2005). Here, we showed that in juvenile offspring from freshly collected snails there are strain differences in their ability to form LTM. Thus, our initial hypothesis that all juvenile offspring from freshly collected snails would be capable of forming LTM is not consistent with our data. Strains that exhibit the average learning phenotype as adults (i.e. TC2 and TC3 snails) are incapable of forming LTM as juveniles. These two average strains are therefore comparable to the Dutch inbred strain. The data from juvenile offspring of the smart strains (i.e. TC1 and WSL) are consistent with our second hypothesis that these juveniles are also smart.

Based on our data from the F1 TC2 and freshly collected TC3 juveniles, the inability of Dutch juveniles to form LTM following operant conditioning of aerial respiration (McComb et al., 2005) is not a result of many (~60) years of being lab bred. The Dutch juveniles as well as the TC2 and TC3 juveniles exhibit an inability to withhold a behavioural response even though they have received training that should lead to a withholding of the response (i.e. perseveration). The term perseveration was first used to describe non-pathological behaviours by Muller and Pilzecker in 1900 (see Lechner et al., 1999) in their studies on memory consolidation. Later on, the term may have come to signify reverberating neuronal activity that occurred as part of a 'dual process' in memory consolidation (McGaugh, 2000). However, the term now is typically used in descriptions of more pathological behaviours occurring as a result of damage to specific circuits in the CNS

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(Sotres-Bayon et al., 2004; Son et al., 2013). But we do not believe that the three strains of juveniles tested that exhibit what was referred to as perseverance in the late 1970s and 1980s (e.g. Mattingly and Zolman, 1980) exemplify a pathological state.

Interestingly, there are strain differences in mouse models of perseverance too (Thomas et al., 2016), and it has also been shown that growing up in an enriched laboratory environment (i.e. a more natural environment) lessens perseveration (Campbell et al., 2013). We did not observe a similar finding in regards to an enriched environment. Neither the TC3 juveniles, which hatched and developed in a natural environment, nor the F1 TC2 juveniles, reared from eggs hatched from freshly collected adults, exhibited LTM. We did, however, observe strain differences in the lack of perseveration in that F1 juvenile TC1 and WSL strains did not exhibit it.

Both McComb et al. (2005) and Sunada et al. (2010b) thought that juvenile *Lymnaea* perseverated because the memory for the task was occluded as a result of a lack of specific inhibitory circuits. This appears to be a recurring theme in the literature across species. Such behaviours included habituation (Peretz and Lukowiak, 1975; Lukowiak, 1980), suppression of attack responses (Dickel et al., 1997), passive avoidance (Blozovski and Cudennec, 1980; Mattingly and Zolman, 1980), spatial discrimination (Bronstein and Spear, 1972) and the classical conditioning of *Lymnaea*'s whole-body withdrawal response (Ono et al., 2002). However, this does not mean that the nervous system in each of the three juvenile strains (TC2, TC3 and Dutch) is incapable of mediating LTM formation. Most germane to this discussion is that juvenile Dutch *Lymnaea* are capable of forming LTM following classical conditioning of an appetitive behaviour (Yamanaka et al., 2000). As both TC1 and WSL juveniles form LTM, perseverance is not the 'default' state in *Lymnaea* juvenile snails and should not be considered as some sort of behavioural pathology.

Both perseveration and stereotypic behaviour in mammals are thought to be the result of the 'lack of inhibitory pathways' in the CNS (Aron, 2007). In *Lymnaea*, McComb et al. (2003) showed that there was significantly less suppressive input in the CNS of juveniles than there was in adults. Specifically, they studied RPeD1; this neuron has been shown to be a necessary site of LTM formation following operant conditioning of aerial respiration (Scheibenstock et al., 2002). Additionally, Braun et al. (2012) showed significant differences in properties of RPeD1 between smart versus average *Lymnaea*. RPeD1 in smart snails is significantly less excitable than in average snails. It appears that RPeD1 in smart snails is 'primed' to rapidly form memory compared with RPeD1 in the TC2 snails. These data are consistent with the idea that there may be a lack of 'inhibition' in nervous systems where perseveration (i.e. no LTM) is observed. Future experiments, when juvenile smart snails are available, will determine whether the activity of RPeD1 in smart-strain juveniles is different from that in average-strain juveniles. Additionally, these experiments will determine whether RPeD1 activity in smart strains is more adult like.

The lack of inhibition found in juvenile Dutch snails (McComb et al., 2003) is due to a deficit in suppressive inhibitory input from peripheral structures (e.g. osphradial ganglion; Inoue et al., 2001) to the CNS. As the Dutch strain matured, the suppressive input to the respiratory central pattern generator (CPG) increased and this increase in suppressive input allowed snails to form LTM. It is unclear how this occurs. It is known that exposure to CE in juvenile Dutch snails counteracts the increased excitability of RPeD1 and forces the network into a more 'adult-like' configuration, thereby allowing operant conditioning to result in LTM formation (Sunada et al., 2010b; Forest et al., 2016). CE's effects in snails are blocked by DNA methylation blockers (Lukowiak et al., 2014) and the long-lasting effects (at least 4 weeks) of training juvenile snails in CE are also dependent on DNA methylation (Forest et al., 2016). Our working hypothesis is that the peripheral inhibitory influence over RPeD1 develops sooner in the juvenile smart snails and is comparable to that seen in adult smart snails.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization, T.S. and K.L.; Methodology, T.S., A.P., E.H., C.S. and E.S.; Investigation, T.S., A.P., E.H., C.S., E.S., K.S.L. and I.P.; Writing - original draft, T.S. and K.L.; Writing-review and editing, T.S., E.H. and K.L.; Funding acquisition K.L.; Supervision, K.L.

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