ISJ 17: 129-137, 2020 ISSN 1824-307X

RESEARCH REPORT

Redefining operant conditioning of escape behaviour in *Lymnaea stagnalis*

C Benatti[1,2], V Rivi[3], C Colliva[1,2], G Radighieri[3], F Tascedda[1,2], JMC Blom[2,3*]

[1]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy
[2]Centre of Neuroscience and Neurotechnology, University of Modena and Reggio Emilia, Modena, Italy
[3]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy

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Abstract

The escape behaviour is one of the many behavioural responses that can be operantly conditioned in a stimulus-dependent manner in both vertebrates and invertebrates. By exposing the pond snail *Lymnaea stagnalis* repeatedly to a negative reinforcement its natural tendency to explore its surroundings can be operantly conditioned in both adult and aged snails. When adult snails were trained with 100 mM of KCl their number of escapes was significantly decreased and the latency to first escape was significantly increased. Our behavioural protocol allowed us to investigate memory acquisition, consolidation, and retrieval in pre- and post-training sessions over different days. From the 3rd day of training the learned response was strengthened: the number of the escapes in the post-test session remained significantly reduced even when animals were presented with distilled water. Moreover, adult snails exposed to the negative reinforcement for at least 4 days started to escape significantly less than the control group also in the pre-test session. This effect became more pronounced in the following days and was accompanied by a significant increase in the latency to first escape at the beginning of the pre-test on day 6 and 7. Aged snails, instead, showed selective deficiencies when operantly conditioned: memory retention appeared only after 7 days, while memory retrieval could not be induced. This redefined paradigm can help unravelling a variety of sophisticated cognitive phenomena in *L. stagnalis* and could be employed also to study the basis of memory impairment occurring during neuro-aging.

Key Words: associative learning; memory; behaviour; pond snails; aging

Introduction

Escape behaviour is the result of a complex integration of information from sensory systems, internal states and expectations arising from experience and prior beliefs (Evans *et al.,* 2019). From a neuroscience perspective, escape is a behavioural response that can be operantly conditioned in a stimulus-dependent manner, allowing to study complex cognitive processes, such as learning, memory and decision-making not only in vertebrates, but also in invertebrate model systems (Kemenes and Benjamin, 1989a). In the last decades, in fact, the behavioural, cellular and molecular basis of associative learning and memory have been found to be basically similar throughout the animal kingdom, suggesting that these processes are so fundamental that they appeared early in evolution and are fairly conserved over time (Carew *et al.,* 1981; Kemenes and Benjamin, 1989; Rivi *et al.,* 2020). This has led to the recognition of invertebrates as a more flexible model for the study of conserved basic and advanced cognitive processes (Carew *et al.,* 1981). Among the invertebrate models, the pond snail *Lymnaea stagnalis* has been extensively used to study memory and learning (Kemenes and Benjamin, 1989a; Kojima *et al.,* 1996; Lukowiak *et al.,* 1996; Swinton *et al.,* 2019; Benjamin, 2000). In fact, thanks to the dynamicity of their behaviours, snails can be trained to respond to a wide variety of stimuli, acquiring different forms of associative learning, such as single- or multi-trials, aversion or reward, operant or classical conditioning (Alexander *et al.,* 1984; Kojima *et al.,* 1996; Lukowiak *et al.,* 1996; Kemenes *et al.,* 1997; Andrew and Savage, 2000; Kawai, 2004; Fulton *et al.,* 2005; Ito *et al.,* 2013).

Corresponding author:
Joan MC Blom
Department of Biomedical, Metabolic and Neural Sciences
University of Modena and Reggio Emilia
Via Campi 287, 41125, Modena, Italy
E-mail: joan.blom@unimore.it

