Anim Cogn (2012) 15:483–494
DOI 10.1007/s10071-012-0472-3

ORIGINAL PAPER

Alternate behavioural measurements following a single operant training regime demonstrate differences in memory retention

Sarah Dalesman · Ken Lukowiak

Received: 26 September 2011 / Revised: 5 January 2012 / Accepted: 3 February 2012 / Published online: 22 February 2012

Abstract Frequently studies of learning and memory measure a single focal behaviour; however it is likely that any learning paradigm will alter multiple behavioural traits in the same animal. We used video footage of the great pond snail (*Lymnaea stagnalis*), collected immediately prior to both training and testing for memory in response to operant conditioning to reduce aerial respiration, to measure two additional alternate behavioural traits: reducing the size of the pneumostome (breathing orifice) opening and shell tilt to cover the pneumostome. Typically, the training regime used here results in memory to reduce the number of breathing attempts lasting 24 h but not 72 h. However, memory duration when measured using the two additional behavioural traits differed significantly; shell tilt was short-lived lasting less than 1 h following training, whereas the reduction in pneumostome size was still apparent 72 h following training. Therefore, conclusions about the ability of *L. stagnalis* to retain memory in response to a single type of training regime will differ significantly depending on the focal behavioural trait measured. A significant correlation between the reduction in opening attempts and visible pneumostome area indicated that these behavioural traits are co-specialised, whereas pneumostome opening and shell tilt behaviour varied independently.

Keywords Behavioural plasticity · *Lymnaea stagnalis* · Memory · Operant conditioning

S. Dalesman (✉) · K. Lukowiak
Department of Physiology and Pharmacology, Hotchkiss Brain Institute, University of Calgary, 2104 Health Sciences Centre, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada
e-mail: sarah.dalesman@ucalgary.ca

Introduction

Changes in behavioural phenotype (i.e. behavioural plasticity) can occur rapidly in response to environmental stimuli, allowing an organism to closely match phenotype to current conditions (Mery and Burns 2010). However, costs associated with behavioural plasticity may limit the extent or duration that an alternate phenotype is shown (reviewed in: Relyea 2002; Auld et al. 2010). One way in which behavioural phenotype can be altered is via learning and memory; the duration that the memory is retained will alter how long the resulting behavioural change lasts. Short-term and intermediate-term memory, lasting a few minutes to a few hours, respectively, will allow an animal to respond to immediate surroundings whilst potentially preventing an unnecessary response once the cause of the change in behaviour is no longer present, that is, adaptive forgetting (Kraemer and Golding 1997). Long-term memory, lasting from a day or so to many weeks, will allow an animal to continue to maintain the behavioural response in the absence of reinforcement, potentially then enabling it to demonstrate an appropriate behaviour immediately if the stimulus is repeated.

Frequently, when learning and memory is assessed, there is a specific target for training, and it is only changes in this specific focal trait that is measured. However, measuring a single trait only constitutes one possible target of the training procedure; therefore, failure to see a response in one behavioural trait does not provide adequate evidence that the subject has failed to form an association (Rescorla and Holland 1982; Wasserman 1981; Spear et al. 1990). Non-focal changes in behaviour occurring as a consequence of the training regime may be of importance in interpretation of results, but this importance may be missed if they are not recorded. Behavioural traits may be

co-specialised, such that animals exhibiting the strongest response and expression of memory retention in one behavioural trait may do so in all traits associated with a particular learning regime. This could be due to the underlying neuronal changes following memory formation altering multiple traits simultaneously. For example, in the pond snail, *Lymnaea stagnalis*, right pedal dorsal 11 (RPeD11) is an interneuron that can alter activity in the central patter generator (CPG) controlling aerial respiration, but is also coupled to motoneurons in the foot and body wall, modulating whole-body movements (Syed and Winlow 1991; Inoue et al. 1996). Therefore, following training to alter aerial respiration, we may also see changes in body posture and movement if training is directly altering activity in RPeD11. By measuring multiple behavioural traits, we can develop a greater understanding of the control mechanisms of different behaviours. For example, strong correlation amongst traits may indicate a common neuronal basis. This could either indicate co-selection on a suite of behaviours or evolutionary constraints on the ability of the animal to alter one behavioural trait without altering other related traits. Alternatively, a lack of covariance between traits would indicate disparate control mechanisms underlying different behaviours, and indicate that the animal can alter each trait independently.

As well as co-specialisation, animals may also demonstrate trait compensation, whereby those individuals that appear not to be responding strongly in the focal trait may demonstrate an enhanced response in other non-focal traits. In aquatic gastropods, trait compensation has been demonstrated comparing behavioural and morphological responses, particularly in response to predator threat (reviewed in Covich 2010). Those animals that demonstrate the strongest induced morphological defences against predation are frequently those that demonstrate weaker behavioural responses to predation threat (e.g. Rundle and Brönmark 2001). In this case, if only behavioural traits were measured, an observer could incorrectly assume that morphologically defended species lacked antipredator defences. If multiple behavioural traits are also compensatory, measuring a single behavioural trait in response to a particular learning paradigm may not give a true indication of an animal's ability to learn and form memory, that is, a lack of response in the focal trait may be due to changes in alternate traits. Therefore, it is important to consider changes in multiple behavioural traits if we are to accurately assess whether an animal has responded to a particular stimulus.

The great pond snail *L. stagnalis* is frequently used as a model species to study learning and memory, due to a simple set of behaviours that are easily observable and altered through experience, and a relatively simple nervous system that allows the neural correlates for these behavioural changes to be determined (Parvez et al. 2006; Benjamin and Kemenes 2008; Lukowiak et al. 2003). Learning and memory in this species, as with most model systems used to assess cognitive ability, is commonly assessed by measuring changes in a single focal trait. However, in the only study where multiple behavioural traits were recorded in the same animal, in response to food aversion training, other traits were altered alongside the focal feeding behaviour (Kita et al. 2011). In this study, traits were only recorded at a single time point, so it is unknown whether changes in different traits following learning were retained for different periods of time.

We use operant conditioning to reduce aerial respiration, where the animal is gently poked on the pneumostome (respiratory orifice) to reduce aerial breathing attempts in hypoxic conditions, to assess behavioural and neural correlates of memory in *L. stagnalis*. In the past, we have only measured a single trait to assess memory formation, that of a reduction in the number of pneumostome opening attempts; however, it has been observed subjectively that the snail also alters the way in which the pneumostome opens when it does attempt to breathe during training and testing (first noted in Lukowiak et al. 1996). These observations indicated that the snail appears to reduce the area of the pneumostome visible to the trainer via tilting the shell to cover the pneumostome.

Here, we used video footage taken immediately prior to and following the training and testing procedures, to allow quantification of two additional behavioural traits, shell tilt behaviour and reduction in pneumostome area, to assess how the snail alters these behaviours in response to training (see Fig. 1 for a diagram of these changes). Both inter-individual variation within a population and also inter-population variability in the retention of memory to reduce pneumostome opening attempts have been found in the past (Dalesman et al. 2011b; Orr et al. 2009b). We therefore wanted to assess whether these additional behaviours that had been noted previously are compensatory, that is, the snails with apparently poor learning and memory are in fact demonstrating memory via these alternate behavioural traits.

