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OPERANT CONDITIONING OF AERIAL RESPIRATORY BEHAVIOUR IN *LYMNAEA STAGNALIS*

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Summary

In this study, we operantly conditioned the aerial respiratory behaviour of the freshwater snail *Lymnaea stagnalis*. Aerial respiration in *Lymnaea stagnalis* is accomplished by the spontaneous opening and closing of its respiratory orifice, the pneumostome, at the water surface. Weak tactile stimulation of the pneumostome area, when the pneumostome is open, evoked only the pneumostome closure response, which is one aspect of the escape–withdrawal reflex. Pneumostome stimulation resulted in its closure and the termination of aerial respiratory activity. A contingent tactile stimulation paradigm was used to operantly condition the animals. Stimulation of the pneumostome whenever the animal attempted to breathe resulted in significantly fewer attempts to open the pneumostome as training progressed. The latency of the first breath (subsequent to stimulation), the number of breaths and the total breathing time were measured before and after each training period. Significant, quantifiable changes in these behavioural parameters were observed only in the operant conditioning group animals. Control animals receiving tactile stimulation to their pneumostome not contingent upon pneumostome opening movements (yoked controls) or those that were physically prevented from surfacing to breathe (hypoxic controls), did not exhibit significant changes in these behavioural parameters. Our data provide the first direct evidence for operant conditioning of respiration in any animal.

Key words: *Lymnaea stagnalis*, operant conditioning, associative learning, aerial respiratory behaviour, hypoxia, molluscan model system, snail.

Introduction

Most animal behaviours are not fixed but rather they exhibit a high degree of plasticity and can be altered in the face of changing environmental conditions. Animals are capable of making associations between external stimuli and their behavioural responses to those stimuli. This capability allows them to make predictions about what stimuli are likely to occur on the basis of a memory of these associations, and thus they alter their behaviour appropriately. This alteration of behaviour is what we call learning. Some events are learned only when stimuli occur in close temporal contiguity and in a fixed sequence (associative learning), while other learned responses do not require a pairing of events (non-associative learning). Non-associative learning is thought to be the simplest and most primitive type of learning, and includes such phenomena as habituation and sensitization. Associative learning, in contrast, is of a more complex nature and is thought to be the basis of most of human learning.

Typically, associative learning is divided into various categories, two of which are classical and operant conditioning. In classical conditioning, an initially ineffective conditional stimulus (e.g. the bell used for Pavlov's dogs) after pairing with an effective unconditional stimulus (e.g. food powder) eventually comes to evoke the unconditional response (e.g. mouth watering). In contrast to classical conditioning, operant conditioning is response-contingent. That is, the reinforcing stimulus is presented only if the animal performs a specific behaviour. The reinforcing stimulus can be negative, in which case the animal learns to avoid engaging in the behaviour, or the reinforcing stimulus may be positive and the operantly conditioned animal will spontaneously perform the behaviour more often. The animal learns an association between its behaviour and the reinforcing stimulus and, as a consequence, its behaviour is altered (see Mackintosh, 1974, for a complete description of the types of learning in animals).

Compared with vertebrates, relatively few studies of operant conditioning have been performed on invertebrates (Carew and Sahley, 1986) and, of these, even fewer progressed to the point where it was possible to examine the underlying changes in

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nervous system activity accompanying the associative learning. Early studies in *Aplysia californica* suggested the possibility that this animal was capable of being operantly conditioned (e.g. Preston and Lee, 1973). More recently, Susswein *et al.* (1986) have described a type of associative learning in *A. californica* that is related to operant conditioning. In these studies, hungry *A. californica* were offered food encased in plastic netting. Since the food was inedible, the animals learned not to attempt to eat it. In a more recent study involving operant conditioning in *A. californica*, Cook and Carew (1989*a*,*b*,*c*) demonstrated that the head-waving response could be operantly conditioned. One other study of potential importance reported that the gill withdrawal response of *A. californica* could be operantly conditioned (Hawkins *et al.* 1985).

Attempts have also been made in a number of other invertebrate species to operantly condition various behavioural responses (see Carew and Sahley, 1986). Some examples are leg-lifting in an insect (Horridge, 1962; Hoyle, 1982) and *Drosophila* (Booker and Quinn, 1981) and eye-stalk positioning in a species of crab (Abramson and Feinman, 1987). In each of these examples, animals learned to position an appendage on the basis of the contingent presentation of an adequate reinforcing stimulus.

In most of the above instances, the behaviours which were operantly conditioned were relatively simple and reflexive. Whether an important homeostatic behaviour, such as respiration, is capable of such operant conditioning needed to be determined (see Mortola, 1995; Thomas *et al.* 1992, 1995). This problem was investigated in the present study using the freshwater pond snail *Lymnaea stagnalis*.

