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Juvenile *Lymnaea* ventilate, learn and remember differently than do adult *Lymnaea*

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Summary

Adult snails are capable of learning associatively not to perform aerial respiration and then to consolidate the acquired behaviour into long-term memory (LTM). Juvenile *Lymnaea*, however, perform aerial respiration significantly less often and the three-neuron circuit that drives this behaviour operates significantly differently than in it does in adults. We asked whether these ontogenic behavioural and neurophysiological differences are manifested as an altered ability of juveniles to learn and/or form LTM. We found that juvenile snails learn significantly less well than adults and are, as a group, incapable of forming LTM. To control for the possibility that the poor learning and inability to form memory were the result of juvenile's receiving on average fewer reinforcing stimuli because they perform aerial respiration less often than adults we subjected juveniles to an enforced period of hypoxia to 'motivate' juveniles. Motivated juveniles perform aerial respiration as often as adults; yet these 'motivated' juveniles continue to be poor learners and still cannot form LTM. Additionally, a small percentage of juveniles perform aerial respiration as often as adults (i.e. high responders). When these 'high-responders were trained they still exhibited poorer learning ability compared with adults and could not form LTM. We conclude that juvenile snails have a more difficult time learning and remembering to suppress aerial respiratory activity than do adults.

Key words: aerial respiration, learning and memory, *in vitro* semi-intact preparation, *Lymnaea*, operant conditioning, associative learning, long-lasting memory.

Introduction

Adult *Lymnaea* are bimodal breathers, satisfying their respiratory needs by both cutaneous and aerial (i.e. ventilatory) respiration (Jones, 1961; Lukowiak et al., 1996; Taylor et al., 2003). In well-aerated (i.e. eumoxia; P_{O_2}>9.9 kPa; 6 ml O_2 l^{-1}) conditions, most of the snail's oxygen requirements are obtained *via* respiration through the skin (i.e. cutaneous respiration, Taylor and Lukowiak, 2000; Taylor et al., 2003). However, when the oxygen content in the water is reduced (i.e. hypoxia; P_{O_2}<9.3 Pa; <0.1 ml O_2 l^{-1}), the frequency of ventilatory behaviour in adult snails increases significantly (Lukowiak et al., 1996; Taylor et al., 2003). To perform aerial respiration, *Lymnaea* visits the surface of the water and opens its respiratory orifice, the pneumostome. This is often repeated several times before the animal re-submerges (Syed et al., 1991; Inoue et al., 2001). Aerial respiratory behaviour in *Lymnaea* thus resembles the periodic breathing behaviour of some species of amphibians, reptiles and diving mammals (Milsom, 1990). Aerial respiratory behaviour in *Lymnaea* is a well-defined, easily monitored, important homeostatic behaviour (Taylor and Lukowiak, 2000; Taylor et al., 2003). A three-neuron central pattern generator CPG drives ventilatory behaviour, and this is one of the few studied behaviours where both the sufficiency and necessity of the neurons comprising a CPG has been experimentally demonstrated (Syed et al., 1990, 1992; McComb et al., 2003).

In adult *Lymnaea* aerial respiratory behaviour can be operantly conditioned and this learning can be consolidated into long-term memory (LTM; Lukowiak et al., 1996, 2003a). Neural correlates of memory formation have been found in RPeD1 (Spencer et al., 1999, 2002), one of the three CPG neurons that drive this behaviour. Moreover, this neuron is a necessary site for the processes of memory consolidation, reconsolidation, extinction and forgetting (Scheibenstock et al., 2002; Sangha et al., 2003a-c, 2005) Interestingly in juvenile *Lymnaea* RPeD1 spontaneous activity is significantly higher than in adults (McComb et al., 2003). Whether this higher level of RPeD1 activity is coincident with altered learning and memory ability has not been experimentally tested.

Typically, younger animals (vertebrate and invertebrate) 'perseverate' (the inappropriate or unintentional repetition of a response or behaviour) (Cider, 1997) on learning tasks where they are required to withhold a behavioural response (Denenberg and Kine, 1958; Peretz and Lukowiak, 1975;

Blozovski and Cudennec, 1980; Mattingly and Zolman, 1980; Dickel et al., 1997, 2000). The neuronal basis of behavioural perseveration is not known. Since adult trained snails learn and remember not to perform aerial respiratory behaviour and since RPeD1 activity in juveniles is different than it is in adults we hypothesize that juvenile *Lymnaea,* on this specific task, will learn and remember more poorly than adults.

Age-dependent changes in neural circuitry have previously been shown to affect learning and memory abilities in a wide variety of preparations. In *Aplysia*, for example, the ability of the gill withdrawal reflex to undergo habituation, dishabituation and sensitization is subject to a specific ontogenic timetable (Rankin and Carew, 1987; Rankin et al., 1987; Carew, 1989; Marcus et al., 1994; Mauelshagen et al., 1996; Stark and Carew, 1999). In vertebrates, the inability to withhold responding is ascribed to the presumed delay in the maturation of inhibitory control over frontal brain structures (Mabry and Campbell, 1973; Myslivecek and Hassmannova, 1983; Band and van der Molen, 2000). By contrast, learning and memory ability, in both vertebrates and invertebrates (including *Lymnaea*) on essential tasks (e.g. taste aversion) or where animals are required to 'perform' a specific behaviour in response to a positive reinforcing stimulus may be similar across ontogenesis (Solyom and Miller, 1965; Roberts, 1966; Yamanaka et al., 1999). In addition to the differences in learning ability they are also age-related differences in the ability to consolidate the acquired behaviour (i.e. learning) into memory. For example, Liston and Kagan (2002) have shown in human infants that long-term retention (>24 h) of a learned event increases during the second year of life coincident with the maturation of the hippocampus and frontal lobe. We show here that juvenile *Lymnaea* exhibit poorer learning compared with adults and are unable to form LTM.