Materials and methods

Adult *L. stagnalis*, 25 ± 1 mm spire height, were raised under standard rearing conditions at the University of Calgary (Dalesman and Lukowiak 2010) from stock originally obtained from Vrije Universeit in Amsterdam, originating from wild snails collected in the 1950s from canals in a polder located near Utrecht. Snails were transferred to the laboratory and maintained at room temperature (20 ± 1°C) at a stocking density of 1 snail per

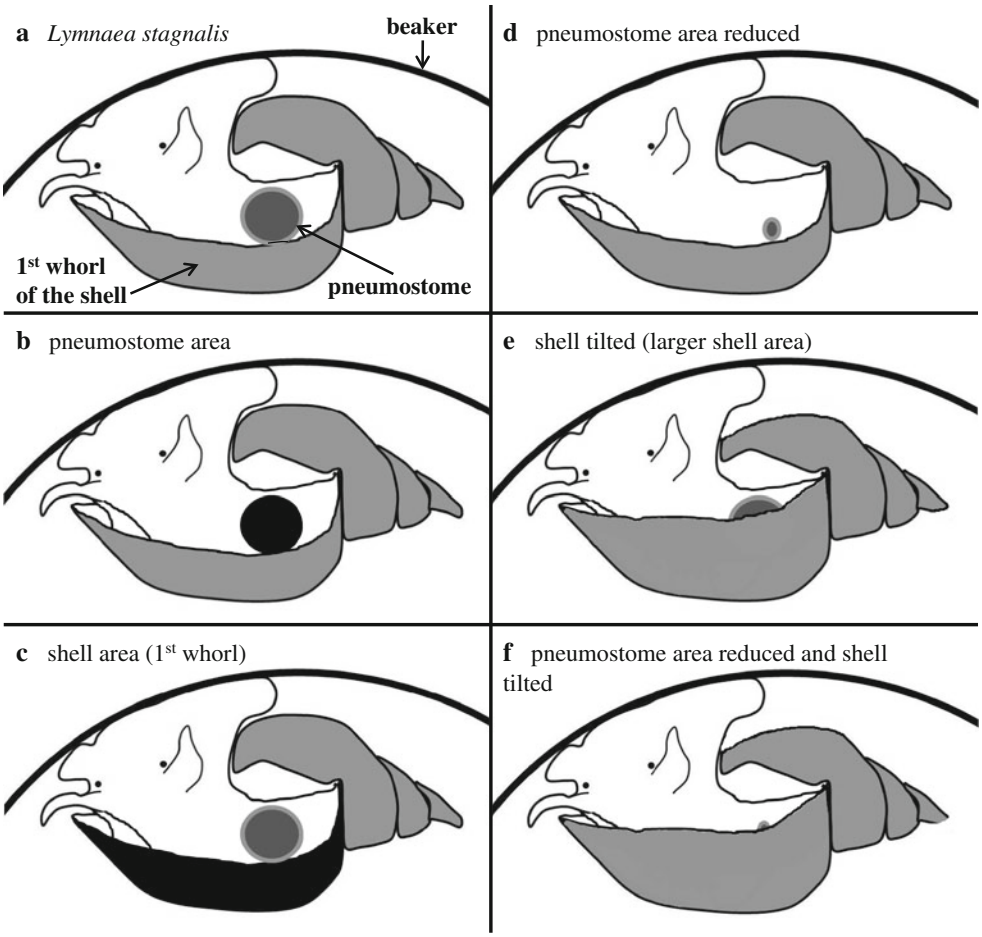

Fig. 1 Diagram of *Lymnaea stagnalis* during aerial respiration, showing **a** basic diagram of *L. stagnalis* attached to the inside wall of the beaker, with pneumostome open at the water's surface; **b** visible area of the pneumostome measured from digital images is shaded in *black*; **c** visible area of the first whorl of the shell measured is shaded in *black*; **d** example of how the visible pneumostome area can be reduced by the snail restricting the opening without changing shell orientation; **e** example of how the visible pneumostome area is reduced without the opening being restricted, by altering shell orientation alone; and **f** example of how the visible pneumostome area is reduced by altering both pneumostome restriction and shell orientation

litre and fed romaine lettuce ad libitum in standard artificial pond water (0.26 g/l Instant Ocean®, Spectrum Brands Inc.) with additional calcium sulphate dehydrate added to provide 80 mg/l for a minimum of 1 week prior to training.

Training protocol

Lymnaea stagnalis are pulmonates, that is, they possess a basic lung. In eumoxic conditions, they breath primarily cutaneously, absorbing oxygen directly from the water via their skin; however, in hypoxic conditions, they move to the water's surface and breath using a basic lung, opened to the atmosphere via a respiratory orifice, the pneumostome (see Fig. 1a; Lukowiak et al. 1996). Snails can be operantly trained to reduce the number of times they attempt to open their pneumostome in hypoxic conditions by gently poking the pneumostome each time they attempt to open it (Lukowiak et al. 1996, 1998, 2000). This physical contact results in the snail closing its pneumostome, but not in full body withdrawal.

Snails were individually labelled 24 h prior to training to avoid labelling stress affecting results. Prior to training, to increase aerial respiration, we first made water hypoxic by vigorously bubbling N_2 through 500 ml of artificial pond water in a 1-l beaker for 20 min. N_2 was continuously bubbled through the water at a reduced rate throughout the training period to maintain hypoxic conditions, though bubbling was stopped during the observation periods to allow uninterrupted video images to be obtained. Snails were introduced to a beaker in cohorts of 6 individuals and allowed to acclimate for 10 min prior to a training session; this period was then used as the observation period (Obs). Following the observation period, snails were trained for 30 min by gently poking the pneumostome with a sharpened wooden stick each time the snail attempted to open it. Following the first 30-min training session (TR1), snails

were returned to their home aquaria in eumoxic conditions for 1 h and then trained again for a further 30 min (TR2) following the identical protocol, after which they were returned to eumoxic aquaria until testing. Long-term memory following training to reduce the number of pneumostome opening attempts was tested 24, 72 h or 1 week following training (test). We used 2 cohorts of 6 animals to test each memory duration, 12 in total per treatment group. No individual snail received more than one testing session. Operant training to reduce the number of pneumostome opening attempts following two half-hour training sessions in pond water using our standard laboratory population typically results in long-term memory lasting 24 h, but not 72 h.

To ensure that any reduction in pneumostome opening attempts was the result of training rather than exposure to the hypoxic environment or a general response to repeated physical contact, we also carried out yoked controls. In this case, the methods are identical to those outlined for training above, except that the pneumostome (or area adjacent if the pneumostome is closed) was poked when the snail to which the control snail was yoked (paired with) opened its pneumostome. In other words, yoked snails are poked the same number of times as trained snails, but the 'poke' is not contingent with pneumostome opening during training sessions. As the snail to which they are yoked reduces the number of attempted pneumostome openings during the second training session (TR2), the number of times the yoked snail is poked during this second training session is also reduced. Again we tested snails in two cohorts of 6 individuals (12 in total), poking the pneumostome contingent to pneumostome opening during the testing phase 24 h later (test).

