

**The adverse effects of chronic social stress on learning and the role of
serotonin quantified by a binary logistic regression model
in individual crickets (*Gryllus bimaculatus*)**

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The ability to learn and change future behaviour based on past experiences is crucial for the life and survival of animals. For various behaviours exhibited by animals it is clear that in a seemingly homogeneous population not all individuals behave the same way, even in invertebrates. In crickets (*Gryllus bimaculatus*), a model system for the mechanisms of intra-specific aggression, agonistic experiences with the underlying impact of neuromodulators have been identified as a cause of inter-individual differences. For mammals and humans, the experience of adversity and stress can have detrimental effects on cognitive abilities and chronic defeat stress is used as a model for depression. In crickets the equivalent, the chronic social defeat stress paradigm, has been established. This thesis first sets out to construct a new model for measuring a conditioned response from multiple behavioural aspects and quantify learning in individual crickets. Video tracking of responses revealed behavioural variables that were included in a binary logistic regression analysis, whereas the resulting multi-variable model proves to be superior to other models constructed and can give the probability of an individual exhibiting a conditioned response. With this, learning indices can be calculated for each individual trained in a differential appetitive olfactory paradigm. With the method at hand, this thesis reveals that the experience of chronic social stress impairs learning in crickets, susceptible and resilient to defeat stress alike. The experience of multiple wins, however, does neither improve nor decrease learning abilities, but a long-term winner effect on aggression could be shown. Although inter-individual differences in learning are present, the aggressive state of crickets is not correlated to the learning indices. The application of serotonergic drugs that block receptors or act as re-uptake inhibitors reveal the influence of serotonin on learning within this paradigm. In addition to maintaining reduced aggressiveness, serotonin promotes the impairment of learning after the experience of chronic social defeat stress.

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Leipzig, 26.01.2022



Kim J. Borstel

Abbreviations

aggr	aggressive
AICc	Akaike information criterion for small sample sizes
AM	amyl acetate
AUC under ROC	area under the receiver operating characteristic curve
avg.	average
°C	degree(s) Celsius
CI	confidence interval
cm	centimetre(s)
cm/s	centimetre(s) per second
Coef.	coefficient
CS	conditioned stimulus
CS°	unrewarded stimulus
CS ⁺	rewarded stimulus
DA	dopamine
def	defeat/defeated
DMSO	dimethyl sulfoxide
eds.	editors
e.g.	exempli gratia / for example
EPV	events per variable
Fisher	Fisher's exact test
flu	fluoxetine treatment
h	hour(s)
HL- <i>p</i>	Hosmer Lemeshow goodness-of-fit propability
5HT	serotonin
i.e.	id est / that is
IQR	interquartile range
ket	ketanserin treatment
<i>LI</i>	learning index
LTI	long term isolated
LxWxH	length * width * height
MER	maxillary palpi extension response
met	methiothepin treatment
min	minute(s)
ml	millilitre(s)
MLR	maxilla-labia response

mm	micrometre(s)
mmol/l	millimole(s) per litre
model	binary logistic regression model
mol/l	mole(s) per litre
MWU	Mann-Whitney U-test
N	number of values
NaCl	sodium chloride
Na ₂ HPO ₄ ·2H ₂ O	disodium hydrogen phosphate dihydrate
Na ₂ HPO ₄ ·H ₂ O	disodium hydrogen phosphate monohydrate
NaOH	sodium hydroxide
NO	nitric oxide
no def	no defeat
no win	no winning experience
n.s.	not significant
OA	octopamine
OCT	1-octanol
p	p-value/probability
PER	proboscis extension reflex
P _{resp}	probability of a cricket exhibiting a conditioned response to an odour
r	Spearman correlation coefficient
s	second(s)
SE	standard error
Seq.	sequence
Spearman	Spearman's Rank-Order Correlation
STI	short term isolated
subm	submissive
t	t statistic
U	U statistic
US	unconditioned stimulus
veh	vehicle treatment
VIF	variance inflation factor
W	W statistic
Wald	Wald statistic
Wilcoxon	Wilcoxon signed-rank test
μl	microlitre(s)

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1 INTRODUCTION

Insects, as small as they might be, offer endless possibilities to understand the basic principles of biological processes. Their nervous systems, consisting of a reduced number of neurons in comparison to vertebrates, have the advantage of being very accessible to various experimental tools while simultaneously offering insights in a rich repertoire of behavioural and cognitive abilities (Chittka and Niven, 2009). These range from stereotypical behaviour, like fights between male crickets (Hofmann and Stevenson, 2000), to complex social learning (Leadbeater and Chittka, 2007) and finding new solutions in object moving tasks in bumblebees (Loukola et al., 2017). One characteristic of learning is that experiences change an individual's behaviour in a repeatable way (Papj and Prokopy, 1989) which makes the process of learning crucial for many important aspects of an animal's life and survival. In research concerning animal behaviour, it has become evident that conspecifics show consistent inter-individual differences in behaviours to the extent that correlations of specific traits can be defined as a behavioural syndrome (review: Sih and Bel, 2008; Wilson et al., 2019). A coupling of variations in cognitive abilities and other traits has little supportive evidence in non-human animals (Sih and Del Giudice, 2012). To gain further insight into these variations, individuality in learning abilities has to be considered in behavioural experiments. In honey bees it has been shown that the standard measurement of learning in a group of animals does not adequately represent the behaviour of individuals (Pamir et al., 2011) and furthermore two cognitive phenotypes, fast and slow bees, have been found with some additional traits correlating with the phenotypes, others not (Tait and Naug, 2020). In crickets it has become evident that agonistic experiences during development forge inter-individual differences and support for a proactive-reactive syndrome has been found (Balsam and Stevenson, 2021). In general, social subjugation and its consequences influence future aggression in crickets (Rose et al., 2017b; Rillich and Stevenson, 2018; Balsam and Stevenson, 2020, 2021) and in mammals induce depression-like symptoms (Carnevali et al., 2012). Social defeat is also proposed as a model for depression in mammals (Hollis and Kabbaj et al., 2014) and can have various detrimental effects (Yu et al., 2016) to the extent that it disrupts reward learning (Der-Avikan et al., 2017). Even in humans, early adversity broadly effects development and life (Hanson et al., 2017). As crickets are an excellent model system (Horch et al. (eds.), 2017) for both, studies of aggression (review: Stevenson and Rillich, 2019) with resulting potentially life-long influences causing inter-individual differences (Rose et al., 2017b; Balsam and Stevenson, 2020, 2021) as well as learning (Mizunami et al., 2013; Mizunami and Matsumoto, 2017). The influence of chronic

social defeat stress on learning and memory in crickets and the existence and causes of inter-individual differences in learning capability are major research topics.

Interactions with conspecifics are often aggressive in various species and expressed in diverse forms (Huntingford and Turner, 1987). Crickets exhibit a stereotypic fighting behaviour consisting of six levels of increasing escalation that can be terminated at each level by the retreat of one opponent (Hofmann and Stevenson, 2000). After a single fight losers act submissively, do not immediately reengage in a fight and take 0.5 h to up to 3 h to regain their aggressiveness (loser effect), whereas winners exhibit a short time of increased aggressiveness (winner effect) directly after the fight (Alexander, 1961; Adamo and Hoy, 1995; Hsu et al., 2006; Iwasaki et al., 2006; Stevenson and Rillich, 2012, 2013; Rillich and Stevenson, 2011, 2014). A long-term depression of aggression in crickets is achieved by subjecting the animals to chronic social defeat, i.e. multiple successive losses (Iwasaki et al., 2006; Rose et al., 2017b; Rillich and Stevenson, 2018). Aggression in crickets is modulated by various biogenic amines and nitric oxide. In brief, it has been shown that octopamine (OA) acts as a neuromodulator that enhances aggressiveness in crickets (Stevenson et al., 2005; reviews: Stevenson and Rillich, 2012, 2019). Dopamine (DA) is necessary for the recovery of aggressiveness after defeat (Rillich and Stevenson, 2014). Nitric oxide (NO) has a major influence in the animal's decision to flee from a fight and also in depression of aggression after an agonistic experience (Stevenson and Rillich, 2015, 2017). Serotonin (5HT) is known to act through various receptor subtypes (Vleugels et al., 2015) and therefore its role is difficult to unravel (De Boer et al., 2015). So far, no influence on normal fighting behaviour of naive crickets could be found (Stevenson et al., 2000; Rillich and Stevenson, 2015, 2017). The impact of serotonin is revealed after an agonistic encounter and chronic social defeat in which it maintains the depressed aggressiveness of the loser of the fight (Rillich and Stevenson, 2018). In other invertebrates it has been shown to have functions that partially can have contrary effects to those in crickets, like promoting aggression in stalk-eyed flies (Bubak et al., 2014).

The value that invertebrates hold for understanding learning and memory has been revealed by numerous studies conducted in various species (reviews - *Drosophila*: Boto et al., 2020; *Drosophila* larvae: Gerber and Stocker, 2007; honey bees: Menzel, 2012; cockroaches and crickets: Mizunami et al., 2013; paper wasps: Tibbetts and Sheehan, 2013; various: Leadbeater and Chittka, 2007; Giurfa, 2015). A basic principle of associative learning is classical or pavlovian conditioning which could be implemented for invertebrates, where a neutral stimulus is presented in temporal proximity to an unconditioned stimulus, i.e. the reinforcer, in the training after which an association is formed between conditioned stimulus (CS) and

unconditioned stimulus (US) that can be recalled later after presentation of the CS alone (Menzel, 1983). In studies of learning in invertebrates, various odours are often used as the CS resulting in various olfactory conditioning paradigms (Bitterman et al., 1983; Matsumoto and Mizunami, 2000; Scherer et al., 2003). The US can be rewarding (appetitive), for example sugar water (Bitterman et al., 1983) or punishing (aversive), for example salt water (Matsumoto and Mizunami, 2000) or electric shock (Tully and Quinn, 1985), but if an reinforcer has an aversive or appetitive effect is additionally influenced by the timing of the presentation. If the US is not given in temporal proximity to the CS, the animals cannot form an association (unpaired training; Hammer and Menzel, 1995). But also, the effect of timing dependent valence reversal has been observed, meaning that for example the termination of a generally punishing stimulus is perceived as appetitive (Vogt et al., 2015; Gerber et al., 2019). The conditioned response in invertebrates can be observed in multiple forms and is manifested in many different learning paradigms. It is for example defined as the active movement towards the CS (*Drosophila* larvae: Neuser et al., 2005; Saumweber et al., 2011), time probing the CS (Matsumoto and Mizunami, 2000) or as specific and readily identified response, such as the proboscis extension reflex (PER) in honey bees (Bitterman et al., 1983) and the maxilla-labia response (MLR) in cockroaches (Arican et al., 2020). Methodologically, learning in invertebrates is typically reported as a group response, e.g. the percentage of animals showing a PER to an odour (Bitterman et al., 1983) or the number of animals visiting the odour site (Apostolopoulou et al., 2013) but as mentioned before, the performance of individuals may not be adequately represented in classical conditioning (Pamir et al., 2011). Nonetheless, the learning capacity of individuals in invertebrates has been evaluated by calculating the relative portion of correct choices in multiple trials (*Drosophila* larvae: Neuser et al., 2005; Saumweber et al., 2011; bumble bee: Muller and Chittka, 2012), time spent probing an odour source (Matsumoto and Mizunami, 2002) or time until a spot is located in a thermal-visual place learning paradigm (Scheiner et al., 2020). By using group performances, the risk of overlooking the spectrum of individual performances and behavioural subtleties arises. In vertebrates, variations in individual learning are well documented (e.g. Dukas, 2004; Groothuis and Carere, 2005) and also studies for invertebrates, including insects, are published (Dukas, 2008). In blow flies selective breeding of good and poor learners (McGuire and Hirsch, 1977), or in honey bees and cockroaches consistency in intra-group performance differences over successive trials underline the existence of individual variations (Pamir et al., 2011, 2014; Arican et al., 2020). Measures of an animal exhibiting a conditioned response are typically binary based in the form of specific behaviours that are or are not exhibited and these can be manifold. For this, the

extension of mouthparts in response to a stimulus (honey bees: Bitterman et al., 1983; ants: Guerrieri and d’Ettorre, 2010; cockroaches: Arican et al., 2020), choosing the correct path in a maze (Giurfa et al., 1999; Dupuy et al., 2006) or the presentation site of two presented odours (Scherer et al., 2003) is often documented. But with these measures a gradient of individual learning capacity cannot be quantified and with the knowledge of the complexity of a conditioned response (Onodera et al., 2019) in mind, the possibility of an individual’s response potential being overlooked, arises.

The aptitude of crickets in studies of learning and memory has been demonstrated (review: Mizunami and Matsumoto, 2017) with two paradigms that are applied. First, an appetitive-aversive differential olfactory conditioning paradigm where the animals are trained to associate an initially repellent odour (peppermint) with a water reward and an initially attractive (vanilla) odour with a saline solution punishment. In the test they are placed in an arena with both odours presented and after actively seeking and finding the odour sources the time probing each site is measured and a preference index calculated (Matsumoto and Mizunami, 2000, 2002). A second paradigm is based on the maxillary palpi extension response (MER). This is used either for appetitive or aversive conditioning. A group of animals is trained in a differential conditioning paradigm in which one odour is rewarded or punished respectively, and a second odour is not rewarded or punished. The percentage of animals exhibiting the MER is observed over the training trials and the retention performance monitored one day after conditioning (Matsumoto et al., 2015).

As is true for aggression and is also known to be true for learning amongst other things, biogenic amines are important regulators for behaviour of invertebrates (Blenau and Baumann, 2001; Scheiner et al., 2006; Mancini et al., 2018; Stevenson and Rillich, 2019). By utilizing applications of the substances or drugs allowing investigation of direct effects (Mancini et al., 2018) and consequences of blocking receptors (Rillich and Stevenson, 2011, 2014, 2018) or optogenetical tools for individually identified neurons in *Drosophila* (Riemensprenger et al., 2016; Schleyer et al., 2020), the significance of neuromodulators for learning in invertebrates has been determined. DA is involved in various learning processes, in reward-seeking behaviour as well as in modulating aversive and appetitive learning (Selcho et al., 2009; review: Verlinden, 2018). OA is generally linked to appetitive learning and reward responses (review: Perry and Barron, 2013). It is difficult to describe the role of serotonin in learning, as it functions through various receptor subtypes with often contrary effects on behaviour (Vleugels et al., 2015), but it has been revealed to be important for salt reinforcement processing and aversive olfactory learning (Huser et al., 2017) and is in general linked to aversive reinforcement

(Sitaraman et al., 2017). Besides biogenic amines, it is suggested that the gaseous neurotransmitter NO is playing a role in long-term memory in crickets (Matsumoto et al., 2006, 2009). For these animals it has been revealed that blocking of OA receptors impaired appetitive, but not aversive olfactory conditioning whereas blocking of DA receptors had the opposite effect (Unoki et al., 2005). The same influences are also present for the memory recall in appetitive and aversive paradigms (Mizunami et al., 2009; Mizunami and Matsumoto, 2017). The role of serotonin in cricket learning and memory remains unclear. Besides experimental manipulation, there are multiple processes that can influence learning and memory in animals. For example, it has been shown that age-related memory impairment is present in multiple insect species (crickets: Matsumoto et al. 2016; *Drosophila*: Mery, 2007; honey bees: Behrends and Scheiner, 2010). An immune response (Alghamdi et al., 2008) or external factors like pollution can impair learning (Leornard et al., 2019). In mammals, adverse experiences in adolescence can decrease cognitive function in adult rats (Novic et al., 2013). In order to measure influences of agonistic experiences on the learning capacity of crickets and reveal individual differences, it is important to quantify learning as a gradient in individual animals, therefore binary logistic regression analysis can be employed.

Binary logistic regression analysis describes the impact of various explanatory (independent) variables on the dependent (response) variable, resulting in modelling the chance of an outcome based on these individual characteristics (Bewick et al., 2005; Sperandei, 2014). The dependent variable in this analysis is binary, like for example death or survival in a group of patients and takes the values 1 and 0 and in this instance, modelling can be used to predict the outcome by multiple metabolic markers (Bewick et al., 2005) or the like. In order to build a model for future predictions a dataset is needed where the dependent variable is known for each of the corresponding independent variables (Osborne, 2012) and the goal is to find the most parsimonious model that still accurately reflects the data (Hosmer and Lemeshow, 2000). In order to perform a binary logistic regression analysis with an existing dataset of the dependent and various independent variables, preparative steps are necessary before starting the analysis. It is advised to start with a large number of potential independent variables and narrow down the variable selection before modelling (Stoltzfus, 2011; Sperandei, 2014). One commonly used method for this is fitting univariate models with a relaxed alpha for each variable (Hosmer and Lemeshow, 2000; Stoltzfus, 2011; Sperandei, 2014). It is also advised to not disregard the experimental importance of variables, despite the outcome of the univariate analysis (Stoltzfus, 2011). All pre-selected variables are then entered into a binary logistic regression analysis and fitted to a model with differing building strategies possible to use, for example a

stepwise procedure that includes or excludes the independent variables based on statistical probabilities (Hosmer and Lemeshow, 2000). Nonetheless, a careful purposeful selection is advised to include clinically or biologically relevant variables (Hosmer and Lemeshow, 2013). The resultant model then gives all independent variables retained and estimates the coefficients for each of them additionally to the intercept. With this the probability of the outcome can then be calculated (Stoltzfus, 2011; Osborne, 2012; Sperandei, 2014).

All models fitted should be assessed carefully afterwards to evaluate their goodness-of-fit, appropriateness, adequacy and usefulness (Hosmer and Lemeshow, 2000; Bewick et al., 2005). There are several methods and tests that should be considered. First the significance of the individual coefficients in the model is tested with the Wald statistic (Hosmer and Lemeshow, 2000; Bewick et al., 2005). It is advisable to test the variables for strong interdependences (multicollinearities) and therefore to rule out influences on the model interpretation and results by using the variance inflation factor (Thompson et al., 2017). The calculated allocation to the binary groups of dependent variables can be compared to the actual allocation with classification tables (Hosmer and Lemeshow, 2000). A model's goodness-of-fit can be assessed using the Hosmer-Lemeshow test, which evaluates whether the observed frequencies are significantly different to the estimated expected frequencies of the dependent variable (Hosmer and Lemeshow, 2000). The area under the receiver operating characteristic curve (Hosmer and Lemeshow, 2000) can assess a model's ability to discriminate between the two outcomes of the dependent variable via sensitivity and specificity (Austin and Steyerberg, 2012). Out-model data with known variables can be additionally used to assess the fit of the model (Hosmer and Lemeshow, 2000; Cullen et al., 2010) and compare the actual allocation to the binary group. Different models can be compared for their relative prediction accuracy with the Akaike information criterion for small sample sizes (Akaike, 1974) and therefore the superiority over one another be tested. One issue that should be considered is the event per variable (EPV) ratio which means how many observations in the smaller of the binary group are included for each independent variable and is often advised to be at least 10 (Peduzzi et al., 1996), but the threshold is also subject of discussion (van Smeden et al., 2016). In general, this concept is used to prevent building an unreliable model when the EPV is too low (Ott, 2018). Binary logistic regression modelling is a powerful tool often used in medicine to predict recovery (Lucas et al., 2019) and survival for example in cancer research (Ahmad et al., 2018; Nourelahi et al., 2019), for analysing health risk factors (Bewick et al., 2005; Zhang et al., 2011), to calculate the risk of traffic crashes for patients with sleep disorders (Bharadwaj et al., 2021) or in social sciences to investigate the causes of school dropout (No et al., 2012; Osborne,

2012). It has also successfully been applied to characterise gregarious and solitary living locusts based on behavioural variables in order to understand mechanisms of phase change (Roessingh et al., 1993; Simpson et al., 2001; Anstey et al., 2009; Gray et al., 2009; Cullen et al., 2012; Ott et al., 2012; Rogers et al., 2014).

This thesis aims to investigate the intriguing possibility of chronic social defeat stress not only influencing future aggression (Rose et al., 2017b, Balsam and Stevenson, 2021), but also learning and memory in crickets, *Gryllus bimaculatus*, and possible inter-individual differences in learning capacity being linked to the aggressive state of an animal. Therefore, a method for the quantification of individual learning scores for freely moving animals was developed and tested. From observations of a cricket's conditioned responses in an appetitive olfactory paradigm it became clear that the MER (Matsumoto et al., 2015) is only part of a searching behaviour that is more complex than previously assumed. In order to capture and measure this response, video tracking was employed to extract the behavioural components. These behavioural variables were then tested and used for the construction of multiple binary logistic regression models. The best fitting and most parsimonious model was evaluated using various statistical tests and then used to calculate a probability for each individual cricket that it exhibited a conditioned response to an odour (P_{resp}). In a differential appetitive olfactory conditioning paradigm, individual learning indices were calculated and the learning capacity of a group of crickets assessed. It was hypothesised and tested that the multi-variable analysis will prove superior to a single metric for estimating individual learning capacity, the head-bobbing response. With this method to measure learning, the hypothesis that the experience of chronic social defeat stress impairs learning could be assessed in groups of animals originating from differing rearing types. Multiple winning experiences were also tested for their influence on learning and aggression. Finally, the influence of serotonin on learning in the chronic social defeat paradigm was revealed using the 5HT-receptor blockers ketanserin and methiothepin which have differing binding affinities to receptor subtypes (Thamm et al., 2013; Vleugels et al., 2015). Additionally, the re-uptake inhibitor fluoxetine was used to investigate the influence of serotonin in a non-chronic defeat paradigm, in which the animals were only subjected to two successive defeats (Rillich and Stevenson, 2018).