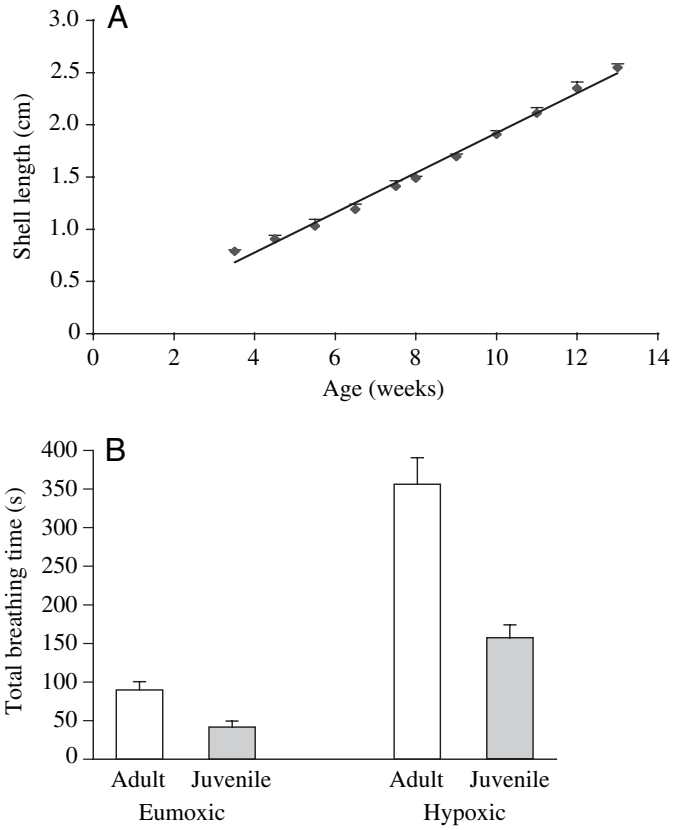

Fig. 1 Shell length, age and ventilatory behaviour in *Lymnaea.* (A) The growth of snails (as measured by shell length) was linear between 3.5–13 weeks of age (post-hatching). Snails were maintained in standard conditions and a cohort (N=33) of randomly selected snails from the over 200 snails in the aquarium were measured weekly. The snails displayed continuous shell growth at a rate of 0.25 mm day^{-1}. Based on this growth curve adults (2.5 cm) and juveniles (1.5 cm) are approximately 13 and 8 weeks old, respectively. (B) Increased aerial respiration in hypoxia. Both adult (N=43 each) and juvenile snails significantly (P<0.01 in both cases) increase their total breathing time when exposed to the hypoxic environment. However in both eumoxia and hypoxia the total breathing time in adults was significantly greater (P<0.001) than in juveniles.

Materials and methods

Lymnaea

Snails (*Lymnaea stagnalis* L.) used in these studies were obtained from the snail raising facility at the Faculty of Medicine, University of Calgary using stocks originally obtained from Vrije Universeit of Amsterdam, The Netherlands and were maintained in the laboratory at room temperature (20–22°C) on an approximately 18 h:6 h L:D cycle throughout the year.

Newly hatched *Lymnaea* (N~200) were maintained in an aquarium lined with crushed oyster shells and filled with well-aerated pond water (de-chlorinated City of Calgary tap water). They were fed on molluscide-free Romaine lettuce *ad libitum.* Every week 33 snails from this population were randomly selected and their shell lengths measured before they were returned to the aquarium. An experimenter 'blind' to the purposes of the study performed the weekly selection of animals to be measured. An age *vs* growth curve for snails reared in the laboratory was constructed (Fig. 1). Newly laid egg masses were never observed in aquaria until snails reached an average shell length of 2–2.5 cm, which we took to indicate that they were now adults.

In eumoxic conditions (i.e. atmospheric air was continuously bubbled into the test beaker; P_{O_2}>9.9 kPa) there is approximately 6 ml O_2 l^{-1} and adult Lymnaea perform aerial respiration infrequently, once every 10–20 min. Under hypoxic conditions, when 100% N_2 is continuously bubbled into the test beaker for at least 20 min, there is less than 0.1 ml O_2 l^{-1} and both the frequency and amount of aerial respiratory behaviour increase significantly in adults under such hypoxic conditions (P_{O_2}<0.9 kPa).

Breathing observations were performed in either eumoxic or hypoxic conditions. For both conditions, a 1 l beaker was filled with 500 ml of pond water and either atmospheric air or N_2 was bubbled through the beaker continuously during the observation period. Individually labeled animals were placed

into the pond water and given a 10 min acclimatization period during which they could freely open their pneumostome. At the end of this period, all snails were gently pushed under the water, signaling the beginning of the 45 min observation session. The opening and closing times for each breath were recorded and the number of breaths, the average duration of each breath, and the total breathing time (calculated by the sum of the duration of each individual breath) were obtained.

