The Journal of Experimental Biology 211, 2807-2816
Published by The Company of Biologists 2008
doi:10.1242/jeb.020172

Comparing memory-forming capabilities between laboratory-reared and wild *Lymnaea*: learning in the wild, a heritable component of snail memory

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Accepted 19 June 2008

SUMMARY

We set out to determine whether the ability to form long-term memory (LTM) is influenced by laboratory rearing. We investigated the ability of four populations of *Lymnaea stagnalis* to form LTM following operant conditioning both in the freely behaving animal and at the electrophysiological level in a neuron, RPeD1, which is a necessary site for LTM. We hypothesized that laboratory rearing results in a decreased ability to form LTM because rearing does not occur in an 'enriched environment'. Of the four populations examined, two were collected in the wild and two were reared in the laboratory – specifically, (1) wild Dutch snails; (2) their laboratory-reared offspring; (3) wild Southern Alberta snails (Belly); and (4) their laboratory-reared offspring. We found that Belly snails had an enhanced capability of forming LTM compared with Dutch laboratory-reared snails. That is, the Belly snails, which are much darker in colour than laboratory-reared snails (i.e. blonds), were 'smarter'. However, when we tested the offspring of Belly snails reared in the laboratory we found that these snails still had the enhanced ability to form LTM, even though they were now just as 'blond' as their laboratory-reared Dutch cousins. Finally, we collected wild Dutch snails, which are also dark, and found that their ability to form LTM was not different to that of their laboratory-reared offspring. Thus, our hypothesis was not proved. Rather, we now hypothesize that there are strain differences between the Belly and Dutch snails, irrespective of whether they are reared in the wild or in the laboratory.

Key words: *Lymnaea*, long-term memory, wild, electrophysiology, operant conditioning, environmental enrichment.

INTRODUCTION

Despite a rich history of exploration, investigating how genetic predisposition versus environmental experience affects the ability to learn and form memory remains poorly understood. The positive effects of 'environmental enrichment' on brain function (van Praag et al., 2000), including improved memory formation (Berardi et al., 2007; Fischer et al., 2007; Harburger et al., 2007; Rosenzweig et al., 1993) as well as neuroanatomical, neurochemical and behavioral consequences (Renner and Rosenzweig, 1987; Rosenzweig, 1979), have been described for a wide range of species and sensory systems. Evidence exists in vertebrates and invertebrates demonstrating an innate 'hardwired' component to cognitive processing as well as a parallel network that is adaptable for associative learning processes (Kobayakawa et al., 2007; Suh et al., 2004; Tobin et al., 2002). The interaction between 'hardwired' behaviors and how they are modified by experience is unclear.

Few model systems exist where the: (1) essential neural circuit mediating a behavior is known; (2) behavior is easily observable, interesting and tractable; and (3) opportunity exists to investigate both laboratory-reared and naturally occurring wild populations. One system that meets these criteria is aerial respiratory behavior in the pond snail *Lymnaea stagnalis*. In *Lymnaea*, the aerial respiratory behavior is driven by a three-neuron central pattern generator (CPG) whose sufficiency and necessity has been documented (Syed et al., 1990; Syed et al., 1992). The behavior exhibits associative learning and long-term memory (LTM) (Lukowiak et al., 1998; Lukowiak et al., 1996; Lukowiak et al., 2003b; Martens et al., 2007a; Martens et al., 2007b; Parvez et al., 2006b). Moreover, not only have electrophysiological correlates of memory formation been demonstrated in a single neuron, RPeD1, that is a member of the three-neuron CPG (McComb et al., 2005; Spencer et al., 2002; Spencer et al., 1999) but it has also been shown that this neuron is a necessary site for formation of LTM, reconsolidation, extinction and forgetting (Sangha et al., 2003a; Sangha et al., 2005; Sangha et al., 2003b; Scheibenstock et al., 2002). Finally, *Lymnaea* is a cosmopolitan species that can be easily collected in the wild and whose progeny can then be maintained in the laboratory for many generations. The snails collected in the wild (either in The Netherlands or Southern Alberta, Canada) are much darker in colour than snails reared in the laboratory (Fig. 1). We therefore referred to the laboratory-reared snails as 'blonds'.

We performed a series of pilot experiments using locally obtained *Lymnaea* (i.e. *Lymnaea* from the Belly river drainage in Southern Alberta; see Materials and methods) and found to our amazement that these snails had a significantly enhanced ability to form LTM compared with our laboratory-reared 'blond' snails. Our 'blond' snails are descended from snails that were originally collected in Utrecht Province in The Netherlands in the 1950s. Thus, we hypothesized that rearing snails in the laboratory not only resulted in their colour lightening but also resulted in snails that had a diminished capacity to form LTM. Possibly, this cognitive disability arose because they were reared in an 'unenriched environment' compared with snails reared in the wild. We first tested this hypothesis by 'enriching' the laboratory environment by introducing the presence of a sympatric predator, crayfish to the laboratory-reared snails. We demonstrated that our laboratory-reared *Lymnaea* maintained the ability to detect and respond to the scent of a crayfish with multiple predator-avoidance behaviors and changes in the electrophysiological properties of RPeD1 (Orr et al., 2007). Thus, this instinctual behavior was maintained in these laboratory-reared

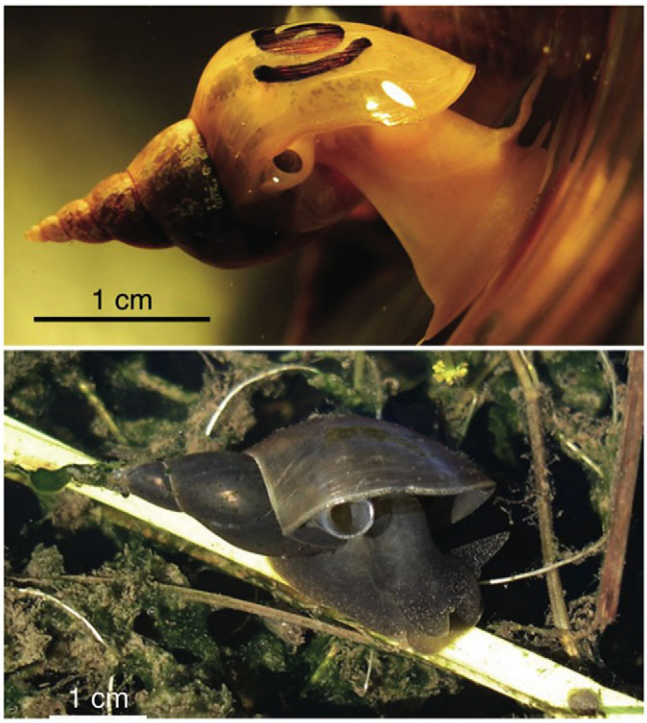

Fig. 1. (A) Laboratory-reared snail performing aerial-respiratory behavior at the air–water interface. Note that the markings on the dorsal shell are utilized in the laboratory for identifications purposes. (B) A wild *Lymnaea stagnalis* aerial-respiring in its native habitat. The bars indicate that the wild snail is approximately twice the size of the laboratory snail.

snails. We further demonstrated that this predator-detection enhanced formation of LTM both behaviorally and electrophysiologically in RPeD1 (Orr and Lukowiak, 2008).