2 MATERIALS AND METHODS

2.1 Experimental animals

Mature, adult male crickets (*Gryllus bimaculatus*, de Geer) were used for conducting the experiments. The animals were obtained from a commercial breeder (Bugs-International GmbH, Irsingen/Unterfeld, Germany) either as adults or as nymphs of a later instar or they were bred from these animals. They were reared under standard conditions in transparent plastic boxes (LxWxH: 35 x 19 x 30 cm), with sand covered floor and egg cartons, at the animal housing facility at Leipzig University at 22-24 °C with a relative humidity of 40-60 % and under a 12 h:12 h light-dark cycle. The animals were fed on protein flakes (Tetra GmbH, Melle, Germany), carrots and with water given *ad libitum*. Prior to the experiments the animals were isolated in individual glass jars (H: 10 cm; diameter: 8 cm) with a sand covered floor. Two different isolation protocols were used:

Short term isolated (STI) animals were socially isolated for 48 h. Thus, they had social contact to mature individuals of both sexes prior to isolation.

Long term isolated (LTI) animals were isolated as last nymphal instar just before their final moult and kept in isolation until they sexually matured after 10 - 14 days (Alexander, 1961). Until the start of an experiment, these animals had no contact to an adult individual.

All animals were deprived of water 24 h prior to conditioning and only fed protein flakes. This work is based on the analysis of 594 animals, of which non participated twice in different experiments. After the experiments all animals were taken care of until the natural end of their lives. All procedures conformed to the Principles of Laboratory Animal Care and German Law on Protection of Animals (Deutsches Tierschutzgesetz).

2.2 Appetitive olfactory conditioning

2.2.1 Odour application and rewarding

For the experiments conducted, two odours served as the conditioned stimulus (CS). 1-octanol (OCT; Merck KGaA, Darmstadt, Germany) was applied pure and amyl acetate (AM; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was diluted 1:10 in paraffin oil (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). It is important to notice, that a separate dedicated odour syringe was used for the application of each odour. The technique to apply the odour was based on a 10 ml syringe, that could be opened with a removable plunger as shown in Figure 1A. While working under a conventional laboratory fume hood a filter paper (1 x 1 cm) soaked with

5 μ l odour substance was transferred into the syringe. The plunger was then closed and positioned at the 6 ml mark. To ensure a greater and precisely controllable odour signal, a plastic funnel (diameter: 15 mm) was fashioned from a disposable pipette that was cut-open on both ends and shortened to a length of 7.5 cm and the thinner end (diameter: 7 mm) fixed to the syringe via the existent thread (Figure 1A). The filter paper was renewed after five uses. To avoid startling the animal by sudden movements of the experimenter, the syringe was positioned in the vicinity of the animal approximately 30 s before the odour application. The funnel was positioned circa 2 cm above the animal's antennae and the odour was applied directly onto the antennae by depressing the plunger from the 6 ml to the 0 ml mark in a smooth steady motion within 1.5 s (Figure 1B).

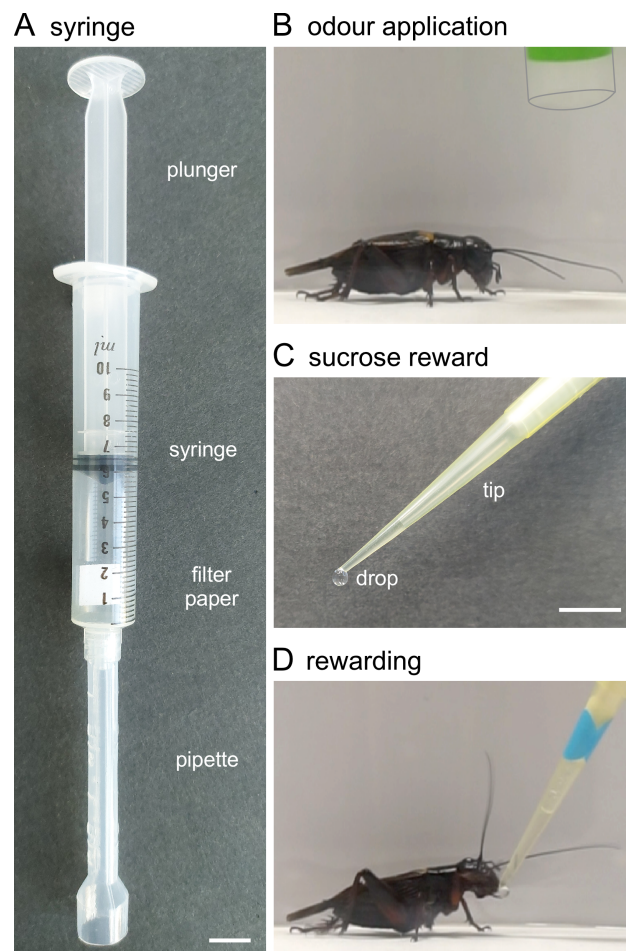


Figure 1 | Tools for conditioning and application process. **A** For odour application a tool was fashioned from a 10 ml syringe with a modified pipette forming a funnel. A filter paper soaked in odour substance was placed inside the syringe via the removable plunger. **B** Odour application directed to the antennae of the animal. The end of the funnel of the odour syringe is traced in grey. **C** For rewarding a drop (7 – 10 μ l) of sugar water was formed on the tip of a 20 μ l pipette. **D** The cricket was rewarded by being allowed to drink a drop of sugar water. Scale bar: 1 cm (both). Taken and partly modified from (Borstel and Stevenson, 2021).

Care was taken that the stream of air out of the funnel was as fast as possible but gentle enough to not startle the animal. It was also important that the application only started when the animal was stationary for, e.g. acquiring comparable measures for the beginning of a response to the odour application. Between successive odour applications and experiments with different animals an additional fan was used to clear the air from the work area, additionally the experiments were conducted in a room with a ventilation system.

A sucrose reward served as the unconditioned stimulus (US). For this, sucrose (Nordzucker AG, Braunschweig, Germany) was dissolved in tap water (1 mol/l). A drop of sucrose solution (7 - 10 μ l) was formed on the tip of a 20 μ l pipette (Figure 1C), manually positioned in front of the animal's palps and the animal allowed to drink from this *ad libitum* (Figure 1D).

2.2.2 Absolute conditioning paradigm

In absolute conditioning, only a single odour, either AM or OCT, was used as the CS. In general, prior to conditioning, the animals were presented with the odour to elicit initial odour responses for the experimental naive animals in all groups. For paired training (CS+US-paired) the animal was first presented with the odour followed by the reward. All forms of trainings in absolute conditioning were always repeated six times, with intervals of 5 min.

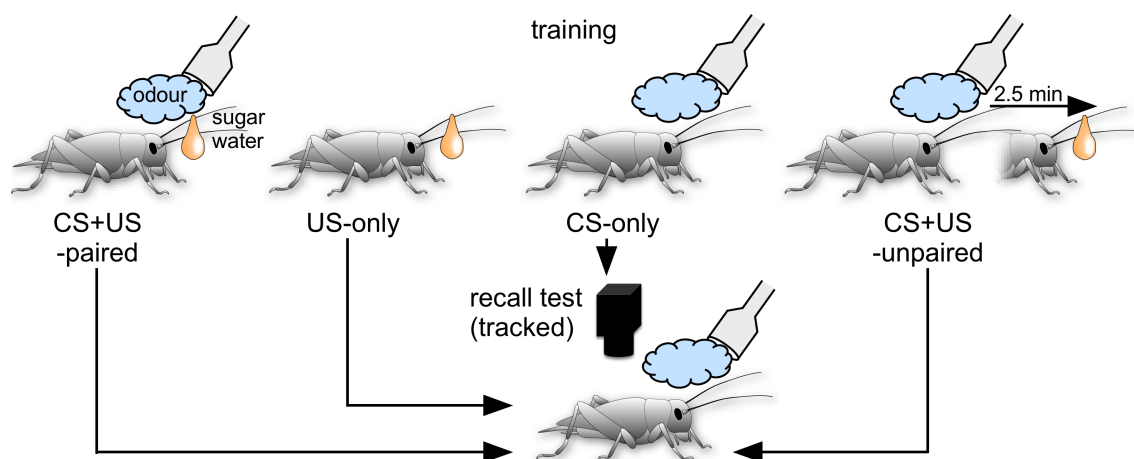


Figure 2 | Absolute conditioning paradigm. Depending on the experimental group in the training either odour was presented alone (CS-only), the animal was given sugar water alone (US-only), odour presentation was followed directly by the sugar water reward (CS+US-paired) or with a interposing pause of 2.5 min (CS+US-unpaired). Between each of the 6 repetitions of the training was a pause of 5 min. The recall test, where the animals' response to the odour was tracked, started after a pause of 30 min. Taken and modified from (Borstel and Stevenson, 2021).

Several additional groups in this paradigm were required for model building (for details see 2.4) and control purposes. In unpaired training (CS+US-unpaired) the animal received the odour followed by the reward after a pause of 2.5 min in between, followed by an additional 2.5 min pause before starting the next sequence of the six training repetitions. The stimuli were also presented alone, consequently this resulted in two more groups. In the CS-only group the animal was presented with the odour six times with 5 min intervals. In the US-only group the animal was only given the sugar reward six times with 5 min intervals. All groups are illustrated in Figure 2.

The recall test, where the same odour was presented to each animal of each group and the response examined, was performed after a pause of 30 min. Animals were discarded from any experiment if they did not drink from the sucrose reward or died within two days following the experiment.

2.2.3 Differential conditioning paradigm

In the differential conditioning paradigm, both odours OCT and AM were used as CS. The initial presentation of both odours in succession to the animal was followed by training. Here, the animal was presented with both odours alternately with a pause of 5 min in between, whereby one odour was rewarded with sugar water (CS⁺) and the other was not rewarded (CS[°]). This training was repeated three times at 5 min intervals. The recall test was performed after a pause of 30 min. During the latter, each odour was presented only once, with a pause between (3 min) and the response to each observed for 2 min. The sequence in which each odour was presented and rewarded in the training and in the recall test was varied systematically to give eight different permutations (Table 1) to test for possible effects there. Animals were discarded from any experiment if they did not drink from the sucrose reward or died within two days following the experiment.

Table 1 | Sequences in the differential conditioning paradigm. Animals were presented with two odours in succession (amyl acetate, AM and 1-octanol, OCT), each once before training (naive), each three times in succession during training, whereby one odour (bold and underlined) was paired with a sugar water reward. Finally, each odour was presented once more at the recall test, in which the response of the focal animal was filmed and tracked. Pauses between applications are indicated. The actual sequence of when and which odour was applied and rewarded was varied to give eight different permutations (1-8). Taken and modified from (Borstel and Stevenson, 2021).

Seq.	Naive				Training 3x				Recall test		
	1 st	3 min	2 nd	5 min	1 st	5 min	2 nd	30 min	1 st	3 min	2 nd
1	AM		OCT		<u>AM</u>		OCT		AM		OCT
2	AM		OCT		<u>OCT</u>		AM		OCT		AM
3	AM		OCT		OCT		<u>AM</u>		AM		OCT
4	AM		OCT		AM		<u>OCT</u>		OCT		AM
5	OCT		AM		<u>OCT</u>		AM		AM		OCT
6	OCT		AM		<u>AM</u>		OCT		OCT		AM
7	OCT		AM		AM		<u>OCT</u>		AM		OCT
8	OCT		AM		OCT		<u>AM</u>		OCT		AM

2.3 Experimental setup for video-tracking

After isolation, the animals were carefully transferred individually from the glass jars to circular arenas fashioned from an acrylic glass cylinder (diameter: 15 cm, height: 5 cm) that were placed upon a floor of cardboard covered with white paper that was exchanged regularly. Throughout one experiment the animals were never removed from the arena and when one had to change location it was always with the arena to minimise effects of handling. Each cricket was then carefully marked with a dot of yellow acrylic paint (C. Kreul GmbH & Co. KG, Hallerndorf, Germany) on the head or pronotum depending on the accessibility and allowed to acclimatise for 20 min after the transfer.

Video tracking was performed in a recording chamber (80 x 60 cm and 79 cm high) that was lined on three sides with reflective card and illuminated by LED light panels (NL480, Neewer, Luo hu district, Shenzhen, Guangdong, China) arranged to minimise shadows cast by the animal. The light was set to a medium brightness level that was sufficient for the video tracking but did not disturb the animals. Each animal's behaviour as response to odour application was filmed from above with a digital video camera (Basler acA1920-155uc, Ahrensburg, Germany, 60 frames/s; Figure 3A), then stored and analysed using commercial video-tracking software (EthoVision XT14, Noldus, Wageningen, Netherlands) running on a computer (Dell Precision 3620, Round Rock, Texas, USA) with Windows system software (10, Microsoft, Redmond,

Washington, USA). Regions of the visual space that were filmed, were defined using EthoVision. First the tracking arena, the space within the circular acrylic arena including the walls, was determined. This tracking arena was then virtually partitioned into eight equally sized arcs used later to define where the animal was situated when the stimulus was presented and accordingly in which region the odour was applied (Figure 3B). Additionally, the centre of the arena (diameter: 10 cm), the part where the animal has to actively leave the wall, was defined. It could be observed that the crickets tend to walk along the wall with one antenna touching it (Chapman and Webb, 2006). The colour-marker tracking routine of EthoVision was used to detect the yellow marker on the animal during life filming from above with the camera. After an acclimation time of 2 - 5 min in the recording chamber, the recording started with the application of the odour and the simultaneous press of the record command as soon as the animal was stationary. In order for the odour application syringe to be removed from view and to give time for the system to detect the marker after it was possibly hidden behind the syringe and hand, recordings always started 1 s after the command. The recordings stopped automatically after 2 min.

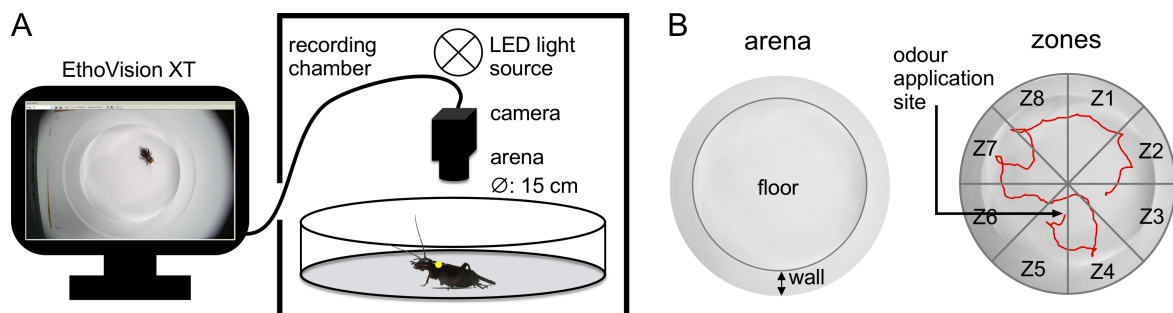


Figure 3 | Video-tracking setup. **A** For the recordings of the animals with a digital video camera from above, a tracking software (EthoVision XT) running on a computer was used. The focal animal was marked with a yellow dot and placed in a circular arena (diameter: 15 cm). In combination with the recording chamber (80 x 60 cm and 79 cm high) being lined on three sides with a reflective wall, the LED light panel illuminated the arena with little shadow casting. **B** Top view of the arena. Left side: When filmed from above the floor and the wall of the arena are visible. Right side: Exemplary track (red line) of an animal moving in the arena for 2 min. The arena was virtually partitioned to identify the odour application site. Scale bar: 3 cm. Taken and modified from (Borstel and Stevenson, 2021).

2.4 Binary logistic regression model

2.4.1 Binary groups for model building

After being isolated (STI) and transferred into the circular arena, each animal was marked with the yellow dot. After a pause of 20 min the arena together with the animal was carefully moved into the recording chamber. After the acclimatisation time of 2 min and when the animal was stationary, the odour was presented (see 2.2.1) and the recording of the naive response to the

odour started (as in 2.3). In a separate working area, the absolute conditioning was performed after a pause of 10 min with the animal still in the circular arena. For constructing a binary logistic regression model, animals for each of the CS+US-paired (N = 75) and US-only (N = 75) groups pictured in Figure 2 were trained six times with 5 min intervals. Individuals of both groups were trained and tested in parallel to exclude day dependent effects. Additional groups for model testing were CS-only (N = 25) and CS+US-unpaired (N = 25) trained animals. Following a pause of 30 min after the last training the animal was again moved into the recording chamber for the recall test and after 5 min the according odour was applied, and the recording with EthoVision started. A random number generator (Excel: Mersenne Twister-Algorithm) was used to select 50 of the 75 CS+US-paired and 50 of the 75 US-only trained animals to build the model with. The remaining 25 animals of each group were used to test the model.

2.4.2 Variables of a behavioural response

The tracking program EthoVision offers wide variation of variables that can be extracted and calculated from the video tracks to evaluate movement, the position of the focal subject within the coordinate system, which is virtually projected onto the arena and manually scored behaviours, further defined in the results. The tracking was based on a X/Y coordinate system calibrated in cm and the tracking area included the floor and the walls of the arena. Additionally the area was virtually divided in 8 pie shaped zones and the centre of the arena defined. To reduce jitter from ventilation movements of the animal and minimal shifts in detection of the centre of the yellow marker, a minimum distance moved filter, set to 0.05 cm below which no movements were registered, was applied. EthoVision provided the raw data as one value per variable for each 1/60 s frame over 2 min for each individual in the form of an Excel sheet (version 16.30, Microsoft, Redmond, Washington, USA) and this resulted in 13 variables measured. The names and definitions for all independent variables are described in the result section (see 3.1.1). The manual scoring function in EthoVision was used to measure the variable *bobbing* with each animal's group identity and treatment blinded. The dependent variable *conditioning* was coded as CS+US-paired = 1 and US-only = 0. All 13 independent variables were then separately pre-analysed and further selected for model building by fitting univariate models and using Wald statistics (Hosmer and Lemeshow, 2000). This univariate fitting tests the significance of each single independent variable contributing significantly to the model predicting the dependent variable. The p-value for further consideration of the variables was relaxed and set to 0.1 for this particular analysis which is advised as pre-selection strategy

(Stoltzfus, 2011; Sperandei, 2014). Eight variables were therefore retained for model building and potentially describing the differences between the CS+US-paired and the US-only groups. The other five were not used further in the actual model building (see 3.1.1). All the respective variables and results of this analysis are explicitly stated in the results in Table 2.

2.4.3 Calculating a conditioned odour response probability (P_{resp})

The behavioural variables extracted from the video tracks of the 50 US-only and 50 CS+US-paired animals and retained for model building were entered in an analysis program (SPSS Statistics, Version 25, IBM, Armonk, New York, USA) to perform a binary logistic regression analysis. The goal of this analysis was to find a model that can differentiate between the two groups best using as few independent variables as possible (parsimonious). The CS+US-paired animals were coded as 1 and US-only as 0 (binary response categories) in the analysis. Three models (see 3.1.2) were calculated using the forward stepwise conditional method, where the independent variables are added one at a time to a model containing only the constant and are retained or dismissed based on statistical probabilities (Hosmer and Lemeshow, 2000). The probability levels for stepwise entry and removal of a variable were set to 0.05 and 0.1, respectively. One model was calculated using the “enter” method, in which the model contains all variables that were selected by the experimenter.

Each of the calculated models provides a means of calculating a single metric giving the predicted probability of an individual belonging in the category 1 meaning in this context of this work that it exhibited a conditioned response to an odour, P_{resp} , given by the logistic algorithm:

$$P_{\text{resp}} = \frac{e^{\eta}}{(1 + e^{\eta})}$$

whereby η is the sum of each behavioural independent variable (x) found to be a strong predictor of a conditioned odour response, each weighted by a coefficient β and an intercept β_0 as given by the regression equation:

$$\eta = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_k x_k$$

An ideal model should correctly discriminate the individuals regarding the binary groups, whereby individuals with a resultant P_{resp} greater than the cut off value of 0.5 are allocated to

the CS+US-paired group (coded as 1), and individuals scoring less than 0.5 are assigned to the US-only group (coded as 0). The correct assignment of each animal is then assessed by comparing the calculated P_{resp} with the actual treatment. For a group of animals this then gives the degree of correct assignment by the model as a percentage.

The four resulting models that ought to be considered to use for further experiments contained different variables and had to be validated and compared to pick the model that describes the data best. The Wald statistic was used to test for the significance of contribution of each coefficient and variable to the model (Bewick et al., 2005) and in this analysis had no relaxed p -value ($p < 0.05$). To test for multicollinearity, which is defined as interdependence of independent variables in a regression model, which could negatively influence variable estimates and statistical significance of contribution (Thompson et al., 2017), the variance inflation factor (VIF) was calculated using Prism 8 (GraphPad Software Inc., La Jolla; CA, USA). Each model's goodness-of-fit was assessed using the Hosmer-Lemeshow test (Hosmer and Lemeshow, 2000). Based on the predicted probabilities each individual was sorted into the two categories based on the cut off value. The test gives the probability that the model's actual allocation of individuals to the binary groups is significantly different to the expected distribution that is known for each animal; therefore, a good fit should have a p -value > 0.05 . The Akaike information criterion for small sample sizes (AICc; Akaike, 1974) was calculated using Prism 8, to estimate their relative prediction accuracy comparing the models against each other, whereby a lower AICc score indicates a superior model. The area under the receiver operating characteristic curve (AUC under ROC, Hosmer and Lemeshow, 2000) was used to measure the model's ability to discriminate between animals of the US-only and CS+US-paired group by assessing sensitivity and specificity of the classifications (Austin and Steyerberg, 2012), whereby an AUC under ROC of ≥ 0.9 is considered as outstanding discrimination. To further ensure the reliability of the resulting model, the events per variable (EPV) were calculated from the number of animals in one outcome group relative to the number of regression coefficients (van Smeden et al., 2016). Finally, an additional measure of model quality was to test the discriminating power using variables from animals not used for model construction with a known training regime and compare it with the actual allocation by the model. For this, the remaining 25 animals of the CS+US-paired and US-only groups, as well as the two additional control groups that received CS-only and CS+US-unpaired ($N = 25$ each) during training were considered.

