Universidad de Oviedo

Departamento de Psicología

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TESIS DOCTORAL

Programa de Doctorado de Psicología

BIOLOGICAL FUNCTION OF CLASSICAL CONDITIONING IN *CORNU ASPERSUM* AND *LYMNAEA STAGNALIS:* EFFECTS ON FOOD FINDING, NUTRITION AND MATING

Beatriz Álvarez Díaz

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Oviedo
2017

FUNDACION PARA EL FOMENTO EN ASTURIAS
DE LA INVESTIGACION CIENTIFICA APLICADA
Y LA TECNOLOGIA

That snail,

One long horn, one short,

What's on his mind?

(Yosa Buson)

Agradecimientos

A todos aquellos que han hecho posible la elaboración de esta tesis y que me han acompañado a lo largo del camino. A Nacho, Karen, Joris, Joaquín, Susi, Félix, Eu, Paula, Diego, y Ana. De manera aún más especial, a mi madre y a mis abuelos.

Gracias

Contents

Index of Figures

Index of Tables

Declaración

Los experimentos recogidos en el capítulo 2 de esta tesis han sido publicados como:

Loy, I., Alvarez, B., Strempler-Rubio, E. C., & Rodríguez, M. Coordinating associative and ecological accounts of learning in the garden snail *Cornu aspersum.* *Behavioural Processes, in press*

Resumen

El estudio de la cognición animal es importante no solo para conocer cuáles son y cómo funcionan los procesos psicológicos que hacen que los animales capten, procesen, recuerden y utilicen información del medio ambiente, sino también para determinar cómo dichos procesos psicológicos permiten que se adapten mejor a su entorno, incrementando su supervivencia y su éxito reproductivo o fitness (e.g., Mackintosh, 1974). El condicionamiento clásico es uno de los procesos de aprendizaje que permite a los animales predecir eventos biológicamente relevantes y su estudio ha despertado un gran interés dentro de la psicología comparada. Sin embargo, a pesar de que el objetivo principal de la psicología comparada es conocer cómo han ido evolucionando los distintos procesos que determinan la inteligencia (Mackintosh, 1988), la mayor parte de la investigación en este campo se ha llevado a cabo con especies cordadas (especialmente ratas, palomas y humanos; Beach, 1949; Shettleworth, 2009), lo que dificulta el desarrollo de una psicología que sea verdaderamente comparada y representativa del árbol filogenético animal. De forma similar, los trabajos experimentales realizados sobre la función biológica o los beneficios adaptativos del condicionamiento clásico se han hecho mayoritariamente con animales pertenecientes casi exclusivamente al filo de los cordados, siendo muy pocas las excepciones en las que se han utilizado especies de otros filos.

Los moluscos constituyen el segundo mayor filo dentro del reino animal (Ponder & Lindberg, 2008) y han sido varios los autores que han desarrollado procedimientos experimentales para el estudio comparado del aprendizaje asociativo en distintas especies dentro de este filo (e.g., véase Gelperin, 2013 para algunos ejemplos en gasterópodos). En el caso del caracol común *Cornu aspersum,* Ungless (1998) desarrolló un

procedimiento de condicionamiento que posteriormente fue ampliamente utilizado para el estudio comparado del aprendizaje asociativo. No obstante, aunque con dicho procedimiento se han descrito varios fenómenos de aprendizaje asociativo, la función biológica del condicionamiento clásico sigue sin haber sido explorada en esta especie. Parte del trabajo que se presenta a continuación tiene como objetivo analizar cuáles son los beneficios adaptativos del condicionamiento clásico en términos de búsqueda de comida y de ingesta y crecimiento en dicha especie. Por otro lado, también se explora la función biológica del condicionamiento clásico en el apareamiento de una especie distinta, el caracol de agua dulce *Lymnaea stagnalis*. Esta especie fue elegida para el estudio de la función biológica del condicionamiento clásico por dos razones. La primera es que, a pesar de que se conoce mucho sobre su comportamiento reproductivo (e.g., Koene, 2010; Nakadera et al., 2015) y sobre los aspectos fisiológicos involucrados en su apareamiento, no hay estudios que evalúen la interacción entre condicionamiento clásico y apareamiento en esta especie. La segunda razón es que, hasta la fecha, no se ha podido demostrar que esta especie se pueda beneficiar de la experiencia a la hora de aparearse (Koene & Cosijn, 2012).

En cuanto a la búsqueda de comida, los resultados obtenidos mostraron que, para *Cornu aspersum,* la experiencia previa con la comida es necesaria para que ésta sea encontrada y que el condicionamiento clásico aumenta la probabilidad de que encuentren estímulos que han sido emparejados con comida anteriormente pero no la de que encuentren la propia comida. Con el mismo procedimiento se observó que los caracoles juveniles, y también los adultos jóvenes, aunque en menor medida, ven incrementada la cantidad de alimento ingerido. Cuando los caracoles juveniles disponían de poco tiempo para comer (2min/día), el consumo más eficiente de la comida resultó en un mejor mantenimiento de su peso corporal comparado con el grupo control. El condicionamiento

2

clásico también resultó beneficioso para los caracoles de la especie *Lymnaea stagnalis* a la hora de aparearse. Cuando los dos caracoles estaban motivados para aparearse en el rol de macho y, por tanto, había un conflicto sobre la ejecución del rol sexual masculino, aquellos para los que el olor señalaba la presencia de un individuo de la misma especie tenían más posibilidades de aparearse en dicho rol. En cambio, el condicionamiento clásico favoreció un apareamiento más rápido cuando se eliminó dicho conflicto sobre la ejecución de uno u otro sexual.

Abstract

The study of animal cognition is important not only to know what are the psychological processes that allow animals to obtain, process, remember and use information from their environment and how those processes work but also to determine how theses psychological processes permit animals to better adapt to their surroundings, increasing their survival and their reproductive success or fitness (e.g., Mackintosh, 1974). Classical conditioning is one of the learning processes that allows animals to predict the occurrence of biologically relevant events, and its study has aroused great interest within the field of comparative psychology. However, although the main aim of comparative psychology is to know how intelligence and its determining processes have evolved (Mackintosh, 1988), most of the research conducted in this field has used chordate species (especially rats, pigeons, and humans; Beach, 1949; Shettleworth, 2009), which hinders the development of a true comparative psychology representative of the animal phylogenetic tree. Similarly, the experimental work that has focused on the biological function or adaptive benefits of classical conditioning has almost exclusively employed animals that belong to the chordate phylum, with very few exceptions that have used animals of other phyla.

Molluscs are the second largest animal phylum (Ponder & Lindberg, 2008) and there have been several authors who have developed experimental procedures for the comparative study of associative learning in different species (e.g., see Gelperin, 2013 for some examples in gastropods). In the case of the garden snail *Cornu aspersum,* Ungless (1998) developed a conditioning procedure that was largely employed for the comparative study of associative learning. Despite the fact that with such procedures several associative learning related phenomena have been described, the biological

function of classical conditioning remains unknown in this species. Part of the work presented here aims to analyse what are the adaptive benefits of classical conditioning in terms of food finding, food intake and growth in this species. In addition, the biological function of classical conditioning is also explored in a different species, the great pond snail *Lymnaea stagnalis,* in terms of mating. This species was chosen for the study of the biological function of classical conditioning for two reasons. The first one is that, although much is known about its reproductive behaviour and the physiological aspects involved in mating (e.g., Koene, 2010; Nakadera et al., 2015), there are no studies in which the relationship between classical conditioning and mating is assessed. The second reason is that, to date, this species has not been shown to benefit from experience when it comes to mating (Koene & Cosijn, 2012).

Regarding food finding, the results obtained showed that *Cornu aspersum* snails require a previous experience with a given food to find it, and that classical conditioning increases the likelihood of finding stimuli that have been previously paired with food but not the food itself. With the same procedure it was also shown that juvenile snails, and young adults to a smaller extent, can increase their food intake. This efficient consumption of the available food in juveniles resulted in a better weight maintenance compared to a control group when the amount of time to feed was limited (2 min/day). Classical conditioning also provided snails of the species *Lymnaea stagnalis* with mating advantages. When both snails attempt to mate in the male sexual role, and thus, there was a conflict over sex role performance, the snails for which an odour signalled the presence of a conspecific became more likely to perform the male role. Alternatively, when such a conflict over the performance of the male role did not exist, classical conditioning resulted in a faster mating.

Chapter 1- Introduction

Classical conditioning can be described as the learning process in which a cue (conditioned stimulus or CS) becomes to signal biologically relevant events (unconditioned stimulus or US) for the animals. Predicting such important events has a biological function, which is to increase animals' adaptation to the environment (e.g., Bouton, 2007; Mackintosh, 1974). Indeed, when a signal reliably predicts the presence of a relevant event, animals can respond or become ready to respond before the stimulus arrives. This adaptiveness of learning can be easily inferred in virtually all classical conditioning preparations. For example, conditioned place preference for a context in which a cue had been previously paired with access to food indicates that the animal anticipates where the food is going to be available. On the contrary, a control group that is not able to orientate its searching strategies towards a significant cue would be less efficient in terms of foraging.

The particular benefits that animals obtain through learning may also be quantified as it has already been done, especially in the research conducted by Karen Hollis with blue gouramis (*Trichogaster trichopterus*) and by Michael Domjan with Japanese quail (*Coturnix japonica*). In blue gouramis, the lightning of a red bulb was used as the cue (CS) to signal the biologically relevant event (US) (the presence of a competitor male or the presence of a female). When the red light signalled the presence of a female, animals receiving the experimental treatment responded less aggressively to the females. They delivered significantly fewer bites to the females and spent more time in the courtship appeasement posture compared to the males that were not able to rely on a cue to predict the arrival of the female (Hollis, Cadieux, & Colbert, 1989). This training not only resulted in less aggressive behaviour toward the female but also it increased male blue

gouramis' paternity success: experimental males took less time to induce spawning in the female and they also produced more offspring than control males (Hollis, Pharr, Dumas, Britton, & Field, 1997). On the other hand, when the lightning of the red light was used to signal the presence of another male, the learning males showed an increased number of aggressive responses (frontal displays, bites and tail beatings) toward the intruder (Hollis, 1984). This work shows how classical conditioning in the blue gourami can help in the identification of a conspecific (male or female) as well as in the selection of the right behavioural repertoire to be displayed in a given encounter (courtship or aggressive behaviour). Furthermore, this training has been shown to have a great impact on the reproductive and paternity success of this species as revealed by the larger amount of offspring sired by the males who were able to use the red light as a predictor of a female.

In Japanese quail, different stimuli (lights, contexts or even toys) were used to signal a mating encounter. Males who received this treatment were significantly faster at mating (Domjan, Lyons, North, & Bruell, 1986) and released larger ejaculates and a larger number of spermatozoa (Domjan, Blesbois, & Williams, 1998). Similarly, females for whom a context had been paired with mating with a male, showed an increase in the duration of squatting, which indicates female sexual receptivity (Gutiérrez & Domjan, 1997). Moreover, as in blue gourami fish, classical conditioning resulted in an increased reproductive fitness as measured by the number of fertilised eggs laid by the female (Adkins-Regan & MacKillop, 2003; Mahometa & Domjan, 2005).

A common feature of studies in the field of comparative psychology generally, and in particular of studies that explore the biological function of classical conditioning, is that they have primarily focused on species of the chordate phylum. Regarding comparative psychology, the most commonly employed research species have long been rats, pigeons and humans (Beach, 1949; Shettleworth, 2009). On the contrary, only a few

species belonging to other phyla such as platyhelminthes, nematodes, molluscs, annelida and arthropods have been studied (see Alvarez, Loy, & Prados, in press, for a review). Likewise, there are very few studies in which the biological function of classical conditioning has been quantified in phyla other than chordates. Particularly, only arthropods and molluscs have been employed as experimental subjects.

Adaptive benefits of classical conditioning in arthropods

In arthropods, two studies in which grasshoppers and antlions have been used as experimental subjects, have most clearly shown the importance of classical conditioning for optimal foraging and growth (Dukas & Bernays, 2000; Guillette, Hollis, & Markarian, 2009). For grasshoppers and antlions, and insects in general, food intake during the larval stage has important consequences since a certain level of growth is necessary before reaching pupation and adulthood. In the experiment with grasshoppers (*Schistocerca americana*), the experimental group was exposed to a combination of cues, involving spatial location, taste and colour, paired with a particular diet type: one diet was balanced, and the other was not. By contrast, the control group was not able to rely on any of the cues for choosing between the two kinds of food. The animals in the experimental group were able to use the predictable cues to visit the balanced diet more frequently and ate more of the balanced diet. Consequently, these animals grew at a faster rate and pupated sooner than those animals in the control group (Dukas & Bernays, 2000). Similar results have been obtained in a more recent study conducted with antlions (the larvae of an insect of the Myrmeleontidae family). During their larval stage, these animals passively wait for prey to fall inside a conical pit that they dig in sandy soils, and they are able to spend long periods of time at the bottom of the pit without eating. In the study by Guillette, Hollis,

and Markarian (2009), the authors showed that a vibration could be used as a cue for signalling the delivery of a prey item. Antlions that received the learning treatment were more efficient at feeding compared to the control group in which the vibration cue and the prey delivery were never presented together. In a subsequent study, it was also shown that this same training led to a faster growth of the antlions. This resulted in a shortening of the larval stage of the antlions, who pupated significantly sooner than those in the control group (Hollis, Cogswell, Snyder, Guillette, & Nowbahari, 2011).

Another instance of biological adaptation mediated by classical conditioning can be found in the reproductive success of a parasitoid wasp, *Biosteres arisanus*. For these wasps, finding an appropriate host is very important so that the eggs laid can grow until hatching takes place. In a study conducted by Dukas and Duan (2000), wasps were able to develop a conditioned preference for a particular fruit that had contained host's eggs: that is, the wasps become more efficient at selecting a good nest site after training. In a second and third experiment, wasps of the experimental group had an initial sampling trial in which they were allowed to examine two different fruits (CSs), only one containing hosts. After a single conditioning trial, wasps were exposed to a series of 6 tests in which they had to choose between the two fruits. Once they had made a choice they were transferred to their home cage where they remained for three hours with the fruit of their choice, which contained hosts or not, depending on the condition (i.e., the fruit employed as CS+ contained hosts whereas the fruit used as CS- contained no hosts). The same series of tests were carried out for the control group except that the wasps had the same probability of finding hosts with either type of fruit). Animals in the learning treatment parasitized significantly more eggs than their counterparts in the control group (Dukas & Duan, 2000, Experiment 2); moreover, significantly more larvae of the learning group moulted into adult wasps compared to the control group (Dukas & Duan, 2000,

Experiment 3). These experiments show that classical conditioning provides extra benefits to *Biosteres arisanus* in terms of locating and finding a good host substrate, which results in an increased number of parasitized eggs and more adult offspring.

Adaptive benefits of classical conditioning in molluscs

In molluscs, two studies have explored the biological function of classical conditioning, one in the garden snail *Cornu aspersum* and another one in the great pond snail, *Lymnaea stagnalis.* In *Cornu aspersum* (formerly *Helix aspersa*), classical conditioning has been studied by means of an experimental procedure in which an odour (CS) is paired with access to food (US), training that results in an increased tentacle lowering response (i.e., conditioned response or CR) (Ungless, 1998). Interestingly, despite being a robust procedure that has allowed for the study of several associative learning phenomena such as acquisition, second order conditioning, latent inhibition (Loy, Fernández, & Acebes, 2006), blocking (Acebes, Solar, Carnero, & Loy, 2009) conditioned inhibition (Acebes, Solar, Morís, & Loy, 2012) and extinction (Alvarez, Morís, Luque, & Loy, 2014), this training failed to reveal any adaptive benefits (Ungless, 2000).

The tentacle lowering response of snails has long been linked to their food finding behavioural repertoire (e.g., Chase & Croll, 1981), and authors such as Ungless (2001) have hypothesized that tentacle lowering conditioning would lead to an increased success in food finding. Contrary to this initial hypothesis, the available data suggests that tentacle lowering conditioning and food finding are not so tightly related to one another since no advantages in food finding were observed after training. The results reported by Ungless (2001) are rather striking because, as just mentioned, there is a wide scientific literature

available that supports the relationship between snails' posterior tentacles and food finding. For example, Peschel, Straub and Teyke (1996) reported that snails lower their tentacles as they approach an edible substance, and behavioural and physiological studies (e.g., Chase, 1986) have shown that the snails' posterior tentacles are involved in food detection (through olfaction) and food finding. Thus, the biological function of this robust response remains to be determined.

In the other study, snails of the species *Lymnaea stagnalis* were trained by means of a naturalistic conditioning procedure in which the scent of a natural predator (the tench fish *Tinca tinca*) was paired with the alarm cues emitted from crushed conspecifics. Animals trained in this condition responded quicker and showed a greater escape response by crawling out of the water than snails in the control conditions that had been exposed to the conspecific alarm cue alone, to the predator cue alone, or to untreated artificial pond water (Dalesman, Rundle, Coleman, & Cotton, 2006). This study shows how learning can enhance antipredator behaviour, which would be expected to increase the chances of survival upon a real encounter with a predator.

This review shows that there are few studies in which the biological function of classical conditioning in species other than chordates has been demonstrated. Three clear examples of adaptive benefits have been reported for arthropods and only one for molluscs. Moreover, of the studies conducted with molluscs, one (Ungless, 2001) questions the adaptive value of classical conditioning in molluscs.

Thesis outline

The aim of the research presented here was to set the ground for the assessment of the biological function of classical conditioning in two different species of molluscs (*Cornu aspersum* and *Lymnaea stagnalis*) and in three different domains: food finding

(Chapter 2), nutrition measured in terms of food intake and growth (Chapter 3), and mating (Chapter 4).

