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RESEARCH ARTICLE

The temperature sensitivity of memory formation and persistence is altered by cold acclimation in a pond snail

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ABSTRACT

There are reports on the inability of inbred, laboratory-reared *Lymnaea stagnalis* to perform feeding and aerial respiration in the cold. It has also been suggested that laboratory-bred snails have an inability to perform aerial respiration in winter months in the laboratory. Here, we used an inbred, laboratory-reared strain of *Lymnaea* (the S-strain) to demonstrate that the snails are capable of performing those behaviours in a cold (4°C) environment after a 2 day acclimation period. In addition, the inbred snails were able to perform aerial respiration during winter months at room temperature (20°C) in the laboratory. The persistence of long-term memory (LTM) was extended for at least 4 weeks by placing S-strain snails into a 4°C environment following training. Typically, the cold block (CB) procedure (1 h at 4°C) immediately after a training session blocks LTM formation in the S-strain but not in a freshly collected strain. Four weeks at 4°C transformed the S-strain phenotype into one resisting the CB procedure. Thus, with a 4 week cold spell snails gain a resistance to the CB procedure, and that would explain why freshly collected snails are resistant to the procedure. However, we found that F1 progeny of a freshly collected strain reared in the laboratory were resistant to the CB procedure. This suggests that an unknown selection resulted in the S-strain being susceptible to the CB procedure.

KEY WORDS: *Lymnaea*, Strain transformation, Cold block, Memory formation, LTM persistence

INTRODUCTION

Lymnaea inhabit northern Eurasia and North America. There snails encounter substantial changes in both temperature and photoperiod. Here, we focus on temperature changes, specifically temperatures of 4°C, in a laboratory-reared inbred strain (S-strain) and three freshly collected strains (TC1, TC2 and Margo Lake) of *Lymnaea*. Laboratory-reared F1 offspring of the Margo Lake snails were also used. We explored the consequences of a 4°C environment (i.e. a cold spell) on homeostatic aerial respiration, the persistence of long-term memory (LTM) and the ability of the cold block (CB) procedure to prevent consolidation or reconsolidation from occurring. Finally, we determined whether a difference between the inbred S-strain and freshly collected strains regarding behaviours following the CB procedure is due to the snails experiencing a cold spell.

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At our collection sites in Alberta and Saskatchewan (Hughes et al., 2017; Dodd et al., 2018; Kagan and Lukowiak, 2019), water temperatures in ponds range from ∼4°C after the ice comes off the ponds in April/May to close to 30°C on some days in late August (temperature data loggers at White Sand Lake pond in Saskatchewan). During August, the temperatures in the shallow ponds around Calgary vary from 8°C at night to close to 29°C over the course of a single day. In winter, these ponds have ice and snow on them from October to late April/May. These ponds do not freeze to the bottom, and the water temperature during this period is ∼4°C. As *Lymnaea* is a poikilotherm, temperature alters both its physiology and behaviour. Vaughn (1953) states that *Lymnaea* growth ceases at a temperature of 10°C. Boag and Perrlstone (1979) made a similar observation in freshly collected snails in Southern Alberta ponds.

By contrast, our inbred laboratory-reared snails (the S-strain) rarely, if ever, experience a fluctuation in temperature greater than 1°C over the year and are maintained at ∼20°C. We have subjected inbred, laboratory-reared S-strain to different thermal stressors: (1) a brief step increase in temperature (1 h at 30°C) from 20°C, termed the heat shock stressor, which enhanced LTM formation; and (2) a brief step decrease from 20°C to 4°C for 1 h, termed the CB procedure, which obstructs either the memory consolidation or the reconsolidation processes (Sangha et al., 2003a,b,c,d; Martens et al., 2007; Teskey et al., 2012; Takahashi et al., 2013). We found in freshly collected *Lymnaea* that the heat shock stressor did not alter LTM formation (Hughes et al., 2016). Recently, in preliminary experiments on freshly collected Alberta and Saskatchewan *Lymnaea* strains, applying the CB procedure did not block LTM formation. It therefore seemed appropriate to more fully determine whether outbred, freshly collected Canadian *Lymnaea* respond in the same manner as the inbred, laboratory-reared snails. In addition, we wished to determine whether the S-strain snails can be transformed by experiencing the cold environment (i.e. a cold spell) to exhibit the behavioural phenotype possessed by the freshly collected snails.

Here, we test four hypotheses. (1) Laboratory-reared inbred *Lymnaea* (the S-strain) subjected to a cold environment (4°C) are capable of performing important homeostatic behaviours (i.e. feeding and aerial respiration) at that temperature after a short period of acclimation. That is, it is possible to induce physiological plasticity in these snails with this cold spell. (2) The S-strain can be transformed by a 4 week cold spell (4°C) to resemble the behavioural phenotype exhibited by freshly collected Canadian *Lymnaea*. Because of this S-strain transformation, the CB procedure no longer obstructs LTM formation. (3) LTM persistence in the S-strain can be extended for at least 4 weeks by placing the snails into the 4°C cold environment following operant conditioning of aerial respiratory behaviour. (4) Experiencing a cold spell is a requirement to obtain the CB-resistant phenotype.

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MATERIALS AND METHODS

Snails

Laboratory, inbred *Lymnaea stagnalis* (Linnaeus 1758), the S-strain, were bred and raised in the snail facility at the Cumming School of Medicine, Hotchkiss Brain Institute at the University of Calgary. They were derived from a founder colony set up originally in Leiden in the 1950s. Our colony was established in the 1980s using snails obtained from the Dutch founder colony. We also used freshly collected snails from Alberta (the TC1 and TC2 strains; Dodd et al., 2018) and Saskatchewan (the Margo Lake strain). The freshly collected strains used (2.5–3.0 cm shell length) were maintained at room temperature (~20°C) for at least 2 weeks after being collected in the spring and summer, and had continuous access to lettuce in their home eumoxic aquaria. Finally, we used the F1 offspring from the Margo Lake strain. The eggs were laid in August 2020 in our laboratory and developed and matured under laboratory conditions.

Homeostatic aerial respiration

Lymnaea stagnalis are bimodal breathers; they obtain oxygen necessary for life via cutaneous and aerial respiration. In eumoxic conditions with normal air pressure (1100 m elevation) and temperature (20°C), this results in the O_2 content in a 1 l beaker filled with 500 ml pond water (PW) to be measured as 6 ml O_2 l^{-1}. At this concentration of O_2, snails satisfy their oxygen needs primarily through cutaneous respiration (Lukowiak et al., 1996). To create a hypoxic environment (measured as <0.1 ml O_2 l^{-1}), we bubbled 100% N_2 gas into a 1000 ml beaker filled with 500 ml PW. In this hypoxic environment, snails preferentially perform aerial respiration.

To perform aerial respiration, the snail comes to the water surface and opens its pneumostome, the respiratory orifice that allows gas exchange with the atmosphere. In a 0.5 h observation session in hypoxic PW, we measured the duration of each breath and the number of breaths, and thus the total breathing time (TBT) of each snail in the session is calculated in hypoxia. We performed this at room temperature (20°C) and at 4°C at various times after snails were placed into the cold environment (Figs 1 and 2). In a second experiment (Fig. 2C), we measured TBT before and after 4 weeks in 4°C after a gradual rewarming (3 h) in the hypoxic environment at 20°C.

