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XXXIII CICLO DEL DOTTORATO DI RICERCA IN NEUROSCIENZE E SCIENZE COGNITIVE

THE IMPACT OF BRAIN DEVELOPMENT, CONTEXT CHANGE, INDIVIDUAL DIFFERENCES AND ENTROPY ON ANIMAL HABITUATION

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Abstract

In a world full of information, animals have evolved cognitive mechanisms to ignore irrelevant stimuli and selectively focus on important ones. Among these mechanisms, those controlling habituation allow the suppression of the response elicited by irrelevant stimuli that repeat over time.

To better understand this behavioral and phenomenon, we capitalized on the domestic chick (*Gallus gallus*), whose brain develops almost completely in the egg, which makes this animal mobile and independent from parental cares soon after hatching. In Chapter 2, by testing chicks from the first day after hatching we found that the neural mechanisms underlying habituation are immediately active in chicks, but newborn chicks can habituate to a greater extent than older animals. This shows an early period of increased plasticity in the chick brain that rapidly decreases in the first three days after hatching. Together with habituation, we found that dishabituation is also fully developed in newborn chicks. Comparator theories of habituation predict that both mechanisms stem from a common memory mechanism. Our results represent the first evidence to support this conclusion using a developmental approach.

Previous studies have shown that habituation is context-specific and cannot be entirely transferred to a new context. In Chapter 3, we reviewed the evidence in support of this conclusion and demonstrated that the neural mechanisms underlying context-specific habituation are functional in chicks' embryos and modulate the postnatal recalling of a habituated response acquired prenatally. We found context-specific habituation also in an invertebrate species phylogenetically distant from the domestic chick, the bumblebee (*Bombus*

terrestris). This result adds to the existing evidence that the cognitive mechanisms underlying context-specific habituation are widespread in the animal reign, irrespective of the complexity of the nervous systems considered. Before exploring context-specific habituation in bumblebees, we developed a new paradigm to study habituation and dishabituation of the defensive behaviour in this species.

In Chapter 4, by testing several behavioural traits of inexperienced chicks and measuring their body size, we found that more exploratory chicks have greater variability of habituation rate than less exploratory ones. This variability is linked to the body size of the more exploratory chicks. Bigger chicks are more responsive to a loud sound but habituate to a greater extent than smaller ones. Since in our experiments chicks were born and reared in a controlled environment, our results suggest that an innate biological factor may be involved in the covariation between exploration, body size and habituation.

We studied the generalization capacity of the invasive crayfish *Procambarus clarkii*. By using a habituation-dishabituation paradigm we found that crayfish can transfer a habituated response to a new stimulus. Generalization takes place after a single trial in which the new stimulus is presented following the habituation one and last for at least 45 days. Generalization may contribute to the superior behavioural flexibility of this invasive crayfish compared to native species.

Finally, in Chapter 5 we developed a new model of rate-sensitivity of habituation. This model accounts for the information conveyed by the temporal sequence of stimulus repetitions exploiting the notion of entropy. We found that the amount of habituation of the freezing response elicited by a loud sound decreases as a function of the entropy apported by a new stimulus repetition.

Overall, our findings support comparator theories of habituation and provide strong evidence in support of the associative nature of habituation.

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Chapter 1

General introduction

Focusing limited cognitive resources on relevant stimuli requires that organisms attend to some stimuli while ignoring others. There are several strategies by which organisms filter out irrelevant stimuli (for a review, see Chelazzi et al., 2019), but perhaps the most straightforward way is reducing the response elicited by a repetitive stimulus. Such a decremental phenomenon can be an instance of habituation, that represents a form of experience-dependent plasticity, widely preserved across different animal species. It reflects a set of cognitive processes involving learning, memory, and prediction that allows an organism to disengage from repetitive sensory input (Ramaswami, 2014; Thompson, 2009). This is present in many domains of our everyday life. Habituation affects our response to irrelevant distractors (Turatto & Pascucci, 2016) as well as the preference for motivational stimuli like food (Epstein et al., 2009) and the choice of a sexual partner (Daniel et al., 2019). Habituation is also involved in the development of addiction and affects several aspects of drug tolerance (Siegel, 1977) as well as drug relapse (Lloyd et al., 2014).

Habituation can affect a wide range of behaviours so the number of studies directly or indirectly concerning habituation has increased since the sixties (see, Figure 1). In addition, the test for habituation is very simple: it is sufficient to present the same stimulus several times and record specific aspects of the subject's response to that stimulus. Alternative decremental phenomena - like motor fatigue or sensory adaptation - must be excluded. To distinguish between these alternatives, it is sufficient to record the subject's response to a different stimulus. If a new stimulus restores the response, then it can be assumed that the decrease in response was not due to a general lack of energy or fatigue. This test is also known as the stimulus specificity/stimulus generalization test (Rankin et al., 2009). The new stimulus may increase the subjects' response to a new repetition of the habituated stimulus. This phenomenon is known as dishabituation. It excludes the fact that mere adaptation of specific receptors is involved. Hence, an effective paradigm to study habituation must include two different stimuli: one for the habituating response, the other to dishabituate that response.

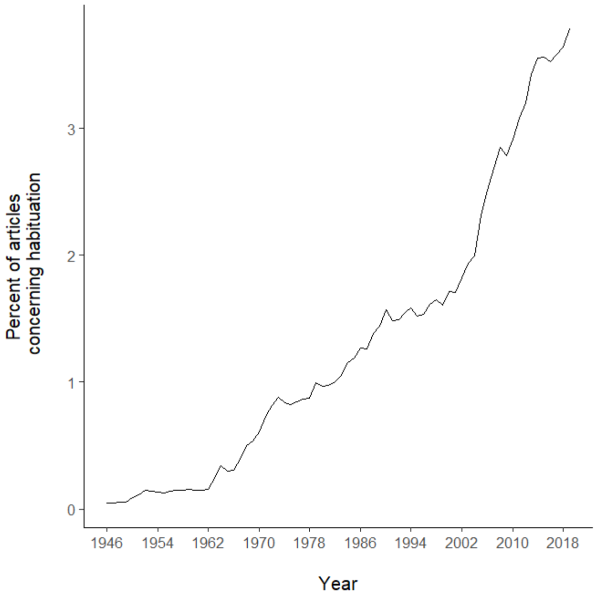

Figure 1. Percent of papers concerning habituation by year. The percent is computed out of the total number of papers concerning habituation on PubMed. The interest on habituation has been increasing since the sixties. This also reflects the versatility of habituation that can be used as a tool to study other cognitive processes.

1.1 Defining properties of habituation

Stimuli, responses, and organisms may be quite different, but the characteristics associated with habituation are remarkably similar across phylogeny. Thompson and Spencer (1966) first described nine defining properties of habituation, mostly based on their study of spinal reflexes in cats. A broader group of scientists in 2009 (Rankin et al., 2009) updated the list of characteristics to accommodate the increasing number of new responses and preparations that have been used to study habituation since 1966. The most relevant updates were a clearer definition of the generalization test to distinguish between habituation and motor fatigue, and the inclusion of a tenth characteristic that distinguishes between a short- and the long-term form of habituation. For this thesis, I have reported only five characteristics that will be addressed in the following chapters but see Appendix I for the full list.

> **#2.** If the stimulus is omitted after the response decrement, the response recovers at least partially when the stimulus is reintroduced (spontaneous recovery). This characteristic acknowledges that when the habituating stimulus is withheld, the habituating response spontaneously recovers completely or in part within the time frame considered.
>
> **#4.** Other things being equal, the higher the frequency of stimulation, the more rapid and/or more pronounced is the response decrement, and the more rapid is the spontaneous recovery (frequency-dependent habituation). This characteristic concerns the rate sensitivity of habituation, namely the fact that more frequent stimulation results in more rapid and/or more pronounced response decrement, and more rapid spontaneous recovery.
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> **#7.** Within the same stimulus modality, the response decrement shows some stimulus specificity. To test for stimulus specificity/stimulus generalization, a different stimulus is presented, and a comparison is made between the changes in

the responses to the habituated stimulus and the new stimulus. This point describes the stimulus specificity/stimulus generalization test that distinguishes between the decremental effect resulting from habituation and motor fatigue.

#8. The presentation of a different stimulus results in an increase of the habituated response to the original stimulus (dishabituation). This characteristic can then rule out other alternative explanations of the response decrement, specifically an adaptation process of the receptors that are under repeated stimulation.

#10. Some stimulus repetition protocols may result in a response decrement that lasts hours, days or weeks (long-term habituation), introducing the possibility that some protocols may produce a long-lasting form of response decrement. But long-term habituation involves different biological processes than short-term habituation (Carew et al., 1972).

1.2 Models of habituation

Theories of habituation can be divided into two general types: comparator theories and non-comparator theories. Comparator theories including those of Sokolov (1960, 1963), Wagner (1976, 1978, 1981) and Hall and Rodriguez (2017, 2020) postulate that the nervous system builds a model of the habituating stimulus in the memory system that is compared to the afferent stimulation. The magnitude of the response decrement is directly related to the degree of disparity between the internal model and incoming information. Non-comparator theories, like the dual-process theory of Thompson and colleagues (Groves & Thompson, 1970; Thompson & Spencer, 1966), do not assume any specific memory model of previous stimulation. Rather they suggest that habituation follows the interaction of two independent processes: the decrement of the activity of the habituating stimulus-response pathway (i.e., habituation) and the increased general activity of the organism (i.e., sensitization).

1.2.1 The "Stimulus-Model Comparator" theory

Sokolov developed this model based on his habituation experiments of the orienting reflex (OR), an ensemble of physiological and behavioural response that allows animals to focus their attention on target stimuli (see also Pavlov, 1927). The core notion of this theory is that the brain forms a memory model of a repetitive stimulus. If the model matches the stimulus characteristics, an inhibitory signal is sent to the response system that controls the OR. The system in turn decreases the intensity of the OR, preventing future repetitions of the same stimulus from eliciting an OR again. The memory model proposed by Sokolov encodes all the stimulus dimensions including intensity, duration, sensory modality, spatial location, and timing, and can account for all the characteristics of habituation.

The stimulus model is not static, rather it is constantly updated to accommodate new stimulus changes. The dynamic updating of the stimulus model is central to classical computational theories of learning (Kamin, 1969; Pearce & Hall, 1980; Rescorla & Wagner, 1972) and recent Bayesian models of habituation (Itti & Baldi, 2009; Sekoguchi et al., 2019; Ueda et al., 2021). These models assume that habituation results from a decrement in information gain brought about by the repetition of a stimulus. Information gain is a measure of the divergence between the prior model of the stimulus, and the incoming sensory information, namely the core mechanism of Sokolov's stimulus-model comparator (1969).

1.2.2 The "Sometimes Opposite Processes" theory

Developed by Wagner and colleagues (Wagner, 1981) under the framework of the general Rescorla-Wagner model of conditioning (Rescorla & Wagner, 1972), this theory is based on an associative structure of memory, where stimuli are represented by nodes connected by excitatory or inhibitory links. The different combinations by which the links activate or inhibit the nodes within the memory system can explain a wide range of phenomena predicted by the Rescorla-Wagner model. This theory offers a broader framework for understanding habituation than Sokolov's model while maintaining the comparison between sensory input and the stimulus memory model as the main outcome of the memory mechanisms leading to habituation.

The core of this theory illustrates the response elicited by a new stimulus decreases if the representation of the stimulus is already active (or primed) in short-term memory (STM). This happens both when a stimulus is repeated at closely spaced intervals (i.e., self-generated priming), or when a cue associatively retrieves the stimulus representation from long-term memory (LTM, i.e., retrieval-generated priming). Wagner hypothesized that representations in STM can be in different states of activity. When a new stimulus enters STM in a primary state of activity (A1), a stimulus is processed with higher priority and is more likely to elicit a response. However, its representation decays rapidly from this primary state to a secondary state of activity (A2). The representation, now active in A2 and the new repetition of that stimulus can no longer enter the A1 state (i.e., self-generated priming), preventing the stimulus from eliciting a response again (i.e., short-term habituation). Wagner proposed a second mechanism by which representations can enter A2. He suggested that within the memory network, a node can spread its activation to an associated node, priming the activation of the corresponding representation in A2 state (i.e., retrieval-generated priming). Such an associative mechanism would form the basis for long-term habituation.

This associative account of long-term habituation has been extensively studied in relation to context-specific habituation. It is generally assumed that the repetition of the habituating stimulus in a specific context results in the conditioning of the contextual cues to the stimulus itself. That is, long-term context-specific habituation takes place because contextual cues prime the stimulus representation in STM. This associative mechanism can explain other learning phenomena related to habituation, such as the conditioned diminution of the unconditioned response (UR) (Kimble & Ost, 1961; Kimmel, 1966). It consists of a response decrement to an unconditioned stimulus (US) when it is anticipated by a conditioned stimulus (CS) that signal its occurrence, compared to the case in which US is unsignaled. The conditioned diminution of the UR is predicted by Wagner's theory since the CS can associatively prime the US in STM, reducing its effectiveness. Specific preparations have revealed more complexities. When a CS elicits the same emotional response as the US, the UR may be facilitated by the superimposition of the similar CR, called "conditioned emotional response". A classic example is fear-potentiated startle (Davis, 2006), where a CS is first conditioned to an aversive response and then paired with an equally aversive US eliciting startle. The US induces a stronger startle reaction than in the absence of the CS. This phenomenon challenges the core assumption of the SOP that the effectiveness of a stimulus wanes as it becomes fully predicted by preceding cues. The SOP does not consider the possibility that emotional representations of stimuli may modulate the response. To fill this gap, Wagner and Brandon (1989) created an extended version of the SOP model. The new model AESOP (see also, Vogel et al., 2019; Wagner & Vogel, 2010)preserved the core associative nature of the SOP but assumed that stimuli are represented by separate sensory and emotive nodes and that both can directly or associatively enter an A1/A2 state. Critically, the activity of the emotional representations in A2 can increase the activity of the sensory nodes. The SOP and AESOP models will be more extensively discussed in Chapter 3.

1.2.3 The "Habituation as a No learning event" theory

Hall and Rodriguez's theory (2017, 2020) is based on the notion that habituation arises because the habituating stimulus is not followed by any significant event, creating the preconditions for extinction of the response. Moving from the Pearce-Hall model of conditioning (Pearce & Hall, 1980) this theory assumes that a new stimulus induces an expectation that an event may follow. This expectation initially increases the salience of the stimulus, which will come to evoke a response from the organism. However, when a stimulus is repeatedly presented alone, an inhibitory learning process (extinction) reduces its effective salience producing habituation of the organism's response. This inhibitory learning process would account for other forms of learning based on stimulus repetition. Zajonc proposed a similar inhibitory account of the mere exposure effect (Zajonc, 2001) in which participants are exposed to a set of stimuli and the experimenter records the developing preference for those more frequently repeated. In Zajonc's research, the participants expected that something would follow the stimulus. This uncertainty then induced aversive reactions in the participants. The aversion is extinguished as participants observed that the stimuli are repeated without any consequence. Both theories capitalize on a similar inhibitor process to explain two opposite phenomena. Mere exposure and habituation share similar behavioural characteristics. For example, both are stimulus-specific and rate-sensitive (Montoya et al., 2017). To date, habituation and mere exposure have never been studied using the same preparation, but past theories give rise to the interesting possibility that the two may coexist.

Regarding habituation, Hall and Rodriguez' theory (2017, 2020) explains context-specific habituation assuming that contextual cues act as an occasion-setter that facilitate the association between a conditioned stimulus (CS) and an unconditioned one (US). It claims that the habituating response recovers when contextual cues change and that this is the result of an increment of both the associability and salience of the habituating stimulus elicited by a new context. That is, they counterbalance the inhibitory process of habituation. This model is less comfortable in explaining other context effects. For example, the repeated presentation of the context without habituating stimulus would weaken the associative strength between the two representations (extinction), causing a spontaneous recovery of the response when the habituating stimulus is presented again in the same context. But the mere exposure to context alone is not expected to decrease its occasion-setting properties (Fraser & Holland, 2019).

In dealing with conditioned diminution/potentiation of a response, this model provides a complementary explanation of the diminution of a CR that parallels the UR during conditioning. It assumes that a CS stimulus may lose its effective salience as it predicts the upcoming event and decreases its effectiveness in eliciting a CR. To support this conclusion Hall and Rodriguez reported a study by Honey and colleagues (Hall & Rodríguez, 2020). A group of rats was trained with a sequence of stimuli AX-food or BY-food and then tested with a BX-food and AY-food. The appetitive response to food evoked by X and Y was greater when the untrained sequences (BX-food and AY-food) were used. Both X and Y reliably predicted food in both conditions. The effectiveness of a stimulus was reduced when it predicted the consequence. This response facilitation can only be explained in terms of the increased salience of A and B.

1.2.4 The "Dual-process theory"

The dual-process theory (Groves & Thompson, 1970) took a different approach to response habituation. Rather than assuming that responses were elicited by a mismatch between a memory model and the information available at the time, it assumed that responses were a result of the combination of two processes, one decremental/habituation and one incremental/sensitisation, that developed independently in the central nervous system. Thompson and Groves (1970) suggested that habituation arose because the stimulus repetition activated several synaptic mechanisms that decreased the excitability of the stimulus-response pathway involved in the stimulation. Sensitization stems from enhanced excitatory transmission in multiple synapses that increased the general state of activity of the organism.

The behavioural outcome of the two processes depends on the frequency and the intensity of the repetitive stimulus. Response sensitization is initially promoted by high-intensity stimuli but then decays at a rate that is inversely proportional to the frequency of stimulation. Response habituation occurs from weak stimuli and is directly proportional to the frequency of stimulation. When the stimulus is withheld, the inhibitory processes of habituation decay and the sensitization processes restore the response (i.e., spontaneous recovery). The response can be equally increased when a new stimulus raises the general level of excitation of the animal. The resulting superimposed sensitisation process decays at different time rates according to the intensity of the stimulus, with stronger stimuli producing a greater amount of sensitisation. Therefore, due to a carry-over effect, sensitisation spreads to the following repetition of the habituating stimulus, resulting in dishabituation.

This model is based on data from several physiological responses including spinal reflexes. Therefore, it provides an accurate description of the neurobiological underpins of habituation for basic reflexes. There is strong neurobiological evidence supporting the existence of both habituation and sensitisation at a neuronal level in both vertebrates and invertebrates (e.g., Castellucci & Kandel, 1974; Pinsker et al., 1970). However, its explanatory power is very limited when it comes to explaining more sophisticated phenomena linked to habituation such as the context effects and conditioned diminution of the UR.

1.3 Neural bases of habituation

Habituation observed in a single-celled protozoan (Wyers et al., 1973) cannot be sustained by the same biological substrates as that underlying the habituation of food preference in humans (Epstein et al., 2009). Multiple sites of plasticity are involved according to the specific behavioural system (McDiarmid et al., 2019).

The first description of the molecular mechanisms of habituation was made by Kandel and colleagues on the sea snail *Aplysia* (e.g., Castellucci & Kandel, 1974). They observed that the stimulation of the siphon from a water jet elicited a strong retraction of both the gill and the siphon due to the L7 motoneuron (i.e., the gill-withdrawal reflex, GWR). This reflex progressively waned as the stimulations continued. Kandel and colleagues demonstrated that a homosynaptic depression occurred of excitatory neurotransmission induced by desensitization of the channels controlling the calcium (Ca+). Subsequent biological studies questioned the possibility that habituation was due to a depression in the sensory axon terminals alone, suggesting instead a network-level potentiation of the inhibitory neurotransmission (e.g., Bristol & Carew, 2005).

The discovery of cellular mechanisms of habituation has facilitated the development of computational models of conditioning. For example, Hawkins and Kandel (1984) hypothesized that conditioning is mechanistically related to basic cellular mechanisms like synaptic depression and facilitation. The same mechanisms also underlie habituation and sensitization. Their intuition was correct. Indeed, a series of simulation studies demonstrated that, by combining few basic cellular mechanisms, it is possible to explain a number of high-order features of the conditioning of the gill-withdrawal reflex in *Aplysia,* like blocking and latent inhibition (Gluck & Thompson, 1987; Hawkins, 1989). These studies indicate that the sophisticated neural machinery of conditioning may have evolved from the combination of computationally simpler cellular processes. Advances in the neurological understanding of conditioning have in turn served to support cognitive models of habituation, like the SOP. Wagner and Donegan (1989) found a plausible set of correspondences between SOP and the neural eyeblink conditioning circuit in rabbits (*Oryctolagus cuniculus*). The correspondence involved broader neuroanatomical structures than those considered by Hawkins and Kandel (1984). For example, the initial processing of a stimulus (the primary activity state, A1), is activated by the input fibers ascending from the brainstem to the cerebellum. Here, a secondary processing of the stimulus (the secondary activity state, A2) inhibits the ascending A1 pathway, via descending fibers from the cerebellum. The inhibition produces a refractory-like mechanism that initiates the habituation process.

More recently, the neural bases of olfactory habituation has been incorporated into the computational model by Ramaswami (2014) in *Drosophila Melanogaster.* Ramaswami and colleagues (2014) found that an ensemble of afferent olfactory neurons in the antennal lobe of *D. Melanogaster* was involved in olfactory habituation. The recurrent inhibition of the afferent neurons that encodes a repetitive external odour created an inhibitory “negative” image of the original odour. The negative image dampened the transmission of the odour-evoked activity in the brain areas, reducing the elicited response. The negative image represents an instance of a memory model assumed by the stimulus-comparator models of habituation (Sokolov, 1960) that nervous systems form during repetitive exposure to the same stimulus to filter the incoming information. The negative image may have also represented the biological mechanism underlying the cognitive priming described by Wagner (1976), suggesting a link between cognitive and biological theories of habituation.

An innovative application of machine vision together with genotypic analysis of the flat worm *Caenorhabditis Elegans* and the zebrafish *Denio Rerio* has provided the possibility to quantitatively measure online behaviours of a considerable number of animals at one time. This has allowed researchers to identify genetic mutations resulting in different behavioural phenotypes, advancing our understanding of the genetic bases of habituation (Ardiel et al., 2017; McDiarmid et al., 2019) and their link with neurodevelopmental disorders (McDiarmid et al., 2017).

Machine vision together with genotypic analysis allows also to investigate the possibility that different aspect of a habituating response may involve different biological mechanisms (McDiarmid et al., 2019). This hypothesis was initially proposed by Broster and Rankin (Rankin & Broster, 1992) based on the observation that habituation of the withdrawal response elicited by a mechanical tap in *C. Elegans* was faster following training with shorter ISIs but also that spontaneous recovery from habituation was more rapid following training with shorter ISIs. Recent evidence (Ardiel et al., 2018) suggests that different molecules involved in calcium regulation (c*mk-1* and *ogt-1*) promote habituation to a mechanical tap at 60s ISI, while inhibiting habituation at 10s intervals. The story is complicated since *amk-1* affects primarily the response speed, while *ogt-1* its duration. Randlett et al. (2019) found that eight different components of the escape of zebrafish to a flashing light habituated at different rates and to different extents. This suggests that a different aspect of the habituating response can be dissociable in biological mechanisms.

An interesting question for future research is why having multiple mechanisms to control habituation is an advantage. This question remains open to several possibilities. It may provide animals with different behavioural strategies to cope with a stimulus before learning to ignore it. That is, may promote behavioural differentiation within a species, increasing adaption to new stimulus contingencies.

1.4 Our contribution to the state of art

1.4.1 The domestic chick as a new animal model for habituation

Habituation has been a valuable tool for developmental psychologists to investigate a wide range of cognitive processes in pre-verbal infants (Colombo & Mitchell, 2010). To understand the development of more complex learning and cognition, it is important to study the change in habituation mechanisms across the lifespan. Studying the development of habituation can be easier in laboratory animals that are born and grow under experimental control than in human infants. Researchers have classically relied on altricial species as their model. The nervous system of altricial species is quite immature at birth and fully develops after time. As a result, researchers must study habituation by changing their paradigm according to the various stages and pace of development of each altricial animal model. This hinders the replicability of the same testing conditions across life, as aspects of the response or the sensory system develop later in life, changing the habituation outcome.

We capitalized on a precocial avian species, the domestic chick (*Gallus gallus*). The brain of young chicks develops almost completely in the egg and chicks are mobile and independent from parental cares after hatching. The chicks can be tested from the first day of life, recording a wide range of responses that are comparable with more mature individuals (Chiandetti & Vallortigara, 2008). They are equipped with a fast motor and sensory development and have been a valuable animal model to study early learning.

A paradigmatic example is the phenomenon of imprinting, namely their ability to learn the characteristics of their proximate social companions (Horn, 1981). This ability declines in the first week of life suggesting the presence of a critical period in which the brain is more sensitive to the specific properties of the stimulation that modulate imprinting, like biological motion (Vallortigara et al., 2005), agency (Mascalzoni et al., 2010), or consonant intervals (Chiandetti & Vallortigara, 2011). This line of research has revealed the pivotal role of memory in the early stages of the chick's life and several studies capitalized on the chicks to study the neural base of memory consolidation. An example would be the one-trial passive avoidance learning tasks (Gibbs et al., 2008).

In my PhD project, the previous knowledge about the early learning capacity of domestic chicks has been extended to habituation and dishabituation. The benefit for developmental research is double: first, to further explore cognitive processes, as developmental psychologists do in infants; and second, to investigate the broader development of the memory system itself. An example would be the development of the STM memory capacity that supports habituation (Davis, 1970).

1.4.2 Developmental study of habituation

Our research provides the habituation community with a new animal model to study habituation and dishabituation mechanisms in developing organisms. Chapter 2 contains a literature overview of developmental studies of habituation in non-human animals. The results of the studies are discussed in the frame of Wagner's priming theory (1976) as it offers a broad view of the cognitive processes affecting the development of habituation, specifically the maturation of STM and LTM. The section also includes two experiments regarding the development of short- and long-term-habituation/dishabituation in domestic chicks. These two studies present a new and reliable paradigm to study habituation that we adapted to test a wider range of related phenomena in the following chapters. These studies provide new evidence that habituation and dishabituation arise from a common STM mechanism.

1.4.3 Associative nature of habituation

A recurrent topic in the literature on habituation is its associative nature. Since this possibility is explicitly predicted by Wagner (1976, 1978, 1979), an interesting question arises: how does the associative mechanism underlying habituation develop? In Chapter 3, we review the evidence to support that habituation is associative in nature. Previous research demonstrated that habituation of the freezing response in 3- and 4-day-old chicks is context-specific (Chiandetti & Turatto, 2017). Chicks' nervous system is essentially fully developed on the hatching day. We tested the hypothesis that context-specific habituation occurs for stimuli experienced in the prenatal context (i.e., the egg) and modulates the transfer of prenatal habituation to the post-natal context.

Context-specific habituation is perhaps the most tested of the predictions stemming from Wagner's (1976). A fruitful approach to test this theory would be an experiment involving the manipulation of discrete contextual cues that animals can then learn to predict the habituating stimulus. As foreseen by Wagner, the response to the habituated stimulus should decrease as it comes to be fully predicted by the cue, but it should recover in the absence of that predictor. This simple prediction is complicated when contextual stimuli elicit an emotional response that opposes the response that the habituating stimulus is trying to evoke. Contextual cues may modulate both the acquisition of habituation and its retrieval in the long term, as predicted by AESOP model (Brandon & Wagner, 1989)

Experiment 4 addresses this experimental topic in bumble bees (*Bombus terrestris*). This Hymenoptera has been extensively studied to understand the neural basis of associative learning in the case of simple reflexes such as the proboscis extension. But so far, no paradigm is available to study the complex antipredator behaviour in habituation. We have filled this gap and established a novel habituation-dishabituation paradigm for bees.