2.5 Evaluation of learning with the binary logistic model

The most parsimonious and best fitting model was applied to measure individual learning scores in a differential olfactory conditioning paradigm. After being placed into the circular arena and marked, each animal was presented with both odours (AM and OCT in various sequences) with a pause of 3 min in between. The training was performed as described in 2.2.3 also with the eight different permutations of odour and reward presentation (Table 1). After the pause of 30 min the animals were transferred to the recording chamber and the response to both odours consecutively tracked for 2 min using EthoVision with a pause of 3 min between presentations. This resulted in two video-tracks from the recall test for each animal for the two presented odours of which one was unrewarded (CS°) and one was rewarded (CS^+). Each video-track was then analysed, the according response variables extracted and the P_{resp} values for the CS^+ and the CS° calculated from the binary logistic regression model that had previously been determined to be the best model for describing the conditioned response (3.1.2). From these two measures the individual learning index for each animal was calculated from the difference: $LI = P_{\text{resp}} CS^+ - P_{\text{resp}} CS^\circ$. Since the P_{resp} values are based on probabilities, a difference of $LI > 0.05$ is assumed as being significant, hence here learning is defined as the correct discrimination between the two presented odours with a stronger response and appetitive association to the CS^+ , this results in a $LI > 0.05$, also referred to as positive LI . Animals that did not learn showed no discrimination between the odours and thus scored a $(-0.05) \leq LI \leq 0.05$, also referred to as zero LI . Animals showed an incorrect discrimination between the odours when the response to the CS° was stronger resulting in a $LI < (-0.05)$, also referred to as negative LI .

An initial experiment to first evaluate and examine appetitive learning in crickets using the binary logistic regression model was performed according to the description above with a group of 64 animals which led to 8 animals per regime (Table 1) to control for effects there. Additionally, for this dataset the variable *bobbing* was analysed as a single variable for measuring a conditioned response and additionally compared with the P_{resp} values and the LI in terms of effectiveness of measuring learning in crickets. To obtain a better comparison, the *bobbing* data for the entire dataset (CS° and CS^+ combined) was normalised so that the lowest value was 0 and the highest value was 1. A *bobbing-LI* was calculated from the difference between the bobbing to the CS^+ and the CS° : $\text{bobbing-LI} = \text{bobbing } CS^+ - \text{bobbing } CS^\circ$.

2.6 Evaluation of aggression with a standardised fight

Aggressive behaviour in crickets can be assessed using two standardised measures that can be observed when two adult male crickets engage in stereotypical fighting behaviour. The first describes the increasingly aggressive actions when two males come in contact and is referred to as “level of aggression” (Alexander, 1961). This behaviour can be scored on a scale of 0-6 whereas the single levels were previously described in detail (Hofmann and Stevenson, 2000; Hofmann and Schildberger, 2001; Stevenson et al., 2000, 2005, Stevenson and Rillich, 2012):

Level 0: Both animals show mutual avoidance without aggression.

Level 1: One cricket attacks, the other retreats immediately.

Level 2: The crickets antennae touch, and they perform antennal fencing.

Level 3: One of the crickets spreads its mandibles.

Level 4: Both of the opponents spread their mandibles but do not touch with them.

Level 5: The crickets engage and interlock their mandibles.

Level 6: They grapple each other and in an all-out fight try to turn the opponent on the back.

The fight can be terminated at any of these levels by one animal retreating and the maximal escalation level of the fight was thereby measured. The animal retreating from the fight is considered the loser, the other the winner.

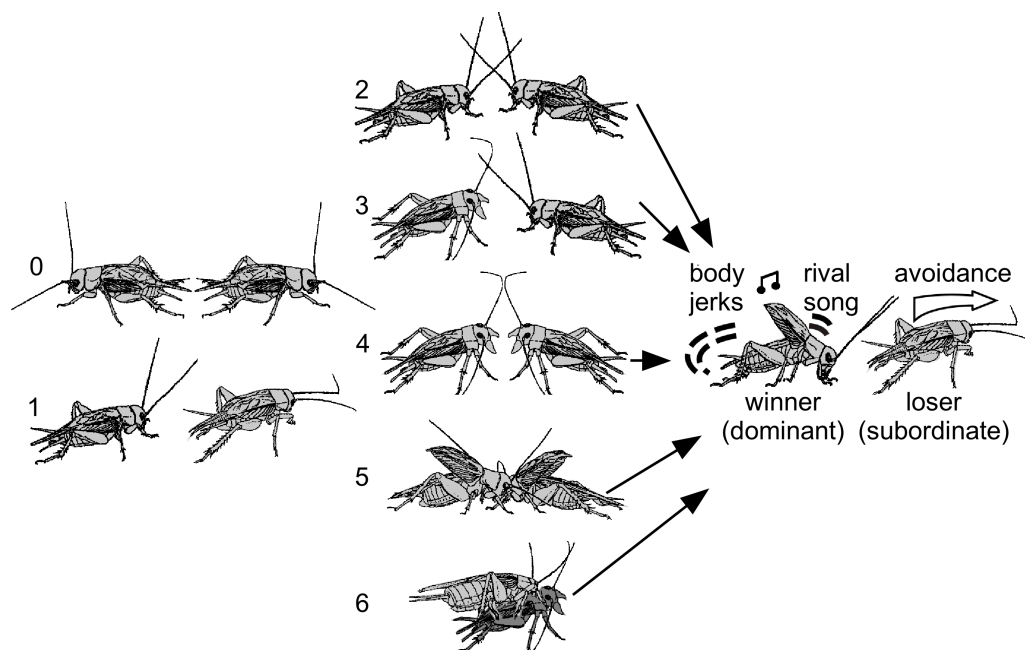


Figure 4 | Stereotypical behaviour in a fight between two male crickets characterised as increasing levels of aggression. Level 0: mutual avoidance with no interaction. Level 1: attack of one animal, avoidance of the other animal (pre-established dominance). Level 2: antennal fencing. Level 3: mandible spreading of one animal. Level 4 mandible spreading of both animals. Level 5: Mandible interlocking with animals pushing. Level 6: Grappling (all-out fight). The encounter can be terminated at each level by the escape of one animal which is the loser of the fight. Adapted from (Stevenson et al. 2000, 2005).

The second measure is the duration of the fight, which was measured with a stopwatch and rounded to full seconds. If there was a short interruption of the fight, the clock was stopped, and the measurement continued when the fight continued. When one animal retreated from the fight the clock was stopped. To test if the fight had really been decided, the animals were separated by removing the winner and reunited after 1 min, as actual defeated animals then behave submissive towards the opponent and retreated immediately (loser effect, Iwasaki et al., 2006; Hofman and Stevenson, 2000; Hsu et al., 2006). If not, the measurement continued, and if yes, the match duration was noted.

In a normal fight between random animals either of the opponents could retreat at any point of the fight. To precisely measure level of aggression and duration of the encounter for a focal animal it needed to be matched against a standard hyper-aggressive opponent that was most likely to win the match because of increased aggressiveness (Rillich and Stevenson, 2011). Consequently, the focal animal determined the end of the fight and therefore its specific level of aggression and match duration (persistence) was measured. Hyper-aggressive animals were generated by holding it in the breeze of a fan (AKA electric, QL I) and allowing it to fly for about three minutes (as in Stevenson and Rillich, 2015) after being fixated with a short thin wire glued to the pronotum for easy relocation and elevation. The animals in one match had a maximal weight difference of 5%. The fight took place in the circular arena where the focal animal was already located and the hyper-aggressive was placed in the arena. Level of aggression and match duration for the focal animal were noted.

2.7 Multiple agonistic experiences

2.7.1 Chronic social defeat stress

For this set of experiments STI (N = 30) and LTI (N = 40) animals were subjected to chronic social defeat stress after isolation by fighting against a standard hyper-aggressive opponent (weight matched) and losing multiple consecutive matches. The focal animals were placed in the circular arena and on the first day they were defeated 6 times in 1 h intervals starting in the morning (Rose et al., 2017b). Their initial aggression (level of aggression and match duration) was evaluated in the first match (see 2.6). The focal animals remained in the arena overnight with protein flakes to eat. On the second day, 24 h after the first fight, the animals were again subjected to defeat three times at 1 h intervals. If a focal animal won against the hyper-aggressive animal in any of the matches, it was discarded from the experiment. Animals of the control groups (STI: N = 20; LTI: N = 20) were not subjected to chronic defeat stress and were placed individually into the circular arenas directly after the ninth fight of the other animals in

order to perform the following steps of the experiment parallel with all experimental groups to exclude day dependent influences. The identity in regard to the treatment was masked. After a pause of 3 h following the last fight, the animals were trained in the standard differential conditioning paradigm (according to 2.2.3) to determine their learning capacity. The aggression of each animal was evaluated by staging a test match against a hyper-aggressive opponent 10 min after the recall test. Focal animals that terminated this match at a level of aggression of ≤ 2 (antennal fencing) were defined as submissive, animals that fought at a level ≥ 4 were defined as aggressive (mandible threat, both animals). In this paradigm level 3 (mandible threat unilateral) was obsolete because the measurement of the level referred to the focal animal and in level 3 the focal animal would show a mandible threat and the hyper-aggressive would retreat. This would be the reason to exclude the focal animal from the experiment.

2.7.2 Multiple wins

This paradigm resembled the process of the chronic defeat paradigm with the difference that the STI animals (N = 20) experienced multiple wins by allowing them to fight against a weight matched opponent taken from a breeding colony. If the focal animal won the first match, the experiment was pursued until it experienced 6 consecutive wins in an interval of 1 h on the first day and 3 more on the following day 24 h later. Level of aggression and duration of the fights were not noted as the opponent and not the focal animal determined the end of the fight. If a focal animal was defeated by the opponent, it was discarded from the experiment. Control animals (N = 20) that were tested parallelly had no fighting experience. The learning capacity of the animals was then determined in the differential conditioning paradigm after a pause of 3 h following the last fight. After a pause of 10 min following the recall test, the aggression of each animal was evaluated by staging a fight against a hyper-aggressive opponent to determine level of aggression and match duration. Here, the focal animal lost the fight accordingly.

2.8 Serotonin

2.8.1 Pharmacological treatments

Three serotonergic drugs were used for this set of experiments with different effects on the serotonergic system. The effective dosages influencing aggression without general detrimental effects had already been analysed and employed in previous studies (Rillich and Stevenson, 2017, 2018). To block serotonin receptors ketanserin (+)-tartrate salt (10 mmol/l; ketanserin; Tocris Bioscience, Bristol, United Kingdom), a 5HT-receptor antagonist with a potentially

higher affinity to the receptor subtype 2 and low binding affinity to other subtypes (Thamm et al., 2013; Vleugels et al., 2015), and methiothepin mesylate salt (10 mmol/l; methiothepin; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), a non-selective 5HT-receptor antagonist (Vleugels et al., 2015), were used. Furthermore, fluoxetine hydrochloride (1 mmol/l; fluoxetine; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), a 5HT re-uptake inhibitor was used. Each of the drugs was first dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and then diluted in phosphate buffered saline (saline; components in mmol/l: NaCl 86 (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), Na₂HPO₄-2H₂O 79 (Grüssing GmbH, Filsum, Germany), Na₂HPO₄-H₂O 23 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), regulated with NaOH (1 mol/l, Merck KGaA, Darmstadt, Germany) to pH 7.4) to finally have the drug soluted in 5% DMSO in saline. Utilizing a microliter syringe (HAMILTON bonaduz AG, Bonaduz, Switzerland) 20 µl of each drug solution or of the vehicle were injected directly into the haemocoel through the pronotal shield (as in Rillich and Stevenson, 2017, 2018). The saline with 5% DMSO served as a vehicle control and was dosed and handled the same as the drug solutions.

2.8.2 Methiothepin and ketanserin

For this set of experiments STI animals were subjected to chronic social defeat stress as described in 2.7.1. Two hours after the ninth defeat the drug treated animals were either injected with methiothepin (N = 20) or ketanserin (N = 20). Animals of one group, which served as control, were injected with the vehicle (N = 20). To control for effects of the drug/vehicle alone three additional groups (N = 20 each) were placed in the circular arenas parallel to the chronic defeat subjected groups but did not experience defeat and were then injected with the respective drug or vehicle. Following a pause of 1 h after the injection, which adds up to 3 h after the last defeat, the animals were trained and tested in the standard differential conditioning paradigm. It is important to mention that their group affiliation regarding drug or vehicle injection was masked before the training began. 10 min after the recall test, the aggression was evaluated by letting them fight against a hyper-aggressive male.

2.8.3 Fluoxetine with non-chronic defeat

For this experiment STI animals were placed into the circular arenas and two groups (N = 20 each) were directly injected with fluoxetine and one control group with the vehicle (N = 20). After a pause of 1 h the animals of one fluoxetine treated, and the vehicle control group were subjected to two consecutive defeats against a hyper-aggressive male with a pause of 1 h in

between defeats. In contrast to the ketanserin and methiothepin treated animals, in this experiment the animals were subjected to non-chronic defeat, meaning only two consecutive fights with only short depression of aggression in comparison to chronic defeat (Rillich and Stevenson, 2018). The second fluoxetine treated group did not experience any defeat and acted as an additional control for the effects of fluoxetine. The group identity of the individuals was blinded and after 24 h the animals of the three groups were trained and tested in the differential conditioning paradigm and 10 min after the recall test the aggression of the animals was evaluated.

2.9 Additional data analysis and statistic

In general, statistical tests and data analysis were performed with Prism 8, the binary logistic regression model was built using SPSS, both running on a Macintosh computer (Mac mini, Apple, Cupertino, CA, USA). The Shapiro-Wilk test was used to test if the data were normally distributed. If the data were not normally distributed the according statistical tests and analysis with the median, interquartile range (IQR) are given for each dataset. Whiskers show the 10 + 90 percentiles and to some extent the individual values are given in the graphs. To test for significant differences between two unpaired datasets, the Mann-Whitney U-test (MWU) was used. Differences between paired datasets were tested with the Wilcoxon matched-pairs signed-rank test (Wilcoxon). The Wilcoxon signed rank test was used to test the dataset against a hypothetical value. Normally distributed, unpaired data were tested with the Student's two-tailed unpaired t-test and the means given with the 95% confidence interval (CI; Hazra, 2017). N indicates the number of animals. To test for statistically significant differences in *LIs* between the eight different permutations of the differential conditioning paradigm (Table 1) a one-way ANOVA on ranks (Kruskal-Wallis test) was performed. The significance level alpha was set to $p < 0.05$. When the same dataset was used in two statistical analyses the Bonferroni correction was used and accordingly alpha was set to 0.025. For which comparisons the alpha-level was corrected, is stated explicitly in the text or legends. A bimodality coefficient was calculated for two datasets to test for a bimodal distribution according to Pfister et al. (2013). Fisher's exact test with Freeman-Halton extension was used to compare relative frequencies in a 2x3 contingency table (in SPSS). To test the aggression and the *LI* data for correlations Spearman rank correlation was used. The figures were created with canvas DRAW 5 for MAC (Version 5.0.2, ACD Systems International Inc., Victoria, BC, Canada).

Parts of this thesis are published, and elements have been adapted or taken verbatim or in spirit from Borstel and Stevenson, 2021. Corresponding figures and tables are indicated.

3 RESULTS

3.1 Binary logistic regression model for quantifying learning

3.1.1 Behavioural variables of a conditioned odour response

When presented with a novel odour, crickets showed a range of responses to this new sensational input. A considerable number of the animals did not react to an odour naively and stayed still for the observation time of 2 min or walked around in the arena casually in any direction. Normally, when crickets walk, they touch the ground with their palps and the antennae wave constantly with a relatively low frequency touching the ground and the walls of the arena (Figure 5). The crickets noticed the novel odour stimulus and therefore some waved their antennae in the direction of the odour and started walking around to further investigate. Typically, after paired appetitive conditioning, which is the presentation of the odour (conditioned stimulus, CS) reinforced with a sugar water reward (unconditioned stimulus, US) given from above with a pipette, the animals response to the presentation of this odour subsequently changed.

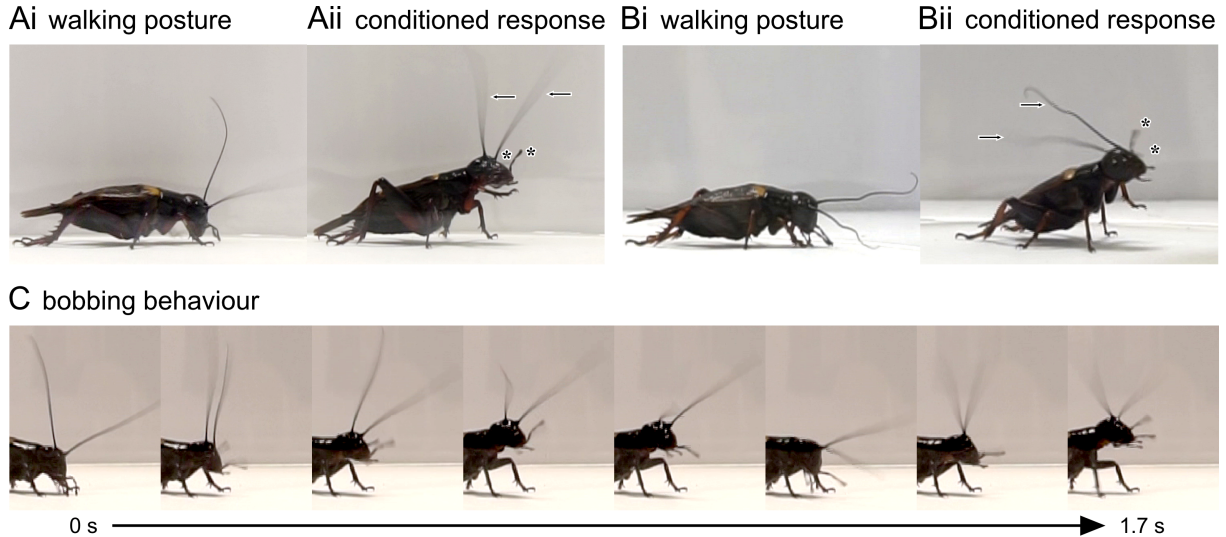


Figure 5 | Conditioned odour responses. **Ai, Bi** Normal walking posture of two example animals **Aii, Bii** Snapshot of the conditioned response of the respective example animals. The head is elevated with extended palps (asterisks) and waving antennae (arrows). **C** Image sequence of bobbing behaviour. The animal moves its head up and down repeatedly with extended palps and waving antennae. Sequence: 1.7 s.

They responded to that odour with searching behaviour. This conditioned response is characterised by the animal moving around the arena proximate to the odour application site, while waving their antennae rapidly, as if searching for the reward. A frequent and prominent part of the conditioned response was bobbing behaviour (Figure 5) which is characterised by the animal raising the head up and down repeatedly, with extended palps and waving of the antennae. The intensity and duration of this behaviour varied between the animals and the individual occurrences but seemed to be a strong indicator for a conditioned odour response.

The aim to measure and quantify the conditioned response and the learning capacity of individual crickets incorporated the intention to automatize this procedure by using video tracking in combination with binary logistic regression modelling. First, to build a model, two groups of animals were trained to differ in their odour responses. These animals received training in an absolute appetitive conditioning paradigm in which individuals of one group were presented with the odour paired with a sugar water reward (CS+US-paired) six times and the others received only the reward six times (US-only). Additional control groups received either the odour alone six times (CS-only) or the CS and US presented with a pause of 2.5 min in between (CS+US-unpaired). The video tracking and analysing of the odour responses over 2 min with EthoVision revealed 13 behavioural variables that came into consideration for further analysis (*bobbing, initial distance, initial velocity 0.2 s, initial velocity 0.5 s, initial velocity 1 s, movement at boundary, time in centre, time moving in odour area, time moving in odour zone, time to move, total distance, total time moving, velocity*) based on the movement or the placement of the animal in the arena, whose definitions are further specified in Table 2. By averaging the data of *total time moving, time to move* and *bobbing* for each group and selecting the track that resembles these values most, representative tracks of US-only (Figure 6A), CS-only (Figure 6B), CS+US-unpaired (Figure 6C) and CS+US-paired (Figure 6D) trained animals are displayed. Differences between the responses of these example animals already become visible, some animals only walked short distances (US-only, CS-only, CS+US-unpaired vs. CS+US-paired), some walked more in close proximity to the odour application site (e.g. CS-only vs. CS+US-unpaired) and the walking paths were more straight forward (US-only and CS+US-unpaired) or the animals were going back and forth (CS-only and CS+US-paired).

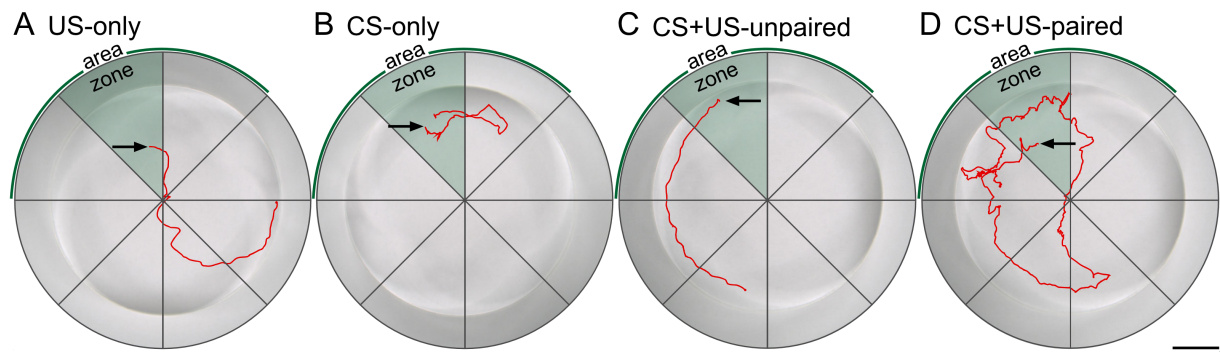


Figure 6 | Representative EthoVision tracks of **A** US-only, **B** CS-only, **C** CS+US-unpaired and **D** CS+US-paired trained animals. The track of the animal is traced in red, arrows mark the start position of the animal and additionally the odour application site. The partition that represents the odour application zone is marked in green and the partitions that represent the odour application area are indicated by a green arc. Scale bar: 3 cm (A-D).