Operant conditioning training

Operant conditioning of aerial respiration (Figs 3–8) was conducted under hypoxic conditions as previously described (Lukowiak et al., 1996, 2000). Prior to each training session (TS) and memory test session (MT), 100% N_2 gas was vigorously bubbled into 500 ml PW in order to create the hypoxic environment. This bubbling was maintained at a lower rate during all TSs and MTs. A 10 min acclimation period was given before each TS and MT to allow the animals to acclimate to this new hypoxic environment. Immediately prior to each TS and MT, all animals were gently pushed underwater in the test beaker.

Two different training protocols were employed to operantly condition aerial respiratory behaviour. The first consisted of two 0.5 h TSs (TS1 and TS2) spaced 1 h apart with a MT administered 24 h after TS2. This procedure is necessary to produce LTM lasting 24 h in *Lymnaea* strains possessing the average cognitive memory-forming phenotype (i.e. the S-strain and TC2 strain; Braun et al., 2012). 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 cognitive strains but not in average cognitive strains (Orr et al., 2008, 2009a,b; Dalesman et al., 2011).

The TSs and MTs consisted of delivering a tactile stimulus to the pneumostome area as the snail attempted to open it while at the PW surface. Following the training session, snails were returned to their eumoxic home aquarium. Operant conditioning was not performed on snails in the cold environment.

Operational definition of memory

Memory formation was operationally defined as in previous studies (Lukowiak et al., 1996, 2000). When two training sessions were administered to average cognitive phenotype snails, LTM was present when (1) the number of attempted pneumostome openings performed during the MT was significantly lower than the number observed during TS1; and (2) the number of attempted pneumostome openings during the MT was not significantly greater than that seen in TS2. When only a single TS was administered to smart cognitive phenotype snails, 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 declared.

Fig. 1. *Lymnaea stagnalis* in the cold. Cold-adapted S-strain *Lymnaea* shown eating and defecating (left), and performing aerial respiration at 4°C (right). The same snail is shown in both pictures. The beaker was filled with 4°C pond water (PW) and was placed into a tray filled with melting ice chips and water to maintain the temperature at 4°C. In the picture on the left, the top arrow points to the mouth of the snail eating lettuce. The bottom arrow points to the anal area of the snail showing the elimination of food waste (i.e. faeces). In the picture on the right, the same snail is at the water surface in hypoxic, cold PW and its pneumostome is open (arrow) in order to perform aerial respiration. In both pictures, we have labelled the ice in the melting ice-water bath.

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The prolonged cold environment procedure

Snails were kept in aquaria in a cold room maintained at 4°C for 1–4 weeks (Figs 2, 4 and 8). We refer to this as a cold spell. During this time, fresh 4°C PW was exchanged every 7 days. Fresh lettuce was added at the same time, with the previous partially eaten lettuce removed. For taking photographs of snails (Fig. 1) in the cold, a 1.5 l beaker filled with 1000 ml PW was pre-chilled and maintained at 4°C.

Following the cold spell, snails were rewarmed to room temperature (20°C) over the course of 3 h in eumoxic PW. In the experiments shown in Fig. 4, 1 h following TS2, snails were placed into pre-chilled aquaria (4°C) in the cold room to experience the cold spell for the times specified (2 or 4 weeks).

The CB procedure

A 1 l beaker filled with 0.5 l eumoxic water was pre-chilled and maintained at 4°C to serve as the cooling apparatus for the CB procedure (Figs 5–8). A step change from 20°C to 4°C, and after 1 h at 4°C a step change back to 20°C, after operant conditioning training or reactivation of memory obstructs the consolidation or reconsolidation process (Sangha et al., 2003b,c,d).

Data analyses

For the breathing observation experiments (Fig. 2A,C) a paired *t*-test was performed for the room temperature experiment. For the breathing observation experiments in Fig. 2B, a Friedman test was used, because on days 1 and 2 there were 0 attempted breaths and thus the data are not normally distributed. An ANOVA followed by Tukey's *post hoc* test was used for the data in Figs 3 and 5–8. In Fig. 4, we used a mixed-effect analysis. In the ANOVAs and mixed-effects model (REML) analyses, a Tukey's *post hoc* test followed to determine which sessions were significantly different from each other. The size effect statistic is also reported in each analysis. Statistics were performed using GraphPad Prism (v.9 for Mac OS 11.2.1, GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

In Fig. 1, 10 inbred laboratory-reared strain (S-strain) *Lymnaea* were placed into the cold (4°C) environment (i.e. a cold spell) for 4 weeks. We photographed (Fig. 1) a randomly picked snail in a beaker of hypoxic PW maintained at 4°C after experiencing a 1 week cold spell. This snail is eating lettuce and performing aerial respiration. The top arrow in the picture on the left shows the mouth