1.4.4 Individual and species-specific differences in habituation

A largely unexplored topic is how the outcome from habituation changes for each individual. Habituation reflects the presence of filtering mechanisms in prioritizing incoming information. Differences in these mechanisms can shape early interactions with the perinatal environment and influence future behaviour. Experiment 5 in Chapter 4 explores the presence of individual differences in habituation in newborn chicks with a limited experience of the external world. Compared to classical models that focus on stimulus "novelty" as the main determinant of habituation, we show that several innate behavioural and morphological traits are associated with the rate at which newborn chicks learn to ignore stimuli. We also studied the invasive crayfish *Procambarus Clarkii* to demonstrate for the first time a long-lasting form of generalization of the habituated response following a single trial event. This extraordinary capacity of *P. Clarkii* may reflect a form of plasticity underlying the invasive success of this species. These two experiments will emphasize that habituation has important fitness-related consequences for animals.

1.4.5 Rate sensitivity of habituation

Experiment 7 in Chapter 5 presents a new approach to quantify the information conveyed by the temporal sequence of stimulus repetitions based on Shannon's entropy notion. As suggested by Sokolov (1969), the model of the habituating stimulus contains a description of all its characteristics, including the temporal information. The repetition rate of a stimulus affects the habituation outcome as described by characteristics number 7 concerning rate sensitivity of habituation. Other models have been proposed to explain rate sensitivity (Staddon & Higa, 1996), but our entropy-based approach has the advantage to explain this characteristic within a wider information theory of learning and memory (Shannon, 1997). We adopted a top-down approach to validate this model in inexperienced new-born of domestic chicks to test the hypothesis that the neural mechanism involved is part of the innate endowment of this species. Our approach may be useful in investigating the biological and behavioural correlates of rate sensitivity in different species.

Chapter 2

The development of habituation in domestic chicks (*Gallus gallus*)

The material in this chapter has been adapted from the following papers:

Chiandetti, C., **Dissegna, A.**, & Turatto, M. (2018). Rapid plasticity attenuation soon after birth revealed by habituation in newborn chicks. *Developmental psychobiology*, 60(*4*), 440-448.

Dissegna, A., Turatto, M., & Chiandetti, C. (2018). Short-term memory in habituation and dishabituation of newborn chicks' freezing response. *Journal of Experimental Psychology: Animal Learning and Cognition*, 44(*4*), 441.

Habituation is important because it represents a gating mechanism for rejecting repetitive information (Ramaswami, 2014), and represents a precondition for a wide range of cognitive processes (Schmid et al., 2015). It has been studied in relation to children's cognition and learning (Colombo & Mitchell, 2010) because a disruption in habituation has been linked to several neurodevelopmental (Lloyd et al., 2014; McDiarmid et al., 2017) and psychiatric disorders (e.g., Ludewig et al., 2003). Habituation is an early form of learning that emerges prenatally in infants (Hepper et al., 2013). Studying its development can help clinicians identify early predictors of dysfunctional developmental trajectories. Studying the development of habituation in humans can be difficult due to the lack of appropriate control on the many factors affecting children's development. A comparative approach can overcome these limitations; several methodological aspects should be considered. Habituation depends on the maturation of the intrinsic stimulus-response pathway. Habituation may be both present and absent from a developing organism based on the behaviour recorded. A clear example comes from the developmental dissociation between habituation of basic reflexes, that appear early in life, and complex behaviours, like exploratory activity, that take more time to develop. For example, juvenile rats (15days old) habituate as mature rats (36days old) when tested for habituation of the startle reflex but take more time to decrease their exploratory activity in a new context (Williams et al., 1975). Rubel and Rosenthal (1975) found that chicks of *Gallus gallus* of 1 and 3-4 days old decreased their eye-opening reflex elicited by a repetitive sound to the same extent, while Zolman, Sahley, and Mattingly (Zolman et al., 1978) found that 1-day-old chicks did not decrease their exploratory activity as did 4-day-old chicks.

The developmental dissociation of habituation of simple and complex behaviour is more relevant in altricial species (like mammals) than in precocial species (e.g., the chick). Young altricial species take some time to develop complex behaviour like locomotion, while young precocial species are mobile from the day of hatching. Younger chicks take longer to suppress their exploratory activity; this may be due to factors other than the development of the motor system itself. Zolman et al. (1978) found that 1-day-old chicks could habituate to the new environment as did older individuals but required more time in a new context. This reveals that animals' neophobia, namely the fear reaction to novel objects or environments, can mask the presence of habituation by increasing the general arousal of an organism. Habituation is inversely related to the arousal level (Mackintosh, 1987). It is crucial that young animals have enough time to habituate to the context in which the stimulation occurs before tests regarding the effects of neophobia.

An organism's reactivity to new stimuli decreases with age. This finding has been further supported by Rattan and Peretz (1981) in *Aplysia*. They compared the habituation rate of the gill-withdrawal reflex (GWR) elicited by a tactile stimulation between mature (120-day old) and old (190 to 200-day old) sea snails. The results revealed that older snails are less sensitive to the stimulation and suppress their response quicker than younger snails. This result shows that behavioural plasticity of animals, modifying behaviour to accommodate new environments changes across the lifespan. Old sea snails were less responsive to the stimulation from the beginning; they stopped decreasing the response before the youngest group. This group was less “plastic” in adapting its behaviour.

While behavioural plasticity may decrease during the lifespan, other cognitive mechanisms underlying habituation may improve with age. Rankin and Carew (Rankin & Carew, 1987) found a systematic relationship between age and the ability of *Aplysia* to habituate. Progressively older animals could habituate to stimuli presented at progressively longer intervals. The authors proposed that this progression could be due to the development of STM, necessary in rehearsing the stimulus representation in the absence of sensory information. If the stimulus is not rehearsed, then its memory representation will rapidly decay and make the organism responsive again to future repetitions of the stimulus.

The same authors emphasized the theoretical benefit of a developmental approach to the study of habituation. They argued that learning processes during different stages of development can be related to separate neural mechanisms. It is possible to adopt this approach to test habituation, dishabituation and sensitization’s dependence on memory process development. Rankin and Carew (1987, 1988) found that habituation of the GWR in *Aplysia* emerges a few days before dishabituation while sensitization develops several weeks later. They concluded that habituation and dishabituation may involve the same STM mechanisms as predicted by Wagner’s (1976). Contrary to the Dual-process theory (Groves & Thompson, 1970), dishabituation does not coincide with sensitization. They could not demonstrate that dishabituation and habituation can be concurrently present at the same developmental stage.

Several questions remain unanswered. If habituation depends on the development of specific behavioural systems, it is important to capitalize on animal models that allow us to reliably compare the same response across the entire lifespan. The ontogenetic dissociations in habituation that have been reported may reflect the slow development of altricial species compared to precocial species, rather than different habituation mechanisms. Whether habituation and dishabituation arise from a common memory mechanism is unclear. In the following two experiments we adopted a developmental approach to explore how habituation develops in a precocial species like the domestic chick.

2.1 Experiment 1

In this experiment, we compared the freezing response elicited by a loud sound in 1-, 2- and 3-day old chicks to study the development of short-term and long-term habituation.

2.1.1 Subjects

We tested 54 (27 males) newly hatched domestic chicks (*Gallus gallus*) of the Ross 308 (Aviagen) broiler strain. The fertilized eggs were supplied by a local commercial hatchery (Agricola Berica s.c.r.l., Montegalda, Vicenza, Italy) and housed in incubators under controlled temperature (37.7 °C) and humidity (about 50–60%) conditions. Chicks hatched in individual compartments made of an opaque plastic material (*polionda*®, 10 cm^3) marked with a number. The hatching moment was recorded by a camera set inside the incubator to date chicks' hatching hour.

2.1.2 Apparatus

The chicks were tested in a running wheel (30 cm in diameter) placed on the rear end of a black rectangular arena (45 × 50 × 160 cm, width, height, depth) as depicted in Figure 2. Two loudspeakers were positioned on the top of the running wheel, at about 30 cm from the chick's head. A red cylinder (measuring 6 × 7.5 cm, diameter, height) hanging from above in front of the wheel was used as the imprinting object so that chicks were motivated to run toward it. The red cylinder was the first conspicuous item experienced by the chicks. It represented an imprinting object that chicks tried to reach by running in the wheel (McCabe, 2013). The mean luminance of the room was 145 cd/m^2. The time and distance of running in the wheel were computed by *Arduino* circuit and displayed on a monitor. Both the running-wheel and the monitor were recorded by a video camera from above the arena.

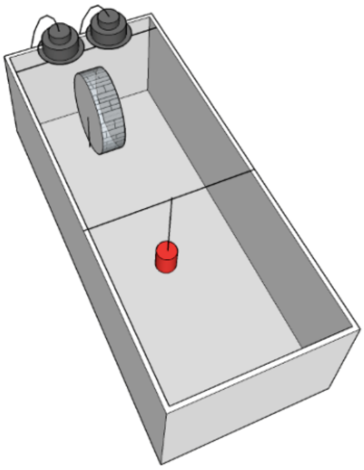


Figure 2. The layout of the experimental apparatus. The imprinting object hangs from above in front of the running-wheel. The chick, attempting to approach the red object, causes the wheel to spin. The acoustic stimuli are administered *via* two loudspeakers placed above the running-wheel.

2.1.3 Procedure

On the hatching day, chicks were randomly assigned to one of the following three groups defined by their Age during the first testing day: Day 1–2 (n = 18) included chicks tested during the 1st and 2nd day after hatching; Day 2–3 (n = 18) consisted of chicks tested during the 2nd and 3rd day after hatching; Day 3–4 (n = 18) comprised chicks tested during the 3rd and 4th day after hatching. Until the test, chicks were kept in darkness within the incubators at a constant temperature of 31 °C. Each chick was tested individually for habituation on two consecutive days (session 1 and session 2) and the stimulation consisted of 2 acoustic sequences each Day (sequence 1 and sequence 2 in session 1; sequence 3 and sequence 4 in session 2), 1 hr apart. The acoustic sequences consisted of five bursts of white noise, each of 250 ms duration, 90 dB, with a semi-random inter-stimulus interval ranged from 30 s to 60 s to reduce the possibility of observing complete habituation after the first day of stimulation in case of a regular presentation. The stimulation was manually started by the experimenter once the chick ran for a minimum distance of 1000 cm to reach the red cylinder. This took approximately 8 min (see Chiandetti & Turatto, 2017 for the same procedure). During this period, chicks were familiarized with the new testing context. Habituation was defined as the waning in the freezing response to the repetitive burst of noise. The number and duration of the freezing response in each trial were scored.

2.1.4 Results

Proportion of freezing

A mixed ANOVA with one within-subject factor (Sequence, with four levels: 1, 2, 3, and 4) and one between-subjects factor (Age, with three levels: Day 1–2, Day 2–3, ad Day 3–4) revealed a significant main effect of Sequence $F(3, 51) = 176.50$, $p < .001$, $\eta_p^2 = .78$, indicating that the chicks' freezing response decreased with training. The overall proportion of stops duringngning did not change with Age $F\,(2, 51) = 0.61$, $p = 0.55$, whereas the Sequence X Age interaction was significant $F(6, 51) = 3.08$, $p = .01$, $\eta_p^2 = 0.11$ (Figure 3, panel A). To qualify the nature of this interaction we ran separate analyses for the 2 days of stimulation, session 1 (sequences 1 and 2) and session 2 (sequences 3 and 4). In session 1, the ANOVA showed a significant effect of Sequence $F(1, 51) = 63.40$, $p < .001$, $\eta_p^2 = 0.55$, and Age $F(2, 51) = 3.52$, $p = .03$, $\eta_p^2 = 0.12$, whereas the interaction Sequence X Age was not significant $F(2, 51) = 0.257$, $p = 0.77$. The decrement in the chicks' freezing response between sequence 1 and sequence 2 was significant for each Age: Day 1–2 $t(17) = 6.43$, $p < .001$, Day 2–3 $t\,(17) = 5.02$, $p < .001$, and Day 3–4 $t(17) = 3.70$, $p = .002$. A post-hoc Tuckey test on the factor Age showed that the youngest chicks froze significantly more than the older ones during sequence 1 (respectively, Day 1–2 vs. Day 2–3: $p = .039$; Day 1–2 vs. Day 3–4: $p = .005$; Day 2–3 vs. Day 3–4: $p = 0.731$) but not during sequence 2 (respectively, Day 1–2 vs. Day 2–3: $p = 0.735$; Day 1–2 vs. Day 3–4: $p = 0.197$; Day 2–3 vs. Day 3–4: $p = 0.580$). Hence, overall this pattern of results demonstrated that in session 1 the youngest group showed the greatest learning rate. In session 2, the ANOVA revealed a significant effect of Sequence $F(1, 51) = 35.36$, $p < .001$, $\eta_p^2 = 0.41$, and a significant Sequence X Age interaction $F(2, 51) = 3.31$, $p = .044$, $\eta_p^2 = 0.11$. The difference in freezing response between sequence 3 and 4 was significant for chicks of different ages: Day 1–2 $t(17) = 7.42$, $p < .001$, Day 2–3 $t(17) = 3.85$, $p < .001$; by contrast, the difference in the proportion of stops between sequence 3 and 4 for the oldest chicks (Day 3–4) was not significant $t(17) = 1.19$, $p = 0.249$, which shows that these animals reached an asymptotic performance level earlier than chicks of 2 and 3 days of age. Moreover, in sequence 4 the difference in freezing response between Day 1–2 and Day 3–4 groups reversed and approached significance ($p = .051$), indicating that at the end of the experiment the youngest chicks froze less than the oldest ones and showed the greatest amount of learning.

Duration of freezing

The mixed ANOVA showed a significant effect of Sequence $F(3, 51) = 85.47$, $p < .001$, $\eta_p^2 = .63$, Age $F(2, 51) = 14.86$, $p < .001$, $\eta_p^2 = .37$, and a significant Sequence x Age interaction $F(6, 51) = 11.29$, $p < .001$, $\eta_p^2 = .31$ (Figure 3, panel B).

In session 1, the ANOVA revealed a significant main effect of Sequence $F(3, 51) = 71.40$, $p < .001$, $\eta_p^2 = .58$, Age $F(2, 51) = 15.56$, $p < .001$, $\eta_p^2 = .38$, and a significant Sequence x Age interaction $F(6, 51) = 8.56$, $p < .001$, $\eta_p^2 = .25$. The decrease in the duration of freezing response between sequence 1 and 2 was significant for each group: Day 1-2: $t(17) = 6.05$, $p < .001$; Day 2-3: $t(17) = 4.73$, $p < .001$; Day 3-4: $t(17) = 4.32$, $p < .001$. A *post hoc* Tuckey test on the factor Age showed that during sequence 1, Day 1-2 chicks froze for a longer period than the older animals: Day 1-2 vs Day 2-3: $p < .009$; Day 1-2 vs Day 3-4: $p < .001$; on the contrary, Day 3-4 chicks showed the shortest time of freezing (Day 3-4 vs Day 2-3: $p < .001$).

In session 2, the ANOVA revealed a significant main effect of Sequence $F(1, 51) = 18.11$, $p < .001$, $\eta_p^2 = .26$, and a significant Sequence x Age interaction $F(2, 51) = 5.51$, $p < .001$, $\eta_p^2 = .18$. The difference in the duration of stops duration between sequence 3 and 4 was significant for Day 1-2 chicks $t(17) = 3.32$, $p = .004$, and Day 2-3 chicks $t(17) = 3.69$, $p = .002$. As shown in Figure 2 (panel B), Day 3-4 chicks reached an asymptotic level before others groups as the difference between sequence 3 and 4 was not significant: $t(17) = 0.68$, $p = .51$. There was no significant difference between groups in sequence 4.

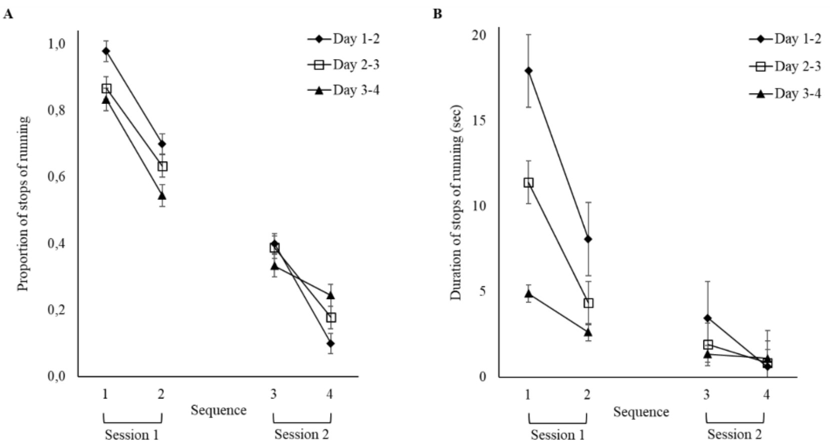


Figure 3. Panel A depicts the proportion of stops of running in the running-wheel for Day 1-2, Day 2-3 and Day 3-4 chicks across the four sequences. Panel B depicts the corresponding duration of the stops of running in the same conditions.

2.2 Experiment 2

The previous experiment has shown that habituation is already present 1 day after hatching, revealing that the neural mechanisms underlying this form of plasticity are immediately active in chicks. In the following experiment, we studied the age at which dishabituation develops. According to comparator models (Sokolov, 1963; Wagner, 1981), habituation and dishabituation stem from a common memory mechanism. Hence, dishabituation should be present from the first day after hatching like habituation.

2.2.1 Subjects

Thirty-six (20 males) domestic chicks (*Gallus gallus*) Ross 308 (Aviagen) hatched from eggs supplied by a local commercial hatchery (Agricola Berica s.c.r.l., Montegalda, Vicenza, Italy) in our laboratory under controlled temperature (37.7°C) and humidity (about 50-60%) conditions. Once hatched in the dark, the animals were reared singly at a controlled temperature (31°C) in rectangular cages (22 x 30 x 40 cm, width, height, depth) with a red cylinder (measuring 6 x 7.5 cm, diameter, height) hanging from above as a social companion.

2.2.2 Apparatus

Animals were tested within a running wheel (30 cm in diameter) located at the rear end of a uniformly black rectangular arena (45 x 50 x 160 cm, width, height, depth). A red cylinder was suspended, by a thin thread, 40 cm in front of the running wheel to motivate the chicks' running behaviour. Time, distance and direction of the run were computed by *Arduino* circuit and displayed on a monitor. Both the running wheel and the monitor were recorded by a video camera from above the arena.

2.2.3 Stimuli

During the experiment, a sequence of 16 consecutive stimuli was presented. The habituating stimulus was repeated from the 1st to the 10th trial, and from the 12th to the 16th trial. On the 11th trial a dishabituator was presented. The habituating stimulus was a 1200-ms Red-tailed hawk (*Buteo jamaicensis)* call (90 dB of average intensity), administered at a pseudo-random inter-stimulus interval ranging from 30 to 60 s. The dishabituator was a burst of 1200-ms white noise (90dB) administered 30 s after the previous trial.

2.2.4 Procedure

After hatching, each chick was randomly assigned to one of two groups according to the age at the testing day: the 1-day-old group (n=18) included chicks tested 1 day after hatching; the 3-day-old group (n=18) consisted of chicks tested 3 days after hatching.

The stimulation was manually delivered by the experimenter once the chick had run for a minimum distance of 1000 cm. As in the previous experiment, we scored the number and duration of stops of running in response to the acoustic stimulation.

2.2.5 Results

Habituation was evaluated by analysing the chicks' response to the first 10 trials. The results showed strong habituation of the freezing response in both groups, attested by the systematic decrement in the probability and duration of the freezing response to the acoustic stimulation (see Figure 4).

Proportion of freezing

Data were analysed with a mixed ANOVA with one within-subjects factor (Trial, 10 levels) and one between-subjects factor (Age, 2 levels: 1-day-old and 3-day-old). There was a significant main effect of the factor Trial, $F(9, 306) = 24.49$, $p < .001$, $\eta_p^2 = .42$, 95% CI = .32, .47, whereas the factor Age, $F(1, 34) = 3.13$, $p = .086$, $\eta_p^2 = .09$, 95% CI = 0, .28, and the Trial x Age interaction, $F(9, 306) = .44$, $p = .915$, $\eta_p^2 = .003$, 95% CI = 0, .01 were not significant (see Figure 4, panel A). The decrement in the stops of running across trials significantly fitted a linear trend, $F(1, 34) = 557.65$, $p < .001$, $\eta_p^2 = .94$, 95% CI = .90, .96, whereas the Age x linear trend interaction was not significant, $F(1, 34) = .30$, $p = .586$, $\eta_p^2 = .01$, 95% CI = 0, .15.

Duration of freezing

A mixed ANOVA with the same factors as before, revealed a significant main effect of the factors Trial, $F(9, 306) = 37.47$, $p < .001$, $\eta_p^2 = .52$, 95% CI = .44, .57, and Age, $F(1, 34) = 14.32$, $p = .001$, $\eta_p^2 = .30$, 95% CI = .07, .49, as well as a significant Trial x Age interaction, $F(9, 306) = 3.16$, $p = .001$, $\eta_p^2 = .09$, 95% CI = .01, 011 (see Figure 4, panel B). Data significantly fitted a linear trend, $F(1, 34) = 149.67$, $p < .001$, $\eta_p^2 = .81$, 95% CI = .69, .87, but there was also an Age x linear trend interaction, $F(1, 34) = 12.60$, $p = .001$, $\eta_p^2 = .27$, 95% CI = .05, .47, thus indicating that stimulus repetition had different effects on the duration of stops of running depending on the chicks' age. Pairwise comparisons (*t* tests) of the initial duration of stops of running revealed that 1-day-old chicks stopped for a longer period than the 3-day-old chicks during trial 1, $t(34) = 2.25$, $p = .031$, $d = .75$, 95% CI = .08, .43, whereas the two groups stopped for the same time during trial 10, $t(34) = -.706$, $p = .480$, $d = -.23$, 95% CI = -.89, .42.

Stimulus specificity was evaluated by comparing the chicks' responses from trial 10 to trial 11, when the dishabituator occurred (i.e. the white noise). Both groups of animals showed that habituation was stimulus-specific as both showed a marked increase of the freezing response from trial 10 to trial 11.

Proportion of freezing

A mixed ANOVA with one within-subjects factor (Trial, 2 levels: trial 10 and trial 11) and one between-subjects factor (Age, 2 levels: 1-day-old and 3-day-old) revealed a significant effect of the factor Trial $F(1, 34) = 170.00$, $p < .001$, $\eta_p^2 = .83$, 95% CI = .71, .88, whereas the factor Age, $F(1, 34) = .70$, $p = .411$, $\eta_p^2 = .02$, 95% CI = 0, .18, and the Trial x Age interaction, $F(1, 34) = .00$, $p = 1.000$, $\eta_p^2 = .00$, 95% CI = 0, .0 were not significant (see Figure 4, panel A).

Duration of freezing

A mixed *ANOVA* with the same factors as before revealed a significant effect of the factor Trial $F(1, 34) = 138.83$, $p < .001$, $\eta_p^2 = .80$, 95% CI = .66, .86, whereas the factor Age, $F(1, 34) = 1.26$, $p = .269$, $\eta_p^2 = .04$, 95% CI = 0, .21, and the Trial x Age interaction, $F(1, 34) = 1.70$, $p = .200$, $\eta_p^2 = .05$, 95% CI = 0, 23 were not significant (see Figure 4, panel B).

Dishabituation was assessed by comparing the chicks' response to the predator call in the 10th and 12th trial (i.e., after the burst of noise). The results showed that the unexpected burst of white noise caused a marked increase in the proportion and duration of stops to the predator call in both groups of chicks (see Figure 4).

Proportion of freezing

Data were analysed with a mixed ANOVA with one within-subjects factor (Trial, 2 levels: trial 10 and trial 12) and one between-subjects factor (Age, 2 levels: 1-day-old and 3-day-old). There was a significant main effect of the factor Trial, $F(1, 34) = 38.84$, $p < .001$, $\eta_p^2 = .53$, 95% CI = .28, .67, whereas the factor Age, $F(1, 34) = .70$, $p = .407$, $\eta_p^2 = .02$, 95% CI = 0, .28, and the Trial x Age interaction, $F(1, 34) = .09$, $p = .768$, $\eta_p^2 = .003$, 95% CI = 0, .11 were not significant (see Figure 4, panel A).

Duration of freezing

A mixed ANOVA with the same factors as before, revealed a significant main effect of the factors Trial, $F(1, 34) = 27.95$, $p < .001$, $\eta_p^2 = .45$, 95% CI = .19, .61, whereas the factor Age, $F(1, 34) = .52$, $p = .474$, $\eta_p^2 = .01$, 95% CI = 0, .13, and the Trial x Age interaction, $F(1, 34) = .11$, $p = .738$, $\eta_p^2 = .01$, 95% CI = 0, .12 (see Figure 4, panel B).

Habituation after *dishabituation* was evaluated by analysing the chicks' response in the last 5 trials. The results showed a robust habituation of the freezing response in the two groups, as both the probability and duration of the freezing response to the acoustic stimulation decreased (see Figure 4).

Proportion of freezing

Data were entered into a mixed ANOVA with one within-subjects factor (Trials, 5 levels) and one between-subjects factor (Age, 2 levels: 1-day-old and 3-day-old) revealed a significant main effect of the factors Trial, $F(4, 136) = 10.56$, $p < .001$, $\eta_p^2 = .24$, 95% CI = .10, .33, and Age, $F(1, 34) = 4.12$, $p = .050$, $\eta_p^2 = .11$, 95% CI = 0, .31, with the 1-day-old chicks overall less responsive to the stimulation than the 3-day-old ones (see Figure 1, panel A). The Trials x Age interaction was not significant, $F(4, 136) = .12$, $p = .977$, η_p^2

$= .01$, 95% CI = 0, .01. Data significantly fitted a linear trend, $F(1, 34) = 39.28$, $p < .001$, $\eta_p^2 = .54$, 95% CI = .28, .68, whereas the Age x linear trend interaction was not significant, $F(1, 34) = .25$, $p = .619$, $\eta_p^2 = .007$, 95% CI = 0, .14.

Duration of freezing

The analysis of the duration of stops revealed that dishabituation was significant at each age, $F(1, 34) = 27.95$, $p < .001$, $\eta_p^2 = .45$, 95% CI = .19, .61 and that the duration of stops of running on trial 12 was the same in both groups, $F(1, 34) = .11$, $p = .740$, $\eta_p^2 = .01$, 95% CI = 0, .12. A mixed ANOVA with the same factors as before revealed a reliable main effect of the factor Trial, $F(4, 136) = 14.31$, $p < .001$, $\eta_p^2 = .30$, 95% CI = .16, .39. The factor Age, $F(1, 34) = 1.66$, $p = .207$, $\eta_p^2 = .05$, 95% CI = 0, .22 and the Trials x Age interaction, $F(4, 136) = .12$, $p = .975$, $\eta_p^2 = .01$, 95% CI = 0, .01 were not significant. Data significantly fitted a linear trend, $F(1, 34) = 30.96$, $p < .001$, $\eta_p^2 = .48$, 95% CI = .22, .63, revealing a linear decrement of the duration of stops of running during the last 5 trials. The Age x linear trend interaction was not significant $F(1, 34) = .01$, $p = .919$, $\eta_p^2 = .01$, 95% CI = 0, .06.