In Ungless' (2001) study, a key aspect of snails' food finding behaviour has been overlooked and, thus, one might argue that the experimental design employed may have confounded the results (see Chapter 2 for a detailed explanation). Behavioural studies in gastropods have shown that prior experience with a particular food item is a critical variable for snails to recognise an edible food as such and to successfully locate it (e.g., Teyke, 1995). In Ungless (2001), the same food source was used as both the CS and the US, and therefore, it is not possible to know whether snails were searching for the food-US or for the food-CS. If, according to previous literature (e.g., Teyke, 1995), food finding was due to experience with the food-US, the fact that snails located it regardless of their rate of tentacle lowering conditioning (a response that would be evoked by the food-CS), does not necessarily lead to the conclusion that tentacle lowering conditioning and food finding are dissociated. The second chapter aims to address this question by examining the ecology of food finding behaviour.

The previous literature review on the biological function of classical conditioning demonstrates that the same single procedure (i.e., the pairing of one given CS with a particular US) provides animals with advantages at different levels. For example, in the case of male blue gouramis, exposure to a red light followed by the presence of a female resulted in 1) an increase in the appeasement courtship posture, 2) faster spawning, and 3) larger number of offspring (Hollis et al., 1989; Hollis et al., 1997). Similarly, foraging is a complex behavioural system in which different processes are involved, and therefore, the pairing of an odour (CS) with access to food (US) could have multiple effects other than tentacle lowering or food finding. Just as in the experiments with grasshoppers and antlions, classical conditioning could result in an improvement in food intake and growth.

13

This question is explored in the third chapter, in which the experimental procedures employed in the studies conducted with insects have been adapted to the procedure typically employed in *Cornu aspersum.*

Finally, in the fourth chapter, the effect of classical conditioning in a mating context is examined in a different mollusc species, the great pond snail, *Lymnaea stagnalis.* Although this species has been widely used as a model for the study of learning and memory (e.g., Lukowiak et al., 2003), and of hermaphroditic mating (e.g., Koene, 2010), the relationship between classical conditioning and mating has not been explored in this or in any other hermaphroditic species. *Lymnaea stagnalis* is a simultaneous hermaphrodite that mates unilaterally, which means that all individuals are functionally both male and female at the same time, but that at each mating encounter they can adopt only one sex role at a time. Several variables have been shown to affect this decision, for example, the amount of available seminal fluid or the size of the snail (e.g., Nakadera et al., 2015), but little is known about the effect that learning can have on mating in this species. A study conducted by Koene and Cosijn (2012) showed that prior mating experience did not have any effects on mating success in a second mating encounter. However, no studies have assessed the relationship between classical conditioning and mating in this or any other hermaphroditic species. The research reported in Chapter 4 explores whether classical conditioning training could have different effects in this hermaphroditic species' reproductive success in two different mating scenarios: one in which there is a conflict over sex role performance and another one in which there is not such a conflict.

Chapter 2- Classical conditioning and its effect on food finding

In snails, tentacle lowering has long been thought to be involved in food detection and food finding. Indeed, when posterior tentacles of *Achatina fulica* are ablated, the snails of this species are not able to find food. On the contrary, ablation of the anterior tentacles does not produce any impairment in food orientation and snails succeed at locating the food (Chase & Croll, 1981). Peschel, Straub, and Teyke (1996) also noted that *Helix pomatia* snails that failed to locate a food source in an open field arena locomoted with their posterior tentacles held upright whereas those who were able to find it lowered their tentacles as they approached it. A study conducted by Friedrich and Teyke (1998), in which posterior tentacles of *Helix pomatia* where anesthetised, also confirmed the results obtained by Chase and Croll (1981): posterior tentacles are necessary for food finding.

These results suggest that tentacle lowering conditioning could result in an improvement in food finding. However, when this hypothesis was tested in *Cornu aspersum*, such improvement did not occur (Ungless, 2001). In that study, food finding was tested in two groups of snails, one that had been kept naïve (i.e., the control group) and another that had been fed on apple. For the experimental group, the same food source (apple) served as both CS and US, as it had been previously shown that just feeding snails on a particular food resulted in an increased tentacle lowering response to the odour of that same food (Ungless, 1998). After this training, animals were tested in an open field arena in which a piece of apple had been located 55º to the right of the animal and at a distance of 12 cm. Tentacle lowering responses were also registered. To examine whether tentacle lowering conditioning could improve food finding or not, Ungless divided

animals within the experimental group into two subgroups according to their tentacle lowering response: those that had shown a high rate of tentacle lowering and those that responded at a lower rate. Ungless found that conditioned snails were more likely to find the food source than naïve subjects, but he did not observe any differences in terms of food finding between high responders and low responders. Thus, high-tentacle-lowering snails did not have any advantages over low-tentacle-lowering snails and such results led Ungless to conclude that conditioning and food finding are dissociated (Ungless, 2001).

The results obtained by Ungless (2001) indicate that food finding and tentacle lowering are not so tightly related to one another as originally thought. However, one of the reasons why no relationship was observed between tentacle lowering responding and food finding may be the fact that the same substance (apple) was employed as CS and US. Under this experimental design, it is not possible to know whether, at the test, snails were looking for the food (US) or for the cue that signalled the presence of the food (CS).

Indeed, food finding has been reported to depend on experience. Research conducted in different laboratories with different species, showed that experience with the food source is necessary for snails to find it. For example, in *Achatina fulica,* their preference for carrot or cucumber was dependent on prior experience (Croll & Chase, 1977; Croll & Chase, 1980). In *Helix pomatia*, only 12.5% of the snails that had not fed on either potato or carrot were able to locate that food source in a subsequent open field test (Teyke, 1995; see also Friedrich & Teyke, 1998 and Peschel et al., 1996). However, after feeding them with one of the substances, 87.5% of the snails located the food. Thus, whether snails in the Ungless (2001) study found the piece of apple on the food finding test could be solely explained by the fact that they were familiar with the food and not by any tentacle lowering conditioning effect. This variable could be confounding the results and the conclusions drawn, and thus, examination of experience and conditioning on the

food finding behaviour of *Cornu aspersum* is needed, but to achieve this goal, the mechanisms of food finding of the garden snail *Cornu aspersum* first must be determined.

The aim of this series of experiments was to analyse some of the ecological variables that may play important roles in food finding in this species. In the first two experiments, the effect of prior experience of different modalities was explored, whereas the third experiment focused on the role of classical conditioning in food detection.

General Method

Subjects and Housing

Subjects were experimentally naïve adult snails that were individually housed in plastic cages (50 x 50 x 100 mm), which had air holes and contained a small amount of water. The boxes were placed in a dark room in which temperature was held constant at 22ºC. Snails were deprived of food for 10 days prior to the start of the experiment, which is a short period of deprivation for this species since snails typically hibernate for up to 3 or 5 months during winter in European countries (or in summer when facing drought periods or high temperatures; Chevallier, 1992). During hibernation they seal themselves within the shell and live off the stored fat. Deprivation lasted for 10 days to match the experimental procedure used in comparable experiments (e.g. Ungless, 1998; Loy et al., 2006). After the experiments, snails were given food ad libitum and placed back into the wild in a different location to that in which they had been collected (at least 20 km apart).

Apparatus and Stimuli

The experimental setting used was a plastic perforated surface (390 mm X 360 mm; with 5.5 mm diameter holes roughly 2 mm apart from one another) placed above the surface of a table at a distance of 65 mm. The experimental room was maintained at 22ºC

and illuminated with a red light (20 W). Carrot and apple were used as stimuli unless otherwise stated.

Procedure

Before each trial, animals were placed on their sides and sprayed with fresh tap water to induce activity. Once the snails had emerged from their shells, they were placed on the perforated surface and the trial started.

Four different types of trials were used in the experiments on food finding, namely feeding, smelling, conditioning and unpaired trials. Feeding trials consisted of access to a piece of carrot or apple for a period of 120 s. Once the trial had finished, animals were removed from the experimental setting and put back in their cages. This treatment was repeated 6 times with an intertrial interval (ITI) of 1 hour. In smelling trials, animals were exposed to the odour alone: a dish containing either apple or carrot slices was placed on top of a plastic dish situated below the perforated surface. The distance between the odour source and the snails was 20 mm and subjects were allowed to smell the food source for 120 s. After this time, they were removed from the experimental setting and put back in their cages. Smelling trials also were carried out six times per day with an ITI of one hour.

In conditioning trials, a dish containing an odour source, the CS, was placed below the perforated surface and a piece of food, the US, was placed in front of the snail. Animals were allowed to eat the US for a period of 120 s (the 120 s period began 5 s after the presentation of the food; by then, the snails had usually started to eat). On unpaired control trials animals were exposed to the US for 120 s and, half an hour later, to the CS for another 120 s (see below for specific details).

In food finding tests animals were placed onto the perforated surface where a piece of apple and a piece of carrot had been placed. Whether each of the substances was to the

left or to the right of the snail was counterbalanced. The distance between the two food sources and between each food source and the snail was 7 cm (i.e., forming an equilateral triangle). A choice was considered to be made when the snail touched the piece of food with its head (feeding was prevented immediately upon contact). If after 7 minutes, snails had not located either of the food items, a second test trial was conducted immediately after the first one. In such cases, before the second test trial begun, the snail was momentarily removed and the perforated surface was cleaned.

Analyses

All analyses were run using SPSS v21 (SPPS Inc., Chicago, IL, U.S.A.).

Experiment 1

Previous experiments showed that experience with a particular food is necessary for snails of different species (Friedrich & Teyke, 1998; Teyke, 1995 in *Helix pomatia;* Croll & Chase, 1977; 1980 in *Achatina fulica*) to find that food in a subsequent open field test. The aim of this experiment was to assess whether the same is true for snails of the species *Cornu aspersum.* To do so, a food finding test was conducted with snails that had been either fed or kept naïve.

Method

Subjects

Subjects were 25 snails taken from the wild with a mean shell diameter of 29.2 mm (range 27.3-33.4 mm). Snails were randomly divided into three different groups: Carrot (*N*=8), Apple (*N*=8) and Naïve (*N*=9).

Procedure

Following the procedure above described for feeding trials, snails in the group Carrot were fed on carrot, and snails in the group Apple were fed on apple. As in Ungless (2001), snails in the Naïve group were left untreated: they stayed in their cages until the test. The next day, a food finding test was conducted for all animals.

Results and Discussion

All subjects from Carrot and Apple groups found a food source in the choice test. In both groups, 75% of the Experienced snails found the same food that they had previously eaten regardless of whether it had been apple or carrot, and the remaining 25% found the other food source (see Figure 1). A cumulative binomial test showed that

previous experience significantly affected animals' choice, as the difference between the percentages was found to be statistically significant [p=0.038].
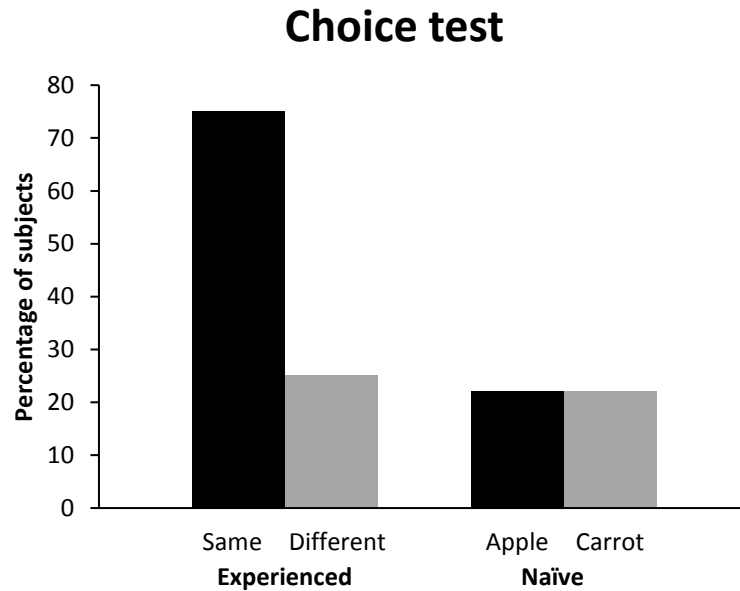
Choice test

Figure 1: Results of Experiment 1. Percentage of animals that found the same (or different) food to which they had been exposed (Experienced) and percentage of Naïve animals that found one of the two food sources (apple or carrot).

In contrast, as depicted in Figure 1, 22% of the Naïve snails found one of the two food sources (carrot) and another 22% found the other one (apple). The remaining 56% of the snails in the Naïve group did not find any of them.

These results corroborated the results reported by Teyke (1995; see also Friedrich & Teyke, 1998) and by Croll and Chase (1977; 1980): successful food finding is significantly enhanced by the previous experience with the food employed in the test. On the contrary, animals lacking experience behaved at random. Thus, as in *Helix pomatia* and *Achatina fulica,* experience with food is a critical factor for food finding in *Cornu aspersum*.

Experiment 2

The previous experiment showed that *C. aspersum* snails require prior experience with food to find it. However, it could also be possible that just exposure to the odour of an edible substance would be enough for them to successfully locate it. Teyke (1995) tested this hypothesis by exposing *Helix pomatia* snails to the smell of carrot for 12 hours. He observed that only 8.3% of the snails found the food (see also Friedrich & Teyke, 1998). Likewise, in *Achatina fulica,* exposure to a food odour for 48 hours was not enough for animals to locate it (Croll & Chase, 1980). It could be argued that such a long exposure to the odour could have resulted in a sort of habituation learning, decreasing the food searching activity of the snails. The aim of this experiment was to test this hypothesis in *Cornu aspersum* by comparing the food finding behaviour of snails that had been fed on a substance or that had been exposed just to its odour. To rule out any habituation effect, the amount of exposure to the odour source was shorter than that employed by Teyke (1995) and by Croll and Chase (1980).

Method

Subjects

Subjects were 47 adult snails with a mean shell diameter of 31.2 mm (range 29.2-33.1 mm) at the start of the experiment. They were randomly divided into four groups: US_1-Smelling ($N=12$), US_1-Eating ($N=11$), US_2-Smelling ($N=12$), and US_2-Eating ($N=12$).

Procedure

Animals in the smelling condition were exposed to either the smell of carrot or apple whereas animals in the eating condition were allowed to feed on one of the two substances. After this treatment, snails in all groups were tested in a food finding test.

Results and Discussion

In the choice test trial, 7 out of the 47 seven snails did not choose. Two of them belonged to the group that had been exposed to the smell of carrot; one belonged to the group that had been fed apple; and four, to the group exposed to apple odour. These snails were eliminated from the data analysis.

As depicted in Figure 2, and similar to the results of Experiment 1, snails that had been allowed to feed on either carrot or apple found the same substance they had eaten (86.36%). On the contrary, only 33.33% of the snails located the same source of food the smell to which they had been exposed [χ^2=11.88, p<0.001]. The statistical analysis revealed that the differences observed were significant. A cumulative binomial test showed that eating [p<0.001], but not smelling [p=0.119] had significantly affected the choice made by the animals.
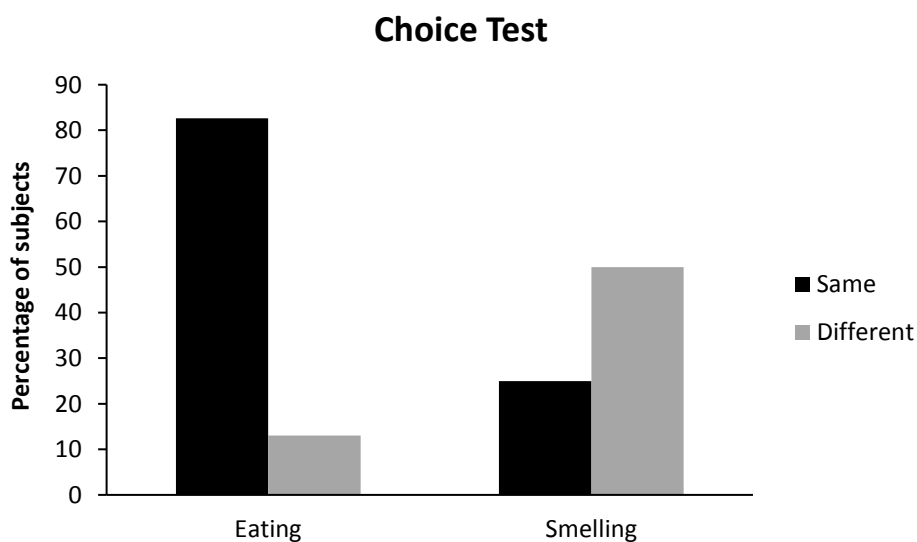

Figure 2: Results of Experiment 2.Percentage of animals that chose the same (or different) food source on which they had been previously fed or to which odour they had been previously exposed.

As in Teyke (1995), exposure to the odour was not enough for animals to find the food. Thus, in both species *Helix pomatia* and *Cornu aspersum,* snails were unable to

locate a food source without experience or after been exposed only to its odour. Ungless (2001) reported that conditioned snails were significantly more likely to find the piece of apple than those who were naïve, a result that can be attributed to the experience with the US and not to the conditioning training. This fact does not fully invalidate the main claim reported by Ungless (2001), namely that conditioning and food finding are dissociated, because he later split the snails that had received conditioning training into two different groups depending on their rate of tentacle lowering in the test. Nonetheless, given that the same substance was employed as both CS and US, it is unclear whether the snails were searching for the food itself (i.e., US) or for the odour that had been paired with the food (i.e., CS).