The rationale for selecting *L. stagnalis* for this study was based on several considerations. (1) *L. stagnalis* is a bimodal breather; gaseous exchange occurs *via* transpiration across the skin or with the atmosphere *via* the pneumostome and lung when the animal becomes hypoxic. This bimodal nature of respiration allows the investigator to disturb only one aspect of respiration (i.e. skin *versus* aerial respiration) without seriously jeopardizing the survival of the animal. (2) Aerial respiration occurs only at the water surface when the animal opens its pneumostome. This behaviour is easily observed and quantified (Jones, 1961). (3) In *L. stagnalis*, aerial respiratory behaviour is mediated by a well-characterized central pattern generator (CPG) and a network of identified sensory and motor neurones (Syed *et al.* 1990; Syed and Winlow, 1991) which will allow investigators the opportunity to examine the neural basis of any learned behaviour.

In the present study, we demonstrate that aerial respiratory behaviour in *L. stagnalis* can be operantly conditioned and we establish a reliable learning paradigm which can be used in future studies to examine underlying neuronal mechanisms of this operant conditioning. To be effective, reinforcing stimuli must elicit motivationally significant behaviours, that is, behaviours that are important to the animal, such as escape behaviours (Dickinson, 1980). We chose tactile stimulation to the pneumostome area as the negative reinforcing stimulus. This stimulus reliably causes the animal to close its pneumostome and to stop aerial respiratory behaviour. This pneumostome closure response is one component of the escape–withdrawal response (see also Ferguson and Benjamin, 1991) which is at the apex of the behavioural hierarchy pyramid of *L. stagnalis* (Syed and Winlow, 1991; Winlow *et al.* 1992). Some of the results presented here have been reported in abstract form (Lukowiak *et al.* 1994).

Materials and methods

Animals

Laboratory-reared *Lymnaea stagnalis* (L.), originally derived from the stocks of the Vrije University in Amsterdam, were used in this study. Animals were 25–30 mm in shell length, had continual access to food (lettuce supplied twice per week), and were maintained at room temperature (20–22 °C) on a Calgary summer light–dark schedule (18 h:6 h L:D).

Aerial respiration

Aerial respiration is defined as the spontaneous opening and closing of the pneumostome (the respiratory orifice) at the water surface. Under normoxic conditions, as occurs when atmospheric air is bubbled through the aquarium, animals surface and open their pneumostome infrequently, once every 15–20 min (Moroz *et al.* 1993; see below).

Hypoxia

When animals are maintained in a hypoxic environment, the frequency of aerial respiration increases (Moroz *et al.* 1993; see below). To create a hypoxic environment, 100 % N_2 was continuously bubbled into the test beaker for 10 min prior to placing the animals in it and for the entire duration of the experiment.

Pneumostome closure response

Pneumostome closure was evoked by applying a weak tactile stimulus to the pneumostome area. The stimulus was delivered to the pneumostome area by an investigator using a sharpened wooden applicator stick. We did not attempt to quantify the intensity of this stimulus used in our studies. It was, however, always sufficient to elicit pneumostome closure without evoking a whole-animal withdrawal response. An explicitly stronger intensity tactile stimulus was given to one of the yoked control group of animals. This was done to control for any unconscious attempt on the part of the experimenter to give a stronger intensity stimulus to the operant group.

Training procedures

All animals (N=65) were individually identified by a series of specific coloured markings on their shells. Animals (N=45) were randomly assigned to one of the following groups: (1) the operant conditioning group; (2) the hypoxic control group; or (3) the yoked control group. Groups received five 30 min training sessions, two sessions per day, with each days' session separated by 2 h.

Experiments were performed by two investigators. Individual A monitored all movements of the animal, signalling to investigator B which animal opened its pneumostome. Investigator A also applied the tactile stimulus to the pneumostome area (see below). Investigator B kept track of the following aerial respiratory parameters: (1) the latency of the first breath, (2) the duration of each breath, and (3) the number of breaths.

Prior to experimentation, animals were taken from their home aquarium and placed into a 1 l beaker filled with 500 ml of hypoxic pondwater and allowed a 10 min acclimation period to explore their new environment (see Kemenes and Benjamin, 1994). The animals were then touched with a pair of forceps which propelled them to the bottom of the beaker. This signalled the start of the pre-test period (see below).

Operant conditioning paradigm

There were three phases to each experiment: pre-test, training period and post-test. The pre-test period, which lasted 30 min, began immediately following the gentle forceps stimulation at the end of the acclimation period. In this period, the latency of the first breath, the number of breaths and the duration of each breath, i.e. total breathing time (calculated by the arithmetic summation of the duration of each individual breathing episode) were recorded. At the end of this period, all animals (N=15) were again touched with forceps.

The animals now entered the training period, which also lasted for 30 min. During this period, whenever the snail attempted to open its pneumostome, it received a tactile stimulus to the pneumostome area. This stimulus was of sufficient intensity to result in closure of the pneumostome. In some instances, the snail immediately descended from the water surface, but usually it remained at the surface with its pneumostome closed. The time at which each tactile stimulus was applied was recorded. At the end of the training session, all animals were again touched by forceps to propel them to the bottom of the beaker.

The animals now entered the post-test period, which again lasted 30 min. No tactile stimuli were applied during this period, and the first breath latency, number of breaths and total breathing time were monitored as described above.