Operant conditioning: training and testing procedures

Briefly, animals were placed in a 1 l beaker filled with 500 ml of pond water, made hypoxic (<0.1 ml O_2 l^{-1}) by bubbling N_2 through it 20 min prior to, and during, training sessions. Before each training session, snails were given a 10 min acclimatization period during which they were free to open their pneumostomes. At the end of this 10 min period, animals were gently pushed beneath the water surface, signaling the beginning of the training session. During each training and test session, animals received a gentle tactile stimulus to the pneumostome each time it began to open. This stimulus resulted in the immediate closure of the pneumostome but did not induce the snail to retract its body into the shell, known as the whole-body withdrawal behaviour. Snails typically remain at the surface following the application of the tactile stimulus. The time of each stimulus was recorded and tabulated. With operant conditioning training, animals learned to associate pneumostome opening with the negative reinforcement of receiving a tactile stimulus. In the interval between training and test sessions, animals were placed in eumoxic pond water where they could open their pneumostomes freely (see Lukowiak et al., 1996, 2003b; Spencer et al., 1999; Sangha et al., 2005 for details).

Tactile stimuli delivered to adults and juveniles

Juvenile snails exhibited a stronger behavioural response compared with adults when they were given tactile stimuli of the same intensity. The force of the stimulation to the pneumostome area was therefore adjusted in the experiments dealing with juveniles so that the same behavioural responses were elicited in both juveniles and adults. That is the stimulus did not cause the snail to withdraw into the shell.

Training procedure for long-term memory (LTM)

Snails received two 45 min training sessions separated by a 1 h interval. A 'savings' test session (MT) was given 24 h after the last training session to test for LTM. Long-term memory in adults was present at least 24 h after training (Lukowiak et al., 2000).

Yoked controls

Yoked control experiments were performed to demonstrate that the changes in aerial respiratory behaviour with training were the result of operant conditioning. The day before training commenced, all snails (those to be conditioned and those to serve as yoked controls) received a 45 min pre-test session in hypoxia. During this session, snails received a stimulus to the pneumostome each time it began to open. It was previously shown that one 45 min hypoxic training session is not sufficient to produce long-term memory (Lukowiak et al., 2000). On the following day, snails either received the operant conditioning training procedure (see above) or the yoked control procedure. In the yoked control procedure animals received a tactile stimulus to the pneumostome area every time the snails to which they were 'yoked' attempted to open their pneumostomes. One hour after session 2 all animals (i.e. operantly trained and yoked) were given a 45 min post-test session in hypoxia. During this session, animals received a tactile stimulus to the pneumostome each time it began to open. To determine if tactile stimuli alone (i.e. non-contingent) resulted in a diminution of the response, the number of tactile stimuli pre-test and post-test sessions were compared. This type of yoked control procedure has been used successfully before (e.g. Lukowiak et al., 2003a).

Enforced submersion experiment

Animals were placed in an aquarium filled with pond water made hypoxic by bubbling N_2 through it, prior to and during the experiment. Snails were prevented from reaching the water surface and performing aerial respiration by a submerged, perforated barrier. Animals were submerged for 30 min immediately prior to performing breathing observations or associative training. This enforced submersion 'caused these snails to perform aerial respiration significantly more often; hence we term these animals 'motivated-snails'.

Operational definitions of learning and memory

Associative learning was defined as being present if: (1) there was a significant effect of training on the number of attempted pneumostome openings, and (2) the number of attempted openings in the final training session was significantly less than the number of attempted openings in the first training session.

LTM was defined as being present if: (1) the number of attempted pneumostome openings in the memory-test session was not significantly greater than the last training session; and (2) the number of attempted openings in the memory-test session was significantly less than the number of attempted openings in the first training session.

Statistics

To determine whether operant conditioning training had an effect when compared with the yoked control group a repeated measures one-way ANOVA was performed for both juvenile and adult snails testing both between (e.g. yoked *vs* operantly conditioned groups) and within group differences (Zar, 1999). If the ANOVA was significant ($P<0.05$) a *post-hoc* Fisher's LSD *t*-test was performed to show which individual sessions were significantly different from each other [i.e. for learning session 1 *vs* session 2; for memory the savings test session (MT) *vs* session 1 and MT *vs* session 2]. Significance was at the $P<0.05$.

Results

Shell length vs *age*

Weekly measurements of shell length (N=33 for each measurement) of randomly selected snails from a eumoxic aquarium (Fig. 1A) were made. At 3.5 weeks post-hatching the average shell length was found to be 0.79±0.01 cm. Snails displayed linear and continuous shell growth between 3.5 (0.79±0.01 cm) and 13 (2.5±0.04 cm) weeks post-hatching, at a rate of 0.25 mm day^{-1}. Shell length was found to be a useful measure of age until snails reached shell lengths of approximately 2.5 cm. Snails were classified as 'juveniles' or 'adults' and had shell lengths of 1.5 cm (7.5 weeks post-hatching) and 2.5 cm (13 weeks post-hatching), respectively (a 5.5 week difference in age). We settled on the smallest shell length of 1.5 cm in this study because this size of snail could easily be subjected to handling without causing damage to the snail and allowed easy observation of aerial respiratory behaviour.