Experiment 3

Feeding snails on a particular food has been shown to increase the tentacle lowering response to the odour of that food (Ungless, 1998). However, as just argued above, whether the snails are searching for the food itself (goal tracking) or for the odour that has been paired with the food (sign tracking) remains unclear. Indeed, in a study conducted by Friedrich and Teyke (1995), 83% of the snails that had been fed on filter paper while exposed to the odour of carrot successfully located the carrot-odour source even though they had not consumed it. The fact that they were able to find the carrot juice employed in the test despite not having consumed it could be explained by a CS-searching and not a US-searching strategy. The aim of this experiment was to address this question in a within-subjects experiment in which different substances were used as CS and US.

Method

Subjects

Subjects were 20 adult snails with a mean shell diameter of 28.6 mm (range 26.3-31.2 mm)

Apparatus and stimuli

The stimuli used were two solutions, one of mango and the other of coconut (22% v/v each) as CSs. Carrot and apple were employed as the USs.

Procedure

Table 1 illustrates the experimental design employed in this experiment. Subjects were exposed to two types of trials: conditioning and unpaired trials.

Training	US preference	CS preference
CS_1-US_1	CS_1 (US_1 vs. US_2)	CS_1 vs. CS_2
CS_2/US_2	CS_2 (US_1 vs. US_2)	

Table 1: Experimental design of Experiment 3. The CSs employed were a solution of mango and coconut, counterbalanced. US_1 indicates that carrot was used as a reinforcer. US_2 was access to a piece of apple, which never was paired with any of the CSs.

In conditioning trials an odour was paired with access to food (CS_1-US_1). In unpaired trials (CS_2/US_2) subjects were exposed to a different odour (CS_2) for 2 minutes and half an hour later they were exposed to a different food (US_2). The role of mango and coconut as CS_1 or CS_2 was counterbalanced. In order to avoid differences in conditioning due to the palatability or nutritional value of the US, carrot was always used as a reinforcer (i.e., US_1) and apple was always used as the US_2. On conditioning trials, a dish containing four essence-impregnated cotton pads of CS_1 (mango or coconut, counterbalanced) was placed below the perforated surface and a piece of carrot was placed in front of the snail. On unpaired trials, snails were exposed to the other odour alone (CS_2) for two minutes. Half an hour later they were given access to a piece of apple for another two minutes. This treatment was repeated three times per day on each of four days.

On Day 5, animals' conditioned preference for the US was tested in two food finding tests: once in the presence of CS_1 and another one in the presence of CS_2. The order in which the tests were run was also counterbalanced. If conditioning favours food finding (goal tracking), the presence of the CSs would be expected to affect the outcome of the food finding test. Particularly, if CS_1 signals the presence of US_1, a larger percentage of snails would be expected to find US_1 than US_2. By contrast, such a bias in food finding would not be expected in the presence of CS_2.

On Day 6, snails' conditioned preference for the CSs was tested. The procedure used was the same as the one employed in previous food finding tests except for that, in this case, snails were placed between two pieces of cotton that had been impregnated with the CSs. Whether mango or coconut was to the right or left of the snail was counterbalanced. If conditioning results in a preference for the odour that signals the availability of food (sign tracking), then a higher proportion of snails would be expected to choose CS_1 over CS_2.

Results and Discussion

US-conditioned preference: As is illustrated in Figure 3, in the presence of CS_1, 50% of the snails chose US_1 and 44% chose US_2. Similarly, in the presence of CS_2, 50% chose US_1 and 56% chose US_2. Thus, snails chose carrot and apple equally and independently from the CS that was present at the test [$\chi^2=0.72$, $p=0.4$].
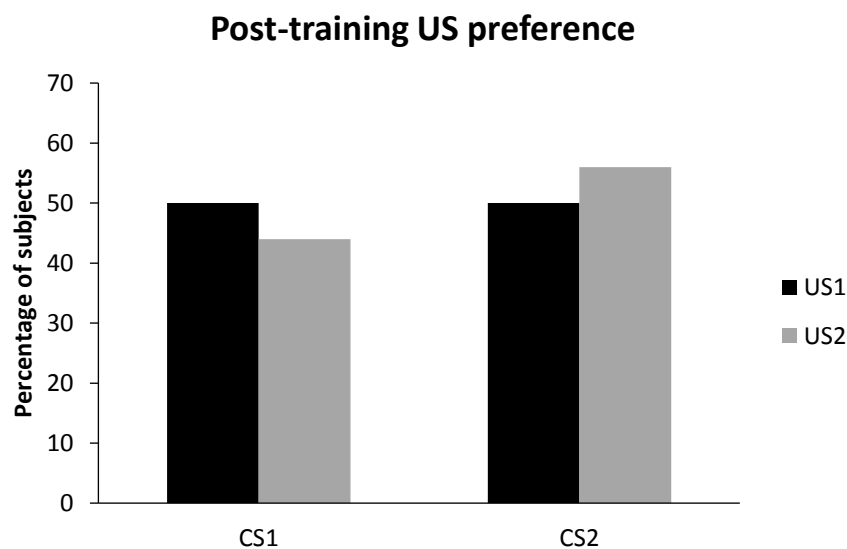

Figure 3: Percentage of snails in Experiment 3 that chose one of the two USs previously employed in classical conditioning training. The choice test between the USs was conducted in the presence of CS_1 or CS_2. US_1 had been paired with CS_1 ($CS_1 \rightarrow US_1$), whereas US_2 had been used as a control (CS_2/US_2).

CS-conditioned preference: As depicted in Figure 4, 63% of the snails chose the CS that had been paired with food (i.e., CS_1) and only 11% chose the CS that had not been paired with food (i.e., CS_2). This difference was statistically significant [χ^2=7.14, p=0.008]. The remaining snails (26%) did not choose.
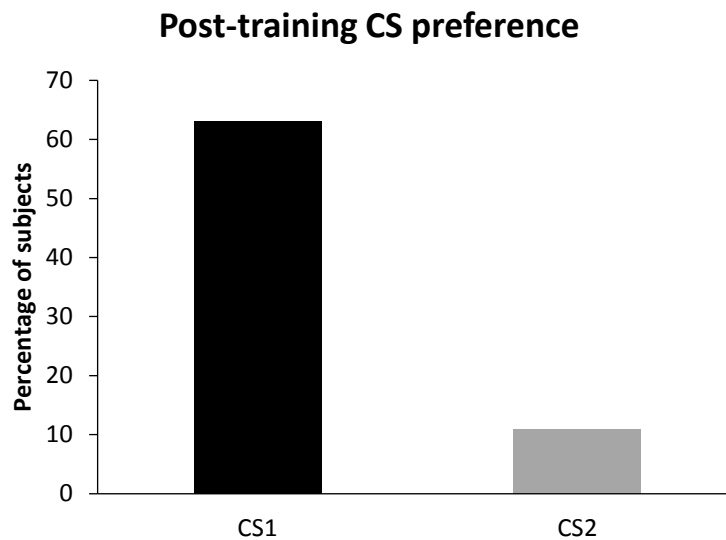

Figure 4: Results of Experiment 3. Percentage of snails that chose the CS that reliably signalled the presence of a US ($CS_1 \rightarrow US_1$) or the CS that served as a control (CS_2/US_2).

The results suggest that the CS did not have any effect on US preference, since snails chose both USs equally and independently of whether one CS or the other was present. Notwithstanding, when a subsequent choice test between the two CSs was conducted, snails showed a clear preference for the CS that had been paired with a US (i.e., CS_1), which would indicate that classical conditioning affects signal tracking but not goal tracking.

One of the shortcomings of this experiment is that the order in which the tests were carried out was not counterbalanced and these results are thus preliminary. Although the tests were not counterbalanced, the fact that the CS preference test (with positive results) was conducted after the US preference test (with negative results), reduces the

possibility that the effect obtained is an experimental artefact. Thus, the results obtained seem to indicate that food searching (goal tracking) is not the same as food-signal searching (sign tracking).

Discussion

Experiments on classical conditioning with snails have shown that pairing an odour (CS) with food (US) results in an increase of the tentacle lowering response (CR) in the presence of the CS. Nevertheless, an explanation of the biological significance of this classical conditioning preparation requires a link between the elements involved in conditioning experiments (CS, US and CR) with the natural activity of the snails when they look for food. In agreement with the results reported by Croll and Chase (1977; 1980), Friedrich and Teyke (1998) and Teyke (1995), just having fed the snails with a given food increased the percentage of snails that found that same food item whereas snails with no experience were much less efficient. Ungless (2001) found that just feeding snails on a particular food increased tentacle lowering but he failed to find any advantages of the tentacle lowering response in terms of food finding. However, the role that the CS plays in food finding independently of the role played by the US was not examined in his study.

The data obtained in this study shows that experience with a given food is what determines food finding (Experiment 1) and not odour exposure (Experiment 2) or conditioning per se (Experiment 3). Regardless of whether the US has been experienced together with or independently of the CS, snails will find it in a food finding test (Experiment 3, Figure 4). Nonetheless, although conditioning does not affect the choice between two familiar USs, it does affect snails' choice when they are presented with two

CSs that signal different outcomes. In this situation, snails will show a preference for the CS that had been paired with a US and not for the CS that had been presented alone (Experiment 3, Figure 5). These results imply that the searching strategy of snails is different depending on the stimulus that is present in the test (the CS or the US) and questions the results reported by Ungless (2001) since the same substance was employed as a CS and as a US. The experimental procedure used by Ungless (2001) does not allow separating goal-tracking and sign-tracking behaviour and therefore, no definitive conclusions can be drawn from the results reported by Ungless (2001). Although the results of the studies here reported do not address all the possible variables that could potentially be confounding the conclusions by Ungless (2001), they clearly indicate that there is a series of factors that have not been taken into account but that should be more carefully examined given their relevance in the ecological aspects of the foraging behaviour of snails.

One of the limitations of the experiments presented in this chapter is the use of a naïve group in Experiment 1. Such an experimental design does not control for differences in food deprivation and motivation, context exposure or even induced activity. The fact that naïve snails (i.e., non-fed snails that would be expected to search for food more actively) were less successful in food finding than fed snails most likely implies that differences in food deprivation and motivation have not affected the results obtained. However, how context exposure could have affected food finding behaviour remains unknown. Furthermore, because the naïve group was treated as in Ungless (2001), snails were not equated in terms of activity: snails fed apple or carrot were sprayed with water and awakened 6 times per day whereas naïve snails' activity was not controlled. Although snails in the Naïve condition were sprayed with fresh tap water at least 20 min before the test and showed normal levels of activity before and during the test, the effects that the

differences in activity could have had on the results is unknown. Regarding Experiment 3, it remains to be explored whether the order in which tests (CS and US preference tests) were carried out affected the results obtained, and future investigation should also include a test for conditioned tentacle lowering response.

Chapter 3- Classical conditioning and its effects on food intake and growth

Classical conditioning has been shown to be of great importance for the growth and development of both grasshoppers and antlions (Dukas & Bernays, 2000 and Hollis et al., 2011, respectively). Certainly, in both insect species, signalling the presence of food resulted in greater food intake and faster pupation. In molluscs, however, this adaptive function of classical conditioning has not been explored.

The aim of this series of experiments was to assess whether food signalling would enhance food intake and weight maintenance in the garden snail (*C. aspersum*) in a situation of food scarcity. To do so, the experimental procedure employed by Guillette et al. (2009) with antlions was adapted to *Cornu aspersum.* Evidence of the biological function of conditioning would be provided if snails subjected to classical conditioning showed a higher intake of food or a higher growth rate compared to subjects in the unpaired control group. This hypothesis was tested in two sets of experiments in which the amount of food access was manipulated. Both juvenile and young adult snails were used as subjects to examine whether the effects of classical conditioning could vary depending on the snails' developmental stage.

General Method

Subjects and Housing

Subjects were snails taken from the wild. They were individually housed in plastic cages (50 x 50 x 100 mm), which had air holes and contained a small amount of water. The boxes were placed in a dark room in which temperature was held constant at 22ºC.

Snails were deprived of food for 10 days prior to the start of the experiment. After the experiments, snails were given food ad libitum and placed back into the wild.

Apparatus and stimuli

The experimental setting used was a plastic perforated surface (390 mm X 360 mm; 5.5 mm diameter holes, roughly 2 mm apart from one another) placed 65 mm above the surface of a table, allowing a dish containing a source of odour to be placed beneath the perforated surface. The experimental room was maintained at 22ºC and illuminated with a red light (20 W). The conditioned stimulus was a solution of mango essence (0.22% v/v) which was poured onto four cotton pads (2 ml per pad), and the unconditioned stimulus was a piece of corn, a nutritious food source that has been reported to be important for snails' growth (Chevallier, 1992). The US was weighed before and after it had been provided to the subjects to measure the amount of food eaten by the snails in each conditioning trial.

Procedure

Before the start of an experimental session, animals were placed on their sides and sprayed with fresh tap water to induce activity. Once the snails had emerged from their shells, they were placed onto the perforated surface and the experimental trial started. In all trials, the distance between the odour cue and the perforated surface was 65 mm. On conditioning trials, a dish containing four mango-impregnated cotton pads was placed below the perforated surface. Animals were allowed to smell the CS for a period of 120 s. The two-minute exposure to the CS was followed by another two or four minutes of access to the US (a piece of corn placed on top of a plastic lid that was situated on top of the perforated surface. The CS was removed after the snail had started to eat the US. In a typical conditioning experiment, snails were given a total of 10 or 12 minutes of daily food access (e.g., Ungless, 1998; Acebes et al., 2009). Because the aim of these

experiments was to examine the effect of classical conditioning in a situation of food scarcity, snails were allowed to feed for either 2 or 4 minutes per day. After feeding, animals were removed from the experimental setting and put back in their containers and the piece of corn employed as the US was cleaned and weighed.

To examine the effect of classical conditioning on food intake and weight maintenance, conditioning trials needed to be conducted individually and thus, the experimental procedure used was that employed by Ungless (1998) in which only one conditioning trial per day was conducted. Thus, this treatment was carried once every day 5 days per week, from Mondays to Fridays, over a three-week period for a total of 15 treatment days. The control group was exposed to both stimuli but in an unpaired fashion. Animals of both groups received the US at the same time of the day, but snails belonging to the control group were exposed to the CS either after or before US exposure. The time interval between CS and US presentation ranged from 5 minutes to 5 hours.

Following Ungless (1998), conditioning would be expected to occur after 10 days of training. Therefore, the tentacle lowering response was measured during the 2 minutes of the CS exposure on the first 10 days. As in Ungless' procedure (see Figure 2 in Ungless, 2001, p. 99), each movement of the tentacle below an imaginary horizontal line parallel to the top of the head was scored as one response throughout the 2 min session. The snails were randomly selected and tested individually in random order. To simplify the test procedure, only movement of the snail's left posterior tentacle was recorded, as described by Ungless (1998, 2001).

Snails' growth was also measured at the beginning and at the end of each of the three weeks of the experiment. Weight proportions were calculated to accurately measure

weight changes by dividing each weight measurement by the initial weight of the animal before the start of the experiment.

Analyses

All analyses were run using SPSS v21 (SPPS Inc., Chicago, IL, U.S.A.). The statistics of effect size reported were Cohen's *d'* (Cohen, 1992) and η_p^2 (Cohen, 1973) for *t* tests and ANOVA, respectively.

Experiment 4A

This experiment attempted to test the biological function of classical conditioning in terms of food intake and growth in juvenile snails (*C. aspersum*). Snails were given either signalled or non-signalled access to 2 minutes of food access.

Method

Subjects

Subjects were 18 juvenile snails with a mean initial weight of 2.81 g (range 1.75 - 4.58 g). They were matched by weight and randomly divided into two groups: Pavlovian (PAV; $N=9$) and Control (CON; $N=9$).

Procedure

Animals in the PAV group were allowed to eat for two minutes following the presentation of the CS. Animals in the control group were exposed to both the CS and the food but in an unpaired fashion.

Results and Discussion

Tentacle lowering: As shown in Figure 5, animals that received paired presentations of the CS and the US showed a higher mean level of conditioned responses than animals that did not receive such training. A repeated measures analysis showed an effect of Group [$F(1,16)=12.897$, $p=0.002$; $\eta^2p=0.446$], of Days [$F(9,144)=2.261$, $p=0.021$, $\eta^2p=0.124$], and of Group x Days interaction [$F(9,144)=2.492$, $p=0.011$, $\eta^2p=0.135$]. Statistically significant differences in tentacle lowering between groups appeared on Days 4, 5, and 8 [$ts(16)>3.049$, $ps<0.008$ $d's>1.52$] but not on the first two days of training [$ts(16)<1.069$, $ps>0.301$, $d's<0.54$]. These results indicate that conditioning differed between the two groups.
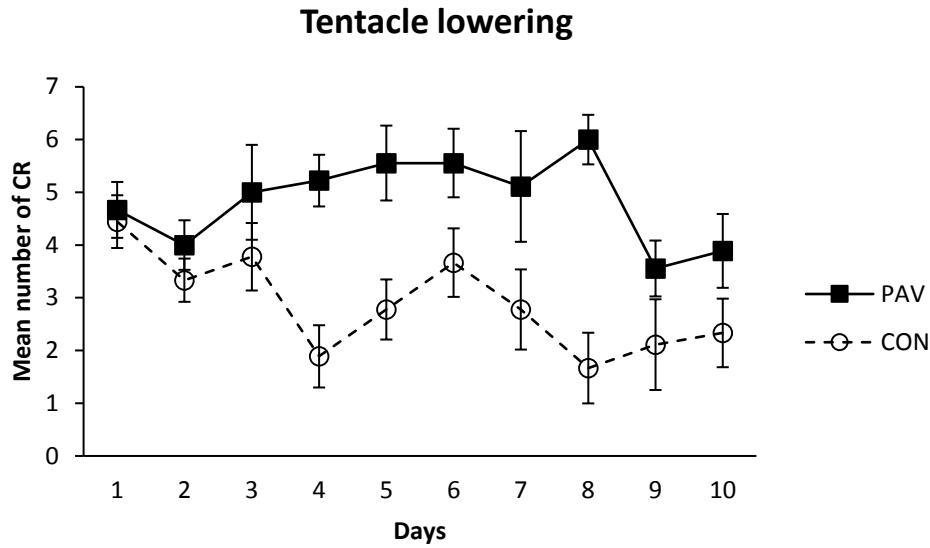
Tentacle lowering

Figure 5: Mean number of tentacle-lowering responses (CR) made by the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 4A. Vertical bars represent SEMs.