Assessment of breathing related morphological measurements

In addition to assessing the number of pneumostome opening attempts during the training and testing periods, we also assessed the visible pneumostome area (Fig. 1b: mm^2) and visible area of the first whorl of the shell (Fig. 1c: mm^2) during observation periods (Obs) in the time period during which we would normally acclimate the snail immediately prior to training and testing. In order to measure whole-animal behaviour in relation to pneumostome opening, snails were videoed in 2 cohorts of 6 individuals during the 10-min observation period immediately prior to the first (Obs1) and second (Obs2) training sessions (TR1 and TR2) and the test session (Obs3), 24, 72 h or 1 week following training (see Fig. 2). This allowed the memory in terms of pneumostome opening attempts to be compared directly to the additional data on visible pneumostome and shell area collected for each animal. Video recording was made using a Kodak EasyShare Z950 12 MP Digital Camera (Kodak Canada Inc., Toronto, Canada) using HD video recording. The camera was mounted directly above the beaker using a short tripod attached to a clamp stand. N_2 aeration was ceased during video recording to prevent small bubbles interfering with the captured image, but restarted once training recommenced at the end of the observation period. Whilst snails were being videoed, the timing of the initial two pneumostome openings during the observation period was noted, to ensure that results were comparable across all conditions. Noting times of pneumostome opening enabled the video to be rapidly forwarded to the correct time to capture still images for analysis. This also allowed the viewer to identify when the pneumostome was open, but not visible on the video image.

Videos were played back using Topaz Moment v3.5 (Topaz Labs, Dallas, USA) enabling still images to be captured from the video. Some degree of subjectivity was used to assess the maximum opening visible during each pneumostome opening event: however, the same person carried out the choice of image to capture, blind to the training stage they were observing, to maintain a similar degree of precision throughout. Two images were captured for each individual snail during each observation period, representing the first two separate pneumostome openings. Therefore, during the observation period prior to each training session (Obs1 and Obs2) and also prior to the testing session (Obs3), a total of 6 images per snail, two from each observation period, were obtained.

Images were analysed using Uthscsa Image Tool 3.0 (University of Texas Health Science Center, San Antonio, USA). Two measurements were taken from each image shown diagrammatically in Fig. 1; visible pneumostome surface area (Fig. 1b) and visible area of the first whorl of the shell was used to represent the angle at which the shell is held (Fig. 1c). In pre-trained animals, the pneumostome is normally fully visible and opened to its full extent (Fig. 1a). When the snail is trained, it may reduce the visible pneumostome area in different ways demonstrated diagrammatically in Fig. 1d–f: by contracting the size of the opening without changing shell orientation (Fig. 1d), by tilting the shell to partially cover the pneumostome whilst maintaining the opening size (Fig. 1e) or a mixture of these 2 methods (Fig. 1f). Alteration in the pneumostome size alone would suggest the snail is not altering the orientation of the shell to cover the pneumostome. However, if there is also an increase in the visible area on the first whorl, it would suggest the snail is making an effort to cover the pneumostome with its shell. Previous subjective observations indicated that both these behavioural phenotypes were visible during training (Lukowiak et al. 1996; Dalesman pers. obs.).

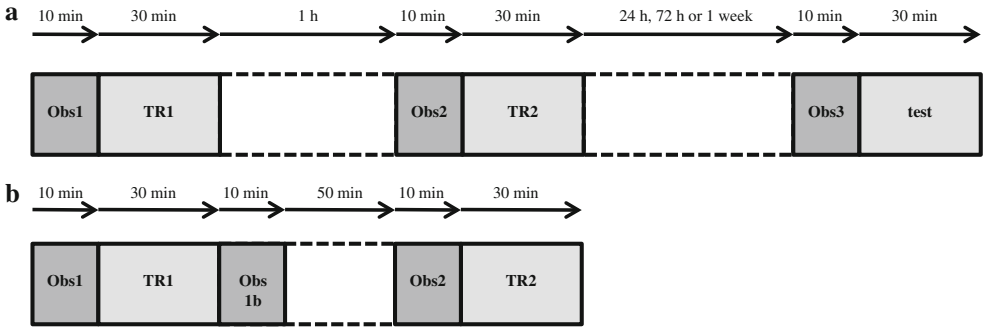

Fig. 2 Timeline for experimental design. **a** Timeline for individuals in which pneumostome and shell area were assessed immediately before training and testing only and **b** timeline for individuals in which pneumostome and shell area were assessed both before and after the first training session. Timeline shows the sequence of observations during which images were taken to assess pneumostome and shell area (*dark grey* Obs1, Obs1b, Obs2 and Obs3) and training and test sessions (*pale grey* TR1, TR2 and test) during which the attempted number of pneumostome openings were recorded

In addition to the data collected above, where pneumostome and shell area could be directly compared to the number of opening attempts during training and testing, we used an additional group of 12 trained snails and 12 yoked snails (2 cohorts of 6 individuals per treatment group) to assess the behavioural response immediately following the first training session (Obs1b; Fig. 2b). Assessment of pneumostome area or shell tilt was not carried out immediately following the training previously as exposure to the hypoxic environment in the absence of training can result in extinction of the learnt behaviour (McComb et al. 2002). However, this further work was carried out to determine whether shell tilt altered during training, but was too short-lived to be measured during normal observation periods (Obs2 and Obs3). The snails were videoed during the first observation period (Obs1) and then trained for 30 min as before, but then also videoed for 10 min immediately following training (or yoked training), during Obs1b (Fig. 2b). The snails were then videoed again 50 min later for 10 min to assess intermediate-term memory in body posture changes. As before, still images were captured from the video data, using the first two pneumostome opening attempts during each recording period, and again analysed using Uthscsa Image Tool.

Statistical analyses

The effect of duration between training and testing on memory following training to reduce the number of pneumostome opening attempts during the first (TR1) and second (TR2) training sessions and during the test session (test) was compared using repeated measures ANOVA in SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Initially, each group was tested separately using cohort as the between-subject factor and training session (TR1, TR2 or test) for within-subject comparison. No effect of cohort was found, so cohort data were grouped within each test group for further analysis. The timing of testing (24 vs. 72 h vs. 1 week: Fig. 3a–c) was subsequently used as the between-subject factor and training session (TR1, TR2 or test) as the within-subject factor.

To confirm that any reduction in pneumostome opening attempts is due to the training procedure experienced by the snails, that is, that they have to experience the poke contingent with pneumostome opening rather than responding in a more generalised way to physical contact, we compared the data from the group tested at 24 h with the yoked controls also tested at 24 h. Again we used repeated measures ANOVA in SPSS with training regime (trained vs. yoked: Fig. 3 a, d) as the between-subject factor and training session (TR1, TR2 or test) as the within-subject factor.

The measurements of pneumostome area and shell area were taken from two individual photographs for each animal within each time period. The mean area derived from both measurements at each time period was then used for all further analyses. To assess whether pneumostome area or area of the first whorl of the shell altered in response to training, the averaged data for each morphological measurement were analysed separately within each treatment group (yoked vs. trained and tested at 24 h vs. trained and tested at 72 h vs. trained and tested at 1 week). These data were analysed separately rather than within a single ANOVA as initial morphological shape shows significant variability amongst individuals. Data were analysed using repeated measures ANOVA with cohort (2 per analysis) as the between-subject factor and observation session (Obs1, Obs2 or Obs3) as the within-subject factor.