In 45 min observation periods (see Materials and methods) we examined aerial respiratory behaviour in juvenile and adult snails in both eumoxic and hypoxic conditions (Fig. 1B). In eumoxic conditions the mean number of breaths in adults (N=43; 4.33±0.47) was significantly greater (P<0.001) than in juveniles (N=43; 2.3±0.32). The total breathing time for adults in eumoxia (89.8±10.6 s) was significantly greater (P<0.001) than it was in juveniles (41.5±7.9 s); but the average time per breath in eumoxia in adults (22.4±1.9 s) was not significantly different (P>0.05) from that in juveniles (17.5±2.4 s).

In hypoxia, somewhat similar results were obtained. That is, the mean number of breaths in adults (10.67±0.47) was significantly greater (P<0.001) than in juveniles (6.69±0.57); the total breathing time for adults (356.42±34 s) was significantly greater (P<0.001) than it was in juveniles (157.3±15 s) but now the average breathing time was also significantly (P<0.05) greater in adults (41.6±7.7 s) than it was in juveniles (23.8±2.2 s).

We conclude that juveniles perform aerial respiration less often than adults in both hypoxic and eumoxic conditions. However, in hypoxia juveniles too, significantly increase aerial respiratory behaviour compared with their performance in eumoxia.

To demonstrate that the changes in aerial respiratory behaviour that result from our training procedure (see Materials and methods) are a *bona fide* example of associative learning we compared the data from operantly conditioned and yoked control preparations in both adults and juveniles (Fig. 2). We first examined four cohorts (N=12 in each) of snails (two juvenile groups – yoked and contingent and two adult groups – yoked and contingent). We compared, as we have previously done (Lukowiak et al., 2003a) the number of attempted openings in the pre-training session to the number of attempted openings in the post-training session. Thus, both between and within group comparisons were made. The ANOVA for the complete data set (i.e. the four cohorts) showed that there was a significant effect of training

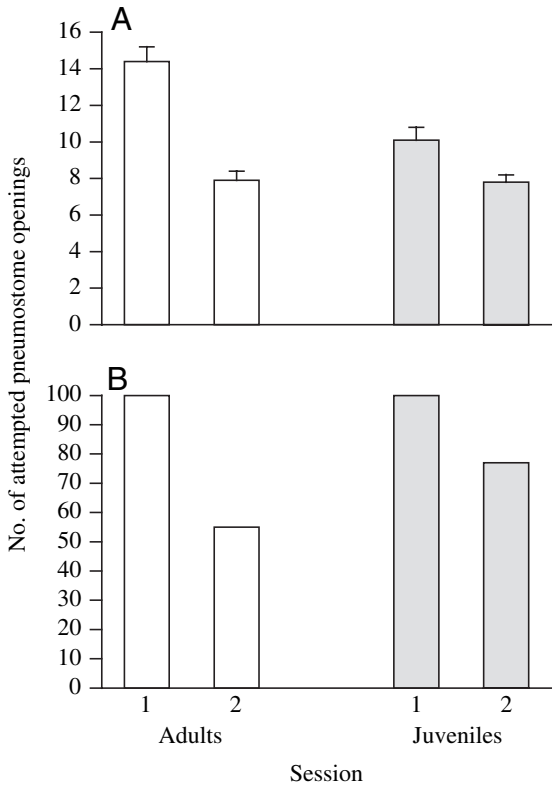

Fig. 2. Juveniles exhibit reduced learning ability compared with adults. (A) Adults and juveniles (N=280 each) were trained using two 45 min training sessions separated by 1 h. Both age groups met the criteria for learning (i.e. a significant reduction in the number of attempted openings between sessions 1 and 2; P<0.001). (B) Normalization of the data plotted in A. The adults show a larger percentage decrease in the number of attempted openings in session 2 compared with juveniles.

($F_{56,7}$=155.5801, P<0.001). We then performed a *post hoc* Fisher's LSD protected t-test on the various test sessions making both within and between group analyses. Consistent with previous findings we found in adult snails that there was a significant decrease in the number of attempted pneumostome openings between the pre-test session and the post-test session (P<0.01) while in the yoked control cohort the post-test session was not significantly less than the pre-test session (P>0.05). Furthermore, the number of attempted openings in the pre-test session was not different between the yoked control and contingent cohort (P>0.05) while the number of attempted openings in the post-test session of the operantly trained cohort was significantly less than in the yoked control cohort (P<0.01). Similar findings were found for the juvenile snails. That is, in the operantly conditioned cohort the number of attempted pneumostome openings in the post-test session was significantly smaller than in the pre-test session (P<0.01); while in the yoked control the post-test session was not significantly less than the pre-test session (P>0.05). When we compared the number of attempted openings between the yoked and operant conditioning juvenile cohorts we found that there was no significant difference