Food intake: As is depicted in Figure 6, snails that received the Pavlovian conditioning treatment, consumed more corn than those who belonged to the control group. The statistical analysis confirmed an effect of Group [$F(1,16)=18.849$, $p=0.001$, $\eta^2p=0.541$], Days [$F(14,224)=27.902$, $p=0.000$, $\eta^2p=0.636$], and of the interaction Group x Days [$F(14,224)=2.727$, $p=0.001$, $\eta^2p=0.146$]. As Figure 6 also shows, the amount of food consumed across the first five days of the experiment is larger than that of the remaining days. This difference is due to the fact that when removing the piece of corn from the snail's mouth, corn dust and small corn pieces had gotten attached to the mucus produced by the snail. Because it was not possible to separate the corn dust from the mucus to dry and weigh them, the data provided must be considered carefully. Nevertheless, there were no statistically significant differences on the first two days of training [$ts(16)<0.724$, $ps>0.479$, $d's<0.37$]. Statistically significant differences were found on Day 3, 5, 13, 14, and 15 [$ts(16)>2.2$, $ps<0.043$, $d's>1.23$]. These results indicate that Pavlovian conditioning affects food consumption.
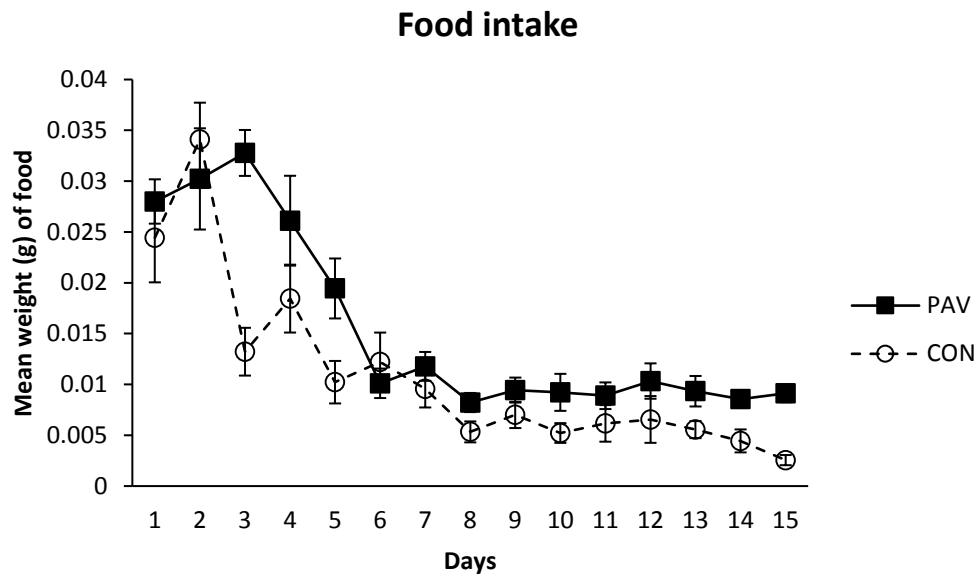
Food intake

Figure 6: Mean weight of corn grams eaten by the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 4A. Vertical bars represent SEMs.

Growth: As can be seen in Figure 7, both groups decreased their weight as days of training progressed. However, snails that were exposed to the CS followed by the US showed a slower loss of weight compared to the control subjects. A repeated measures analysis showed an effect of Group [$F(1,16)=5.011$, $p=0.04$, $\eta^2p=0.238$], of Days [$F(6,96)=27.522$, $p=0.000$, $\eta^2p=0.632$], and of a Group x Days interaction [$F(6,96)=3.179$, $p=0.007$, $\eta^2p=0.166$]. Statistically significant differences were observed only on the fourth measurement of snails' weight proportion [$t(16)=4.1$, $p=0.001$, $d'=0.42$]. These results show that classical conditioning had an effect on weight maintenance.
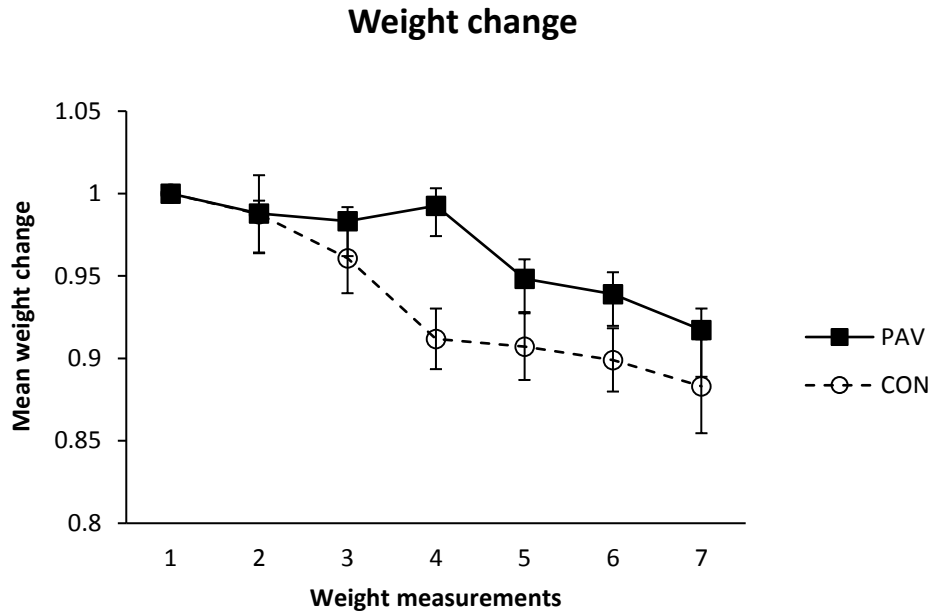
Weight change

Figure7: Mean weight change of the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 4A. Measurement 1 corresponds to the initial weight of animals before the experiment begun. Vertical bars represent SEMs.

Taken together, the results of this experiment show that juveniles (weighing less than 5 g) that learnt to anticipate the arrival of food by means of conditioning showed a better use of the limited time for food ingestion, which ultimately translated into a less weight loss compared to the control group.

Experiment 4B

The aim of this experiment was to assess whether young adult snails of *Cornu aspersum* species would be subjected to the same effects of classical conditioning as juveniles. To do so, the same procedure as in Experiment 4A was used.

Method

Subjects

Subjects were 14 juvenile snails with a mean weight of 7.63 g (range 5.36-10.43 g) at the start of the experiment. They were matched by weight and randomly divided into two groups: PAV (*N*=7) and CON (*N*=7). Housing and maintenance of the animals were as in Experiment 4A. One snail belonging to the PAV group was removed from the experiment because it did not eat. The data of another subject from the CON group was also eliminated because it died during the experiment.

Procedure

As in Experiment 4A, all animals were allowed to eat for 2 minutes. Snails in the PAV condition received the food following the presentation of the CS whereas for the control group food access and CS exposure were never paired together.

Results and Discussion

Tentacle lowering: As shown in Figure 8, animals that received paired presentations of the CS and the US showed a higher mean level of tentacle lowering compared to animals that did not receive such training. However, a repeated measures analysis showed no effect of Group [$F(1,10)=3.638$, $p=0.086$; $\eta^2p=0.267$], of Days [$F(9,90)=1.506$, $p=0.158$, $\eta^2p=1.31$], or of the Group x Days interaction [$F(9,90)=0.655$, $p=0.747$, $\eta^2p=0.061$]. In spite of the statistical analysis, the fact that this procedure has

been previously shown to be a robust conditioning preparation together with the pattern of the results shown in the figure, the marginally significant effect of Group and the rather small sample size, could be interpreted as a weak and slower conditioning effect. The repeated measure analysis conducted with the tentacle lowering responses of the last three days showed only a significant effect of Group [$F(1,12)=5.334$, $p=0.4$, $\eta^2p=0.308$], which could indicate that snails reached an asymptotic level of conditioning since the means of both groups coincide with normal values of tentacle lowering conditioning.
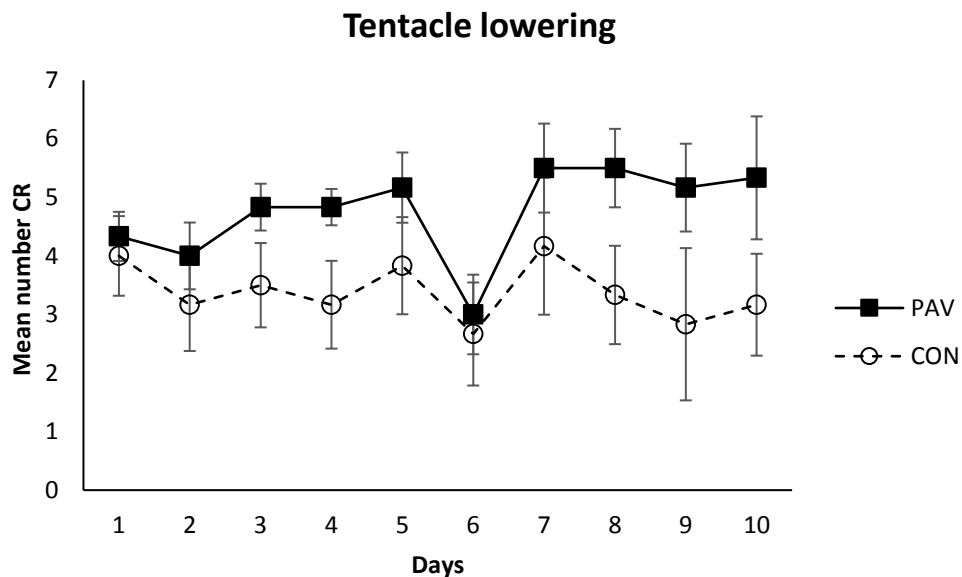

Figure 8: Mean number of tentacle-lowering responses (CR) made by the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 4B. Vertical bars represent SEMs.

Food intake: As depicted in Figure 9, snails that received the classical conditioning treatment, consumed more corn than those receiving the control group treatment. The statistical analysis confirmed an effect of Group [$F(1,10)=8.392$, $p=0.016$, $\eta^2p=0.456$], of Days [$F(14,140)=12.617$, $p=0.000$, $\eta^2p=0.558$], but not of the Group x Days interaction [$F(14,140)=1.366$, $p=0.178$, $\eta^2p=0.120$]. Since this experiment was run in parallel with Experiment 4A, the same error measurement can be observed across the first days of training. Food intake varied similarly in both groups across days, but as can

44

be noted in the figure, groups already differed in their intake on Day 1 [$t(10)=2.453$, $p=0.034$, $d'=1.69$]. Although animals were randomly assigned to each of the groups, the significant differences observed on the first day of training could be due to other non-controlled variables and thus, the results reported for this experiment should be considered carefully.
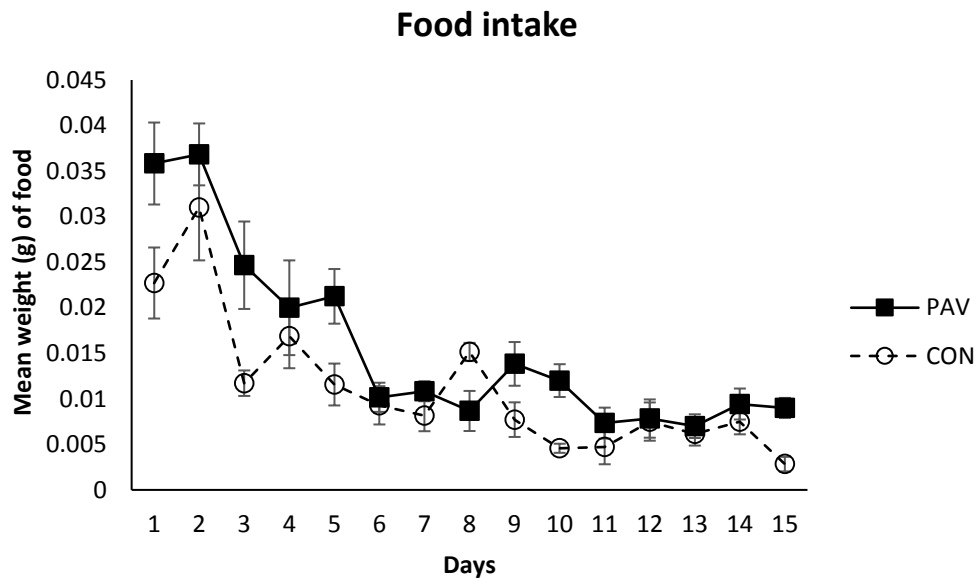
Food intake

Figure 9: Mean weight of food eaten by the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 4B. Vertical bars represent SEMs.

Growth: As it can be seen in Figure 10, both groups experienced a decrease in their weight across training and no differences were observed between the two groups. The decrease of weight was confirmed by a repeated measure analysis in which an effect of Days was observed [$F(6,60)=8.045$, $p=0.000$, $\eta^2 p=0.446$]. This analysis also confirmed the lack of differences between the two groups since no effects of Group [$F(1,10)=0.546$, $p=0.477$, $\eta^2 p=0.052$] or of the Group x Days interaction [$F(6,60)=0.464$, $p=0.832$, $\eta^2 p=0.044$] were found. These results showed that classical conditioning did not have any effect on weight maintenance in young adult snails of the species *C. aspersum* with the parameters used in this experiment.
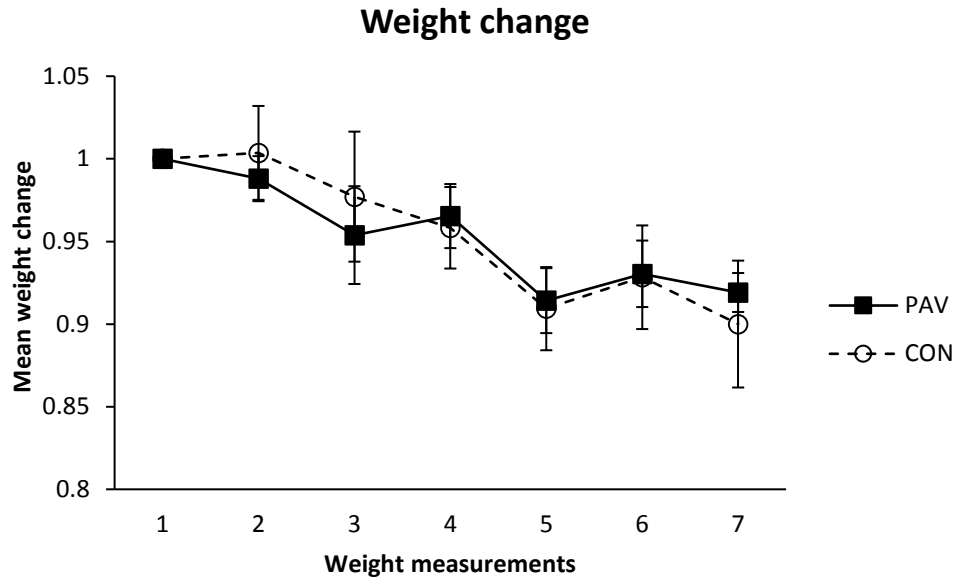
Weight change

Figure 10: Mean weight change of the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 4B. Measurement 1 corresponds to the initial weight of animals before the experiment begun. Vertical bars represent SEMs.

The results obtained in this experiment in which young adult snails were used as experimental subjects showed that paired presentations of the CS and the US increased tentacle lowering conditioned responses in a weak manner. The fact that differences were already found between the groups on the first day of the experiment questions whether the Group effect was due to learning or to other uncontrolled variables. Additionally, conditioning did not have any effects on snails' weight maintenance.

The weak effect of conditioning and food intake observed and the absence of differences obtained in weight maintenance between the two groups do not allow concluding that classical conditioning has no adaptive benefits in young adult snails. Two minutes of food access may have not been long enough for adult snails to show a strong acquisition of the conditioned tentacle lowering response or a strong improvement in feeding, giving rise to the differences observed between juvenile and adult snails. For this reason, another set of two experiments, in which an increased time of food access was

used, was conducted. Juvenile and young adult snails were used again in order to compare how conditioning could be affecting feeding at different age intervals. Moreover, to avoid food-weight measurement problems, in the two following experiments, the corn grains employed as USs were carefully cleaned before each trial.

Experiment 5A

The aim of this experiment was to test whether, in juvenile snails, feeding could be improved when an odour cue was used to signal four minutes of food access. As in Experiment 4A, juvenile snails were exposed to either paired or unpaired presentations of the CS and the US but in this experiment 4 minutes of food access (instead of two minutes) were used. Since the time for feeding was increased, snails' weight would be expected to decrease more slowly than in Experiment 4A. In addition, longer training may be necessary to test if classical conditioning can exert any effects on weight maintenance and thus, for this reason, 25 training sessions (instead of 15) were conducted.