In order to estimate which of these variables were suitable to be considered for model building with binary logistic regression analysis, a univariate analysis was performed, and the resulting Wald statistic (with relaxed significance level: $p < 0.1$) considered. Therefore, the data for the 50 US-only (*conditioning* coded: 0) and 50 CS+US-paired (*conditioning* coded: 1) animals were analysed. Most of the independent variables contribute significantly to a model predicting the dependent variable *conditioning* and have a Wald statistic with $p < 0.1$ (Table 2). The variables *initial velocity 0.2 s* (Wald, $p = 0.166$), *initial velocity 0.5 s* (Wald, $p = 0.111$), *initial velocity 1 s* (Wald, $p = 0.177$) and *time in centre* (Wald, $p = 0.163$) had no significant Wald statistic and were therefore discarded from the further analysis. *Movement at boundary* (Wald, $p = 0.000$) was also discarded, although it was significant, because the spot in the arena where the animals started their movement was inconsistent (sat in the centre or at the boundary) and therefore a comparability between video trials was not given. The remaining eight variables, defined in Table 2 and further described in statistical details later, were retained for model building.

Table 2 | Description of the behavioural response variables extracted from video tracks. Wald statistics, the corresponding p -values (p set to < 0.1 , boldface when significant) of the univariate logistic regression analysis and the decision to consider for further model building are given.

<i>Variable, unit</i>	Definition	Wald	p	Retain
<i>Bobbing, s</i>	Cumulative time spent showing head-bobbing behaviour	9.712	0.002	yes
<i>Initial distance, cm</i>	Sum of the distance moved in each time frame (1/60 s) for the first 0.4 s after the start of movement (<i>time to move</i>)	2.830	0.093	yes
<i>Initial velocity 0.2 s, cm/s</i>	Mean velocity in the first 0.2 s after the start of movement	2.469	0.116	no
<i>Initial velocity 0.5 s, cm/s</i>	Mean velocity in the first 0.5 s after the start of movement	2.546	0.111	no
<i>Initial velocity 1 s, cm/s</i>	Mean velocity in the first 1 s after the start of movement	1.820	0.177	no
<i>Movement at boundary, s</i>	Time moving near the wall (width 2.5 cm)	18.981	0.000	no
<i>Time in centre, s</i>	Time the animal spent in the centre (with + without moving)	1.943	0.163	no
<i>Time moving in odour area, s</i>	Time moving in the odour- and 2 adjacent zones	24.006	0.000	yes
<i>Time moving in odour zone, s</i>	Time moving in the zone where odour was applied	21.764	0.000	yes
<i>Time to move, s</i>	Time until a movement sequence began after odour application. Videos were manually checked to exclude data for movements (e.g. body jerks) that were not the beginning of a clear movement sequence and the value taken when a clear movement began. Individuals that did not move during recording session were allocated the maximum value of 2 min.	13.177	0.000	yes
<i>Total distance, cm</i>	The total distance moved during the entire 2 min recording	21.716	0.000	yes
<i>Total time moving, s</i>	Total time spent moving during the entire 2 min recording	23.344	0.000	yes
<i>Velocity, cm/s</i>	Mean velocity for time frames when the animal was moving	5.244	0.022	yes

With one exception the retained variables differed significantly between the animals that received CS+US-paired in training and those that received US-only. Table 3 gives statistical details of all eight variables and p -values given from Mann-Whitney U tests. After odour presentation at the recall test the CS+US-paired trained animals started to move quickly, with a low *time to move*, in comparison to the US-only animals that started moving significantly later, or not at all during the 2 min recording session ($p < 0.0001$; Table 3, Figure 7A). Even though the *initial distance* moved in the first 0.4 s after the start of the movement did not differ between the two groups ($p = 0.264$; Table 3, Figure 7B), it was considered to be included in the model building process because it was significant in the univariate analysis with relaxed p -values for Wald statistic (Table 2). The *total distance* the animals walked during recording session was significantly longer for the CS+US-paired group compared to US-only ($p < 0.0001$;

Table 3, Figure 7C), also the *total time moving* was longer for the CS+US-paired ($p < 0.0001$; Table 3 Figure 7D). Similarly, the durations recorded for *time moving in odour zone* and *time moving in odour area* were all significantly longer for the CS+US-paired group ($p < 0.0001$ for both variables; Table 3, Figure 7 E and F) in comparison to the US-only animals. When the *velocity* was only considered when the animals were moving, the CS+US-paired crickets were faster than the US-only group ($p = 0.045$; Table 3, Figure 7G). One main aspect of the conditioned response, *bobbing* behaviour was performed by the majority of CS+US-paired animals (78%) and for more than half of them this lasted longer than 10 s. The US-only crickets showed significantly less *bobbing* ($p < 0.0001$; Table 3, Figure 7H) and it was shown by only 10% of the animals, for maximally 3.8 s.

Because animals of each group had alternately either been conditioned with AM or OCT, possible naive preferences for the odours had been controlled for by analysis and examination of the naive preference of the animals that had been tracked before the training. Therefore, the behavioural variables of naive responses of the first 100 animals in the absolute paradigm that were presented with AM (N = 50) were compared to those presented with OCT (N = 50), with no regard to the following training regime (US-only or CS+US-paired) or the categorisation in test or model animals. At the used concentrations, none of the variables differed significantly between AM and OCT (U and p given from MWU) in the naive response to the odours:

time to move (AM: median: 17.08 s, IQR: 4.93 s to 37.55 s; OCT: median: 21.35 s, IQR: 7.84 s to 62.55 s; U = 1065, $p = 0.2021$),

initial distance (AM: median: 0.33 cm, IQR: 0.16 cm to 0.62 cm; OCT: median: 0.36 cm, IQR: 0.15 cm to 0.93 cm; U = 1156, $p = 0.5193$),

total distance (AM: median: 23 cm, IQR: 5.95 cm to 50.37 cm; OCT: median: 24.96 cm, IQR: 3.63 cm to 78.05 cm; U = 1189, $p = 0.6764$),

total time moving (AM: median: 6.06 s, IQR: 1.58 s to 12.42 s; OCT: median: 5.78 s, IQR: 1.06 s to 19.9 s; U = 1212, $p = 0.7927$),

time moving in odour zone (AM: median: 1.175 s, IQR: 0.34 s to 2.39 s; OCT: median: 1.108 s, IQR: 0.2 s to 3.32 s; U = 1226, $p = 0.8677$),

time moving in odour area (AM: median: 2.48 s, IQR: 1.39 s to 5.95 s; OCT: median: 2.45 s, IQR: 0.73 s to 7.08 s; U = 1223, $p = 0.8542$),

velocity (AM: median: 3.91 cm/s, IQR: 3.61 cm/s to 4.1 cm/s; OCT: median: 3.99 cm/s, IQR: 3.42 cm/s to 4.43 cm/s; U = 1111, $p = 0.3399$),

bobbing (AM: median: 0 s, IQR: 0 s to 0 s; OCT: median: 0 s, IQR: 0 s to 0 s; U = 1226, $p = 0.8724$).

Table 3 | Statistical data for response variables. Median and interquartile range (IQR) are given (N = 50 each); *p* and U values are given from the Mann-Whitney U test (boldface when significant). Taken from (Borstel and Stevenson 2021).

<i>Variable, unit</i>	US-only Median (IQR)	CS+US-paired Median (IQR)	<i>p</i> (U)
<i>Time to move, s</i>	25.5 (10.3-86.7)	1.7 (0.9-4.7)	<0.0001 (266)
<i>Initial distance, cm</i>	0.3 (0.1-0.6)	0.2 (0.2-0.4)	0.264 (1087)
<i>Total distance, cm</i>	10.7 (2.1-25.4)	52.2 (29.4-99.1)	<0.0001 (322)
<i>Total time moving, s</i>	2.9 (0.6-6.4)	14.1 (8.5-23.6)	<0.0001 (299)
<i>Time moving in odour zone, s</i>	0.9 (0.3-1.5)	4.1 (1.9-6.4)	<0.0001 (296)
<i>Time moving in odour area, s</i>	2 (0.6-3.3)	8.4 (3.8-14)	<0.0001 (261)
<i>Velocity, cm/s</i>	3.7 (3.4-4)	3.9 (3.7-4.1)	0.045 (959)
<i>Bobbing, s</i>	0 (0-0)	10.4 (2.3-27.1)	<0.0001 (308)

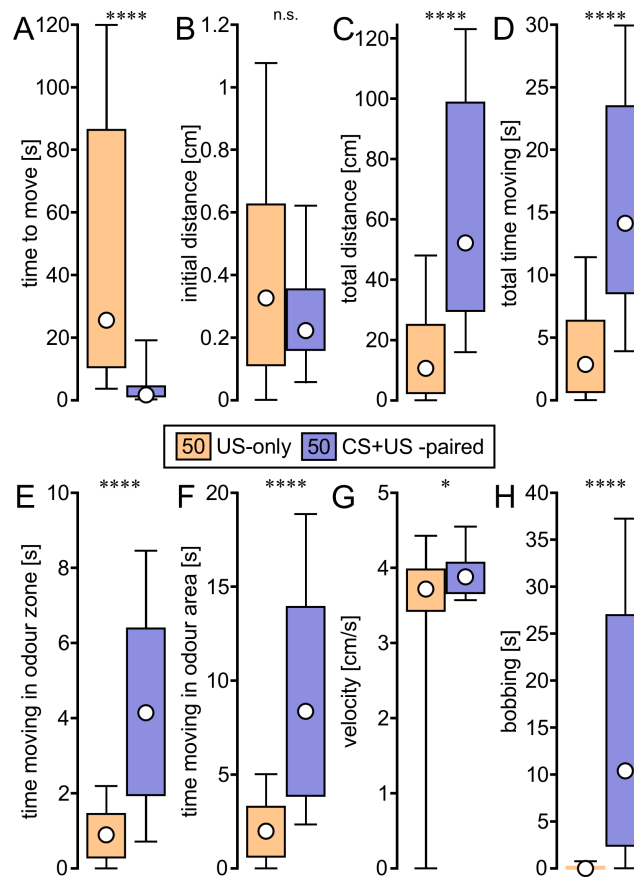


Figure 7 | Box plots of variables extracted from video tracked odour responses and used for binary regression analysis. The animals were trained in the single odour (absolute) conditioning paradigm and received either US-only (orange) or CS+US-paired (purple). Circle: median; box: interquartile range; whiskers: 10th and 90th percentile; N = 50 for each group. **A** *Time to move*. **B** *Initial distance*. **C** *Total distance*. **D** *Total time moving*. **E** *Time moving in odour zone*. **F** *Time moving in odour area*. **G** *Velocity*. **H** *Bobbing*. Asterisks indicate significant differences: Mann-Whitney U test: **** $p < 0.0001$; * $p < 0.05$; n.s. not significant. Taken and adapted from (Borstel and Stevenson, 2021).

3.1.2 Model building and selection

The data of the eight variables retained for model building for the binary groups (dependent variable *conditioning*: US-only = 0 and CS+US-paired = 1, N = 50 each) were included in a forward stepwise conditional binary logistic regression analysis to calculate the first model. The detailed coefficients, data and statistics for all models calculated are depicted in Table 4.

Model-1 retained the variables *time to move*, *time moving in odour area*, and *bobbing*. The Hosmer-Lemeshow test indicated a satisfactory goodness-of-fit ($p = 0.988$) for the allocation of individuals to the CS+US-paired or US-only groups in comparison to their actual classification and the model on average assigned 91% of the animals to their correct groups (US-only 96%, CS+US-paired 86%). The Akaike information criterion for small sample sizes (AICc) score was at 48.9, but in order to make statement about this measure, the AICc scores of alternative models have to be compared to this score. It is important to notice that only two of the three variables were retained with a significant Wald statistic (*time to move* and *bobbing*) and the insignificance of the Wald statistic ($p = 0.094$) for *time moving in odour area* indicates that this variable does not contribute significantly to the model. The model was tested for multicollinearity between the variables which has the potential to negatively influence the model and the statistical contribution of variables. By using the variance inflation factor (VIF) and with values for *time to move* = 1.351, *time moving in odour area* = 1.755 and *bobbing* = 1.476, no collinearities were detected.

For its insignificant contribution, *time moving in odour area* was discarded from the dataset for further analysis and again a forward stepwise conditional binary logistic regression analysis with the remaining seven variables was performed.

Model-2 also retained *time to move* and *bobbing*, but added *total time moving* as third variable. According to Wald statistic, all of the three variables contributed significantly to the model ($p < 0.05$). The Hosmer-Lemeshow goodness-of-fit test yielded a p -value greater than 0.05 ($p = 0.988$), which indicated a good fit. On average, 93% of the animals were classified to the correct group by the model (US-only 96%, CS+US-paired 90%). The AICc score for this model was 45.35 and the VIF values for the independent variables were low and not hinting at multicollinearities (VIF: *time to move* = 1.373, *total time moving* = 1.43, *bobbing* = 1.218).

The goal for a further model analysis was to construct a four-variable model to test if an additional variable could improve the assignment of animals. Therefore, a fourth variable was added to the variables retained in *Model-2*. This time by using the method “enter” that enters all variables given into the binary logistic regression analysis.

Model-3 consists of the variables *time to move*, *total time moving*, *bobbing* and *velocity*. It allocated on average 94% of the animals into their correct groups (US-only 98%, CS+US-paired 90%) and had a good fit (Hosmer-Lemeshow, $p = 0.999$), but it is important to notice, that the contribution of *velocity* to the model was insignificant (Wald: $p = 0.256$). The AICc-score was at 46.11. Regarding multicollinearities, the variable *time to move* had the highest VIF (2.065) of all analyses but the other variables did not hint at multicollinearities (*total time moving* = 1.534, *bobbing* = 1.284, *velocity* = 1.919).

Because *bobbing* has to be evaluated using the manual scoring function in EthoVision, the goal for the analysis of the fourth model was to achieve an automatized approach. Therefore, *bobbing* was excluded from the dataset and the remaining seven variables were included in a forward stepwise conditional binary logistic regression analysis.

Model-4 included three variables: *time to move*, *initial distance* and *time moving in odour area*. The Hosmer-Lemeshow goodness-of-fit test was insignificant but had a very low p -value ($p = 0.114$). Additionally, the model had a high AICc score of 74.68. Even so, the Wald statistic indicated that all three variables made a significant contribution to the model and 87% of the animals were allocated correctly (US-only 88% and CS+US-paired 86%). For this model, the VIF values are not hinting to multicollinearities (VIF: *time to move* = 1.464, *initial distance* = 1.099, *time moving in odour area* = 1.418).

The resulting three- and four-variable models have 16.6 and 12.5 events per variable (EPV), respectively. For all four models the area under the receiver operating characteristic curve (AUC under ROC) was over 0.935 indicating that all models show an outstanding discrimination between the animals of the US-only and CS+US-paired group (AUC under ROC: *Model-1* = 0.970, *Model-2* = 0.975, *Model-3* = 0.976, *Model-4* = 0.936; Table 4).

Regarding the data described here, the preferred model for future analysis of conditioned responses was *Model-2* for several reasons. It assigned the most animals to their correct group (*Model-2* was superior to *Model-1* and *Model-4*) while simultaneously only including variables that contributed significantly to the model (*Model-2* was superior to *Model-1* and *Model-3*). A strong indicator to opt for *Model-2* was also the AICc. A lower AICc score indicates a superior model in a direct comparison between several models and *Model-2* has the lowest AICc score of all four Models. Additionally, it is the most parsimonious model.

Table 4 | Comparison of binary logistic regression models evaluated for calculating the probability of an animal showing a conditioned odour response (P_{resp}). *Model-1*: suggested by SPSS; *Model-2*: the most parsimonious; *Model-3*: has an additional, 4th, variable; *Model-4*: excludes *bobbing*. The table gives the variables retained in a model plus the regression intercept for each of the coefficients (Coef β), the standard error (SE) and Wald statistic (Chi^2 and p , boldface when variable contribution is significant). The percentage of animals assigned correctly to the US-only or CS+US-paired (CS+USp) group (N = 50 each) and the average (Avg.) is given for animals included in the model, and in parentheses for out-model test animals (N = 25 each). The Hosmer-Lemeshow test gave the goodness-of-fit probability (HL- p) for each model. Additionally, the Akaike information criteria score (AICc) and the area under the receiver operating characteristic curve (AUC, in parentheses for out-model test animals) is given for each model. Adapted from (Borstel and Stevenson, 2021).

Model	Variable	Coef		Wald stat.		Assignment, %			HL- p	AICc	AUC	
		β	SE	Chi^2	p	Avg.	US-only	CS+USp				
-1	<i>time to move</i>	-0.148	0.059	6.351	0.012	91	96	86	0.988	48.9	0.970	
	<i>t-mov. odour area</i>	0.199	0.119	2.813	0.094	(86)	(84)	(88)				(0.955)
	<i>bobbing</i>	0.933	0.363	6.627	0.010							
	<i>intercept</i>	-0.458	0.759	0.363	0.547							
-2	<i>time to move</i>	-0.156	0.061	6.523	0.011	93	96	90	0.988	45.35	0.975	
	<i>total time moving</i>	0.152	0.067	5.166	0.023	(90)	(92)	(88)				(0.960)
	<i>bobbing</i>	0.985	0.377	6.838	0.009							
	<i>intercept</i>	-0.973	0.818	1.417	0.234							
-3	<i>time to move</i>	-0.156	0.064	5.880	0.015	94	98	90	0.999	46.11	0.976	
	<i>total time moving</i>	0.205	0.084	6.012	0.014	(90)	(92)	(88)				(0.938)
	<i>velocity</i>	-1.598	1.406	1.292	0.256							
	<i>bobbing</i>	0.956	0.375	6.507	0.011							
	<i>intercept</i>	4.826	5.151	0.878	0.349							
-4	<i>time to move</i>	-0.064	0.028	5.053	0.025	87	88	86	0.114	74.68	0.936	
	<i>initial distance</i>	-1.529	0.745	4.217	0.040	(82)	(84)	(80)				(0.904)
	<i>t-mov. odour area</i>	0.340	0.107	10.00	0.002							
	<i>intercept</i>	-0.018	0.755	0.001	0.981							

3.1.3 Odour response probabilities (P_{resp})

For each tracked response of an animal a probability of exhibiting a conditioned odour response (P_{resp}) could now be calculated by using the coefficients β for each variable in the model-2 (Table 4) from:

$$P_{\text{resp}} = \frac{e^{\eta}}{(1 + e^{\eta})}$$

whereby,

$$\eta = (-0.973) + (0.152 * \textit{total time moving}) - (0.156 * \textit{time to move}) + (0.985 * \textit{bobbing})$$

A P_{resp} of 0 indicates that an animal did not respond to the odour, whereas a P_{resp} of 1.0 means an absolutely certainty that the animal exhibited a conditioned response. In general, the P_{resp} is the predicted probability that the animals show a response comparable to the CS+US-paired conditioned animals. The cut off value for the P_{resp} is set at 0.5 with a $P_{\text{resp}} < 0.5$ indicating an

allocation to the US-only group and a $P_{\text{resp}} > 0.5$ indicated the allocation to the CS+US-paired group and the animals showing a conditioned response.

Depicted in Figure 8A with statistical details in Table 5 are the resultant P_{resp} values for the data from animals used to build the model. Animals that received US-only and thus had no opportunity to associate the odour with the reward, in general showed no conditioned odour response and therefore low P_{resp} values (median: 0.025; Table 5, Figure 8A). Only two individuals exhibited a P_{resp} greater than the cut-off at 0.5. Data from experimentally naive US-only animals before training (N = 50), that also showed low P_{resp} values (median: 0.005; 7 animals over cut off 0.5, Figure 8A), revealed that the sugar water presentation during training alone had no significant effect on the P_{resp} value (Wilcoxon: $p = 0.835$; Table 5). Compared to the US-only trained animals, the P_{resp} for CS+US-paired trained group was significantly greater (median: 1.0; MWU: $p < 0.0001$; Table 5, Figure 8A). The majority of individual animals score very high P_{resp} nearly 1.0, only five animals having a P_{resp} below the cut-off at 0.5.

With Model-2 a clear separation between the US-only and CS-only could be achieved and additionally the P_{resp} of the majority of the individuals are at the minimum and maximum of 0 and 1, respectively. Important for the evaluation of a model is also the predictive power for test-animals that were not used for model construction, but for which the respective training regime was known. Here the predictive power for additional training regimes could also be tested to further underpin the true measurement of a conditioned odour response with association of CS and US (CS-only, US-only, CS+US-unpaired, CS+US-paired; N = 25 each; Table 5, Figure 8B). *Model-2* allocated 92% of the US-only and 88% of the CS+US-paired (avg. 90%) of the out-model animals to their correct groups. The comparison to the other models constructed reveals its superiority to *Model-1* (avg. 86%, US-only 84%, CS+US-paired 88%) and *Model-4* (avg. 82%, US-only 84%, CS+US-paired 80%) and an equal allocation for *Model-3* (avg. 90%, US-only 92%, CS+US-paired 88%; Table 4). The following data was calculated using *Model-2*. Out-model animals that received odour only during training (CS-only) showed low P_{resp} values (median: 0.022, Table 5, Figure 8B) with three animals scoring a P_{resp} of over 0.5. Here again, US-only showed generally low P_{resp} values (median: 0.086, Table 5, Figure 8B), with only two animals exhibiting a response greater than the cut-off value of 0.5. Test animals that received the odour and the reward unpaired (CS+US-unpaired) had equally low P_{resp} scores (median: 0.085; Table 5, Figure 8B), with six animals showing a P_{resp} greater than 0.5. Comparing these neither the CS-only group differed from the US-only (MWU: $p = 0.295$; Table 5), nor did the responses of the CS+US-unpaired animals differ from the US-only group (MWU: $p = 0.758$, Table 5). In contrast to the three other out-model groups but in line with the model

data, out-model CS+US-paired trained animals scored high P_{resp} values (median: 0.997; Table 5, Figure 8B) with only three animals scoring under 0.5, which were in general significantly higher in comparison to the US-only animals (MWU: $p < 0.0001$; Table 5).