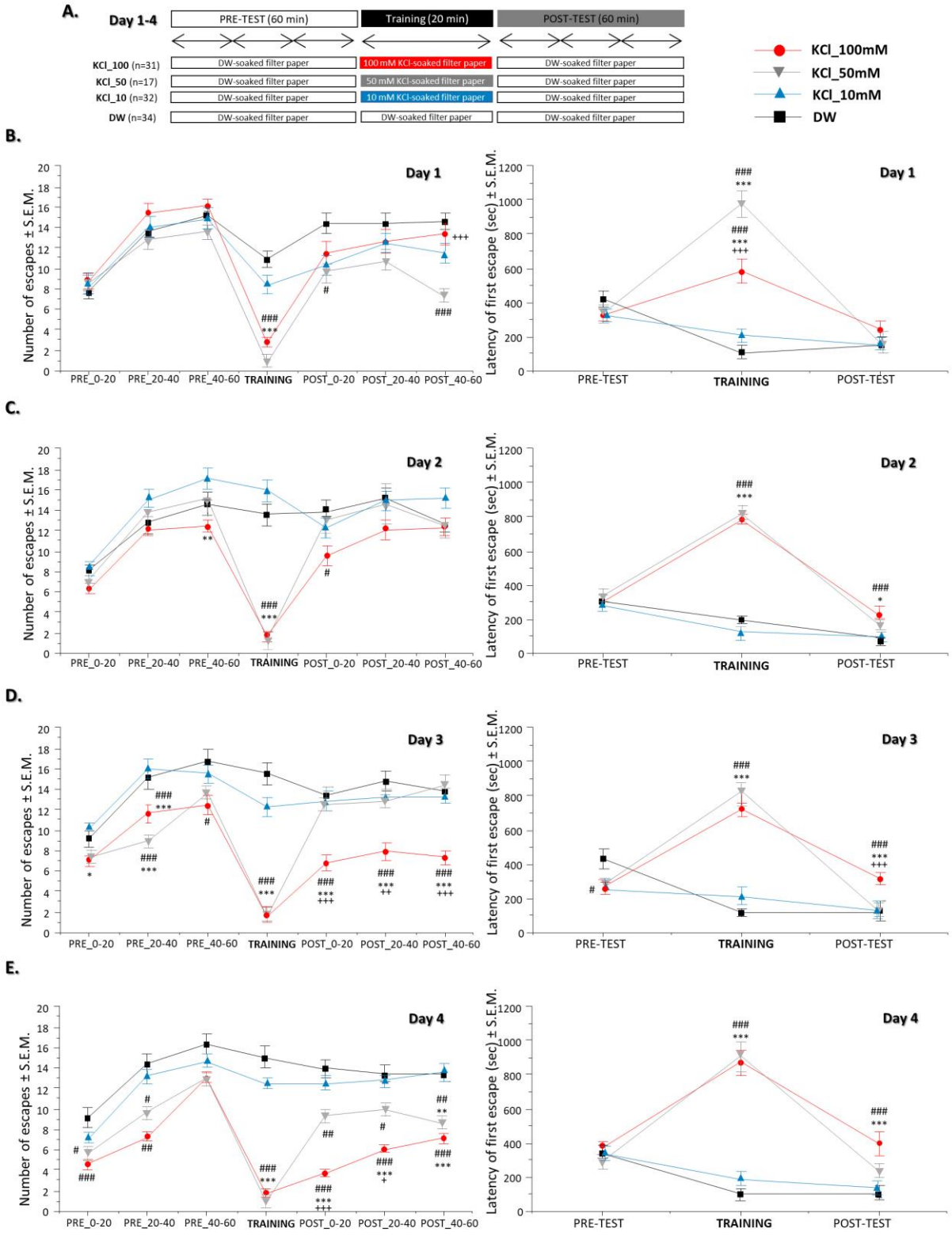

Fig. 1 Experimental groups and behavioural procedure of experiment 1 (A). Escapes performed every 20 minutes and latency to first escape for each session were evaluated on day 1 (B), day 2 (C), day 3 (D), day 4 (E) in adult *L. stagnalis*. Each snail was placed in a 35 mm water container filled with distilled water (DW) set on a filter paper, during training the filter paper was soaked with KCl 100 mM, KCl 50 mM, KCl 10 mM or DW while in the other sessions the filter paper was soaked with DW. Data are expressed as mean ± SEM. Comparisons were made by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test; ### $p < 0.0001$, ## $p < 0.01$, # $p < 0.05$ vs DW; *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$ vs KCl_10mM; +++ $p < 0.0001$, ++ $p < 0.01$, + $p < 0.05$ vs KCl_50mM

In a preliminary study Kobayashi and colleagues observed that the repeated exposure to an aversive stimulus suppressed the natural tendency of snails to explore their surroundings without eliciting a long lasting memory retention (Kobayashi *et al.,* 1998). The chosen aversive stimulus, potassium chloride (KCl) acted as a strong stressor stimulus eliciting the whole-body withdrawal response of the snails into their shells (Kojima *et al.,* 1996; Ito *et al.,* 2015). Previous studies, in fact, demonstrated that the exposure to KCl 100 mM was more aversive than lower concentrations, causing snails to remain in their shells for a prolonged period of time (Kojima *et al.,* 1996).

The purpose of this study is to extend Kobayashi findings and investigate memory acquisition, consolidation, and retrieval and test the hypothesis if repeated exposure to a negative reinforcement results in a long-lasting behavioural alteration manifested even when KCl is not present in the external environment.

Moreover, *Lymnaea* has been identified as valid model system for exploring age-related behavioural, cellular and molecular changes, including memory impairment and decline (Kemenes *et al.,* 2006; Hermann *et al.,* 2007, 2020; Pirger *et al.,* 2014; Watson *et al.,* 2012, 2013; Fodor *et al.,* 2020). So, we employed the paradigm of operant conditioning of escape behaviour in adult and aged snails to assess age-related differences in memory formation and retrieval.

Materials and Methods

Ethics statement

Pond snails *Lymnaea stagnalis* are very abundant in the northern continents of the world and are not endangered nor a protected species. Experiments on pond snails are not subject to the approval of our ethics committee. Nonetheless, every effort was made to maximise the wellbeing of the snails during the behavioural procedures.

Snails and colony maintenance

Laboratory-reared *L. stagnalis* (Linnaeus 1758), originally derived from a stock generously donated by the Vrije University in Amsterdam (The Netherlands), were used in this study. Animals were maintained in aquaria at the University of Modena and Reggio Emilia (Italy) at 21-23 °C in well-aerated dechlorinated tap water on a 12/12 h light/dark cycle (lights on at 08:00 a.m.), animals were fed pesticide-free lettuce twice a week.