Here, we test the hypothesis that laboratory rearing reduces the capability of snails to form LTM following operant conditioning of aerial respiratory behavior. However, the data presented here are inconsistent with this hypothesis and caused us to formulate another set of hypotheses: first, laboratory rearing does not alter the ability of snails to form memory and, second, there are significant strain differences in memory capability between Dutch and Belly snails, which are stable regardless of rearing conditions. That is, there is a heritable component to this memory ability.

MATERIALS AND METHODS

Snails

The great pond snail *Lymnaea stagnalis* L. is a cosmopolitan species found in temperate regions worldwide. In this investigation, we utilized four different populations of snails, two wild and two laboratory reared. We examined two geographically distinct wild populations of snails: first, wild snails collected from polders near Utrecht in Amsterdam (wild Dutch; latitude: 52°16′ N; longitude: 5°17′ E; elevation: –1 m) and, second, wild snails collected from six seasonally isolated ponds in the Belly river drainage in Southern Alberta, Canada (termed Belly snails; latitude: 49°31′ N; longitude: 113°16′ W; elevation: 961 m). *Lymnaea stagnalis* in Alberta were identified by using previously established criteria (Clarke, 1981; Clifford, 1991) as well as descriptions from other published works of snails in a similar locality (Boag and Pearlstone, 1979; Boag et al., 1984). In order to ensure further that both the Albertan and Dutch snails were in fact the same species, cross-breeding experiments between wild Albertan and Dutch snails were conducted to ensure that the progeny themselves produced viable offspring. As this was the case, we concluded that these were in fact the same species. A representative specimen of each population is shown in Fig. 1.

We also collected egg sacs from Belly snails and reared them in separate aquaria in the snail facility at the University of Calgary until adulthood (referred to as Belly F1s). Finally we also used laboratory-reared snails, which have been maintained in Calgary since 1988 (a gift of Vrije Universeit, Amsterdam, The Netherlands). The original Amsterdam colony was established in the mid-1950s from snails collected in a polder near Utrecht. Cohorts of 10–14 adult snails with a shell length of 30–55 mm for wild snails and 21–26 mm for F1 snails were labeled and maintained within home eumoxic aquaria (P_{O_2}>9975 Pa) at room temperature (≈20°C) until training.

Breathing observations

To ensure that aerial respiratory behavior between these populations was directly comparable, we measured several aerial respiratory parameters of naive snails from each population, which is also the same hypoxic challenge the snails experience during operant conditioning (see below). Briefly, snails were placed in 500 ml of room temperature hypoxic pond water (P_{O_2}<931 Pa) and the time, duration and number of pneumostome (the respiratory orifice) openings were noted during a 0.5 h period. From these measurements, the number of openings, total breathing time and average breathing time for the different populations of snails were calculated.

Operant conditioning

Snails were removed from their home aquaria and placed into a 1-liter beaker containing 500 ml of hypoxic pondwater (PW). PW is made hypoxic by bubbling N_2 gas through the water for 20 min before introducing the snails. The animals are given a 10 min acclimatization period before the 30 min training session. By subjecting snails to a hypoxic challenge, the animals increase their rate of aerial respiration (Lukowiak et al., 1998; Lukowiak et al., 1996). The animals are operantly conditioned by applying a gentle tactile stimulus with a sharpened wooden applicator to their pneumostome as the pneumostome begins to open. The stimulus is strong enough to cause the snails to close the pneumostome yet gentle enough that the snails do not perform the full body-withdrawal response. The contingent stimulation is given during both the training session (TS) and during the test for memory (TM). This pneumostome-closure response is a graded part of the whole-snail escape response (Inoue et al., 1996). Every time the snail opens its pneumostome and receives the stimulus during the training period, the time is recorded for future use in yoked control experiments. Yoked controls (see below) were performed for all behavioral and electrophysiological experiments. All behavioral experiments were run concurrently and were performed ‘blind’, where the person performing the training paradigm was unaware of the status of the cohort being tested. Because there is an obvious difference in the size of wild and laboratory-reared snails, the ‘blind’ testers were able to discriminate between wild and laboratory-reared snails; however, the testers were unable to discriminate between the origin of either the wild Alberta or Dutch snails as well as either of the laboratory-reared cohorts during training.

The operant conditioning procedure we utilized consists of a single 30 min TS, after which the snails are returned to their home aquaria. The snails are then tested for memory (TM; i.e. a ‘savings-

test') using a test similar to that of the training session, or the group is then subject to electrophysiological testing in lieu of the TM. The time of the TM or recording is indicated as time after the TS.

Yoked control experiments

During the training period, yoked control snails received exactly the same number and sequence of stimuli as those of the operant conditioning group; however, the stimuli were not contingent upon their pneumostome opening. However, these yoked control snails did receive a contingent stimulus to the pneumostome during the savings test session (TM). Snails that received yoked training were treated in an identical manner as that outlined in the 'yoked operant conditioning procedure' used previously (Lukowiak et al., 2000; Lukowiak et al., 1998; Lukowiak et al., 1996; Lukowiak et al., 2003c).

Semi-intact preparation and electrophysiological recordings

The *Lymnaea* semi-intact preparation used here is similar to those used previously (Inoue et al., 2001; McComb et al., 2003; McComb et al., 2005; Spencer et al., 2002; Spencer et al., 1999) except that only the penis was removed, the head–foot complex and buccal mass being left fully intact (Orr et al., 2007). Preparations were pinned down in individual recording dishes with their dorsal sides uppermost. The central ring ganglia (CNS) were pinned to the dish directly through the foot musculature, with the dorsal-side up. The outer sheath surrounding the CNS was removed using fine forceps. Enzymatic softening of the sheath was not used in any of our recordings. Standard electrophysiological techniques were used, as described in the above-referenced reports. Intracellular recordings were obtained using sharp glass microelectrodes filled with saturated K_2SO_4 solution. The tip resistances of the microelectrodes used for recordings ranged from 20 to 30 MΩ. Intracellular signals were amplified by means of a NeuroData amplifier and displayed simultaneously on a Macintosh PowerLab/-4SP (AD instruments, Colorado Springs, CO, USA) and a Hitachi oscilloscope. Recordings were analyzed and stored using the PowerLab software. Complete details have been published elsewhere (McComb et al., 2003; McComb et al., 2005). Once RPeD1 was successfully impaled, the cells were given a minimum stabilization period of 10 min after which a 600 s trace was used for analysis. Nine electrophysiological characteristics were measured for each recording and are as follows: (1) total number of action potentials (APs) per 600 s, (2) total frequency, (3) resting membrane potential, (4) number of APs per burst, (5) burst frequency, (6) after hyperpolarization of the first AP in each burst, (7) average AP peak of each burst, (8) burst duration and (9) the number of bursts per 600 s.