Table 5 | P_{resp} values for all groups of animals used to construct (in-model) and test (out-model) the binary logistic regression model (*Model-2*). Values for p are boldface where significant, U is from Mann-Whitney U tests, and W from Wilcoxon signed rank tests, alpha is set to 0.025 due to two comparisons using the same data set. For each group median, interquartile range (IQR), 10th and 90th percentiles and number of values (N) are given. Taken and modified from (Borstel and Stevenson, 2021).

	Test group	Median	IQR	10%-90%	N	p	U /W
in-model	naive US-only	0.005	0-0.186	0-0.919	50	0.835	W -42
	US-only	0.025	0-0.192	0-0.432	50		
	CS+US-paired	1	0.932-1	0.439-1	50		
out-model	CS-only	0.022	0-0.184	0-0.694	25	0.295	U 258
	US-only	0.086	0-0.294	0-0.623	25	0.758	U 296
	CS+US-unpaired	0.085	0-0.44	0-0.859	25	<0.0001	U 36
	CS+US-paired	0.997	0.818-1	0.372-1	25		

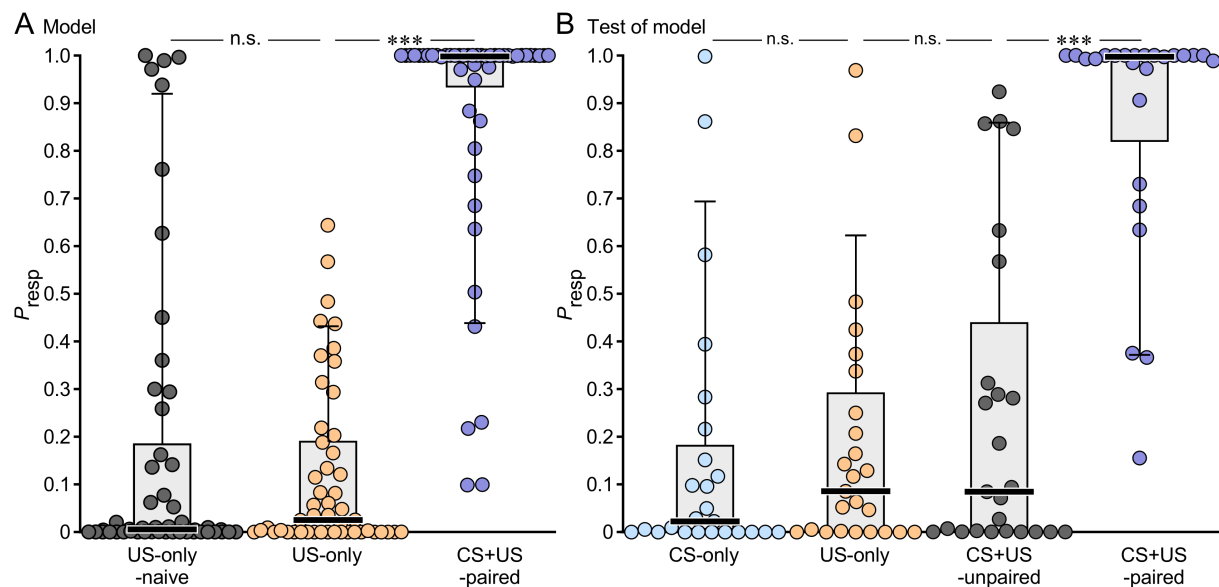


Figure 8 | Box plots giving the individual probabilities of showing a conditioned odour response (P_{resp}) as derived from binary logistic regression modelling. **A** P_{resp} to odour for animals used to construct the model (US-only, CS+US-paired, N = 50 for each plot), together with data for US-only animals before training when experimentally naive (naive US-only, N = 50). **B** P_{resp} data for animals used to test the model (N = 25 for each plot). Prior to testing they received either CS-only (blue), US-only (orange), CS+US-unpaired (grey) or CS+US-paired (purple). Circles: individual values; bar: median; box: interquartile range; whiskers: 10th and 90th percentiles. Asterisks indicate significant differences: Wilcoxon signed rank test (US-only, -naive), Mann-Whitney U test (all other groups), corrected for two comparisons; *** $p < 0.0005$; n.s. not significant. Taken and adapted from (Borstel and Stevenson, 2021).

In the analysis of the out-model groups *Model-2* remains the best model to differentiate between US-only animals that did not show a conditioned response to the odour and CS+US-paired trained animals exhibiting a conditioned odour response. Hence this model was used for all further analysis and is now only referred to as logistic regression model or model.

3.1.4 Application of the regression model to assess the quantification of learning

The binary logistic regression analysis provides a tool to measure a conditioned odour response based on the P_{resp} . The model was applied to a new group of animals that were trained in a differential appetitive olfactory conditioning paradigm in order to evaluate the model's ability to quantify the conditioned response and consequently learning in individual crickets. For this, individual crickets ($N = 64$) were presented with two odours, one was rewarded with sugar water (CS^+) in the training, the other not (CS°). This was repeated three times. In the recall test both odours were presented successively, the video-tracks of the responses to the CS^+ and CS° analysed and the variables *bobbing*, *time to move* and *total time moving* extracted. The P_{resp} value for the response to each of the odours for each animal was then calculated from the regression model and the learning index calculated ($LI = P_{\text{resp}} \text{CS}^+ - P_{\text{resp}} \text{CS}^\circ$).

As it cannot be ruled out that the presentation sequence of odour and reward has an influence on learning in this paradigm, it was important to control for this and therefore the animals were trained in the eight different sequences (Table 1). Comparing all eight permutations among each other, the *LIs* did not differ significantly from each other (Median: Seq. 1: 0.043, Seq. 2: -0.036, Seq. 3: 0.703, Seq. 4: 0.128, Seq. 5: 0.684, Seq. 6: 0.212, Seq. 7: 0.377, Seq. 8: 0.637; $N = 8$ each, Kruskal-Wallis test, $p = 0.334$, $H = 7.985$; sequences compare to Table 1; not illustrated). The 64 animals were then pooled and sorted into two groups in respect to the analysed aspect five times. Detailed statistical values for each combination are depicted in Table 6. There was no significant difference in *LI* between the animals that received AM first in the training to those that received OCT (MWU: $p = 0.132$; Table 6). The same applies to the presentation sequence of odours presented in the recall test, with no difference in *LIs* between the animals presented AM or OCT first (MWU: $p = 0.526$; Table 6). The identity of the odour rewarded (AM or OCT) had no influence on the *LI* (MWU: $p = 0.826$; Table 6). Also, there was no effect on the *LI* if the first or the second CS in training was rewarded (MWU: $p = 0.221$; Table 6). It made no significant difference whether the rewarded odour was presented first in the recall test or second (MWU: $p = 0.132$; Table 6). Despite the multiple uses of the data from the animals for statistical tests a Bonferroni correction was not applied, because all differences

were insignificant in the first place. Since there were no significant differences found for any sequential effects, the data for the eight regimes were pooled for further analyses.

Table 6 | Data from tests for differences in learning index (*LI*) based on the P_{resp} values for the eight permutations of the differential olfactory conditioning paradigm (Table 1). For each of the five tests performed, the entire data set for 64 animals was divided into two, but in different ways to generate five pairs of data sets, which differed in only one aspect regarding either the identity or presentation sequence of the rewarded odour during training and at the recall test. Significant differences were not found (Mann-Whitney U test, $p > 0.05$ in all cases). The last column gives data for the entire group of 64 animals (Wilcoxon signed rank test with hypothetical value = 0.05; $p < 0.0001$; $W = 1434$). Adapted from (Borstel and Stevenson, 2021).

	Odour ...	Median/IQR	10% - 90%	p (U)
... given 1 st in training	AM	0.096 (0.003-0.690)	-0.013 - 0.995	0.132 (399)
	OCT	0.626 (0.217-0.949)	-0.228 - 0.995	
... given 1 st at recall	AM	0.436 (0.013-0.906)	-0.216 - 0.998	0.526 (464)
	OCT	0.365 (0.001-0.858)	-0.084 - 0.990	
... rewarded in training	AM	0.479 (0.010-0.847)	-0.013 - 0.985	0.826 (495)
	OCT	0.314 (0.002-0.972)	-0.228 - 0.997	
... rewarded, given in training	1 st	0.238 (0.001-0.906)	-0.228 - 0.987	0.221 (420)
	2 nd	0.526 (0.058-0.890)	0.000 - 0.998	
... rewarded, given at recall	1 st	0.096 (0.000-0.837)	-0.302 - 0.998	0.132 (399)
	2 nd	0.526 (0.208-0.949)	0.001 - 0.991	
All data		0.395 (0.005-0.890)	-0.332 - 0.999	<0.0001

The pooled data was used for further analyses of the general learning capacity of crickets in this differential learning paradigm and the benefit of using the model to measure learning over a single variable indicating a conditioned response. For the first assessment of the data regarding the P_{resp} values the cut-off value of 0.5 is again used to categorise the responses. In response to the CS° , two thirds (67 %) of the 64 animals exhibited a P_{resp} lower than the cut-off value 0.5 (CS° median: 0.204; Table 7, Figure 9A). But in general, the P_{resp} values were distributed broadly with some animals even reaching high values close to 1. For the odour that was rewarded (CS^+), 78% of the of the animals showed a P_{resp} of over 0.5 (CS^+ median: 0.983, Table 7, Figure 9A). Nonetheless, when the P_{resp} values to the CS° and the CS^+ in individual animals are considered, the majority of animals scored a greater P_{resp} to the CS^+ than to CS° (Wilcoxon: $p < 0.0001$, Table 7), as indicated by adjoining lines in Figure 9A. The difference

between CS⁺ and CS[°] yielded a median *LI* of 0.395 (Figure 9C), with a very broad distribution of individual values ranging from animals that reached a *LI* of 1 to even negative values. For the majority of the animals (69%) the *LI* is > 0.05, indicating a significant appetitive learning effect with 28% even scoring very high *LIs* > 0.8. Some of the individuals (20%) did not show differences in responses between CS[°] and CS⁺ and therefore showed no significant learning ($-0.05 \leq LI \leq 0.05$). A small percentage of the animals (11%) reacted stronger to the CS[°] in comparison to the CS⁺ and therefore scored *LIs* < (-0.05). Despite of some animals not being able to learn the odour or show the correct responses, a Wilcoxon signed rank test against the hypothetical value of an *LI* of 0.05 ($p < 0.0001$; Table 6) indicated that this group of animals showed an appetitive learning.

Table 7 | P_{resp} values and resultant learning indices (*LI*) for the animals in the differential conditioning paradigm calculated from the binary logistic regression Model (*model*) and from normalised *bobbing*-only (*bobbing*). Values for p are boldface where significant, W is from Wilcoxon signed rank tests. For each group median, interquartile range (IQR), 10th and 90th percentiles and number of values (N) are given. Taken and modified from (Borstel and Stevenson, 2021).

Group		Median	IQR	10%-90%	N	Comparison	p	W
P_{resp}								
differential conditioning	CS [°]	0.204	0-0.708	0-0.992	64	CS [°] vs. CS ⁺	<0.0001	W 1716
	<i>model</i> CS ⁺	0.983	0.654-1	0.059-1	64			
differential conditioning	CS [°]	0	0-0.019	0-0.066	64	CS [°] vs. CS ⁺	<0.0001	W 1130
	<i>bobbing</i> CS ⁺	0.054	0-0.216	0-0.455	64			
Learning index								
<i>model</i>	LI	0.395	0.005-0.890	-0.081-0.994	64	<i>model</i> vs. <i>bobbing</i>	<0.0001	W 1390
<i>bobbing</i>	LI	0.054	0-0.184	-0.017-0.446	64			

The variable *bobbing* appears to be the strongest predictor of a conditioned response to an olfactory signal after appetitive conditioning and therefore it is important to check whether this variable alone is sufficient to evaluate learning or if the logistic regression model is superior. In order to reach a better comparability, the entire dataset for *bobbing* was first pooled (CS[°] and CS⁺) and then normalised so that the lowest value was 0 and the highest 1, but the non-normalised original values are also indicated in Figure 9B (same relative distribution). In response to the CS[°] animals seldom show any bobbing behaviour, therefore the normalised *bobbing* values were very low (median: 0; Table 7, Figure 9B) and for all animals were smaller than 0.5. The majority of animals also responded to the CS⁺ with higher scores, but only 5 animals even exceeded values of 0.5 (median: 0.054; Table 7, Figure 9B). In general, these

scores were very low, with 28% showing no bobbing behaviour at all. Despite this, the difference between the bobbing responses to the CS^o and the CS⁺ of individuals, indicated by adjoining lines in Figure 9B, was significant (Wilcoxon: $p < 0.0001$; Table 7). The differences in the *bobbing* values to the CS⁺ and CS^o yielded a median *LI* of 0.054 (Figure 9C) with the majority of animals scoring low *LIs* and only a few scoring high *LIs*. Further investigation into the data reveals that 53% of the animals score a *bobbing-LI* > 0.05 but only 3% > 0.8 , whereas 45% of the animals did not show a different bobbing response to the CS⁺ in comparison the CS^o ($-0.05 \leq LI \leq 0.05$) and 2% a $LI < -0.05$). However, it should be noted that these limits are originating from the model calculations.

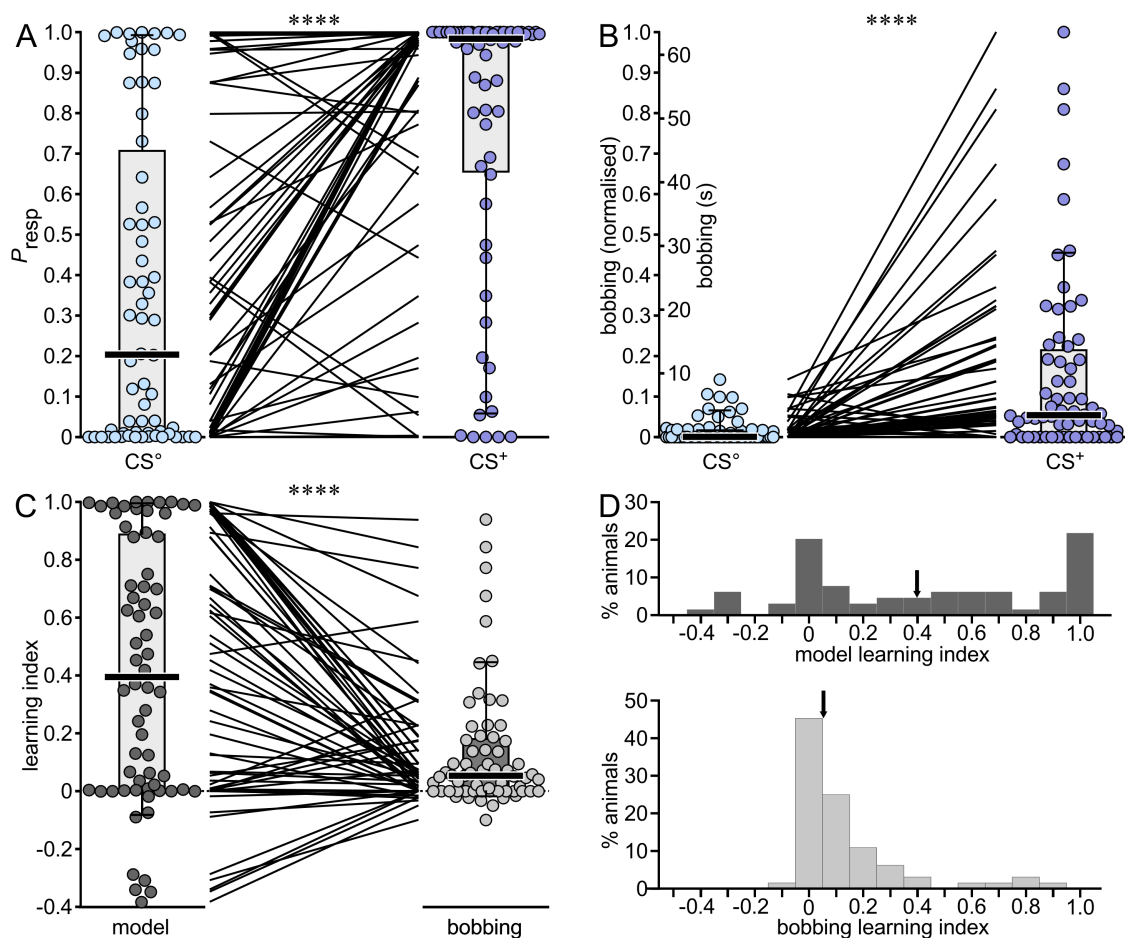


Figure 9 | Performances of individual crickets in the differential olfactory conditioning paradigm: A comparison of the binary regression model with *bobbing*. **A** Boxplots giving P_{resp} values derived from the binary regression model for the non-rewarded and rewarded odour (CS^o and blue circles, respectively CS⁺ and purple circles; bars: median; boxes: interquartile range; whiskers: 10th and 90th percentiles, N = 64). The interposing lines between plots indicate the difference in response for each individual. **B** As for A, but for *bobbing*, for which the duration was normalised for the entire dataset (CS^o and CS⁺: 0 = lowest value; 1 = highest; the original data scale *bobbing* (s) is also depicted for comparison). **C** Comparison of the learning indexes given by *Model-2* and from normalised *bobbing* alone (CS⁺ minus CS^o values from A and B respectively). Asterisks in A-C indicate significant differences: Wilcoxon signed rank test; **** $p < 0.0001$. **D** Histograms showing the distribution of the model learning index (top) and the *bobbing* learning index (bottom). Arrows indicate the medians. Taken from (Borstel and Stevenson, 2021).

When the *LIs* given by the *model* and from *bobbing* alone were compared, the statistical analysis revealed a significantly lower score for *bobbing* (Wilcoxon: $p < 0.0001$, Table 7, Figure 9C). The difference between the two ways to determine learning is also illustrated in the frequency histogram of Figure 9D. The distribution of animals in the histograms looked completely different. The *model-LI* had two modes at 0 and 1.0, whereas the data for the *bobbing-LI* resembled a skewed distribution, with only one mode at 0. In order to test if the bimodality presumed for the distribution of the *model-LI* is true, a coefficient of bimodality of 0.558 was calculated (values exceeding the critical value of 0.555 indicate significant bimodality (Pfister et al., 2013)).

The bimodal distribution opened the question whether this is an artefact of the binary regression analysis due to the binary characterisation of 0 and 1 with a cut-off at 0.5, or if the model really is able to capture differences in learning capacity of individual animals. The individual P_{resp} values to the CS° and the CS^+ were pooled and arranged in ascending order and then plotted (the same was done for the *bobbing* values). This resulted in a sigmoid curve with mainly 0 and 1 scores, but also many, evenly distributed intermediate scores (Figure 10A) with more CS^+ values on the upper half and more CS° values on the lower half of the graph, but for the intermediate scores there was a composition of CS^+ and CS° assigned values. When the *model-LI* were sorted in ascending order a more linear distribution with relatively evenly spaced individual *LI* values was diagrammed (Figure 10A), with two clusters at 0 and 1.0, but in general the spectrum of values was between an *LI* of -0.4 and 1.0. The plot for the individual normalised *bobbing* values for CS° and CS^+ sorted in ascending order yielded an exponential curve, with the most CS° values being very low and then slowly increasing values with finally some exceptional high values (mostly for the CS^+ , Figure 10B), whereas the majority of all values were situated in the lower half (< 0.5). The *bobbing-LI* values sorted in ascending order also resemble an exponential curve ranging from -0.1 to 0.938 (Figure 10B) with the majority of values smaller than 0.2.

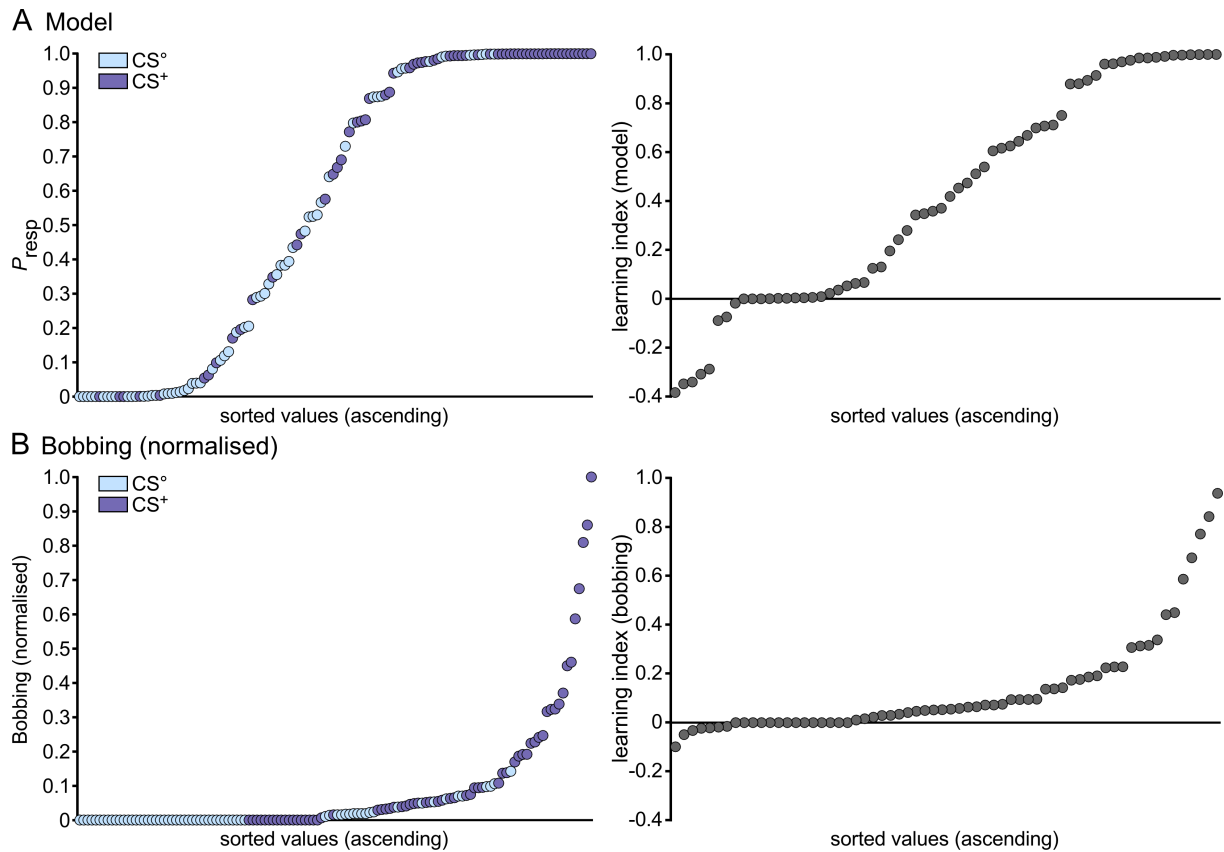


Figure 10 | Plots of individual values. **A** Values derived from the binary logistic regression model. Left graph: P_{resp} values for the non-rewarded and the rewarded odour (CS^o and blue circles, respectively CS⁺ and purple circles) pooled and then sorted in ascending magnitude. Right graph: the resultant learning indices sorted in ascending magnitude. **B** As for A, but for *bobbing* with normalised duration values. Left graph: normalised *bobbing* duration to CS^o and CS⁺. Right graph: *bobbing* learning index. Modified from (Borstel and Stevenson, 2021, Supplementary Figure1).