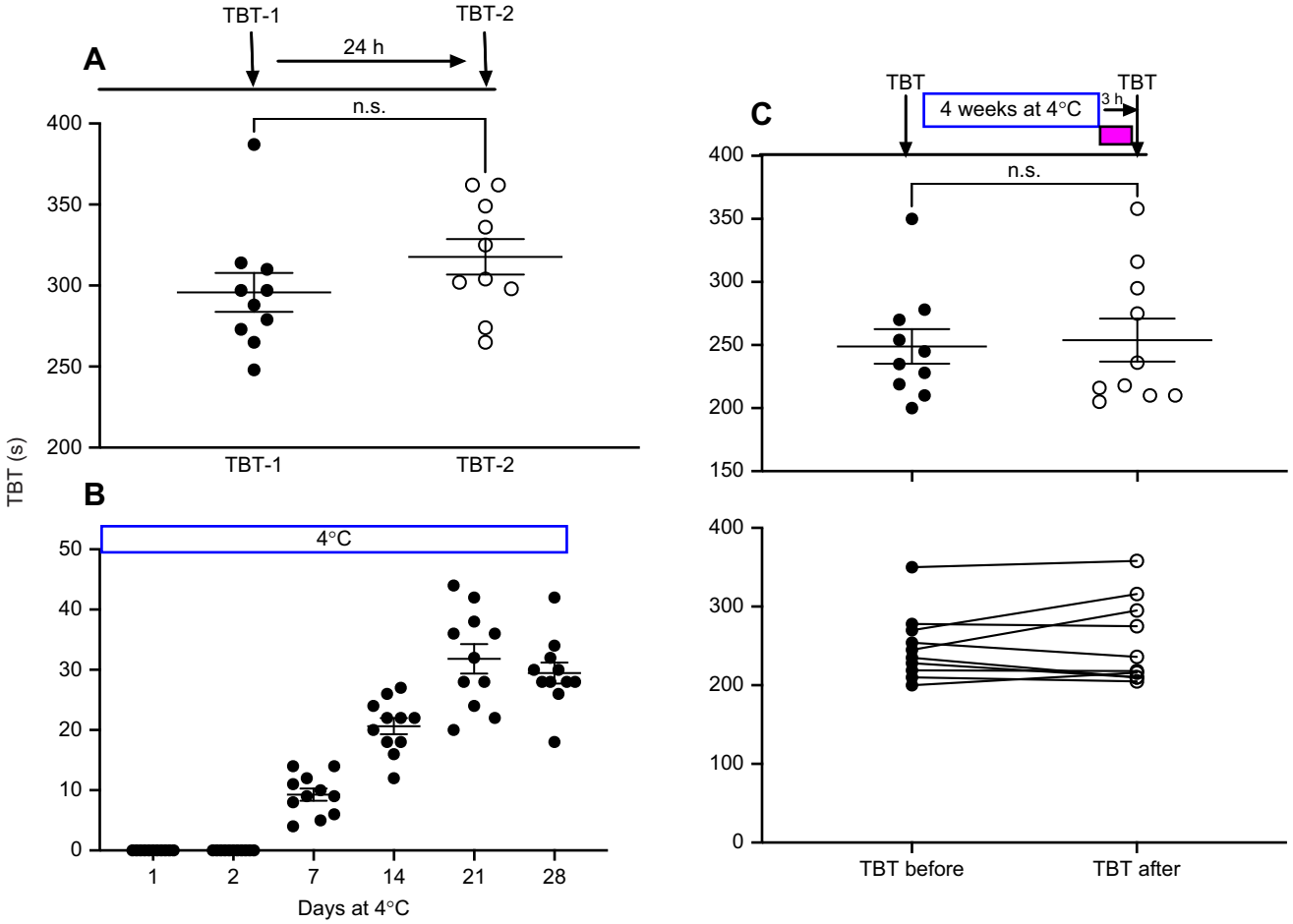

Fig. 2. Total breathing time (TBT) in S-strain *L. stagnalis* at room temperature, in a cold (4°C) environment and following a 4 week cold spell. The timelines of the experiments are shown above each data set. (A) The TBT in a naive cohort of S-strain snails (*N*=9) in two observation sessions 24 h apart at room temperature (20°C) in hypoxic PW. (B) The TBT in S-strain snails (*N*=11) placed into the cold (4°C) environment measured at the indicated times on the *x*-axis. The TBTs were determined in hypoxic cold (4°C) PW. There were significant differences in TBT between day 7 and days 1 and 2. There was also a significant difference in TBT between day 7 and day 14. In addition, there were significant differences in TBT between day 14 and days 21 and 28. (C) TBT before and after experiencing the 4 week cold spell. Following the 4 week cold spell, snails were allowed to gradually warm to room temperature (3 h) before measuring TBT. There was no significant difference (n.s.) in TBT between the before and after observation sessions. These data are replotted below, showing the TBT of each individual snail before and after they experienced the cold spell.

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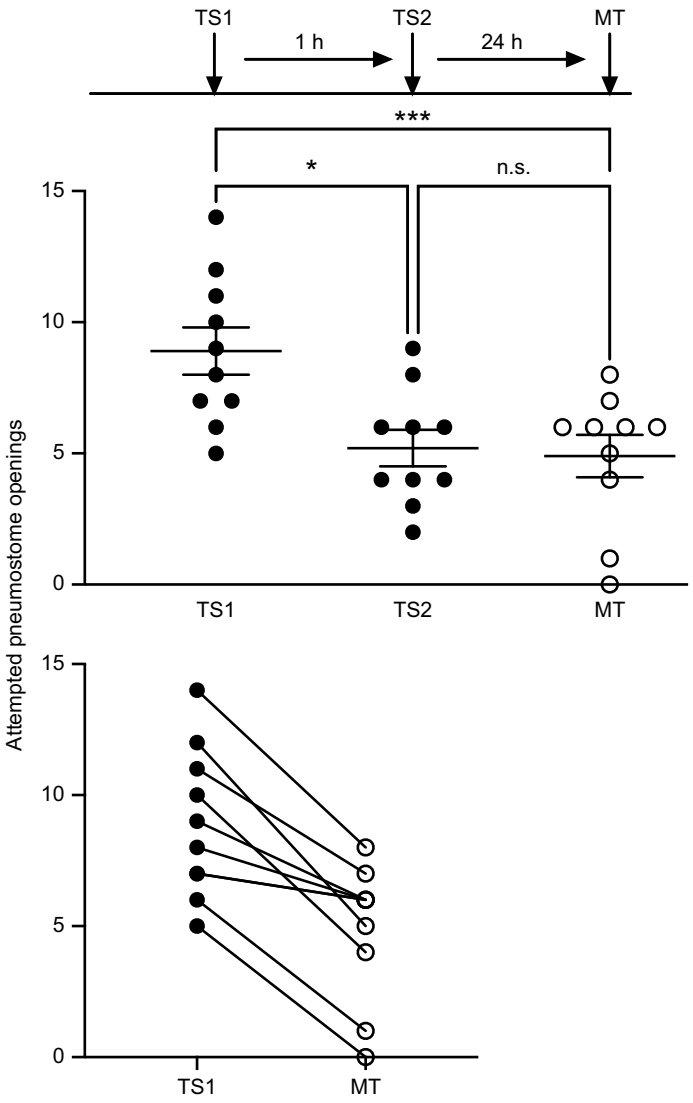

Fig. 3. Learning and long-term memory (LTM) formation in S-strain *L. stagnalis*. The timeline of the experiment is shown above the data. A naive cohort (N=10) of S-strain snails was subjected to the operant conditioning training procedure. They received two 0.5 h training sessions (TS1 and TS2, filled circles) 1 h apart. They were then tested for LTM formation 24 h later (MT, open circles). There was a significant decrease in the number of attempted pneumostome openings in TS2 compared with TS1, indicating that associative learning had occurred. The number of attempted pneumostome openings in MT was significantly less than that in TS1 and was not significantly greater than the number in TS2 (P=0.9519). The data from the TS1 and MT sessions are replotted below, showing the response of each individual snail. *P=0.0156; ***P=0.0006; n.s., not significant (Tukey's *post hoc* test).

open; the bottom arrow shows faeces being extruded from the snail's anus. In the picture on the right, the arrow shows the pneumostome open as the snail performs aerial respiration at the surface of the hypoxic PW beaker. Thus, these laboratory-reared, inbred snails after a 1 week cold spell eat, defecate and perform aerial respiration in 4°C hypoxic PW.

We next examined homeostatic aerial respiratory behaviour in hypoxia in S-strain snails. We measured (Fig. 2A) TBT at room temperature (20°C) in hypoxic PW (N=10) in two sessions (TBT-1 and TBT-2) 24 h apart. There was no significant difference in the TBT between the two sessions (paired t-test; t=1.992; d.f.=9; P=0.0776; r=0.5440).

We then measured TBT in another cohort of S-strain snails (N=11; Fig. 2B) over the course of their 4 week period in 4°C PW. Each snail was subjected to a 0.5 h breathing observation session at the same time in hypoxic 4°C PW on the days indicated. As the data were not normally distributed, the Friedman test followed by Dunn's multiple comparisons *post hoc* test showed that there was a significant effect of days at 4°C on TBT (Friedman test, P<0.0001; Friedman statistic=52.13). We then statistically compared the TBTs between sessions (Dunn's multiple comparisons test). To summarize: (1) there was no significant difference in aerial respiratory behaviour between day 1 and day 2; (2) the TBT in each subsequent 0.5 h observation session (days 7–28) was statistically greater than that in either of the first two sessions; and (3) with the exception of the TBT in the day 21 session and the day 28 session (which were not significantly different), as time in the cold increased, the TBT statistically increased (e.g. the TBT in the 14 day session was greater than that in the 7 day session, etc.).