To assess short-term changes in body posture, the pneumostome area and visible shell area immediately prior to (Obs1) and following (Obs1b) the first training session were compared. The area for each trait was averaged across two measurements taken at each time period as before, then analysed using repeated measures ANOVA, with cohort (2 per analysis) as the between-subject factor and training

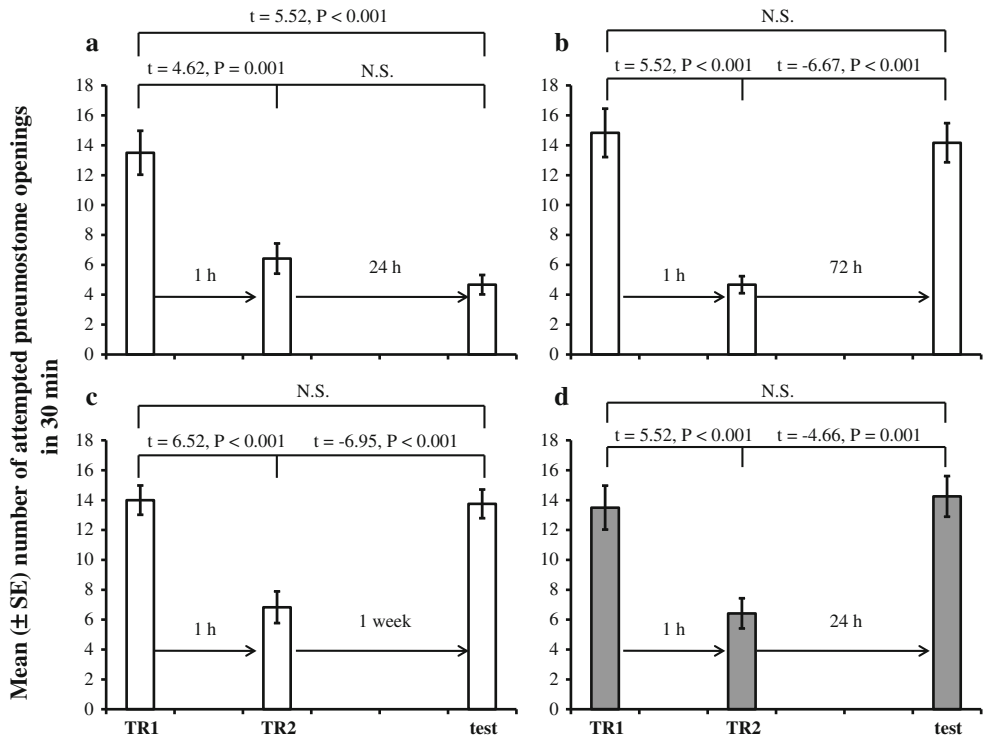

Fig. 3 Mean number of pneumostome opening attempts during training (TR1 & TR2) and testing in trained (*white columns*) and yoked (*grey columns*) animals: **a** trained and tested at 24 h; **b** trained and tested at 72 h; **c** trained and tested at 1 week; and **d** yoked snails tested at 24 h. *Horizontal bars* above the columns indicate significant post hoc within-subject differences as found in the pneumostome area (paired *t* test; *t* value and *P* value given when significant; $N = 12$ for all comparisons)

session (Obs1, Obs1b or Obs2; Fig. 2) as the within-subject factor. As no significant effect of cohort was found, cohort data were grouped to compare trained with yoked animals, then analysed using repeated measures ANOVA with training session (pre TR1, post TR1 or pre test 1 h following TR1) as the within-subject factor and training regime (trained vs. yoked) as the between-subject factor.

For all ANOVA analyses, data were assessed using Mauchly's test for sphericity prior to analysis to confirm homogeneity of variance. The more conservative Greenhouse–Geisser *P* values were used where the assumption of homogeneity of variance was not met. Tukey's HSD test was used to carry out between-subject post hoc comparisons, primarily used to confirm that the number of pneumostome openings, or area of pneumostome and shell, did not vary significantly amongst treatment groups in naïve animals. Paired *t* tests (with the *P* value required for significance adjusted to 0.0167 for multiple comparisons) were used to carry out post hoc within-subject comparisons where overall significance was found to confirm where traits had altered relative to the naïve state.

In order to assess consistency with individual response to training over time, we used Pearson's correlations to compare the relative change in each trait between the first and second training sessions (TR1 vs. TR2 and Obs 1 vs. Obs2) and between the first training period and the test (TR1 vs. test and Obs1 vs. Obs3) and to assess whether these changes were consistent within individual. In other words, did individuals that demonstrated the largest change in each trait between the first and second training or observation sessions also demonstrate the largest change between the first training or observation period and the test session? In addition, we used Pearson's correlations to assess whether there was any interrelationship between these relative changes in the three variables measured, comparing whether animals that demonstrated the greatest change in one trait also demonstrated the greatest change in other behavioural traits within each time period. In addition to testing these variables during the standard training and testing, we also tested whether there was any relationship between shell tilt and pneumostome area immediately following TR1 (Obs1b).

Results

Number of pneumostome opening attempts

To assess whether any change in pneumostome opening attempts was due to memory formation, we compared

trained animals at 24 h with yoked controls (Fig. 3a, d). The number of times the snails attempted to open their pneumostome 24 h following training was significantly lower in the trained group relative to the yoked control group (Tukey's HSD test: $P < 0.05$), demonstrating a significant decline in the trained group during the test period relative to the first training session (Fig. 3a: TR1 vs. test paired t test: $t = 5.52$, $P < 0.001$, $N = 12$) but no such decline in the yoked controls (Fig. 3d; TR1 vs. test paired t test: $t = -0.35$, $P = 0.73$, $N = 12$; repeated measures ANOVA: 2-way interaction between time and training method; $F_{2,44} = 12.04$, $P < 0.001$). This indicates that decline in pneumostome openings during the test phase relative to the first training session is due to long-term memory (LTM) formed due to the training procedure as found previously as a decline is only seen when the 'poke' is contingent with pneumostome opening (Sangha et al. 2003).

To assess how long memory to reduce pneumostome opening attempts was retained, we compared training and memory tests at 24, 72 h and 1 week. Overall, there was a significant effect of the timing of the test period (Fig. 3; repeated measures ANOVA: 2-way interaction between response to training and time at which memory was tested; $F_{4,66} = 9.13$, $P < 0.001$). This is due to the number of pneumostome opening attempts at 24 h following training being significantly lower than both the number of attempts during the first training session (TR1 vs. test at 24 h paired t test: $t = 5.52$, $P < 0.001$, $N = 12$), and also significantly lower than the number of attempts during the testing phase at either 72 h or 1 week (Tukey's HSD test: $P < 0.05$ for both comparisons). The number of attempted openings during the test session did not decline relative to the first training session when the test was carried out either 72 h or 1 week following training (paired t test: $P > 0.05$ for both comparisons). This result is directly comparable to previous work using the Dutch population of snails, where two half-hour training sessions separated by 1 h result in LTM to reduce the number of pneumostome openings that lasts 24 h, but not 72 h (Sangha et al. 2003).