Method

Subjects

Subjects were 14 juvenile snails with a mean weight of 2.85 g (range 2 - 4.74 g) at the start of the experiment. They were matched by weight and randomly divided into two groups: PAV (*N*=7) and CON (*N*=7). Housing and maintenance of the animals were as in the previous experiments.

Procedure

In this experiment animals had 4 minutes of access to the food (after the presentation of the CS for the PAV group and in an unpaired fashion for the CON group). A total of 25 conditioning sessions were conducted.

Results and Discussion

Tentacle lowering: As shown in Figure 11, animals that received paired presentations of the CS and the US showed a higher mean level of conditioned responding than animals that did not received such training. A repeated measures analysis showed an

effect of Group [$F(1,12)=15.14$, $p=0.002$; $\eta^2p=0.558$], but not of Days [$F(9,108)=0.982$, $p=0.459$, $\eta^2p=0.076$], or of the interaction Group x Days [$F(9,108)=0.309$, $p=0.970$, $\eta^2p=0.025$]. The effect of Group could be taken as an index of a weak learning effect although the small sample size used in this experiment does not allow fully support this conclusion. It could be also possible that other variables may be affecting the results, such as differences between the groups due to randomisation errors in subjects' group assignment. In this particular case, no statistically significant differences between the groups were obtained for the first two days of training and thus, the lack of a clear effect of learning may be more likely due to the small sample size used in this experiment.
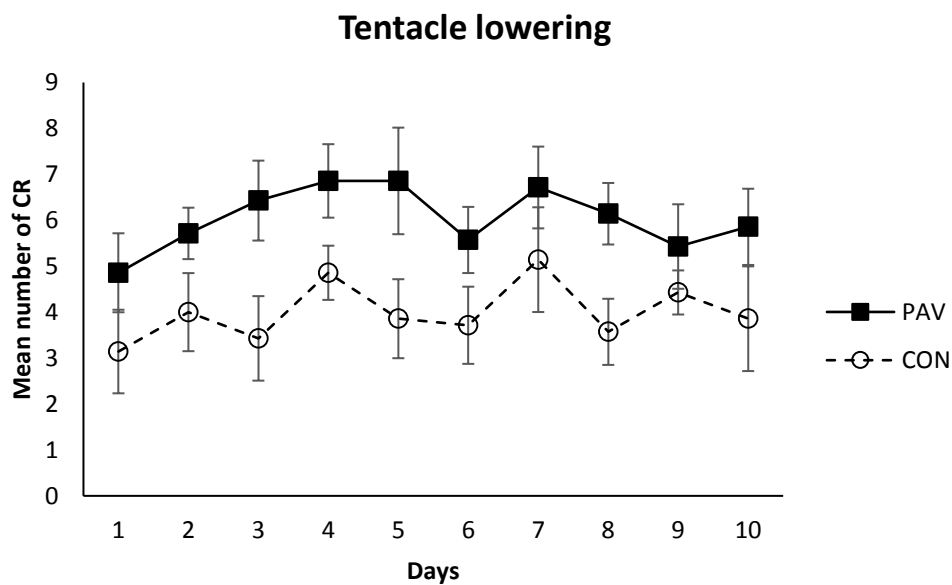
Figure 11: Mean number of tentacle-lowering responses (CR) made by the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 5A. Vertical bars represent SEMs.

Food intake: As it is depicted in Figure 12, snails that received the Pavlovian conditioning treatment, consumed more corn than those receiving the control group treatment. The statistical analysis confirmed an effect of Group [$F(1,12)=19.559$, $p=0.001$, $\eta^2p=0.62$], Days [$F(24,288)=1.982$, $p=0.005$, $\eta^2p=0.142$], and of the interaction

Group x Days [$F(24,288)=2.386$, $p=0.000$, $\eta^2p=0.166$]. Groups significantly differed on Day 5, 8, 12, 16, 18 and 24 [$ts>2.349$, $ps<0.037$, $d's>1.364$]. These results show that PAV snails ate significantly more than control subjects.
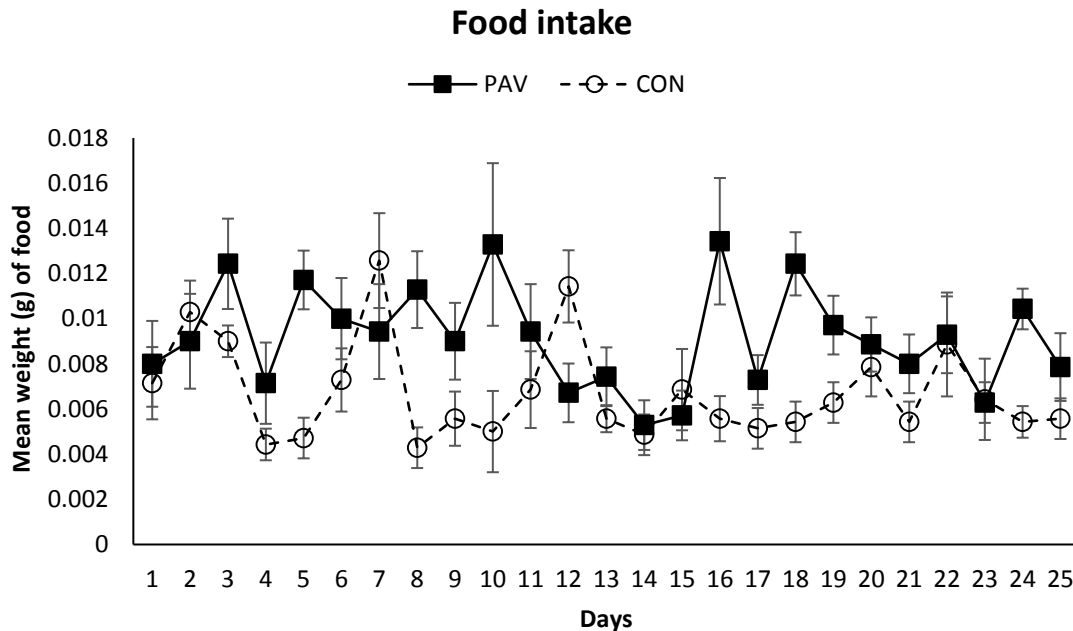
Figure 12: Mean weight of food eaten by the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 5A. Vertical bars represent SEMs.

Growth: As can be seen in Figure 13, snails of both groups experienced a decrease in their weight across the experiment regardless of whether they had been exposed to paired or unpaired presentations of the CS and the US. A repeated measures analysis confirmed these results: there was an effect of Days [$F(10,120)=6.709$, $p=0.000$, $\eta^2p=0.359$], but not of the Group [$F(1,12)=1.081$, $p=0.319$, $\eta^2p=0.083$] or of the interaction Group x Days [$F(10,120)=0.905$, $p=0.531$, $\eta^2p=0.07$].

Weight change

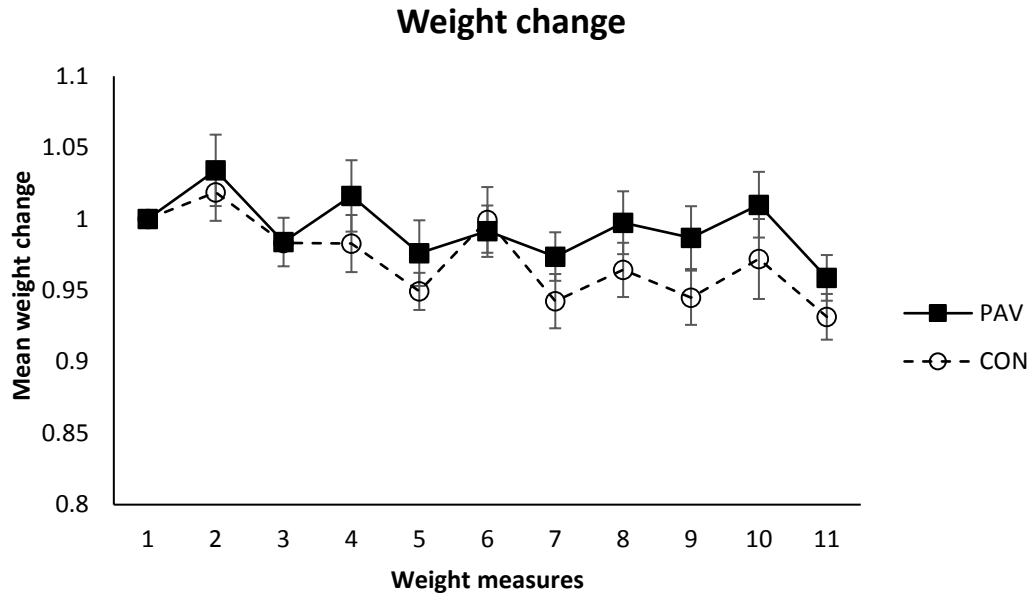**Figure 13**: Mean weight change of the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 5A. Measurement 1 corresponds to the initial weight of animals before the experiment begun. Vertical bars represent SEMs.

The results obtained in Experiment 5A showed that pairing an odour with 4 minutes of access to food did not clearly increase the tentacle lowering response but it did result in a greater consumption of food. However, despite the differences in food intake, no differences were observed in terms of weight change. The results reported here replicated only to a certain extent the results described for Experiment 4A and raised an interesting question about the relationship between tentacle lowering conditioning and food intake. Because groups differed in their food intake but the effect of training on the conditioned tentacle lowering response was not very strong, it could be argued that these two responses are not necessarily related to one another. Yet other possibilities would be that the sample size was very small, or that four minutes of daily food access is not as critical for their survival as 2 minutes of access. Notwithstanding, the fact that differences were observed in the amount of food eaten, renders this last possibility unlikely.

Experiment 5B

The aim of this experiment was to assess whether feeding could be improved after Pavlovian conditioning in young adult snails. Experiment 5B is a replicate of Experiment 5A with two differences: snails had four minutes of food access instead of two, and a total of 25 conditioning sessions were conducted.

Method

Subjects

Subjects were 14 adult snails with a mean weight of 7.40 g (range 5.14 - 9.42 g) at the start of the experiment. They were matched by weight and randomly divided into two groups: PAV ($N=7$) and CON ($N=7$). Housing and maintenance of the animals were as in previous experiments.

Procedure

As in Experiment 5A, animals were allowed to eat for 4 minutes (after the presentation of the CS for the PAV group and in an unpaired fashion for the CON group), and 25 conditioning sessions were conducted.

Results and Discussion

Tentacle lowering: As shown in Figure 14, animals that received paired presentations of the CS and the US showed a higher mean level of conditioned responding than animals that did not received such training. A repeated measures analysis showed an effect of Group [$F(1,12)=7.524$, $p=0.018$; $\eta^2p=0.385$], and of the Group x Days interaction [$F(9,108)=2.181$, $p=0.029$, $\eta^2p=0.154$], but not of Days [$F(9,108)=1.665$, $p=0.106$, $\eta^2p=0.122$]. Differences between the groups were found in Day 5, 6 and 9 [$ts(12)>2.305$, $ps<0.040$, $d's>1.18$]. These results show that pairing an odour with food

access resulted in tentacle lowering conditioning of the experimental group compared to the control group.
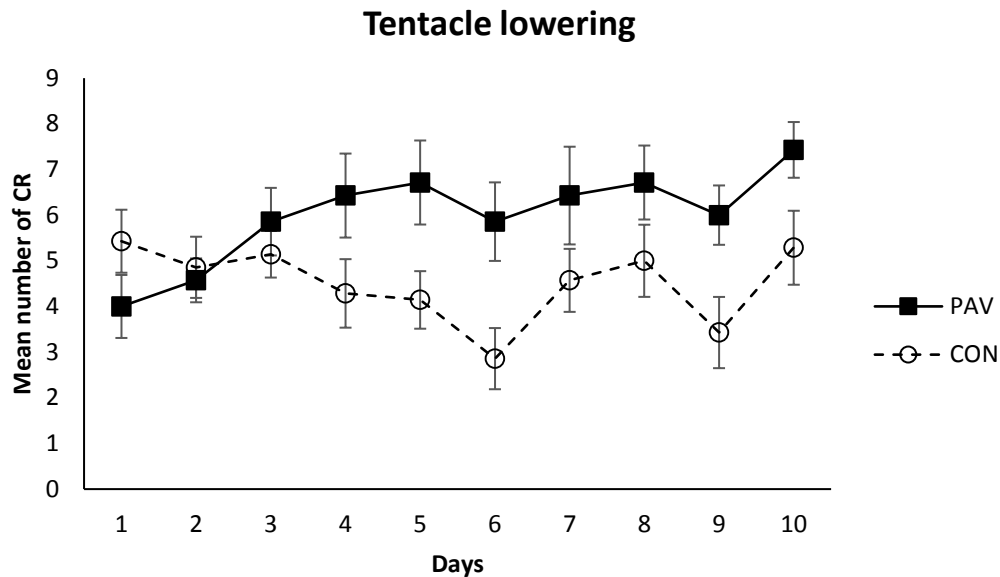
Tentacle lowering

Figure 14: Mean number of tentacle-lowering responses (CR) made by the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 5B. Vertical bars represent SEMs.

Food intake: As is depicted in Figure 15, snails that received the classical conditioning treatment consumed more corn than those receiving the control group treatment. The statistical analysis confirmed an effect of Group [$F(1,12)=15.033$, $p=0.002$, $\eta^2 p=0.556$], and of Days [$F(24,288)=2.192$, $p=0.001$, $\eta^2 p=0.154$], but no effect of the Group x Days interaction [$F(24,288)=1.395$, $p=0.107$, $\eta^2 p=0.104$] was obtained. Although conditioning was expected to affect food intake across the entire experiment, it could be possible that training had a different effect at the beginning or at the end of the experiment. In fact, Groups significantly differed on Days 4, 5 and 6 [$ts(12)>3.486$, $ps<0.004$, $d's>2.026$]. These results could indicate that conditioning had a greater effect on food intake at the beginning of the training than at the end.

Food intake
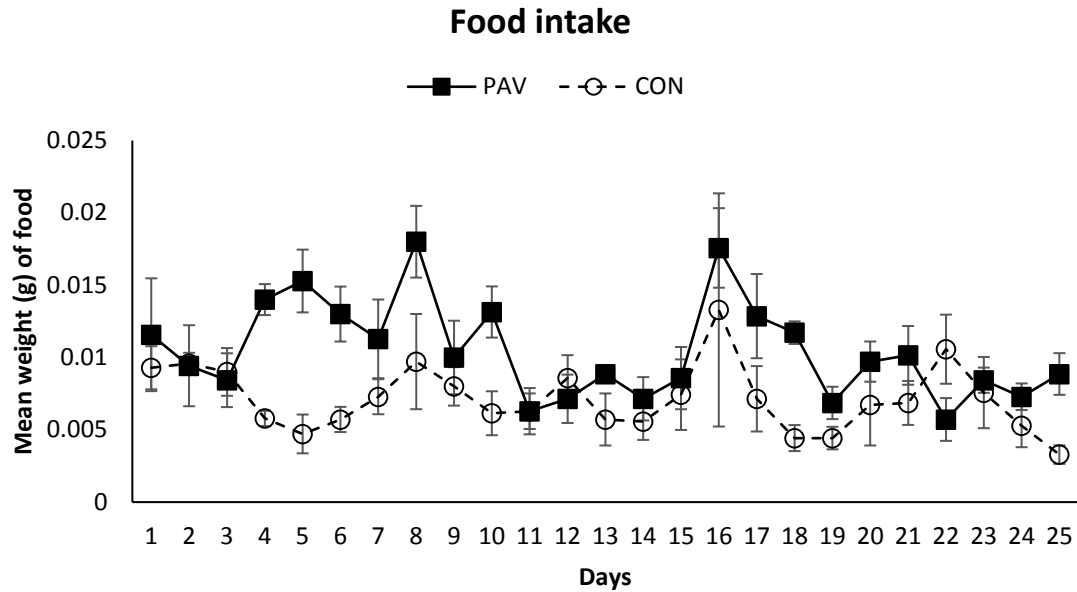
Figure 15: Mean weight of food eaten by the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 5B. Vertical bars represent SEMs.

Growth: As can be seen in Figure 16, snails' weight slightly decreased across training in both groups. A repeated measure analysis showed an effect of Days [$F(10,120)=8.252$, $p=0.000$, $\eta^2p=0.407$], but no effect of Group [$F(1,12)=0.909$, $p=0.359$, $\eta^2p=0.07$], or of the interaction Group x Days [$F(10,120)=0.916$, $p=0.521$, $\eta^2p=0.071$] were found.
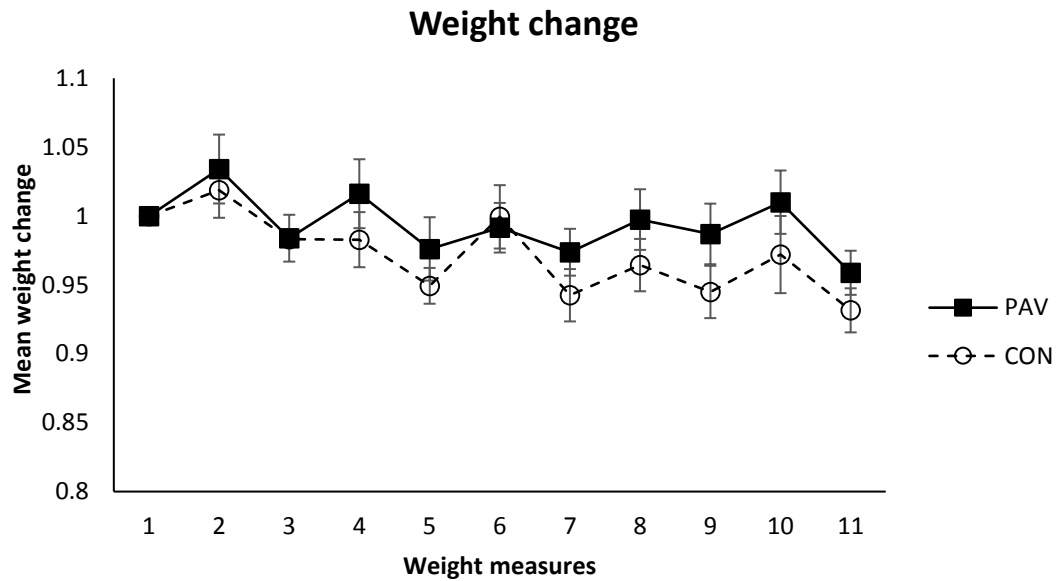
Weight change

Figure 16: Mean weight change of the PAV (Pavlovian conditioned) and the CON (control) groups in Experiment 5B. Measurement 1 corresponds to the initial weight of animals before the experiment begun. Vertical bars represent SEMs.