Yoked control group

In this group of animals (N=15), the pre- and post-test periods were exactly the same as described above. During the training period, however, yoked snails received exactly the same number of stimuli using the same pattern of stimulation as those of the conditioning group; however, the stimuli were not contingent upon the animal making pneumostome opening movements. If the pneumostome area was not readily accessible, the stimulus was applied in the proximity of the pneumostome.

Hypoxic control group

The pre- and post-test periods were as described above. During the training period, these animals (N=15) were prevented from surfacing by a plastic mesh. The openings in the mesh were large enough to allow the N_2 bubbles to escape; but prevented the snails from surfacing. These snails were submerged in hypoxic pondwater for 30 min without the opportunity to breathe, but they did not receive a tactile stimulus to their pneumostome area. Animals could tolerate such treatment for at least 36 h before any noticeable effects on mortality were observed (Moroz *et al.* 1993).

Data analysis

A paired t-test was used to determine whether hypoxic pondwater significantly increased aerial respiratory behaviour.

We plotted first breath latency, number of breaths and total breathing time in the pre- and post-test periods for each group (operant, yoked and hypoxic) for the five sessions. In addition, for the operant conditioning group snails, the average number of stimuli received during each training period was plotted. In each of the graphs, the standard error of the mean (S.E.M.) has also been plotted.

Pre- and post-test values of first breath latency, number of breaths and total breathing time were analyzed using a three-factor analysis of variance (ANOVA, according to Winer *et al.* 1991). The ANOVA encompassed one between-subjects factor corresponding to the treatment (denoted by 'group') and two within-subjects factors which will be referred to as 'session' and 'test'. Preliminary analyses of the variables mentioned above indicated that all of them were somewhat skewed. In order to improve upon normality and homoscedasticity, the data were transformed prior to ANOVA adopting methods described by Sokal and Rohlf (1981) and Winer *et al.* (1991). The effect of the transformations on normality was judged by means of probability plots and one-sample Kolmogorov–Smirnov tests. Homoscedasticity of the transformed data was examined by means of an F-max test (Winer *et al.* 1991). In addition, analyses of the residuals of the transformed data indicated no outliers. The transformations $\sqrt{(1 + \text{number of openings})}$, $\log_e(10 + \text{total breathing time})$ and $\log_e(\text{first breath latency})$ were found empirically to result in optimal distributions. In those cases where an animal did not open its pneumostome during the test period, a value of 1800 s was assigned for first breath latency.

The number of tactile stimuli delivered to the trained animals over the course of the five sessions was analyzed using a one-factor repeated-measures design. In this case, only the within-subjects factor 'session' was considered. Preliminary analyses of the distribution of the number of tactile stimuli indicated a strongly skewed distribution. Hence, the number of tactile stimuli was transformed by $\log_e(10 + \text{number of tactile stimuli})$.

Analyses of the residuals obtained by ANOVA of any of the transformed variables gave no reason to suspect violations of the assumptions of random error.

In the cases of first breath latency, number of breaths and total breathing time, the overall ANOVA indicated a

significant third-order interaction. Unless mentioned, statements of significance will therefore be based upon an additional analysis of simple main effects and simple interactions rather than overall main effects and overall interactions. Comparisons of individual cell means and analysis of trends were performed using orthogonal contrasting methodologies (see Winer *et al.* 1991, chapter 7).

Results

Aerial respiration in hypoxic versus *normoxic pondwater*

Initial experiments were performed to determine whether aerial respiration was significantly enhanced in hypoxic pondwater compared with normoxic pondwater. We recorded first breath latency, number of breaths and total breathing time under hypoxic and normoxic conditions. In each condition, a 10 min acclimation period was included. Animals were returned to their home tank after exposure to each situation. Two different groups of animals were subjected to these tests: in one group (N=10), they were first exposed to the hypoxic pondwater then to normoxic pondwater; in the second group (N=10) the order of exposure was reversed. At least 1 h elapsed between each test situation. These data are presented in Fig. 1. As expected, we found that snails had a significantly shorter first breath latency, an increased number of breaths and an increased total breathing time in the hypoxic pondwater condition than in the normoxic condition (P<0.001 for all three parameters).

Operant conditioning

In the operant training group, we first determined whether there was a significant change in the number of tactile stimuli over the course of the five sessions. These data are plotted in Fig. 2 and show that the number of tactile stimuli decreased significantly from 7.5±1.1 in session 1 to 2.3±0.3 in session 5 ($F_{4,36}$=14.04, P<0.001). Thus, operant conditioning training resulted in significantly fewer attempts to breathe.

First breath latency

In Fig. 3, the pre- and post-test first breath latency data for each of the three groups are shown. These data show that the profiles of first breath latency developing over the five training sessions differ significantly between the groups ($F_{8,108}$=2.595, P<0.012). The operant conditioning group differed from the two control groups in showing a significant trend in the post-test values of first breath latency ($F_{4,216}$=5.233, P<0.001). In this group, the post-test first breath latency significantly increased from 447±94.4 s on the first session to 1473±174.2 s on the fifth and last session ($F_{1,216}$=13.08, P<0.001). Neither of the control groups showed such a trend in their post-test first breath latency values.