Operational definition of learning and memory

As described previously (Lukowiak et al., 2000; Lukowiak et al., 1998; Lukowiak et al., 1996) for the single 0.5 h training session, memory is defined as a significant reduction in the number of attempted pneumostome openings in the memory test session (TM) compared with the training session (TS). That is, TM must be significantly less than TS, and the TM of the corresponding yoked cohort must not be significantly different from the TS.

Snail grades

Another measure of memory that we have previously used is to assign a 'mark' to each snail whether they performed extremely well or very poor in TM. That is, individual snails were given grades based upon their performance (Lukowiak et al., 2003c; Rosenegger et al., 2004). Briefly, an 'A' grade was given if there was greater than a 50% reduction in the number of attempted pneumostome openings in the TM compared with the TS, whereas a 'B' grade was given for a 35–49% reduction; a 'C' grade for a 20–34% reduction and an 'F' grade was given if the decrease was less than 20%.

Statistics

We analyzed operant conditioning effects on snail behavioral data with repeated-measures ANOVA, where the within-subject factors of populations were used and the between-subject factor of Interval (time in days) was used. All repeated-measures data were tested for equal variance using Mauchly's test for sphericity. In cases where sphericity could not be assumed, we used the conservative adjusted Greenhouse–Geisser *P*-values. For cases in which we identified a significant interaction between the repeated factor and the population, we used repeated contrasts to identify which treatment pairs differed significantly. Electrophysiological data were analyzed using ANOVA with a Tukey's *post hoc* test to detect cases in which we identified a significant interaction. Nonhomogenous data (number of spikes per 600 s interval, spikes per burst, burst duration and number of bursts) were log transformed to homogenize between treatment data before ANOVA. Grade distributions (i.e. 'marks') were compared using a chi-squared (χ^2) comparison of proportions test. In cases where the number of samples, *N*, between populations was uneven (grades comparison), a random selection of animals was used from each population to match the *N* value of the smallest sample in the analysis. In all analyses reported here, a type I error rate of 0.05 was used. All statistics were run on SPSS Macintosh OSX version 11.0.4 (SPSS Inc., Chicago, IL, USA).

RESULTS

Considering that all of our previous work on *Lymnaea* has utilized a strain that was originally derived from canals in a polder located near Utrecht in the early 1950s and has been reared in the laboratory ever since, we thought it would be interesting to sample snails (e.g. test their ability to learn and remember) from: (1) a local wild population (Belly snails), (2) their laboratory-reared offspring (Belly F1s) and (3) a wild population (wild Dutch) from the area where the founding Amsterdam colony was originally collected in order to see how they compared with the established laboratory population (referred to as 'laboratory snails') reared in our laboratory.

Aerial respiratory behavior of wild and laboratory-reared snails

To ensure that aerial respiratory behavior between the four different snail populations was similar and therefore directly comparable, we measured aerial respiratory behavior between both wild (Belly and Dutch) and both laboratory-reared (Belly F1s and 'laboratory snail') populations. We found no significant difference between the wild and the laboratory-reared populations in the number of pneumostome openings, total breathing time or average breathing time (Table 1). We concluded that the aerial respiratory behavior is similar between the Belly and Dutch populations of snails we sampled regardless of whether they were reared in a natural or laboratory setting.

Behavioral memory profile of the wild and laboratory-reared populations

After confirming that the aerial respiratory behavior of all four populations of snails was similar, we could begin to test the hypothesis that laboratory rearing results in a diminished capacity to form LTM following operant conditioning of aerial respiration.

Table 1. The mean (±s.e.m.) number of pneumostome openings, total breathing time and mean breathing time of snails from each of the four populations

	Population	*N*	Mean ± s.e.m.
Number of pneumostome openings	Wild Belly	14	8.29±1.495
	Belly F1	15	6.93±1.422
	Wild Dutch	12	8.92±1.598
	Lab Dutch	15	8.53±0.729
Total breathing time (s)	Wild Belly	14	230.50±44.178
	Belly F1	15	175.40±24.733
	Wild Dutch	12	237.17±19.472
	Lab Dutch	15	222.13±31.191
Mean breathing time (s)	Wild Belly	14	28.14±4.309
	Belly F1	15	22.27±3.517
	Wild Dutch	12	32.92±5.336
	Lab Dutch	15	26.20±2.196

No significant difference was found between any populations for each of the measures of breathing characterized.

We first compared the memory-forming abilities of the Belly snails and the Dutch derived laboratory-reared snails. When the Belly snails were subjected to the single 0.5 h training session (TS), we found that these snails formed LTM (Fig. 2A, black bars). In distinct contrast, laboratory-reared snails (Fig. 2B) receiving the same training procedure did not exhibit LTM. That is, Belly snails formed LTM following a single 0.5 h TS, whereas laboratory-reared (i.e. Blond snails) did not.

We next sought to determine the how long the LTM persisted in the Belly snails following the single 0.5 h TS. We found that LTM persisted for at least 72 h. That is, there was a significant reduction in the number of attempted pneumostome openings at the 72 h TM compared with the TS. Yoked control Belly snails did not demonstrate a significant reduction in the number of attempted openings at the 72 h TM (Fig. 2, black faded bars). Belly snails, however, tested one week after the single 0.5h TS did not show LTM. A between-groups comparison of the 24 h and 72 h TM sessions demonstrated that the numbers of attempted pneumostome openings were also significantly reduced compared with the yoked controls at the same time point and the one-week TM. These data are consistent with our hypothesis that snails reared in the laboratory experience an unenriched environment that results in a diminished ability to form LTM.

Considering that we found such a dramatic increase in LTM duration in the Belly snails compared with that which we have reported previously (e.g. Parvez et al., 2005) using laboratory-reared snails (i.e. no LTM with the single 0.5 h TS), we hypothesized that one of two possibilities could account for these observed differences. The first is that the Belly snails had developed in an enriched environment compared with that of the laboratory population and that it was the enriched environment during ontogeny that accounted for the difference in LTM formation. The second possibility was that there could be differences between the original wild populations in their inherent memory-forming capabilities. That is, strain differences between the wild Dutch and Belly snails exist, and this phenotype persisted regardless of rearing conditions. To differentiate between the two hypotheses, we needed to perform two experiments: first, rear the offspring of Belly snails in our laboratory under conditions identical to those of our laboratory-reared Dutch snails and, second, resample wild Dutch snails from the same locations that our laboratory populations were derived from over 50 years ago.

We therefore collected 'wild Dutch' snails from polders near Utrecht from which our laboratory population was originally derived. We also collected egg sacks from Belly snails in the wild and reared them to adulthood in separate aquaria in our laboratory. After successfully crossbreeding these two populations of snails to ensure compatibility, we then proceeded to measure the ability of these different snail populations (i.e. wild Dutch and Belly F1s) to form LTM following the single 0.5 h training session.