Finally, we measured TBT in a new cohort of S-strain snails (Fig. 2C; N=10) at room temperature (20°C) before and after spending 4 weeks at 4°C. We found that, after spending 4 weeks at 4°C, the TBT was not different from that measured at room temperature (20°C) (paired t-test; t=0.0.6127, d.f.=9; P=0.5553; r=0.8810).

Thus, normal homeostatic aerial respiration driven by a hypoxic environment occurs in cold-adapted snails at 4°C in the inbred laboratory-reared S-strain, albeit at a much lower rate than at room temperature. These data are consistent with the hypothesis that induced physiological plasticity (e.g. a change in metabolism) occurs in this inbred, laboratory-reared strain of *Lymnaea*. The data also show that there are no long-lasting changes in homeostatic aerial respiratory activity in snails after they 'recover' from a cold spell.

The S-strain exhibits the average cognitive memory-forming phenotype (see Materials and Methods). These snails (Fig. 3; N=10)

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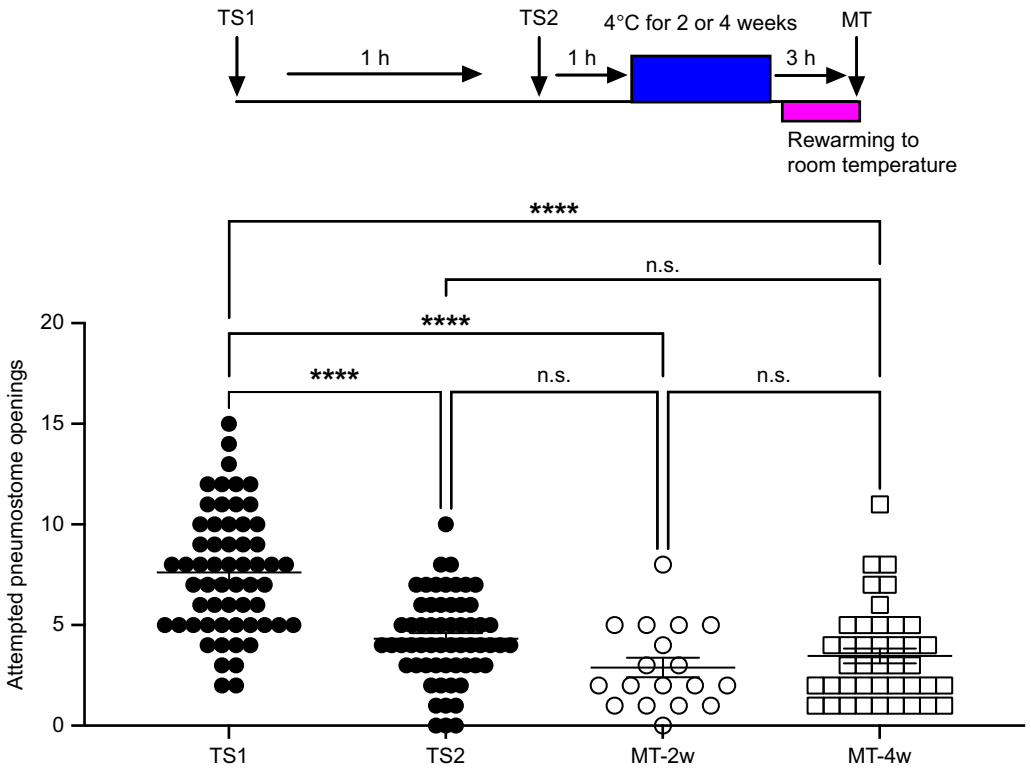

Fig. 4. The cold environment extends LTM persistence in S-strain *L. stagnalis*. The timeline of the experiment is shown above the data. The important statistical comparisons are indicated. All of the S-strain snails (N=59) received two 0.5 h training sessions (TS1 and TS2) at room temperature. One hour following TS2, snails were placed into the cold environment (4°C). Two weeks later, a cohort of snails (N=18; filled circles) was tested for memory (MT-2w); the remaining snails (N=41, open circles) were tested for memory 4 weeks after TS2 (MT-4w). In both cases, the memory test (MT-2w or MT-4w) occurred after a 3 h gradual rewarming to room temperature. LTM formed and was present both 2 and 4 weeks after TS2. Thus, placing snails into the cold environment extends LTM persistence for at least 4 weeks. ****P<0.0001; n.s., not significant (Tukey's *post hoc* test).

exhibit learning and LTM formation. An ANOVA ($F_{1.655,14.89}$=11.64; P=0.0014; R^2=0.3592) shows that there is a significant effect of training and testing for memory. A Tukey's *post hoc* test indicated that the number of attempted pneumostome openings in MT was significantly less than that in TS1, and that MT was not significantly greater than TS2. Thus, the criteria for associative learning and memory formation are met.

We next asked (Fig. 4) whether placing S-strain snails in 4°C PW (N=59) for 2 (N=18) or 4 (N=41) weeks extends the persistence of LTM. Snails were placed into the 4°C environment 1 h after TS2, and were then tested for memory either 2 weeks (MT-2w, open circles) or 4 weeks (MT-4w, open squares) later. Snails were tested for LTM 3 h after being moved from the 4°C environment to the 20°C room temperature. REML analysis ($F_{2.570,98.51}$=34.65; P<0.0001; Geisser–Greenhouse's epsilon=0.8566) indicated that there is a significant effect of training and testing for memory. Importantly, when we compared the various responses (Tukey's multiple comparisons *post hoc* test), a number of results were found. (1) Learning occurred, as the number of attempted pneumostome openings in TS2 was significantly less than that in TS1. (2) LTM was present after 2 weeks in the cold. That is, there were significantly fewer attempted pneumostome openings in MT-2w than in TS1, and MT-2w was not significantly greater than TS2. (3) LTM was present after 4 weeks in the cold. That is, there were significantly fewer attempted pneumostome openings in MT-4w than in TS1, and MT-4w was not significantly greater than TS2. (4) There was no significant difference between the number of attempted pneumostome openings in MT-2w versus MT-4w. Together, these data indicate that placing snails into the cold 4°C environment 1 h after TS2 extends LTM persistence for at least 4 weeks.

A step change from room temperature PW to 4°C PW for 1 h immediately following training at room temperature, the CB procedure, obstructs the memory consolidation process (Sangha et al., 2003a). Here (Fig. 5), we tested whether the CB procedure is effective at obstructing the memory consolidation process in the laboratory-reared S-strain (N=22) and freshly collected TC2 strain (N=10). The TC2 snails were collected in July and were maintained in the laboratory at 20°C for 2 weeks before being used. Both the S-strain and the TC2 strain require two 0.5 h training sessions to cause the formation of LTM. In both strains, the CB procedure (red arrows) occurred immediately after TS2. An ordinary one-way ANOVA ($F_{5,9}$=10.04; P≤0.0001; R^2=0.3603) indicated that there is a significant effect of training and testing for memory. Importantly, when we compared the various responses with a Tukey's multiple comparisons *post hoc* test, a number of results were found. (1) In the S-strain (circles), associative learning occurred (i.e. TS2-s was significantly less than TS1-s) but LTM did not form, as MT-s (open circles) was not significantly less than TS1-s. Thus, the CB procedure obstructed the memory consolidation process. (2) In the TC2-strain (squares), associative learning occurred (i.e. TS2 TC2 was significantly less than TS1 TC2), and because MT TC2 was significantly less than TS1 TC2 and was not greater than TS2 TC2, LTM formed. Thus, the CB procedure in TC2 strain snails did not impede the memory consolidation process. The individual responses of the S-strain and TC2 strain in TS1 and the MTs have been plotted with connecting lines at the bottom of Fig. 5.