Six- and nine-month old snails having shell lengths of 20-25 mm were used in these experiments. Because snails survive for 9-10 months in our breeding facility, we selected two age categories, 6 and 9 months old, based on the age categorization by Hermann and co-workers (2007), to compare learning performance among these cohorts (Hermann *et al.,* 2007). We refer to these two groups as “adult” and “aged” snails in the rest of the text, but these terms should be interpreted as indicating middle and late stages of *Lymnaea*’s life cycle under the current breeding conditions of our lab. The term “aged” as it is used here should not be generalized to mean the maximal chronological age this species can attain.

Twenty-four hours before the behavioural test the animals were food deprived, and 12 hours prior to start the behavioural test, animals were kept in 12 L tanks supplied with 6 L of distilled water (DW) and 6 L of water of aquaria (15-18 animals per tank). All experiments were performed between 10 am and 1 pm local time.

Conditioning paradigm

Snails were placed in the middle of a 35 mm Petri dish (Sarstedt, Germany) with 2 mm depth of DW. That was enough for the snails to be fully submerged.

In the pre-test session, the Petri-dish lids containing *Lymnaea* were set on a sheet of DW-soaked filter paper and every time snails escaped from the lids and touched the soaked paper with their lips, the experimenter moved the snail back into the lid. Snails have been excluded from the study if the total number of escapes during the pre-test on day 1 were < 12 (in 60 minutes). Next, the lids containing *Lymnaea* were transferred on a sheet of filter paper soaked with either DW or different concentrations of KCl for training. When snails escaped and touched the negative reinforcement with the lips, the withdrawal response of their bodies into their shells was elicited. In the post-test session, the Petri-dish lids containing *Lymnaea* were transferred on a new sheet of DW-soaked filter paper. For each session, the number of escapes performed in a 20-minute interval and the time necessary for the first escape to occur were annotated by an experimenter blind to the experimental condition. During the procedure, snails were kept wet minimalizing any form of suffering. At the end of the training procedure, snails were fed *ad libitum* with fresh lettuce until 12 hours prior to the start of the next-day procedure.

Experiment 1 Effect of different concentration of KCl on escape conditioning

One hundred and nine six-month old snails were used in this experiment and divided into 4 experimental groups:

1. KCl 10 mM: snails exposed to KCl 10 mM during the training session (n= 29);
2. KCl 50 mM: snails exposed to KCl 50 mM during the training session (n= 16);
3. KCl 100 mM: snails exposed to KCl 100 mM during the training session (n= 31);
4. DW: snails exposed to DW during the training session (n= 33).

The conditioning procedure consisted in (1) a pre-test session lasting 60 minutes, (2) a training session lasting 20 minutes and (3) a post-test session lasting 60 minutes, repeated over 4 days.

Experiment 2 Effect of massed training on escape conditioning

While in the spaced procedure snails were exposed to KCl for 20 minutes a day repeated for 4 days, the exposure to the negative reinforcement in the massed training procedure occurred only in Day 1 and lasted 80 minutes.

Sixty-seven six-month old snails were used in this experiment, articulated over 3 days:

Day 1: (1) a pre-test session lasting 60 minutes, (2) a training session lasting 80 minutes and (3) a post-test session lasting 40 minutes. During the pre and post-test sessions, the filter paper was always soaked with DW. During the training session, the filter paper was soaked with a KCl 100 mM solution for the conditioning group (KCl group) (n= 31), and with DW for the control group (DW group) (n= 36).

Day 2 and 3: observational session lasting 60 minutes, where all snails were exposed to DW.

Experiment 3 Effect of aging on the efficacy of escape conditioning

Thirty six-month old snails and 45 nine-month old snails were used in this experiment and divided into 4 experimental groups:

1. Aged DW: nine-month old snails exposed to DW during the training session (n= 22);
2. Aged KCl: nine-month old snails exposed to KCl 100 mM during the training session (n= 23);
3. Adult DW: six-month old snails exposed to DW during the training session (n= 15);
4. Adult KCl: six-month old snails exposed to KCl 100 mM during the training session (n= 15).

The conditioning procedure consisted in (1) a pre-test session lasting 60 minutes, (2) a training session lasting 20 minutes and (3) a post-test session lasting 60 minutes, repeated over 7 days.

Statistics

Behavioural data were expressed as the mean number of escapes in a 20-minute interval or the latency to first escape from the water container (seconds) for each session ± S.E.M. (Standard Error of the Mean). Exclusion of extreme outliers was made prior to statistical analysis using the boxplot tool in SPSS (more than 3x the interquartile range outside of the end of the interquartile box). Behavioural data were tested for significant differences by a univariate analysis of variance (ANOVA) followed by Tukey (with $p < 0.05$ significance level). Analyses were conducted using SPSS for Windows® v.26 (SPSS Inc., Chicago, USA).

Results

In experiment 1, a spaced training procedure similar to that employed by Kobayashi *et al.* (1998) was used: animals were divided in four groups with a similar mean number of escapes in the pre-test of day 1 and trained either with KCl 100 mM, KCl 50 mM, KCl 10 mM or DW for 4 days (Fig. 1A). During training, from day 1 to day 4, snails exposed to 50 and 100 mM KCl performed less escapes and showed a longer latency to first exit than their DW and KCl 10 mM counterparts (see Table 1A for a detailed summary of the main effects, One-way ANOVA; Fig. 1B-1E). In the day-1 post-test session the number of escapes and the latency to the first exit were restored after the negative reinforcement was removed, with the sole exception of animals exposed to KCl 50 mM whose performance remained impaired with respect to DW and KCl 10 mM groups (Fig. 1B). No difference was observed between the performance of DW and KCl 10 mM either during training or during the other sessions throughout experiment 1 (Fig. 1B-1E).