First, we determined whether the freshly collected wild Dutch snails formed LTM following the single 0.5 h TS. That is, are the wild Dutch snails as capable as the Belly snails in forming LTM following the single 0.5 h TS? Or, put another way, are wild Dutch snails better able to form LTM than the laboratory snails, which are descendants of snails collected from the same Utrecht polder? We also tested yoked-control wild Dutch snails. We were surprised to find that wild Dutch snails did not demonstrate memory 24 h after the single TS session (Fig. 3). That is, there was no significant reduction in the number of attempted pneumostome openings in the 24 h TM session compared with the TS. Yoked-control wild Dutch snails also did not demonstrate LTM. These data are similar to our previous findings demonstrating that 'laboratory snails' do not exhibit LTM at 24 h following a single 0.5 h TS (Lukowiak et al., 2000; Lukowiak et al., 2003a; Lukowiak et al., 2003b; Parvez et al., 2006a; Parvez et al., 2006b; Taylor and Lukowiak, 2000). These data are not consistent

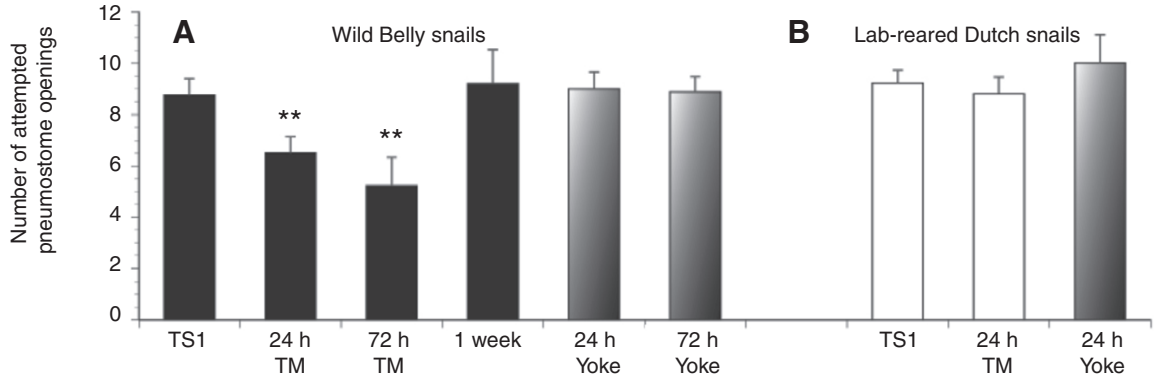

Fig. 2. Behavioral response of wild Belly (left black bars) and laboratory snails (descendents of wild Dutch snails; right white bars) after a single 0.5 h training session (TS). (A) Operant conditioning of wild Belly snails results in an LTM that persists for 24 and 72 h (24 h TM, N=53, P<0.05; 72 h TM, N=23, P<0.05). Yoked control snails do not demonstrate memory at these same time periods (faded bars; 24 h yoke, N=26, P=0.36; 72 h yoke, N=25, P=0.45). Snails did not demonstrate memory after 1 week (1 week TM, N=32, P=0.24). (B) Laboratory snails do not demonstrate LTM after a single 0.5 h training session (TS; 24 h TM, N=30, P=0.55; 24 h yoke, N=15, P=0.42). All results shown as means + s.e.m. **P<0.001.

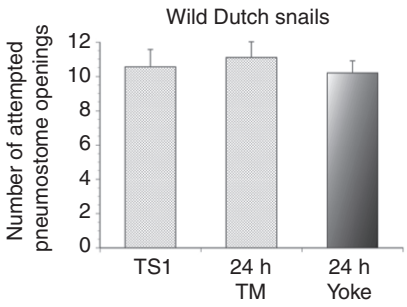

Fig. 3. Behavioral response of wild Dutch snails after a single 0.5 h training session (TS). Wild Dutch snails do not demonstrate LTM when tested 24 h after operant conditioning (left light-gray bars, 24 h TM, N=22, P=0.60). Yoked controls do not demonstrate altered behavior after conditioning (right faded-black bar, 24 h yoked, N=16, P=0.57). Results are shown as means + s.e.m.

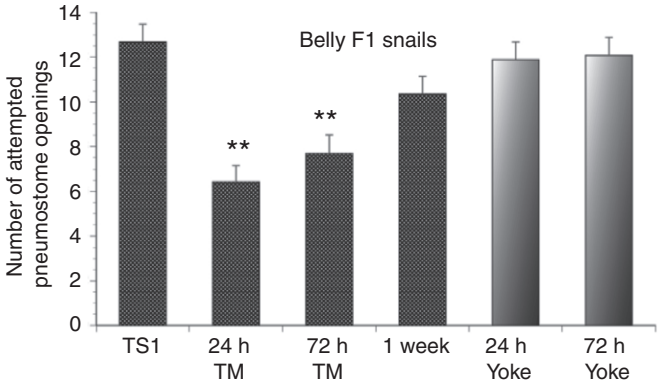

Fig. 4. Behavioral response of Belly F1 snails after a single 0.5 h training session (TS). Operant conditioning of Belly F1 snails results in an LTM that persists for 24 and 72 h (24 h TM, N=26, P<0.05; 72 h TM, N=26, P<0.05). Yoked control snails do not demonstrate memory at these same time periods (faded-black bars, 24 h yoke, N=24, P=0.55; 72 h yoke, N=25, P=0.73). Snails did not demonstrate memory after 1 week (1 week TM, N=25, P=0.82). Results are shown as means + s.e.m. **P<0.001.

with our original hypothesis regarding laboratory rearing. That is, wild Dutch snails, presumably developing in an enriched environment compared with the laboratory-reared snails (originally derived from wild Dutch snails), do not exhibit superior memory-forming capabilities compared with laboratory-reared snails. These data, however, are consistent with the hypothesis that there are strain differences between Dutch and Belly snails in their capability to form LTM. However, we still had to demonstrate that the offspring from Belly snails reared in the laboratory have a superior capacity to form LTM compared with that of wild Dutch and laboratory-reared snails that were derived from an original Dutch population.

We thus subjected Belly F1s (i.e. snails reared from eggs collected in the Belly river drainage), when they reached a length of 2–2.5 cm and had begun laying eggs of their own, signifying maturity (McComb et al., 2003; McComb et al., 2005), to a single 0.5 h TS and determined whether they had the capability to form LTM. We found that these laboratory-bred Belly snails (Belly F1s) had a similar memory profile to that of the wild parental population. That is, the Belly F1s demonstrated memory at 72 h but not at 1 week (Fig. 4). The yoke control Belly F1 snails did not demonstrate memory at either 24 or 72 h. A between-groups comparison of the 72 h TM session demonstrated that the number of attempted pneumostome openings was also significantly reduced compared with that of the yoked controls at the same time point and the one-week TM. Thus, laboratory rearing of Belly snails did not result in a diminished capacity to form LTM. These data are also in agreement with our data regarding Dutch snails, which also showed that laboratory rearing did not alter their inherent ability to form LTM.