We then wished to determine whether the CB procedure was also ineffective in the freshly collected TC1 strain (Fig. 6). The TC1 strain snails exhibit the smart cognitive phenotype and only require a single 0.5 h TS to cause LTM formation (Braun et al., 2012). Thus, in these snails (N=9), following the single 0.5 h TS, we first tested memory formation 24 h later (MT-1) to confirm that the TC1 snails continue to exhibit the smart cognitive phenotype, and then applied the CB procedure immediately after MT-1 to determine whether the reconsolidation process was blocked (i.e. MT-2). We then tested this 24 h after the CB (i.e. MT-2). An ANOVA ($F_{1.616,12.92}$=13.47; P=0.0011; R^2=0.6274) indicated that associative learning had

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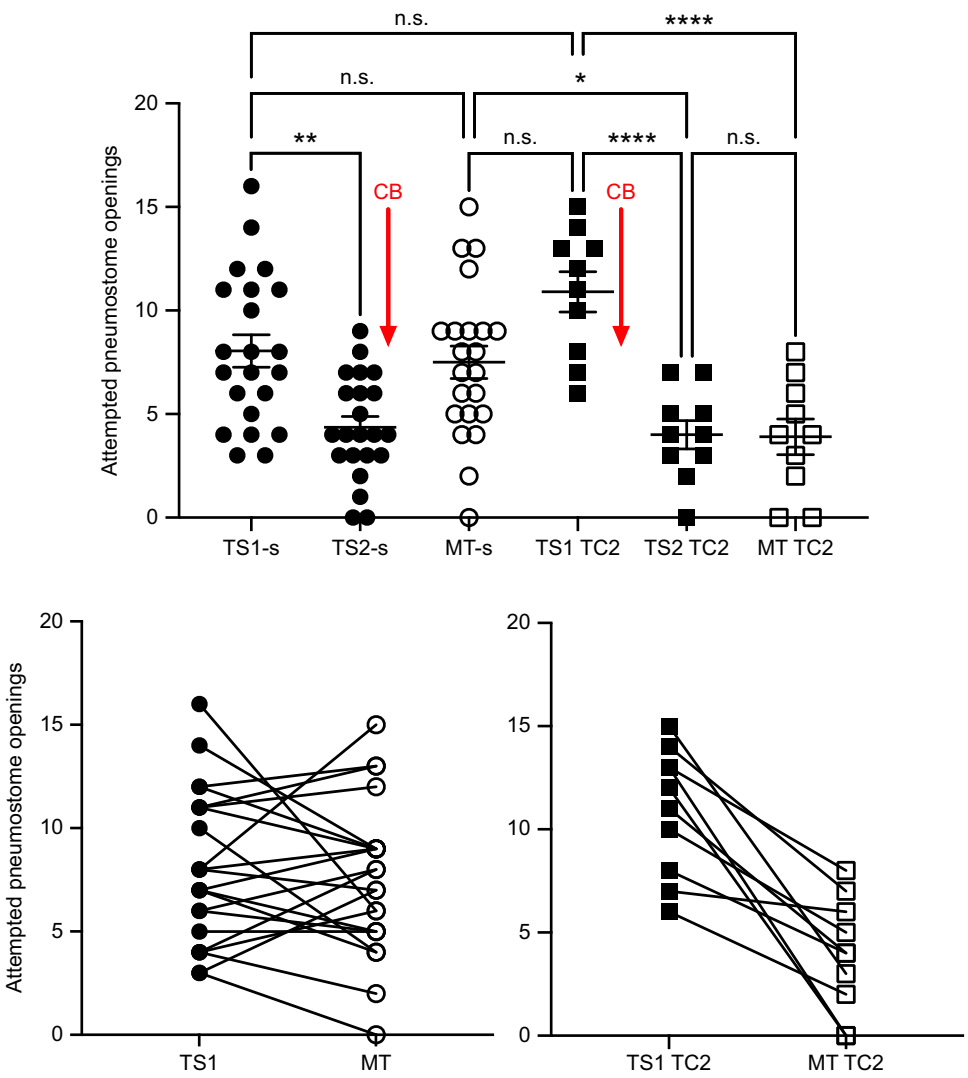

Fig. 5. The cold block (CB) procedure obstructs LTM formation in S-strain but not TC2 strain *L. stagnalis*. A naive cohort of S-strain snails (*N*=11, circles) received operant conditioning training (two 0.5 h training sessions; TS1-s and TS2-s). Immediately after TS2, snails received the CB procedure (red arrows). Memory was then tested 24 h later (MT-s, open circles). A similar experiment was performed on TC2 (filled squares for TS1 TC2 and TS2 TC2) snails collected in June/July (*N*=10). In this freshly collected strain, the CB procedure was ineffective, as memory was present (MT TC2, open squares). In the S-strain, LTM did not form (i.e. MT-s was not significantly different from TS1-s). In the TC2 snails, LTM formed; that is, MT TC2 was significantly less than TS1. These data are replotted below, showing the response of each individual snail in TS1 and MT. **P*=0.0132; ***P*=0.0025; *****P*<0.0001; n.s., not significant (Tukey's *post hoc* test).

occurred and LTM had formed. A Tukey's *post hoc* test indicated that the number of attempted pneumostome openings in MT-1 was significantly less than that in TS, and that MT-2 was also significantly less than TS. Finally, there was no significant difference between MT-1 and MT-2. These data show that, in the TC1 snails, applying the CB procedure immediately after MT-1 did not impede the reconsolidation process.

We next determined (Fig. 7) whether the F1 offspring of freshly collected snails conceived, born, hatched and reared under our laboratory conditions would also exhibit the ineffectiveness of the CB procedure seen in the freshly collected snails. These F1 snails had not experienced a cold spell. We first present data from Margo Lake snails freshly collected in the summer of 2020, which had thus experienced a cold spell (Fig. 7A). An ANOVA ($F_{2,60}$=7.830; P=0.0010; R^2=0.2070) indicated that associative learning had occurred and LTM had formed. A Tukey's *post hoc* test indicated that the number of attempted pneumostome openings in MT-1 was significantly less than that in TS, and that MT-2 was also significantly less than TS. Finally, there was no significant difference between MT-1 and MT-2. Thus, in the Margo Lake smart cognitive phenotype snails, the CB procedure was not effective at blocking the reconsolidation process. The F1 Margo Lake snails (Fig. 7B) received the same training and testing procedure as the freshly collected Margo Lake snails. An ANOVA ($F_{1.977,17.79}$=28.56; P≤0.0001; R^2=0.3130) indicated that associative learning had occurred and LTM had formed. A Tukey's *post hoc* test indicated that the number of attempted pneumostome openings in MT-1 was significantly less than that in TS, and that MT-2 was also significantly less than TS. Finally, there was no significant difference between MT-1 and MT-2. Thus, these F1 Margo Lake snails exhibit the smart cognitive phenotype, and the CB procedure did not block the reconsolidation process. These data show that it was not necessary for snails to experience a cold spell in their life to be resistant to the CB procedure.