Table 1 Summary of statistical analysis of experiment 1 (A), experiment 2 (B), and experiment 3 (C).Main effects on escapes performed every 20 minutes and latency to first escape for each session were evaluated using one way analysis of variance (ANOVA), all mean differences were considered statistically significant if $p < 0.05$ (bold).

A)

EXP 1	Number of escapes							Latency to first escape		
	Pre-test			Training	Post-test			Pre-test	Training	Post-test
Day	0'-20'	20'-40'	40'60'	0'-20'	0'-20'	20'-40'	40'60'			
1	F(3;104)=0.266 p=0.850	F(3;104)=1.348 p=0.263	F(3;104)=2.206 p=0.092	**F(3;99)=37.596 p< 0.0001**	**F(3;103)=4.238 p=0.007**	F(3;104)=1.781 p=0.155	**F(3;105)=8.256 p<0.0001**	F(3;102)= 1.456 p=0.231	**F(3;103)= 52.209 p<0.0001**	**F(3;102)= 2.783 p=0.045**
2	F(3;102)=1.976 p=0.122	F(3;99)=1.616 p=0.190	**F(3;100)=3.618 p=0.016**	**F(3;101)=72.975 p<0.0001**	**F(3;103)=3.676 p=0.015**	F(3;101)=2.100 p=0.105	F(3;100)=2.254 p=0.087	F(3;98)= 0.439 p=0.726	**F(3;101)= 47.547 p<0.0001**	**F(3;100)=6.748 p<0.0001**
3	F(3;103)=2.980 p=0.035	**F(3;102)=9.212 p<0.0001**	**F(3;101)=3.531 p=0.018**	**F(3;101)=59.037 p<0.0001**	**F(3;102)=12.738 p<0.0001**	**F(3;100)=11.463 p<0.0001**	**F(3;102)=19.181 p<0.0001**	**F(3;101)=3.935 p= 0.011**	**F(3;100)= 29.935 p<0.0001**	**F(3;100)= 15.342 p<0.0001**
4	**F(3;99)=8.428 p<0.0001**	**F(3;97)=21.592 p<0.0001**	**F(3;101)=4.348 P=0.006**	**F(3;96)=82.100 p<0.0001**	**F(3;98)=36.338 p<0.0001**	**F(3;98)=22.669 p<0.0001**	**F(3;98)=18.642 p<0.0001**	F(3;96)= 0.595 p=0.620	**F(3;98)= 59.661 p<0.0001**	**F(3;98)= 11.851 p<0.0001**

B)

EXP 2	Number of escapes									Latency to first escape		
	Pre-test			Training				Post-test		Pre-test	Training	Post-test
Day	0'-20'	20'-40'	40'60'	0'-20'	20'-40'	40'60'	60'-80'	0'-20'	20'-40'			
1	F(1;66)=0.674 p=0.414	F(1;65)=0.001 p=0.975	F(1;65)=2.238 p=0.139	**F(1;65)=62.98 p<0.0001**	**F(1;65)=114.4 p<0.0001**	**F(1;66)=82.66 p<0.0001**	**F(1;66)=181.4 p<0.0001**	**F(1;64)=10.456 p=0.002**	F(1;65)=1.16 p=0.286	F(1;66)=0.198 p=0.658	**F(1;65)=9.824 p=0.003**	F(1;65)=3.079 p=0.084
	Observation									Observation		
2	**F(1;63)=8.808 p=0.004**	**F(1;63)=6.615 p=0.012**	**F(1;63)=5.104 p=0.027**							F(1;64)=0.197 p=0.659		
3	F(1;61)=1.876 p=0.176	F(1;61)=0.092 p=0.763	F(1;61)=1.993 p=0.163							F(1;63)=0.011 p=0.915		

C)