3.2 The influence of agonistic experiences on aggression and learning

3.2.1 Chronic social defeat stress

From the examination of the learning capacity in the previously described paragraph, it became clear that in a randomly chosen untreated group of crickets the learning capacity can vary hugely between individuals. Previous studies revealed, that in a group of multiple individuals that have male to male contact, the group will likely not be homogenous, but consists of individual animals that are behaving differently based on their previous social experiences, for example regarding aggression and proactiveness/reactiveness (Rose et al., 2017a; Balsam and Stevenson, 2020, 2021). Social chronic subjugation has been shown to have a huge influence on the future behaviour of crickets, in the form that it induces long-term depression with resulting submissiveness in males (Rose et al., 2017b; Rillich and Stevenson, 2018). The first goal of the following set of experiments was to investigate whether the learning capacity of crickets is influenced by chronic social defeat. Therefore, animals were subjected to chronic

defeat by losing nine matches against a standard hyper-aggressive opponent, trained in the differential learning paradigm and finally had their learning capacity and aggressiveness evaluated. Additionally, the experimental procedure described in detail in Figure 11A, was performed with short-term isolated (STI, pre-experimental contact to adult males) and long-term isolated (LTI, no pre-experimental contact to adult males) animals to gain information for possible differences there.

First, the baseline of aggression in the first fight after being isolated for 48 h (STI) for all animals was observed (pooled, $N = 30$) and this match escalated to a median level of aggression of 5 (IQR: 2 to 6, 10%-90%: 2 to 6; Figure 11B) with a median duration of 4 s (IQR: 1.75 s to 11.25 s, 10%-90%: 1 s to 18.9 s; Figure 11B). After being subjected to chronic defeat and being trained and tested in the conditioning paradigm, the focal animals were either behaving submissively (level of aggression ≤ 2) or aggressively (level of aggression ≥ 4) in the test match more than 4.5 h after the last defeat and were classified respectively. As was to be expected from the previous classification of the focal animals, the submissive animals showed a significantly reduced level of aggression but also a reduced match duration at the test match (level: median: 1, IQR: 1 to 2, 10%-90%: 1 to 2; duration: median: 1 s, IQR: 1 s to 2 s, 10%-90%: 1 s to 4.9 s, $N = 20$; Figure 11B) in comparison to the control animals which experienced no defeat (level: median: 5, IQR: 4 to 6, 10%-90%: 2 to 6, MWU: $U = 16$, $p < 0.0001$; duration: median: 7.5 s, IQR: 5.25 s to 12.75 s, 10%-90%: 2.1 s to 20 s, MWU: $U = 17.5$, $p < 0.0001$, $N = 20$; corrected for two comparisons; Figure 11B). The level and duration of the test fight did not differ between the aggressive animals (level: median: 4, IQR: 4 to 5, 10%-90%: 4 to 5.9; duration: median: 5.5 s, IQR: 4 s to 7.25 s, 10%-90%: 3.1 s to 17 s, $N = 20$; Figure 11B) and the animals that experienced no defeat (level: MWU: $U = 77.5$, $p = 0.2818$; duration: MWU: $U = 68.5$, $p = 0.17$; corrected for two comparisons). When the data for the first match of the submissive and aggressive animals were sorted in their respective groups retrospectively, the animals were already showing different characteristics. At the first match, the prospective aggressive animals fought with a significantly higher level of aggression, almost exclusively on level 6, and had a longer match duration (level: median: 6, IQR: 6 to 6, 10%-90%: 2.4 to 6; duration: median: 12 s, IQR: 10.75 s to 19.25 s, 10%-90%: 5.5 s to 29 s, $N = 10$; Figure 11B) compared to the animals that were allocated to the submissive group (level: median: 4, IQR: 2 to 5, 10%-90%: 2 to 5, MWU: $U = 20.5$, $p = 0.0001$; duration: median: 2 s, IQR: 1 s to 4 s, 10%-90%: 1 s to 8.7 s, MWU: $U = 8.5$, $p < 0.0001$, $N = 20$; Figure 11B).

A different outcome could be observed when the animals were LTI with no previous contact to adult males and then subjected to chronic defeat. In the first match, regarding the pooled data

of all focal animals (N = 40) the median level of aggression was 6 (IQR: 5 to 6, 10%-90%: 4.1 to 6) and the median match duration was 11 s (IQR: 6.25 to 16.75, 10%-90%: 5.1 s to 21 s, Figure 11C). It is also true for the LTI that after being subjected to chronic defeat, some animals reacted aggressively, and some animals reacted submissively in the test fight. In comparison to the control group with no defeat (level: median: 6, IQR: 5 to 6, 10%-90%: 4 to 6; duration: median: 12.5 s, IQR: 8.25 s to 18.75 s, 10%-90%: 6 s to 24.9 s, N = 20; Figure 11C) the level and duration of the match was significantly reduced for the submissive animals (level: median: 2, IQR: 1 to 2, 10%-90%: 1 to 2, MWU: U = 7, $p < 0.0001$; duration: median: 2 s, IQR: 1 s to 3 s, 10%-90%: 1 s to 5 s, MWU: U = 4.5, $p < 0.0001$; N = 20; corrected for two comparisons; Figure 11C). The level and duration of the test fight for the animals that remained aggressive after multiple defeats (level: median: 5, IQR: 4 to 6, 10%-90%: 4 to 6; duration: median: 7.5 s, IQR: 5 s to 12.5 s, 10%-90%: 3.1 s to 26.4 s, N = 20; Figure 11C) was also significantly lower in comparison to the no defeat control, even after correcting for two comparisons (level: MWU: U = 123.5, $p = 0.0213$; duration: MWU: U = 117, $p = 0.0237$). The data revealed that when the pooled data of the first fight was classified retrospectively for the future aggressive (level: median: 6, IQR: 5 to 6, 10%-90%: 5 to 6; duration: median: 12 s, IQR: 6 s to 15.25 s, 10%-90%: 5.1 s to 21 s; Figure 11C) and submissive animals (level: median: 5.5, IQR: 5 to 6, 10%-90%: 4 to 6; duration: median: 10 s, IQR: 7 s to 18.5 s, 10%-90%: 5.1 s to 23.6 s; Figure 11C), no differences in level and duration prior to the chronic defeat were present (level: MWU: U = 183, $p = 0.6788$; duration: MWU: U = 194.5, $p = 0.8879$). It is important to notice, that the experiment for STI and LTI animals was repeated until the group of submissive animals reached 20 animals, because these were the fundamental group for this experiment and should be compared to the 20 control animals that experienced no defeat.

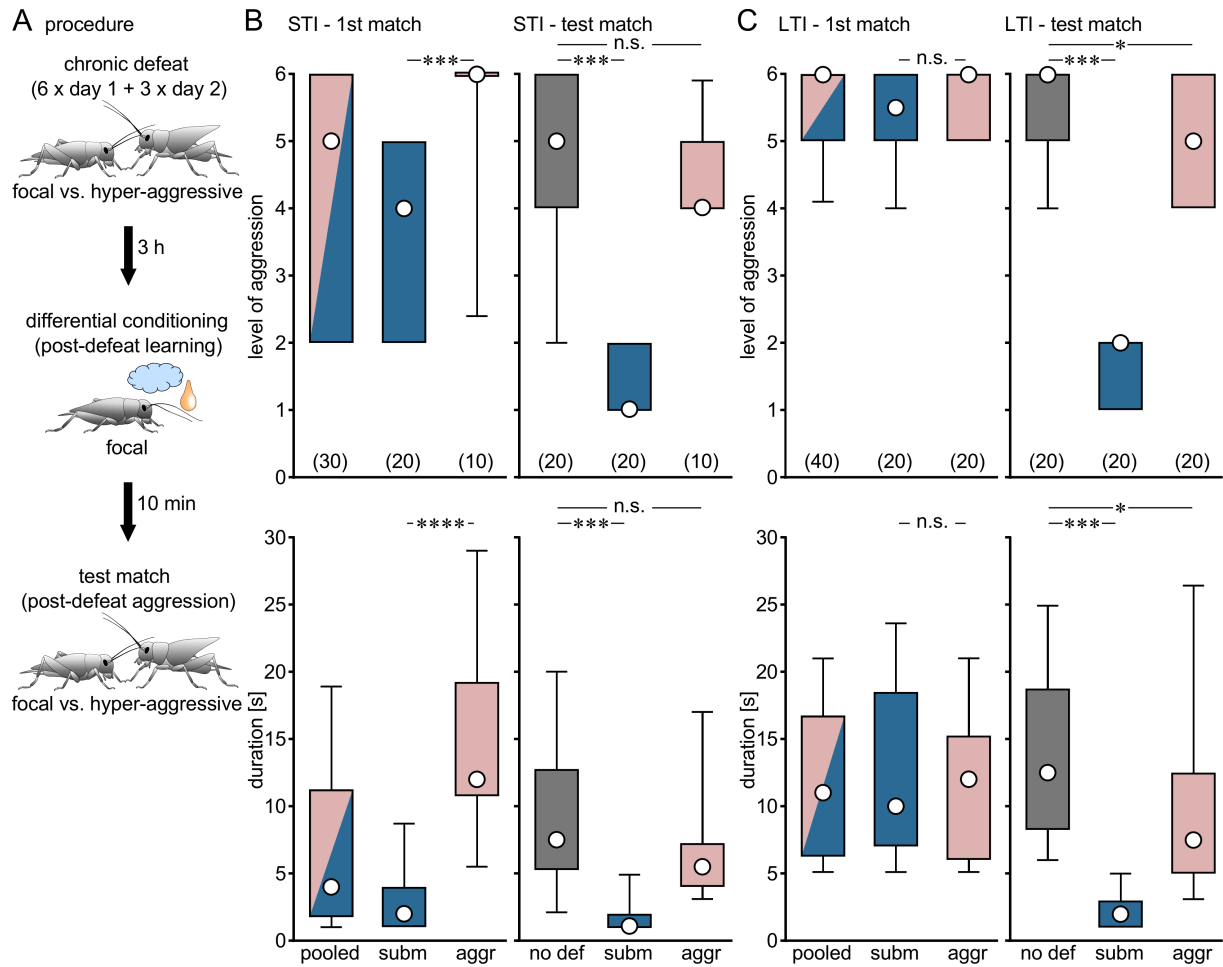


Figure 11 | Effects of chronic social defeat on aggression. **A** Procedure: Each focal animal was subjected to 9 defeats by matching it with a standard hyper-aggressive opponent (6 defeats on day 1, 3 on day 2 at 1 h intervals). This was followed 3 h after the last defeat by differential conditioning to determine post-defeat learning capacity and subsequently by a final test match after a pause of 10 min to determine post-defeat aggressiveness. **B** Box plots give the level of aggression (top) and match duration (bottom) of short term isolated crickets (STI), before chronic defeat at the first match (left) and at the test match after chronic defeat (right). Blue boxes: animals that were submissive > 3 h after chronic defeat (subm). Pink boxes: animals that were aggressive > 3 h after chronic defeat (aggr). Blue/pink boxes: pooled data. Grey boxes: control animals without prior chronic defeat (no def). Circle: median; box: interquartile range; whiskers: 10th and 90th percentile; N = indicated in parenthesis above the x-axis. **C** As for B, but for long term isolated crickets (LTI); Significant differences between groups yielded by Mann-Whitney U-tests are indicated by asterisks: 1st match: **** $p < 0.0001$; *** $p < 0.001$; n.s., not significant; test match, with alpha corrected for 2 comparisons: *** $p < 0.0005$; * $p < 0.025$; n.s., not significant.

Since it has been confirmed that chronic social defeat has an influence on future aggression, but at the same time not all animals are influenced in the same way due to some animals behaving aggressive in the test match in contrast to those that behave submissive, the data from the differential conditioning paradigm was also examined in these groups. Therefore, the data from the video tracked response to the CS^o and the CS⁺ at the recall tests after training was extracted and the P_{resp} for both calculated. From the difference, the LI for each individual was calculated and the results of detailed statistical analysis (median, IQR, 10%-90%, N, MWU, U, Wilcoxon, W) of the P_{resp} and LIs are depicted in Table 8 for STI and Table 9 for LTI animals. The data for the sequences depicted in Table 1 were again pooled for all following experiments. STI animals that experienced no defeat scored significantly lower P_{resp} values to the CS^o (median: 0.205) compared to high P_{resp} values to the CS⁺ (median: 0.804, Wilcoxon: $p = 0.0012$; N = 20; Table 8, Figure 12Ai). This is also indicated by the increasing adjoining lines in Figure 12Ai. When the animals had been subjected to chronic defeat and behaved submissive at the test match, the P_{resp} to the CS^o was distributed very broadly and almost exclusively near the maximum (1.0) and minimum (0) to the extent that almost half of the animals showed a high P_{resp} to the CS^o and the other half a low P_{resp} (median: 0.647). In addition to this, they scored low P_{resp} values to the CS⁺ (median: 0.225), that in result there was no difference between CS^o and CS⁺ (Wilcoxon: $p = 0.1231$; N = 20; Table 8, Figure 12Bi) with some individuals even scoring lower P_{resp} to the CS⁺ indicated by decreasing lines in Figure 12Bi. For the animals that were subjected to chronic defeat and behaved aggressively at the test match, both the P_{resp} to the CS^o (median: 0.267) and the CS⁺ (median: 0.161) were comparatively low without a significant difference (Wilcoxon: $p = 0.7695$; N = 10; Table 8, Figure 12Ci). The resultant LIs of the animals that experienced no defeat were at a medium of 0.247. Compared to this the animals that were submissive after chronic defeat had significantly impaired LIs with a median at -0.006 (MWU: $p = 0.0002$; corrected for two comparisons; Table 8, Figure 12Di) and in general did not show learning with some animals even reacting stronger to the CS^o which resulted in negative LIs . The animals that behaved aggressively also were impaired in learning (median: -0.024) in comparison to the no defeat control group (MWU: $p = 0.0146$; corrected for two comparisons; Table 8, Figure 12Di).

Table 8 | P_{resp} values and resultant learning indices (LI) for the effect of chronic social defeat on learning in short term isolated animals. Values for p are boldface where significant, W is from Wilcoxon signed rank tests, and U from Mann-Whitney U tests, for the latter, alpha is set to 0.025 due to two comparisons using the same data set. For each group median, interquartile range (IQR), 10th and 90th percentiles and number of values (N) are given.

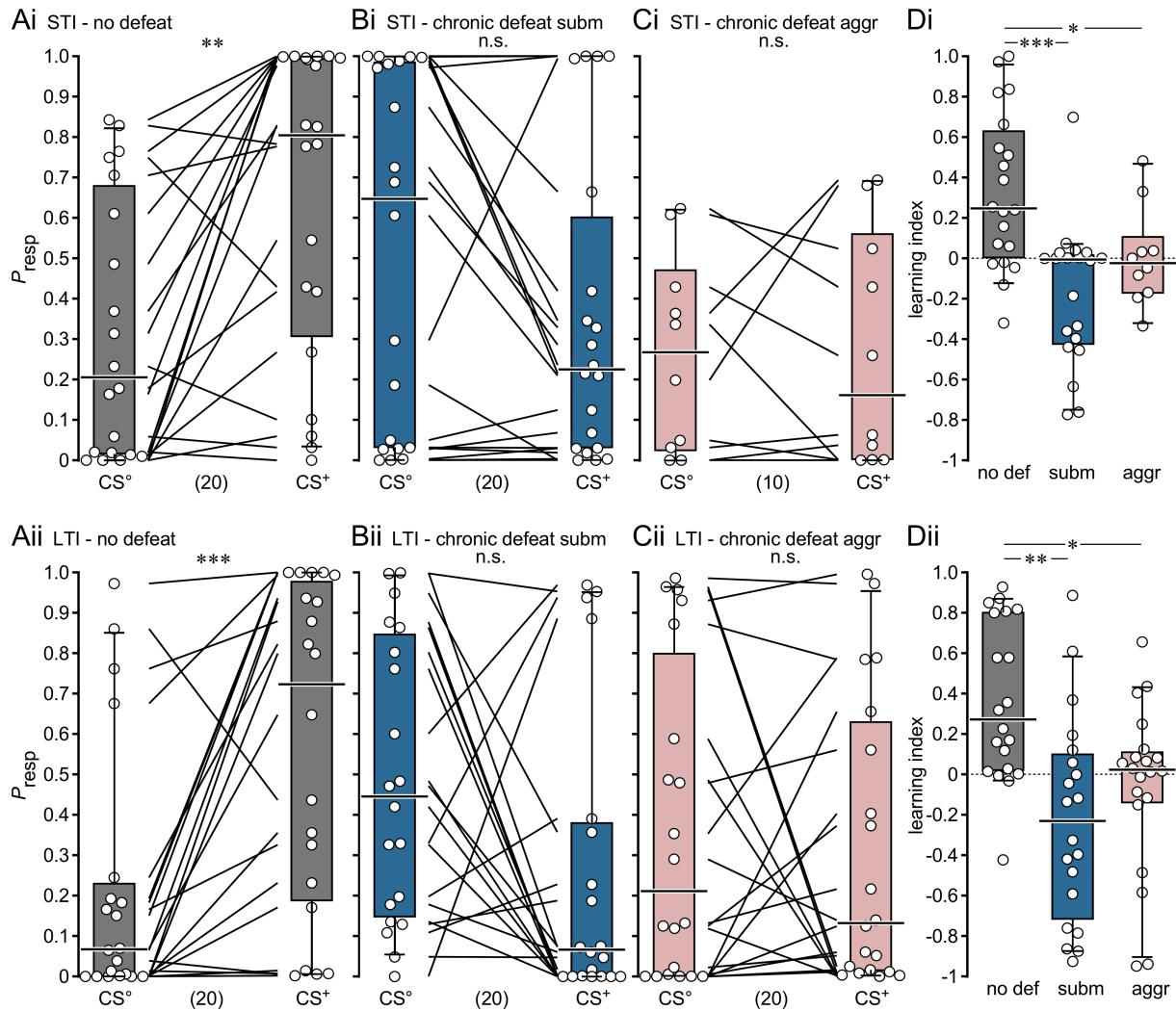
Group		Median	IQR	10%-90%	N	Comparison	p	W/U
<i>P_{resp}</i>								
no defeat	CS [°]	0.205	0.015-0.682	0-0.822	20	CS [°] vs. CS ⁺	0.0012	W 164
	CS ⁺	0.804	0.305-0.996	0.035-1	20			
submissive	CS [°]	0.647	0.03-0.986	0-0.999	20	CS [°] vs. CS ⁺	0.1231	W -84
	CS ⁺	0.225	0.03-0.603	0-1	20			
aggressive	CS [°]	0.267	0.024-0.474	0-0.621	10	CS [°] vs. CS ⁺	0.7695	W -7
	CS ⁺	0.161	0.001-0.563	0-0.692	10			
Learning index								
no defeat	LI	0.247	0-0.633	-0.123-0.959	20			
submissive	LI	-0.006	-0.429-0.021	-0.749-0.071	20	no defeat vs. subm	0.0002	U 69
aggressive	LI	-0.024	-0.176-0.11	-0.321-0.467	20	no defeat vs. aggr	0.0146	U 45

For the LTI animals chronic social defeat had a quite similar effect on the P_{resp} values and the LIs . Animals that did not experience defeat scored low P_{resp} values to the CS[°] (median: 0.067) and significantly higher P_{resp} values to the CS⁺ (median: 0.723; Wilcoxon: $p = 0.0006$; N = 20; Table 9, Figure 12Aii). When the animals had been subjected to chronic defeat and behaved submissive at the test match, they showed a broad spectrum of individual P_{resp} values with a median at 0.446 and, comparable to the STI animals, strongly impaired P_{resp} values to the CS⁺ (median: 0.066). There was no significant difference between these two responses, but the majority of animals scored a lower P_{resp} to the CS⁺ compared to the CS[°] resulting in decreasing adjoining lines in the Figure 12Bii (Wilcoxon: $p = 0.0759$; N = 20; Table 9, Figure 12Bii). The aggressive animals score low P_{resp} values to the CS[°] (median: 0.211) and the CS⁺ (median: 0.132) with no significant difference (Wilcoxon: $p > 0.9999$; N = 20; Table 9, Figure 12Cii). Additionally, there is no clear picture of whether the animals individually (lines in Figure 12Cii) react stronger to the CS⁺ or to the CS[°]. The LIs for the LTI animals that had experienced no defeat were comparable to those of the STI, with a median at 0.272. The median LI of the submissive animals was negative (median: -0.231) and strongly impaired compared to the no defeat control (MWU: $p = 0.0005$; corrected for two comparisons; Table 9, Figure 12Dii). The median LI for the aggressive animals (median: 0.023) was also significantly lower compared to the no defeat control (MWU: $p = 0.0056$; corrected for two comparisons; Table 9, Figure

12Dii). In both the STI and LTI groups, there were some submissive animals exhibiting a conditioned-like response to the CS^o including bobbing behaviour. Because of the similarity between the STI and the LTI animals regarding the effect of chronic social defeat on learning, following experiments were conducted with STI animals.