Finally, we asked whether we could transform the laboratory-reared S-strain snails to express the CB procedure-resistant phenotype exhibited by the TC1, TC2, Margo Lake and F1 Margo Lake strains. We subjected the S-strain snails to a 1 or 4 week cold spell (Fig. 8; *N*=24) before operant conditioning. In both cohorts, immediately after TS2, the CB procedure (red arrows) was applied, and the snails were tested for LTM 24 h later (1w-MT or 4w-MT). An ordinary one-way ANOVA ($F_{5,66}$=5.22; P≤0.0001; R^2=0.6564) indicated that there is a significant effect of training and testing for memory. When we compared the various responses with a Tukey's multiple comparisons *post hoc* test, the following were found: (1) in snails subjected to a 1 week cold spell, associative learning occurred, as 1w-TS2 was significantly less than 1w-TS1;

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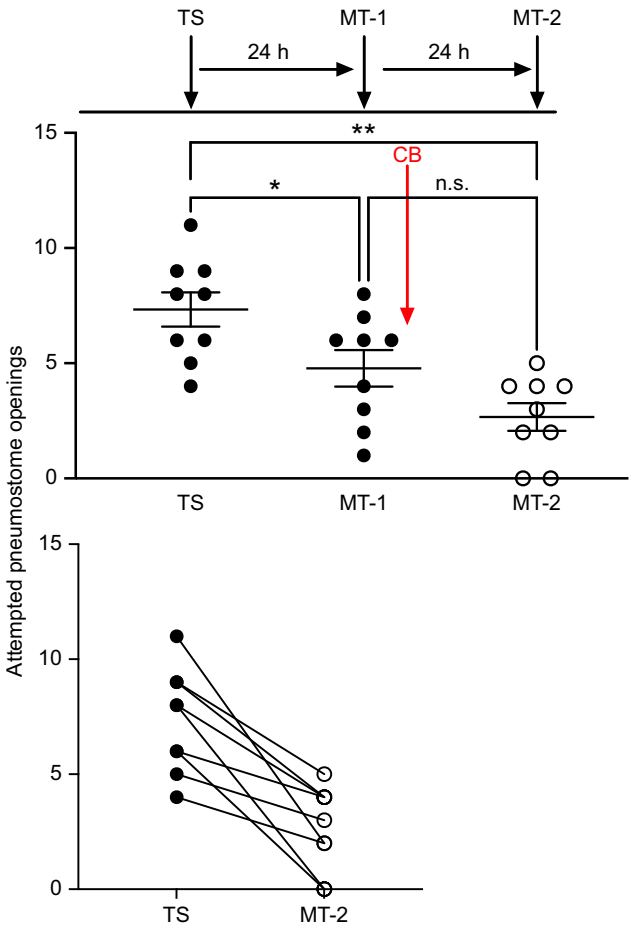

Fig. 6. The CB procedure does not obstruct LTM reconsolidation in freshly collected TC1 smart cognitive phenotype *L. stagnalis*. The timeline of the experiment is shown above the data points. The important statistical comparisons are indicated. A cohort of naive TC1 snails (*N*=9) received a single 0.5 h TS. Memory was tested 24 h later (MT-1). Immediately after MT-1 snails received the CB procedure (red arrow). Memory was again tested 24 h later (MT-2). The number of attempted pneumostome openings in MT-2 was also significantly less than that in TS, indicating that the CB procedure did not interfere with the memory reconsolidation process. Data are replotted below, showing the response of each individual snail in TS and MT-2. **P*=0.0170; ***P*=0.0017; n.s., not significant (Tukey's *post hoc* test).

and (2) LTM was not present, as the number of attempted pneumostome openings in 1w-MT was not significantly less than that in 1-w TS1 and was significantly greater than that in 1w-TS2. Thus, the CB procedure continued to be effective in the cohort of S-strain snails experiencing a 1 week cold spell.

However, in the snails subjected to a 4 week cold spell, the CB procedure did not block LTM formation. That is, associative learning had occurred, as the number of attempted pneumostome openings in 4w-TS2 was significantly less than that in 4w-TS1; and LTM was present as there were significantly fewer attempted pneumostome openings in 4w-MT than in 4w-TS1, and MT-4w was significantly greater than 4w-TS2 (*P*=0.0398). Thus, the CB procedure was ineffective at obstructing the memory consolidation process in the cohort of S-strain snails experiencing a 4 week cold spell.

DISCUSSION

We tested four hypotheses relating to how an inbred, laboratory-reared strain (the S-strain) of *Lymnaea* responds to a cold environment. Our data are consistent with our first three hypotheses; however, importantly, our data negate the fourth hypothesis. That is, to be resistant to the CB procedure snails must experience a cold spell.

We showed here a number of findings. (1) The S-strain snails maintained at 4°C for more than 2 days eat and perform homeostatic aerial respiration in a hypoxic, 4°C environment. With spending longer time in the cold, aerial respiration (TBT) increased. (2) In S-strain snails, LTM persisted for 24 h, but the persistence of LTM was extended to 4 weeks by maintaining snails at 4°C following operant conditioning. (3) The CB procedure obstructs the memory consolidation in S-strain snails. (4) In freshly collected, outbred snails, the CB procedure neither blocks consolidation nor reconsolidation. (5) In S-strain snails experiencing a 1 week cold spell, the CB procedure continues to block LTM. (6) In S-strain snails experiencing a 4 week cold spell, the CB procedure no longer blocks LTM. Thus, a 4 week cold spell transforms these snails to possess a phenotype similar to that of freshly collected snails. Together, these findings are consistent with three of our hypotheses. However, our finding that the CB procedure was ineffective at obstructing the reconsolidation process in the F1 Margo Lake snails hatched and reared under laboratory conditions is incompatible with our fourth hypothesis. We believe that, at some point after the founder colony was established, genetic drift or an inadvertent selection process occurred, resulting in a CB-susceptible phenotype in the S-strain.

Our observations on S-strain snails are at odds with the Winlow and Polese (2014) report that *Lymnaea* neither feed nor perform aerial respiration in a cold environment. We do not doubt the observations of that report; nor do we doubt the data reported by Copping et al. (2000). Those data sets tell us that there are strain-specific differences in homeostatic behaviours, even between inbred strains derived from the same founder colony. A similar situation exists in mice (Crabbe et al., 1999). The study of Crabbe et al. (1999) concerned specific behavioural differences observed in mice from data originating from three laboratories. The authors reported that using the exact same inbred strain of mouse, sourced and shipped at the same time from the same supplier, and tested at the same circadian time in the three different laboratories, large effects of site-specific differences on 'standard' behavioural tests were seen. They concluded that there can be important influences of 'local' environmental conditions on specific behaviours, even in a defined genetic strain of mouse.