In this experiment, experimental subjects showed an increased tentacle lowering response. They also ate more corn than controls at the beginning of the training but not throughout the entire experiment. As a whole, these results suggest that a classical conditioning preparation in which a CS is followed by four daily minutes of food access is more meaningful for young adult snails than an equivalent training in which animals are allowed to eat for only two minutes (as in Experiment 4B), but still not enough to observe a robust effect of learning on both conditioned tentacle lowering and food intake.

Discussion

When an odour cue was used to signal access to food, juvenile snails significantly increased their food intake compared to a control group for which the odour and the food were unpaired. This result was consistent regardless of the amount of time animals were allowed to eat (2 or 4 minutes in experiments 4A and 5A, respectively). However, only

when the odour cue was used to signal a short period of food access did training have a significant effect on weight maintenance. Similarly, only in that situation was tentacle lowering conditioning clearly observed. These results show that juvenile *Cornu aspersum* can learn to predict food access and to better use their limited time to feed. The fact that no differences in weight were observed when animals were given 4 minutes of food access rather than 2, could further indicate a greater motivation to learn (i.e., due to food scarcity) for animals in this developmental stage. It could be argued that food signalling is beneficial when food is scarce but when it is more abundant it does not add extra advantages. In order to clarify this question, different food access intervals should be employed.

Contrasting to the results obtained with juveniles, the two experiments run with young adult snails failed to show effects of conditioning in weight maintenance (Experiments 4B and 5B). Only when a long feeding interval was used (i.e., 4 min, Experiment 5B) were the results indicative of adaptive effects of conditioning in terms of feeding. The absence of strong conditioned tentacle lowering in Experiment 4B and of strong effects of learning on food intake (in both experiments 4B and 5B) could be due to the short food access time employed. In the most commonly employed experimental procedure, snails are given 6 conditioning trials per day, which adds up to 12 minutes of food access (e.g., Acebes et al., 2009). Experiments in which only one conditioning trial per day was run snails were given 10 minutes of food access (e.g., Ungless, 1998; Loy et al., 2006). Thus, it could be possible that the amount of food consumed during the experiments 4A and 4B was not significant enough for adult snails to observe a clearer conditioning effect.

When taken together, the differences observed between juveniles and young adults indicate that the consequences of learning differ depending on the developmental

stage of the experimental subjects and on the experimental parameters adopted; a question that requires further research. In this sense, further parametric experiments in which the duration of the US is explored would be necessary to shed more light on this issue. Additionally, such experiments may be also helpful for determining the critical circumstances under which classical conditioning can affect not only tentacle lowering and food intake but also weight maintenance.

Another interesting result is that in Experiment 5A there were significant differences between the experimental and control groups in the amount of food eaten but the effect of conditioning on the tentacle lowering response was not as strong as on food intake. These results could imply that tentacle lowering and food intake are not necessarily related to one another, even though they may co-occur as in Experiment 4A. Nonetheless, the small sample size used in that experiment does not allow to firmly conclude that such dissociation exists between tentacle lowering and food intake.

As a whole these experiments indicate that snails can become more efficient at feeding due to classical conditioning. Notwithstanding, there is a number of shortcomings that should be taken into account for future research. First, the sample sizes used in all the experiments was small and thus, the individual differences observed between subjects led to a high variability in the registered dependent variables. Second, both food intake and weight measurement need to be improved. Especially, snails can greatly vary in their body water content affecting their total weight (e.g., Burton, 1964). Although weight measurement can be used to assess the adaptive benefits of classical conditioning under situations of food scarcity, a better index of growth would be that of dry weight for adults (Burton, 1964), shell size for juveniles (e.g., Dan & Bailey, 1982; Noshita, Shimuzu, & Sasaki, 2015) or shell regeneration for either adults or juveniles (e.g., Wagge, 1951). Finally, since the results differed between juveniles and young adults, age or

developmental stage should also be taken into consideration. An improvement in all these aspects is required for an accurate assessment of the biological function of classical conditioning in this species with this procedure.

Chapter 4- Classical conditioning and its effect on mating[1]

As already mentioned in the introduction, classical conditioning has been shown to importantly increase reproductive success in, especially, two species: the blue gourami fish (*Trichogaster trichopterus*) and the Japanese quail (*Coturnix japonica*). For males of both species it has been shown that pairing a conditioned stimulus (e.g., a light or a context) with the presence or access to a female, resulted in an increased reproductive success. Blue gouramis became more successful at mating as they showed less aggressive behaviour towards the female, they started courtship faster (Hollis et al., 1989), and they took less time to induce spawning in the female, siring more offspring than the control males, who were not able to rely on any sort of cues to predict the upcoming mating encounter (Hollis et al., 1997). Similarly, in the Japanese quail, classical conditioning resulted in larger ejaculates and larger number of spermatozoa released by the treatment group compared to the control group (Domjan et al., 1998), as well as in an increase in the number of fertilised eggs (Adkins-Regan & MacKillop, 2003).

These studies show that classical conditioning can facilitate several stages of the mating sequence (courtship, mating, and fertilization), but its effects on hermaphrodites have not been tested. The great pond snail *Lymnaea stagnalis* is a simultaneous hermaphroditic snail species that mates unilaterally. This means that even though all individuals are functionally both male and female throughout their life, they can only perform either one role or the other within one mating interaction. Given that they are able to swap roles after the first mating, they can both gain male and female reproductive fitness but they have to choose roles in each mating encounter. Whether *Lymnaea*

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stagnalis mates in the male (hereafter also referred to as "sperm donor") or the female role (hereafter also referred to as "sperm recipient") has been shown to depend on several variables, being the availability of seminal fluid in the prostate gland one of the most determinant factors (see Nakadera & Koene, 2013 for a review). When seminal fluid is available snails show an increased motivation to mate in the male role (Van Duivenboden & Ter Maat, 1985). This can lead to an especially conflictive situation when both individuals are willing to mate as sperm donors. On the other hand, no conflict over sexual role performance is expected when one of the two snails has recently mated. In that case, the prostate gland of the recently mated snail will be virtually empty and thus the female role will be preferred (Van Duivenboden & Ter Maat, 1985).

It is interesting to point out that, when two snails are motivated to mate in the male role, the first sperm donor gets higher reproductive success due to two known physiological mechanisms that snails of this species are endowed with. The first one is the presence of (at least) two identified seminal fluid (LyAcp5 and LyAcp8b) that affect the male function of the sperm recipient (Nakadera, al., 2014). When the snail that has been inseminated (i.e., has played the female role) is going to mate in the male role (i.e., in a second mating encounter), it transfers about half the amount of sperm to its next partner compared to a snail that has not been previously inseminated (Nakadera et al., 2014). Moreover, having received sperm prior to mating in the male role has also been shown to decrease the recipient's paternity success when it mates in the male role (Nakadera et al., 2014). This effect of reducing both the quantity of sperm transferred and the paternity (i.e., the male function) of the recipient can last for up to one day after insemination (no longer intervals have been tested). As explained in Nakadera et al. (2014) the snail that mates first as a male could be gaining two possible benefits. By reducing its partner's male function, the sperm donor is enhancing its own male function

-less sperm competition- as well as its own female reproductive success –it avoids receiving excessive amounts of sperm and unwanted substances such as seminal fluid proteins (Nakadera et al., 2014).

The second benefit snails of this species obtain by mating first in the male role is that sperm transfer can induce the recipient to allocate its reproductive resources to the female function. As shown in other studies, another seminal protein (LyAcp10) causes a delay in egg laying (Koene et al., 2010) that seems to be related to a higher investment per egg (Hoffer, Schwegler, Ellers, & Koene, 2012). This would not only potentially increase the donor's paternity success, but also the overall fitness since its offspring may be more likely to survive. Thus, in a mating encounter between two snails that are motivated to mate as sperm donors, performing the male role first would seem to be the best reproductive strategy.

One aspect that could be affecting sex role decision is classical conditioning. Previous research with this species has shown that *Lymnaea stagnalis* is able to learn via operant (e.g., Lukowiak et al., 2003) and classical conditioning procedures (Audesirk, Alexander, Audesirk, & Moyer, 1982; Kemenes & Benjamin, 1989). In classical conditioning preparations, an appetitive procedure in which a CS (either a tactile stimulus or a neutral odour) was paired with sucrose led to an increase in conditioned feeding responses (opening of the mouth, rasping and closing of the mouth). However, the role of classical conditioning in mating has not been tested in this species.

The aim of this work was to assess whether classical conditioning can improve mating in *Lymnaea stagnalis* under two different circumstances: one in which there is a medium level of conflict over the performance of the male role and another one in which such a conflict does not exist. In the first situation, by signalling the presence of a possible

mate, snails in the learning condition may be able to better overcome sex role conflicts compared to those snails that are not able to predict the mating encounter. Particularly, since mating first in the male role provides greater overall reproductive fitness, snails in the learning condition are expected to be more likely to perform the male role first compared to a control group. Contrarily, in a second scenario in which there is no conflict over sex role performance because one of the snails has already mated, learning would be expected to provide different advantages. For example, as for the blue gouramis, conditioning could be expected to reduce the total amount of time invested in mating. In order to address this question, a second experiment in which there was not conflict over the sperm donor role was conducted.

General Method

Subjects

Subjects were sexually mature adult snails (about four months old) obtained from the laboratory culture of the Vrije Universiteit in Amsterdam. Snails were housed, either individually or collectively, in plastic pots (measuring 85 x 100 x 80 mm) that were allocated in the same large tank with running low-copper water at 20ºC. Each plastic pot had 22 slits that allowed for water exchange. They were kept under a 12-hour light schedule, starting at 7 am. All snails were daily given access to food *ad libitum* and were marked with nail polish for identification purposes. Snails were divided into two groups: Pavlovian (hereafter referred to as PAV) and Control (hereafter referred to as CON) groups. Snails belonging to the PAV and CON groups were isolated 10 days prior to the start of the experiment to increase their motivation to mate in the male role, and they were matched by their body size. Body size is a variable that also influences the preference for mating in either the male or the female role and thus needs to be controlled for (Nakadera

et al., 2015). Additionally, other snails were used as the unconditioned stimulus. Snails employed as USs were either kept isolated (Experiment 6) or in groups (Experiment 7) and they were also matched by size.

Apparatus and stimuli

Conditioning and testing was conducted in closed pots which were of the same characteristics of the ones used as the home container except for that they did not have slits. Following Audesirk at al. (1982), the stimulus used as the conditioned stimulus (CS) was amyl acetate (an oily substance that smells like banana; 0.004% v/v) since it has already been shown to work as a CS in *Lymnaea stagnalis*. The US was the introduction of another snail.

Procedure and Experimental setting

The procedure used for this study was similar to that employed by Audesirk et al. (1982) with some differences. As in Audesirk et al. (1982), training for the PAV group consisted of 15 training trails conducted over a period of three days, 5 trials per day, with an intertrial interval of 90 min. Prior to the start of a conditioning trial, snails were individually transferred from their home tank to a plastic pot, situated on a table, which had been filled with fresh running low-copper water at 20ºC and with no food available. They were left undisturbed for 10 minutes so they could acclimatise to the new space. Following this acclimation period, snails in the PAV group were exposed to the CS for two min. The CS consisted of the presentation of 50 ml of a 0.004% solution of amyl acetate that was gently poured into the plastic pot. The CS was distributed throughout the pot while pouring but not directly on the snails. Subjects were allowed to move around in the solution for 2 min, after which a US snail was introduced into the pot (the time of CS exposure differs from that used in Audesirk et al., 1982, where the US was presented 15 seconds after the presentation of the CS). Both snails were allowed to move around

together for 2 minutes. After training, target PAV subjects were individually and briefly transferred (for 30 seconds) to a rinsing plastic pot containing clean water, and then to another clean training pot where they waited for the next trial. All the pots in which conditioning and rinsing took place were thoroughly cleaned, so that all the smells were removed, and filled again with fresh water. This treatment was repeated 5 times per day, with an intertrial interval of 90 minutes, during three days. The snails used as a US were also placed in a rinsing pot for 30 seconds before being transferred to a clean new pot. All the US snails were presented to all the target snails (PAV and CON) so they all had equivalent experience with all the snails. For snails belonging to the control group, the treatment was the same except for that the CS and the US were never presented together. Rather, they were given unpaired pseudorandom presentations of the CS and the US in such a way that no more than two consecutive presentations of the same stimuli were allowed. The length of CS and of US presentations was also 2 minutes.

Conditioning was tested by presenting the CS to each of the target animals for two minutes, after which a US snail was introduced inside the pot. Animals were allowed to interact with each other and mate. The test lasted for 5 hours in which the experimenter registered the behavioural mating sequence every 5 minutes. The behaviours recorded were mounting, circling, positioning, partial eversion, full eversion, probing, and intromission (see Table 2 for a clearer description of each of them; see also De Boer, Jansen, & Ter Maat, 1996, p. 168 and 169 for a description and illustrations of the mating sequence).

Behaviour	Description
Mounting	Snail climbs on top of the other snail's shell.
Circling	Snail starts to describe circles in a counter clockwise fashion on top of the other snail's shell.
Positioning	Snail stops circling on the right edge of the other snail's shell and remains motionless for at least10 seconds.
Partial Eversion	Snail's preputium becomes visible but is not fully everted.
Full Eversion	The preputium is fully everted.
Probing	Snail searches for the female opening with the preputium fully everted.
Intromission	Snail introduces the penis into the vagina of the partner

Table 2: Description of the behavioural mating sequence of Lymnaea stagnalis

These behaviours were recorded for both the target subjects (more likely to perform the male role) and the US subject (more likely to perform the female role), and the latency to perform each of the behaviours was estimated. Both PAV and CON groups were tested at the same time and the experimenter was unaware of their previous training history. In order to facilitate the observation of the mating sequence, experiments were run in replicates of 10 subjects each (5 PAV and 5 CON).

Analyses

All the analyses were run using SPSS v21 (SPPS Inc., Chicago, IL, U.S.A.). The size effect reported was η_p^2 (Cohen, 1973).

Experiment 6

The aim of this experiment was to test the role of classical conditioning in a mating situation with a medium level of conflict over the performance of the male role. To do so, snails were either isolated for a long period (14 days) or a short period (4 days) before mating. Given the benefits of performing the male role in the first place, a conflict over performing this role is expected to ensue. Snails in the learning treatment are hypothesised to benefit from classical conditioning and to be more likely to mate first as males compared to the snails in the control group.

Method

Subjects

Subjects were 80 sexually mature adult snails. They were randomly assigned to two different groups: PAV (N=20) and CON (N=20). Another 40 snails were used as USs. Snails belonging to PAV and CON groups were isolated for ten days before the start of the experiment and paired by size (mean size of 29.46; SEM= 0.24, for both groups). The snails employed as USs were kept in groups of five snails until the start of the experiment and they had a mean body size of 29.1 (SEM= 0.16). From Day 1 onwards they were kept isolated so that on Day 4 (when the test took place) they had been isolated for 4 days. This isolation period was chosen because the motivation to mate as a male is slightly increased (after 8 days they prostate gland if full) but they still are motivated to mate as females (e.g., Nakadera et al., 2015).

Results and Discussion

Two snails belonging to the CON group and one belonging to the PAV group were discarded from the analysis since they did not mate. One experimental subject from the PAV group died during training. Thus, each group consisted of 18 subjects each.

As it can be seen in Figure 17, a larger percentage of the snails that were exposed to a conspecific signalled by an odour, mated first in the male role (12/18 or 67%) compared to those snails that were exposed to both the odour and the conspecific but in an unpaired fashion (6/18 or 33%). This difference in their preferred role performance was statistically significant [χ^2=4; p=0.045]. This result shows that learning to predict the presence of a conspecific increases the likelihood of mating first as a sperm donor in a signalled mating encounter.
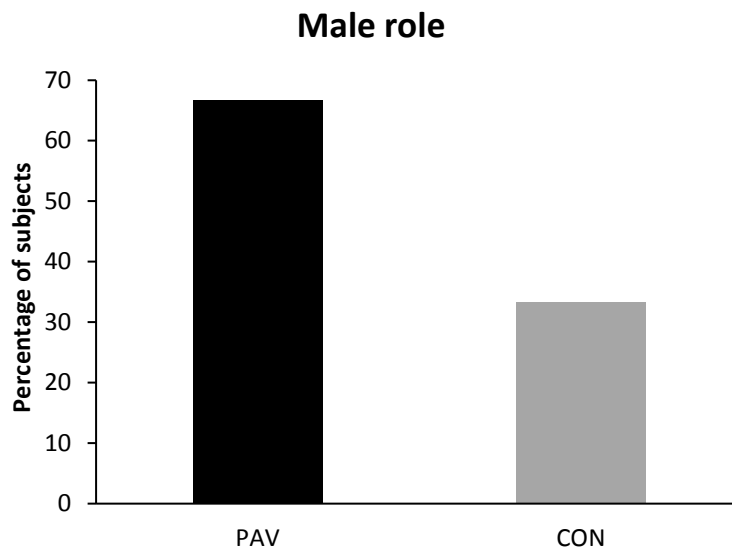
Male role

Figure 17: Percentage of PAV (Pavlovian conditioned) and CON (control) subjects that mated first in the male role in Experiment 6.