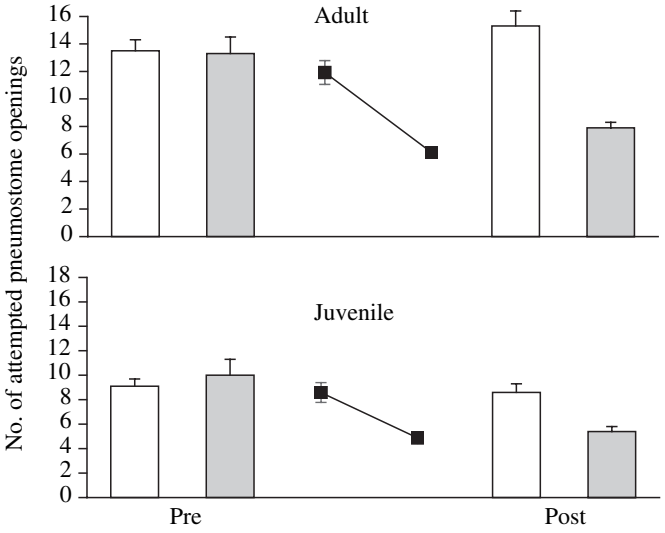

Fig. 3. Yoked control data for adult and juvenile snails. Plotted are the number of attempted pneumostome openings in the 'pre' yoked training session and the 'post' yoked control-training sessions for both operantly conditioned (grey bars) and yoked control (white bars) preparations. Also shown are the results of operant training on the number of attempted pneumostome openings during the course of training. The number of attempted openings in the 'post' session 1 h after the session 2 of both adult and juvenile snails did not significantly decrease in the yoked control snails as it did for the snails that received the operant conditioning training procedure.

between the cohorts in the pre-test session (P>0.05); while the number of attempted openings in the post-test session of the operantly conditioned juveniles was significantly smaller than in the yoked control cohort (P<0.01). Finally, the number of attempted openings in both of the adult pre-test sessions was significantly greater than the number of attempted openings in the pre-test sessions of the juvenile cohorts (P>0.01 for both comparisons). These analyses showed that associative learning occurs in both adults and juveniles.

To begin to determine whether there were age-related differences in learning ability between juvenile and adult *Lymnaea* we subjected a relatively large number of juvenile and adult snails (N=280 snails each) to the operant conditioning procedure. In both adult and juvenile snails learning was demonstrated (Fig. 3A). That is, the number of attempted pneumostome openings in session 2 was significantly less than in session 1 (P<0.01). Normalized data of learning ability revealed that adults and juveniles reduced their attempted openings in the second training session by 46% and 30%, respectively (Fig. 3B).

Learning and memory, while related, are not a unitary process. We therefore determined if they were also age-dependent differences in the ability to consolidate the associative learning into LTM (Fig. 4). We hypothesized that the reduced learning ability in juveniles (only a 30% *vs* a 46% change) compared with adults would result in poorer memory retention. To investigate the retention of LTM, adults and juveniles were given two 45 min training sessions (separated

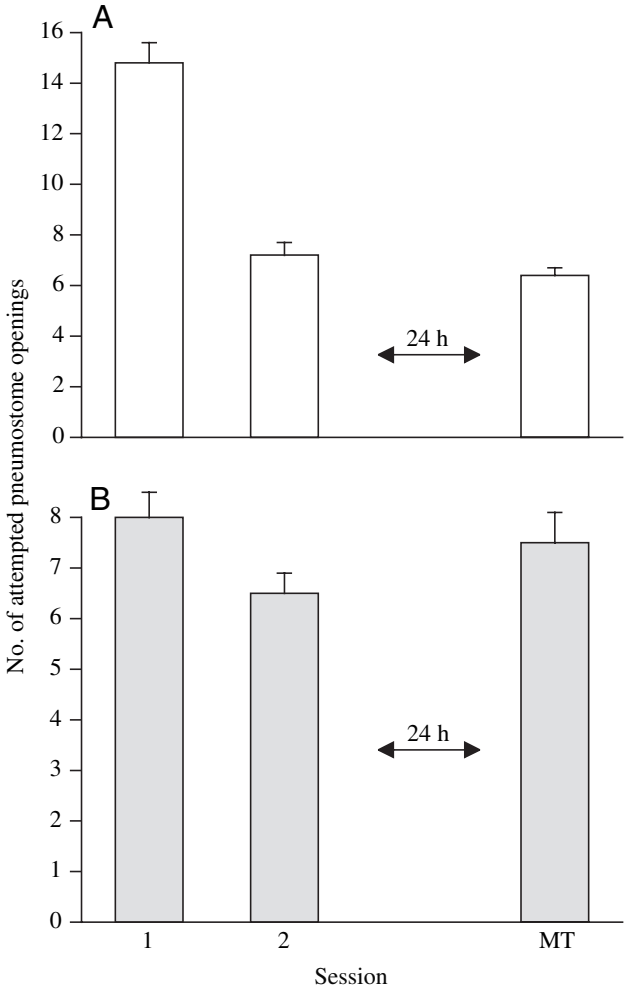

Fig. 4. Adults exhibit LTM but juveniles do not. (A) Adult (N=75) snails subjected to the LTM training procedure exhibited LTM when tested 24 h after the last training session (MT). That is, the criteria for LTM (the number of attempted openings in MT was significantly less than in session 1 but was not significantly greater than in session 2) were met. (B) Juvenile (N=75) snails trained and tested in the same manner as the adults in A did not meet the criteria necessary for LTM.

by 1 h) followed by a savings-test 24 h later. Adults, but not juveniles, met the criteria for long-term memory when tested 24 h after the last training session. That is, in adult snails the number of attempted openings in the savings-test session was significantly different from the number of openings in session 1 (P<0.01), but was not significantly different from the number of openings in session 2 (P>0.05). In juveniles, however, there was no significant difference in the number of attempted openings between the savings-test session (MT) and session 1 (P>0.05; i.e. memory not demonstrated), while there was a significant difference between the number in MT and session 2 (P<0.01). Thus while the criteria for memory were met in adults they were not met in juvenile snails. Adults were found to show a 51% reduction in their attempted openings between session 1 and MT. By contrast, juveniles demonstrated only a slight decrease (6%) in the number of attempted pneumostome