Pneumostome area

The pneumostome area was altered significantly during the experiments in all trained groups (Fig. 4a–c; repeated measures ANOVA: main effect of time period; 24 h: $F_{2,20} = 31.67$, $P < 0.001$; 72 h: $F_{2,20} = 35.93$, $P < 0.001$; 1 week: $F_{2,20} = 19.54$, $P < 0.001$), though there was no

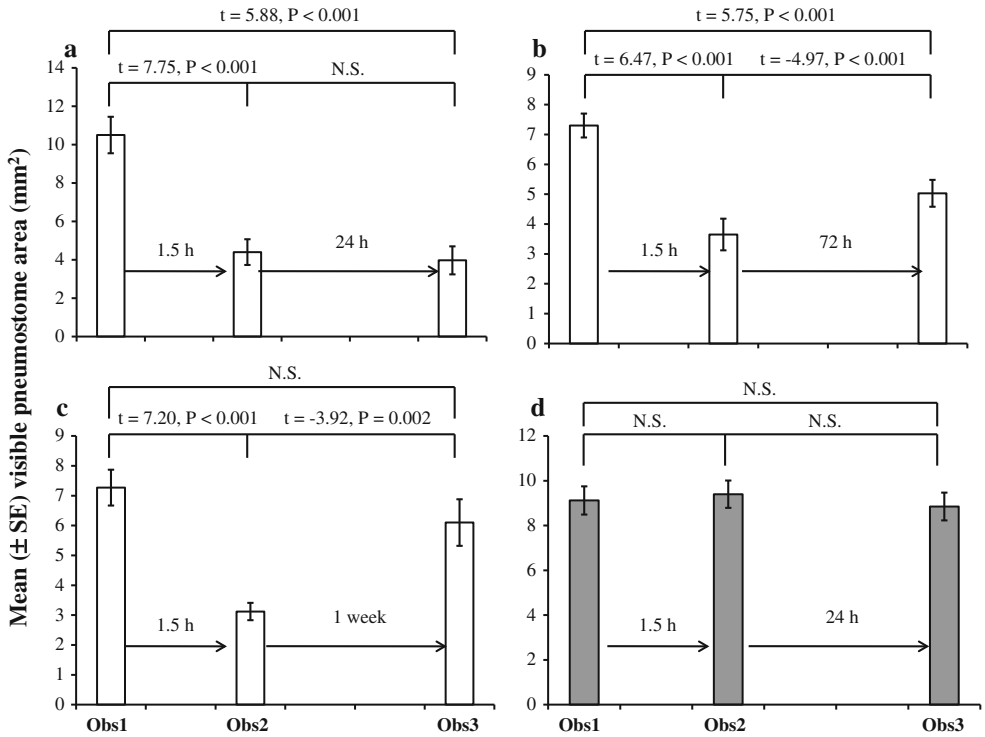

Fig. 4 Mean visible pneumostome area in the 10-min observation period prior to TR1 (Obs1), during the 10-min observation period prior to TR2 (Obs2) and during the 10-min observation period prior to the test (Obs3) in trained (*white columns*) and yoked (*grey columns*) animals: **a** standard training, test at 24 h; **b** standard training, test at 72 h; and **c** standard training, test at 1 week and **d** yoked control, test at 24 h. *Horizontal bars* above the columns indicate significant post hoc within-subject differences as found in the pneumostome area (paired t test; t value and P value given when significant; $N = 12$ for all comparisons)

significant effect of training on the visible pneumostome area in yoked control snails (Fig. 4d). There was no effect of cohort tested, or interaction between cohort and time period for any of the experiments (trained or yoked controls). For all trained snails, the visible pneumostome area declined significantly between Obs1 (before any training took place) and Obs2 when the snails had received a single half-hour training session followed by an hour in their home aquaria in all trained groups (24 h: $t = 7.75$, $P < 0.001$; 72 h: $t = 6.47$, $P < 0.001$; 1 week: $t = 5.75$, $P < 0.001$; $N = 12$ for each test). However, when the pneumostome area during Obs1 is compared to that during Obs3, immediately prior to the test phase, not all groups showed the same pattern. Snails tested at 24 h demonstrated a significant decline in pneumostome area between Obs1 and Obs3 ($t = 5.88$, $P < 0.001$, $N = 12$), but no difference between the visible pneumostome area in Obs2 versus Obs3 ($t = 0.55$, $P = 0.59$, $N = 12$). This indicated that they demonstrated both intermediate-term memory (ITM) 1 h following initial training and LTM at 24 h for this behaviour. Snails tested at 72 h also demonstrate a significant decline in visible pneumostome area between Obs1 and Obs3 immediately prior to testing 72 h later ($t = 5.75$, $P < 0.001$, $N = 12$); however, there was also a significant increase in pneumostome area between Obs2 and Obs3 at 72 h ($t = -4.97$, $P < 0.001$, $N = 12$). This indicates that whilst this group does show LTM at 72 h, with a significant decline in pneumostome area relative to when they were naïve, the effect of training on the visible pneumostome area during Obs3 is not as substantial as that seen during the Obs2 when they demonstrate ITM. Snails tested 1 week following training demonstrated no significant decline in the pneumostome area during Obs3 before testing, relative to Obs1 (paired t test: $P > 0.05$), with the pneumostome area also increasing significantly between Obs2 and Obs3 ($t = -3.92$, $P = 0.002$). Therefore, 1 week following training, the snails did not show a behavioural expression of LTM to reduce pneumostome area, but did show ITM during training.

Shell area

The visible area of the first whorl of the shell did not change significantly in any of the treatment groups (trained or yoked; Fig. 5), nor was there any significant effect of cohort on visible shell area. This indicates that during the

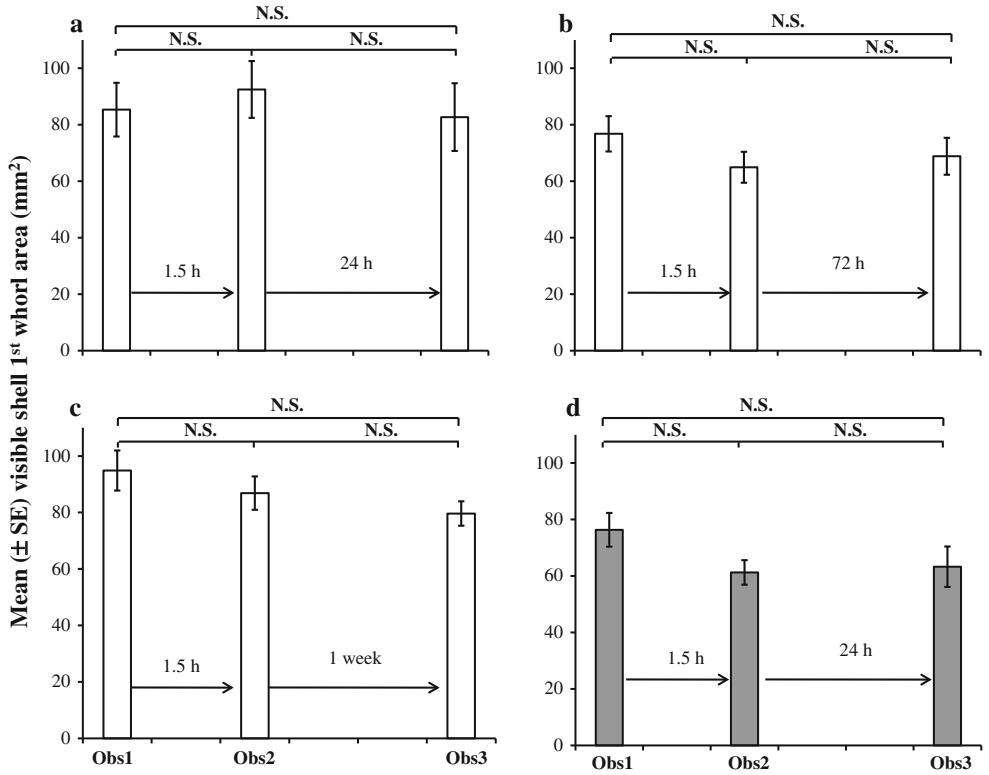

Fig. 5 Mean visible first whorl of the shell area in the 10-min observation period prior to TR1 (Obs1), during the 10 min observation period prior to TR2 (Obs2) and during the 10-min observation period prior to the test (Obs3) in trained (*white columns*) and yoked (*grey columns*) animals: **a** standard training, test at 24 h; **b** standard training, test at 72 h; **c** standard training, test at 1 week and **d** yoked control, test at 24 h. *Horizontal bars* above the columns indicate significant post hoc within-subject differences as found in the pneumostome area (paired t test; t value and P value given when significant; $N = 12$ for all comparisons)