The pre-test first breath latency values did not significantly differ between the experimental and two control groups ($F_{2,54}$=1.698, P>0.025); nor did any of the groups exhibit a significant trend in first breath latency values as training progressed (operant conditioning group $F_{4,216}$=1.394, P>0.025; yoked control group $F_{4,216}$=1.289, P>0.025; hypoxic control group $F_{4,216}$=0.858, P>0.025). However, the changes in the pre-test first breath latency values in the conditioning group appeared to be more pronounced than in either control group.

We conclude that the operant training paradigm significantly delayed the occurrence of the first breath in the post-test session and that this effect on latency cannot be explained by the application of non-contingent tactile stimuli to the pneumostome area or by hypoxia.

Number of breaths

We next examined whether the operant conditioning paradigm had any effect on the number of breaths taken in the pre- and post-test periods. These data are presented in Fig. 4. The three groups differ significantly ($F_{8,108}$=4.783, P<0.001) in their profiles of number of breaths. The ANOVA indicated

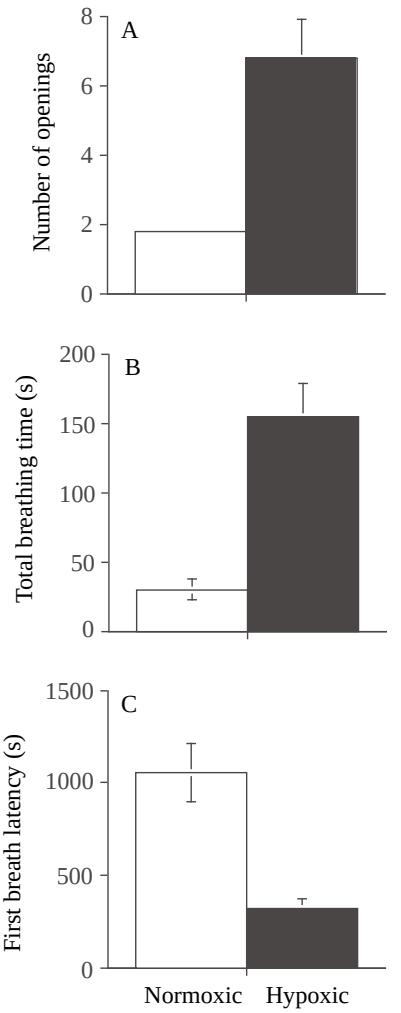

Fig. 1. Hypoxic pondwater significantly increased aerial respiratory behaviour in *Lymnaea stagnalis*. Pooled data (N=20) from animals which were either first exposed to hypoxic pondwater, given a 1 h rest and then exposed to normoxic pondwater (N=10) or *vice versa* (N=10). The number of breaths (openings), total breathing time and first breath latency in hypoxic and normoxic pondwater conditions are plotted as means ± S.E.M. A paired t-test showed that each of the aerial respiratory behaviours was significantly different in the two conditions (P<0.001).

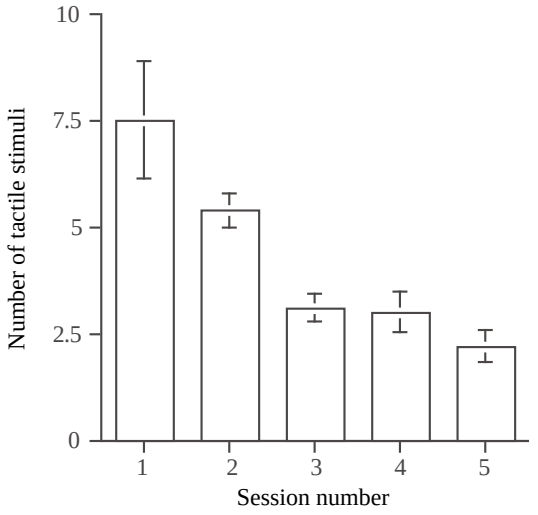

Fig. 2. Operant conditioning training resulted in fewer breathing attempts as training progressed. The mean numbers (± S.E.M.) of tactile stimuli delivered to the 15 snails during each training session have been plotted. An ANOVA showed that there was a significant effect of training on the number of breathing attempts as training progressed. Thus, snails attempted to breathe significantly less often with training.

an effect of repeated sessions on the behaviour of the operant conditioning group ($F_{4,243}$=10.098, P<0.001), but not on either the yoked ($F_{4,243}$=0.083, P>0.90) or hypoxic ($F_{4,243}$=0.831, P>0.40) control groups.

In the operant conditioning group, the strongest effect of the training paradigm was on the value of number of breaths in the post-test period. Number of breaths in the post-test period decreased significantly with training ($F_{4,216}$=12.3, P<0.001). Moreover, post-test data from the operant conditioning group show that the number of breaths taken during session 1 was significantly greater than that in either of the two control groups ($F_{2,270}$=6.213, P<0.004). The number of breaths then decreased rapidly in sessions 2 and 3, becoming even smaller than those observed during the pre-test period. Thus, in sessions 2 and 3 the values of the number of breaths are *not* different from those observed in the control groups ($F_{2,270}$=1.008, P>0.25, and $F_{2,270}$=2.752, P>0.05, respectively). However, the number of breaths continued to decrease so that by session 4 the number of breaths taken by the operant group animals was significantly smaller than that of either of the two control groups ($F_{2,270}$=3.709, P<0.05). Finally, on session 5, the number of breaths was reduced even further compared with that of the two control group animals ($F_{2,270}$=14.309, P<0.001). In the pre-test period over the course of the experiment, there was no statistically significant change in the number of breaths ($F_{4,216}$=1.328, P>0.20).