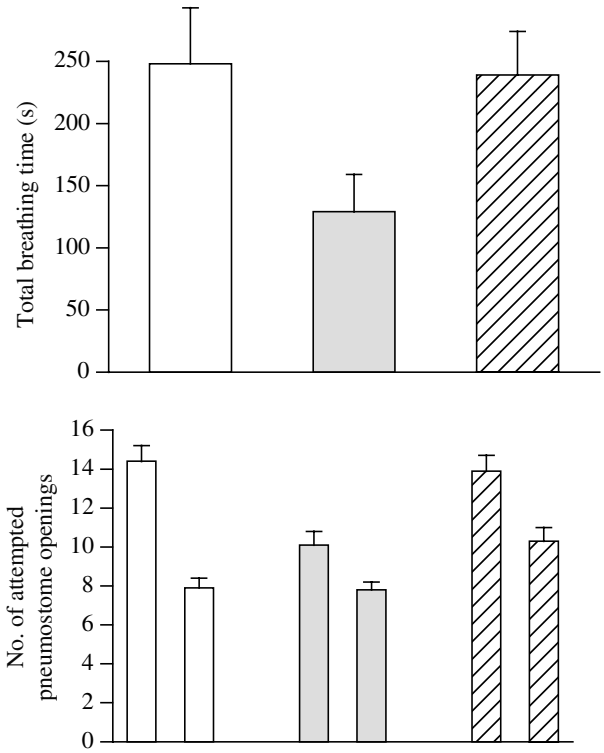

Fig. 5. Motivated juveniles do not exhibit better learning than control juveniles. To motivate juvenile snails we submerged them in hypoxic pond water for 30 min before training. (A) Preventing juvenile snails from performing aerial respiration for 30 min (i.e. submerged juveniles) before the observation period significantly increases their total breathing time. Plotted are the total breathing time (mean ± S.E.M.) for adults (N=15, clear bar), juveniles (N=15, grey bar) and submerged juveniles (N=12, striped bar). The submerged juveniles breathe significantly longer than control juveniles (P<0.01) and statistically the same as adults. (B) Submerged juveniles (N=28, striped bars) received operant conditioning training immediately after being submerged. The number of attempted openings of the submerged juveniles in session 1 was not significantly different to adults (P>0.05). These snails had a 27% reduction in the number of attempted openings in session 2 compared with session 1. Control juvenile snails exhibited a 24% reduction in session 2 compared with session 1.

openings between session 1 and MT. We conclude that only adults possess the capacity of consolidating the learned behaviour into LTM.

On inspection of the data in the previous figures it can be readily seen that juveniles perform, or attempt to perform, aerial respiration less often than adults and, thus, receive fewer reinforcing stimuli. It could be argued this could be a reason why there is 'less' robust learning and no memory in juveniles compared with adults. To determine whether the differences in learning ability and LTM formation between juveniles and adults were due solely to differences in the number of reinforcing stimuli delivered during training, we performed additional experiments and additional re-analyses of the data shown above.

To increase the number of reinforcing stimuli delivered to juvenile snails to levels approximating those in adults, juveniles were submerged in hypoxic conditions for 30 min prior to performing breathing observations. Submersion (i.e. the prevention of aerial respiration; see Materials and methods) significantly increased total breathing time in juveniles to a level that was not significantly different from adults (P>0.05) (Fig. 5A). In these 'motivated' juvenile snails the number of attempted openings in session 1 was not significantly different from adults (P>0.05) (Fig. 5B). However, while submersion significantly increased the number of reinforcing stimuli received by the 'motivated' juvenile snails it did not affect learning ability in juveniles. That is, learning ability was still found to be significantly poorer in the motivated snails compared with adults (P<0.05). Moreover, the 'motivated' juvenile snails did not learn any better than the 'un-motivated' juvenile snails (P>0.05). Submerged and non-submerged juveniles showed a 27% and 24% reduction in attempted openings between sessions 1 and 2, respectively. Based on these results we conclude that the difference in learning between juveniles and adults was not caused by the juveniles receiving fewer reinforcing stimuli than adults during training.

We also re-examined the data presented in Fig. 4 but *post-hoc* extracted and re-analyzed the top 25% juvenile responders (N=20; 'high responders'). These data are plotted in Fig. 6, as well as data from adults (N=20) randomly chosen from the adult cohort used in Fig. 4. We first performed a two-sample t-test comparing the number of attempted pneumostome openings in session 1 between the juvenile high responders (N=20) and the randomly chosen adults (N=20) and found that statistically they were not different (P>0.05). Thus, the juvenile 'high responders' received statistically the same number of reinforcing stimuli as the randomly chosen adults. We then determined if the juvenile 'high performers' have the ability to form LTM. An ANOVA showed that there was a significant effect of training on the two cohorts (high responders and randomly chosen adults; ($F_{39,5}$=156.6676, P<0.001). We then performed a *post hoc* Fisher's LSD protected t-test on the various test sessions making both within and between group analyses. Confirming our previous analyses we found that the adult snails have the ability to form LTM. That is, the number of attempted openings in the memory test session (MT) was significantly less than the number of attempted openings in session 1 (P<0.01) but was not significantly greater than the number in session 2 (P>0.05). On the other hand, the within group analysis of the juvenile 'high-responders' showed that memory was not formed. That is the number of attempted openings in MT was not significantly less than the number in session 1 (P>0.05) while the number of attempted openings in MT was significantly greater than session 2 (P<0.01). The criteria for us to conclude that memory was present were not met. The between group analyses showed that the responses in session 1 between adults and these juveniles were statistically similar (P>0.05), but the responses in MT were different, the randomly chosen adults received significantly fewer reinforcing stimuli (P<0.01) than the juvenile high responders. That is, the adult snails exhibited