EXP 3	Number of escapes							Latency to first escape		
	Pre-test			Training	Post-test			Pre-test	Training	Post-test
Day	0'-20'	20'-40'	40'60'	0'-20'	0'-20'	20'-40'	40'60'			
1	F(3;73)=1.594 p=0.199	F(3;71)=0.685 p=0.564	F(3;71)=1.254 p=0.297	**F(3;72)=21.396 p<0.0001**	F(3;74)=0.234 p=0.872	F(3;72)=0.886 p=0.453	**F(3;71)=2.871 p=0.048**	F(3;73)=1.834 p=0.149	**F(3;71)=13.971 p<0.0001**	F(3;71)=2.318 p=0.083
2	**F(3;71)=3.295 p=0.026**	F(3;74)=0.305 p=0.822	F(3;74)=0.198 p=0.898	**F(3;71)=97.282 p<0.0001**	**F(3;74)=11.439 p<0.0001**	**F(3;72)=4.172 p=0.009**	**F(3;71)=3.989 p=0.011**	F(3;71)=0.165 p=0.919	**F(3;71)=53.447 p<0.0001**	F(3;71)=1.309 p=0.279
3	F(3;67)=2.075 p=0.112	**F(3;71)=9.415 p<0.0001**	**F(3;71)=3.451 p=0.021**	**F(3;71)=66.751 p<0.0001**	**F(3;71)=7.788 p<0.0001**	**F(3;71)=9.082 p<0.0001**	**F(3;71)=9.224 p<0.0001**	**F(3;70)=3.158 p=0.030**	**F(3;70)=24.652 p<0.0001**	F(3;71)=2.113 p=0.107
4	**F(3;64)=9.684 p<0.0001**	F(3;64)=1.679 p=0.181	**F(3;64)=2.882 p=0.043**	**F(3;66)=120.382 p<0.0001**	**F(3;65)=7.607 p<0.0001**	**F(3;64)=7.037 p<0.0001**	**F(3;66)=6.392 p=0.001**	F(3;65)=0.219 p=0.883	**F(3;66)=179.868 p<0.0001**	**F(3;65)=7.607 p<0.0001**
5	**F(3;63)=6.646 p=0.001**	**F(3;63)=5.694 p=0.002**	**F(3;63)=4.696 p=0.005**	**F(3;62)=56.218 p<0.0001**	**F(3;65)=4.238 p=0.009**	**F(3;65)=7.041 p<0.0001**	**F(3;65)=5.507 p=0.002**	**F(3;61)=3.315 p=0.026**	**F(3;61)=152.088 p<0.0001**	**F(3;63)=6.718 p=0.001**
6	**F(3;63)=6.793 p=0.001**	**F(3;63)=2.310 p=0.085**	**F(3;63)=3.495 p=0.021**	**F(3;63)=70.078 p<0.0001**	**F(3;63)=13.860 p<0.0001**	**F(3;63)=16.126 p<0.0001**	**F(3;63)=19.625 p<0.0001**	**F(3;59)=9.847 p<0.0001**	**F(3;62)=66.824 p<0.0001**	**F(3;63)=3.573 p=0.019**
7	**F(3;63)=6.200 p=0.001**	**F(3;63)=10.338 p<0.0001**	**F(3;63)=9.346 p<0.0001**	**F(3;63)=424.356 p<0.0001**	**F(3;63)=21.904 p<0.0001**	**F(3;63)=8.364 p<0.0001**	**F(3;63)=31.251 p<0.0001**	**F(3;62)=10.397 p<0.0001**	**F(3;61)=56.167 p<0.0001**	**F(3;60)=14.348 p<0.0001**

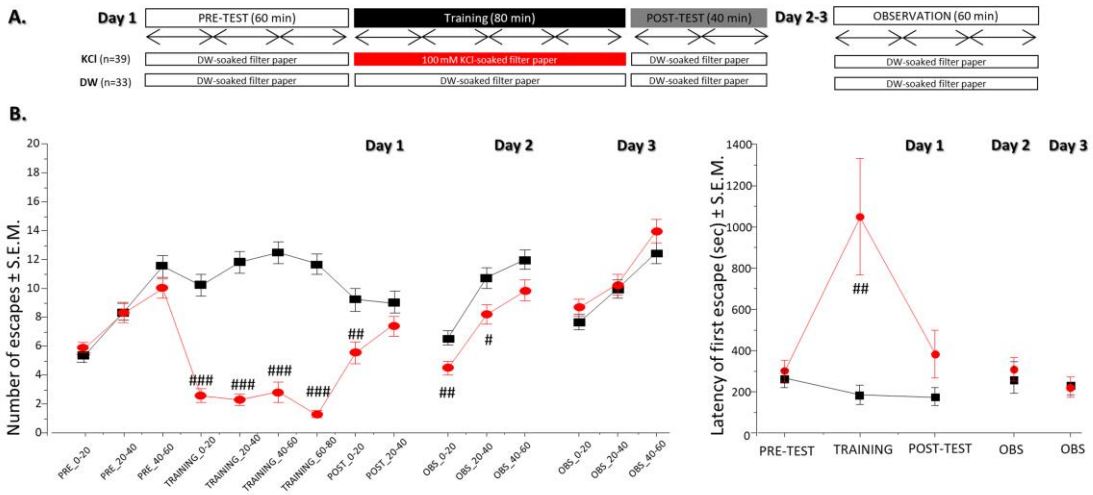

Fig. 2 Experimental groups and behavioural procedure of experiment 2 (A). Escapes performed every 20 minutes and latency to first escape for each session were evaluated in *L. stagnalis* (B). Each snail was placed in a 35 mm water container filled with distilled water (DW) set on a filter paper, during training the filter paper was soaked with either KCl 100 mM or DW while in the other sessions the filter paper was soaked with DW. Data are expressed as mean ± SEM. Comparisons were made by one-way analysis of variance (ANOVA) ### $p < 0.0001$, ## $p < 0.01$; # $p < 0.05$