Another method used to determine how 'good' a memory is makes use of individual marks given to each snail (Lukowiak et al., 2003c; Rosenegger et al., 2004). We therefore determined the grade distribution for each wild Dutch snail and each 'laboratory snail' based on their performance following the single 0.5 h TS. We found no difference in the grade distribution between the wild Dutch and the laboratory snails 24 h after TS. That is, the percentage of snails given 'A' grades in the wild Dutch cohort (27%) was not significantly different from the number that the laboratory snails earned (16%). The number of 'F' grades given was also similar between these populations, with 44% of the wild Dutch snails failing the test and 57% of laboratory snails receiving 'F' grades (χ^2, N=89, P=0.335). Thus, we conclude that the behavioral memory profiles of wild Dutch snails and their laboratory-reared cousins are similar. Thus, it appears that laboratory rearing has not altered the ability (or inability) of the populations to form LTM.

We also compared the individual grade distributions between the wild Belly snails and their laboratory-reared offspring. We found no difference in the grade distribution between these two populations. That is, the percentage of snails given 'A' grades in the wild Belly snails (40%) was not significantly different from the number that the Belly F1 snails earned (50%). The number of 'F' grades given was also similar between these populations, with 36% of the wild Belly snails failing the test and 35% of Belly F1 snails receiving 'F' grades (χ^2, N=51, P=0.712). Thus, we conclude that being reared in the 'simple' environment of the laboratory does not affect the behavioral memory profile of the Belly snails.

To dissect further the differences between the wild Dutch, laboratory snails, wild Belly snails and Belly F1 snails, we compared the individual grade distributions in each separate population. We found that the wild Belly snails and Belly F1 snails received significantly more 'A' grades and significantly fewer 'F' grades than the wild Dutch or laboratory snails (χ^2, N=104, P=0.022). Together, all of the data we collected regarding the ability to form LTM between the different populations of snails are consistent with the hypothesis that there are strain-specific differences between Dutch and Belly snails, with the Belly snails having superior memory-forming capabilities regardless of their rearing conditions.

Electrophysiological profile of RPeD1

In RPeD1, the neuron that both initiates aerial respiratory behavior and is a necessary site of LTM formation, we have recently demonstrated electrophysiological changes associated with enhanced LTM formation that parallel the duration of the behavioral phenotype following predator detection (Orr and Lukowiak, 2008). We therefore hypothesized that, given the differing memory capabilities between the Dutch and Belly snail populations, there would be predictable electrophysiological differences in RPeD1 activity following the single 0.5 h TS between these two snail populations.

We have also recently shown that, 24 h after the single 0.5 h TS, laboratory snails do not demonstrate memory, and the electrophysiological characteristics of RPeD1 are not different from the naive state (Orr and Lukowiak, 2008). We therefore first sought to determine whether the electrophysiological properties of RPeD1 in wild Dutch snails were altered when sampled 24 h after the single 0.5 h TS. However, before we could make this comparison,

we needed to determine whether the activity recorded in RPeD1 from semi-intact preparations prepared from naive wild Dutch and laboratory snails was similar. We found that there were no significant differences in any of the nine electrophysiological measurements made from 'naive' RPeD1s in these two groups of snails (data not shown, but see Materials and methods for descriptions of the electrophysiological properties measured).

Next, we trained both wild Dutch and laboratory snails with the single 0.5 h TS and then 24 h later recorded from RPeD1 in semi-intact preparations. We found, as in the behavioral experiments, that there was no difference in any of the measured electrophysiological parameters in RPeD1 24 h after TS compared with the naive state (Fig. 5). That is, 24 h after TS1, RPeD1 activity is indistinguishable from that seen before training.

Considering that we observed a dramatic difference in the ability of wild Belly snails and Belly F1s to form LTM compared with wild Dutch and laboratory snails, we sought to determine whether changes in RPeD1 activity would parallel the behavioral changes in these wild Belly snails. We therefore trained naive cohorts of wild Belly and Belly F1 snails with the single 0.5 h TS and 3 days later prepared semi-intact preparations from these snails. We found in both the wild Belly (Fig. 6) and the Belly F1s (Fig. 7) significant changes in the measured electrophysiological parameters of RPeD1 72 h after TS. Specifically, the number of spikes per 600 s, the number of spikes per burst, frequency of spikes per burst, burst duration and total numbers of bursts per 600 s were all reduced in the trained groups at 72 h compared with the naive state. Yoked control groups did not demonstrate significant changes at 72 h compared with the naive state (Figs 6 and 7 for each population respectively). Thus, we conclude that single 0.5 h TS has similar effects on the electrophysiological characteristics of both wild Belly and Belly F1 snails and that these changes parallel the observed behavioral phenotype. Thus, unlike the case with wild Dutch and laboratory snails, significant electrophysiological differences are seen in RPeD1 from wild Belly snails and Belly F1 snails for 72 h after the TS, which parallel the behavioral memory.

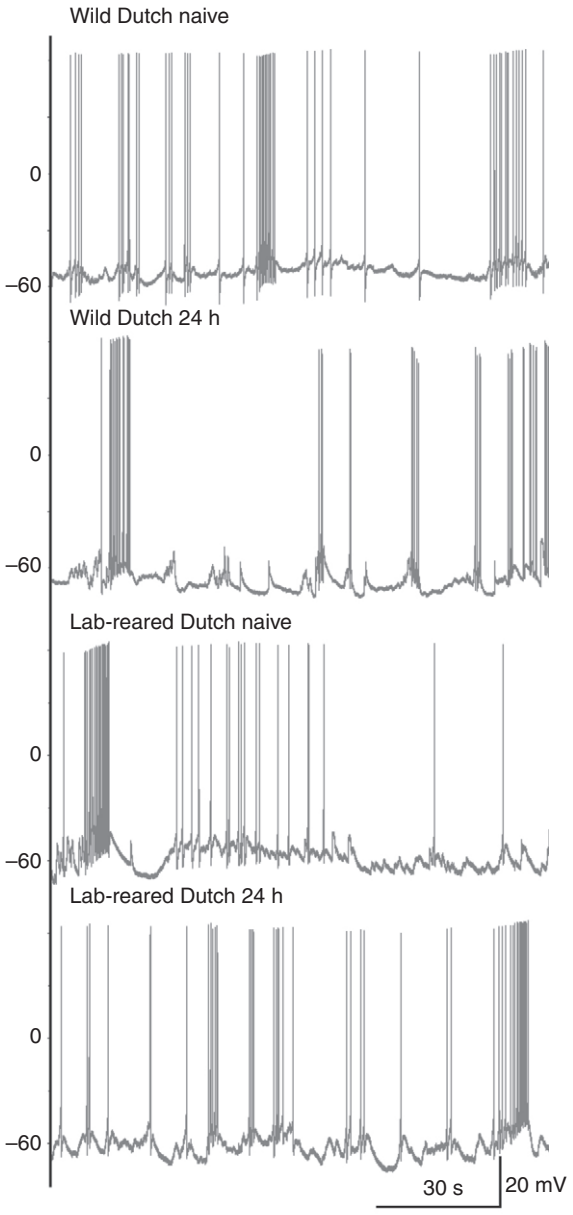

Fig. 5. Representative electrophysiological recordings from RPeD1 in semi-intact preparations taken from wild Dutch and laboratory snails before (naive) and 24 h after operant conditioning. No significant differences were found between any of the measured electrophysiological characteristics (wild Dutch naive, *N*=8; wild Dutch 24 h, *N*=8; laboratory-reared Dutch naive, *N*=9; laboratory-reared Dutch 24 h, *N*=8).