observation periods immediately prior to TR2 (Obs2) and also immediately prior to the test period (Obs3) any change seen in pneumostome area is not a result of change in shell orientation by the snail, that is, ITM and LTM resulting in a decline in pneumostome area is not due to the snail tilting its shell to cover the pneumostome. Whilst this 'tilting' behaviour was observed subjectively during training, it appears not to be the primary source of the snail reducing the visible pneumostome during memory tests.

Relationship between pneumostome opening attempts, pneumostome area and shell area during memory tests

The relative decrease in both pneumostome opening attempts between TR1 and TR2 and between TR1 and test was significantly positively correlated over time (pneumostome opening attempts: $r = 0.75$, $P < 0.001$). Additionally, the relative change in both pneumostome and shell area between Obs1 and Obs2 and between Obs1 and Obs3 was also significantly positively correlated (pneumostome area: $r = 0.46$, $P = 0.005$; shell area: $r = 0.82$, $P < 0.001$). This indicates that snails were consistent between time periods in the degree to which they altered all three traits following training. Within each time period (i.e. TR2/Obs2 and test/Obs3), there was no correlation between the relative change in shell area and the change seen in the other two variables. However, the relative change in the number of pneumostome opening attempts and the relative change in pneumostome area were significantly correlated with one another, both during TR2/Obs2 ($r = 0.56$, $P < 0.001$) and test/Obs3 ($r = 0.41$, $P = 0.014$).

Pneumostome area and shell area immediately following training

There was no significant effect of cohort on either the visible pneumostome area or the area recorded for the first whorl of the shell in either trained or yoked snails. As found previously, the visible pneumostome area decreased significantly following training relative to the pre-training size (Fig. 6a; repeated measures ANOVA: $F_{2,20} = 24.39$, $P < 0.001$), both immediately following training (Fig. 6a; paired t test: $t = 5.86$, $P < 0.001$, $N = 12$) and also when tested 1 h following training (Fig. 6a; paired t test: $t = 5.29$, $P < 0.001$, $N = 12$). However, unlike during the memory test (Obs3), a significant change in the area of the first whorl of the shell was also apparent (Fig. 6c; repeated measures ANOVA: $F_{2,20} = 5.39$, $P = 0.013$), that is, the

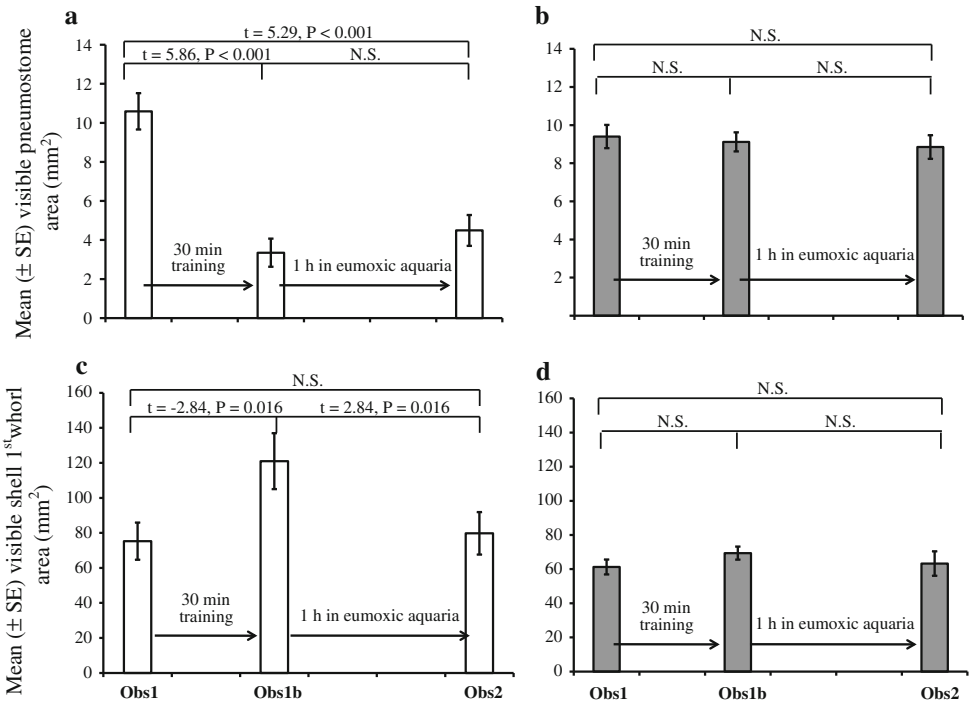

Fig. 6 Mean visible pneumostome area in the 10-min observation period prior to TR1 (Obs1), immediately following the first training session (Obs1b) and during the 10-min observation period prior to TR2 (Obs2) 1 h later following standard training (**a**—*white columns*) and yoked control training (**b**—*grey columns*) and mean visible first whorl of the shell area in the 10-min observation period prior to TR1 (Obs1), immediately following the first training session (Obs1b) and during the 10-min observation period prior to TR2 (Obs2) following standard training (**c**—*white columns*) and yoked control training (**d**—*grey columns*). *Horizontal bars* above the columns indicate significant post hoc within-subject differences as found in the pneumostome area (paired t test; t value and P value given when significant; $N = 12$ for all comparisons)

visible shell area increased during Obs1b relative to Obs1 (Fig. 6c; paired t test: $t = -0.84$, $P = 0.016$, $N = 12$), but not during Obs2 1 h following training (Fig. 6c; paired t test: $t = -0.29$, $P = 0.774$, $N = 12$). Yoked snails demonstrated no significant difference in either pneumostome area (Fig. 6b) or shell area (Fig. 6d), either immediately post-training (Obs1b) or pre-testing 1 h following yoked training (Obs2) compared to the visible area in naïve snails during Obs1. It therefore appears that the shell tilting behaviour, previously observed subjectively during training, is due to operant learning, memory of which rapidly diminishes within 1 h of the training procedure. There was no significant correlation between the change in visible shell area and change in pneumostome area between Obs1 and Obs1B, indicating that these two variables are altered independently.