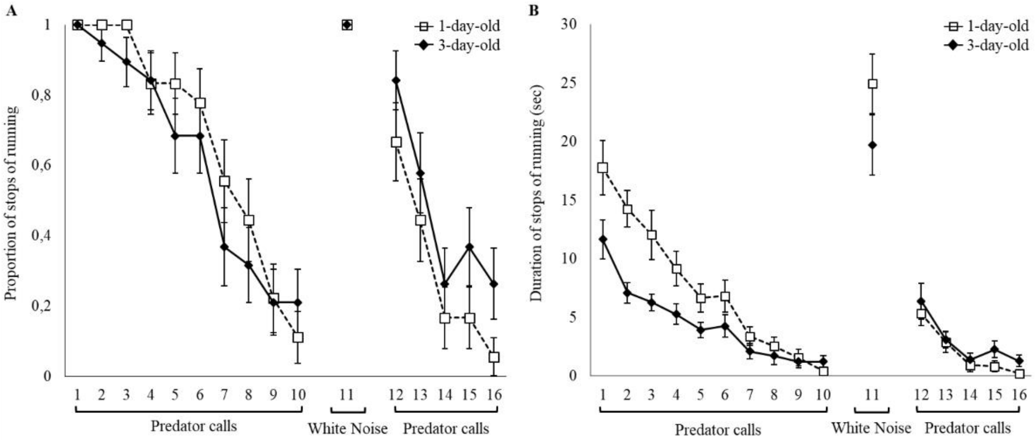


Figure 4. Panel A depicts the proportion of stops of running in the running wheel for 1-day-old and 3-day-old chicks. Panel B depicts the corresponding duration of the stops of running.

2.3 General Discussion

The habituation mechanism operates like a filter reducing the responsiveness to repetitive events to facilitate the processing of novel information (Cowan, 1988; Ramaswami, 2014; Sokolov, 1963). Given the highly adaptive value of habituation, what is the development of this ancestral form of behavioural plasticity? In Experiment 1 we have shown that 1-day-old chicks can habituate their defensive behaviour within the same number of stimulus repetitions (i.e., 10) as 2- and 3-day-old chicks do. This result supports the conclusion that younger and older chicks take the same time to develop habituation when potential arousing effects of the context are controlled (Hall & Channell, 1985; Mackintosh, 1987). The arousing effects of the context could mask habituation of younger chicks in the previous study by Zolman (1978), disrupting the ongoing habituation of their exploratory behaviour. By contrast, the effect of the context was reduced in our paradigm because all chicks had enough time to familiarize themselves with the new context. Please note, the responses measured in Zolman's experiment were significantly different. We scored a defensive response, namely freezing, while Zolman scored an exploration behaviour, that of the locomotor activity of chicks. Habituation of complex motor behaviour may require more time to develop than freezing. This could account for the different results. While this explanation holds true for altricial species, precocial species like chicks are mobile from the hatching moment and can immediately explore their surroundings like mature individuals. We controlled that chicks' locomotor activity before starting the experiment was the same at all ages (they ran for a minimum of 10 metres). Therefore, there is substantial evidence against this alternative account.

We have also found that newborn chicks are generally more plastic in adapting their behaviour to the stimulus. During the second day of stimulation, 1-day-old chicks decreased their response to the stimulus suggesting greater behavioural plasticity.

Rattan and Peretz (1981) obtained similar results in *Aplysia*; the behavioural plasticity underlying habituation may follow similar developmental trajectories in different species. Indeed, in many species, synaptic plasticity peaks relatively soon after birth and then declines, at variable rates, with increasing age (Hübener & Bonhoeffer, 2014). Different species show distinct phases of enhanced plasticity for specific sensorimotor experiences – e.g., imprinting for birds (Lorenz, 1935), language acquisition in humans (Kuhl, 2010), and development of visual brain areas (Hubel & Wiesel, 1963; Wiesel & Hubel, 1963b, 1963a). Habituation is a ubiquitous phenomenon among animals that affects different types of physiological (Barry, 2009) and motor responses (Rankin & Carew, 1987), as well as cognitive functions (Schmid et al., 2015; Turatto et al., 2017; Turatto & Pascucci, 2016). Hence, it may represent a broader form of behavioural plasticity beyond the species-specific differences. We suggest that habituation represents a model phenomenon to study life-long plasticity in different species. An important advantage of studying habituation could be the possibility of using modern high throughput behavioural analysis (McDiarmid et al., 2018) to study data derived from multiple levels at different ages, to identify a reliable marker of cognitive decline.

Newborn chicks may benefit from their enhanced plasticity to adapt their behaviour to environmental changes. Their motor system develops fast enough that immediately after hatching they can explore and forage independently from their parents. Suppressing a defensive response for long periods of time can be risky for chicks that may encounter potential threats during their early explorations. Experiment 2 shows that newborn chicks are very selective when decreasing their defensive behaviour. In fact, the presence of a new stimulus immediately restores chicks’ responsivity to both the unfamiliar stimulus and the familiar stimulus they had previously ignored, a phenomenon known as dishabituation.

While there is a general consensus among habituation theories regarding the role of the memory trace (a neural model in Sokolov's terminology) of the recent stimulation against which the current sensory input is compared (Konorski, 1967; Sokolov, 1963), it is less clear when it comes to dishabituation. Groves and Thompson argued that dishabituation does not exist and that the increased response level results from the process of sensitization superimposed on the habituated response level (Groves & Thompson, 1970). According to Wagner and colleagues (Terry, 1979; Wagner, 1976a; Whitlow, 1975) dishabituation reflects the disruption of the habituation model stored in STM. They proposed that the efficiency with which an incoming signal is processed, and consequently the probability that it elicits an unconditioned response, depends on whether it is already represented (or primed) in STM. The more a stimulus is primed in STM by its previous occurrences, it triggers a less vigorous response - a mechanism that would provide the basis of short-term habituation. The stimulus representation in STM decays spontaneously with time, but it can also be cancelled (or at least perturbed) by the occurrence of a new stimulus, called dishabituator, which displaces the previous stimulus representation from STM. In the latter case, the new stimulus elicits an unconditioned response itself, but, crucially, it also produces a recovery of the previously habituated response when the old stimulus is presented again, i.e., the dishabituation phenomenon.

Does the STM mechanism governing dishabituation appear concurrently with the manifestation of habituation, or at later stages of development? Previous studies on the ontogeny of habituation, dishabituation and sensitization in *Aplysia* found that these phenomena appear at different stages of development (Rankin & Carew, 1987, 1988; see Figure 5 for a comparison between altricial and precocial species). This pattern of results suggests two main conclusions: first, dishabituation requires a further step of development of the STM neural circuitry; second, that dishabituation is not a special case of sensitization, but rather, in accordance with Wagner's theory, represents a perturbation of the neural model of habituation (Terry, 1979; Whitlow, 1975). This is a conclusion in agreement with human studies on the electrodermal orienting reflex (Steiner & Barry, 2011, 2014). Our results fit the possibility that dishabituation represents a disruption of the habituation model stored in STM (Wanger, 1976). They show that dishabituation must not necessarily appear at later stages of development, compared to habituation (Rankin & Carew, 1987). The two phenomena are concurrently present in the first day of a chick's life.

Some alternative explanations must be considered. We do not know when habituation and dishabituation develop during chick embryogenesis. It is possible that the two phenomena develop at distinct stages of the embryogenesis and are both fully functional after birth. Hence, the ontogenetic dissociation between habituation and dishabituation might have occurred at an earlier age than in our test.

We could not entirely exclude the possibility that the dishabituator had simply aroused the chicks, increasing the probability of freezing to a following event. According to Thompson and Groves (1966), a sensitization process can overlap with the ongoing habituation process resulting in dishabituation. This mechanism does not require any memory representation of the habituation stimulus, rather it follows an arousal state of the organism. Perhaps future studies can adopt our paradigm to disentangle the development of dishabituation from sensitization following a similar ontogenetic approach of Rankin and Carew (1987).

Still, the results of Experiment 2 can support the theoretical framework proposed by Wagner (1976), which provides a comprehensive explanation of a common STM mechanism for habituation and dishabituation.

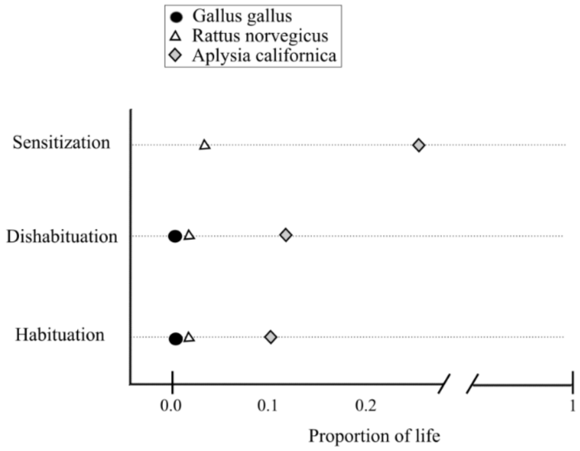


Figure 5. Habituation, dishabituation and sensitization emerge at different developmental stages in *Gallus gallus*, *Rattus norvegicus* and *Aplysia californica*. For age normalization, the number of days at which the three processes arise was divided by the maximum life span for each species and expressed as the proportion of life. The three forms of learning are present early in *Gallus gallus* and *Rattus norvegicus*, whereas they appear at different stages of maturation in *Aplysia californica*.

Accordingly, it has been found that the response decrement produced by the recent stimulus repetition tends to be disrupted when the ISI exceeds the capacity of the STM, and by temporally adjacent presentation of a dishabituator that competes for access to the STM (Terry, 1979; Whitlow, 1975). The simultaneous presence of habituation and dishabituation a few hours after hatching in the newborn chicks indicates an early development of the STM in this precocial avian species.

Our results suggest that a unitary STM mechanism of habituation and dishabituation appears to be immediately available after a chick hatch. If the habituation mechanism operates like a filter to reject the unwanted non-noxious stimulation, for example by reducing the orienting of attention toward the irrelevant distractors (e.g., Gati & Ben-Shakhar, 1990; Neo & Chua, 2006; Pascucci & Turatto, 2015; Sokolov, 1963; Turatto et al., 2017; Turatto & Pascucci, 2016; Waters et al., 1977), dishabituation may serve the function of resetting the habituation of attentional capture when a new extraneous alerting stimulus is encountered (but see, Ben-Shakhar, Gati, Ben-Bassat & Sniper, 2000), making the attention system sensitive again to a previously rejected distractor.

Chapter 3

The associative nature of habituation in domestic chicks and in bumblebees (*Bombus terrestris)*

Part of the material in this chapter has been adapted from the following papers:

Turatto, M., **Dissegna, A.**, & Chiandetti, C. (2019). Context learning before birth: evidence from the chick embryo. *Biology letters*, 15(*7*), 20190104.

Dissegna, A., Turatto, M., & Chiandetti, C. (2021). Context-Specific Habituation: A Review. *Animals*, 11(*6*), 1767.

We perceive the world in scenes, where objects are automatically embedded as a unified representation with their context (Bar, 2004). This cognitive capacity to associate objects within their context is fundamental for animals, so they can exploit context features to remember the properties of objects. We have all experienced, for example, that it is easier to remember the location of a lost object, *where* we last saw it. The fact that recall is better if the environment of learning is the same at retrieval has been demonstrated by a seminal work of Godden and Baddeley (Godden & Baddeley, 1975). In this experiment, participants learnt a list of words either on land or underwater. After a few minutes, they had to recall all the words they remembered. According to Godden and Baddeley's hypothesis, individuals rely on contextual cues to remember items. Their results showed that what was learned under water was best recalled under water and vice versa.

Contextual cues facilitate the remembering of a planned action to perform in the future (e.g., Cook et al., 2005). They also facilitate the suppression of unnecessary responses to stimuli that we have learned to ignore. When exposed to a repetition of a distractor in the same context, our attentional system learns to utilize the predictive properties of the context to anticipate its future occurrence, decreasing the likelihood that the distractor will capture our attention again (Turatto et al., 2017; Turatto, Bonetti, et al., 2019). The predictive properties of the environment help organisms to disengage from irrelevant stimuli quicker without requiring further exposures to the stimulation. Context learning increases survival; some precocial animals like domestic chicks develop this ability early in life (Chiandetti & Turatto, 2017).

How does a new context restore animals' responsivity to a previously habituated stimulus? There are several notable explanations. First, a different surrounding can alter the perception of some properties of a stimulus, disrupting habituation (Mackintosh, 1987). This may occur in the case of colours when, for example, the simultaneous contrast of adjacent items can change our perception so that the same stimulus may be perceived as unfamiliar. This can be controlled, however, by confirming a participant can recognize the stimulus irrespective of its context.

Second, the habituating response may be restored by a general sensitization process induced by the novel context. Hall and Channell (1985), found that once a stimulus was habituated in one context, the elicited response increases when it is presented in a second context not seen before by the subjects. The presentation of the stimulus in the second context had no effect if the animal had been in that context before. To account for this second possibility, animals were usually allowed to become familiar with both contexts before starting the habituation test.

Third, the repeated presentation of a stimulus in the presence of the same contextual cues promotes associative linkage between their corresponding memory representations (Vogel et al., 2019; Wagner, 1976, 1978, 1981). This associative account of habituation was suggested by Wagner in the context of the general Rescorla-Wagner (Rescorla & Wagner, 1972) model of conditioning. According to this model, the cues of the training context (CS) associatively activate the node of the habituating stimulus (US) in a secondary state of activation A2, resulting in long-term habituation. Only this latter explanation supports an associative account of habituation. In the next section, we review the literature to provide an overview of the current evidence for this account.

3.1 A review of evidence to support Wagner's SOP

Methods to Investigate Context-Specific Habituation

Three different approaches have been traditionally used to show that habituation can be specific for the context of stimulation. The most straightforward is the context-change method, whereby the context changes from the training to the test phase. Evidence of context-specific habituation is found when the response habituated during training recovers in a different context during the test. A second approach to reveal the associative nature of habituation relies on the extinction phenomenon. Here the logic is that during training the stimulus automatically forms associations, stored in the LTM, with the surrounding environmental cues (Wagner, 1976). When the context is further encountered in the future, these associations prime the stimulus (or anticipate its arrival) in the STM, thus maintaining previous habituation. However, if after habituation the organism is repeatedly exposed to the same context without the stimulus (the extinction condition), the previously formed associations will extinguish, and the habituated response will recover when the stimulus is reintroduced in the same context. By contrast, if the stimulus is omitted altogether with the context (the control condition), the stimulus-context associations will be retained in the LTM, so that habituation will remain effective when the stimulus occurs again in the same context. A third method is based on the latent inhibition phenomenon reported in conditioning studies (Lubow & Moore, 1959). In this phenomenon, the association between the conditioned and the unconditioned stimuli (CS and US) is delayed when the CS is pre-exposed in isolation before being paired with the US. One of the viable explanations is that, during the pre-exposure phase, the CS becomes associated with contextual cues, which then reduce its ability to enter in association with the US. Alternatively, during the pre-exposure phase, the animal learns that the context predicts no consequences, namely that no US would follow. In the same vein, if habituation is context-specific, then presenting the context alone in a pre-exposure phase will be less associated with the to-be habituated stimulus during training. Hence, at the test, the context would not activate or would activate to a lesser extent, the stimulus representation in the STM, and the retention of habituation will be compromised as compared to when the pre-exposed context is different from that used during training.

To conclude this brief overview of the rationale behind the main methods used to address the associability of habituation, it might be worth mentioning that at least two alternative phenomena can explain the disruption of habituation when the context changes. One is that the presentation of a stimulus in a different environment may simply alter the stimulus representation. This would make the stimulus essentially new to an organism, resulting in a disruption of the ongoing habituation. A second one is that a sudden change of the context of stimulation might lead to a neophobia reaction. This in turn increases the animal's arousal and its propensity to respond to any stimulus, a result that could mimic a context-specificity effect (Hall & Channell, 1985; Hall & Honey, 1989; Honey et al., 1992). To rule out this possible confound, in some studies animals have been given the possibility to familiarize themselves with the new context before the test (see Table 1).

Behavioral Response	Species	Method	Evidence of Context Specificity	Study
Drug tolerance	*Rattus norvegicus*	CC	✓	(Siegel, 1977)
Escape	*Lumbricidae*	CC *	✓	(Reyes-Jimnez et al., 2020)
	Chasmagnathus granulate	CC *, LI, Ext	✓	(Hermitte et al., 1999; Tomsic et al., 1993, 1998)
	Caenorhabditis elegans	CC *, LI, Ext	✓	(Lau et al., 2012; Rankin, 2000)
Gene expression	*Taeniopygia guttata*	CC	✓	(Kruse et al., 2004)
Neophobia	*Rattus norvegicus*	CC *	×	(Honey et al., 1992)
Orienting response				
Attentional capture	*Homo sapiens*	CC, Ext	✓	(Turatto, et al., 2019; Turatto et al., 2017)
Skin conductance	*Homo sapiens*	CC	×	(Churchill et al., 1985; Schaafsma et al., 1989)
Light approach	*Rattus norvegicus*	CC *, LI	×	(Hall & Channell, 1985)
Head orienting	*Rattus norvegicus*	Ext	✓	(Jordan et al., 2000)
Startle	*Rattus norvegicus*	CC *	×	(Jordan et al., 2000; Marlin & Miller, 1981)
	Mus musculus	CC *	×	(Pilz et al., 2014)
Suppression				
Bar press	*Rattus norvegicus*	CC *	✓/×	(Hall & Honey, 1989; Jordan et al., 2000)
Licking	*Rattus norvegicus*	CC *, Ext	✓/×	(Evans & Hammond, 1983; Jordan et al., 2000; Leaton, 1974)
Wheel running	*Gallus gallus*	CC *	✓	(Chiandetti & Turatto, 2017; Turatto, Dissegna, et al., 2019)
Vasomotor activity	*Oryctolagus cuniculus*	Ext	✓	(Wagner, 1976)

Table 1. Evidence of context specificity as a function of behavioral response in different species. CC = Context change; LI = Latent inhibition; Ext = Extinction. * indicates that, to attenuate neophobia, animals were exposed to the new context before testing, as suggested in Hall and Channel (1985). ✓ evidence; × absence of evidence; ✓/× mixed results.

Context-Specific Habituation in Humans

Evidence of context-specific habituation in humans (*Homo sapiens*) is rather scant. In one of the few studies that have addressed this issue, Turatto, Bonetti, and Pascucci (Turatto et al., 2017) investigated whether habituation of attentional capture, a covert component of the OR, is context-specific or generalizes across different contexts. In three consecutive days, participants performed a visual discrimination task in focused attention, while a visual onset distractor appeared in the display, which also defined the context of stimulation. The results showed that on day 1 the capture of attention triggered by a repetitive visual onset distractor was subject to habituation. Then, on day 2, participants were divided into two groups: the extinction group performed the same visual discrimination task of day 1, but without the distractor, whereas the control group suspended the task. On day 3, both groups resumed the visual task with the distractor. While habituation of capture was still present in the control group, the attentional capture response recovered in the extinction group, a result consistent with a context-specific habituation view (Wagner, 1976, 1978, 1981). Evidence in favour of context-specific habituation of attentional capture emerged also in a subsequent study (Turatto et al., 2019). Here the stimuli used to measure habituation of capture were presented over a background consisting, for example, of a countryside landscape. The next day, one group of participants continued the same task in the same context, whereas for another group the context changed, with the background image now depicting an industrial landscape. Habituation of capture was retained in the same-context group but was disrupted in the different-context group.

Studies investigating the context-specific habituation of different electrophysiological responses have provided less consistent results. An electrodermal study by Churchill, Remington, and Siddle (Churchill et al., 1985) recorded the skin conductance response of participants repeatedly exposed to a geometrical shape projected on a monitor. The authors found the same level of long-term habituation when either local contextual cues—e.g., the background image of the monitor—or global contextual cues—e.g., the furniture in the experimental room—changed between the training and test sessions, thus showing generalization of habituation across different contexts. Similarly, an extinction session with the trained context did not produce a recovery of the habituated response. Schaafsma, Packer, and Siddle (Schaafsma et al., 1989) studied the role of context in the long-term habituation of the skin conductance response to stimuli with different motivational value. Specifically, all participants were exposed to tones and vibrations as habituation stimuli. The authors manipulated the motivational value of either tones or vibrations by instructing participants to press a microswitch at the offset of one of the two types of stimuli. The hypothesis was that instructing participants to perform a specific action in response to one of the two habituation stimuli would increase the amount of processing that one stimulus received from the STM relative to the other. Since stimuli that receive more processing in the STM are more likely to consolidate their association with contextual cues, the authors expected that the context change would produce less long-term habituation retention for the motivationally significant stimulus than for the other one. However, the authors found no effect of context for either of the stimuli.

Context-Specific Habituation in Non-Human Mammals

Initial evidence of context-specific habituation was reported by Wagner (Wagner, 1976) in an unpublished work on rabbits. The author reported lower retention of long-term habituation of the vasoconstriction response to a repeated tone in rabbits that remained in the experimental apparatus between the training and test session compared to animals that returned to their home cage, a pattern of results indicating a disruption of habituation due to the extinction of the context-stimulus association. Several subsequent studies have used the rat as an animal model to study context-specific habituation. Of remarkable importance is the discovery made by Siegel (1977) that the associative link between context and habituation is implicated in the regulation of drug tolerance. Tolerance is an instance of habituation because some of the drug effects decrease with its administration. For example, in rats the analgesic effects of morphine declines after repeated injections, leading rats to increase the amount of narcotic to resist pain. Siegel (1977) demonstrated that rats injected with morphine in a given context developed a context-specific tolerance for that drug, but tolerance dropped significantly when rats were given the shot in a new context. He also demonstrated context-specific morphine tolerance using latent inhibition and extinction, thus attesting that the associative nature of habituation can interact with the biological processes underlying addiction.

The scenario, however, is a bit more intricate, as some responses appear to be more prone to show context-specific habituation than others. In particular, habituation has been shown to depend on the context in the case of inhibitory responses (e.g., lick suppression or bar-press suppression Evans & Hammond, 1983; Jordan et al., 2000; see Hall & Honey, 1989; Leaton, 1974 for a null result), whereas there is no evidence for the context-specific habituation of the startle response (Jordan et al., 2000; Marlin & Miller, 1981) (see also Pilz et al., 2014 for a study on mice), and conflicting results for different aspects of the OR (null results have been reported in Hall & Channell, 1985; a positive result in Jordan et al., 2000). Specifically, the study of Jordan, Strasser, and McHale (2000) has found, in the same animal, evidence of the context-specific long-term habituation of lick and bar-press suppression, but not of the acoustic startle response. Moreover, they found that extinction of context disrupted the long-term habituation of lick-suppression and the OR to a light. These results suggest that different responses supported by independent neural circuits can be differentially sensitive to the context, in agreement with the hypothesis that habituation does not represent a unitary phenomenon affecting all behaviors in the same fashion (McDiarmid et al., 2019; Randlett et al., 2019).

However, as briefly mentioned above, it is important to stress the fact that not all the response increments observed after a context change are instances of context-specific habituation. For example, Hall and Channell (1985) showed that once rats stopped to orient toward a repetitive flashing light, orientation increases again when rats were moved into a new context—apparently supporting the context-specific hypothesis—but this effect disappeared if rats could familiarize themselves with the new context before the test. Hall and Channell (1985) hypothesized that when rats were tested in the new context, the OR increased as a consequence of the neophobia induced by the context change (Mackintosh, 1987). However, contrary to context-specific habituation, response increments due to neophobia or arousal should affect the overall responsivity of the animal (Thompson & Spencer, 1966). Indeed, a similar response sensitization was measured in a new context also in rats tested for habituation of lick-suppression (Hall & Honey, 1989) and neophobia reaction induced by a new flavor (Honey et al., 1992).

A less tested hypothesis is that context-specific habituation might be modulated by the biological significance of the stimulation. Indeed, stimuli that are more relevant for an organism are more likely to form associative links with the surrounding environment, as they have more associative strength (Wagner, 1976, 1981). In line with this hypothesis, Evans and Hammond (1983) showed that long-term habituation of lick suppression elicited by the distress squeal of another rat was context-specific, whereas long-term habituation of the same response caused by an artificial sound with similar acoustical features was not.

Context-Specific Habituation in Birds

Studies with two types of avian species, zebra finches and chicks of domestic fowl, have provided converging evidence of context-specific habituation in birds. Kruse, Stripling, and Clayton (2004) reported for the first time that habituation of a genetic response is context-specific. They measured the expression of the *zenk* gene—a specific immediate early gene (IEG) synthesized in the auditory brain areas of zebra finches (*Taeniopygia guttata*)—in birds repeatedly exposed to a conspecific song. The results showed that the expression of this gene decreased when the same song was repeated in the same context. However, when finches listened to the familiar song under different light conditions, the synthesis of the *zenk* gene spiked again, suggesting that habituation of its expression was specific for the context in which the song was experienced.

The research with domestic chicks has focused on the development of the associative mechanism underlying context-specific habituation. Chicks of domestic fowl (*Gallus gallus*) are precocial birds that develop almost completely in the egg. Thus, after hatching, they already have enough cognitive and motor skills to be independent of parental care. Chiandetti and Turatto (2017) demonstrated that the associative learning process underlying context-specific habituation is also part of this early cognitive equipment. They measured the stop of the wheel-running behavior elicited by a loud sound in 4-day old chicks in four consecutive sessions of stimulation within the same context, comparing this performance with that of groups of chicks for which different aspects of the context were changed after the first two sessions. The degree of generalization vs. specificity of the habituated freezing response to the sound varied with the number of features that the training contexts shared with the test context. In addition, it should be also noted that the increased response observed when the context changed cannot be accounted for by generally increased arousal, as the response measured was the stop of the ongoing activity (i.e., wheel-running behavior).

Furthermore, a recent study by Turatto, Dissegna, and Chiandetti (2019, described in detail as Experiment 3 of this thesis) suggests that the ability to take into account the context of stimulation to filter unwanted sensory input is an innate cognitive ability in chicks. The authors exposed one group of animals in the last stage of their embryonic maturation to a repetitive sound, and then tested their freezing response to an analogous series of sounds in a running wheel two days after hatching. They compared habituation to the sounds in this group of animals with that of another group of chicks exposed to the sounds one day after birth in the running wheel or a different context, namely in the chicks' home cage. The results showed that the prenatal group of chicks had a similar disruption of long-term habituation as the chicks trained in the home cage. Still, however, their degree of habituation to the sounds was higher than that of an untrained group, attesting that the prenatal stimulation had successfully induced habituation and that the level of habituation was comparable to that achieved by the group stimulated and tested in two different contexts.

Context-Specific Habituation in Invertebrates

Despite the relatively simple organization and the modest dimension of invertebrate's nervous systems, the study of habituation in these species has confirmed that this form of learning can be context-specific. Here we will briefly review the main studies conducted with the crab (*Chasmagnathus granulate*), the nematode (*Caenorhabditis elegans*) and, more recently, the earthworm of the *Lumbricidae* family (Reyes-Jimnez et al., 2020).

There exists a long tradition of studies concerning the remarkable ability of crabs to form associations between stimuli and context and to flexibly adapt their escape responses. Tomsic and colleagues (1993) demonstrated that changing the contextual cues between the training and test phase produced a recovery of habituation of the escape response elicited by a paddle moving above the animal. The authors found the same result when crabs were exposed to the context prior to or following habituation training, thus attesting that the escape response was also sensitive to latent inhibition and extinction (Tomsic et al., 1998). Furthermore, the neurobiological mechanisms regulating context-specific habituation were also investigated by injecting crabs with an inhibitor of protein synthesis (*Cycloheximide)*. Interestingly, injection of the drug before the training blocked the context-specificity of habituation in crabs that could fully retain long-term habituation even when the context changed. By contrast, the injection of the inhibitor after the training impaired the formation of long-term habituation in crabs tested either in the same or different contexts (Pedreira et al., 1995, 1996). This pattern of results suggests the presence of two distinct cellular processes that lead to context-specific habituation: one responsible for the formation of contextual memories, that was immediately activated as crabs were placed in the training context; the second deputed to the consolidation of long-term habituation triggered by repeated exposure to the stimulus.