Table 9 | P_{resp} values and resultant learning indices for the effect of chronic social defeat on learning in long term isolated animals. Values for p are boldface where significant, W is from Wilcoxon signed rank tests, and U from Mann-Whitney U tests, for the latter, alpha is set to 0.025 due to two comparisons using the same data set. For each group median, interquartile range (IQR), 10th and 90th percentiles and number of values (N) are given.

Group		Median	IQR	10%-90%	N	Comparison	p	W/U
P_{resp}								
no defeat	CS ^o	0.067	0.001-0.232	0-0.85	20	CS ^o vs. CS ⁺	0.0006	W 172
	CS ⁺	0.723	0.186-0.979	0.006-1	20			
submissive	CS ^o	0.446	0.146-0.848	0.054-0.992	20	CS ^o vs. CS ⁺	0.0759	W -96
	CS ⁺	0.066	0.001-0.382	0-0.951	20			
aggressive	CS ^o	0.211	0.001-0.801	0-0.964	20	CS ^o vs. CS ⁺	>0.9999	W 0
	CS ⁺	0.132	0.013-0.632	0.002-0.954	20			
Learning index								
no defeat	LI	0.272	0.018-0.805	-0.03-0.869	20			
submissive	LI	-0.231	-0.719-0.104	-0.874-0.584	20	no defeat vs. subm	0.0005	U 76
aggressive	LI	0.023	-0.142-0.114	-0.904-0.432	20	no defeat vs. aggr	0.0056	U 99



3.2.2 Multiple experiences of winning

Whether the animals were aggressive or submissive after a chronic defeat did not have a strong impact on the learning capacities as both groups showed significantly lower LI scores in comparison to animals that experienced no defeat. It could be argued that the multiple agonistic experiences of fighting and having close contact to a conspecific could be the cause of impaired learning and not the defeat itself. To test this, STI animals won nine fights against a less-aggressive opponent. Following this they were trained in the standard differential conditioning

procedure to measure learning capacity and were tested for their aggressiveness afterwards, more than 4.5 h after the last winning experience (Figure 13A). In comparison to the control group that experienced no wins (level: median: 5, IQR: 4 to 6, 10%-90%: 2 to 6; N = 20), the nine fold winners fought at a significantly higher level of aggression (level: median: 6, IQR: 6 to 6, 10%-90%: 5 to 6; N = 20, MWU: U = 105.5, $p = 0.0034$; Figure 13B). However, there was no difference between the animals that experienced no win (duration: median: 10 s, IQR: 6 s to 16.5 s, 10%-90%: 4.1 s to 25.4 s; Figure 13B) and nine wins regarding the duration of the match (duration: median: 10.5 s, IQR: 8 s to 18.75 s, 10%-90%: 6 s to 28.8 s, MWU: U = 164.5, $p = 0.3423$, Figure 13B).

All data and statistical analyses for learning in multiple winners are depicted in more detail in Table 10. The P_{resp} values for the individuals that experienced no wins were low to the CS[°] (median: 0.171) and high to the CS⁺ (median: 0.909) and significantly different (Wilcoxon: $p = 0.0007$; N = 20, Table 10, Figure 13C). The multiple winners also scored low P_{resp} values to the CS[°] (median: 0.04) and high P_{resp} values to the CS⁺ (median: 0.99) with 18 animals showing significantly increasing adjoining lines for the CS[°] to the CS⁺ (Wilcoxon: $p < 0.0001$; N = 20; Table 10, Figure 13D). The *LIs* show a typical distribution for animals without treatment, in this case without wins (median: 0.269) with the majority scoring positive *LIs*. The nine-time winners also showed high *LIs* with a median at 0.529 with no animals scoring a higher P_{resp} to the CS[°] in comparison to the CS⁺ that would have resulted in $LI < (-0.05)$. But despite this, there was no significant difference between the learning capacity of the animals that experienced no win and the animals that experienced nine wins (MWU: $p = 0.1918$; Table 10, Figure 13E).

Table 10 | P_{resp} values and resultant learning indices for the effect of multiple wins on learning in short term isolated animals. Values for p are boldface where significant, W is from Wilcoxon signed rank tests, and U from Mann-Whitney U tests. For each group median, interquartile range (IQR), 10th and 90th percentiles and number of values (N) are given.

Group		Median	IQR	10%-90%	N	Comparison	p	W/U
<i>P_{resp}</i>								
no win	CS [°]	0.171	0.007-0.532	0-0.878	20	CS [°] vs. CS ⁺	0.0007	W 170
	CS ⁺	0.909	0.252-0.997	0.006-1	20			
multiple winner	CS [°]	0.040	0.001-0.464	0-0.905	20	CS [°] vs. CS ⁺	<0.0001	W 204
	CS ⁺	0.990	0.453-0.999	0.118-1	20			
Learning index								
no win	LI	0.269	0.005-0.782	-0.084-0.98	20	no win vs. multiple winner	0.1918	U 151
multiple winner	LI	0.529	0.179-0.914	-0.002-0.979	20			

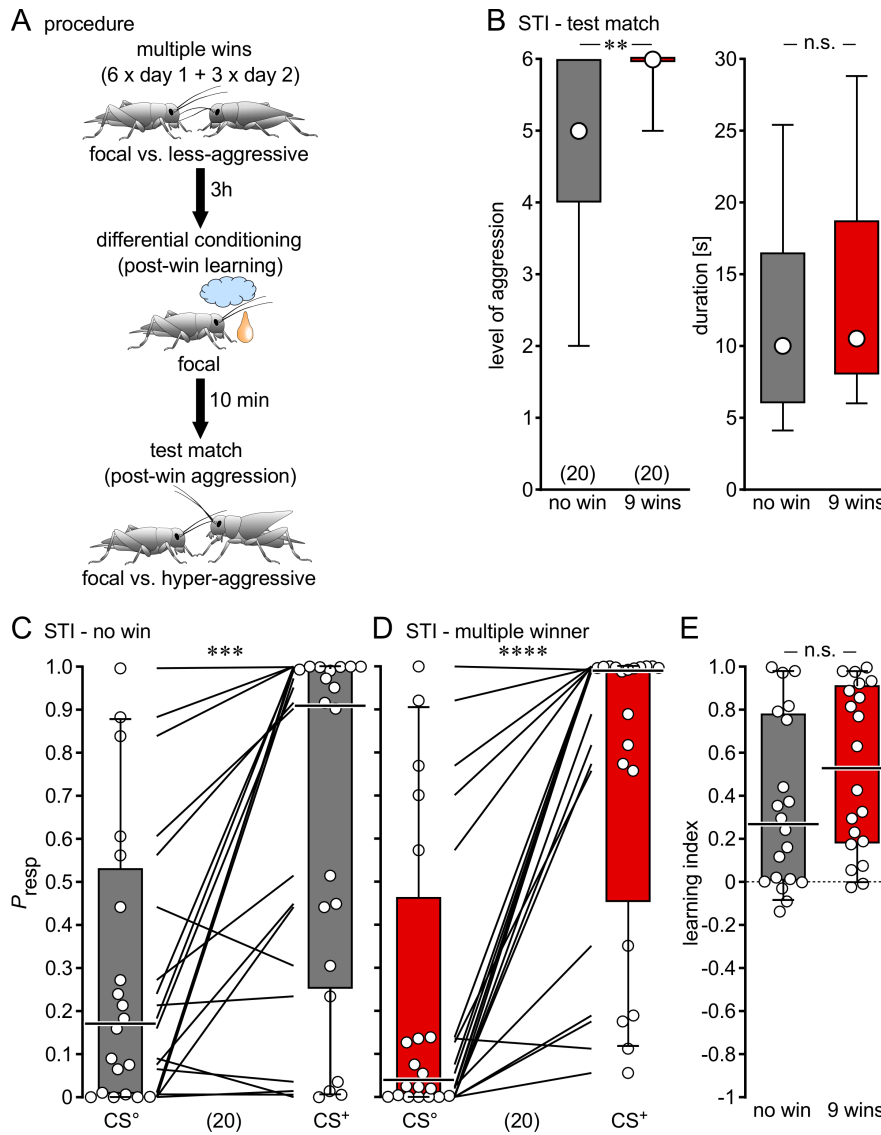


Figure 13 | Effects of multiple wins on aggression and learning of short term isolated animals. **A** Procedure: Each focal animal experienced 9 successive wins by matching it repeatedly against the individual it defeated at the first match, that was hence less aggressive (6 wins on day 1, 3 on day 2 at 1h intervals). This was followed 3 h after the last win by differential conditioning to determine post-win learning capacity and subsequently by a final test match against a hyper-aggressive opponent after a pause of 10 min to determine post-win aggressiveness. **B** Box plots give the level of aggression (left) and match duration (right) at the test match. Grey boxes: data for control animals that had no fighting experience prior to conditioning. Red boxes: data for individuals that experienced multiple wins. Circle: median; box: interquartile range; whiskers: 10th and 90th percentile; N = indicated in parenthesis above the x-axis. **C** Control animals that experienced no wins (grey). Boxplots giving the P_{resp} values for the non-rewarded and rewarded odour (CS° , CS^+). Bars: median; boxes: interquartile range; whiskers: 10th and 90th percentiles; circles: individual values; N = indicated in parenthesis under the graph. The interposing lines between plots indicate the difference in response for each individual. **D** As for C, but for multiple winners (red). **E** Learning indices for the control animals (grey) and multiple winners (red). Asterisks indicate significant differences between groups: B, E: Mann-Whitney U-test; $**p < 0.01$; n.s., not significant. C-D: Wilcoxon signed rank test; $****p < 0.0001$; $***p < 0.001$.

3.2.3 Correlation of aggression and learning

When the LI is calculated for a large group of untreated animals like in Figure 9 (performances of individual crickets in the differential olfactory conditioning paradigm), a bimodal

distribution is indicated. As one possible explanation of this data indicates that these inter-individual differences could have derived from previous agonistic experiences which influence future behaviours of individual animals (Balsam and Stevenson, 2020, 2021), even general not exclusively aggression associated behaviours (Rose et al., 2017a). To test if causes for inter-individual differences in aggressiveness (level of aggression and duration of a fight) also influence learning indices, these values were tested for correlations for the animals participating in the chronic social defeat and multiple win paradigm, because the aggression was documented for each animal here. Data for these analyses are depicted in Table 11. There was no correlation between level of aggression and *LI* or match duration and *LI* for any of the groups (STI no defeat, STI submissive+aggressive, LTI no defeat, LTI submissive+aggressive, STI no defeat as control of multiple winner, STI winner; Table 11). In the test match of the STI animals that were subjected to chronic defeat there were correlations between level of aggression (Spearman: $r = 0.3635$, $p = 0.0483$, $N = 30$; Table 11) and duration (Spearman: $r = 0.3781$, $p = 0.0394$, $N = 30$; Table 11) indicated, but this was insignificant after Bonferroni correction.

Table 11 | Correlations between aggression and learning indices. Level of aggression and duration of the first and test matches in the chronic defeat paradigm of short term (STI) and long term (LTI) isolated animals. The aggressive (aggr) and submissive (subm) animals are pooled. r gives correlation coefficient and p the significance of correlation deriving from Spearman's rank correlation. Due to two comparisons alpha is set to 0.025, whereas p values that are insignificant after correction are underlined.

Group		x-axis	y-axis	r	p
STI no defeat (control chronic defeat)	test	level	LI	0.2174	0.3572
		duration	LI	-0.0144	0.9521
STI subm and aggr	first	level	LI	-0.0535	0.7788
		duration	LI	-0.1064	0.5759
	test	level	LI	0.3635	<u>0.0483</u>
		duration	LI	0.3781	<u>0.0394</u>
LTI no defeat	test	level	LI	-0.2426	0.3027
		duration	LI	-0.0233	0.9222
LTI subm and aggr	first	level	LI	-0.0484	0.7669
		duration	LI	0.0563	0.7301
	test	level	LI	0.1672	0.3025
		duration	LI	0.1072	0.5102
STI no defeat (control multiple wins)	test	level	LI	-0.1494	0.5295
		duration	LI	0.1488	0.5311
STI winner	test	level	LI	-0.1610	0.4977
		duration	LI	-0.0718	0.7636

3.2.4 Summary of learning capacities – multiple experiences

To examine the differences in learning between the groups further and summarise them, the animals were sorted into three categories and statistically assessed by the Fisher's exact test (Fisher): animals that had learned the odour hence scored a positive *LI* ($LI > 0.05$) and reacted stronger to the CS⁺ in comparison to the CS^o, animals that did not differentiate between the odours, referred to as zero *LI* ($-0.05 \leq LI \leq 0.05$) and animals that responded stronger to the CS^o, referred to as negative *LI* ($LI < (-0.05)$), which includes for some animals showing a conditioned-like response to the non-rewarded odour. Generally, for STI animals that are illustrated in Figure 14A, in the control group (no defeat) the majority of animals learned the odour (75%), 15% did not differentiate between the odour and 10% of the animals responded stronger to the CS^o. Compared to the control, the contingency of the nine-times defeated animals that were submissive at the test match was significantly different because of the percentage of animals with a positive *LI* strongly reduced (10%), many animals scoring a zero *LI* (45%) and a comparably increased percentage (45%) showing a negative *LI* (Fisher: $p < 0.001$, N = 20 each; Figure 14A). The nine-times defeated animals that were aggressive (positive *LI* = 20%; zero *LI* = 40%; negative *LI* = 40%) also differed significantly to the control when sorted in the three categories (Fisher: $p = 0.002$, N = 10; Figure 14A).

In the group of LTI animals with no defeat (Figure 14B), again the majority of the animals showed a positive *LI* (70%), 25% scored a zero *LI* and 5% showed a negative *LI*. Here again the contingency of animals subjected to chronic defeat differed significantly to the control (Fisher: $p < 0.001$; N = 20 each; Figure 14B). The learning was impaired with only 30% scoring a positive *LI* and there were 10% of animals that scored a zero *LI* and in this group the majority (60%) of animals scored a negative *LI*. When only evaluated by the three learning categories, the contingency between control and aggressive animals is not significant (positive *LI* = 45%; zero *LI* = 20%; Fisher: $p = 0.065$, N = 20; Figure 14B), but here also the percentage of animals scoring negative *LIs* is increased (35%).

In comparison to the control (positive *LI* = 65 %; zero *LI* = 25 %; negative *LI* = 10%; N = 20; Figure 14C), the relative frequency of the animals in the three learning categories did not differ from the frequency in nine-times winners (Fisher: $p = 0.154$; Figure 14C). This group had the highest percentage of individuals scoring a positive *LI* (90%) and only 10% of the animals scoring a zero *LI* and no animals scoring a negative *LI*, which is exclusive for this experimental group.

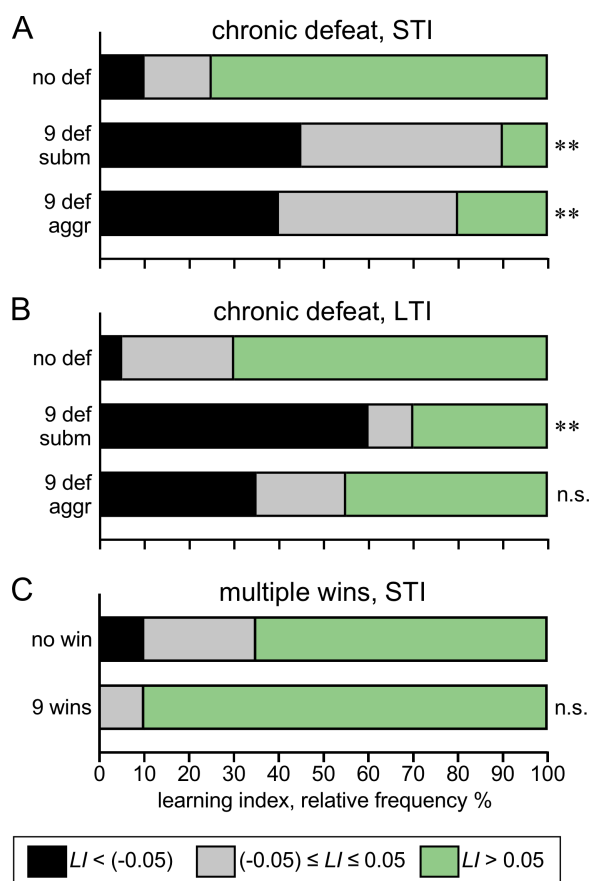


Figure 14 | Summary of the influence of social experiences on learning. **A** Data for short term isolated animals (STI) after either no defeat (no def), chronic social defeat resulting in submissiveness (9 def subm), or chronic social defeat resulting in aggressiveness (9 def aggr). The bars give the percentages of individuals that exhibited either learning indices less than (-0.05) (black), no significant learning (LI between (-0.05) and 0.05, grey) or significant learning (LI greater than 0.05, green) **B** As in **A**, but for long term isolated animals (LTI). **C** As in **A**, but for animals that either had no winning experience (no win) or experienced multiple wins (9 wins). Asterisks indicate significant differences to control data (no def in **A** and **B**, no win in **C**) from Fisher's exact test with alpha correction for 2 comparisons: ** $p < 0.005$; n.s., not significant.

3.3 The influence of serotonergic drugs on learning after chronic defeat

3.3.1 Methiothepin and ketanserin

Serotonin is known to take part in the establishment of long-term depression after chronic social defeat and the effects of serotonergic drugs on the resulting depression of aggression have been examined (Rillich and Stevenson, 2018). In order to test whether serotonin also influences the chronic defeat induced impairment of learning, a new cohort of STI crickets was subjected to chronic defeat by matching it against a hyper-aggressive opponent nine times. As the impairment of learning was present in submissive and aggressive animals, there was no differentiation between the aggressive status in the following experiments. Two hours after the last defeat the animals were injected with 5HT-receptor blockers methiothepin or ketanserin. The injection of the vehicle was used as control. After a pause of 1 h, learning was evaluated

in the differential learning paradigm and the aggressiveness assessed afterwards (Figure 15A). First, to confirm the effects of the serotonergic drugs and also to control for a successful effect of chronic defeat, the aggressiveness of the animals at the first match (pre chronic defeat, pre drug) and the test match (post chronic defeat, post drug) was analysed. At the first match prior to drug treatment the control animals fought with a median level of aggression of 4.5 (IQR: 4 to 5, 10%-90%: 2.2 to 5.9) and a median match duration of 5.5 s (IQR: 4.25 s to 9 s; 10%-90%: 3.1 s to 13 s; N = 20; Figure 15B). The group of animals that would later be given methiothepin did not differ from the vehicle control group at the first match (level: median: 5, IQR: 4 to 5.75, 10%-90%: 2 to 6, MWU: U = 187, $p = 0.6994$; duration: median: 7.5 s, IQR: 3.25 s to 14.75 s, 10%-90%: 2.1 s to 17.7 s, MWU: U = 171, $p = 0.4390$, N = 20; corrected for two comparisons; Figure 15B). Also, the group of animals that were given ketanserin after the chronic defeat paradigm did not differ from the vehicle control group in terms of level and duration of the first match (level: median: 5, IQR: 4 to 6, 10%-90%: 2 to 6, MWU: U = 139, $p = 0.0873$; duration: median: 7 s, IQR: 3.25 s to 10 s, 10%-90%: 2.1 s to 19.2 s, MWU: U = 197.5, $p = 0.9518$, N = 20; corrected for two comparisons; Figure 15B).

Confirming earlier findings, when subjected to chronic defeat the vehicle treated group was generally submissive with the median level of aggression at 2 (IQR: 1.25 to 4, 10%-90%: 1 to 4.9) and the median match duration of 3.5 s (IQR: 1.25 s to 5.75 s, 10%-90%: 1 s to 6.9 s; N = 20; Figure 15C). After receiving nine defeats and methiothepin treatment, these animals did not differ from the vehicle control in level and duration of the test match (level: median: 2, IQR: 2 to 3.5, 10%-90%: 1.1 to 4, MWU: U = 197, $p = 0.9779$; duration: median: 3 s, IQR: 2 s to 6 s, 10%-90%: 1 s to 8.8 s, MWU: U = 189.5, $p = 0.7828$, N = 20; corrected for two comparisons; Figure 15C). In contrast to this and in comparison to the vehicle treated group, ketanserin treated animals fought more aggressive and longer (level: median: 4, IQR: 4 to 5, 10%-90%: 2 to 5, MWU: U = 88, $p = 0.0015$; duration: median: 6 s, IQR: 4 s to 10 s, 10%-90%: 2.1 s to 13 s, MWU: U = 102, $p = 0.0068$, N = 20; corrected for two comparisons; Figure 15C) and showed no chronic defeat induced depression of aggression.