Experiment 7

In the previous experiment, all the snails were motivated to mate as sperm donors and classical conditioning was shown to increase the likelihood of mating in the male role first. However, classical conditioning could have other effects on mating other than sex role decision such as a reduction of the time invested in mating. To test this possibility, the same experiment was conducted again except for that the snails employed as USs were not isolated in order to keep their motivation to mate as sperm donors at a minimum level.

Method

Subjects

Subjects were 60 sexually mature adult snails. They were randomly divided into two different groups: PAV (N=15) and CON (N=15). Another 30 snails were used as USs. Snails belonging to PAV and CON groups were isolated for ten days before the start of the experiment and paired by size (mean size of 28.45; SEM= 0.28, for both groups). Snails employed as USs were kept in groups of five snails throughout the entire experiment and they had a mean body size of 27.88 (SEM= 0.15).

Results and Discussion

Two snails died during the experiment (one belonging to the experimental group and the other one belonging to the control group). Out of the 14 subjects left, four snails in the experimental group and five in the control group did not mate and they were excluded from the analysis. Mating did not occur because none of the snails showed any interest in mating or because the snail employed as the US (and thus expected to perform the female role) avoided mating by crawling out of the water.

As it can be seen in Figure 18A, snails that were exposed to a solution of amyl acetate followed by the introduction of another snail took less time (5.5 min; SEM=1.46) to mount than those snails that had been exposed to both stimuli but in an unpaired pseudo-random fashion (13.3; SEM=6.613). The same pattern of results was obtained for all the remaining behaviours, namely circling (Figure 18B), positioning (Figure 19), partial eversion (Figure 20A), full eversion (Figure 20B), probing (Figure 21A) and intromission (Figure 21B). Although snails in the PAV condition always showed a lower latency to perform any of the behaviours registered, statistically significant differences were found only for some of them. Particularly, differences were statistically significant for behaviours displayed at the end of the mating sequence (i.e., partial and full eversion, probing and intromission) but not for those performed at the beginning of the mating sequence (i.e., mounting, circling and positioning).
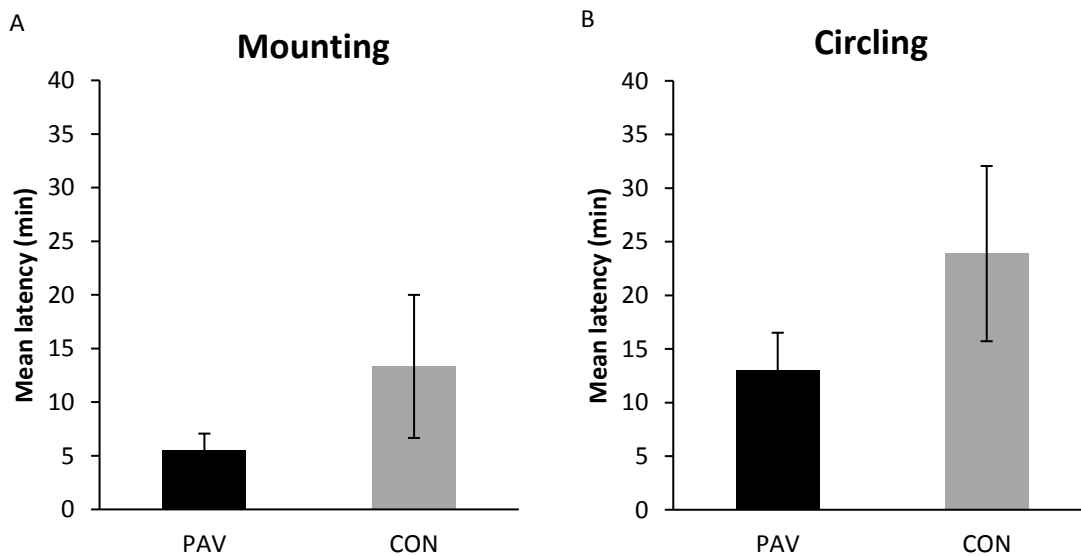

Figure 18: (A) Mean latency of PAV (Pavlovian conditioned) and CON (control) subjects to mount the other snail's shell and (B) mean latency to start circling in Experiment 7. Vertical bars represent SEMs.
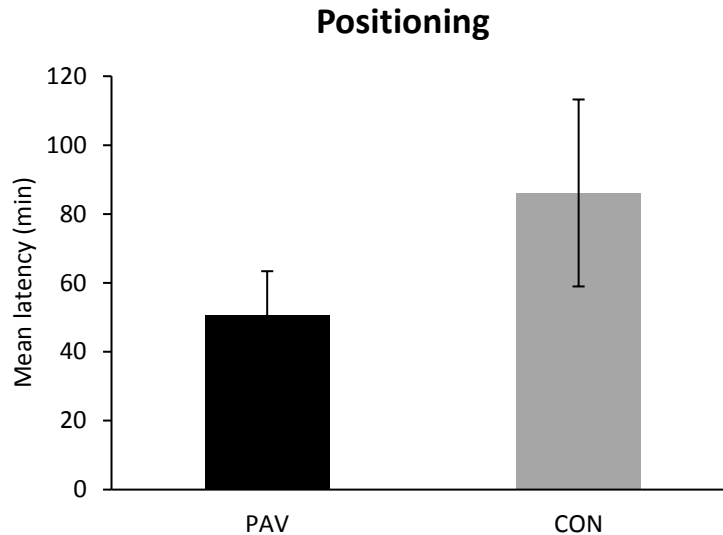
Positioning

Figure 19: Mean latency of PAV (Pavlovian conditioned) and CON (control) subjects to position in Experiment 7. Vertical bars represent SEMs.

The statistical analysis showed that the differences between both groups in the time to mount [$F(1,17)=1.45$, $p=0.245$, $\eta^2=0.079$], to circle [$F(1,17)=1.766$, $p=0.201$, $\eta^2=0.094$], and to position [$F(1,17)=1.502$, $p=0.237$, $\eta^2=0.081$] were not significant. However, the differences in the latency to show partial [$F(1,17)=5.318$, $p=0.034$, $\eta^2\eta^2=0.238$] and full eversion [$F(1,17)=6.364$, $p=0.022$, $\eta^2=0.272$], to probe [$F(1,17)=5.874$, $p=0.027$, $\eta^2=0.257$] and to intromit [$F(1,17)=6.271$, $p=0.023$, $\eta^2=0.269$] were statistically significant.
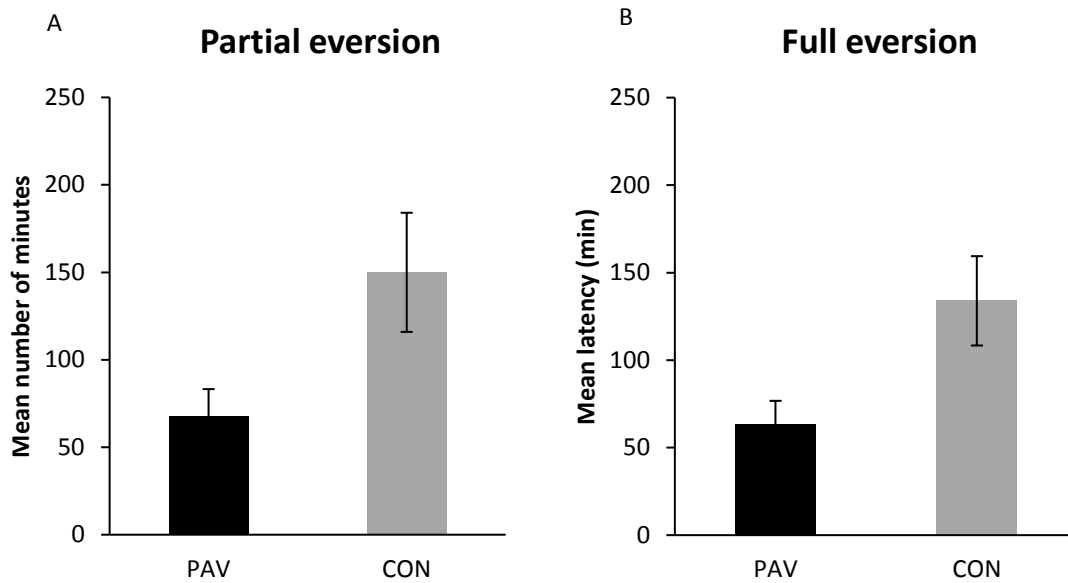
Figure 20: (A) Mean latency of PAV (Pavlovian conditioned) and CON (control) subjects to show partial eversion and (B) to show full eversion in Experiment 7. Vertical bars represent SEMs.

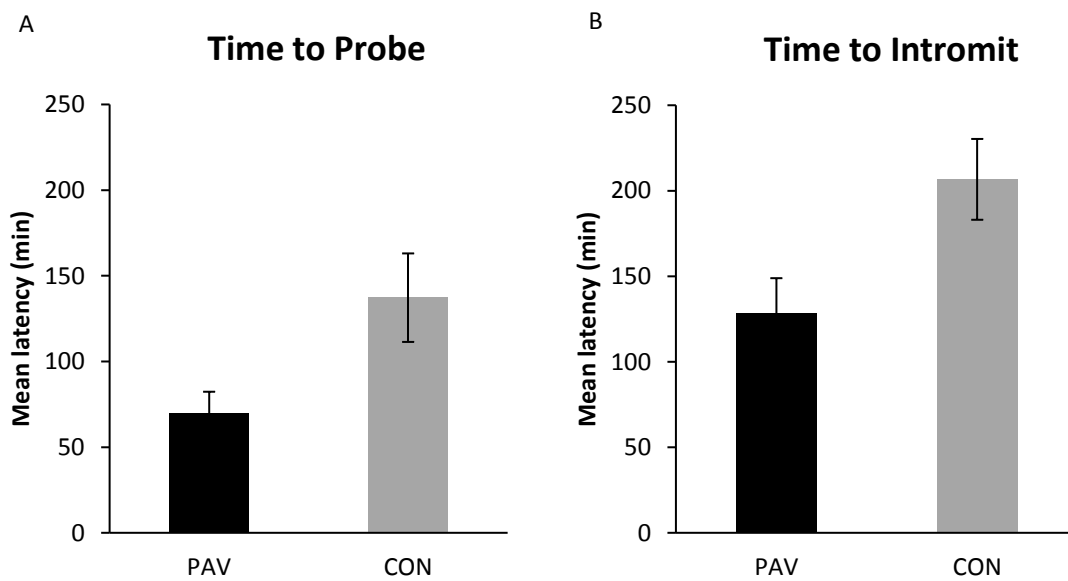**Figure 21**: (A) Mean latency of PAV (Pavlovian conditioned) and CON (control) subjects to start probing and (B) to intromit in Experiment 7. Vertical bars represent SEMs.

These results show that signalling the presence of a potential mate reduces the overall amount of time invested in mating, especially at the end of the mating sequence.

Discussion

The aim of this study was to test the role that classical conditioning could play in mating in *Lymnaea stagnalis* when there is and when there is not a conflict over the performance of the sperm donor role. In both cases, animals in the experimental group were exposed to paired presentations of a cue (i.e., amyl acetate) followed by the presence of a conspecific, whereas animals in the control group were exposed to the same stimulation except for that these two events were never occurring together.

In the first experiment of this chapter, a medium conflict level was arranged by isolating the target snails during 10 days prior to training and the snails employed as USs during 4 days prior to the test. The results obtained showed that classical conditioning led to an increased male role mating performance for the experimental group compared to the control group (Experiment 6), which is more adaptive for this species. As reported in the introduction section of this chapter, when both animals are motivated to mate in the male role, being the first one to receive sperm entails receiving seminal proteins that decrease the male investment in the next insemination and that also reduce paternity success (Nakadera et al., 2014). An extra disadvantage is that reciprocal mating may not even occur since "the sperm recipient cannot assure its role as a male in the second insemination prior to being inseminated, because the alternation position can be established only after the first insemination has started" (Koene & Ter Maat, 2005, p. 848). In other words, the sperm recipient, who has produced and stored sperm for a few days, may not be able to inseminate its partner; a situation that would imply even a higher cost for the male function. Additionally, receiving sperm proteins can be costly for the female function, since they can cause a delay in egg laying and a higher investment in egg production.

In Experiment 7, no differences were observed in terms of snails performing the male role. This was expected given that the snails used as USs had not been isolated prior to the mating encounter so their prostate gland was most likely depleted. However, classical conditioning did have an effect on the time employed in mating. The effect of learning was not significant for the early behavioural displays of the mating sequence (i.e., mounting, circling, positioning) but it was for the last ones (i.e., partial and full eversion, probing, and intromission).

As for the blue gouramis or the Japanese quails, classical conditioning has been shown to affect different aspects of the mating behaviour in *Lymnaea stagnalis.* The two experiments here reported show that classical conditioning has a positive effect on mating in this species, and that the particular benefits that snails of this species will get will depend on other situational variables such as the physiological state or mating deprivation of their partner. Furthermore, this study constitutes the first demonstration of the adaptive effects of classical conditioning in an hermaphroditic species.

Chapter 5- General Discussion

This thesis attempted to explore the adaptive benefits of classical conditioning in two mollusc species in three different domains of their behavioural ecology. In *Cornu aspersum,* paired presentations of an odour (CS) and food (US) were used to test whether snails of this species will gain any advantages in terms of food finding (Chapter 2) or of food intake and growth (Chapter 3). In the fourth chapter, the great pond snail *Lymnaea stagnalis* was employed for the analysis of the adaptive benefits of classical conditioning in terms of mating.

Regarding food finding behaviour, the research conducted focused on determining some of the variables that affect food finding as a first necessary step for a future evaluation of the benefits of classical conditioning for food finding. The results obtained showed that prior experience (Experiment 1), and not just odour exposure (Experiment 2), is necessary for *Cornu aspersum* to locate a distant food. Furthermore, it was shown that snails can learn to search for an odour that has been paired with food when the food is not present in the test but that they do not rely on such conditioned cues when the food they had experienced is present (Experiment 3). These results could be indicating the existence of two different food finding mechanisms or simply that snails follow the most reliable cue for food: when the scent of an already known edible substance can be detected, that food source would be expected to be nearby and they search for it regardless of other odours' presence. On the contrary, in a situation in which two odours are present and none of them stems from a recognised food source but one of them has been paired with the presence of food, animals' best food searching strategy will be to orientate the search to that odour. In this sense, tentacle lowering conditioning would provide snails with a better food finding strategy. Nonetheless, these findings do not completely address

the issues raised by Ungless (2001) since tentacle lowering was not further examined in the experiments conducted in Chapter 2. It remains, therefore, to test whether those snails who display a greater conditioned response are also better food-sign finders.

The same tentacle lowering procedure was also used in Chapter 3 to test whether conditioning could provide snails with advantages other than food finding in a situation of food scarcity, and food intake and weight were measured in four different experiments conducted with both juvenile and young adult *Cornu aspersum* snails. In juveniles, the results obtained indicate that pairing an odour with limited food access resulted in a larger intake regardless of whether a robust effect of conditioned tentacle lowering occurred. Importantly, this learning training had more relevant consequences in terms of weight maintenance when the amount of time allowed for feeding was the shortest (2 min; Experiment 4A). For young adults, this same training resulted in an increased food intake, especially at the beginning of the training, when the animals were allowed to eat for a longer period of time (4 min; Experiment 5B). However, no effect on weight change was observed whatsoever (Experiments 4B and 5B). The results showed that the adaptive benefits of classical conditioning can depend on the developmental stage of the animals under study (e.g., juvenile vs. young adults; Experiments 4A and 4B). Moreover, the demonstration of the adaptive benefits of classical conditioning on food intake or weight maintenance may vary depending on other variables such as the level of deprivation (e.g., 2 min vs. 4 min of food access; Experiments 4A and 5A). On the other hand, a deeper assessment of the biological function of classical conditioning on nutrition requires further improvements in the experimental procedure. Experiments conducted with juvenile snails in which larger sample sizes and refined measurements of the dependent variables (e.g., shell growth/shell regeneration) are used seem to be most suitable for the analysis of this question,

Finally, the aim of Chapter 4 was to explore whether classical conditioning training could facilitate mating in the great pond snail *Lymnaea stagnalis.* In this set of experiments, it was observed that learning can either increase the likelihood of mating first as a sperm donor (Experiment 6) or decrease the total amount of time invested in mating (Experiment 7). In the first situation, classical conditioning would be enhancing the male reproductive function by reducing sperm competition and by increasing its partner's reproductive investment (e.g., Nakadera et al., 2014), whereas in the second one, snails would benefit by reducing the amount of time spent in mating. The particular outcome of each of the experiments depended on a variable that was external to the target snails (conditioned or control) given that it was the level of motivation to mate in the male role of the snails used as US that had been manipulated. This finding illustrates that classical conditioning does not imply the display of a fixed behavioural pattern but that it allows animals to confront different situations in a flexible way.