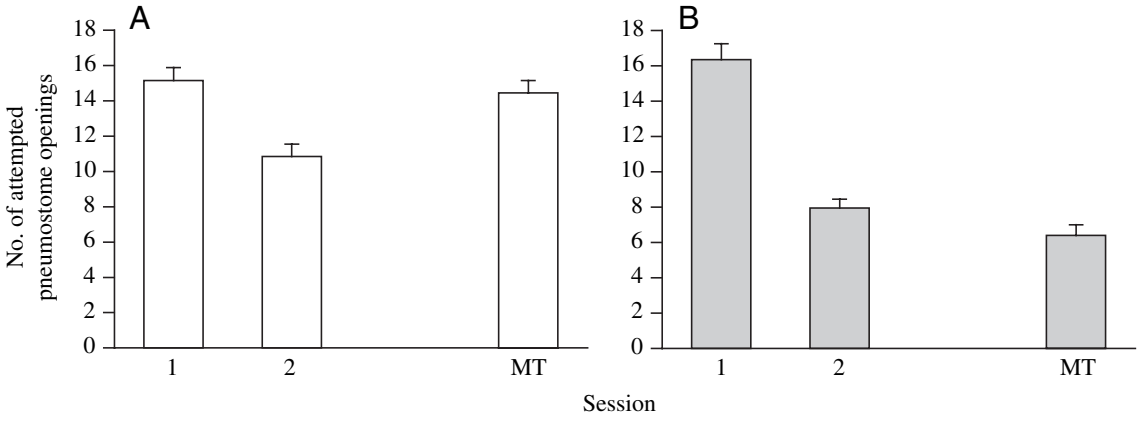

Fig. 6. Juvenile 'high responders' do not exhibit LTM. (A) Data from the 20 juvenile 'high responders' previously plotted in Fig. 4 do not demonstrate LTM even though they received the same number of reinforcing stimuli as the adults in B. (B) The data from 20 randomly chosen adult snails from the data in Fig. 4 show LTM when tested 24 h after the last training session (MT).

memory whereas the juvenile high responders did not exhibit memory.

Approaching the argument from the other direction we also re-analyzed the data from adult 'low-responders' (i.e. the 25% that received the fewest number of stimuli in session 1). The number of reinforcing stimuli these adult snails received in session 1 was 6.78±0.65. Yet, these snails when tested in MT (4.1±0.32) exhibited LTM. That is, the number of attempted openings in MT was significantly less than in session 1 ($P<0.01$) but was not significantly different than the number of attempted openings in session 2 (3.9±0.29; $P>0.05$). Thus, these adult 'low responders' have the capacity to form LTM.

Discussion

As previously reported by others (Janse et al., 1989), shell length was found to be a useful measure of age. We used snails (shell length) measuring 1.5 cm (juvenile) and 2.5 cm (adult) respectively, which differ in age by 5.5 weeks. We classified the 1.5 cm snails as juveniles since they were not observed to engage in copulatory behaviour nor did we ever observe an egg mass in their aquaria. Copulatory behaviour and egg masses were observed in aquaria housing snails that were 2–2.5 cm in shell length, hence they were classified as adults.

We first tested the hypothesis that juveniles show different aerial respiratory behaviour in both eumoxic and hypoxic conditions compared with adults. Juveniles exhibited significantly less ventilatory behaviour compared with adults during 45 min breathing observations in both hypoxia (0.1 ml O_2 l^{-1}) and eumoxia (6 ml O_2 l^{-1}). That is, total breathing time, the average time per breath, and the mean number of breaths were reduced in juveniles compared with adults. We conclude that juvenile snails satisfy their respiratory needs to a lesser extent than adults *via* aerial respiration. That is, in juveniles cutaneous respiration satisfies their respiratory needs to a greater extent than it does in adults.

These results are not surprising, considering juvenile *Lymnaea* have a larger surface area to volume ratio than adults and this could result in more efficient cutaneous respiration. That is, juveniles presumably obtain a higher percent of their ongoing oxygen requirements *via* cutaneous respiration, reducing their need to perform ventilatory behaviour. In eumoxia, adult *Lymnaea* on average spend approximately 4% of their time performing aerial respiration whereas juveniles spend less than 15% of their time engaged in this activity. Adult *Lymnaea* spend about 15% of their time performing aerial respiration in hypoxic conditions; under similar conditions juveniles spend only ~5% of their time engaged in aerial respiration. In a hypoxic environment there is less dissolved oxygen in the pond-water causing inadequate cutaneous respiration in both juvenile and adult snails. Thus both juvenile and adult *Lymnaea* when challenged with hypoxic conditions must significantly increase ventilatory behaviour in order to preserve respiratory homeostasis.