DISCUSSION

The original purpose of this study was to test the hypothesis that laboratory rearing of *Lymnaea* results in 'blond' snails that are challenged with respect to formation of LTM. Our initial pilot experiment on locally obtained wild *Lymnaea* (i.e. Belly snails, which are darker in color; Fig. 1) found that these snails had superior memory-forming capabilities compared with our 'blond' laboratory-bred snails. Consistent with this hypothesis were the data obtained from a series of experiments on exposing these blond snails to a sympatric predator (crayfish) in an attempt to 'enrich' the laboratory-rearing environment. We found (Orr et al., 2007; Orr and Lukowiak, 2008) that these 'blond' snails instinctually responded to the presence of the predator with specific predator-avoidance behaviors, including an enhanced ability to form LTM. This enhanced ability to form LTM, as a result of predator detection, was strikingly similar to the pilot data from the Belly snails. Thus, we were encouraged to perform a series of experiments to test our hypothesis directly that an enriched environment, such as that provided in the snails' natural habitat, results in snails with superior memory-forming capabilities. The data presented here, however, did not support this hypothesis; rather, the data support an alternative hypothesis that there are heritable differences in memory-forming ability between two geographically distinct populations of *Lymnaea*.

From this study, we have drawn three important conclusions: first, we have identified two naturally occurring, geographically separate, wild populations of *Lymnaea stagnalis* that have different capacities for forming LTM following operant conditioning of aerial respiratory behavior; second, rearing of the progeny of wild snails under laboratory conditions does not significantly alter their memory-forming abilities – that is, there is an inherent and heritable capacity for memory formation within each population that is maintained regardless of rearing in either natural or artificial conditions; and, third, this 'hardwired' memory capability, which differs between stains of *Lymnaea stagnalis*, is encoded within a neural network that is itself malleable – that is, significant physiological changes occur within this neural network during memory formation that are directly correlated with behavioral modification. Support for these conclusions was obtained at both

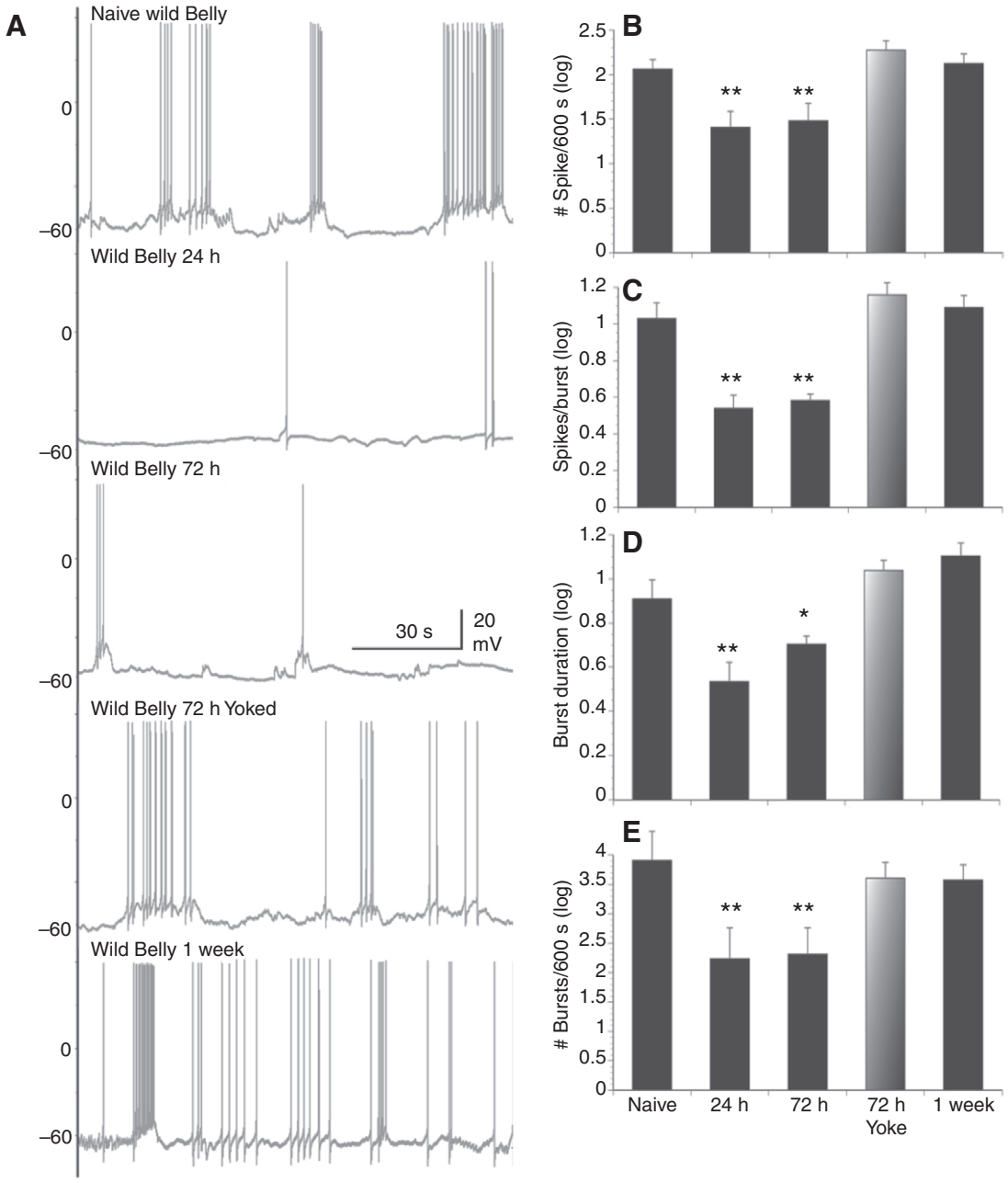

Fig. 6. RPeD1 activity in wild Belly snails following the single 0.5 h training session. (A) Representative recordings from RPeD1 in wild Belly snails starting at top with a naive snail (naive wild Belly), 24 h after the operant conditioning procedure, 72 h after conditioning, 72 h yoked control and 1 week after conditioning. (B) Summary data for mean (+s.e.m.) spiking activity per 600 s. (C) Number of spikes per burst. (D) Burst duration. (E) Number of bursts per 600 s. In all measured characters presented here, both the 24 h and 72 h operantly conditioned groups demonstrated significantly lower activity than the naive state and are significantly lower than the 72 h yoke and 1 week groups. 72 h yoke and 1 week groups are not significantly different from the naive state. All data represent log-transformed values. *P<0.05; **P<0.001. Naive N=9; 24 h N=9; 72 h N=7; 72 h yoke N=7; and 1 week N=8. No significant differences were detected between treatments in other electrophysiological parameters measured (see Materials and methods).