The research on crabs has also revealed that the frequency of stimulation is also critical for the emergence of context-specific habituation. For example, it has been suggested that the longer a stimulus representation remains active in the STM before being displaced by the next stimulus occurrence, the more likely it will consolidate its association with the representations of contextual cues in the LTM (Davis, 1970). In line with this, Hermitte et al. (1999) demonstrated that context change produced a recovery of the habituated response in a group of crabs trained with a 171 s inter-stimulus-interval (ISI), but not in a group of crabs trained with a 0 s ISI. Moreover, the injection of the protein inhibitor *Cycloheximide* impaired the retention of long-term memory only in crabs trained at a 171 s ISI, attesting that training with long and short ISIs recruited separate cellular processes (for a replication of this result, see Pereyra et al., 2000).

However, it remains unclear whether only training with long ISIs results in context-specific habituation. Of considerable importance to this topic is a study in which Rankin (2000) investigated context-specific habituation of the tap-withdrawal response in the *C. Elegans*, with stimulations delivered with either a 10 or a 60 s ISI. The context was defined by the presence of a given chemical substance (sodium acetate, $NaCh_3COO$) in the petri-dish housing the nematode. The results showed greater retention of habituation at both 10 and 60 s ISI in animals trained and tested in the presence of $NaCh_3COO$, as compared with a group trained in the same context but tested in a different one (i.e., plain agar). Rankin also found that context-specific habituation was abolished by latent inhibition and extinction if the animals were exposed to the chemosensory context in the absence of the tap. The fact that context-specific habituation emerged also with the shorter ISI is surprising because with a 10 s ISI long-term habituation is usually not observed in *C. Elegans* (Beck & Rankin, 1997). This paradoxical result was found also by Lau, Timbers, Mahmoud, and Rankin (Lau et al., 2012) in which another chemical substance (diacetyl, $C_4H_6O_2$) was used as the context. Lau et al. also compared several mutant strains of *C. Elegans* to identify genes involved in context-specific habituation. They found that worms with a mutation in the *nmr-1* gene (NMDA-type glutamate receptor subunit) showed comparable long-term habituation to the non-mutant group when trained and tested in a plain petri-dish, but they showed a lack of context-specific effect in the presence of the olfactory cue, suggesting a deficit in their capacity to associate the tap with the context. This suggests that context-specific habituation and long-term habituation involve different biological mechanisms, that long-term habituation does not necessarily require context-stimulus associations, and that the mechanisms underlying context-specific habituation are activated by both short and long ISIs, whereas those responsible for long-term habituation are activated only by long ISIs.

Conclusions

Here we have briefly summarized evidence, from phylogenetically distant species, showing that habituation can be context-specific for a large set of responses (see Table

1), and irrespective of the complexity of the nervous systems considered (see Figure 7). In spite of other theories that could also be compatible with context-specific habituation (Hall & Rodríguez, 2020; Sokolov, 1963), the model proposed by Wagner (Vogel et al., 2019; Wagner, 1981) offers a straightforward explanation of context-specific habituation. A single memory mechanism – the association between context and the habituating stimulus – can explain the results obtained with the three main approaches have been traditionally used to show context-specific habituation (*i.e.,* context change, latent inhibition, and extinction). With respect to this, Wagner's theory is perhaps the most exhaustive one in accounting for habituation, including by its nature the feature of being, for certain types of responses, context-specific.

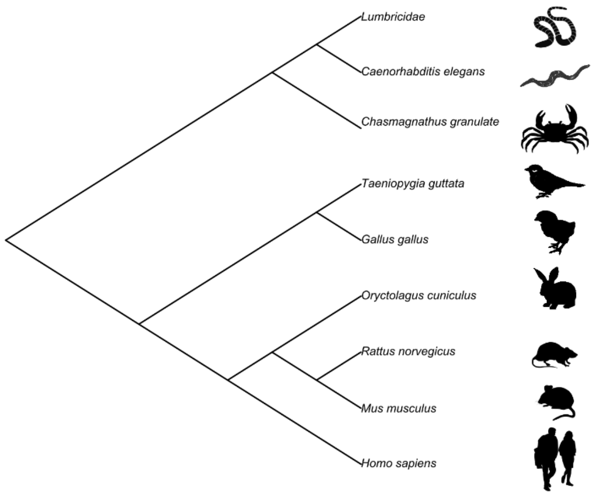


Figure 6. Cladogram of the species showing context-specific habituation.

3.2 Experiment 3

In this experiment we exploited the embryonic development of domestic fowl chicks in a closed environment (*i.e.*, the egg) to test Wagner's hypothesis that contextual cues can prime the memory representation of a habituating stimulus, resulting in long-term habituation. We hypothesized that this memory mechanism is developed in chicks' embryo modulating post-natal transferring of memories acquired before birth.

3.2.1 Subjects

Domestic chicks (N = 120; males = 58) of the Ross 308 (Aviagen) broiler strain hatched singly in individual opaque compartments of 10 cm^3 from fertilized eggs incubated in our laboratory under controlled temperature (37.78C) and humidity (about 50–60) conditions. The hatching moment was recorded by a camera set inside the incubator. The auditory stimuli were administered by two loudspeakers positioned on the ceiling of the incubator. In the exposure phase, the temperature of the incubator was lowered to 31.58C for the different-context group. Illumination was kept constant across conditions at 0 lx.

3.2.2 Apparatus

The test setting was the same as in Experiment 1 and 2 and consisted of a running wheel (30 cm in diameter) located at the rear of a black arena (45 x 50 x 160 cm, width, height, depth). A red cylinder (6 x 7.5 cm, diameter, height) was hung from above in front of the running-wheel to elicit the chicks' running behaviour. In the testing room, the temperature was 28°C; illumination within the apparatus varied from 3 lx in the running-wheel to 14 lx in the proximity of the red cylinder. The auditory stimuli were delivered by two loudspeakers positioned on the top of the running-wheel, at about 30 cm from the chick's head. Time, distance and direction of the runs within the wheel were computed by an Arduino circuit and displayed on a monitor. Both the running-wheel and the monitor were recorded by a video camera from above the arena.

3.2.3 Procedure

Each sequence of stimulation consisted of five bursts of 250 ms white noise (90 dB SPL) delivered at a pseudo-random interstimulus interval, ranging from 30 to 60 s. Four experimental groups of 30 chicks each were used: same context, different context, *in ovo* and control (see Figure 8). In the exposure phase, all except the control group received two sessions of stimulation 1 h apart, with each session consisting of a sequence of stimulation. The groups of chicks differed as a function of the context in which they received the acoustic stimulation: in the same-context group, each chick was individually stimulated in the running-wheel (as in the test phase); chicks in the different-context group were collectively stimulated in the incubator within 24 h after hatching; chicks in the *in ovo* group were collectively stimulated in the incubator in the last 24 h before hatching; chicks in the control group were not submitted to the exposure phase.

During the test phase, all chicks were tested individually in the running-wheel from 24 to 48 h after hatching. The acoustic stimulation was identical to the one delivered in the exposure phase. Hence, in the test phase, the context of stimulation was identical for all chicks. The stimulation in the running-wheel was started manually by the experimenter once the chick, in the attempt to reach the red object in front of it, showed a consistent wheel-running behaviour (i.e., they run for a minimum distance of 10 m). During this period, the animals had also the possibility to familiarize themselves with the running-wheel context.

Habituation to the acoustic stimulation was evaluated by scoring the number of freezing responses to the bursts of white noise during the test phase. For chicks in the same-context group, the same type of data was collected also during the exposure phase.

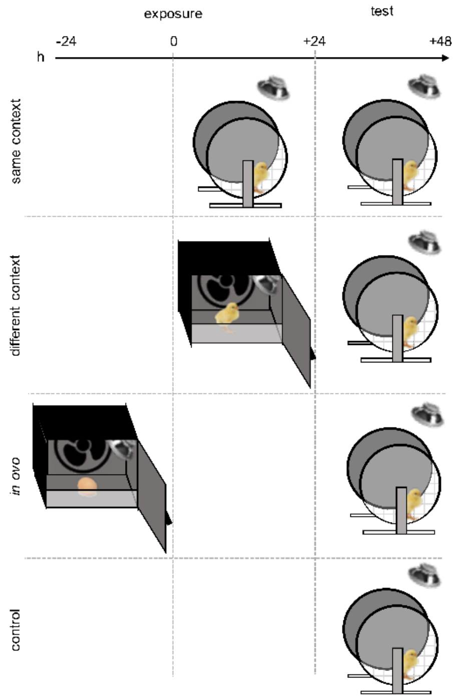


Figure 8. Schematic representation of the experimental design and conditions. The exposure (-24/+24) and the test (+48) phases took place in the same or different contexts. The freezing response to the bursts of white noise (90 dB) was measured as a stop of the wheel-running behaviour.

3.2.4 Results

The main results are illustrated in Figure 9. The proportions of freezing responses were not normally distributed so data were analysed with non-parametric statistical tests (Kruskal–Wallis and Wilcoxon signed-rank tests).

The same-context group showed a reliable habituation of the freezing response from the exposure to the test phase ($p < 0.001$, $r = 0.73$). The rate of freezing in the first session of the test phase was different among groups ($\chi^2(3) = 41.897$, $p < 0.001$, $\eta^2=0.33$, Kruskal–Wallis H test). Post hoc comparisons showed that the different-context group had a higher freezing rate compared to the same-context group ($p = 0.001$, $r =0.40$), which confirmed previous results of context-specific habituation in chicks (Chiandetti & Turatto, 2017). The same result emerged for the *in ovo* group ($p < 0.001$, $r =0.33$). This indicates that habituation was context-specific, even before hatching, for chick embryos. Chicks could encode the surrounding context forming associations with the acoustic stimuli. Both groups showed a similar freezing rate (p =0. 854, r =0.02), but a lower rate compared to the control group (different-context group: p =0.010, r =0.22; *in ovo* group: p =0.012, r =0.21). This meant that during the exposure phase, chicks partially habituated to the sounds regardless of context.

The overall pattern of results is evidence that context learning takes place in the chick embryo. The response of the test for the *in ovo* group was only coincidentally like that of the different-context group, and higher than that of the same-context group. This was caused by the longer interval between the exposure and test phase in the *in ovo* group, which then lead habituation to decay more rapidly. This interpretation is less reliable because it requires more assumptions than the context-learning one to explain the overall pattern of results. But we cannot rule it out completely. An alternative related manipulation to eliminate the role of temporal differences between exposure and test phases among groups, if any, would be to further stimulate the *in ovo* group on the running-wheel, comparing performance directly to the *in ovo* group stimulated in the incubator. Further studies are needed to clarify these issues.

As shown in Figure 9 (b), habituation was effective for all chick groups in the two sessions of the test phase (same-context group: $p < 0.001$, r =0.79; different-context group: $p < 0.001$, r =0.78; *in ovo* group: $p < 0.001$, r =0.76; control group: $p < 0.001$, r =0.79), confirming that our paradigm was adequate to elicit a reliable habituation of the freezing response in this avian species.

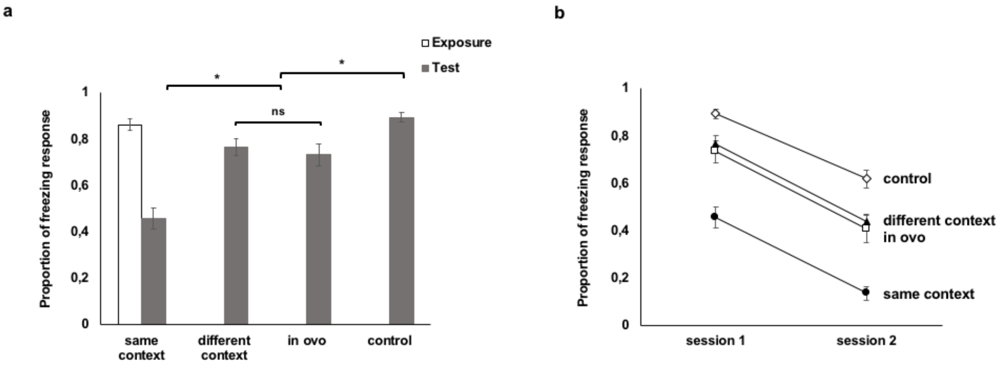


Figure 9. (a) Amount of freezing in chicks exposed to the bursts of noise. In the test phase, data are from the first session of stimulation. The rate of freezing increased in chicks stimulated in a different context (*in ovo* or after hatching in the incubator) during the previous exposure phase. (b) During the test phase, all groups showed a reliable habituation of the freezing response across the two sessions of stimulation.

3.3 Experiment 4

The previous experiment has shown that the neural mechanisms underlying context-specific habituation are functional in chicks' embryos. As mentioned in the literature review (Section 3.1 of this book), evidence in support of contextual sensitivity of habituation has since been concerned with three main classes of associative phenomena: latent inhibition of the context (e.g., Jordan et al., 2000), extinction of the context (e.g., Turatto et al., 2017) and context-specificity (e.g., Tomsic et al., 1998). A more direct demonstration of this theory would require recording the response elicited by a target stimulus in the presence of specific cues that do not necessarily predict its presence. A diminution of the elicited response would be expected (by the target stimuli) when anticipated by a predictor. As well an increase of the elicited response would happen in the opposite condition. Kimmel (1967) trained a group of participants by pairing a light (CS) and an electric shock (US) eliciting a Galvanic Skin Response (UR). He found that the intensity of the UR was weaker for this group relative to a group of participants for whom the US was not anticipated by any CS. This study demonstrated a decline in the US elicited a response in conditions of predictability a phenomenon known as the conditioned diminution of the UR (Kimmel, 1966).

A conspicuous corpus of literature (Bombace et al., 1991; Brandon et al., 1991; Brandon & Wagner, 1991; Davis, 1986) has demonstrated that the UR can be potentiated by the presence of specific CS that then induces a conditioned emotional reaction similar to the UR. The most common example is pairing a CS with an aversive stimulus, like a brief electric shock, before being conditioned to an equally aversive US (eliciting a) startling response. The aversive reaction elicited by the CS facilitates the following UR, potentiating the magnitude of the startle, i.e., the fear-potentiated startle (Davis, 1986). Wagner and Brendon (1989) suggested that the cause of the potentiation effect could be the superimposition of an emotional response elicited by the CS (i.e., the conditioned emotional response) to the ongoing one to the US. To support this argumentation, Wagner and Vogel (2010) reported several unpublished experiments by Brandon et al. on the eyeblink response of rabbits. They demonstrated that an UR evoked by the periorbital shock of one eye was greater when the shock was preceded by a CS that was trained with an equally aversive stimulus. The authors then capitalized on the fact that a shock delivered in the left eye produces a response that is lateralized in that eye, and vice versa, to train different CSs to the same aversive US but in distinct eyes. The two CSs shared the same conditioned emotional response associated with the same US but they predicted the occurrence of the US in different eyes. Later, rabbits were tested with a shock delivered to one of the eyes. The shock could be preceded by the “right” or the “wrong” CS, so that the US location was predicted only when the “right” CS was delivered. They disentangled the diminution effect of the CS predictive properties from the emotional response potentiating effect. The results showed that the UR was stronger when a CS signalled the US in the “wrong” eye, revealing the presence of a conditioned emotional response. The correct CS generated a diminution of the UR likely reflecting the two opposite effects of the conditioned diminution and the conditioned emotional response which modulated the UR.

The superimposition of a separate conditioned emotional response to an UR occurs when two following events share a similar emotional state, i.e., when the response to the preceding event mimics the response to the event that follows. Wagner’s classical theory (1976) did not include any emotional representations of stimuli. Wagner and Brandon (1989) formulated an affective extension of the SOP, the AESOP model. AESOP is distinguished between two sets of nodes that are concurrently active when a stimulus occurs: one representing its sensory feature and another its emotive aspect. Both sets of nodes can associatively enter an A2 state when predicted by appropriate cues. However, the associations of the CS with the sensory features of the US control the UR; while the associations between the CS and the emotive aspect of the US modulate the conditioned emotional response. The emotional activity of a US in A2 modulates the activation of the corresponding sensory nodes, affecting animals’ responsivity. When two consecutive events elicit a similar emotional state, the response to the second increases. When two consecutive events elicit an opposite emotional state, the response to the second dampens. To date, the latter hypothesis has not been experimentally tested but can be inferred by the AESOP model. The emotional node of a target stimulus can associatively enter A2 when primed by an affective congruent cue, but associative priming would not be possible for an affective incongruent cue. As a result, the emotional representation of the target stimulus directly enters an A1 state, without superimposing the conditioned emotional response (see Figure 6). This point is in contradiction with the basic notion of the SOP that the amplitude of the response a stimulus evokes is directly determined by how unexpected it is.

Intuitively speaking, the lack of priming of the emotive representation of a stimulus would increase the evoked response to a greater extent than when the representation is primed (1976). This stems from the brain's primary objective to infer the causes of its sensory input by forming a predictive model of external stimuli and reduce uncertainty. When a mismatch between prior expectations and reality arises, a prediction error is generated in the brain (den Ouden et al., 2010). A prediction error reflects the size of the perceptual disparity between the model of the stimulus and the sensory input but also the valence of the mismatch. In other words, the prediction error judges an outcome event as better or worse than expected. When the outcome is better than predicted, a positive prediction error is generated that promotes excitatory learning and approach behaviours. When the outcome is worse than predicted, it leads to a negative prediction error, aversive or avoiding reactions of the object that occurred and inhibitory learning processes (Laurent et al., 2018; Ploghaus et al., 2000). Under the prediction error framework, appetitive and aversive reactions are differently potentiated/inhibited as a function of the mismatch between both the expected sensory and emotive aspects of the upcoming stimulus and reality.

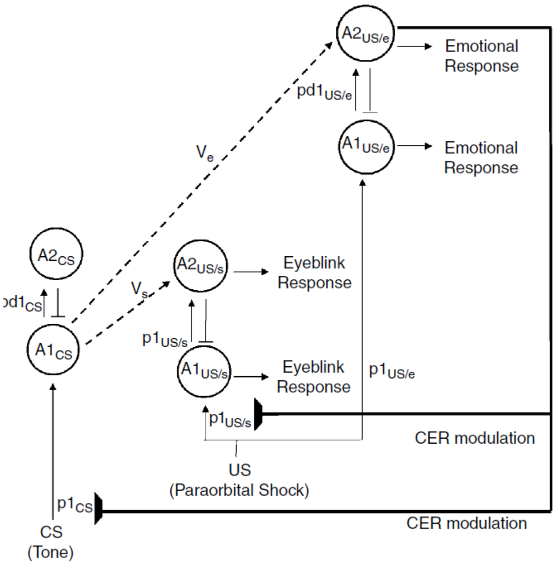


Figure 6. A schematic representation of the AESOP model from Wagner and Vogel (2010, p. 150). The CS is represented by the activity of a primary ($A1_{CS}$) and a secondary activity state ($A2_{CS}$). The US is represented by two sets of units: the sensory units, $A1_{US/s}$ and $A2_{US/s}$, and the emotive units, $A1_{US/e}$ and $A2_{US/e}$. The activity in the primary units is proportional to the p1 values. The self-generated activity of secondary units is expressed by pd1. The associative-generated activity of the US secondary units induced by the CS is represented by the associative links Vs and Ve for the sensory and emotional representations, respectively. The activity of the US secondary sensory unit evokes a discrete UR while the activity of the US secondary emotive units modulates the conditioned emotional responses. The link between the emotive units and the sensory units represents the modulatory effect of the US secondary emotive unit on the activity of the CS and US sensory units. The modulatory effect is assumed to be an increment in the respective p1 values, which is proportional to $A2_{US/s}$.

Appetitive or positive responses are then facilitated by positive prediction errors and inhibited by negative prediction errors; aversive responses are inhibited by positive prediction errors and facilitated by negative prediction errors (Den Ouden et al., 2012). Compared to the AESOP model, the effect of emotional incongruent cues on the response evoked by a target stimulus changes according to the type of response and the valence of the prediction error. Conflicting emotional representations that induce a negative prediction error then potentiate an aversive response, extending the predictions of

AESOP. In the following experiment, we focused on bumblebees (*Bombus terrestris*) to investigate the effect of emotional cues on the acquisition of both short- and long-term habituation. To this aim, we have developed a new habituation-dishabituation paradigm (Experiment 4.1) and tested the associative nature of habituation in bumblebees (Experiment 4.2).

3.3.1 Subjects

Twelve colonies of bumble bees, *B. terrestris* were used for the full series of experiments (Experiment 4.1 included 30 bumble bees from 3 colonies; Experiment 4.2 included 52 bumblebees from 9 colonies). The colonies were raised in the Biobest® standard hives laboratory. All bees were identified by glueing numbered labels of various colours on the thorax. Bees were provided with an ad libitum supply of sucrose solution in the colony, except for the testing days, when foragers fed from plastic flowers within the testing arena.

3.3.2 Apparatus

The main apparatus was similar for both experiments. Each colony was housed in a wooden nest box (30 x 15 x 15, length, width, and height). The box was attached to a rectangular arena 30 x 30 x 11 cm, length, width, and height) by a clear plastic walkway (30 cm). The access to the arena was controlled by a series of 3 gates along the walkway to allow easy identification of bees. Three blue artificial flowers were located at the centre of the arena, containing 5 drops of 5µl 20% sucrose solution each. A black paddle (circumference = 15 cm) was automatically moved overhead by means of an Arduino® circuit, resembling a looming predator. The paddle moved from 0 to π radians and back, after a 1-second interval, at a speed of approximately 1 radian per second. The speed of the movement was chosen to maximize the bees' defensive response (see Figure 10, panel a and b).

In experiment 4.2 we added two cues preceding the habituating stimulus (Figure 10, panel c) and d). A group of bees received a simulated predator attack before entering the arena by means of a spoon trapping mechanism attached between the second and the third gate of the walkway. This mechanism simulated a predator attack by applying constant pressure to bees' thorax for 3-seconds by a stamp-shaped device connected to a micro-servo motor (see also, Solvi, Baciadonna, & Chittka, 2016). A different group of bees instead received a 5μl 60% sucrose solution in the same location. The complete experiment was recorded from above by a camera.

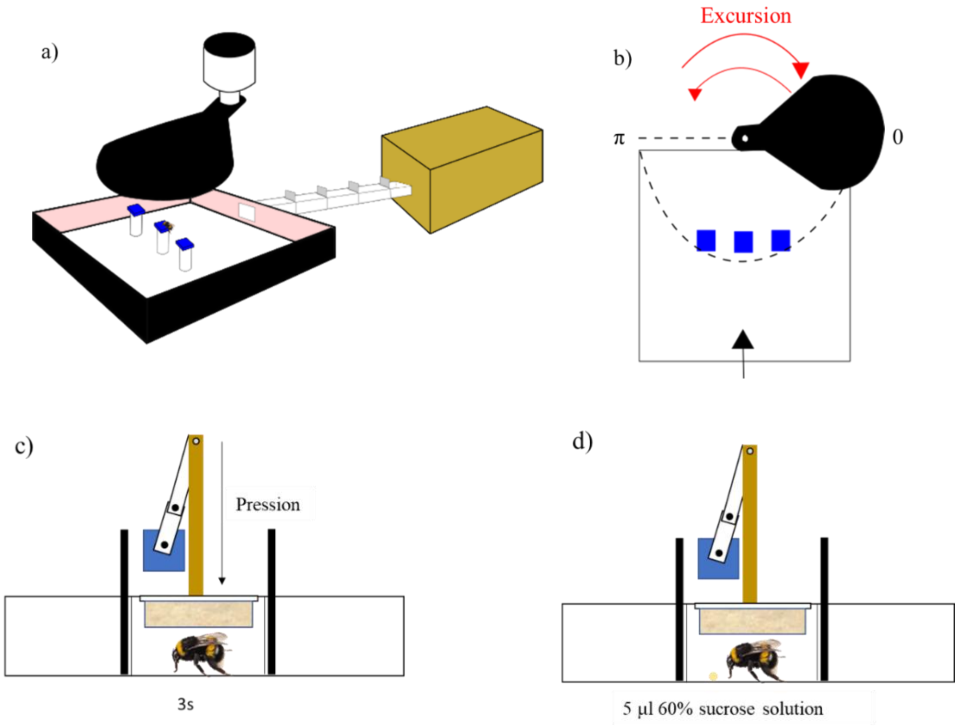


Figure 10. Panel a) represents the overall apparatus. A walkaway connected the nest box with the testing arena. In the testing area, bumble bees could forage from three blue artificial flowers at the centre. A paddle was automatically moved overhead by a servo motor connected to an Arduino circuit. Panel b) shows that the paddle moved from 0 to π radiant and back at a speed of approximately 1 radiant per second, projecting a looming shadow over the artificial flowers. Panel c) and d) represents the additional cues that

preceded the habituation test in Experiment 4.2. A spoon trapping mechanism controlled by an Arduino circuit applied constant pressure on the bee for 3 seconds (Panel c). A drop of sucrose was released in the same site as a positive reward.

3.3.3 Procedure

The main procedure was similar for both experiments. The day before the experiment, bees foraged in the arena to find the sucrose solution on the 3 plastic flowers. Once a bee foraged in a flower, it was number tagged for identification. Then, the bee was placed back in the colony and allowed to return to foraging. During the rest of the day, we noted the number of bouts and selected the most motivated foragers to be tested for habituation. To be selected, a bee had to feed on the flowers in at least 15 consecutive bouts.

In Experiment 4.1, we investigated habituation and dishabituation of bees' defensive response to the looming stimulus moving overhead. The habituation paradigm consisted of 5 blocks of 10 repetitions of the looming stimulus, with random ISI between 5 and 15 seconds. Each bee was tested individually. After each block, bees were placed back into their hive. A new block was started when the bee returned to the arena, usually after 5-10 minutes. Bees that took less or more time were excluded from the sample. Bees of the Dishabituation group received a dishabituator before the fifth block. The dishabituator consisted of a 3 seconds vibratory movement of the flowers. The vibration was strong enough to elicit a consistent escaping response without displacing the bee. As the bee returned to foraging from the flowers, the fifth block was started usually within 10 seconds from the end of the vibration. Bees of the Habituation group were also tested for long-term habituation on the following day (Day 1: Block 1 to 5; and Day 2: Block 6 to 10). For each stimulus repetition, we scored frame-by-frame the duration (ms) and type (escape, disturbance leg-lift, and startle) of defensive response elicited by the looming stimulus. The 3 types of defensive responses considered could be reliably distinguished based on both the sequence of movements involved and their duration. When escaping, a bee flew away from the flower and took approximately 1700 ms (sd = 922 ms) to return. We scored the time elapsed from the beginning of the flight to the moment it returned to one of the flowers. The disturbance leg-lift response (DLR, Varnon et al., 2021) consisted of the posturing of the bee when it raised one or both its middle legs to signal readiness to sting. We scored the time elapsed from the moment the bee raised its leg to the moment the bee lowered it, which usually took 459 ms (sd = 207 ms). For the startle response, we considered the beginning and the end of the rapid receding movement triggered by the stimulus (mean duration = 226 ms, sd = 204 ms).