We conclude that as a result of operant conditioning training the number of breaths taken in the post-test period became significantly smaller and that this effect cannot be explained by the application of non-contingent tactile stimuli to the pneumostome area or by hypoxia.

Total breathing time

Finally, we examined the effect of operant conditioning training, as well as the two control procedures, on total breathing time in the pre- and post-test periods (Fig. 5). These results parallel the number of breaths results presented above. That is, there was a significant effect of repeated sessions on the operant conditioning group ($F_{8,108}$=4.324, P<0.001) but not on either the yoked or the hypoxic control groups.

Furthermore, as was the case with number of breaths, the primary effect of the operant conditioning paradigm on total breathing time was in the post-test period ($F_{4,243}$=9.206, P<0.001). Total breathing time became shorter in the post-test period, whereas total breathing time did not change significantly in the pre-test period. Thus, only operant training had a significant effect on the total breathing time and only in the post-test period.

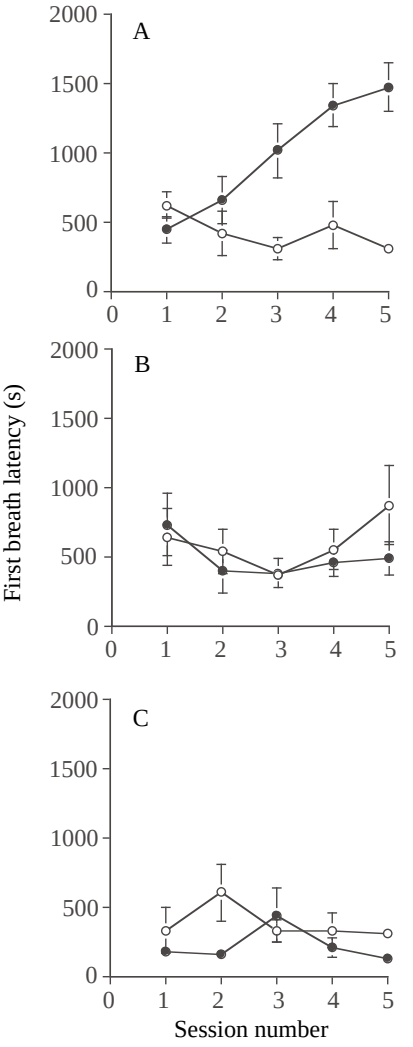

Fig. 3. Comparisons of first breath latency in the pre- (○) and post- (●) test periods in (A) operant, (B) yoked and (C) hypoxic groups of snails. In the operant group snails, there appeared to be a trend, although this was not significant, towards shorter pre-test first breath latency; there was a significant increase in the post-test first breath latency. Neither control group (B,C) exhibited any significant trend in first breath latency in either the pre- or post-test periods.

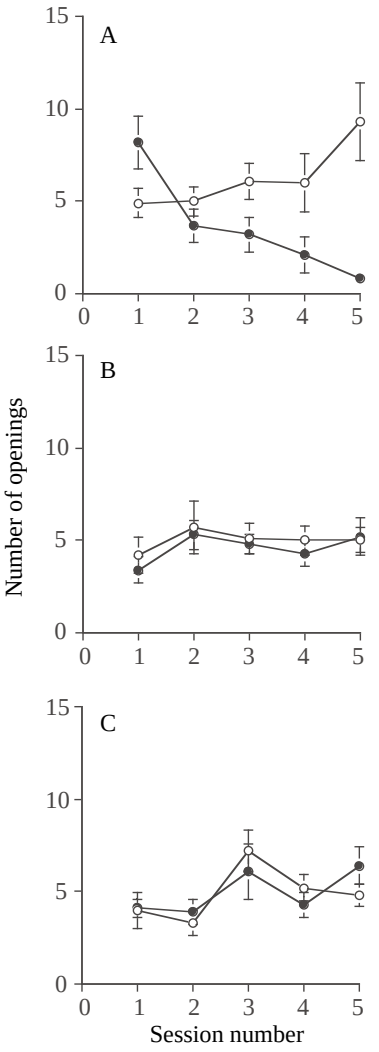

Fig. 4. Comparisons of the number of pneumostome openings in the pre- (○) and post- (●) test periods in (A) operant, (B) yoked and (C) hypoxic groups of snails. In the operant group, there appeared to be a trend, although this was not significant, for a greater number of pneumostome openings in the pre-test period; there was a significant decrease in the number of pneumostome openings in the post-test period. In neither control group (B,C) were there any significant changes in number of pneumostome openings in the pre- or post-test periods over the course of the experiment.