We should have expected strain-specific differences in similar behaviours based on our own published data (e.g. Dodd et al., 2018; Rothwell et al., 2018; Rothwell and Lukowiak, 2019). In the Dodd et al. (2018) study, *Lymnaea* of one strain were able to recognize differences in another strain, even if the other strain was initially derived from the same founder colony. They showed that crowding of a strain with itself results in blocking associative learning and memory formation, whereas crowding with a different species of snail or with a different strain does not. Additionally, the Rothwell et al. (2018) study further demonstrated that a strain known as the B-strain, which was derived from the S-strain used here, underwent a change in its ability to learn and form associative memory after it was moved from Alberta to Ontario some 20 years ago. The S-strain required two 0.5 h training sessions, but the B-strain required four such training sessions. It appears that the local Ontario environment caused that shift. Thus, it may be expected that different laboratories using the same *Lymnaea* strain obtain different results doing the same experiment. However, not everything changes. For example, the smart cognitive phenotype, has remained stable across time in

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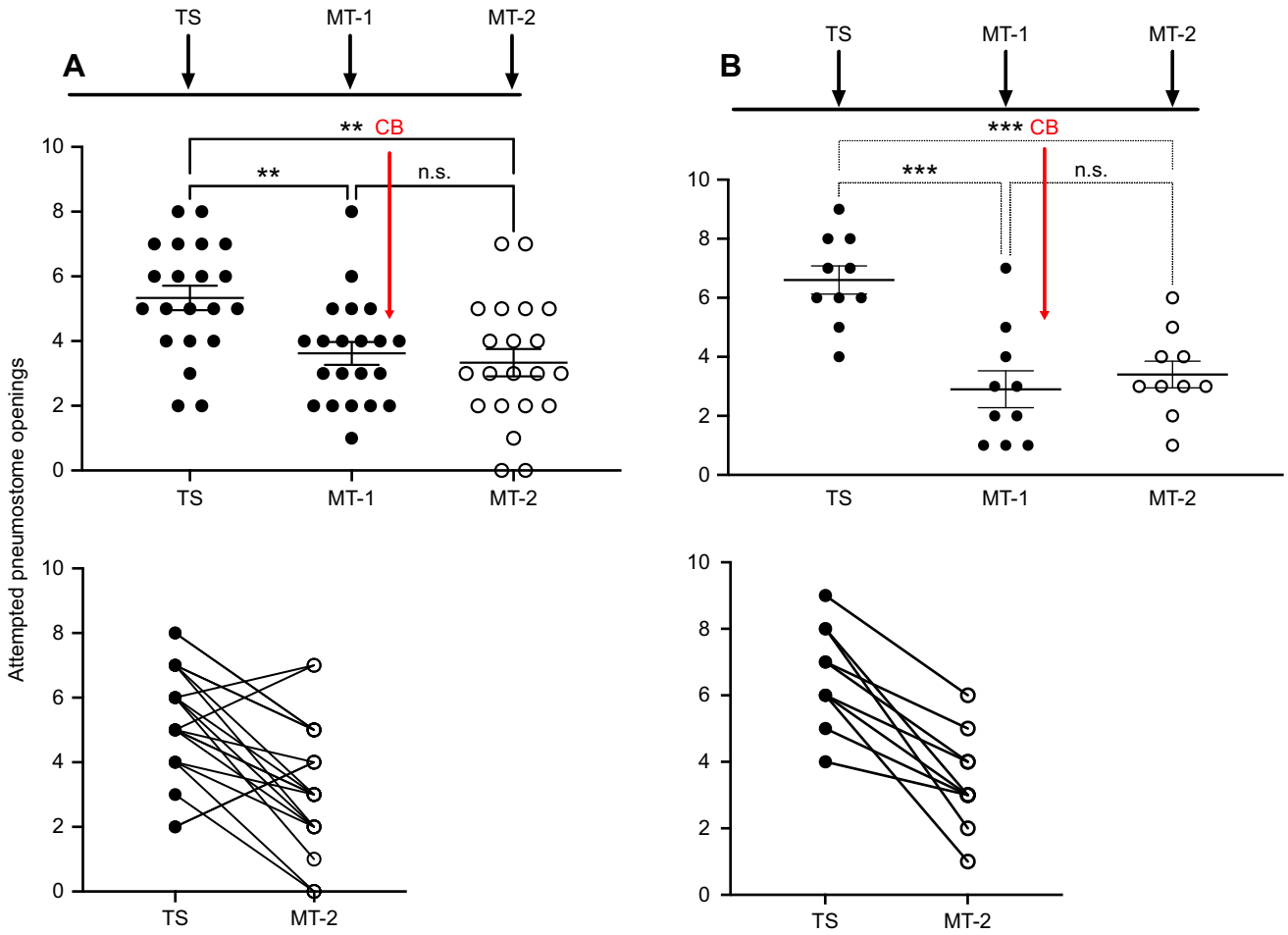

Fig. 7. The CB procedure does not obstruct LTM reconsolidation in freshly collected smart cognitive phenotype Margo Lake and the F1 laboratory-reared Margo Lake *L. stagnalis*. The timeline of each experiment is shown above the data points. The important statistical comparisons are indicated. (A) A cohort of naive Margo Lake snails (*N*=23) received a single 0.5 h TS (TS). Memory was tested 24 h later (MT-1). Immediately after MT-1, snails received the CB procedure (red arrow). Memory was again tested 24 h later (MT-2). The number of attempted pneumostome openings in MT-1 was significantly less than that in TS1, indicating that these are smart cognitive phenotype snails. The number of attempted pneumostome openings in MT-2 was also significantly less than that in TS, indicating that the CB procedure did not interfere with the memory reconsolidation process. (B) As in A, except a naive cohort of F1 Margo Lake snails was used (*N*=10). As with the freshly collected Margo Lake snails, the F1 offspring are smart cognitive phenotype (MT-1 is significantly less than TS), and the CB procedure is ineffective at obstructing the reconsolidation process. That is, MT-2 is significantly less than TS and not significantly different from MT-1. These data are replotted below, showing the response of each individual snail in TS and MT-2. TS–MT-1 ***P*=0.0074; TS–MT-2 ***P*=0.0015; ****P*=0.0003; n.s., not significant (Tukey's *post hoc* test).

snails in their natural habitat (Dalesman et al., 2011; Braun et al., 2012; Hughes et al., 2016, 2017) and in their offspring from eggs laid in the laboratory environment (Fig. 7).

At room temperature, LTM persists for 24 h in S-strain snails, but LTM persistence could be extended for 8 days by maintaining snails at 4°C (Sangha et al., 2003b). Here, we extended those findings to show that LTM could be extended for 4 weeks by placing snails into a 4°C PW environment following training. We hypothesize that this increase in memory persistence was due to two factors. (1) At 4°C, metabolism is slower, which would slow down the active process of forgetting (i.e. the decay of the memory trace hypothesis; see Gates, 1930), as forgetting is an active process depending on altered gene activity and new protein synthesis (Sangha et al., 2005). Thus, the 4°C environment would impede those metabolic processes. (2) In the cold, snails perform less aerial respiration even in hypoxia (see Fig. 2); this would affect the process of retrograde interference (see Jenkins and Dallenbach, 1924; McGeoch, 1932) as a cause of forgetting would be lessened. The retrograde interference hypothesis states that new learning and its consolidation into memory results in conflicting associations that ultimately cause forgetting. The conflicting association in this regard is the opening of the pneumostome without receiving the poke. The data obtained in the Sangha et al. (2005) study lend support to both hypotheses, as do our data here, as in the cold the number of times a bout of aerial respiration occurs without reinforcement is ∼10 times less than at room temperature, and there is a slowing of the metabolism in the cold for the 4 week period.