In Experiment 4.2, we tested the effects of different contextual cues on the acquisition of both short-term habituation (Day 1: Block 1 to 5) and long-term habituation (Day 2: Block 6 to 10). We adopted the same habituation paradigm described above. But this time the bees received a simulated predator attack or an unexpected droplet of sucrose before each habituation block. The contextual cue could elicit an emotional response that was Congruent with the habituating stimulus in the case of the simulated predator attack, or Incongruent with the habituating stimulus in the case of the unexpected drop of sucrose. Bees were tested in the presence of the Same cue or the cue could be Different between Day 1 and Day 2. We obtained 4 different combinations summarized in Table 2.

For bees in a Same-Congruent group (N = 13) and in a Same-Incongruent group (N = 12) the habituation block was preceded by the simulated predator attack and the sucrose solution, respectively, on Day 1 and Day 2. Bees in Different-Congruent group (N = 14) received the simulated predator attack before each block of Day 1 and the sucrose solution before each block of Day 2. The order of the pre-test events was counterbalanced for bees in the Different-Incogruent group (N = 13). We scored the same measures as before as dependent variables.

Group	Cue preceding the looming stimulus	
	Day 1	**Day 2**
Same-Congruent (n = 13)	Predator attack	Predator attack
Same-Incongruent (n = 12)	Sucrose solution	Sucrose solution
Different-Conguent (n = 14)	Predator attack	Sucrose solution
Different-Incongruent (n = 12)	Sucrose solution	Predator attack

Table 2. The table above summarises the conditions. The first word in the label indicates that the same cue preceded the habituation test on both days (Same) or that the cue changed on Day 2 (Different). The second word indicates that the cue elicited a response that was consistent (Congruent) or inconsistent (Incongruent) with the aversive response elicited by the habituating stimulus.

3.3.4 Results

Experiment 4.1

Habituation and dishabituation of the proportion of defensive responses

To explore habituation of bumblebees' response to the stimulus, we focused on the first 4 blocks of stimulation (Block 1 to 4).

The results show that the overall proportion of each type of response was the same for the Habituation and the Dishabituation group (difference in proportion of the escape response: *Habituation – Dishabituation* = .066, W = 1777, p = .587; DLR response: *Habituation – Dishabituation* = .012, W = 1728, p = .790; startle response: *Habituation – Dishabituation* = .019, W = 1917, p = .175). We then pooled the two groups and analysed habituation of each behavioural response separately. The proportion of escape responses decreased from Block 1 to 4 (*Block 1 – Block 4 = .175, W* = 573, *p = .016*), while the proportion of the DLR was stable (*Block 1 – Block 4 = .009, W* = 457, *p = .878*). The number of startle responses increased with stimulus repetitions (*Block 1 – Block 4 = -.060, W* = 297, *p = .044*).

To test if the vibratory stimulus that preceded the fifth block induced dishabituation to the looming stimulus in the Dishabituation group, we compared the proportion of each response to the fourth and fifth block. Only the proportion of escapes increased in the Dishabituation group (difference in proportion of the escape response: *Block 4 – Block 5 =* -.192, *V* = 7, *p* = .023) and not in the Habituation group (difference in proportion of the escape response: *Block 4 – Block 5 =* -.033, *V* = 12, *p* = .799).

Habituation and dishabituation of the duration of defensive responses

We pooled the three types of responses and analysed the overall duration (milliseconds, *ms*) of bees' responsivity to the stimulus. All analyses were performed using robust statistics based on median estimators (Wilcox, 2011) to address the violation of normality assumptions of data. We used an overall median-based generalized linear model (fixed factors: Block, from Block 1 to Block 4; random factors: individual subjects) and pairwise two Wilcoxon and Wilcoxon signed-rank tests to demonstrate the presence of habituation to the looming stimulus.

As with the proportions, habituation was assessed by pooling the performance of the Habituation and the Dishabituation group as both underwent an identical stimulation. There was a significant main effect of the factor Block (*F(4) = 88.90, p < .001; Eta2* $_{(partial)}$ *= 0.20*). Bees significantly reduced the duration of their response from Block 1 to Block 4 (*Block 1 – Block 4* = 613.45 ms, *W* = 3672, *p* = .020).

Dishabituation was attested by an increment in the response duration from Block

4 to Block 5 for the Dishabituation group (*Block 4 – Block 5* = -373.81 ms, *W* = 51, *p* = .031). On the contrary, the Habituation group further decreased its response to the looming stimulus (*Block 4 – Block 5* = 291.92 ms, *W* = 171, *p* = .045) as depicted in Figure 11, panel a. This pattern of results reflected the response decrement in the duration of bees' response to the stimulus was an instance of habituation and is not due to motor fatigue or sensory adaptation.

Long-term habituation of the proportion of each defensive response

Long-term habituation was assessed by comparing the proportion of response of bees in the Habituation group between Block 5 on Day 1 and Block 6 on Day 2. None of the defensive responses recovered (difference in the proportion of the escape response: *Block 5 – Block 6* = -.112, *V* = 10.2, *p* = .214; DLR: *Block 5 – Block 6* = -.090, *V* = 37, *p* = .999; startle response: *Block 5 – Block 6* = .038, *V* = 68, *p* = .064; overall probability of response: *Block 5 – Block 6* = .035, *V* = 45, *p* = .873), attesting the presence of long-term habituation. The proportion of each response did not change during the following blocks (escape: *Block 6 – Block 10* = .112, *V* = 10.2, *p* = .214; DLR: *Block 6 – Block 10* = .015, *V* = 37, *p* = .999; startle: *Block 6 – Block 10* = .024, *V* = 68, *p* = .064; overall probability of response: *Block 6 – Block 10* = .017, *V* = 45, *p* = .873), attesting that bees reached their asymptotic level of response.

Long-term habituation of the duration of the proportion of response

Similarly, the duration of bees' defensive response did not recover from Block 5 to Block 6 (*Block 5 – Block 6 = 36.31 ms, V* = 136, *p = .545*) and there was no further decrement of bees' defensive response to the looming stimulus during the five blocks of Day 2 (*Block 6 – Block 10 = 124.23 ms, V* = 157, *p = .151*).

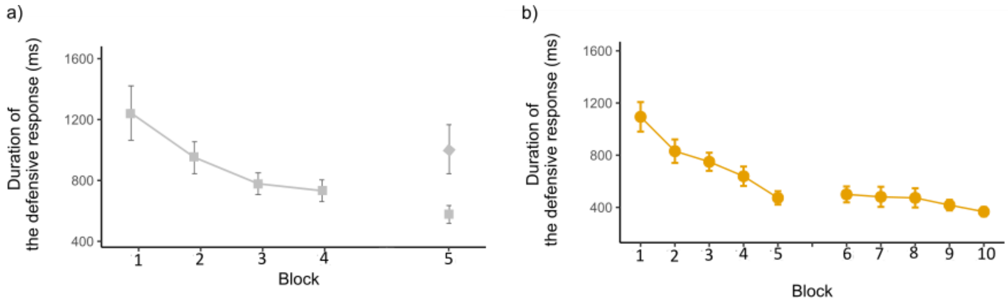


Figure 11. Panel a) shows the habituation curve of bumble bees (Block 1 to 4) and the dishabituation effect induced by the vibratory stimulus (Block 5). The y-axis reports the observed average duration of the defensive response per Block (x-axis). Panel b) depicts the habituation curve of the Habituation group on Day 1 (Block 1 to 5) and long-term habituation on Day 2 (Block 6 to 10). Error bars express 95% C.Is.

Experiment 4.2

Short-term effects of emotional cues on the defensive responses to the looming stimulus on Day 1

We analysed the effect of the emotional contextual cues on short-term habituation by grouping bees into Different-Congruent and Same-Congruent (Emotional Congruency group) and Different-Incongruent and Same-Incongruent (Emotional Incongruency group), because their testing condition on Day 1 was identical. We used generalized mixed linear models with the Block (from *Block 1* to *Block 5*) and Group (*Emotional congruency* and *Emotional Incongruency*) as fixed factors and estimated the random effects for each subject. We used non-parametric Wilcoxon (*W*) and Wilcoxon signed-rank tests (*V*) to compare the responsivity of bees in the two groups.

Proportion of defensive responses

Overall, the bees in the Emotional Incongruency group were more responsive than bees in the Emotional Congruency group (difference in the proportion of response:

Emotional Incongruency – Emotional Congruency = .159, $W = 3135$, $p < .001$). This group showed enhanced flight response (Emotional Incongruency – Emotional Congruency = .093, $W = 5733$, $p = .027$) and startle responses (Emotional Incongruency – Emotional Congruency = .068, $W = 3673.5$, $p = .002$). The proportion of escape and DLR responses decreased in both groups from *Block 1* to *5* (escape: $F(4) = 2.98$, $p = 0.021$; $Eta2_{(partial)} = 0.07$; DLR: $F(4) = 3.73$, $p = 0.006$; $Eta2_{(partial)} = 0.09$). But neither the factor Group nor the Group x Block interactions were significant.

The different reactivity of the two groups can be explained by other factors than the emotional incongruency between the response elicited by the cue and the habituating stimulus. For example, bees that received a sucrose solution could be more "aroused" by the reward which in turn could increase their general level of activity. To control this possibility, we compared how long it took the bees from the time they left the site where the cues were delivered at the time they entered the arena. The results showed that this time was the same (Emotional Incongruency - Emotional Congruency = .224 ms, $W = 425$, $p = .087$).

Duration of defensive responses

There was a significant main effect of the factors Block ($F(4) = 11.59, p < .001; Eta2_{(partial)} = 0.25$), but neither the factor Group nor the Block x Group interaction were significant. Habituation was significant for both groups (Emotional Incongruency: *Block 1 – Block 5* = 1542.312 ms, $V = 608$, $p < .001$; Emotional Congruency: *Block 1 – Block 5 =* 630.224 ms, $V = 353$, $p < .001$).

We further compared the performance of bees in the Emotional Congruency and Emotional Incongruency groups with the bees in Habituation group from the first experiment to test the possibility that both emotional cues may have facilitated their response to the habituating stimulus. Indeed, both groups increased the duration of their defensive response to the looming stimulus during Block 1 (Emotional Congruency - Habituation group = 1407.94 ms, *W* = 99, *p = .042;* Emotional Incongruency - Habituation group = 1738.65, *W* = 55, *p* < .001). Results are depicted in Figure 12, panel a.

Long-term effects of emotional cues on the defensive responses to the looming stimulus on Day 2

We analysed the effect of a change of contextual cues on habituation by pooling bees in the Same-Congruent and Same-Incongruent groups (Same Cue group) and Different-Congruent and Different-Incongruent groups (Different Cue group). With the former group, the contextual cues were the same on Day 1 and Day 2; they were switched in the latter group.

Recovery for individual conditions

We compared the duration of defensive response of Block 5 Day 1 and Block 6 Day 2 for each group. Recovery of the response was significant only for bees in the Different-Incongruent group (*Block 5 – Block 6* = -1501.602 ms, *V* = 33, *p* = .013), attesting that the disruptive effect of changing the contextual cues was greater for the group of bees the associated an emotional incongruent cue with the habituating stimulus.

Duration of defensive responses

Results on the duration of defensive responses show a significant effect of the Block (*F(4) = 3.58, p = 0.008; Eta2* $_{(partial)}$ *= 0.06*), the Group (*F(1) = 5.43, p = 0.021; Eta2* $_{(partial)}$ *= 0.02*) and the Block x Group interaction (*F(4) = 2.43, p = 0.049; Eta2* $_{(partial)}$ *= 0.04*). Bees in the Different Cue group responded longer to Block 6 of the stimulation than bees in the Same Cue group (Different Cue – Same Cue = 1859.164 ms, *W* = 422, *p = .003*), attesting that changing the contextual cues from Day 1 to Day 2 recovered the bees' responsivity to the looming stimulus. Habituation during the following blocks was significant only for the Different Cue group (*Block 6 – Block 10* = 1123.24 ms, *V* = 265, *p*

= .010). Results are depicted in Figure 12, panel b.

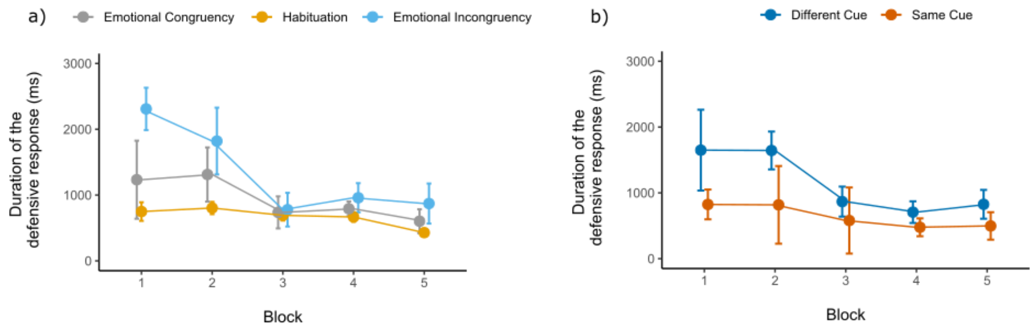


Figure 12. Panel a) shows the habituation curve of bumble bees (Block 1 to 5) in the Habituation, Emotional Congruency and Emotional Incongruency groups. The y-axis reports the observed average duration of the defensive response per Block (x-axis). Panel b) depicts the habituation curve of bumble bees that were tested in presence of the Same Cue or a Different Cue than Day 1 (Block 6 to 10). Error bars express 95% C.Is.

3.4 General discussion

The results of the two experiments (Experiments 3 and 4) show that contextual cues modulate the acquisition of habituation, supporting the associative nature of habituation predicted by Wagner. A lack of appropriate contextual cues restored animals' responsivity to the habituating stimuli when chicks were moved from the training to a different testing context (Experiment 3) and when bumble bees switched the cues that preceded the habituating stimulus between in the two experimental days (Experiment 4).

Experiment 3 shows that chicks *in ovo* can use the limited sensory experiences of the external world to build a unified context for the stimulus. Likely the context representations were limited to the ambient light or sound perceived through the eggshell, or the perceived temperature. Other factors like the hormonal, physiological and proprioceptive state may have played a major role as contextual cues. All these parameters changed after hatching, and particularly when chicks were moved from the egg in the incubator to the running-wheel. The results find a straightforward explanation in Wagner's (1976) model, but an alternative explanation must be carefully considered. The partial recovery of the defensive behavior observed could be accounted for by a general arousal effect or sensitization induced by the shift from a familiar context (e.g., the egg) to a new context (i.e., the running wheel) (Jordan et al., 2000). Enhanced arousal could have increased the general responsiveness of the animals, elevating also the freezing response (Groves & Thompson, 1970). To overcome this limitation, we controlled for the chicks' activity level before starting the test. A chick had to run for a minimum distance of 10 m to be tested. This gave them time to familiarize themselves with the running-wheel context. Chicks can imprint on sounds presented to the egg in the 48 hours before hatching (Impekoven, 1976). This supports the idea that chicks can recognize prenatal sound in the post-natal environment. Still, we found that chicks increased their freezing reaction to the habituating stimulus, suggesting that the disruption in the acquisition of long-term habituation was due to the lack of appropriate associative contextual cues.

Prenatal learning for specific discrete stimuli has already been documented in different taxa (Colombelli-Negrel et al., 2014; Reid et al., 2017), but evidence for context learning was unclear. Evidence of prenatal learning can be interpreted as cases of embryonic contextual learning (Ferrari et al., 2010; Lickliter & Hellewell, 1992). For example, wood frog embryos, exposed to predator cues in two fixed time windows during the day, use this temporal information after hatching to adjust their antipredator response in the same day periods (Ferrari et al., 2010). It is not clear, though, whether this remarkable ability of temporal learning in embryonic amphibians is an instance of temporal conditioning or an instance of temporal context learning. Here we have shown that prenatal context learning is not restricted to temporal information. It was the same for all our groups of chicks and could not account for the results. Our study with chicks showed that prenatal cognition extends to the ability to automatically encode the complex ensemble of background stimuli.

A more straightforward demonstration of the associative nature of habituation stems from Experiment 4. Here, as in Experiment 3, we introduced two discrete contextual cues that preceded the habituating stimulus in a training and testing phase. These cues elicited emotional responses that could mimic or oppose the aversive response of the habituating stimulus. In the latter case, it induced a negative prediction error.

According to the AESOP model (Brandon & Wagner, 1989), the emotional representation of a target stimulus can be associatively primed by an emotional congruent representation of a cue. With priming, the emotional representation modulates a separate conditioned emotional response that increases the activity in the sensory nodes of the target stimulus resulting in response potentiation. By contrast, the representation of an emotional incongruent cue cannot prime the emotional representation of the target stimulus. The response would decrease quicker as the sensory representations of the target stimulus come to be fully predicted by the cue. A separate conditioned emotional response superimposed to be evoked by a stimulus is supported by several experiments. But the prediction of AESOP cannot fully explain our data. Bees in the Emotional Congruency group increased their response to the looming stimulus compared to the neutral Habituation group. Bees in the Emotional Incongruency group enhanced their responsivity to a greater extent, attesting that the sucrose solution facilitated the aversive response to the looming stimulus. This result may be consistent with the effect of a negative prediction error that potentiated the aversive response to the looming stimulus. However, before exploring this possibility it is important to rule out other simpler explanations of the result.

Maybe the rewarded bees were more excited by the sucrose solution which might have increased their general level of activity. To address this point, we compared the walking speed of bees from the moment they left the site where the cues were delivered to enter the arena in both Emotional Congruency and Emotional Incongruency group. Remarkably, the walking speed was the same. A previous study by Solvi, Baciadonna and Chittka (2016) on bumble bees further supports the conclusion that our cues did not affect bees' activity. The authors trained bumble bees to associate stimulus A with a reward (CS+) and stimulus B with the absence of the reward (CS-). A stimulus intermediate between A and B was presented. This stimulus did not elicit any CR in bees. If bees received an unexpected drop of sucrose before the test, they were more likely to approach the stimulus than an unrewarded group of bees. The authors considered the possibility that sucrose may have simply aroused the rewarded bees, resulting in a quicker choice of novel alternatives. To address this point, they measured two additional variables, namely the speed of flight and the thorax temperature of tested bees. The latter measure is a positive predictor of the foraging motivation of bees (Sadler & Nieh, 2011). Similar to our results, they did not find any evidence of a different level of activity between groups of bees, although rewarded bees had a significantly higher thoracic temperature reflecting an increased appetitive drive.

Our bumble bees were not differently aroused by the pre-test events. It may be useful to compare our experiment with others that reported a similar mismatch between the response elicited by the contextual cues and that evoked by a habituating stimulus. For example, Siegel (1977) demonstrated that rats injected with morphine in a specific context developed a context-specific tolerance for that drug (tolerance be an instance of habituation to the effect induced by morphine itself). Siegel suggested that environmental cues regularly paired with the administration of morphine elicit a compensatory reaction that increases rats' sensitivity to pain (hyperalgesia). The narcotic required higher doses to sort its analgesic effects. As a result of the conflicting pattern of responses elicited by contextual cues and morphine, the drug tolerance increased. Siegel (1977) demonstrated there is an association between the contextual cues and morphine by testing for tolerance context-specificity, context-extinction, and latent inhibition of the context. Wagner and Vogel (2010) interpreted the compensatory CR to contextual cues in support of their hypothesis that when a CR opposes the response the US wants to evoke, there is a decrease in the UR. It seemed that the emotional mismatch between contextual cues and morphine had reduced rats' analgesic response to morphine. However, the compensatory CR elicited by contextual cues developed over time attests that rats learn the associative link between the two stimuli. As the associative model was consolidated in memory, the associative strength of morphine decreased as the contextual cues could reliably predict its occurrence. The observed decrement of the analgesic effect was simply explained with the priming theory proposed by Wagner (1976). By assuming the contextual cues primed the morphine representation in STM, it prevented other representations of the same stimulus to elicit the analgesic effects again. In our experiment, we concentrated on response facilitation. The facilitation induced by the emotional conflict between the appetitive response elicited by the sucrose solution and the aversive reaction to the habituating stimulus. We found it arose from the first block of stimulation, namely when bumble bees had just started to learn the contingency between the events. Our results reveal that a different emotional mechanism affected bumble bees' responsivity before they consolidated a memory model of the two events.

Under the prediction error hypothesis (Den Ouden et al., 2012; den Ouden et al., 2010), when the outcome of an event is different from what is expected, the mismatch is interpreted as a prediction error from the brain, that modulates the organisms' reactions. An expected event elicits a weaker response than the previous case because the prediction error signal is reduced.

For an unexpected aversive outcome, the negative prediction errors determine the effectiveness of the stimulus in evoking an aversive response and supporting aversive learning (Li & McNally, 2014; McNally et al., 2011). When an aversive outcome is predicted by proper aversive cues, the intensity of the aversive response evoked is reduced but still significant. Previous studies show that a fear state potentiates the following of aversive response to stimuli (Davis, 1986, 2006). This could be due to the superimposition of two similar emotional responses as suggested by the AESOP (Vogel et al., 2019; Wagner & Brandon, 1989). There is also evidence that the facilitation of aversive responses under fear is driven by increased negative prediction signals in the brain (Robinson et al., 2013). In other words, in fear situations, the brain automatically increases the negative prediction error signals that facilitate aversive reactions.

It must be acknowledged that an important difference between our paradigm and that commonly used in fear-potentiated experiments (Davis, 1986, 2006) concerns the type of cues that preceded the fear response. Fear-potentiation is usually elicited by pairing a neutral cue (CS) with an aversive US. Instead, in our study, both the sucrose reward and the predator attack had an intrinsic biological value and represented two USs. Still, their role of contextual cues was justified by the fact that both of them are known to trigger the expectation that some event will follow biasing bees response to the upcoming stimulus (Solvi, Baciadonna & Chittka, 2016).

Bumble bees in the Emotional Incongruency group showed a stronger aversive reaction to the habituating stimulus than the other group. They were rewarded before starting the habituation test, then the looming stimulus elicited a stronger negative prediction error that could increase their responsivity to a greater extent than the Emotional Congruency group. The Emotional Congruency group, in turn, responded stronger than the neutral Habituation group, in agreement with the fear-potentiated effects reported in the literature (Davis, 1986). This result must be interpreted with caution given that bees of the two groups were tested as part of two separate experiments. Despite the two experiments took place within the same month, in the same arena and involved bees selected from random colonies, several uncontrolled factors might have changed meanwhile (e.g., the seasonal dark:light cycle).

Explaining our pattern of results based on response potentiation agrees with the prediction of classical comparator models of habituation (Sokolov, 1960; Wagner, 1976). That is, the magnitude of the response that a stimulus evokes is determined by how unexpected the stimulus is. This account may conciliate opposite views on the role of stimulus predictability in habituation. While several studies assume that habituation develops as the stimulus is fully predicted (Sokolov, 1960; Wagner, 1976), others have emphasized that habituation relies on what a stimulus predicts (Hall & Rodrguez, 2017; Hall & Rodríguez, 2020). Our approach was based on a clear distinction between the emotional response elicited by the cue and the habituating stimulus and may help to address this question. The results of our study suggest that both aspects may have coexisted in our preparation. The cues that we used played a pivotal role in generating the prediction error as suggested by Pearce and Hall (Pearce & Hall, 1980). But the response to the habituating stimulus then progressively decreased as it came to be fully predicted by these same cues in the following blocks of stimulation as suggested by Wagner and Sokolov (Sokolov, 1960; Wagner, 1976).

In principle, there is a second explanation that might fit our results. One might argue that the sucrose reward and the simulated predator attack might have equally facilitated the response to the habituating stimulus. After the facilitation, a refractory-like effect might have occurred in the Congruent group following a self-generated priming of the habituating stimulus, as predicted by SOP. The simulated predator attack might have activated in the bees of the Congruent group some elements in the node representing the aversive looming stimulus, since both share an aversive predatory nature. The self-generated priming of the looming stimulus might have partially prevented this group of bees from a full response to the looming stimulus. This second explanation cannot be fully disentangled by our experimental design, but does not change the conclusion that bees can use the hedonic value of stimuli to make predictions about future events.

The effect of the cue on the acquisition of long-term habituation was tested on the second experimental day (Day 2). The results reveal that the manipulation of specific cues in this experiment paralleled the context-specific effect induced by changing the broad context in Experiment 3. Switching the cues on Day 2 restored the bumble bees' response to the habituating stimulus as compared to the bumble bees we tested in the presence of the same cue. Our result supports the evidence of context-specificity of habituation and attests that changing specific emotional cues can yield the same effects that could be obtained by broader contextual changes. An analysis of the individual conditions revealed that this effect was driven by bees that were trained in the presence of the sucrose solution and then switched to the simulated predator. While this group-specific effect must be interpreted with caution given the reduced sample size, this result may not be surprising in light of the prediction error hypothesis. Prediction errors have also been proposed to signal the salience of cues (Spratling, 2008). The salience cue is determined by how unexpected its outcome is. Salience increases the associative strength of the cue, facilitating the memory associations with other stimuli (Pearce & Hall, 1980). The negative prediction error of the sweet cue was probably greater than in the other conditions, so the salience and associative strength of this cue may have also increased, promoting stronger memory associations with the looming stimulus during Day 1. The lack of this cue on Day 2 may have impaired the acquisition of long-term habituation to greater extent.

This experiment raises several important questions. Does context-specific habituation arise from a change of specific contextual cues or is this possibility limited to biological relevant cues? How do bumble bees encode the negative prediction error? In animals, dopamine is the main biogenic amines involved in error processing (Schultz, 2016); it may have a similar role in insect brains. Solvi, Baciadonna and Chittka (2016) found that blockade of dopamine receptors in bees abolished the positive interpretation of the ambiguous stimulus induced by the unexpected drop of sucrose. Dopamine may also be involved in coding the emotional representation of stimuli.

Chapter 4

Individual and species-specific differences in learning for domestic chicks and an invasive crayfish (*Procambarus clarkii)*

Part of the material in this chapter has been adapted from the following paper:

Dissegna, A., Caputi, A., & Chiandetti, C. (2020). Long-lasting generalization triggered by a single trial event in the invasive crayfish *Procambarus clarkii. Journal of Experimental Biology*, 223(*22*).

Some individuals take longer to habituate than others. In humans, this variability has been linked to personality traits (Blanch et al., 2014; LaRowe et al., 2006; O'Gorman, 1977; Súilleabháin et al., 2018). For example, individuals with high levels of neuroticism take longer to habituate to fearful stimuli (Mangan & O'Gorman, 1969), which likely reflects increased vigilance. A link between personality and habituation has also been demonstrated by a recent study on baboons (*Papio ursinus griseipes*) (Allan et al., 2020). The authors found that two measures of habituation to a human observer – i.e., visual tolerance and flight initiation distance - correlated strongly within individuals and between contexts and time, suggesting that habituation can represent a personality trait itself that affects individual reactions to novelty. Aside from primate studies, research on inter-individual differences in habituation has focused on profiling good and bad habituators according to the proactivity of their behaviour (Carere & Locurto, 2011; Mazza et al., 2018; Verbeek et al., 1994). Along a proactive-reactive continuum of animals' behaviour, bolder, exploratory and aggressive individuals are usually the first to approach novelties, thus they are expected to habituate to new stimuli quicker than shyer, reactive individuals. However, this hypothesis has received little support. Two studies on lizards have addressed this topic. The first study failed to demonstrate that boldness correlated with habituation to a dummy predator (Rodríguez-Prieto et al., 2010) but revealed that habituation was positively associated with individuals' body size. The second study demonstrated an association between habituation and exploratory tendency of lizards (Rodríguez-Prieto et al., 2011), showing also that habituation was uncorrelated with boldness and sociality. Hence, the authors concluded that individual differences in habituation may be linked to the risk-assessment ability of an individual, a broader trait that may underly both habituation to new stimuli and exploration of a new environment (Verbeek et al., 1994).