Discussion

Lymnaea stagnalis from our Dutch laboratory population demonstrated long-term memory to reduce the number of pneumostome opening attempts following two half-hour training sessions 24 h but not 72 h following training, in agreement with previous findings (Orr et al. 2009a; Sangha et al. 2003). The reduction in the visible pneumostome area lasted significantly longer than this 'standard' test for memory as the pneumostome area was significantly reduced relative to pre-training both 24 and 72 h later. In contrast to the inferences drawn from our subjective observational data (Lukowiak et al. 1996), the reduction in pneumostome size immediately prior to the memory test was not due to the snail tilting its shell to cover the pneumostome based on objective measurement of this trait. The visible area of shell was increased immediately following training but not prior to memory tests. Instead, during the observation period prior to memory tests, the snails reduced the visible area directly by not opening the pneumostome to the same degree (i.e. restricting the opening as in Fig. 1d), and shell orientation did not differ significantly from the naïve state. These data support subjective observations that shell tilting to cover the pneumostome does occur during training (Lukowiak et al. 1996); however, this behavioural change is short-lived and only apparent immediately following training.

It is possible that this short-lived retention of memory to tilt the shell over the pneumostome is due to lack of reinforcement during training. That is, the snail is poked each time the pneumostome is opened, but not each time it tilts the shell in a particular way; hence, the shell angle may only be partially reinforced during training. Partial reinforcement of pneumostome opening, for example, where the snail is only poked every other time it opens the pneumostome, does not result in intermediate or long-term memory to reduce the number of pneumostome opening attempts (Sangha et al. 2002), though in this case, short-term memory was not tested. Alternatively, as the snail is constantly moving the shell relative to its body, it may rapidly forget the memory relating to shell angle,that is, forming a new memory that moving the shell does not result in being poked. If an animal regularly performs an activity without reinforcement from the initial stimulus, it will form new memory that precludes the old memory, for example, that a particular activity is no longer associated with receiving an aversive stimulus; this form of forgetting is termed retroactive interference (Jenkins and Dallenbach 1924). There is considerable empirical evidence supporting the role of retroactive interference in the process of forgetting. If animals (including humans) are prevented from performing alternate behaviours, either by direct inhibition or by natural processes such as sleep, forgetting a previously learnt behaviour is prevented (Wixted 2004). This has been demonstrated in *L. stagnalis* by preventing the snail opening its pneumostome between training and testing sessions, preventing forgetting of learnt behaviour (Sangha et al. 2005). The pneumostome is opened relatively infrequently in the eumoxic conditions in the aquaria, therefore memory to alter this trait may be retained for longer periods due to less interference.

In contrast to the very short time period over which shell tilting is maintained, the reduction in pneumostome opening area via restricting the opening is still apparent 72 h, but not 1 week, following two half-hour training sessions separated by 1 h. When memory for the number of pneumostome opening attempts is tested 72 h after training, this behaviour is no longer apparent, and the snail now opens its pneumostome as often as it does in the naïve state. Therefore, the memory for altering pneumostome area via restriction lasts longer than that to alter the number of opening attempts. As both these related behaviours will be performed an equal number of times whilst the snail is in the eumoxic aquaria between training and testing (unlike the shell tilting behaviour) and therefore undergo a similar number of interference events, this cannot be used to explain the difference in retention times. It is more likely that reducing the number of pneumostome opening occurrences carries a greater cost than reducing the size of the opening. In the former, the snail has to restrict breathing attempts in hypoxia, therefore presumably building up an oxygen debt, whereas in the latter, the snail is still able to aerially respire, though the gas exchange between the external environment and the lung will be slowed. We propose that the reduction in the number of opening attempts carries a greater cost to the snail and will therefore be forgotten faster in the absence of reinforcement. Hence, the same training procedure is altering three

behavioural traits associated with aerial respiration in *L. stagnalis*, shell tilting, pneumostome opening attempts and the degree to which the pneumostome is opened, the memory of which are all retained for different durations following training.

There is evidence that a residual memory for the number of pneumostome opening attempts lasts longer than the behavioural expression of memory. Snails that have undergone a training regime, and apparently forgotten the training demonstrating no behavioural change in the number of pneumostome opening attempts, subsequently form long-term memory following a training regime that only results in intermediate-term memory in naïve animals (Parvez et al. 2005). Additionally, snails demonstrating no behavioural change in response to training do show a change in electrophysiological activity in right pedal dorsal 1 (RPeD1) compared to naïve animals, part of the central pattern generator that controls aerial respiration, though not to the same extent as seen in animals that also demonstrate a behavioural change (Dalesman et al. 2011a; Braun and Lukowiak 2011). It is possible that the neuronal basis for the snail altering its pneumostome area is the same as that responsible for this residual memory of opening attempts. Further work is required to verify whether this is the case. For example, the response to training to reduce the number of opening attempts is predicted to be stronger if there is residual memory from previous training (Parvez et al. 2005). We should therefore be able to test whether the maintenance of this boosting effect from residual memory coincides with retention of the memory to reduce pneumostome area.

Significant variability in the duration of memory retention to reduce the number of pneumostome opening attempts has been found between *L. stagnalis* populations (Orr et al. 2009a; Dalesman et al. 2011b). Potentially, this was due to compensatory behaviour, whereby populations demonstrating poor memory to reduce opening attempts are those that show enhanced memory for behaviour that obscures the pneumostome. However, here we found a strong correlation between the reduction in pneumostome opening attempts and the reduction in visible pneumostome area; that is, snails that show the greatest reduction in opening attempts following training also demonstrate the greatest reduction in visible pneumostome area. Correlation between the number of pneumostome opening attempts and the degree to which the pneumostome area is restricted indicates that these behavioural traits are co-specialised, and therefore trait compensation is unlikely to be the source of inter-population variability.

In addition to the relationship between different behavioural traits, there is also a strong relationship between the response to training seen over time in the number of pneumostome opening attempts, pneumostome area and shell orientation. The snails that show the greatest decline in pneumostome opening attempts during the second training session relative to the first training session also show the greatest relative reduction during the memory test. A similar pattern is seen in reduction in the visible pneumostome area during the observation periods. In other words, the snails are demonstrating consistency over time in their response to the training procedure. Snails also show consistency in the relative shell position, that is, the body posture prior to the second training session is correlated with the body posture prior to the test session, despite this trait showing no response to training relative to naïve snails at these two time periods. Together, these data show that individual snails are consistent in their behavioural responses over time, indicating behavioural syndrome following training (Briffa et al. 2008; Dingemanse et al. 2010).

Previous work using the same training procedure presented here to reduce aerial respiration in *L. stagnalis* has consistently demonstrated that this species forms long-term memory lasting 24 h but not 72 h; when assessing the pneumostome opening attempts, our data support this conclusion. However, when assessing alternate behavioural traits that respond to training, pneumostome area and shell tilt, we found that the memory duration as indicated by behavioural expression differs significantly, lasting either considerably longer or shorter than our 'standard' test depending on the trait measured. These traits may be either co-specialised, as found in the case of pneumostome opening attempts and pneumostome opening area, or independent, as found for shell tilt behaviour. Together, the data presented here demonstrate that conclusions about the ability of an animal to learn and form memory based on behavioural measures of memory in response to a single training regime may be highly dependent on the trait measured, supporting previous findings in mammals.

Acknowledgments We would like to thank 3 anonymous reviewers for their comments that helped improve this manuscript. S.D. is supported by an Alberta Heritage Foundation for Medical Research postdoctoral fellowship from Alberta Innovates—Health Solutions, Canadian Institutes for Health Research support K.L. and the laboratory.