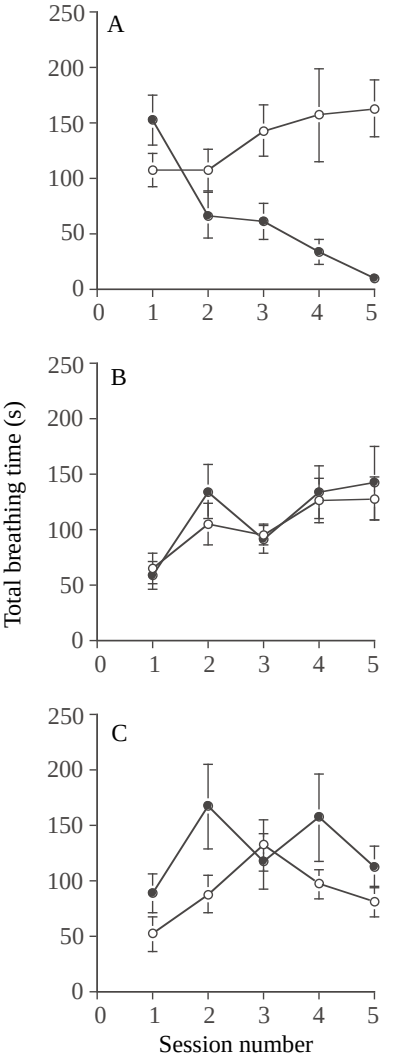

Fig. 5. Comparisons of total breathing time over the course of the experiment in the pre- (○) and post- (●) test periods in (A) operant, (B) yoked and (C) hypoxic groups of snails. The operant group animals appeared to show a trend towards longer total breathing time in the pre-test period (although this was not significant); total breathing time became significantly shorter in the post-test period. Neither control group (B,C) showed any significant trend in total breathing time.

Discussion

The data presented here clearly demonstrate that aerial respiratory behaviour in *L. stagnalis* can be operantly conditioned. That is, as a result of an operant conditioning paradigm, hypoxic animals learned to suppress their aerial respiratory behavioural drive. As training progressed, the animals attempted to open their pneumostome significantly less often (Fig. 2).

First, we tested whether these behavioural changes had been due just to the presentation of repeated tactile stimuli to the area around the pneumostome which elicited the hierarchically more important withdrawal–escape response (Syed and Winlow, 1991; Winlow *et al.* 1993). To determine this, we measured first breath latency, number of breaths and total breathing time in the operant and in the yoked control groups. Significant changes were only observed in these variables in the post-test period in the operant training group. If repeated activation of the pneumostome closure response were sufficient by itself to alter aerial respiratory behaviour, significant alterations in the measured behavioural variables should also have been observed in the yoked control group. This was not the case. A possible unconscious bias could have occurred in terms of the strength of the applied tactile stimulus. To control for this possibility, we employed an explicitly stronger tactile stimulus paradigm to a yoked

control group. Even with this stronger stimulation to their pneumostome area, the measured behavioural variables did not differ from the results obtained with the other yoked control groups.

Next, we asked whether the significant behavioural changes seen in the operant group could have been brought about by repeated periods of hypoxia. Placing the snails in a hypoxic environment produced significant changes in their overall respiratory behaviour (Fig. 1). However, preventing animals from breathing in the hypoxic environment for an equivalent period to that in the training period in the operant group was not sufficient by itself to alter first breath latency, number of breaths or total breathing time significantly. Significant changes in first breath latency, number of breaths and total breathing time (Figs 3–5) were only observed when the tactile stimulus was delivered contingent upon the initiation of pneumostome opening.

In addition to the behavioural parameters (first breath latency, number of breaths and total breathing time) quantified in this study, we observed that snails in the operant conditioning training group also altered the way in which they attempted to open their pneumostome. Naive and all control group snails open their pneumostome so as to maximize gaseous exchange with the atmosphere. They pull back their shell and orient their pneumostome perpendicular to the water surface. The pneumostome remained obviously and conspicuously opened. However, by the second training session, all snails in the operant conditioning group no longer pulled their shells back; instead, the shell was rotated in an attempt to conceal the pneumostome opening. Thus, a change in shell posture was also a result of operant conditioning; neither of the control groups exhibited such postural changes.

'Blind' observers who knew what a pneumostome opening normally looked like, but who were unaware of the training history of the snail, assessed this change in the posture of the various groups of animals after the training. The 'blind' observers had no difficulty determining which snails had received operant training. The results from the 'blind' observers always matched those of the individuals performing the experiment.

There was also a second qualitative change in the behaviour of the operant conditioning group animals; they climbed out of the water and clung onto the sides of the glass beaker (termed climbing out of water behaviour) during both the training and post-test sessions. Snails in the two control groups never exhibited this behaviour. The significance of these qualitative changes in behaviour and their underlying neuronal mechanisms remain to be determined.

As mentioned in the Introduction, there have been relatively few studies performed on molluscs using an operant conditioning paradigm. Moreover, as far as we can determine, this is the first demonstration that *L. stagnalis* are capable of being operantly conditioned. Furthermore, no other study has attempted to demonstrate a similar alteration in respiratory behaviour by employing an operant training paradigm. Thus, it is difficult to compare our results with those of others. Our results do, however, agree with earlier studies (Audesirk *et al.* 1982; Kemenes and Benjamin, 1989, 1994), which show that *L. stagnalis* are capable of associative learning. We hope that the advantages of our preparation listed in the Introduction will allow us to uncover the neuronal mechanisms underlying this form of associative learning.