On the second day of training, some alterations in the behavioural parameters started to emerge only in animals exposed to the highest concentration of the negative reinforcement: the 100 mM KCl group performed less escapes in the final part of the pre-test and in the first twenty minutes of the post-test: when compared to their DW counterparts they showed a significantly increased latency to first exit after training (Fig. 1C). This effect became more pronounced on day 3, where a significant decrease in the number of escapes was evident in KCl 100 mM-exposed animals during the whole pre-test session, while snails exposed to KCl 50 mM performed significantly less escapes than DW and KCl 10 mM-exposed animals only in the first 40 minutes of the pre-test session. After training the latency to first exit and the number of escapes of the 50 mM KCl group was restored, while in animals exposed to the highest concentration of the negative reinforcement the escape behaviour remained significantly compromised: snails exposed to 100 mM of KCl during training preformed significantly less escapes in the post-test and showed a higher latency to first exit with respect to all the other experimental groups (Fig. 1D). In the 4th day, no difference was observed in the behaviour of the DW and KCl 10 mM exposed animals, while training with both the higher concentrations of KCl affected the performance of the animals both in the pre – and post-test sessions. The number of escapes of animals exposed to 50 or 100 mM KCl was significantly decreased with respect to DW or KCl 10 mM groups only in the first 40 minutes of the pre-test, this effect was not present in the last interval of the session. After training, the KCl 100 mM group took significantly more time to first exit from the petri lid than the other groups and the number of escapes they performed remained significantly lower than their 50 mM, 10 mM and DW exposed counterparts for the first 40 minutes of the post-test session. In the last interval the performances of animals exposed to the higher concentrations of the negative reinforcement were not different from each other, while remaining significantly lower than the KCl 10 mM and DW groups. Only training with KCl 100 mM for 4 days significantly affected the latency to first exit in the post-test session (Tab. 1A, Fig. 1E).

In experiment 2 a readapted protocol of a massed training procedure was employed (Fig. 2A, 2B): on day 1, *Lymnaea* were operantly conditioned for 80 minutes using the highest concentration of KCl tested in experiment 1. Initially, 67 snails were monitored for their exploratory activity in the pre-test session and were divided in two groups not statistically different from each other (see Table 1B for a detailed summary of the main effects, One-way ANOVA; Fig. 2A). During the 80-minute long training, the number of escapes was significantly suppressed in KCl exposed animals with respect to the DW control group (Fig. 2B). At the end of the training session, when the animals were re-exposed to DW-soaked filter paper, the number of escapes of the KCl-exposed group remained lower in the first 20 minutes and was recovered 40 minutes after the end of the training session. As observed in the spaced procedure, the latency to first exit at the beginning of the training session was significantly increased in animals exposed to negative reinforcement. A similar, but not statistically significant, trend was observed in conditioned snails

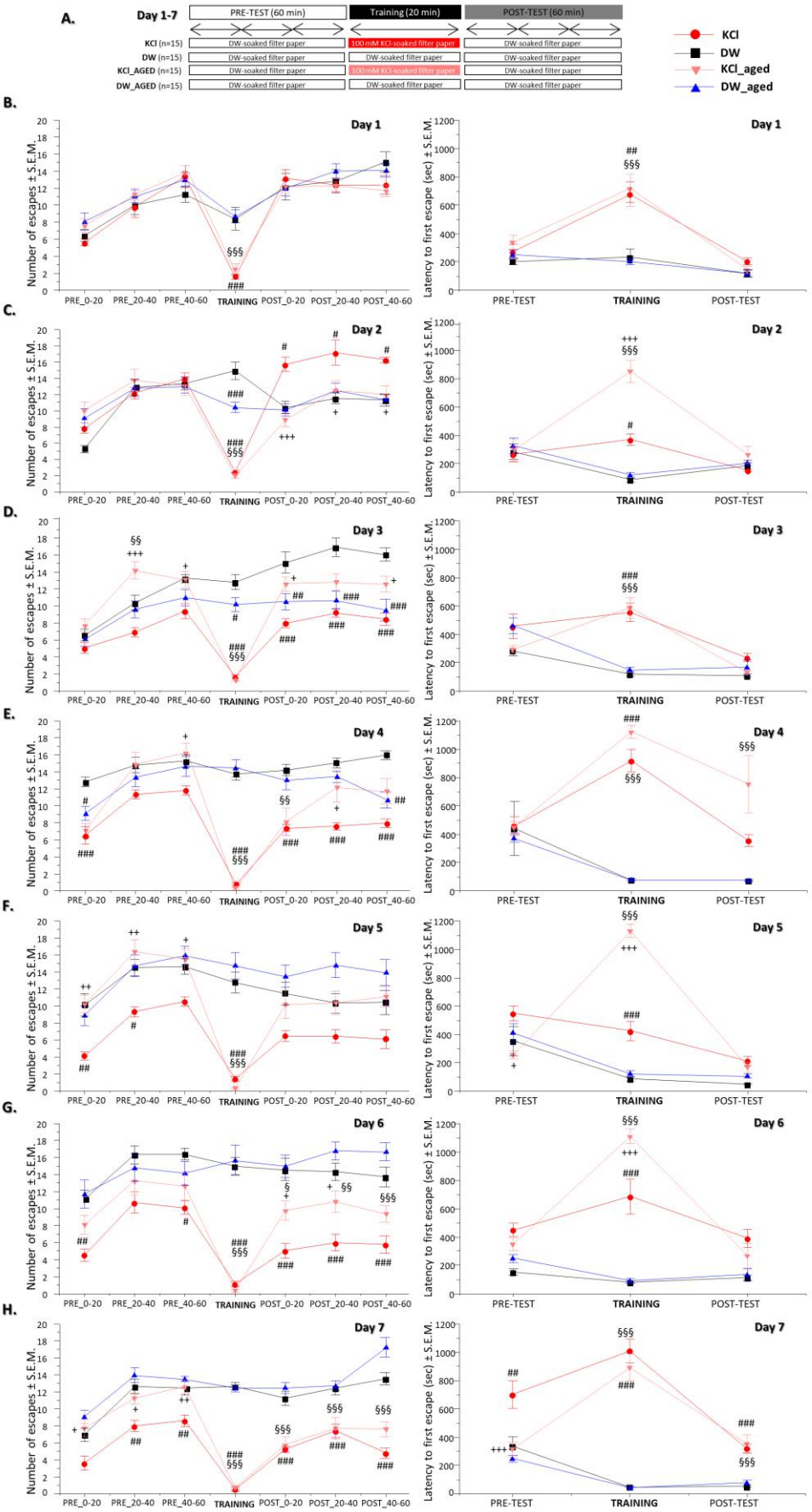
A.
Day 1-7
PRE-TEST (60 min)
Training (20 min)
POST-TEST (60 min)
KCl (n=15)
DW (n=15)
KCl_AGED (n=15)
DW_AGED (n=15)
DW-soaked filter paper
100 mM KCl-soaked filter paper
KCl
DW
KCl_aged
DW_aged
B.
C.
D.
E.
F.
G.
H.
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Number of escapes ± S.E.M.
Latency to first escape (sec) ± S.E.M.
PRE_0-20
PRE_20-40
PRE_40-60
TRAINING
POST_0-20
POST_20-40
POST_40-60
PRE-TEST
TRAINING
POST-TEST