Studies in land snails (Herreid, 1977), air-breathing fish (Munshi and Dube, 1973), and Xanthid crabs (Leffler, 1973) have shown that metabolic rates, measured by oxygen consumption, are higher in smaller animals. Therefore, one would expect small (juvenile) snails to have higher metabolic requirements compared with larger (adult) snails. Yamanaka et al. (1999) examined voluntary activity in *Lymnaea* of different ages and found that 'immatures' (shell length ~1.0–1.5 cm) showed significantly more activity compared with 'adults' (shell length >2.0 cm). Based on the above reports, one would expect juvenile snails to have higher metabolic requirements compared with larger, adult snails. But then the obvious question is: why do juveniles exhibit less aerial respiratory behaviour compared with adults? Even if juveniles do have higher oxygen requirements compared with adults, they have a more favourable surface area to volume ratio and, possibly, more efficient cutaneous respiration, which enables juveniles to meet their respiratory needs without having the need to increase aerial respiratory behaviour. It may be that there is positive survival value (i.e. less predation or less chance to

desiccate) for not having to spend time at the surface opening and closing the pneumostome. Cutaneous respiration may also metabolically 'cost' less than aerial respiration and, thus, juveniles would have to expend less of their energy intake on breathing and more on maturational processes. Higher levels of activity have been found to correspond to higher metabolic rates (Herreid, 1977). Cutaneous respiration must therefore adequately satisfy the presumed increased metabolic needs of the juveniles. It may be that there is a higher positive survival value for not having to spend more time at the surface opening and closing the pneumostome in juveniles than in adults.

Having found an age-related difference in the need to perform aerial respiration we next turned our attention to the question as to whether there would be an age-related impairment in juvenile snail's ability to learn and/or form memory in an associative learning task involving aerial respiratory behaviour. We found that juvenile *Lymnaea* are significantly poorer learners on this task compared with adults and, importantly, as a group are unable to consolidate the learning into LTM. This should not be taken to mean that juvenile *Lymnaea* are incapable of learning or forming LTM. Juvenile *Lymnaea*, as do other animals, have the capacity to learn and form LTM on tasks not involving the withholding of a behaviour. Thus for example, juvenile *Lymnaea* learn and form memory for appetitive food behaviours (Yamanaka et al., 2000).

A possible explanation for explaining why juveniles have a greater difficulty acquiring learning and then consolidating this change into LTM than do adults is that the juveniles do not receive a sufficient number of reinforcing stimuli during the training sessions. We concluded, however, that this was not the explanation. We arrived at this conclusion because we found that: (1) 'motivated' juveniles (i.e. the ones in the submersion experiments) received as many reinforcing stimuli in session 1 as adults yet continue to exhibit poorer learning compared with adults; (2) the 'motivated' juvenile snails do not learn any better than un-motivated' juvenile snails, thus more reinforcing stimuli does not necessarily result in better learning; (3) when we analyzed the ability of the highest responding juveniles (i.e. the 'high responders' in Fig. 4) to form memory, we found that they did not, that is, even though statistically they received the same number of reinforcing stimuli as randomly selected adults, they still did not form memory; (4) adult 'low responders' formed LTM even though they received fewer reinforcing stimuli than the juveniles; (5) finally, Smyth et al., 2002 showed that adult snails that were trained using three 15 min training sessions (each session separated by a 1 h interval) procedure exhibited LTM. These adult snails received fewer reinforcing stimuli (average in session 1 was 4.8) than did the juveniles here, yet LTM was established.

Why, then, can't juveniles form LTM? We do not have a definitive answer but have a number of testable hypotheses. We know in adult *Lymnaea* (Scheibenstock et al., 2002; Sangha et al., 2003a; Sangha et al., 2005) that RPeD1 is a necessary site for LTM formation. We also know that there are significant differences in the levels of spontaneous RPeD1 between juvenile and adults (McComb et al., 2003). RPeD1 is, in juveniles, significantly smaller than it is in adults and its smaller cell size coupled with significant differences in a number of its intrinsic membrane properties (i.e. membrane resistance, time constant and rheobase current) contribute to its increased neuronal excitability. That is, RPeD1 is significantly more active in juveniles than it is in adults. This increased excitability of juvenile RPeD1s paradoxically results in a decreased ability to elicit respiratory rhythmogenesis, as the neuron is firing outside of its optimal range to induce rhythmic activity in the neuronal circuit that controls aerial respiratory behaviour (McComb et al., 2003; Turrigiano, 1999). This increased excitability of RPeD1 in juveniles may contribute to their poor learning and memory formation abilities. That is, since LTM formation depends on altered gene activity and new protein synthesis in RPeD1; it is possible that the transcription factors that must be activated and/or inhibited in RPeD1 to initiate the molecular cascade necessary for the formation of LTM may also be dependent on some optimal level of RPeD1 activity. However, further experimentation (e.g. altering the activity of RPeD1 by the injection of hyperpolarizing current during the course of training) in an *in vitro* preparation will be necessary before we can accept this hypothesis.

Our data concerning the acquisition of learning and its lack of consolidation into LTM are consistent with previous data from juveniles that typically perseverate on tasks where they have to withhold a response, including habituation (Peretz and Lukowiak, 1975; Lukowiak, 1980), suppression of attack responses (Wells, 1962; Dickel et al., 1997), passive avoidance (Blozovski and Cudennec, 1980; Mattingly and Zolman, 1980), spatial discrimination (Bronstein and Spear, 1972) and the classical conditioning of *Lymnaea's* whole-body withdrawal response (Ono et al., 2002). We now have the possibility of determining whether the inability to form LTM for associative learning is dependent partly or completely on differences in a single neuron.

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