The discussed evidence indicates that habituation is linked to the behavioural predispositions of individuals. But what is the origin of this association? Since habituation depends on prior experience with similar stimuli, an intuitive explanation would be that different life experiences affect both habituation and behavioural differences of individuals. For example, habituation to a new environment may prompt its exploration, and a positive outcome of this activity – e.g., the discovery of new resources - would reinforce an animal's exploratory behaviours, resulting in quicker habituation to new environments (Del Giudice & Crespi, 2018; Sih & Del Giudice, 2012). On the other hand, genetic studies (Glowa & Hansen, 1994; Lundgren et al., 2021; Sorato et al., 2018), have demonstrated that both habituation and behavioural predispositions of animals are heritable. This suggests that their association is rooted in the gene pool of an individual and would arise from the early development of organisms and be partly independent of their life experiences.

Recent interest regarding the interplay between the expression of specific behavioural traits and individual differences in learning processing like habituation have reached other fields of research, from evolutionary psychology to behavioural ecology. A fertile application of this research area comes from the study of the invasive success of several species of animals. Invasive species can quickly adapt their behaviour to a wide range of environmental conditions to displace native populations. Increasing evidence attests that many invasive species share similar behavioural traits (Chapple et al., 2012; Sih et al., 2012). Much of it shows enhanced inter- and intraspecific aggressiveness, which in turn is positively associated with boldness, foraging ability and exploratory activity (Duckworth & Badyaev, 2007; Pintor et al., 2008; Rehage & Sih, 2004).

Behavioural flexibility is an important trait for invasive species (Pintor et al., 2009; Sol, 2003; Wright et al., 2010). If animals can flexibly adapt their behaviour to new environments, they can easily survive dispersal and establishment in new territory (Wright, 2010). Behavioral flexibility of invasive species has been linked to several complex behaviours such as social learning or innovation (Audet & Lefebvre, 2017), but so far their presence has been documented in a limited range of animals (e.g, see Brosnan & Hopper, 2014; Caldwell & Whiten, 2002). Innovation and social learning may represent complex behavioural traits entailing other simpler processes (Griffin & Guez, 2014; Heyes, 2012). Hence, other basic mechanisms may have a more direct effect on behavioural flexibility.

Generalization could be a valuable candidate mechanism for this role. It attests the animal's capacity to use past solutions in present situations regarded as similar. It is universal for all animals and is independent of the context, the stimulus-modality or the type of response (learned or innate) (Ghirlanda & Enquist, 2003). Generalization is also a critical test for habituation, as it is used to distinguish habituation from a more general sensory adaptation or motor fatigue (Rankin et al., 2009). But habituation is stimulus-specific and some degree of generalization may occur for stimuli sharing similar features (Gati & Ben-shakhar, 1990). Generalization may be an effective strategy to suppress the response to unfamiliar stimuli that resemble innocuous stimuli in the past.

The experiments of this chapter explore differences in information processing and habituation between individuals and species.

4.1 Experiment 5

In this experiment, we studied the correlation between body size, exploratory activity, boldness, social reattachment and habituation of individual chicks. All the chicks were tested two and three days after hatching under complete experimental control over pre- and post-natal experiences. A correlation between habituation, exploratory activity and body size has been demonstrated in adult lizards (Rodríguez-Prieto et al., 2010, 2011). If we had to find the same covariation in young inexperienced chicks, then the association between habituation, exploratory activity and body size may be rooted in an individual's gene pool.

4.1.1 Subjects

We tested 104 domestic chicks (females = 51) of the Ross 308 strain (Aviagen). Chicks hatched in our laboratory at a temperature of 37.7°C and humidity of 50-60%. On the hatching day, they were housed in individual cages (22 x 30 x 40 cm, width, height, depth) with a red cylinder hanging from above (6 x 7.5 cm, diameter, height) as imprinting object. Chicks had full access to food and water from within their cages. The illumination of the room followed a 12:12 dark:light cycle and the temperature was kept at 31.5°C.

4.1.2 Methods

(b) Behavioural assays (Day 1)

The behavioural tests were conducted when chicks were 2 days of age. The arena consisted of a white square (40 x 40 x 40 cm, width, height, depth) divided in 2 chambers by a transversal plastic wall (polionda ®). The two chambers communicated through a sliding door, that was manually controlled by the experimenter. A set of 16 black cones was placed on the floor of each chamber. The whole experiment was recorded by two cameras located 30 cm above the chicks' head. The chicks' movements were tracked using a custom made opencv-python script. We focused on three main behavioural domains: exploration, boldness, and social reattachment. The set-up changed throughout the behavioural assays to test all three domains in a single trial.

(i) Assay I: Exploratory activity

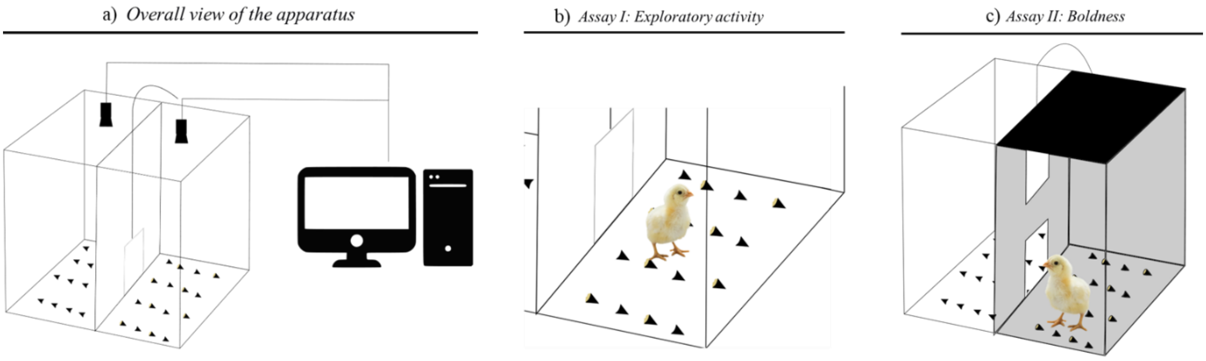
Chicks were gently moved in one of the two chambers of the arena using a cylindric box. Then, they were left free to explore the new environment. Half of the cones on the floor hid food and chicks could feed on cones as they explored the chamber. We measured the latency (s) to move the first step in the chamber, the latency (s) to peck the first cone, the time (s) spent freezing, the time (s) spent pecking at food, the proportion (%) of cones hiding food found and the amount of the chambers explored (%). This first phase lasted 5 minutes. The layout of the experimental arena is depicted in Figure 13 panel b.

(ii) Assay II: Boldness

After the end of the first assay, the first chamber of the arena was covered with an opaque plastic roof. After a minute break, the sliding door separating the two chambers was removed, letting the chicks move to the unfamiliar chamber. We measured the latency (s) to protrude the head from the sliding door and the latency (s) to leave the familiar chamber with the whole body. This second phase lasted for a maximum of 10 minutes. The experimental set is depicted in Figure 13 panel c.

(iii) Assay III: Social reattachment

At the beginning of this phase, the chick was restrained in an opaque cylinder and moved to one corner of the second chamber. Then, both the roof of the first chamber and the plastic wall separating the two chambers were removed. An imprinting object was hung from above on the opposite corner of the chick. We measured the latency (s) to reattach with the familiar object (touch it with the body) after the chick was released. A ceiling was set at 5 minutes for this task. Thereafter, the chick was restrained again with the opaque cylinder and placed on a different corner of the arena. The imprinting object was then moved to one of the two corners perpendicular to that of the chick and an unfamiliar blue rectangular object (8 x 12 x 3 cm, width, height, depth) was hung on the opposite corner. The chick was equidistant from both the imprinting object and the unfamiliar object. Once the chick was released, we measured the latency (s) to reach the imprinting object, the latency (s) to reach the unfamiliar object and a proximity index computed as $I = \frac{dU-dI}{dU+dI}$, where dU is the distance from the unfamiliar object and dI is the distance from the imprinting object. This index ranged from – 1 to +1 and positive values indicate a greater distance from the unfamiliar object, i.e., greater levels of social reattachment. A time limit was set at 5 minutes. The setup of this last behavioural assay is depicted in Figure 13 panel d).



a) *Overall view of the apparatus*

b) *Assay I: Exploratory activity*

c) *Assay II: Boldness*

d) *Assay III: Social reattachment*

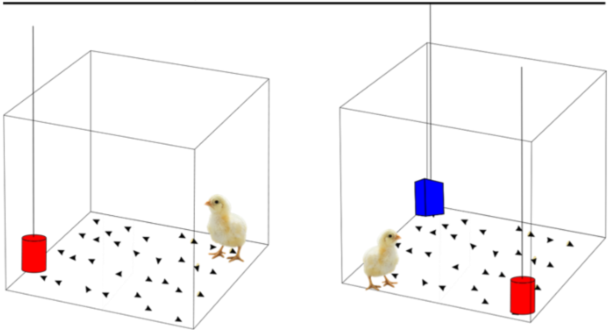


Figure 13. Panel a) represents the modular arena. It consists of two chambers divided by a removable wall and a sliding door. Sixteen cones are placed on the floor of each chamber. The chicks are recorded by two cameras on top of the arena and their movements are tracked by a custom opencv-python program. Panel b) shows a chick in the starting chamber for Assay I. Half of the cones on the floor hide food and the chick have to actively explore the entire chamber to forage. In panel c) the starting chamber is covered reducing the light within. Then, the sliding door is removed, allowing the chicks to move to the second chamber (Assay II). In panel d), the separating wall is removed. The red imprinting object is first presented to the chick. Then, a blue rectangular object is introduced to compare the chick's social reattachment in the presence of an unfamiliar object.

(c) Habituation test (Day 2)

Chicks were placed within a running wheel (30 cm in diameter) located on one of the short sides of a black rectangular arena (45 x 50 x 160 cm, width, height, depth) (see, Chiandetti & Turatto, 2017). Chicks were motivated to run on the wheel attempting to reach their imprinting object. Only chicks that ran for a minimum distance of 10 m within 5 minutes on the wheel were tested. Two loudspeakers played the habituation stimuli at

30 cm above the chick's head.

Chicks were randomized in two habituation conditions before the test. In one condition, the habituation stimulus consisted of a hen cluck (730 ms, 90 dB SPL); in the other, the habituation stimulus was a rooster alarm call (730 ms, 90 dB SPL). In both conditions, the habituation stimulus was repeated 10 times (Trials 1 to 10) at pseudo-random intervals ranging from 30 s to 60 s. The time, distance and direction of the chicks' run were displayed by an *Arduino* circuit. We measured the number of times each chick froze in response to the stimulus and the freezing duration (s). We recorded the entire experiment through a video camera.

4.1.3 Data analysis

First, we inspected the density distributions of behavioural differences. The variables displaying a bimodal distribution were transformed into categorical variables (i.e., the latency to peck the first cone and the proportion of area explored (Assay I), the latency to protrude the head and the body (Assay II) and the proximity index (Assay III). The remaining latencies were log-transformed because positively skewed (i.e., the latency to move the first step (Assay I), the latencies to reach the imprinting object and the unfamiliar one (Assay III)).

A principal component analysis (PCA) was conducted on a mixed correlation matrix (i.e., Pearson correlations for the continuous variables (r), tetrachorics (rtet) for the dichotomous items, and the biserial (rpb) correlations for the various mixed variables) between the 11 variables resulting from the behavioural assays. The resulting components were rotated according to a promax procedure. Factor scores were assigned to each chick using the regression method. Again, we replaced factor scores with a bimodal distribution with categorical variables. We analysed the correlation between chicks' factor scores, sex, and weight (g), using robust correlation coefficients and t-test on maximum-likelihood estimator differences. In order to analyse whether behavioural differences had affected the likelihood of a chick reaching the criterion to begin the habituation test (2 categories: Not Run; Run) and to complete the test (3 categories: Not Tested; Tested) we used a proportion z-test, with s.e. = $\sqrt{\frac{\pi_{H0}*(1-\pi_{H0})}{n}}$, where π_{H0} represents the proportion of cases in each category.

We estimated the latent intercept and habituation rate for chicks that completed the habituation test (n = 46) using latent curve model (LCM). We fitted two models: a liner model with the 10 Trials as observed indicators, and an intercept and a linear slope as latent growth factors; and a quadratic model which also included a quadratic slope as an additional latent factor. The intercept represented the initial duration of the freezing response of chicks; the linear slope missing verb here the monotonical habituation rate, and the quadratic slope accounted for the change of the monotonical rate over Trials (20). The model with the quadratic slope fitted our data significantly better than the model with a linear slope alone (χ2diff [4] = 48.82, p < .001). Therefore, we focused our analysis on this model. We also merged the two stimuli conditions (hen cluck vs rooster alarm call) because there was no significant difference in the slope and intercept of the resulting habituation curves (p = 0.641 for the intercept; p = 0.374 for the slopes). Thus, we tested if differences in chicks' body size (weight) and behaviour (factor scores) predicted their habituation rate. All the analyses were carried out in R 3.6.2. The PCA was carried out using the command principal() on a mixed correlation matrix obtained using the mixedCor() function (both part of the "psych" package); for robust two-samples tests and correlation coefficients we used the "WRS2" package; for LCM we used the "lavaan" package; for the robust regressions we used the "MASS" package.

4.1.4 Results

Based on Keiser's criterion, 3 components were retained from the PCA which in combination explained 70% of the variance of the observed variables. The variables clustered accordingly to the three assays suggest that component 1 represents chicks' level of Exploratory Activity, component 2 their level of Boldness, and component 3 their level of Social Reattachment.

(a) Smaller chicks are more exploratory and bolder than larger ones

There was a positive correlation between factor scores of Exploratory Activity and Boldness ($r_{tet} = 0.31$) and between scores of Exploratory Activity and Social Reattachment ($r_{pb} = 0.32$), but not between Boldness and Social Reattachment ($r_{pb} = 0.10$). Smaller chicks showed greater levels of Exploratory Activity ($t = 3.09$, $p = .036$; $d = .46$) and Boldness ($t = 2.74$, $p = .030$; $d = .40$) than *larger* chicks (see, Figure 14). Chicks' level of Social Reattachment was independent of their weight. There were no significant differences in behaviour between males and females.

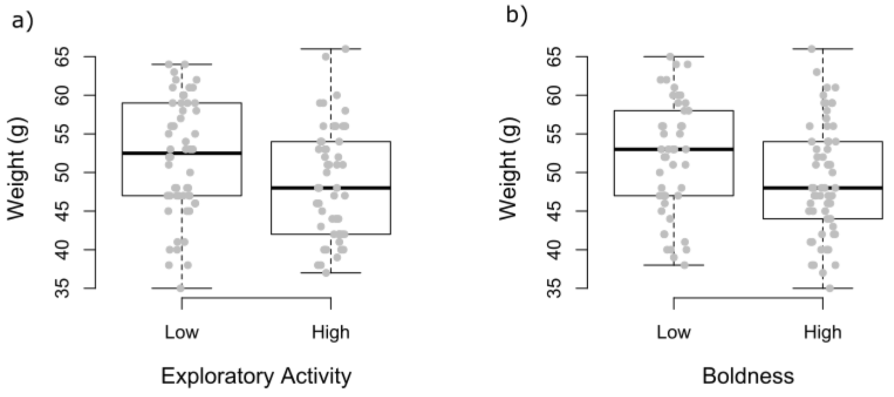


Figure 14. Boxplots for chicks' weight distribution according to their level of Exploratory Activity (panel a) and Boldness (panel b). The categories (Low vs High Exploratory Activity and Low vs High Boldness) refer to chicks' factor score relative to the sample average.

(b) Bolder chicks are more likely to complete a running-wheel test

Chicks with a higher level of boldness were more likely to run on the wheel (n. chicks that run on the wheel = 62, prop. of chicks with a high level of boldness running on the wheel: $\pi = 38/62 = 0.61$, $p = .019$). Bolder chicks were also more likely to complete the habituation test (n. chicks that concluded the habituation test = 43, prop. of chicks with a high level of boldness that completed the habituation test: $\pi = 26/43 = .60$, $p = .042$). The other behaviours and chicks' weight did not affect their probability to run on the wheel.

(c) Exploratory Activity and Body Size effects on chicks' habituation

Overall habituation was attested by a significant decrement of the duration of the chicks' freezing from Trial 1 to 10 ($a = 19.29$, $se = 2.345$, $p < .001$; $b_{lin} = -4.57$, $se = 0.683$, $p < .001$; $b_{quad} = 0.32$, $se = 0.053$, $p < .001$) (see figure 15, Panel a). This result suggests that the mean freezing response of the chicks to the first trial was 19.30 s, with a monotonic decrement of −4.573 s every Trial. The positive quadratic slope of 0.32 suggests that the decrement became flattered at each Trial, namely that animals' learning curve was approaching an asymptotic level. There was a positive correlation between the duration of the initial freezing and the absolute value of the linear slope ($r = -.67$; $p < .001$, Fig 14, panel b). The variance around both the intercept and the linear slope was also significant ($\sigma^2_{\text{int}} = 177.189$, $p < .001$; $\sigma^2_{\text{lin}} = 9.865$, $p = .029$) indicating individual differences in the initial response to the stimulus and the habituation rate.

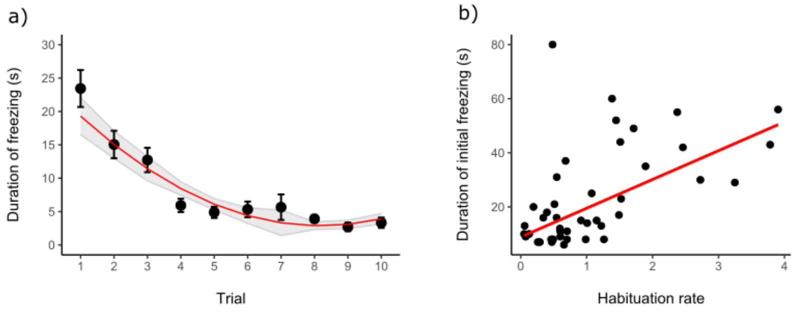


Figure 15. Panel a) shows the habituation curve of chicks. Dots represent the observed average freezing duration (y-axis) per Trial (x-axis). The red line represents the estimated relationship using LCM. Error bars express the S.E.M. Panel b) depicts the relationship between the duration of the initial freezing of chicks and their habituation rate. The habituation rate is expressed in absolute values.

(i) Both Exploratory Activity and Body Size are associated with chicks' initial freezing

Table 3 summarizes the regression models of the relationship between chicks' body size, their behavioral differences, and the duration of the first freezing response to the habituation stimulus. More exploratory chicks stopped significantly longer than less exploratory ones. Similarly, larger chicks stopped significantly longer than smaller chicks.

(ii) The Exploratory Activity x Body Size interaction explains chicks' habituation rate

A set of regression models (Table 3) was run to determine if behavioural differences and body size explained chicks' habituation rate. The effect of the Exploratory Activity on the habituation rate depended on the chicks' Body Size. The interaction plot in figure 16, shows a positive relationship between Body Size and habituation rate only among the most exploratory chicks.

	Initial freezing			***Habituation rate***		
	B	**C.I.**	***p***	**B**	**C.I.**	***p***
Exploratory Activity	0.71	0.16 - 1.26	**.013**	0.57	0.05 – 1.10	**.033**
Boldness	-0.10	-0.65 - 0.45	.709	0.09	-0.43 – 0.62	.716
Social Reattachment	-0.06	-0.44 - 0.12	.258	-0.10	-0.37 – 0.17	.442
Weight	-0.44	0.18 - 0.70	**.001**	0.09	-0.28 – 0.46	.622
Exploratory Activity X Weight				0.61	0.12 – 1.11	**.017**

Table 3. Parameter estimates of the two robust regression models for the Initial freezing and the Habituation rate.

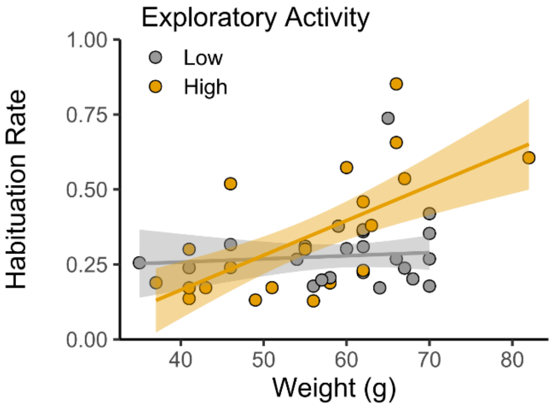


Figure 16 shows the relation between chicks' Body Size, level of Exploratory Activity and habituation rate (*i.e.*, the slope of individuals' habituation curve). The categories (Low *vs* High Exploratory Activity) refer to chicks' factor scores compared to the sample average. The habituation rate is expressed in absolute values. The shaded areas represent S.E.M. The Body Size was positively associated with chicks' habituation rate only in more exploratory chicks.

4.2 Experiment 6[1]

The previous experiment has shown that individual differences in habituation correlate with exploratory activity and body size of inexperienced chicks. This suggests that genetic factors can account for individual differences in habituation. In the next

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[1] *My sincere thanks to Andrea Caputi for collecting the data for this experiment.*

experiment, we studied generalization in the invasive crayfish *Procambarus clarkii*. Generalization is a basic form of learning linked to the superior behavioral flexibility of invasive crayfish (Hazlett et al., 2003). Here, we developed a paradigm to study generalization in *P. Clarkii* by means of a habituation protocol.

4.1.1 Subjects

Red swamp crayfish (*P. clarkii*) (n = 14, m = 3) were collected from an artificial pond called “Bonifica del Brancolo” (45°46' N, 13°30' E, GO, Italy) and transported to our laboratory. On their arrival, the Crayfish were housed in individual plastic tanks (10 x 14 x 12 cm) filled with clean water. The walls of the tanks were opaque to limit their visual experience with the external environment. Illumination was provided following a 12:12 dark:light cycle and water temperature was kept constant at 21°C. Animals’ rostrum to telson length ranged between 9.06 cm and 11.05 cm (mean = 9.94 cm, S.E.= 0.09 cm).

4.1.2 Apparatus

Crayfish were tested within an opaque rectangular arena, as described in Chiandetti and Caputi (2017). An LCD flat screen illuminated the inside of the arena with a white diffused light. On the opposite side, two vaporizers were attached to the wall at about 20 cm from the floor. The arena was surrounded by a black curtain hanging from above to further isolate the tank from the external environment. The experiment was recorded at a frame rate of 29 fps by a camera placed above the arena.

4.1.3 Procedure

Crayfish were tested in 3 individual sessions (Session 1, Session 2 and Session 3) at 15 and 30 days from the first session (Day 1, Day 15, and Day 45). Crayfish could freely explore the new environment at the beginning of each session for 10-minutes. Then, they were placed at the centre of the arena and confined in a clear cylinder. The habituation test consisted in 5 repetitions of the same stimulus – i.e., the habituation stimulus (trials 1 to 5, 7 to 11 and 13 to 17) - followed by a novel stimulus (trials 6, 12 and 18). The habituation stimulus was a jet of vaporized water (VAP) sprayed whenever the crayfish actively explored the illuminated area; the novel stimulus was a direct jet of water (JET, 1 second). The stimulation was manually delivered by the experimenter and never directed toward the crayfish's body. We scored the duration of Crayfish' defensive response - number of frames *per* second – as defined in Chiandetti and Caputi (2017).

4.1.4 Results

Results are shown in Figure 17.

Session 1: Crayfish' response to the VAP stimulus changed from trial 1 to 5: $\chi^2(4)$ = 15.8, *p* = .003, BF_{10} = 4.70. The response was shorter on trial 5 than on trial 1: *mean difference* = 13.3±3.24 frames, *W* = 96, *p* = .007, *d* = 1.07, BF_{10} = 27.42. Stimulus specificity was attested to by a significant increment in crayfish response to the JET stimulus: trial 5 *vs* trial 6: *mean difference* = -11.7±5.97 frames, *W* = 15, *p* = .036, *d* = -.60, BF_{10} =1.83.

Session 2: Crayfish recovered their response to the VAP stimulus when tested after 15 days, trial 5 *vs* trial 7: *mean difference* = -10.8±3.94 frames, *W* = 18, *p* = .033, *d* = -.61, BF_{10} = 1.86. They decreased their response to the following repetitions of the stimulus: $\chi^2(4)$ = 14.4, *p* = .006, BF_{10} = 10.44; trial 7 *vs* trial 11: *mean difference* = 13.7±4.17 frames, *W* = 67, *p* = .031, *d* = .65, BF_{10} = 2.37. This time, crayfish generalized the response decrement to the JET stimulus: trial 11 *vs* trial 12, *mean difference* = 3.33±5.20 frames, *W* = 58, *p* = .40, *d* = .09, BF_{10} = .28.

Session 3: Crayfish' response to the VAP stimulus significantly recovered after 30 days, trial 11 *vs* trial 13: *mean difference* = -20.30±3.42 frames, $W = 1$, $p < .001$, $d = -1.58$, $BF_{10} = 523$. The decrement of their response from trial 13 to trial 17 was significant: $\chi^2(4) = 21.3$, $p < .001$, $BF_{10} = 2759.15$; trial 13 *vs* trial 17: *mean difference* = 21.2±4.69 frames, $W = 89$, $p = .003$, $d = 1.16$, $BF_{10} = 48.20$. Again, crayfish generalized the response decrement to the JET stimulus: trial 17 *vs* trial 18, *mean difference* = -1.16±3.68 frames, $W = 46.5$, $p = .73$, $d = -.01$, $BF_{10} = .27$.

Crayfish's response to the JET stimulus changed across the trials 6, 12 and 18 (Figure 17, panel B): $\chi^2(4) = 21.3$, $p < .001$, $BF_{10} = 32.44$. Their response to trial 6 was greater than their response to trial 12 and 18 (*post-hoc Durbin-Conover*: $T = 5.29$, $p < .001$; $T = 3.44$, $p = .002$).

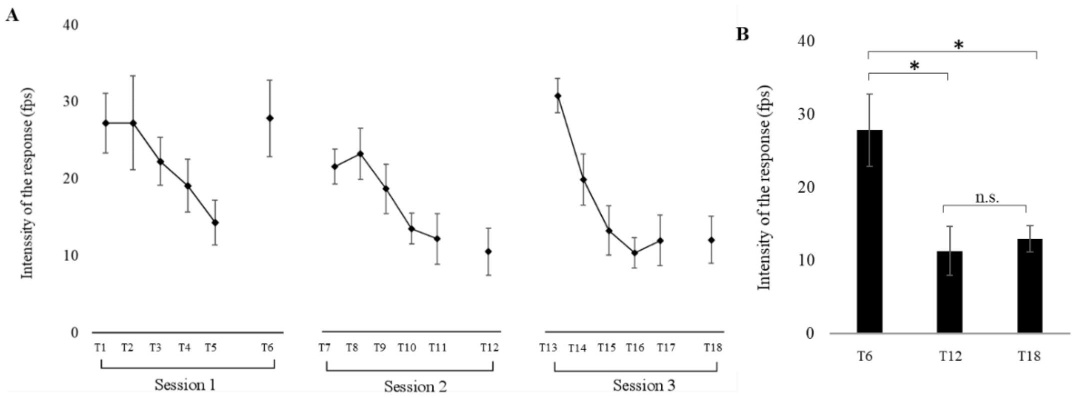

Figure 17. Panel A depicts the intensity of the crayfish' defensive response to the VAP and the JET stimuli in the 3 sessions. Panel B depicts the same response to the JET stimulus only. Bars represent ± 1 S.E.M.