Regarding operant conditioning in gastropod molluscs, perhaps the most convincing studies to date were performed by Cook and Carew (1986, 1989*a*,*b*,*c*). These authors demonstrated that head-waving behaviour in *A. californica* could be operantly conditioned and began a preliminary analysis of the sensory and motor pathways involved in the mediation of these behaviours (see also Cook *et al.* 1991). However, it appears now that an analysis of the neuronal changes which mediate this conditioning was more difficult than first thought. A major difference between our experiments and those of Cook and Carew is that they demonstrated learning following a single training session. In our experiments, we examined the behavioural changes following multiple training sessions over the course of 2.5 days. Initial pilot experiments in which the training period was 1 h suggested that *L. stagnalis* were also capable of associative learning following a single training session (data not shown). However, we chose the multiple training session protocol both because it allowed us to examine the changes more effectively in a number of behavioural patterns over the course of the training period and because it may lead to an increased persistence of the learned response.

One of the great challenges in neuroscience research is to determine the neuronal mechanism(s) which underlie learning and memory. Although great strides have been made towards this goal in recent years using a variety of different preparations ranging from nematodes to mammals (Hawkins *et al.* 1993), this goal still remains unattained. Utilization of this *L. stagnalis* preparation as an experimental system may help to attain this goal. Since the generation of rhythmic neuronal activity which mediates aerial respiration in *L. stagnalis* is the result of emergent properties of a three-cell network (Lukowiak, 1991; Barnes *et al.* 1994), small subtle changes occurring across the network might result in a disruption of the rhythm and, thus, of the behaviour. These changes might include alterations in the synaptic input between the individual neurones making up the circuit, as well as changes in the intrinsic membrane properties of the neurones. Alterations in the pacemaker properties of a neurone were hypothesized by Wollacott and Hoyle (1977) to mediate the operant learning of leg position in the grasshopper.

Preliminary data obtained from our laboratory suggest that the level of spontaneous activity in RPeD1, one of the three cells in the respiratory central pattern generator (CPG), is lower in operantly conditioned animals than in control animals. Since it is a burst of activity in RPeD1 which initiates rhythmic activity (Syed *et al.* 1990), this finding suggests that the rhythm would be less likely to be active in conditioned animals.

Furthermore, in operantly conditioned animals, even when RPeD1 was made to burst, it was more difficult to initiate rhythmic activity than in controls. It is also conceivable that the input from the neurone mediating the escape–withdrawal response, RPeD11 (Syed and Winlow, 1991), which exerts suppressive control over the respiratory CPG (Inoue *et al.* 1995), may increase as a result of the operant paradigm. Thus, neuronal changes both within the CPG network and external to the CPG may constitute the neuronal basis of this form of associative learning.

Further experimentation utilizing semi-intact preparations, as well as the reconstructed *in vitro* CPG, will be necessary to clarify the possible roles of these changes in neuronal activity in the mediation of this learned respiratory behaviour. Data obtained from these studies may also give an indication as to whether the changes in neuronal activity which underlie operant conditioning are similar to those which appear to underlie classical conditioning and sensitization.

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References

ABRAMSON, C. AND FEINMAN, R. (1987). Operant conditioning of eye elevation in the green crab. *Behav. neural Biol.* **48**, 259–277.

AUDESIRK, T. E., ALEXANDER, J. E., AUDESIRK, G. J. AND MOYER, C. M. (1982). Rapid, non aversive conditioning in a freshwater gastropod. I. Effects of age and motivation. *Behav. neural Biol.* **36**, 379–390.

BARNES, S., SYED, N., BULLOCH, A. AND LUKOWIAK, K. (1994). Modulation of ionic currents by dopamine in an interneurone of the respiratory central pattern generator of *Lymnaea stagnalis. J exp. Biol.* **189**, 37–54.

BOOKER, R. AND QUINN, W. (1981). Conditioning of leg position in normal and mutant *Drosophila. Proc. natn. Acad. Sci. U.S.A.* **78**, 3940–3944.

CAREW, T. AND SAHLEY, C. (1986). Invertebrate learning and memory: From behavior to molecules. *A. Rev. Neurosci.* **9**, 435–487.

COOK, D. AND CAREW, T. (1986). Operant conditioning of head-waving in *Aplysia*. *Proc. natn. Acad. Sci. U.S.A.* **83**, 1120–1124.

COOK, D. AND CAREW, T. (1989*a*). Operant conditioning of head-waving in *Aplysia*. I. Identified muscles involved in the operant response. *J. Neurosci.* **9**, 3097–3106.

COOK, D. AND CAREW, T. (1989*b*). Operant conditioning of head-waving in *Aplysia*. II. Contingent modification of electromyographic activity in identified muscles. *J. Neurosci.* **9**, 3107–3114.

COOK, D. AND CAREW, T. (1989*c*). Operant conditioning of head-waving in *Aplysia*. III. Cellular analysis of possible reinforcement pathways. *J. Neurosci.* **9**, 3115–3122.