We further showed here that the CB procedure applied immediately after TS2 in the S-strain snails blocked LTM formation. Again, these data were consistent with earlier data (e.g. Martens et al., 2007). However, the CB procedure did not alter LTM formation in either the freshly collected TC2 snails, which possess the average cognitive phenotype memory-forming capability like the S-strain; nor did the CB procedure obstruct the memory reconsolidation process in freshly collected TC1 and Margo Lake snails, both of which exhibit the smart cognitive phenotype. Unexpectedly, but importantly, the CB procedure did not block

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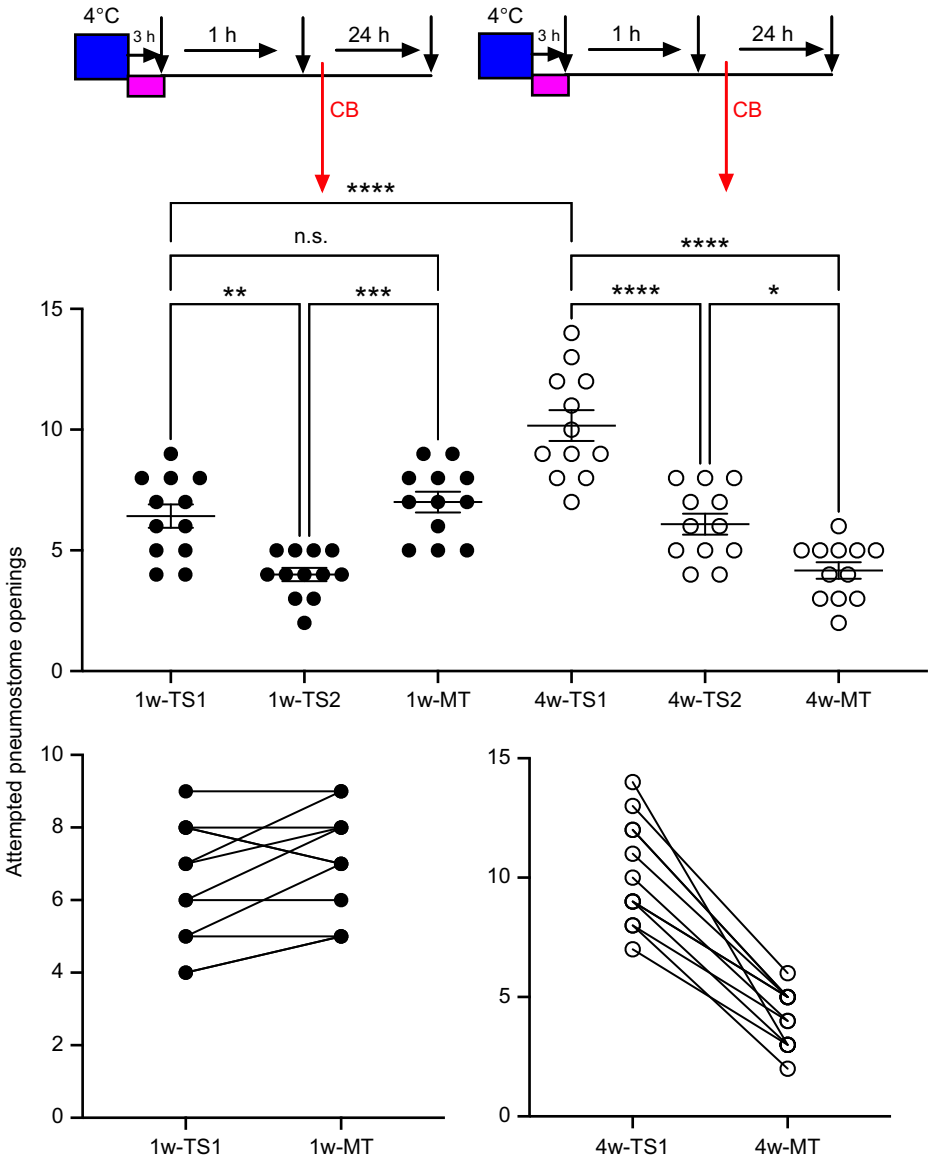

Fig. 8. Cold acclimation in S-strain *L. stagnalis*, and CB procedure effects on LTM formation. The timeline of each experiment is shown above the different cohorts of naive S-strain snails. One cohort (*N*=12, filled circles) was acclimated to the cold environment for 1 week; the other cohort (*N*=12, open circles) was acclimated in the cold environment for 4 weeks. The important statistical comparisons are indicated. In each cohort, following TS2, the CB procedure (red arrows) was used. In the snails cold acclimated for 1 week (filled circles), 1w-MT was not significantly different from 1w-TS1. Thus, the CB procedure in this cohort obstructed LTM formation. In the cohort acclimatized for 4 weeks before training (open circles), the CB procedure did not obstruct LTM formation, as 4w-MT was significantly less than 4w-TS1 and was not greater than 4w-TS2. These data are replotted below, showing the response of each individual snail in TS1 and MT. **P*=0.0194; ***P*=0.0040; ****P*=0.0002; *****P*<0.0001; n.s., not significant (Tukey's *post hoc* test).

reconsolidation in the F1 Margo Lake snails, which developed from eggs laid in the laboratory and thus experienced the same conditions throughout their life as the S-strain snails. We originally hypothesized that the reason the CB procedure did not work in the freshly collected strains was that they had experienced (at least 6 months) a cold spell in their ponds, and thus either their metabolism was adapted for the cold or the CB procedure was no longer perceived as a stressor that impedes memory formation. However, that hypothesis is no longer tenable in light of our F1 Margo Lake results. Our present belief is that, over the years (~50+), traits that make the S-strain snails susceptible to the CB procedure have been inadvertently selected for.

However, the S-strain snails can be metamorphosed into having a CB procedure-resistant phenotype similar to that exhibited by the freshly collected snails. This transformation was brought about by a 4 week cold spell. This suggests to us, but does not prove it, that the CB procedure obstructing LTM formation is probably due to the cold acting as more of a stressor (Lukowiak et al., 2010, 2014) to interrupt the memory consolidation process rather than as a metabolic brake in preventing the consolidation process from occurring. However, how experiencing a cold spell for a 4 week period makes the CB procedure less of a stressor is not understood, although the notion of stress inoculation, which has been observed in molluscs and mice, is an attractive one for future study (Pereira et al., 2020; Ayash et al., 2020). The idea behind stress inoculation is that an earlier exposure to a thermal stressor will enhance stress resilience to a future thermal stressor.

Interestingly, this transformation in the S-strain snails, of an ability typically only seen in freshly collected snails, by a cold spell for a 4 week period serves to remind us that this inbred laboratory-reared strain possesses great plasticity to alter its behaviour in light of changing environmental conditions. This has previously been demonstrated in the S-strain, as there was a metamorphosis from an average cognitive phenotype to a below-average cognitive phenotype when the studied snails had been conceived and reared in a different environment (Rothwell et al., 2018; Rothwell and Lukowiak, 2019). This metamorphosis of cognitive phenotype has persisted for many years, but what drove that transformation is still unknown. Likewise, how a cold spell enables the CB-resistant phenotype described here is also not known. Presently, we also do not know how long this cold-induced metamorphosis will persist.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.L.; Methodology: K.L.; Validation: K.L.; Formal analysis: K.L.; Investigation: M.F., V.R., A.B., K.L.; Resources: K.L.; Data curation: M.F., V.R., A.B.; Writing - original draft: K.L.; Writing - review & editing: M.F., V.R., A.B., K.L.; Supervision: K.L.; Project administration: K.L.; Funding acquisition: K.L.

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