4.3 General discussion

Habituation mechanisms allow the suppression of unnecessary behaviors when stimuli are repeatedly encountered without any relevant consequence. Which biological mechanisms mediate habituation is still debated. While the theory by Groves and Thompson (1970) states that habituation is caused mainly by a depression of the synaptic transmission in the specific response pathway stimulated, recent evidence attests that multiple molecular mechanisms are simultaneously recruited during habituation (McDiarmid et al., 2019). Among the cognitive theories of habituation (Hall & Rodrguez, 2017; Sokolov, 1963; Wagner, 1976), instead, there is general agreement that habituation arises from a cognitive mechanism that matches incoming information with memory models of previously encountered stimuli. The result of this matching would result in the suppression of responses to either a stimulus that can be reliably predicted by the memory model (Sokolov, 1963; Wagner, 1976) or to a repetitive stimulus that is followed by no consequences (Hall & Rodrguez, 2017). Importantly, both these explanations acknowledge that previous experiences of an organism determine its response when a stimulus is encountered again in the future (see also, Bradley, 2009).

Are individual differences in habituation entirely the by-product of previous experience? Our results show that individual differences in habituation, behavior, and morphological factors co-vary from early chicks' life. Because a laboratory-reared newborn chick, whose experiences are intentionally reduced to a minimum, might sample from a limited number of memories established in the first few days of life and is completely naïve to the artificial stimuli presented at test, the possibility that previous experience produced the observed individual differences is negligible. Alternatively, our results suggest a common genetic pathway underlying habituation, exploratory activity and body size of chicks.

Previous studies in adult species have shown that body size and exploratory activity are both associated with habituation. Body size is an important variable associated with the fitness of individuals. It predicts the body condition of an organism and reflects the amount of energy an animal must use to survive (Labocha & Hayes, 2012). It has a positive impact on a range of biological functions including reproduction success

(Thorley et al., 2018), foraging (Ofstad et al., 2016) and social rank (Reed et al., 2019). In general, the increased body size is a positive predictor of individual cognitive performance in different tasks (Boogert et al., 2018). In the specific case of habituation, a study on lizards found that smaller individuals are faster habituators, benefiting from rapid disengagement from repetitive stimuli to gather more resources to improve their body condition (Rodríguez-Prieto et al., 2010). Thus, the relationship between body size and habituation may stem from a trade-off between the benefit of profitable behaviours (e.g., resource gathering) and the reduction in energy costs associated with unnecessary activation of anti-predatory behaviors, including secretion of stress hormones (Pérez-Tris et al., 2004). Additionally, behavioral studies, have found that habituation correlates with risk-assessment behaviors, like the flight initiation distance – the distance at which an individual flees an approaching person (Allan et al., 2020; Blumstein, 2016; Vincze et al., 2016) – and exploration of a new environment (Martin & Réale, 2008; Rodríguez-Prieto et al., 2011; Verbeek et al., 1994).

Several studies have shown that exploratory individuals can adopt different risk assessment strategies. They may assess actual risk quicker than less exploratory ones (Crusio, 2001; Greenberg & Mettke-hofmann, 2001), but they can also show a superior learning capacity even if they spend more time exposed to new stimuli because they can gather more information and adapt their behavior to a greater extent (e.g., by focusing their attention more on the stimulus) (Guillette et al., 2009). The positive association between habituation and exploration we found in chicks, further supports the relationship between exploration and risk-assessment. Moreover, our findings also demonstrate the trait-specific nature of this association, as social reattachment was not correlated with chick habituation rates, whereas boldness influenced the individual likelihood of being tested, rather than habituation.

If previous studies have investigated the co-variation between habituation, body size and exploration individually, in this instance, we show an interaction between these factors for the first time. Specifically, we found that body size was associated with the habituation rate only in the most exploratory chicks. The fact that small exploratory chicks suppressed their freezing response within a few repetitions of the sound, compared to large exploratory chicks, suggests rapid risk-assessment abilities (i.e., the sound was an innocuous distractor in our experiment) in these individuals. However, the moderating effect of exploration on the relationship between body size and habituation rate shows that this difference in risk assessment is evident only in the more exploratory group of chicks. At the speculative level, it is possible that the deleterious effect of repetitive events on less exploratory individuals was so low that having a different habituation rate based on body size might have no relevant effect. Indeed, less exploratory chicks would be expected to be less exposed to unfamiliar stimuli regardless of their body size and would not benefit from body size related risk-assessment strategies as more exploratory individuals do. Greater explorers, by contrast, are known to use distinct strategies to assess the risks depending on different factors, including the frequency of predator encounters (Sommer & Schmitz, 2020), resource availability (Webber et al., 2020) or environmental changes (Thompson et al., 2018). A key point in our experiment was to test whether the different habituation performance of individuals reported by previous studies (Rodríguez-Prieto et al., 2010, 2011) is necessarily the product of relevant life experiences or these differences co-developed with behavioral and morphological aspects genetically available from birth. Our findings demonstrate an early covariation between exploration, body size and habituation in chicks hatched and raised under controlled laboratory conditions, suggesting that a genetic factor may be involved. Obviously, we could not untangle the direction of the relationship between the factors involved. Indeed, it is possible that different habituation capacity may have affected chicks' exploratory capacity and body size condition, but the opposite may also be true. Whatever the causal roles among these factors, we showed that their reciprocal influence shapes the early interaction of chicks with new stimuli in the absence of relevant life experiences. The relationship between exploration, body size and habituation suggest the intriguing possibility that variables related to the fitness of an organism in its early life, such as body size, provide an innate driver to behavioural and cognitive differentiation underlying habituation.

Another extraordinary example of the close link between individual fitness and learning comes from the study of the behavioural flexibility of invasive species. In Experiment 6, we demonstrated that *P. clarkii* – an invasive crayfish - habituated the defensive response to a repeated vaporized water jet, but then the response recovered when a direct water jet was introduced. The response to the vaporized jet showed spontaneous recovery during all the sessions. With time the crayfish were able to generalize the habituated response between the two types of stimuli. The generalization occurred after one learning trial and resisted for up to 45 days.

A possible limitation of this experiment is that the order of the stimuli used in the generalization test was not counterbalanced. We had reasons to conclude that it was not necessary to test more wild animals to control for this factor. Strong stimuli elicit greater response and take longer to habituate (Rankin, 2009; Thompson & Spencer, 1966). A frontal water jet sprayed directly at the animals can be considered a stronger stimulus than a vaporized water jet. We observed that crayfish suppressed their response to the direct water jet over time, revealing that generalization of habituation took place. If habituation generalized from the weakest to the strongest stimulus, the same is expected to occur also in the reverse order.

One may argue that the response to the direct water jet underwent a distinct process of long-term habituation instead of generalization. To the best of our knowledge, habituation after a single exposure has been reported for weak stimuli with no biological relevance and within a single experimental session (e.g., Leaton, 1974). It is unlikely that crayfish did not acquire long-term habituation to the vaporized water jet – which was repeated five times each session – but could form long-term habituation to the direct water jet after a single trial.

In principle, invasive species living in changing environments might benefit from behavioural flexibility more than species living in stable environments because the risk of facing unknown stimuli is higher. A mechanism must have evolved in these species to transfer innate or learned behaviours to new situations. Accordingly, Hazlett and colleagues (2000, 2002, 2003) suggested that invasive species have developed the ability to recognise and respond to a wider range of stimuli than species living in isolation because they have had the opportunity to experience a greater range of habitats in their evolutionary history. This points to rooted flexibility in invasive species. Invasive species must learn through experience how to cope with the challenges posed by the current environment. Behavioural flexibility may entail the capacity to invent new solutions (innovation) or learn these solutions from others (social learning). Animals can exploit past solutions that worked in similar situations, to be generalized to the present one. This solution may be more efficient when innovations and social learning cannot be accomplished rapidly. Crayfish possess some core social abilities, but they seem more a solitary species in which social forms of learning occur in a limited range of situations (Gherardi et al., 2012). We propose that crayfish transferred their defensive response between two different stimuli through a generalization mechanism, obtaining an evident adaptive advantage to solve problems o in new and unknown realities. Whether invasive crayfish have a superior generalization capacity than native species remains unexplored, but our habituation paradigm can be reliably used to address this question in future research. An example is a recent article on the management of invasive crayfish (Manfrin et al., 2021).

Chapter 5

An entropy-based investigation of rate sensitivity of habituation in domestic chicks[2]

Habituation relies on animals' ability to exploit predictive information within the structure of the sensory input to build progressively accurate models of the world. Computational models have been proposed to explain how animals learn to suppress an unnecessary response to repetitive events (e.g., Itti & Baldi, 2009; Sekoguchi et al., 2019). These models share the common assumption that habituation is driven mainly by how "surprising" and "novel" a stimulus can appear based on prior expectations. This notion is perhaps encapsulated in the associative term V (Rescorla & Wagner, 1972) used to refer to the strength of the association between a US and a predictor CS. At the core of these models is the idea that the nervous system tries to reduce the discrepancy (i.e. the error) between the expected input generated by an internal (memory) model of the stimulus, and the incoming sensory information during learning. Once an US is fully predictable (Rescorla & Wagner, 1972) - or a CS can fully predict its consequences (Pearce & Hall, 1980) - asymptotic learning is reached, and an organism filters out the predictable information.

Wagner's (1976) explanation of habituation was based on the hypothesis that the US representation is less likely to be activated by its external stimulus when it is already active in STM (also retrieved from LTM by contextual CS). With repeated spaced presentations, a stimulus loses its effectiveness to evoke a response and habituation occurs. When the time intervals between subsequent presentations of a stimulus are

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[2] *This work is the result of collaboration with Dr. Alessio Perinelli and Prof. Leonardo Ricci.*

sparse or exceed the capacity of STM, habituation may be severely disrupted (Davis, 1970a; Uribe-Bahamonde et al., 2019). This characteristic is known as *rate sensitivity* of habituation (Thompson & Spencer, 1966) and is attested to by the fact that habituation is more rapid when ISIs are shorter. The effects of the ISIs are not only visible during the acquisition of habituation, but also in the recovery of the response to the repetitive stimulus, which is faster with shorter ISIs. The structure of the ISIs between consecutive stimuli play a pivotal role in the acquisition of habituation, perhaps by conveying information that organisms can exploit to predict the stimulus occurrence.

To model the effect of a temporal sequence of ISIs on habituation, Staddon and Higa (Staddon & Higa, 1996) assumed that the effect conveyed by each stimulus repetition is progressively subtracted from the overall stimulus effectiveness by a series of “leaky integrators”. As successive stimuli are presented, the accumulated "charge" of each integrator unit increases. R*esponse output* is the difference between the integrator charge and the constant stimulus input. The response decreases as successive stimuli are presented. If the ISI is too long, the integrator "discharges" in-between stimulus presentations, and the system does not habituate. Rate sensitivity of habituation is represented in this model by assuming that each integrator discharges at different rates, modulating the overall habituation outcome. When repetitions are closely spaced, the faster integrators of the series charge in between stimuli, saturating the stimulus effect without giving time to the slower integrators to charge. With more spaced repetitions, the faster units discharge in between stimulus presentations, allowing a substantial input to charge the slower integrators. This model is quite complex as it relies on multiple units, each constrained to different mathematical parameters. The validity of this model has been questioned by the same authors on a set of data by Rankin and Broster (1992). Except for this simulation study by Staddon and Higa (1966) the general notion of multiple integrators has remained empirically untested.

The SOP model can explain the rate sensitivity of habituation, as well (Davis, 1970; Uribe-Bahamonde et al., 2019; Whitlow & Wagner, 1984). In particular, the refractory-like mechanism responsible for short-term habituation is directly proportional to the frequency of stimulation. According to SOP, each occurrence of the stimulus will elicit a self-generated priming, which decays over time. Hence, short-term habituation is stronger the shorter the inter-stimulus-interval (ISI). The longer the ISI the more the stimulus representation remains active in STM without being disrupted by subsequent stimulation (priming of STM). This will increase the probability that such representation will enter in associations with that of other contextual stimuli, and that these associations will be transferred and consolidated in LTM. Hence, long-term habituation, measured across different sequences of stimulation, should be stronger the longer the ISI (Davis, 1970; Whitlow & Wagner, 1984).

Davis (1970) (see also Rankin & Broster, 1992) demonstrated that variable ISIs sequences produce slower habituation rate than fixed ISIs sequences. He explained this phenomenon with the fact that variable schedules include some long intervals, which allow self-generated priming to decay. However, Davis (1970) proposed an alternative explanation. He speculated that the more predictable the timing of a stimulus, the more habituation will result. Since stimulus timing is hard to predict with variable schedules, habituation should be slower than with a constant schedule.

This explanation is based on the possibility that animals can use the temporal information of stimulus repetitions to habituate. How to exclude the deleterious effect of longer intervals? Our approach was to demonstrate that given a sequence of variable ISIs, the response to a stimulus *n+1* is independent from the exact ISI at which it occurs but depends on its predictability. Namely, on an organisms' capacity to learn from the information conveyed by the temporal sequence when a stimulus will occur again in the future. To quantify this information we capitalized on Shannon's theory of information (Luce, 2003; Shannon, 1997) Information - also referred to as entropy - is measured in terms of the uncertainty contained in the distribution of a random variable: the amount of information associated with an event characterized by a realization of that random variable is proportional to how much the event is surprising or unexpected or, equivalently, to the related prediction error. Within this perspective, learning is a process whereby the nervous system extracts information from the sensory input and adjusts its response accordingly. In an entirely predictable world, nothing is left to learn, the information (entropy) conveyed by any further event is zero, and the prediction error is low.

5.1 Experiment 7

In this experiment, we devised a model that describes how the information content of a temporal sequence controls habituation. The specific role of information content associated with a given stimulus presentation during habituation was directly addressed by introducing a degree of uncertainty in the time (ISIs) of stimulus presentation. The predictions of this model were then validated by recording the habituating response in naïve chicks to test the following hypothesis: the neural mechanism that encodes the information conveyed by a temporal sequence of stimuli is part of the innate abilities of this species.

5.1.1 Subjects

Seventy-five domestic chicks (males = 39, females = 36) of the Ross 308 strain (Aviagen) hatched in our laboratory in complete darkness and standard conditions of incubation (temperature = 37.7 °C, humidity = 50-60%). After hatching, chicks were kept within the same incubator maintained at 31.5°C for 48 hours before being tested.

5.1.2 Apparatus

The experimental setup consisted of a black rectangular arena (45 x 50 x 160 cm, width, height, depth), with a running wheel (diameter 30 cm) placed on one of the short sides. A salient object acting as a social artificial companion was hung in front of the wheel to make the chicks run towards it. Time, distance, and direction of the run were measured by a circuit based on Arduino® and was displayed above the wheel. Two loudspeakers automatically played the stimulus sequence at 30 cm above the chick's head. The scene (the running-wheel, display) was recorded through a video camera.

5.1.3 Procedure

Each habituation sequence consisted of 15 repetitions of a burst of white noise (300 ms, 90 dB SPL). The time intervals between consecutive bursts were computed based on the following equation: $\mathrm{ISI} = \lfloor 25 + 35 \cdot x_i \rceil$, where x represents a random variable distributed as $x \sim \exp(-x)$ and $\lfloor z \rceil$ is the closest integer to z. A custom R script was used to generate the habituation sequences according to the desired values of average and standard deviation for the ISI. We imposed the same average ISI value of 60 to all habituation sequences. Each chick was exposed to the stimulation for 14 minutes. We changed the ISI standard deviation value of the habituation sequence across 3 experimental conditions: a “Fixed" (F) condition, in which the standard deviation was 0 because all the ISI were equal to 60 s; a “Random 30” (R30) condition, in which the standard deviation value was equal to 31 s (range 25 s - 156 s); a “Random 50" (R50) condition, in which the standard deviation was equal to 49.8 s (range 25 s - 228 s). For each experimental condition 25 different habituation sequences were generated, one for each chick. Chicks were randomized between experimental conditions and habituation sequences. For each chick, the test started after the chick had run for 10m on the wheel. The stimulus sequence was controlled by a computer program. The number and duration of the freezing response elicited by each burst were recorded. Chicks that did not run when the stimulus *i* was played, were excluded from data point *i*.

5.1.4 Generation of ISI sequences

We imposed the same ISIs average value of 60s to all the habituation sequences. Hence, each chick was exposed to the stimulation for 14 minutes. We generated the ISIs sequences for the R30 and R50 considitions as follow. First, we generated 14 independent realizations of a random variable x distributed as $x \sim \exp(-x)$. Then, we computed time intervals δt_i as $\delta t_i = \lfloor 25 + 35 \cdot x_i \rfloor$ s. A set of 14 ISIs was selected only if the corresponding average was equal to 60 s and its standard deviation less than 1% different to a chosen value σ. For the dataset R30, σ = 31 s. For the dataset R50, σ = 48.9 s. Figure 18 depicts the distribution of ISIs for the three sequences.

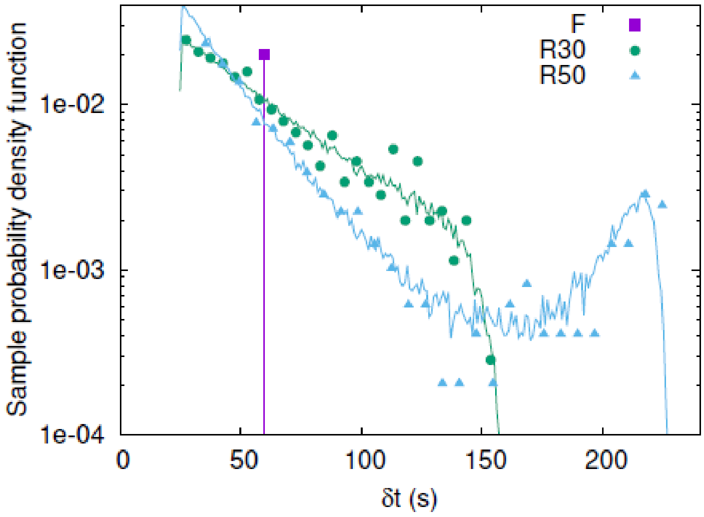
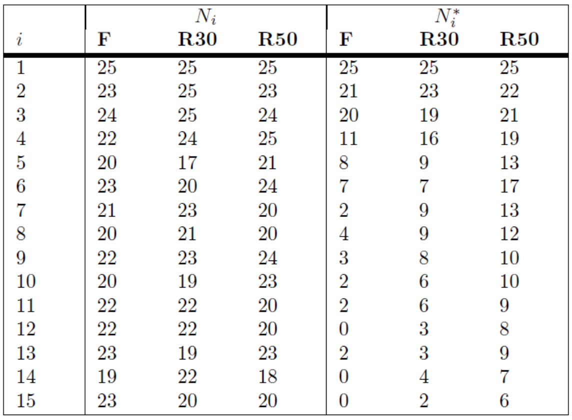


Figure 18. Sample distribution of ISI for the three sequences F, R30, R50. The dots correspond to sample probability densities assessed out of the 25 sequences of 14 ISI used in the experiment and

generated by means of the procedure described in the main text. The bin width is 5 s and 7 s for the R30 and R50 sequences, respectively. The solid lines correspond to 5000 sequences (each made of 14 intervals) generated through the same procedure. In this last case, the bin width is 1 s.

5.1.5 Data analysis

Table 4 reports the number of subjects contributing to each data point *i* under each condition (F, R30, R50): N_i is the number of subjects eligible for the assessment of the freezing rate ($\hat{p}_i$); N^*_i is the number of subjects that exhibited a freezing response at repetition *i*, thus providing $\hat{p}_i > 0$. The observed freezing rate $\hat{p}_i$ at each stimulus repetition is given by $\hat{p}_i = N_i/N^*_i$, where F_i is the number of chicks that froze, and N_i is the number of eligible chicks. The standard error of $\hat{p}_i$ is: $\delta\hat{p}_i = \hat{p}_i\,(1 - \hat{p}_i)/N_i$. Let δt_i represents the time interval elapsed between repetitions *i*-1 and *i*, while ϕ_i represents the freezing time occurring after the presentation of the *i*-th sound ($\phi_i = 0$ in the case of no freezing). An *F*-test was performed to test if the means of the three conditions (F, R30, R50) was significantly different at each data point. We used one-tailed tests, with an alpha level = 0.05 (Table 5).



	N_i			N^*_i		
i	**F**	**R30**	**R50**	**F**	**R30**	**R50**
1	25	25	25	25	25	25
2	23	25	23	21	23	22
3	24	25	24	20	19	21
4	22	24	25	11	16	19
5	20	17	21	8	9	13
6	23	20	24	7	7	17
7	21	23	20	2	9	13
8	20	21	20	4	9	12
9	22	23	24	3	8	10
10	20	19	23	2	6	10
11	22	22	20	2	6	9
12	22	22	20	0	3	8
13	23	19	23	2	3	9
14	19	22	18	0	4	7
15	23	20	20	0	2	6

Table 4. The number of data points used to compute data points and error bars for each condition (F, R30, R50) and repetition i. N_i is the number of subjects eligible for the assessment of $\hat{p}_i$. N^*_i is the number of subjects that exhibited a freezing response.

5.1.6 Fitting procedure

For the Fixed condition, we determined the parameter $k = 0.23 \pm 0.02$ as described in Section "Testing the model on data", the parameter α was determined as follows: given one of the conditions (R30 or R50) and a value of k, α was set within the range $(1 \div 4)*10^{-4} s^{-2}$ with step $2*10^{-6}\ s^{-2}$ (the range was based on preliminary analysis). A hundred synthetic experimental runs were simulated based on the model described by Eq. (3) and fed with a set of δt_i according to the corresponding condition. Each run corresponded to a simulated learning curve that we averaged by groups of 100 runs to produce one synthetic curve. We assessed the match between this synthetic learning curve and the experimental data by means of a chi-square procedure with a single degree of freedom. Figure 19 depicts the tuning of α in the case of $k = 0.23$. For the R30 and the R50 condition, the minimum chi-square was $\alpha = 2.30*10^{-4} s^{-2}$ and $\alpha = 2.06*10^{-4} s^{-2}$, respectively. The statistical error of α, σ_α was the half difference of the values α_2, α_1 at which the chi-square raised above its minimum value by 1. The final values of α and σ_α were then assessed by considering the average between these two values of α computed for R30 and R50. The result is $\alpha = (2.1 \pm 0.1)*10^{-4} s^{-2}$. If we set k to the best-fit, i.e. to 0.23± 0.02, we calculate that the parameter α varies between $1.9*10^{-4} s^{-2}$ and $2.3*10^{-4} s^{-2}$. The green and blue shaded areas reported in Figure 20 are bounded by the average learning process curves corresponding to these two values of α.

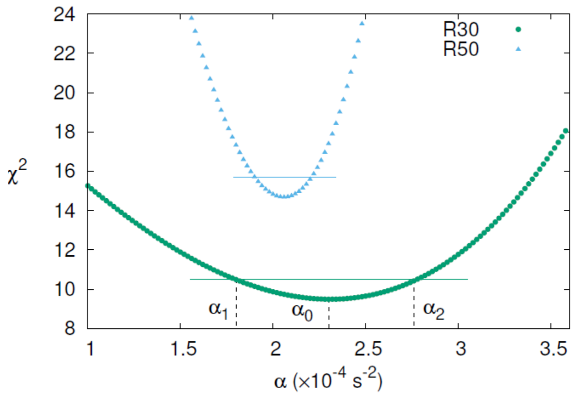


Figure 19. Matching between the average simulated learning process and the experimental data as a function of the parameter α, in the case k = 0:23. The matching is determined by means of a chi-square variable. The horizontal solid lines correspond to $\chi^2_{min} + 1$, where χ^2_{min} is the minimum χ^2 in each of the two conditions R30 (green circles) and R50 (blue triangles).

5.1.7 Results

We expected habituation to develop as exposure to the sequence of sounds progressed irrespective of the condition (F, R30, R50). Figure 20 depicts the decrement in the freezing rate $\hat{p}_i$ as a function of the repetition number, for the three conditions (F, R30, R50). Habituation was significant for each condition and the three conditions become significantly different starting from repetition *i* = 6 (see Table 6).

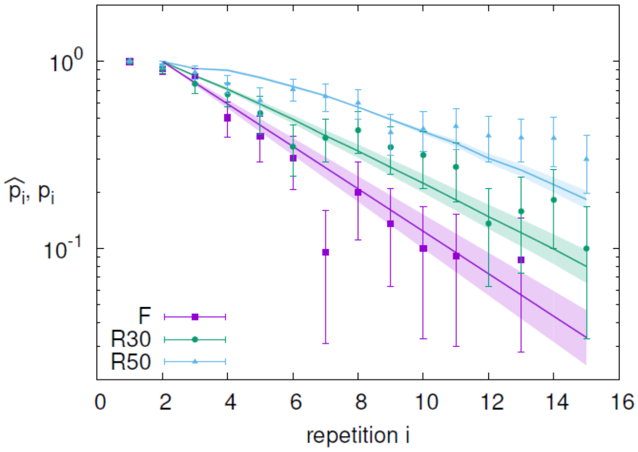
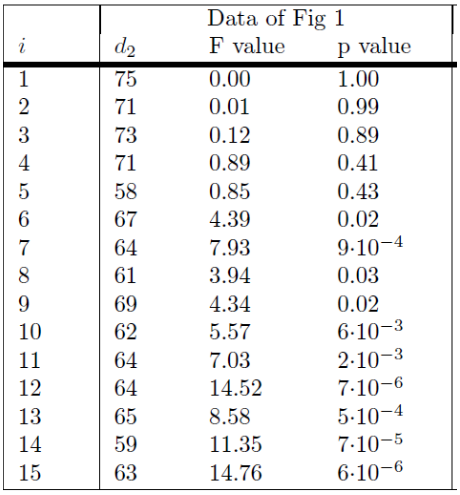


Figure 20. Observed freezing rate $\hat{p}_i$ and model-predicted freezing probability p_i as a function of the repetition number i. Dots and related error bars correspond to the observed freezing rate $\hat{p}_i$ for the three different kinds of sequences (see Table 5 for the number of subjects contributing to each point). Solid

lines and shaded areas correspond to the freezing probability p_i and the related uncertainty estimated by the model of chick habituation described in the main text. In the F condition, i.e. with fixed δt_i, the model produces an exponentially-decaying freezing probability, as predicted by Eq. (1) when $\Delta H_i = 0$ at all repetitions. When ISI are randomly distributed, habituation is affected because prediction errors made by the chick are increased. These errors slow the habituation process as described within Eq. 1.



		Data of Fig 1	
i	d_2	F value	p value
1	75	0.00	1.00
2	71	0.01	0.99
3	73	0.12	0.89
4	71	0.89	0.41
5	58	0.85	0.43
6	67	4.39	0.02
7	64	7.93	$9{\cdot}10^{-4}$
8	61	3.94	0.03
9	69	4.34	0.02
10	62	5.57	$6{\cdot}10^{-3}$
11	64	7.03	$2{\cdot}10^{-3}$
12	64	14.52	$7{\cdot}10^{-6}$
13	65	8.58	$5{\cdot}10^{-4}$
14	59	11.35	$7{\cdot}10^{-5}$
15	63	14.76	$6{\cdot}10^{-6}$

Table 5. Parameters of the F tests performed on the data making up Figure 19.