COOK, D., STOPFER, M. AND CAREW, T. (1991). Identification of a reinforcement pathway necessary for operant conditioning of head waving in *Aplysia californica*. *Behav. neural Biol.* **55**, 313–337.

DICKINSON, A. (1980). *Contemporary Animal Learning*. Cambridge: Cambridge University Press.

FERGUSON, G. AND BENJAMIN, P. (1991). The whole-body withdrawal response of *Lymnaea stagnalis*. *J. exp. Biol*. **158**, 97–116.

HAWKINS, R., CLARK, G. AND KANDEL, E. (1985). Operant conditioning and differential classical conditioning of gill withdrawal in *Aplysia. Soc. Neurosci. Abstr.* **11**, 796.

HAWKINS, R., KANDEL, E. AND SIEGELBAUM, S. (1993). Learning to modulate transmitter release: Themes and variations in synaptic plasticity. *A. Rev. Neurosci.* **16**, 625–665.

HORRIDGE, G. A. (1962). Learning of leg position by headless insects. *Nature* **193**, 697–698.

HOYLE, G. (1982). Cellular basis of operant-conditioning of leg position. In *Conditioning: Representation of Involved Neural Functions* (ed. C. D. Woody), pp. 197–211. New York: Plenum Press.

INOUE, T., TAKASAKI, M., LUKOWIAK, K. AND SYED, N. (1995). Inhibition of the respiratory pattern generating neurons by an identified whole body withdrawal interneuron of *Lymnaea stagnalis*. *J. Neurophysiol.* (in press).

JONES, J. (1961). Aspects of respiration in *Planorbis corneus* and *Lymnaea stagnalis* (Gastropoda: Pulmonata). *Comp. Biochem. Physiol*. **12**, 283–295.

KEMENES, G. AND BENJAMIN, P. (1989). Appetitive learning in snails shows characteristics of conditioning in vertebrates. *Brain Res*. **489**, 163–166.

KEMENES, G. AND BENJAMIN, P. (1994). Training in a novel environment improves the appetitive learning performance of the snail, *Lymnaea stagnalis. Behav. neural Biol.* **61**, 139–149.

LUKOWIAK, K. (1991). Experimental reconstruction of neuronal pattern generators. *Current Opin. Neurobiol.* **1**, 577–582.

LUKOWIAK, K., WILDERING, W., SPENCER, G. AND SYED, N. (1994). Operant conditioning of aerial respiration in *Lymnaea. Soc. Neurosci. Abstr*. **24**, 46.

MACKINTOSH, N. J. (1974). *The Psychology of Animal Learning*. London: Academic Press.

MOROZ, L., HASAN, S., BULLOCH, A., LUKOWIAK, K. AND SYED, N. (1993). Behavioral analysis of respiration in *Lymnaea stagnalis. Soc. Neurosci. Abstr*. **23**, 183.

MORTOLA, J. P. (1995). Invited editorial on 'Modification of conditioned apneas in rats: evidence for cortical involvement'. *J. appl. Physiol*. **78**, 1213–1214.

PRESTON, R. J. AND LEE, R. M. (1973). Feeding behavior in *Aplysia californica*: Role of chemical and tactile stimuli. *J. comp. physiol. Psychol.* **82**, 368–381.

SOKAL, R. R. AND ROHLF, F. J. (1981). *Biometry*. San Francisco: W. H. Freeman and Co.

SUSSWEIN, A., SCHWARTZ, M. AND FELDMAN, E. (1986). Learned changes of feeding behavior in *Aplysia* in response to edible and inedible foods. *J. Neurosci.* **6**, 1513–1527.

SYED, N., BULLOCH, A. AND LUKOWIAK, K. (1990). *In vitro* reconstruction of the respiratory central pattern generator of the mollusk *Lymnaea. Science* **250**, 282–285.

SYED, N. AND WINLOW, W. (1991). Respiratory behaviour in the pond snail *Lymnaea stagnalis*. II. Neural elements of the central pattern generator (CPG). *J. comp. Physiol.* **169**, 557–568.

THOMAS, A., AUSTIN, W., FRIEDMAN, L. AND STROHOL, K. (1992). A model of ventilatory instability induced in the unrestrained rat. *J. appl. Physiol*. **73**, 1530–1536.

THOMAS, A., FRIEDMAN, L., MACKENZIE, C. AND STROHL, K. (1995). Modification of conditioned apneas in rats: evidence for cortical involvement. *J. appl. Physiol.* **78**, 1215–1536.

WINER, B. J., BROWN, D. R. AND MICHELS, K. M. (1991). *Statistical Principles in Experimental Design*. New York: McGraw-Hill.

WINLOW, W., MOROZ, L. AND SYED, N. (1992). Mechanisms of behavioural selection in *Lymnaea stagnalis*. In *Neurobiology of Motor Programme Selection* (ed. J. Kien, C. R. McCrohan and W. Winlow), pp. 52–72. New York: Pergamon Press.

WOLLACOTT, M. AND HOYLE, G. (1977). Neural events underlying learning in insects: Changes in pacemaker activity. *Proc. R. Soc. Lond. B* **195**, 395–415.