Taken together, these experiments show that classical conditioning has a biological function in two mollusc species, just as it has been shown in other chordate or arthropod species (e.g., Hollis, 1999; Hollis et al., 2011). One general weakness of the research presented in Chapters 3 and 4 is the use of an explicitly unpaired control group. As already pointed out in the work of Karen Hollis (1984; see also Hollis et al., 1997) and Michael Domjan (Domjan et al., 1998), animals in the control group were exposed to a cue that actually signalled the non-occurrence of a relevant outcome, which could lead to inhibitory learning (see also Rescorla, 1967 for a discussion on the adequacy of different control groups). If this was the case, the differences then found between the experimental and the control groups would not solely be due to the excitatory learning experienced by the experimental group but also by the inhibitory learning experienced by the control group. Since a truly random group was not included in any of the experiments, it remains

unknown to what extent the differences found in the experiments here reported are due to either learning process. In any case, both excitatory and inhibitory effects are instances of learning and, thus, the lack of a truly random group does not question the results obtained. Notwithstanding, disentangling the excitatory from the inhibitory effects would allow to subsequently investigate the Pavlovian processes that are involved in the adaptations that can be produced by this type of learning.

Future directions

The work here presented is not exempt from criticism and further research is required in order to improve the experimental procedures and designs employed. As already pointed out, there are several variables that need to be controlled for regarding the experiments reported in Chapter 2, such as the levels of food deprivation and activity of the control groups, as well as the familiarity with the context in which testing took place and the analysis of the tentacle lowering response. In Chapter 3 it was observed that the effects of classical conditioning on food intake and growth varied depending on the developmental stage of the experimental subjects but that the amount of food deprivation also played an important role. Experiments in which the amount of food access is varied are necessary in order to determine under what particular circumstances classical conditioning can be of greater or null significance for *Cornu aspersum.* The large variability observed in the data suggests that larger sample sizes should be employed in future experiments in order to address the effect of classical conditioning on nutrition. Furthermore, an improvement in the measurement of food intake (election of the US) and growth (shell-related variables) would be required. Concerning the experiments conducted in Chapter 4, it will be interesting to explore whether classical conditioning can have an effect on the number or size of the eggs laid by both the target snails (PAV

and CON) and the US snails. Importantly, since *Lymnaea stagnalis* stores sperm from different inseminations, genetic analyses to determine paternity success may be needed. Another interesting research question, in which the same experimental procedure can be used, would be to explore whether the female function can be also enhanced after classical conditioning. Furthermore, as just discussed, assessing to what extent the adaptive benefits of classical conditioning are due to excitatory or to inhibitory learning processes would also constitute an interesting research to be conducted.

Disregarding the particularities of the experimental details, procedures or variables employed in this or other series on the adaptive benefits of classical conditioning in different animal phyla, the analysis of such adaptive benefits is of great relevance for several reasons. Not only are they interesting for the development of a comparative psychology but they are also important for understanding how animals adapt to the environment, a determinant factor in evolution (Baldwin, 1896). As pointed out by West-Eberhard (2003), adaptations can be either individual (i.e., they will die with the organism) or they may be transmitted culturally, but in any case they "can be subject to genetic variation, selection and genetical evolution" (p.338). Longitudinal studies in which classical conditioned fitness measured as the number of surviving and reproducing offspring would allow addressing this question.

Finally, another very relevant aspect of the study of the adaptive benefits of classical conditioning is that it can be used as a research tool for the study of cognition in species with low mobility or poor behavioural repertoires such as for example sponges. In some of the studies here presented and in those conducted by other authors (e.g., Domjan et al., 1998; Hollis et al., 2011), animals do not necessarily show an increase of a conditioned response per se but they do show a better physiological response (e.g., greater sperm release in Domjan et al., 1998; or faster pupation into adulthood in Hollis

et al., 2011). Behavioural changes are traditionally measured in experiments on animal cognition because they are indicative of learning. On the contrary, physiological-related changes are not usually registered as indexes of learning. Measuring for the biological function of classical conditioning (e.g., growth or reproductive success) in species with reduced mobility would allow researchers to explore cognition in animal phyla such as porifera (sponges) or cnidaria (corals) but also in species outside the animal kingdom (e.g., see Gagliano, Vyazovskiy, Borbély, Grimonprez, & Depczynski, 2016 for a study on associative learning in plants).

Capítulo 5- Discusión General

El objetivo de esta tesis fue explorar los beneficios adaptativos del condicionamiento clásico en dos especies de moluscos en tres aspectos diferentes de su ecología conductual. En *Cornu aspersum* se emplearon presentaciones emparejadas de un olor (EC) y comida (EI) para comprobar si los caracoles de esta especie pueden obtener alguna ventaja en términos de localización de la fuente de comida (Capítulo 2) o en términos de ingesta de comida y crecimiento (Capítulo 3). En el cuarto capítulo, el caracol de agua *Lymnaea stagnalis* fue empleado para el análisis de los beneficios adaptativos del condicionamiento clásico en el apareamiento.

Con respecto a la localización de la comida, la investigación realizada se centró en determinar cuáles son algunas de las variables que afectan a la búsqueda de comida como un primer paso necesario para una evaluación futura de los beneficios del condicionamiento clásico en términos de búsqueda de comida. Los resultados encontrados mostraron que la experiencia previa con la comida (Experimento 1) y no la simple exposición al olor de la misma (Experimento 2) es necesaria para que *Cornu aspersum* encuentre una fuente de comida distante. Además, se demostró que los caracoles pueden aprender a buscar un olor que ha sido emparejado con comida cuando la prueba se realiza en ausencia de la misma pero que no se guían por dichos estímulos condicionados cuando la comida sí está presente (Experimento 3). Estos resultados podrían indicar la existencia de dos mecanismos distintos de búsqueda de alimento o que, simplemente, los caracoles guían su conducta de forrajeo por la clave más fiable: cuando el olor de una sustancia comestible ya conocida es detectable, se espera que dicha fuente de comida esté presente y, por lo tanto, la buscan independientemente de la presencia de otros olores o claves. Por el contrario, en una situación en la que hay dos olores presentes

y ninguno de ellos proviene de una fuente de comida conocida, pero uno de ellos ha sido emparejado con comida, la mejor estrategia de forrajeo será la de orientar la búsqueda hacia ese olor. En este sentido, el condicionamiento de bajada de tentáculo proporcionaría a los caracoles una mejor estrategia de forrajeo. Sin embargo, los resultados obtenidos no responden de forma completa a las cuestiones planteadas por Ungless (2001) ya que la respuesta de bajada de tentáculo no fue examinada en los experimentos realizados en el Capítulo 2. Por esta razón, queda por determinar si aquellos caracoles que muestran una mayor respuesta condicionada son más eficaces también a la hora de encontrar señales que indican la presencia de comida.

El mismo procedimiento de bajada de tentáculo fue empleado en el Capítulo 3 para evaluar si el condicionamiento clásico puede aportar otras ventajas distintas a la búsqueda de alimento. Para ello se realizaron cuatro experimentos en los que se midió la ingesta de comida y el peso en caracoles juveniles y adultos jóvenes (*Cornu aspersum*). En juveniles, los resultados obtenidos mostraron que el emparejamiento de un olor con un acceso limitado a la comida dio lugar a una mayor cantidad de alimento ingerido independientemente de la observación de un efecto robusto de la respuesta condicionada de bajada de tentáculo. Este entrenamiento tuvo consecuencias más relevantes en términos de mantenimiento del peso de los animales cuando el tiempo empleado para comer fue menor (2 minutos; Experimento 4A). Para los adultos jóvenes, este mismo procedimiento resultó en una mayor ingesta de comida especialmente al comienzo del entrenamiento cuando el tiempo para comer fue mayor (4 minutos; Experimento 5B), pero en ningún caso se observó un efecto del condicionamiento en el peso (Experimentos 4B y 5B). Los resultados muestran que los beneficios adaptativos del condicionamiento clásico pueden depender del nivel de desarrollo de los animales que se estén estudiando (e.g., juveniles frente a adultos jóvenes; Experimentos 4A y 4B). Además, la

demostración de los beneficios adaptativos del condicionamiento clásico en cuanto a la ingesta o al mantenimiento del peso pueden variar en función de otras variables como, por ejemplo, el nivel de privación (e.g., 2 minutos frente a 4 minutos de acceso a comida; Experimentos 4A y 5A). Por otro lado, se requeriría de una mejora en los procedimientos experimentales para poder realizar una evaluación más profunda de la función biológica del condicionamiento clásico. La mejor estrategia sería realizar experimentos con juveniles en los que se emplee tanto una mayor muestra como una mejora en las medidas de las variables dependientes (e.g., crecimiento o regeneración de la concha).

Finalmente, el Capítulo 4 tenía por objetivo explorar si el condicionamiento clásico podría facilitar el apareamiento en el caracol de agua dulce *Lymnaea stagnalis.* En este conjunto de experimentos, se observó que el aprendizaje puede tanto aumentar la probabilidad de que los caracoles se apareen en primer lugar como donantes de esperma (Experimento 6), como disminuir el tiempo total invertido en el apareamiento (Experimento 7). En el primer caso, el condicionamiento clásico beneficiaría la función sexual masculina al reducir la competición espermática y al incrementar la inversión de la pareja en la puesta de huevos (e.g., Nakadera et al., 2014) mientras que, en el segundo caso, se vería reducido el tiempo para aparearse. El resultado concreto de cada uno de los experimentos dependió de una variable externa a los caracoles que se emplearon como sujetos experimentales (condicionados o controles) dado que la variable manipulada fue el nivel de motivación para aparearse en el rol sexual masculino de los caracoles empleados como EI. Este resultado muestra que el condicionamiento clásico no implica el despliegue de un patrón comportamental fijo, sino que permite a los animales enfrentarse a distintas situaciones de forma flexible.

Tomados en su conjunto, estos experimentos muestran que el condicionamiento clásico tiene una función biológica en dos especies de moluscos, al igual que ocurre en

especies pertenecientes al filo de los cordados o de los artrópodos (e.g., Hollis, 1999; Hollis et al., 2011). Una debilidad general de la investigación que conforma los Capítulos 3 y 4 es el uso de un grupo de control explícitamente desemparejado. Tal y como ya señalaron Karen Hollis (1984; véase también Hollis et al., 1997) y Michael Domjan (Domjan et al., 1998), los animales del grupo control fueron expuestos a una clave que, de hecho, señala la no aparición del estímulo relevante, lo que podría dar lugar a un aprendizaje de tipo inhibitorio (véase también Rescorla, 1967 para una discusión sobre la adecuación de los distintos grupos de control). Si este fuera el caso, las diferencias encontradas entre los grupos experimental y control no se deberían solamente al aprendizaje excitatorio experimentado por el grupo experimental sino también al aprendizaje inhibitorio experimentado por el grupo control. Dado que no se incluyó ningún grupo control verdaderamente aleatorio, queda por esclarecer hasta qué punto las diferencias encontradas en estos experimentos son debidas a un proceso de aprendizaje u otro. En cualquier caso, ambos efectos, excitatorio e inhibitorio, son instancias de aprendizaje y, por lo tanto, la ausencia de un grupo verdaderamente aleatorio no cuestiona los resultados obtenidos. No obstante, un ulterior análisis que posibilite separar ambos efectos permitiría investigar qué procesos del aprendizaje Pavloviano están involucrados en las adaptaciones concretas que pueden ser generadas por este tipo de aprendizaje.

Direcciones futuras

El trabajo aquí presentado no está exento de críticas y requiere de investigaciones futuras que permitan mejorar los procedimientos y los diseños experimentales empleados. Tal y como ya se mencionó anteriormente, los experimentos descritos en el Capítulo 2 deberían incluir el control de una serie de variables como los niveles de privación y de actividad del grupo control, así como la familiaridad con el contexto en el que tiene lugar

la prueba y el análisis de la respuesta de bajada de tentáculo. En el Capítulo 3 se observó que los efectos del condicionamiento clásico en la ingesta de comida y el peso varían en función del grado de desarrollo de los sujetos experimentales pero que el nivel de privación también juega un papel importante. Por este motivo, serían necesarios experimentos en los que se varíe la cantidad de comida para poder determinar bajo qué condiciones particulares el condicionamiento clásico puede ser de gran o de ninguna importancia para *Cornu aspersum.* Para poder analizar el efecto del condicionamiento clásico en la nutrición, los experimentos futuros que se realicen sobre esta cuestión, deberían realizarse con muestras experimentales mayores a las aquí empleadas con el objetivo de reducir la gran variabilidad observada en estos experimentos. Además, se deberían mejorar las medidas de ingesta de comida (elección del EI) y del peso (variables relacionadas con la concha). En cuanto a los experimentos del Capítulo 4, sería interesante explorar si el condicionamiento clásico puede tener un efecto sobre el número o el tamaño de los huevos puestos por los sujetos experimentales (PAV y CON) y por los sujetos empleados como EI. Para poder analizar en detalle esta cuestión, debido a que *Lymnaea stagnalis* es capaz de almacenar el esperma recibido en diferentes inseminaciones, podría ser necesario el uso de técnicas de análisis genéticos que permitan determinar el éxito reproductivo. Otro aspecto a investigar, en el que se podría emplear el mismo procedimiento experimental, sería el estudio del efecto del condicionamiento clásico en la función sexual femenina. De forma adicional, tal y como se discutió anteriormente, la evaluación de hasta qué punto los beneficios adaptativos del condicionamiento clásico son debidos a procesos excitatorios o inhibitorios podría constituir otra línea interesante de investigación.

Independientemente de las particularidades de los detalles experimentales, procedimientos o variables empleadas en esta o en otras series de trabajos que versan

sobre los beneficios adaptativos del condicionamiento clásico en distintos filos del reino animal, el análisis de la función biológica del aprendizaje es de gran relevancia por varios motivos. No son solo interesantes para el desarrollo de una psicología comparada, sino que son importantes para comprender cómo los animales se adaptan a su medio ambiente, un factor determinante dentro de la evolución (Baldwin, 1986). Como menciona West-Eberhard (2003), las adaptaciones pueden ser individuales (i.e., mueren con el organismo) o pueden ser transmitidas culturalmente, pero en cualquier caso "están sujetas a variación genética, selección y evolución genética" (p.338). Una forma de evaluar esta cuestión sería mediante estudios longitudinales en los que se examine el éxito reproductivo o fitness obtenido a través del condicionamiento clásico medido como el número de descendientes que sobreviven y se reproducen.

Finalmente, otro aspecto relevante del estudio de los beneficios adaptativos del condicionamiento clásico es que puede ser usado como una herramienta metodológica para el estudio de la cognición en especies con poca movilidad o con repertorios conductuales pobres como, por ejemplo, las esponjas. En algunos de los estudios aquí presentados y en otros desarrollados por otros autores (e.g., Domjan et al., 1998; Hollis et al., 2011), los animales no muestran necesariamente un aumento de una respuesta condicionada, sino que muestran una mejor respuesta fisiológica (e.g., mayor producción de esperma en Domjan et al., 1998; o una aceleración en la metamorfosis en Hollis et al., 2011). Tradicionalmente, el aprendizaje en animales se mide a través de un cambio en su conducta. En cambio, no se suelen registrar cambios en aspectos fisiológicos como índices de aprendizaje. La medición de la función biológica del condicionamiento clásico (e.g., crecimiento o éxito reproductivo) en especies con baja movilidad permitiría a los investigadores explorar los procesos cognitivos en animales pertenecientes a filos como porifera (esponjas) o cnidaria (corales), o incluso en especies que no pertenecen al reino

animal (véase por ejemplo Gagliano, Vyazovskiy, Borbély, Grimonprez, & Depczynski, 2016 para un estudio sobre el aprendizaje asociativo en plantas).

Conclusions

In summary, the conclusions that can be drawn from the results reported are the following:

1. Subjects of the species *Cornu aspersum* that have experienced a given food are better at finding such food source than naïve snails.

2. Mere exposure to the odour of a food source does not improve locating the food itself.

3. Pairing of an odour (CS) with food (US) in a classical conditioning preparation facilitates locating the CS but not the US.

4. When the time for feeding is limited, using an odour to signal the availability of food increases the amount of food ingested by juvenile *Cornu aspersum* snails.

5. When the amount of time for feeding is limited to 2 minutes per day, classical conditioning results in less weight loss in juvenile snails but not in young adults.

6. Pairing of an odour (CS) with access to a conspecific (US) in a classical conditioning preparation with *Lymnaea stagnalis* increased the likelihood of mating first as sperm donors when there is a conflict over the performance of such sexual role.

7. When there is no conflict over sexual role performance, this type of classical conditioning favours mating by reducing the total amount of time invested.

Conclusiones

En resumen, las conclusiones que se pueden extraer de los resultados descritos son las siguientes:

1. Los sujetos de la especie *Cornu aspersum* que tienen experiencia en la ingesta de comida la encuentran posteriormente mejor que los sujetos naïve.

2. La mera exposición al olor de esa misma comida no mejora la localización de esa fuente de alimento.

3. El emparejamiento de un olor (EC) con comida (EI) en una preparación de condicionamiento clásico facilita la localización del EC, pero no la localización del EI.

4. Cuando el tiempo disponible para comer es limitado, la señalización de la disponibilidad de la comida mediante un olor hace que los caracoles juveniles de la especie *Cornu aspersum* incrementen la cantidad de alimento ingerido.

5. Cuando el tiempo para comer se limita a 2 minutos al día el condicionamiento clásico produce una menor pérdida de peso en los caracoles juveniles, pero no en los adultos jóvenes.

6. El emparejamiento de un olor (EC) con el acceso a un congénere (EI) en una preparación de condicionamiento clásico aumenta la probabilidad de que los caracoles de la especie *Lymnaea stagnalis* se apareen en primer lugar como donantes de esperma en una situación en la que existe un conflicto por la ejecución de dicho rol sexual.

7. Cuando no hay conflicto sobre el rol sexual, este tipo de condicionamiento clásico favorece el apareamiento disminuyendo el tiempo total invertido.

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