An entropy-based model of habituation

The curves shown in Figure 20 correspond to the model predictions. The probability of freezing at the stimulus *i* is estimated as follows:

$$p_i = p_{i-1} + \Delta p_i \text{, where} \quad (1)$$

$$\Delta p_i = -k p_{i-1}(1 - \beta \Delta H_i),$$

where k, β are two positive constants. The probability of freezing at repetition *i* is the probability of freezing observed in the previous repletion ($i - 1$) plus a variation Δp_i that depends on the degree of novelty (carried by the past event). The variation Δp_i depends on two factors. The first, $-k\, p_{i-1}$, is a negative factor that determines habituation even when the stimulus occurrence is predicted by the sequence of stimuli (F condition). The constant k expresses how fast learning occurs. The second affects the rate of habituation under uncertainty (R30 and R50 conditions). It is always positive and proportional to the amount of information ΔH_i that is carried by the current event. That is the entropy variation corresponding to the mismatch (i.e. the prediction error) between the model of the stimulus based on prior knowledge and the current sensory information. According to Shannon's interpretation, $\Delta H_i = -\log(\pi_i)$, where π_i is the probability of that event. An event that occurs with probability $\pi_i = 1$ adds no information. The smaller the π_i, the greater amount of information an event carries. In our model, the probability of a new event depends on a time interval δt_i between consecutive repetitions. We assumed that the chicks *learn* the average time interval $\bar{\delta t}_i$ by sampling from an adequate number of repetitions. A new event occurring exactly at δt_i yields no information. On the contrary, information ΔH_i is carried by the difference between δt_i and $\bar{\delta t}_i$. A simple function describes the dependence of ΔH_i to δt_i :

$$\Delta H \propto (\delta t - \bar{\delta t}_\mathrm{i})^2 \qquad (2)$$

The distribution of a probability of δt_i: Gaussian:

$$\pi_i \propto \exp\left[-\frac{(\delta t - \bar{\delta t}\mathrm{i})^2}{2\sigma^2}\right]$$

In other words, the chick's neural system models the δt_i distribution through normal distribution. The model described by Eq. (1) can be written as

$$\Delta p_i = -kp_{i-1}[1 - \alpha\,(\delta t - \bar{\delta t}_\mathrm{i})^2], \quad (3)$$

According to this equation, freezing probability is the result of a binomial process, i.e., a Bernoulli trial, where the probability p_i, reflects the decay of information in memory. *Rate sensitivity* of habituation – the fact that habituation is faster for shorter ISIs - corresponds to higher values of k.

Testing the model on experimental data

We assessed the reliability of this model to explain our data by starting from the Fixed condition. In this case, $\Delta \mathrm{H}_i$ is negligible - approaching zero when the chick learns that δt_i is exactly 60 s. The predicted probability of freezing decays exponentially with the repetitions number. The predicted data points fit the data. The k parameter of Eq. (1) is equal to 0.23±0:02. As depicted in Figure 20 our data fitted the prediction of our model for conditions R30 and R50, as well. For these two conditions, the additional parameter α of Eq. (1) was set to $(2.1 \pm 0.1)*10^{-4}s^{-2}$.

The probability of freezing p_i could depend on the preceding time interval δt_i, rather than on previous knowledge of the average δt_{i-1}. To test this hypothesis, we partitioned the ISI included in the R30 and R50 sequence in bins of 5 s and calculated the average of the observed freezing rate in each bin. The results are shown in Figure 21. The red dashed line is the average of all the bins (mean = 0.56; error = 0.02). A χ^2 test revealed that the probability of freezing was independent of the preceding inter-stimulus interval ($\chi^2_{(13)} = 1.03$, $p > 0.4$). In the presence of ISI variability, the probability of observing a freezing response in the current stimulus does not depend on the time elapsed from the previous one.

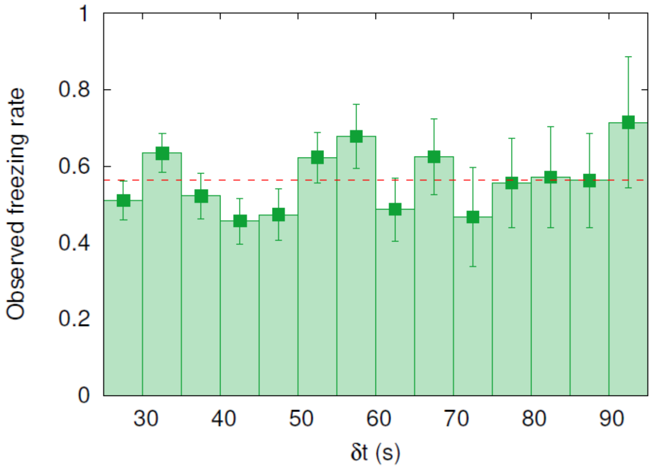


Figure 21. Observed freezing rate as a function of the preceding inter-stimulus interval. The abscissa of each point corresponds to the centre of a δt_i bin having a width of 5 s, whereas its ordinate r is given by the average of the n observed freezing rates recorded for ISI within that bin in both R30 and R50 conditions.

Habituation of freezing times

We analyzed the duration of the freezing response *ϕ_i* in the three conditions. Figure 22 depicts the mean freezing time (T_i) and its standard deviation σ_i. The red line is the average of the repetitions greater than 4 (mean = 4.5s, error = 0.1s). An F-test carried out on each stimulus repetitions suggested that the average freezing time of the three conditions was the same (see Table 6). Unlike the probability of freezing $\hat{p}_i$, which is affected by the predictability of the sequence of stimuli, the duration of the freezing response was independent of the ISI was therefore not constrained by the degree of uncertainty contained in the sequence.

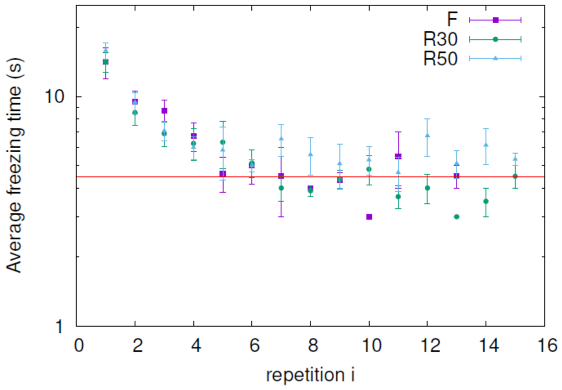


Figure 22. Average freezing time T_i as a function of the repetition number. The red line is the weighted average of the points having repetition number i > 5.

		Data of Fig 4	
i	d_2	F value	p value
1	75	0.11	0.90
2	66	0.08	0.93
3	60	0.32	0.73
4	46	0.04	0.96
5	30	0.18	0.84
6	31	0.00	1.00
7	24	0.51	0.61
8	25	0.33	0.72
9	21	0.05	0.95
10	18	0.15	0.86
11	17	0.13	0.88
12	11	0.16	0.85
13	14	0.18	0.84
14	11	0.23	0.80
15	8	0.01	0.99

Table 6. Parameters of the F tests performed on the data making up Figure 22.

5.2 Discussion

Rate sensitivity is a key characteristic of habituation (Rankin et al., 2009; Thompson & Spencer, 1966). The repetition rate not only affects the acquisition of short-term habituation but also the spontaneous recovery of the habituated response, suggesting that different mechanisms are involved (Davis, 1970; Rankin & Broster, 1992). Recent evidence has demonstrated that specialized molecules control the excitability of synapsis mediate rate sensitivity in *C. Elegans* (Ardiel et al., 2018; McDiarmid et al., 2019). Despite recent discoveries, the information provided by the temporal sequence of stimulation has so far remained elusive. In our experiment, we modelled temporal information by using the notion of entropy as described in Shannon's theory (1963). Our entropy-based model successfully described the experimental data, thus offering a valid explanation of the learning process underlying habituation under different rates of stimulation. Compared to previous models (Staddon & Higa, 1996), it provides a more straightforward explanation of this phenomenon based on a limited number of parameters that arise from the notion of entropy. It offers a broader view of how habituation is driven by the information contained in the timing of stimuli.

We manipulated the degree of entropy in the temporal sequence by using three sequences of auditory stimuli, which had the same average ISI (60 s) but differed in terms of the ISI standard deviation (0, 30 or 50s). When the sequence of stimuli has no temporal variability, the model assumes that habituation depends only on the average ISI. This agrees with previous studies showing that the rate of habituation is constrained by the limited capacity of STM to rehearse the model of the stimulus ISI (Askew, 1970; Geer, 1966; Rankin & Broster, 1992). While the model is active in STM, it prevents another representation of the stimulus to evoke the corresponding response leading to habituation (Sokolov, 1963; Wagner, 1979). The longer the average ISI the higher the probability that the model of the stimulus, or memory of the information extracted from previous stimulation, has degraded by the time the next stimulus is encountered. This mechanism would explain why habituation is slower the longer the ISI.

A more complex scenario emerges when the organism is exposed to stimuli occurring at irregular intervals. To understand how the chick's brain deals with this variability, we added two degrees of variance (R30 and R50 conditions) to the average ISI. We observed a significant reduction of the habituation performance as compared to the fixed condition. To explain why habituation is slower with a variable ISI, the interposition of longer ISI may lead to a decay of the memory model of the stimulus in between the stimulus repetitions (Laming & McKinney, 1990; Rankin & Broster, 1992). There is no experimental evidence supporting this hypothesis. It has been proposed that with a degree of variability in the stimulation, it could take longer for the nervous system to learn the repeated event is the same (Davis, 1970). This view has the merit of acknowledging the role of variability in modulating habituation, but it does not explain *how* an irregular sensory input affects learning. Our model postulates that a key factor is a neural capacity to estimate the information conveyed by each new event in a sequence, given previous events, and consequently to regulate the rate of habituation. The speed of habituation is inversely proportional to the amount of entropy conveyed by each event.

The present study demonstrates for the first time the major role that ISI variability plays in habituation. An idea that was suggested by Davis (1970) but never explicitly disentangled from the effect of ISIs duration by previous models (Staddon & Higa, 1996; Wagner, 1981). The data suggest that after only a few stimuli the chicks’ brain has "coded" the temporal information conveyed by a repetitive stimulus, and use this information to anticipate its occurrence. The role of stimulus predictability in habituation is supported by studies conducted with humans and rats (Kaye & Pearce, 1984; Lovibond, 1969) in which a sequence of compound S1-S2 stimuli was presented, with S2 following S1 with different probabilities. When the probability of S2 given S1 was 0.5, habituation was slower than when S2 followed S1 with either a probability of 0.1 or 0.9. In other words, habituation was found to vary with the degree of uncertainty in the S1-S2 relation. It was a function of whether S1 was a good or poor predictor of S2. Our entropy-based model is perfectly suited to explain such results, as it predicts that the amount of habituation should be inversely related to the amount of variability (i.e., information) in the pair of events considered. Even though the model does not distinguish between predicted and predictive events, the approach of considering a sequence of stimuli as multiple pairs of successive events could be used in future developments to address the following issues: whether habituation develops for stimuli that are progressively more expected (Wagner, 1981), or is determined by how much the stimulus is a good predictor following events (Pearce & Hall, 1980).

The devised model fits nicely with the mechanism of habituation of the orienting response (OR) proposed by Sokolov (1963). It postulates that the habituation rate increases as the stimulus being processed match the expected sensory input. But a deviation from the expected input generates a strong OR. Sokolov tried to explain the OR and its habituation by invoking the notion of information provided by Shannon. He observed that new unexpected stimuli are associated with high entropy; when entropy decreases, habituation emerges, as the same stimulus is repeatedly presented. In Sokolov's proposal, the putative link between habituation and entropy remained at a speculative level, and no formal model was proposed (Sokolov et al., 2002). We provide a mathematical explanation of how information is used to minimize the amount of prediction error, to describe learning from an information theory perspective.

Our model allows some speculations about the role of entropy for recovery and long-term habituation. If the amount of learning is inversely related to the entropy within a sequence, we expect faster recovery of the response the more entropy in a sequences. Unfortunately, literature on the relationship between habituation under variable schedules and recovery rate is scarce. Perhaps, Davis (1970) showed that fixed and variable sequences produce an equal amount of spontaneous recovery. But this result cannot be generalized to our data. The amount of habituation of Davis' rats was the same at the end of the fixed and the variable sequence. Instead, we found that chicks trained with the highest entropy condition (R50) froze significantly more than chicks in the fixed condition (F). A difference indicating that entropy prevented chicks from full habituation to the stimulus. Hence, the prediction that habituation under variable intervals would result in faster recovery.

While we developed the present model to account for habituation, it should be acknowledged that it bears similarities to the well-known model of conditioning proposed by Rescorla and Wagner (1972). Such a similarity is not surprising since both habituation and conditioning reflect a form of learning relying on a prediction error minimization process. The variable of interest described by Rescorla-Wagner model is V_i, namely the *strength of association to stimulus i*. The authors reported that their model was inspired by the Hull linear model of conditioning (Hull, 1952) in which learning is parametrized by the probability of response. The linearity of the Rescorla-Wagner model resides in the variation ΔV_i, namely the change in the strength of association to stimulus i being proportional to the "already known" strength V_i. Our model shares the same property, which accounts for the exponential decay of habituation in the case of the Fixed condition. However, relying on an entropy-dependent term, also describes the learning process when variability is added to the sequence of stimuli.

We were also interested to ascertain whether neural computation reflects an innate cognitive ability. Does the nervous system's capacity to estimate entropy in the stream of sensory input develop ontogenetically after birth, as the organism is exposed to the world or whether such ability is a hard-wired cognitive function present at birth. We used newborn chicks, which passed 3 days the time after hatching in complete darkness within the incubator. A chick reared in these conditions was previously shown to be an ideal animal model to test for the existence of innate cognitive functions. Our data show that the ability to estimate entropy in the incoming sensory input is an innate core capacity. Other cognitive building blocks of cognition have already been shown, in the domains of naive physics and spatial reorientation (Chiandetti & Vallortigara, 2008; Vallortigara, 2012)

To conclude, our study presents an original entropy-based model of habituation, which reveals an innate ability to estimate and use the information entropy conveys by the sensory input to control behavior. The model deals with habituation in a condition of time uncertainty It could be extended to account for habituation in general when the stimuli appear at regular intervals. The entropy-based mode we devised could also be applied to account for other important forms of learning, like conditioning. Artificial neural networks could be used to determine the minimal neural architecture necessary to implement an entropy-based model, thus provide insight into the neural complexity required for entropy estimation during learning.

Chapter 6

General summary and conclusions

Animals learn to ignore repetitive stimuli, likely because they convey less information compared to new stimuli. The result of this learning mechanism is habituation, a widespread and phylogenetically preserved form of plasticity. The aim of this thesis was to empirically investigate this form of learning across development, contexts, individuals, and temporal predictability.

In Chapter 2, we developed of a new habituation-dishabituation tests in chicks, showing that habituation and dishabituation share a similar developmental trajectory supporting the hypothesis that both phenomena arise from a common STM mechanism. Our studies have documented a period of enhanced behavioural plasticity in chicks, that rapidly decays within the first three days of life. This result emphasizes the potential of this precocial avian species to be a translational model for the study of critical periods of enhanced brain plasticity in humans.

In Chapter 3, we have shown that the chick's embryo can form associative memories of prenatal stimuli by using contextual cues. These associative memories modulate the postnatal acquisition of habituation. This experiment revealed that chicks integrate discrete stimuli in a unitary representation with their context before hatching.

In addition, we have developed a new habituation-dishabituation paradigm to study how bumble bees adapt their defensive behaviour to a repetitive looming predator. We capitalized on this paradigm to explore the modulatory effect of emotional contextual cues on short- and long-term habituation. We have found that the response to the looming stimulus is potentiated by the presence of a cue that is emotionally congruent with the aversive effect of the looming stimulus but is potentiated even more by an emotionally incongruent cue. Bumble bee's increased reactivity may be explained in terms of a negative prediction error triggered by a mismatch between the positive outcome induced by the sweet cue and the aversive reaction elicited by the looming stimulus. This opens the possibility of a rudimentary system to encode prediction error in insect brains (Mizunami et al., 2018; Montague et al., 1995)

In Chapter 4, we have shown that individual differences in habituation rate correlate with exploratory activity and body weight of newborn chicks. Bigger chicks show a steeper habituation rate than smaller ones only in the more exploratory group. This result supports the hypothesis that more exploratory chicks used multiple risk assessment strategies, based on their body size. Since newborn chicks were reared in a controlled environment, there is evidence to conclude that this association between body size, exploration and habituation is driven by genetic factors.

In addition, we have documented a long-lasting form of generalization triggered by a single learning event in the invasive crayfish *P. Clarkii*. This capacity may be linked to the enhanced behavioural flexibility of this invasive species compared to native crayfish attesting an evolutionary advantage induced by this simple form of learning.

In Chapter 5, we tested a new entropy-based model that quantifies the information conveyed by the temporal sequence of a repetitive event. We have validated the predictions of this model in inexperienced chicks and demonstrated that their habituation performance decreases as a function of the entropy apported by a new stimulus repetition. Since the notion of entropy has been applied to different learning phenomena, our results can shed light on how the nervous system uses temporal information while learning. In the framework of the new discoveries on the molecules that regulate rate sensitivity of habituation in *C. Elegans*, our entropy-based model can be devised to investigate the neural complexity necessary for entropy estimation.

To conclude, textbooks often describe habituation as a prototypical example of non-associative learning consisting of the simple decrement of basic reflexes for repetitive stimulation (e.g., Castellucci et al., 1978). Despite this simplistic description, habituation is observed for a multitude of pivotal and complex animal behaviors, such as sexual partner choice (Daniel et al., 2019) and food consumption (Epstein et al., 2009). Furthermore, the underlying cognitive mechanisms of habituation have been the focus of a great bulk of the research for almost a century (Thompson, 2009). However, the predominant belief that habituation is an instance of non-associative learning may have considerably limited the research efforts devoted to study whether habituation may, instead, have an associative nature.

Our work provide substantial evidence to support Wagner's theory (Wagner, 1981). In agreement with his predictions, we demonstrated that both habituation and dishabituation arise from a unitary memory mechanism. This mechanism is associative in nature, as suggested by the reviewed evidence and our discovery of context-specific habituation in chicks' embryos and bumblebees.

Still, some of the issues addressed in this thesis remain open. For example, how do variations in habituation contribute to the fitness of individuals and species? Does habituation develops for stimuli that are progressively more expected (Wagner, 1981), or is determined by how much the stimulus is a good predictor of the following events (Pearce & Hall, 1980)? Further studies will hopefully address these questions.

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Appendix I: List of defining characteristics of habituation

The defining characteristics of habituation were originally determined by Thompson and Spencer in 1966. Below, is a full list revised by Rankin et al. (2009).

Characteristic #1

Repeated application of a stimulus results in a progressive decrease in some parameters of a response to an asymptotic level. This change may include a decrease in frequency and/or magnitude of the response. In many cases, the decrement is exponential, but it may also be linear; in addition, a response may show facilitation prior to decrementing because of a simultaneous process of sensitization.

Characteristic #2

If the stimulus is withheld after response decrement, the response recovers at least partially over the observation time "spontaneous recovery".

Characteristic #3

After multiple series of stimulus repetitions and spontaneous recoveries, the response decrement becomes successively more rapid and/or more pronounced this phenomenon can be called potentiation of habituation.

Characteristic #4

Other things being equal, more frequent stimulation results in more rapid and/or more pronounced response decrement, and more rapid spontaneous recovery if the decrement has reached asymptotic levels.

Characteristic #5

Within a stimulus modality, the less intense the stimulus, the more rapid and/or more pronounced the behavioural response decrement. Very intense stimuli may yield no significant observable response decrement.

Characteristic #6

The effects of repeated stimulation may continue to accumulate even after the response has reached an asymptotic level. This effect of stimulation beyond asymptotic levels can alter subsequent behaviour, for example, by delaying the onset of spontaneous recovery.

Characteristic #7

Within the same stimulus modality, the response decrement shows some stimulus specificity. To test for stimulus specificity/stimulus generalization, a second, novel stimulus is presented and a comparison is made between the changes in the responses to the habituated stimulus and the novel stimulus. In many paradigms (e.g. developmental studies of language acquisition) this test has been improperly termed a dishabituation test rather than a stimulus generalization test, its proper name.

Characteristic #8

Presentation of a different stimulus results in an increase of the decremented response to the original stimulus. This phenomenon is termed "dishabituation." It is important to note that the proper test for dishabituation is an increase in response to the original stimulus and not an increase in response to the dishabituating stimulus (see point #7 above). Indeed, the dishabituating stimulus by itself need not even trigger the response on its own.

Characteristic #9

Upon repeated application of the dishabituating stimulus, the amount of dishabituation produced decreases (this phenomenon can be called habituation of dishabituation).

Characteristic #10

Some stimulus repetition protocols may result in properties of the response decrement (e.g. more rapid re-habituation than baseline, smaller initial responses than baseline, smaller mean responses than baseline, less frequent responses than baseline) that last hours, days or weeks. This persistence of aspects of habituation is termed long-term habituation.

Appendix II: List of Figures

Figure 6. Cladogram of the species showing context-specific habituation.

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Figure 7. Schematic representation of the experimental design and conditions. The exposure (-24/+24) and the test (+48) phases could take place in the same or different contexts. The freezing response to the bursts of white noise (90 dB) was measured as a stop of the wheel-running behaviour. Each condition consisted of 30 chicks. (Online version in colour.)

p. 57

Figure 8. (a) Amount of freezing in chicks exposed to the bursts of noise. In the test phase, data are from the first session of stimulation (material and methods). The rate of freezing increased in chicks stimulated in a different context (in ovo or after hatching in the incubator) during the previous exposure phase. (b) During the test phase, all groups showed a reliable habituation of the freezing response across the two sessions of stimulation.

p. 59

Figure 9. A schematic representation of the AESOP model from Wagner and Vogel (2010, p. 150). The CS is represented by the activity of a primary (A1CS) and a secondary activity state (A2CS). The US is represented by two sets of units: the sensory units, A1US/s and A2US/s, and the emotive units, A1US/e and A2US/e. The activity in the primary units is proportional to the p1 values, while the self-generated activity of secondary units is expressed by pd1. The associative-generated activity of the US secondary units induced by the CS is represented by the associative links Vs and Ve for the sensory and emotional representations, respectively. The activity of the US secondary sensory unit evokes a discrete UR while the activity of the US secondary emotive units modulates the conditioned emotional responses. The link between the emotive units and the sensory units represents the modulatory effect of the US secondary emotive unit regarding the activity of the CS and US sensory units. The modulatory effect is assumed to be an increment in the respective p1 values, which is proportional to A2US/s.

p. 63

Figure 10. Panel a) represents the overall apparatus. A walkaway connected the nest box

with the testing arena. In the testing area, bumble bees could forage from three blue artificial flowers at the centre. A paddle was automatically moved overhead by a servo motor connected to an Arduino circuit. Panel b) shows that the paddle moved from 0 to π radiant and back at a speed of approximately 1 radiant per second, projecting a looming shadow over the artificial flowers. Panel c) and d) represents the additional cues that preceded the habituation test in Experiment 4.2. A spoon trapping mechanism controlled by an Arduino circuit applied a constant pressure on the bee for 3 seconds (Panel c). A drop of sucrose was released in the same site as a positive reward.

p. 65

Figure 11. Panel a) shows the habituation curve of bumble bees (Block 1 to 4) and the dishabituation effect induced by the vibratory stimulus (Block 5). The y-axis reports the observed average duration of the defensive response per Block (x-axis). Panel b) depicts the habituation curve of the Habituation group on Day 1 (Block 1 to 5) and long-term habituation on Day 2 (Block 6 to 10). Error bars express the S.E.M.

p. 71

Figure 12. Panel a) shows the habituation curve of bumble bees (Block 1 to 5) in the Habituation, Emotional Congruency and Emotional Incongruency groups. The y-axis reports the observed average duration of the defensive response per Block (x-axis). Panel b) depicts the habituation curve of bumble bees that were tested in presence of the Same Cue or a Different Cue than Day 1 (Block 6 to 10). Error bars express the S.E.M.

p. 74

Figure 13. Panel a) represents the modular arena. It consisted of two chambers divided by a removable wall and a sliding door. Sixteen cones were placed on the floor of each chamber. The chicks were recorded by two cameras on top of the arena and their movements were tracked by a custom opencv-python program. Panel b) shows a chick in the starting chamber for Assay I. Half of the cones on the floor hid food and the chick had to actively explore the entire chamber to forage. In panel c) the starting chamber was covered reducing the light within. Then, the sliding

door was removed, allowing the chicks to move to the second chamber (Assay II). In panel d), the separating wall was removed. The red imprinting object was first presented alone to the chick. Then, a blue rectangular object was introduced to compare the chick social reattachment in the presence of an unfamiliar object.

p. 89

Figure 14. Boxplots for chicks' weight distribution according to their level of Exploratory Activity (panel a) and Boldness (panel b). The categories (Low vs High Exploratory Activity and Low vs High Boldness) refer to chicks' factor score relative to the sample average.

p. 92

Figure 15. Panel a) shows the habituation curve of chicks. Dots represent the observed average freezing duration (y-axis) per Trial (x-axis). The red line represents the estimated relationship using LCM. Error bars express the S.E.M. Panel b) depicts the relationship between the duration of the initial freezing of chicks and their habituation rate. The habituation rate is expressed in absolute values.

p. 94

Figure 16 shows the relationship between chicks' Weight, their level of Exploratory Activity and their habituation rate. The categories (Low *vs* High Exploratory Activity) refers to chicks' factor scores compared to the sample average. The habituation rate is expressed in absolute values. The shaded areas represent S.E.M. The Weight was positively associated with chicks' habituation rate only in more exploratory chicks.

p. 95

Figure 17. Panel A depicts the intensity of the crayfish' defensive response to the VAP and the JET stimuli in the 3 sessions. Panel B depicts the same response to the JET stimulus only. Bars represent ± 1 S.E.M

p. 98

Figure 18. Sample distribution of ISI for the three sequences F, R30, R50. The dots correspond to sample probability densities assessed out of the 25 sequences of 14 ISI used in the

experiment and generated by means of the procedure described in the main text. The bin width is 5 s and 7 s for the R30 and R50 sequences, respectively. The solid lines correspond to 5000 sequences (each made of 14 intervals) generated through the same procedure. In this last case, the bin width is 1 s.

p. 110

Figure 19. Matching between the average simulated learning process and the experimental data as a function of the parameter α, in the case k = 0:23. The matching is determined by means of a chi-square variable. The horizontal solid lines correspond to $\chi^2_{\min} + 1$, where $\chi^2_{\min}$ is the minimum χ^2 in each of the two conditions R30 (green circles) and R50 (blue triangles). As an example, in the R50 case, the abscissa α_0 is graphically highlighted along with the abscissa α_1, α_2 that correspond to the α values at which the χ^2 curve crosses the threshold $\chi^2_{\min} + 1$.

p. 113

Figure 20. Observed freezing rate $\hat{p}_i$ and model-predicted freezing probability pi as a function of the repetition number i. Dots and related errorbars correspond to the observed freezing rate $\hat{p}_i$ for the three different kinds of sequences (see Table 4 for the number of subjects contributing to each point). Solid lines and shaded areas correspond to the freezing probability pi and the related uncertainty estimated by the model of chick habituation described in the main text. In the F condition, i.e. with fixed δt_i, the model produces an exponentially-decaying freezing probability, as predicted by Eq. (1) when ΔHi = 0 at all repetitions. When ISI are randomly distributed, habituation is affected by the fact that the prediction errors made by the chick are increased. These errors slow the habituation process as described within Eq. 1.

p. 113

Figure 21. Observed freezing rate as a function of the preceding inter-stimulus interval. The abscissa of each point corresponds to the center of a δt_i bin having a width of 5 s, whereas its ordinate r is given by the average of the n observed freezing rates recorded for ISI within that bin in both R30 and R50 conditions.

p. 116

Figure 22. Average freezing time T_i as a function of the repetition number (see Table 3 for the number of subjects contributing to each point). The red line is the weighted average of the points having repetition number i > 5.

p. 117

Appendix III: List of Tables