the behavioral level and in the electrophysiological properties of the neuron RPeD1. This is a neuron that is both necessary and sufficient for driving the respiratory network (Syed et al., 1990; Syed et al., 1992) and is a necessary site of LTM formation (Scheibenstock et al., 2002).

We have demonstrated previously that laboratory snails have maintained instinctual defensive responses to a natural predator for over 250 generations of predator-free existence and that operant conditioning in the presence of this predator results in dramatically enhanced LTM (Orr et al., 2007; Orr and Lukowiak, 2008). This instinctual behavioral response is also reflected in RPeD1 activity. Together, these results lend strong support to the idea that there is both an innate ‘hardwired’ component (i.e. that heritable predator defense and memory-capability responses are maintained in both the behavioral phenotype and in the CPG circuit that drives the behavior), whereas the network itself remains adaptable for associative learning and formation of LTM. Thus, behavioral and electrophysiological differences between strains are present in an identified tractable neural network that is inherently malleable and maintained regardless of environmental conditions during ontogeny.

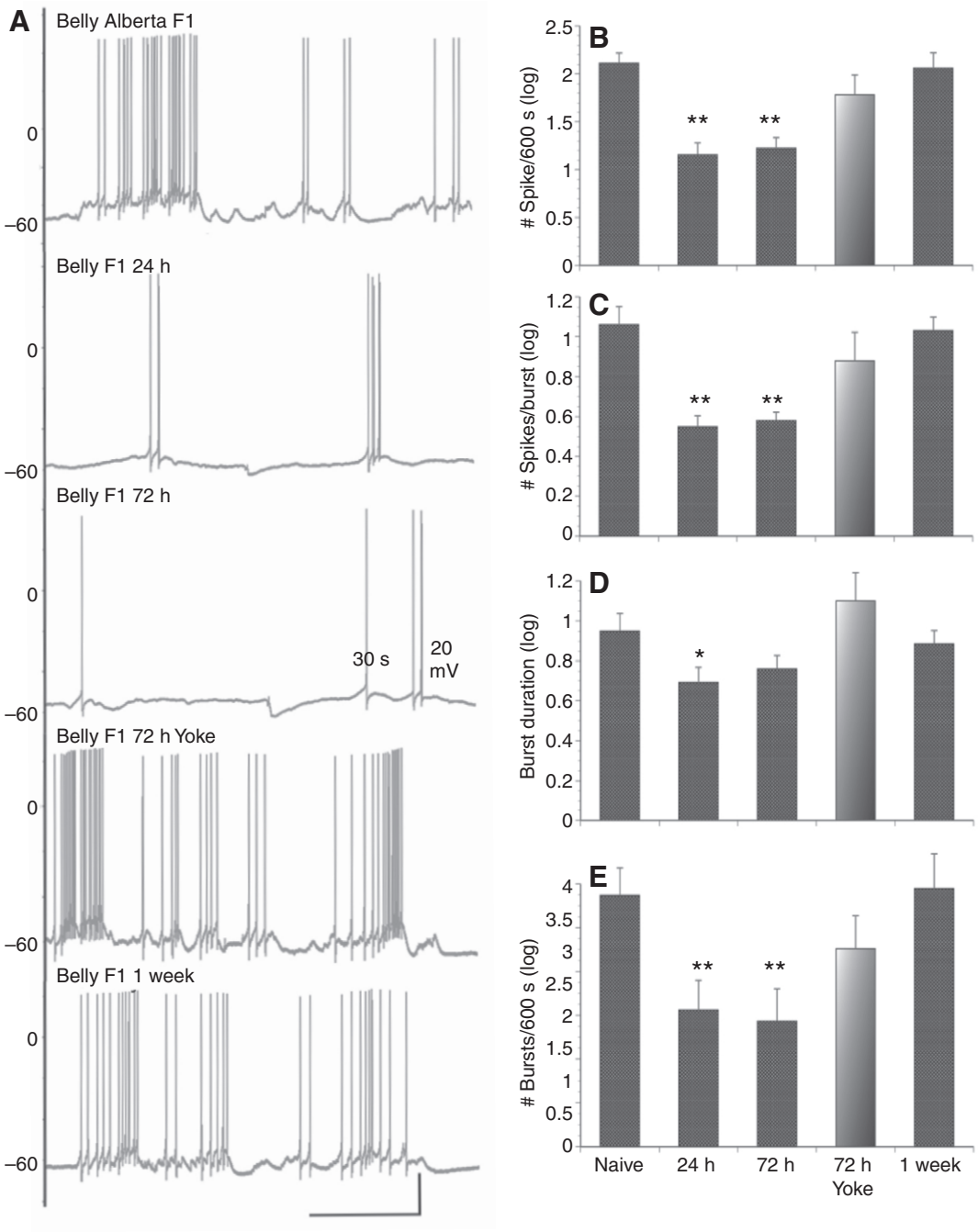

Fig. 7. RPeD1 activity in Belly F1 snails following the single 0.5 h training session (A) Representative recordings from RPeD1 in Belly F1 snails starting at top with a naive snail (naive Belly F1), 24 h after the operant conditioning procedure, 72 h after conditioning, 72 h yoked control and 1 week after conditioning. (B) Summary data for mean (+s.e.m.) spiking activity per 600 s. (C) Number of spikes per burst. (D) Burst duration. (E) Number of bursts per 600 s. In all measured characters presented here, the 24 h and 72 h operantly conditioned groups both demonstrated significantly lower activity than the naive state (except burst duration at 72 h) and are significantly lower than the 72 h yoke and 1 week groups. 72 h yoke and 1 week groups are not significantly different from the naive state. All data represent log-transformed values. $^{*}P<0.05$; $^{**}P<0.001$. Naive $N=8$; 24 h $N=7$; 72 h $N=7$; 72 h yoke $N=8$; and 1 week $N=7$. No significant differences were detected between treatments in other electrophysiological parameters measured (see Materials and methods).