Ethical standard The experiments presented here comply with the law of the country in which they were performed.

References

Auld JR, Agrawal AA, Relyea RA (2010) Re-evaluating the costs and limits of adaptive phenotypic plasticity. Proc R Soc B-Biol Sci 277(1681):503–511. doi:10.1098/rspb.2009.1355

Benjamin PR, Kemenes G (2008) Behavioral and circuit analysis of learning and memory in mollusks. In: Byrne JH (ed) Learning

and memory: a comprehensive reference. Academic Press, Oxford, pp 587–604

Braun MH, Lukowiak K (2011) Intermediate and long-term memory are different at the neuronal level in *Lymnaea stagnalis* (L.). Neurobiol Learn Mem 96(2):403–416

Briffa M, Rundle SD, Fryer A (2008) Comparing the strength of behavioural plasticity and consistency across situations: animal personalities in the hermit crab *Pagurus bernhardus*. Proc R Soc B-Biol Sci 275(1640):1305–1311. doi:10.1098/rspb.2008.0025

Covich AP (2010) Winning the biodiversity arms race among freshwater gastropods: competition and coexistence through shell variability and predator avoidance. Hydrobiologia 653(1): 191–215. doi:10.1007/s10750-010-0354-0

Dalesman S, Lukowiak K (2010) Effect of acute exposure to low environmental calcium alters respiration and locomotion of *Lymnaea stagnalis* (L.). J Exp Biol 213:1471–1476. doi:10.1242/jeb.040493

Dalesman S, Braun MH, Lukowiak K (2011a) Low environmental calcium blocks long term memory formation in a pulmonate snail. Neurobiol Learn Mem 95:393–403. doi:10.1016/j.nlm.2010.11.017

Dalesman S, Rundle SD, Lukowiak K (2011b) Microgeographic variability in long-term memory formation in the pond snail, *Lymnaea stagnalis*. Anim Behav 82:311–319. doi:10.1016/j.anbehav.2011.05.005

Dingemanse NJ, Kazem AJN, Reale D, Wright J (2010) Behavioural reaction norms: animal personality meets individual plasticity. Trends Ecol Evol 25(2):81–89. doi:10.1016/j.tree.2009.07.013

Inoue T, Takasaki M, Lukowiak K, Syed NI (1996) Inhibition of the respiratory pattern-generating neurons by an identified whole-body withdrawal interneuron of *Lymnaea stagnalis*. J Exp Biol 199(9):1887–1898

Jenkins J, Dallenbach K (1924) Obliviscence during sleep and waking. Am J Psychol 35:605–612

Kita S, Hashiba R, Ueki S, Kimoto Y, Abe Y, Gotoda Y, Suzuki R, Uraki E, Nara N, Kanazawa A, Hatakeyama D, Kawai R, Fujito Y, Lukowiak K, Ito E (2011) Does conditioned taste aversion learning in the pond snail *Lymnaea stagnalis* produce conditioned fear? Biol Bull 220(1):71–81

Kraemer PJ, Golding JM (1997) Adaptive forgetting in animals. Psych Bull Rev 4:480–491. doi:10.3758/BF03214337

Lukowiak K, Ringseis E, Spencer G, Wildering W, Syed N (1996) Operant conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*. J Exp Biol 199(3):683–691

Lukowiak K, Cotter R, Westly J, Ringseis E, Spencer G, Syed N (1998) Long-term memory of an operantly conditioned respiratory behaviour pattern in *Lymnaea stagnalis*. J Exp Biol 201(6):877–882

Lukowiak K, Adatia N, Krygier D, Syed N (2000) Operant conditioning in *Lymnaea*: evidence for intermediate- and long-term memory. Learn Mem 7(3):140–150. doi:10.1101/lm.7.3.140

Lukowiak K, Sangha S, Scheibenstock A, Parvez K, McComb C, Rosenegger D, Varshney N, Sadamoto H (2003) A molluscan model system in the search for the engram. J Physiol Paris 97:69–76. doi:10.1016/j.jphysparis.2003.10.008

McComb C, Sangha S, Qadry S, Yue J, Scheibenstock A, Lukowiak K (2002) Context extinction and associative learning in Lymnaea. Neurobiol Learn Mem 78(1):23–34. doi:10.1006/nlme.2001.4041

Mery F, Burns JG (2010) Behavioural plasticity: an interaction between evolution and experience. Evol Ecol 24:571–583. doi:10.1007/s10682-009-9336-y

Orr M, Hittel K, Lukowiak K (2009a) Different strokes for different folks: geographically isolated strains of *Lymnaea stagnalis* only respond to sympatric predators and have different memory forming capabilities. J Exp Biol 212:2237–2247. doi:10.1242/jeb.031575

Orr M, Hittel K, Lukowiak KS, Han J, Lukowiak K (2009b) Differences in LTM-forming capability between geographically different strains of Alberta *Lymnaea stagnalis* are maintained whether they are trained in the lab or in the wild. J Exp Biol 212(23):3911–3918. doi:10.1242/jeb.024281

Parvez K, Stewart O, Sangha S, Lukowiak K (2005) Boosting intermediate-term into long-term memory. J Exp Biol 208(8): 1525–1536. doi:10.1242/jeb.01545

Parvez K, Rosenegger D, Orr M, Martens K, Lukowiak K (2006) Canadian association of neurosciences review: learning at a snail's pace. Can J Neurol Sci 33(4):347–356

Relyea RA (2002) Costs of phenotypic plasticity. Am Nat 159(3): 272–282. doi:10.1086/338540

Rescorla RA, Holland PC (1982) Behavioural-studies of associative learning and animals. Annu Rev Psychol 33:265–308. doi:10.1146/annurev.ps.33.020182.001405

Rundle SD, Brönmark C (2001) Inter- and intraspecific trait compensation of defence mechanisms in freshwater snails. Proc R Soc B-Biol Sci 268(1475):1463–1468

Sangha S, McComb C, Scheibenstock A, Johannes C, Lukowiak K (2002) The effects of continuous versus partial reinforcement schedules on associative learning, memory and extinction in *Lymnaea stagnalis*. J Exp Biol 205(8):1171–1178

Sangha S, McComb C, Lukowiak K (2003) Forgetting and the extension of memory in *Lymnaea*. J Exp Biol 206(1):71–77. doi:10.1242/jeb.00061

Sangha S, Scheibenstock A, Martens K, Varshney N, Cooke R, Lukowiak K (2005) Impairing forgetting by preventing new learning and memory. Behav Neurosci 119(3):787–796. doi:10.1037/0735-7044.119.3.787

Spear NE, Miller JS, Jagielo JA (1990) Animal memory and learning. Annu Rev Psychol 41:169–211

Syed NI, Winlow W (1991) Coordination of locomotor and cardio-respiratory networks of *Lymnaea stagnalis* by a pair of identified interneurons. J Exp Biol 158:37–62

Wasserman EA (1981) Response evocation in autoshaping: contributions of cognitive and comparative-evolutionary analyses to an understanding of directed action. In: Locurto CM, Terrace HS, Gibbon J (eds) Autoshaping and conditioning theory. Academic Press, New York

Wixted JT (2004) The psychology and neuroscience of forgetting. Annu Rev Psychol 55:235–269