Fig. 3 Experimental groups and behavioural procedure of experiment 3 (A). Escapes performed every 20 minutes and latency to first escape for each session were evaluated on day 1 (B), day 2 (C), day 3 (D), day 4 (E), day 5 (F), day 6 (G), day 7 (H) in six-month old (Adult) or in nine-month old (Aged) *L. stagnalis*. Each snail was placed in a 35mm water container filled with distilled water (DW) set on a filter paper, during training the filter paper was soaked with either KCl 100 mM or DW while in the other sessions the filter paper was soaked with DW. Data are expressed as mean ± SEM. Comparisons were made by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test ### $p < 0.0001$, ## $p < 0.01$, # $p < 0.05$ vs DW; §§§ $p < 0.0001$, §§ $p < 0.01$, § $p < 0.05$ vs DW_aged; +++ $p < 0.0001$, ++ $p < 0.01$, + $p < 0.05$ vs KCl

in the post-test session as well (Fig. 2B). On the second and third day all snails were placed over DW-soaked filter papers: while no difference was observed in the latency to first exit between conditioned animals and the control group, the number of escapes of KCl-exposed animals remained significantly lower than their DW counterparts in the first 40 minutes of the observation period and then was restored completely on day 3 (Fig. 2B).

In experiment 3, 30 six-month old snails and 45 nine-month old snails were divided in two groups (not performing differently from each other in the pre-test of day 1). In day 1, the negative reinforcement significantly reduced the number of escapes and the latency to first escape during training in both aged and adult animals (see Table 1C for a detailed summary of the main effects, One-way ANOVA; Fig. 3B). At the end of the session, the escape tendency was restored in KCl-exposed snails irrespective of their age (Tab. 1C; Fig. 3B). From day 2 to day 7, exposure to 100 mM KCl during training resulted in a significant decrease in the number of escapes coupled to an increase in the latency to first escape with respect to control animals in both adult and aged snails (Fig. 3C-H). No difference was observed in the performance of aged and adult animals following the same procedure, with the sole exception of day 3 when older snails exposed to DW performed less escapes during training than their younger counterparts (Fig. 3D). In the post-training session of day 2 the number of escapes was restored in KCl exposed snails and younger snails escaped more than both their older counterparts and DW-controls (Tab. 1C, Fig. 3C). From day 3 onward, in the post-test session, younger snails exposed to KCl continued to escape significantly less than their DW-counterparts, while for aged snails a significant difference in the escape tendency was evident with respect to the DW group after exposure to the negative reinforcement on day 6 and 7 (Tab. 1C, Fig. 3G, 3H). As already observed in experiment 1, adult snails exposed to the negative reinforcement for 4 days started to escape significantly less than their DW-counterparts also in the pre-test session. This effect became more pronounced on the following days and was accompanied by a marked increase in the latency to first escape at the beginning of the pre-test on day 6 and more distinctively on day 7 (Fig. 3F-H).

Discussion

The purpose of this study was threefold. First, we investigated the conditioning effect of different concentrations of KCl in *L. stagnalis*; second, we assessed the optimum concentration of KCl for memory retention, and third we established the number of days of training necessary for memory to occur. Our results showed a concentration-dependent effect of KCl as negative reinforcement: training with either KCl 50 mM or 100 mM resulted in an immediate reduction of the number of escapes and increased latency to first escape, whereas the behavioural outcome induced by the exposure to KCl 10 mM were either transient or minor, possibly because the withdrawal response can be elicited only by concentrations of KCl higher than 10 mM (Kojima *et al.,* 1996; Ito *et al.,* 2015). The experience-derived changes in behaviour observed in snails exposed to the higher doses of KCl may represent a key-step for associative learning and memory acquisition: snails, in fact, associated the negative reinforcement with their escape behaviour, avoiding exiting from their lids. In the first two days of the procedure, once *Lymnaea* recognized that the external environment was safe (i.e. without KCl), the memory of the negative past situation was extinguished and normal escape behaviour was recovered. From the third day of training on, in animals conditioned with KCl 100 mM, the learned response was strengthened: the number of escapes in the post-test session remained significantly reduced and the latency to the first escape was enhanced. Re-exposure to the highest dose of the negative reinforcement consolidated memories and conditioned the tendency of snails to escape from their lids even if they were presented with DW. On the other hand, the behavioural effects elicited by exposure to 50 mM of KCl observed on day-1 and 3 were less consistent. Moreover, on day-3 they were confined to the pre-test session, where they mirrored the effect of the highest concentration. It took 4 days of training with this intermediate concentration of KCl to observe a significant effect on the escape behaviour both in the pre- and the post-test session.