Despite a rich history of exploration, investigating the neural correlates of cognition has led behavioral ethologists to follow generally one of two hypotheses. The first suggests that cognition has been, and continues to be, acted upon by natural selection (Healy and Hurly, 2004). Evidence supporting this theory comes from investigations into optimal foraging theory, where the ability of an animal to choose optimal foraging grounds, remember food caches and incorporate risk assessment results in increased survivorship and reproductive output (Healy and Hurly, 2004; Orr et al., 2007; Shettleworth et al., 1985). However, some researchers have criticized this view (Bolhuis and Macphail, 2001; Macphail and Bolhuis, 2001) and suggest that natural selection has acted only on the 'peripheral nervous system' (by which they mean the neural regions involved in the perception of stimuli) and not on higher learning areas (Healy and Hurly, 2004). Instead, investigators supporting this alternative view have focused on other mechanisms, such as exposure to stress

(Healy and Hurly, 2004; Herberholz et al., 2004; Kim and Diamond, 2002; Martens et al., 2007b; Rundle and Bronmark, 2001; Shors, 2004; Shors, 2006) or environmental enrichment (Berardi et al., 2007; Fischer et al., 2007; Frick et al., 2003; Harburger et al., 2007; Irvine and Abraham, 2005; Martens et al., 2007b) to explain the cognitive variation within and between species. This second hypothesis suggests that the cognitive ability of an organism is dependent on what it experiences during its ontogeny, and it is this ontogenetic adaptation that determines the behavioral fitness of an organism.

We set out to determine whether rearing conditions during ontogeny influenced the ability to form LTM in *Lymnaea* in the hope of using our tractable model to study the interface between environment and memory. Exposure to enriched environments has been shown to result in use-induced cortical plasticity (Hebb, 1950; Hebb, 1951) leading to improved learning and memory (Berardi et al., 2007; Fischer et al., 2007; Harburger et al., 2007; Irvine and Abraham, 2005; Rosenzweig et al., 1993). These studies support a central dogma of neural development – that formation of neural circuits is guided by experience (Feller and Scanziani, 2005). However, we found that our laboratory rearing practices alter neither aerial respiratory behavior nor associative learning and the subsequent formation of LTM. Rather, we found significant behavioral and neurophysiological differences between two distinct geographical populations of *Lymnaea*. The differences were manifest at both the behavioral and neurophysiological level in how much better one population of snails (Belly snails) formed LTM after operant conditioning compared with the other (Dutch snails). Strain differences in memory capability, including neuroanatomical and electrophysiological properties, have been demonstrated between strains of mammalian (Ammassari-Teule et al., 1993; Brooks et al., 2005; Gozzo and Ammassari-Teule, 1983; Ledoux et al., 1983; Reynierse, 1968; Ritzmann et al., 1993; Waddell et al., 2004) and invertebrate models (Hay, 1975; Meller and Davis, 1996). However, here we present the first demonstration we know of where strain differences have been examined from the behavior of natural wild populations to an individual neuron necessary for the behaviors under investigation.

Our finding that LTM formation is not affected by natural or artificial rearing environments is consistent with the findings of others studying the affects of rearing conditions on wild and F1 cohorts. These investigations have found that rearing the offspring of wild animals in artificial conditions has little effect on their behavioral and physiological responses to stress (Kunzl et al., 2003) as well as associative learning or memory formation (Stuermer and Wetzel, 2006). In fact, there is evidence to the contrary demonstrating increased learning by laboratory-reared animals compared with wild animals (Millar, 1975; van der Staay and Blokland, 1996). This finding has been attributed to the effects of domestication in selecting for behaviors that are compatible for laboratory use (Stuermer and Wetzel, 2006), which can occur within a short period of time. It should also be noted that studies examining environmental enrichment are necessarily performed on a single strain to isolate the effects of the enrichment alone, as assessing the relative amount on 'enrichment' of wild strains would be difficult. Our inability to elucidate differences between snails reared in the wild and those in the laboratory does not exclude the possibility that *Lymnaea* respond to the effects of environmental enrichment. It might be that our rearing conditions are simply not 'impoverished' enough or we are examining a behavior that is unaltered by environment challenges during ontogeny. Perhaps, if we reared individual snails in isolated environments, a difference in cognitive ability might be detected. These investigations are ongoing in our laboratory.

Our data demonstrate that Belly snails have the capability of forming LTM with a training procedure that does not usually result in LTM in the wild Dutch and laboratory snails. However, a single 0.5 h TS will in the laboratory-reared blond snails result in LTM if the training is performed in crayfish effluent (Orr and Lukowiak, 2008) or if the TS is immediately preceded with or followed by a sufficiently stressful event [e.g. immersion in 25 mmol l^{-1} KCl for 30 s (Martens et al., 2007a)]. In addition, when individual snails are examined, we found that the 'quality' of LTM in the Belly snails was significantly better than in the Dutch snails. The biological reasons for this difference in memory capability between the Alberta and Dutch populations are unknown to us at this time; however, we can rule out laboratory rearing as a cause.

The two populations are clearly subject to differing predatory regimes as crayfish are not endemic to the Belly River, whereas they are sympatric with Dutch snails in the Utrecht polders. It might be that these populations have undergone differential selection resulting in this altered cognitive phenotype. Certainly, the traits under study in this experiment have at least two of the three required characteristics that would be acted upon by natural selection: a heritable component of the traits measured and variation among the traits (Endler, 1986; Lande and Arnold, 1983). Whether this trait variation results in fitness differences in an individual or between populations remains to be demonstrated.

Now that our model system includes naturally occurring strain differences (i.e. Belly snails versus Dutch), we can begin to investigate the biological reasons why these differences exist. We can also now explore how a neural network, which is governed by an instinctual response, maintains the ability to be adaptable to operant conditioning. For example, in the future, we can begin to determine what differences in neural connectivity or in the constituent molecular processes within this network underlie these strain differences. It is possible that, in Belly snails, the ratio of suppressive and activator isoforms of the cyclic AMP response-element-binding protein CREB (Silva et al., 1998) in CPG neurons (Sadamoto et al., 2003) favors activation, such that LTM formation is triggered more easily. The identification of these two strains of *Lymnaea* presents an exciting new opportunity to investigate the malleability of hardwired networks in a system where a defined behavior is driven by identified neural circuitry, yet separate natural populations within the model demonstrate dramatic variation in the behavior.

Here, we have strong evidence demonstrating that hardwired instinctual behaviors are encoded within a network that is itself inherently malleable. As the molecular events in a single neuron (RPeD1) in *Lymnaea* have been shown to be necessary for formation of LTM, reconsolidation, extinction and forgetting (Lattal et al., 2006; Parvez et al., 2006b; Sadamoto et al., 2003), it is now possible to investigate how a hardwired network can be modified to alter behavior at the level of the single neuron and how this neuronal adaptation affects these animals at the level of populations.

We thank the Orr ranch and the Nelson ranch for allowing this field work and Hyo-jung Orr, David Rosenegger, Kara Martens, Kashif Parvez and Kim Browning for all their help and comments. This research was supported by NSERC.

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