While the latency to first exit in the post test was affected only by KCl 100 mM, the number of escapes was decreased in a concentration-dependent manner.

In the paper by Kobayashi *et al.* (1998) the acquisition of learning in the spaced training

procedure was very brief and extinguish quickly (Kobayashi *et al.,* 1998), whereas in our study we observed that the exposure to negative reinforcement for 4 consecutive days started to affect the behaviour of the snails even in the pre-test session, indicating that the memory of the past situation was enough to impair the escape behaviour also in a safe external environment. This behavioural output suggests that the previously acquired memory has been retrieved. Thus, memory retrieval made snails able to predict what and when stimuli are likely to occur avoiding, in that way, exiting even in the pre-test session.

In our study, we also confirmed that memory persists longer with spaced training than it does with massed training. With the massed training experiments, in fact, we aimed to evaluate the effects of this procedure on the escape behaviour and to confirm what had been already observed by Kobayashi *et al.,* (1998). We observed that the reduction in the number of escapes induced by an 80-minute long training with KCl 100 mM did not persist for more than 24 hours and did not impact the latency to first exit when the aversive stimulus was removed. The spaced procedure was more effective in conditioning the escape behaviour of *L. stagnalis*. This is in accordance with several studies using both classical and operant conditioning paradigms in vertebrates and invertebrates models demonstrating that spaced training is superior at generating long-term memories (Lukowiak *et al.,* 1998; Commins *et al.,* 2003; Takigami *et al.,* 2014).

Finally, we demonstrated that memory retention appeared in aged snails after 7 days of training, three days later than in the younger ones. Interestingly from day 5 to day 7 the escape behaviour of adult snails became more compromised also in the pre-test session. Based on these data, we hypothesized that memory acquisition is not affected by aging, but its retention gradually declines in older *Lymnaea*. Because the same KCl-induced withdrawal responses have been observed in adult snails as in the aged ones, it seems that independent of their age, snails can detect the presence of KCl and modify their behaviour consequently. Similar to what we observed, Hermann and colleagues (2007) demonstrated that the acquisition of appetitive memory was not compromised by aging, but its retention and consolidation become progressively impaired with advancing age (Herman *et al.,* 2007). Moreover, using the appetitive classical conditioning, they correlated the age-associated learning and memory deficits with a reduced electrophysiological excitability in key neurons controlling the feeding behaviour (Herman *et al.,* 2007).

At this point of our research, future studies will be needed to explore which mechanisms may sustain our behavioural results: the impact of age-related changes in electrophysiological activity, motor and/or chemosensory functions and the functionality of biochemical components of memory formation on escape behaviour in aging snails remains to be investigated.

Interestingly, Pirger and colleagues (2014) demonstrated that the age-related memory decline in old snails can be reversed by administrating the Pituitary Adenylate Cyclase Activating Polypeptide 38 (PACAP38), a molecular key-factor involved in synaptic plasticity and memory processes in both vertebrates and in invertebrates (Kiss and Priger, 2013; Pirger *et al.,* 2014). Other studies characterizing age-related changes in “learning ganglia” of the feeding and respiratory network used classical and operant conditioning, respectively (Patel *et al.,* 2006, 2010; Yeoman *et al.,* 2008; Watson *et al.*, 2012, 2013).

Lymnaea has been established as a powerful and suitable platform for the study of evolutionarily conserved age-related modifications (Audesirk *et al.,* 1982; Patel *et al.,*2006; Hermann *et al*., 2007, Yeoman *et al.,* 2008; Deak and Sonntag, 2012; 2020; Watson *et al.,* 2012, 2013; Pirger *et al.,* 2014; Tascedda *et al.,* 2015).

Our data support for this model a more prominent role in this field: the operant conditioning of escape behaviour in *L. stagnalis*, scarcely employed in the last twenty years, could help to unravel a variety of sophisticated cognitive phenomena that were previously thought to be restricted to vertebrates or humans, such as configural learning (Swinton *et al.,* 2019) and goal-directed decision-making (Crossley *et al.,* 2019).

Acknowledgements

This research was supported by Regione Emilia-Romagna "L’invertebrato *L. stagnalis* quale modello per la Medicina Traslazionale" L.R. N. 20/2002 PROGETTI DI RICERCA SUI METODI ALTERNATIVI ALL'UTILIZZO DI ANIMALI; and FAR 2016 Department of Life Sciences, University of Modena and Reggio Emilia. These sources of funding had no involvement in the study design, data collection, analysis, and interpretation, writing of the report, or in the decision to submit the paper for publication. The Authors thank the CONAD “Le Torri” Shopping Mall (Modena, Italy) for generously providing lettuce for the snails’ feeding.

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