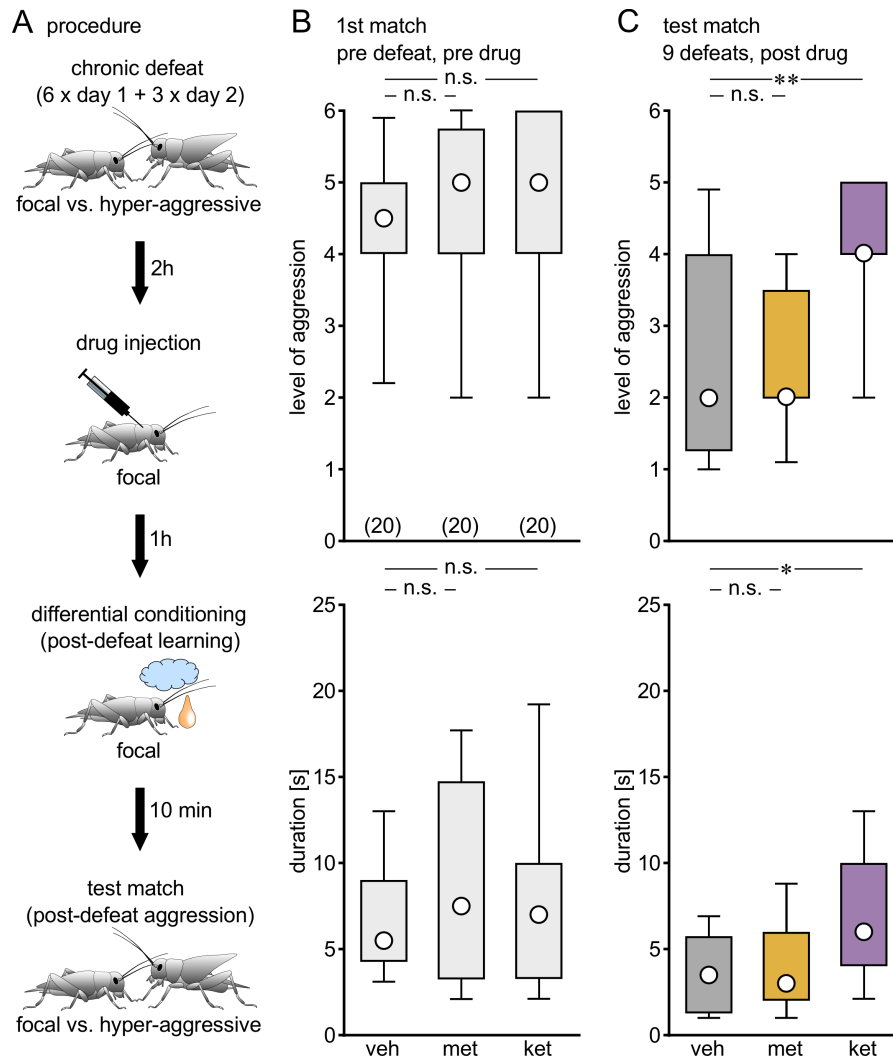


Figure 15 | Effects of 5HT receptor blockers on chronic defeat induced depression of aggression. **A** Procedure: Each focal animal was subjected to 9 defeats by matching it with a standard hyper-aggressive opponent (6 defeats on day 1, 3 on day 2 at 1 h intervals). This was followed 2 h after the last defeat by drug injection and after another 1 h by differential conditioning to determine post-defeat learning capacity. Subsequently, after a pause of 10 min post-defeat aggressiveness was determined by a final test match. **B** Box plots give the level of aggression (top) and match duration (bottom) at the first match before defeat and drug treatment: vehicle (veh), methiothepin (met), ketanserin (ket). Circle: median; box: interquartile range; whiskers: 10th and 90th percentile; N = indicated in parenthesis above the x-axis. **C** As for B, but for the test match after chronic defeat and injection of vehicle (grey), methiothepin (yellow) or ketanserin (purple). Asterisks indicate significant differences to vehicle control: Mann-Whitney U-test, corrected for 2 comparisons: ** $p < 0.005$; * $p < 0.025$; n.s., not significant.

All statistical analyses for the influence of chronic defeat with treatment of the serotonergic drugs methiothepin and ketanserin on learning are depicted in more detail in Table 12 and illustrated in Figure 16. When the animals were treated with the vehicle after chronic social defeat they respond with high P_{resp} values to the CS° (median: 0.762) and the P_{resp} values to the CS^+ (median: 0.552) did not differ significantly from them (Wilcoxon: $p = 0.0759$, $N = 20$; Table 12, Figure 16A). Animals that were treated with methiothepin after chronic defeat did not show a significant difference between the CS° (median: 0.336) and the CS^+ (median: 0.197)

regarding the P_{resp} values (Wilcoxon: $p = 0.8124$, $N = 20$; Table 12, Figure 16B). For the animals that were treated with ketanserin after chronic defeat, the negative effects of chronic defeat on learning were not present. They scored lower P_{resp} values to the CS° (median: 0.332) in comparison to the CS^+ (median: 0.978) resulting in a significant difference in the responses (Wilcoxon: $p = 0.0064$, $N = 20$; Table 12, Figure 16C). When the resulting learning indices were concerned, the vehicle group scored low LIs with the median at (-0.077) (Table 12, Figure 16D). The treatment with methiothepin had no influence on the impairment of learning (LI median: 0) as there is no significant difference to the vehicle group (MWU: $p = 0.1572$, $N = 20$; corrected for two comparisons; Table 12, Figure 16D). In the ketanserin treated group on the other hand, the LI (median 0.146) was significantly higher compared to the vehicle control group (MWU: $p = 0.0013$, $N = 20$; corrected for two comparisons; Table 12, Figure 16D).

Table 12 | P_{resp} values and resultant learning indices for the effects of 5HT-receptor blockers on learning after chronic defeat. Values for p are boldface where significant, W is from Wilcoxon signed rank tests, and U from Mann-Whitney U tests, for the latter, alpha is set to 0.025 due to two comparisons using the same data set. For each group median, interquartile range (IQR), 10th and 90th percentiles and number of values (N) are given.

Group		Median	IQR	10% - 90%	N	Comparison	p	W/U
<i>P_{resp}</i>								
vehicle	CS°	0.762	0.271-0.997	0-1	20	CS° vs. CS^+	0.0759	W -96
	CS^+	0.552	0.206-0.945	0-0.981	20			
methiothepin	CS°	0.336	0.009-0.930	0-0.999	20	CS° vs. CS^+	0.8124	W -14
	CS^+	0.197	0.011-0.981	0-0.999	20			
ketanserin	CS°	0.332	0.005-0.895	0.-0.972	20	CS° vs. CS^+	0.0064	W 142
	CS^+	0.978	0.194-0.999	0-1	20			
Learning index								
vehicle	LI	-0.077	-0.271-(-0.001)	-0.675-0.692	20			
methiothepin	LI	0.000	-0.159-0.098	-0.495-0.582	20	vehicle vs. methiothepin	0.1572	U 147
ketanserin	LI	0.146	0-0.604	-0.218-0.894	20	vehicle vs. ketanserin	0.0013	U 84

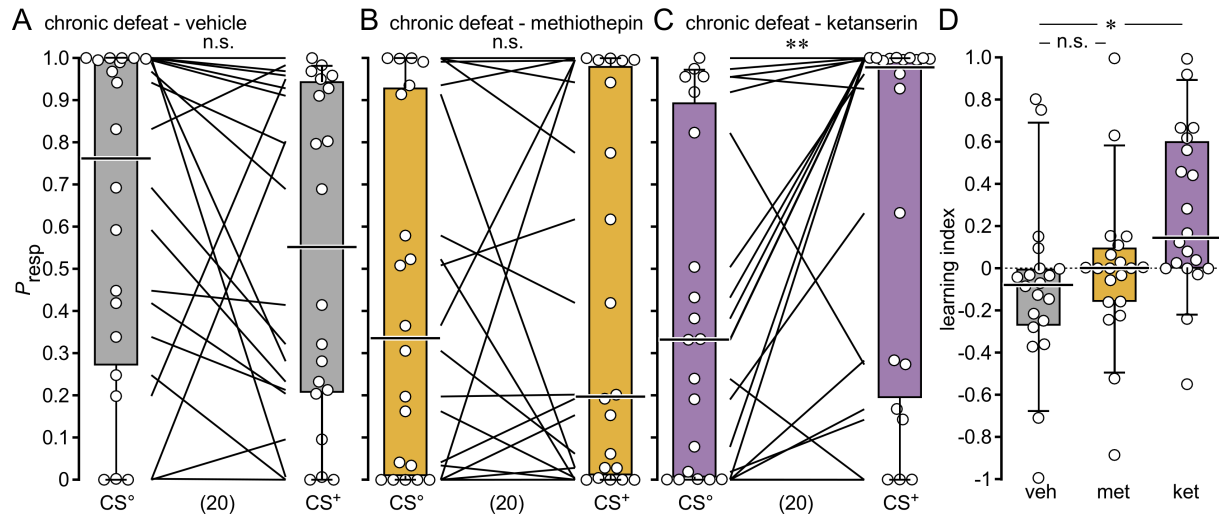


Figure 16 | Effects of 5HT receptor blockers on learning after chronic defeat. **A** Animals that were injected with vehicle after being subjected to chronic defeat and prior to conditioning (grey). Boxplots giving the P_{resp} values for the non-rewarded and rewarded odour (CS° , CS^+). Bars: median; boxes: interquartile range; whiskers: 10th and 90th percentiles; circles: individual values; N = indicated in parenthesis under the graph. The interposing lines between plots indicate the difference in response for each individual. **B** As for A, but for animals that were injected with methiothepin (yellow). **C** As for A, but for animals that were injected with ketanserin (purple). **D** Learning indices for the animals that received vehicle (grey), methiothepin (yellow) or ketanserin (purple). Asterisks indicate significant differences between groups: A-C: Wilcoxon signed rank test; $**p < 0.01$; n.s., not significant. D: Mann-Whitney U-test, corrected for 2 comparisons: $*p < 0.025$; n.s., not significant.

The results of the 5HT receptor blockers on learning in animals subjected to chronic defeat naturally entailed the necessity to test if the drugs have an influence on learning alone without the experience of chronic defeat. This control was performed with STI crickets that experienced no defeat and were treated with either methiothepin, ketanserin or vehicle (control) and then trained in the differential conditioning paradigm. For the control animals that received no defeat and the vehicle, the P_{resp} values to the CS° were low (median: 0.07) and they scored a median P_{resp} of 0.569 to the CS^+ (Table 13, Figure 17A). The difference between the responses was significant (Wilcoxon: $p = 0.0028$, $N = 20$; Table 13, Figure 17A). The methiothepin treated animals that did not receive chronic defeat also score low P_{resp} values to the CS° (median: 0.166) and high values to the CS^+ (median: 0.518) resulting in a significant difference between paired samples (Wilcoxon: $p = 0.0042$, $N = 20$; Table 13, Figure 17B). The same is true for the animals that were treated with ketanserin and experienced no chronic defeat. They score low P_{resp} values to the CS° (median: 0.207) and high P_{resp} values to the CS^+ (median: 0.949) with a significant difference between CS° and CS^+ (Wilcoxon: $p = 0.0028$, $N = 20$; Table 13, Figure 17C). Regarding the resulting *LIs*, there is neither a significant difference between the vehicle (mean: 0.37) and methiothepin treated groups (mean: 0.254; t-test: $p = 0.377$; corrected for two comparisons; Table 13, Figure 17D), nor between the vehicle control and ketanserin treated animals (mean: 0.321; t-test: $p = 0.7203$; corrected for two comparisons; Table 13, Figure 17D).

Table 13 | P_{resp} values and resultant learning indices for the effects of 5HT-receptor blockers on learning without prior chronic defeat. Values for p are boldface where significant, W is from Wilcoxon signed rank tests, and t from Student's two-tailed unpaired t-test, for the latter, alpha is set to 0.025 due to two comparisons using the same data set. For the P_{resp} data median, interquartile range (IQR), 10th and 90th percentiles and for the leaning indices mean and \pm 95% CI. The number of values (N) is given.

Group		Median	IQR	10%-90%	N	Comparison	p	W/t
P_{resp}								
vehicle	CS ^o	0.070	0.001-0.218	0-0.567	20	CS ^o vs. CS ⁺	0.0028	W 131
	CS ⁺	0.569	0.087-0.990	0-0.999	20			
methiothepin	CS ^o	0.166	0-0.642	0-0.956	20	CS ^o vs. CS ⁺	0.0042	W 148
	CS ⁺	0.518	0.208-0.981	0.022-0.999	20			
ketanserin	CS ^o	0.207	0.001-0.785	0-0.949	20	CS ^o vs. CS ⁺	0.0028	W 131
	CS ⁺	0.949	0.097-0.998	0-0.999	20			
Learning index		Mean	95% CI					
vehicle	LI	0.370	0.159-0.580		20			
methiothepin	LI	0.254	0.083-0.425		20	vehicle vs. methiothepin	0.3765	t 0.89
ketanserin	LI	0.321	0.133-0.509		20	vehicle vs. ketanserin	0.7203	t 0.36

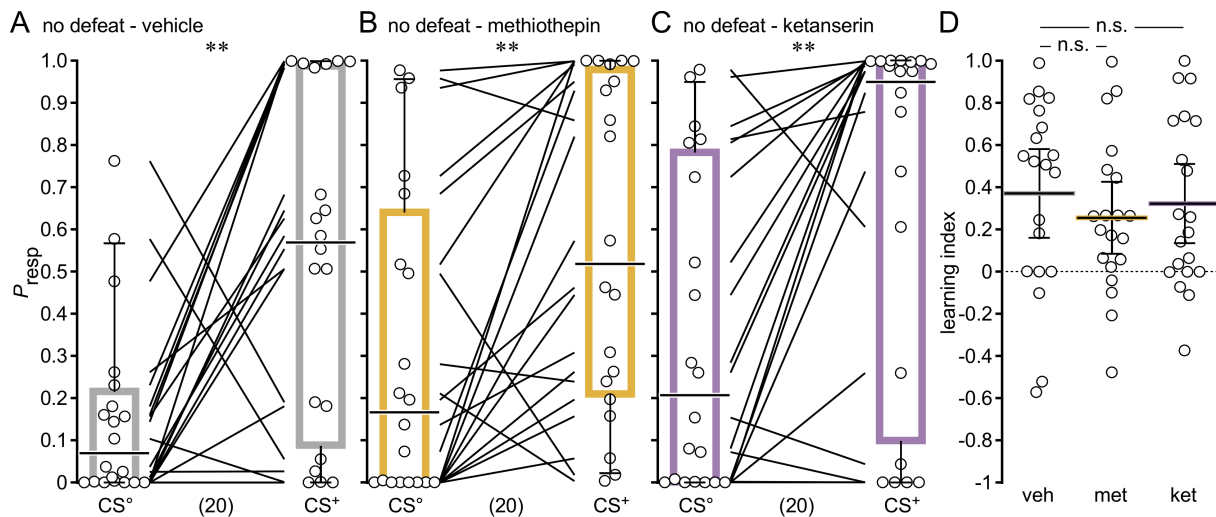


Figure 17 | Effects of 5HT receptor blockers on learning without prior chronic defeat. **A** Animals that were injected with vehicle prior to conditioning (grey outlines). Boxplots giving the P_{resp} values for the non-rewarded and rewarded odour (CS^o, CS⁺). Bars: median; boxes: interquartile range; whiskers: 10th and 90th percentiles; circles: individual values; N = indicated in parenthesis under the graph. The interposing lines between plots indicate the difference in response for each individual. **B** As for A, but for animals that were injected with methiothepin (yellow outlines). **C** As for A, but for animals that were injected with ketanserin (purple outlines). **D** Learning indices for animals that received vehicle (grey), methiothepin (yellow) or ketanserin (purple). Bars: mean; whiskers: 95% confidence interval; circles: individual values. Asterisks indicate significant differences between groups: A-C: Wilcoxon signed rank test; ** $p < 0.01$; D: Student's two-tailed unpaired t-test, corrected for 2 comparisons: n.s., not significant.

3.3.2 Fluoxetine

To further confirm the effect of impaired learning after chronic defeat to be based on serotonin, the 5HT re-uptake inhibitor fluoxetine was tested. From previous studies it is known that two defeats (non-chronic defeat) are sufficient to induce long term suppression of aggression when the animals were treated with fluoxetine (Rillich and Stevenson, 2018). To test this the experimental procedure was adjusted to the protocol used for fluoxetine (Rillich and Stevenson, 2018). The animals were first injected with fluoxetine or vehicle and after 1 h subjected to two defeats. After 24 h their learning capacity was tested in the differential conditioning paradigm and the aggressiveness evaluated. A third group was injected with the drug parallelly but had no defeat experience.

Regarding the aggression, in the first fight after drug injection neither level of aggression nor duration of the fight differed between vehicle control (level: median: 4, IQR: 2 to 5, 10%-90%: 2 to 6; duration: median: 4.5 s, IQR: 3 s to 6.75 s, 10%-90%: 2 s to 17.5 s, N = 20) and the fluoxetine treated group (level: median: 4, IQR: 2 to 5, 10%-90%: 2 to 5.9, MWU: U = 198, $p = 0.9885$; duration: median: 5 s, IQR: 4 s to 7.75 s, 10%-90%: 2.1 s to 8.9 s, N = 20, MWU: U = 190.5, $p = 0.8029$; Figure 18). At the test match the level of aggression of the vehicle group was again at a median value of 4 (IQR: 2 to 5, 10%-90%: 2 to 6), but after only two defeats the fluoxetine treated animals differed significantly from the control with a strongly reduced level of aggression (level: median: 2, IQR: 2 to 2, 10%-90%: 1 to 4, MWU: U = 89, $p = 0.001$; Figure 18). Also, the median duration of the test match for the vehicle group was almost at the previous value with 4 s (IQR: 4 s to 6 s, 10%-90%: 2.1 s to 14.5 s) but here again the duration of the fluoxetine group was significantly reduced (duration: median: 3 s, IQR: 2 s to 4 s, 10%-90%: 1 s to 7.7 s, MWU: U = 99.5, $p = 0.0051$; Figure 18). The animals that received fluoxetine were submissive after only two defeats for more than 24 h.

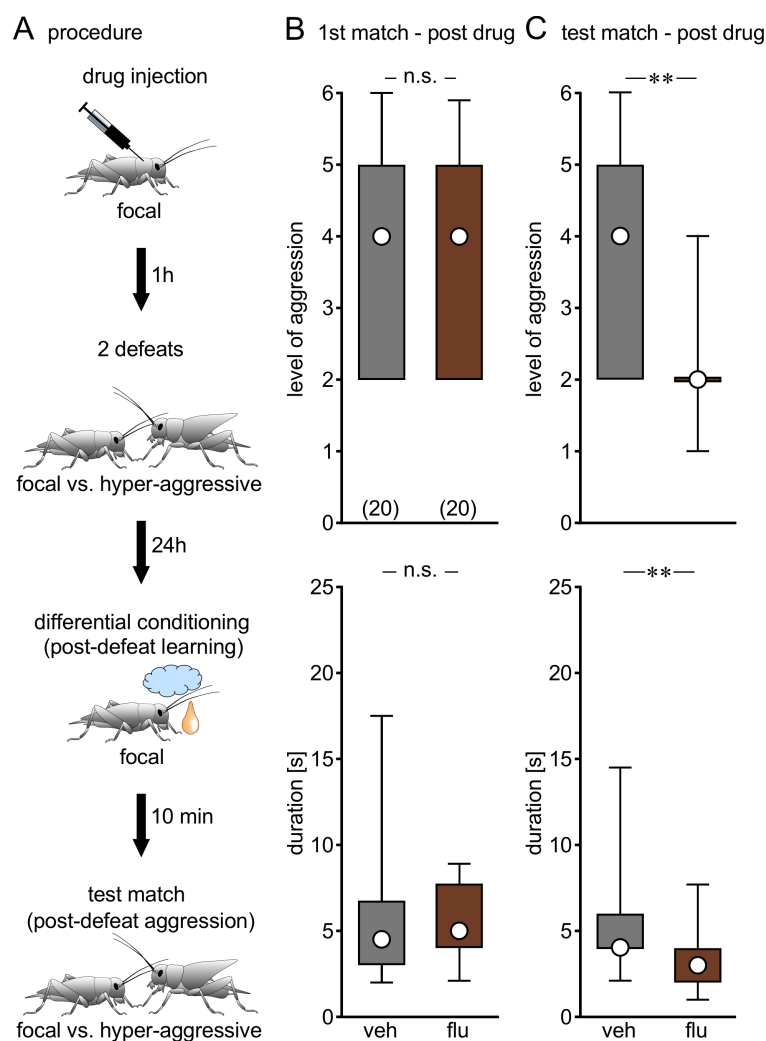


Figure 18 | Effect of 5HT re-uptake inhibitor fluoxetine on aggression after non-chronic defeat. **A** Procedure: Each focal animal was injected with the drug and after 1 h subjected to two defeats by matching it with a standard hyper-aggressive opponent (at 1 h interval). This was followed 24 h after the last defeat by differential conditioning to determine post-defeat learning capacity. Subsequently, after a pause of 10 min post-defeat aggressiveness was determined by a final “test match”. **B** Box plots give the level of aggression (top) and match duration (bottom) at the first match after drug injection: vehicle (veh, grey), fluoxetine (flu, brown). Circle: median; box: interquartile range; whiskers: 10th and 90th percentile; N = indicated in parenthesis above the x-axis. **C** As for B, but for the test match after injection of vehicle (grey) or fluoxetine (brown), two defeats and differential conditioning. Asterisks indicate significant differences to vehicle control: Mann-Whitney U-test: $**p < 0.01$; n.s., not significant.

Animals that received vehicle and then non-chronic defeat responded with low P_{resp} values to the CS° (median: 0.038) and high P_{resp} values to the CS^+ (median: 0.682) resulting in mostly ascending individual lines (Wilcoxon: $p = 0.0042$, $N = 20$; Table 14, Figure 19A). The fluoxetine treatment in combination with non-chronic defeat had a strong influence on the conditioned odour responses of the animals. They show comparatively high P_{resp} values to the CS° with a median at 0.506 and a very broad distribution of individual P_{resp} values. They mostly respond with very low P_{resp} values to the CS^+ (median: 0.133) with the result that the responses a lot of animals are lower to the CS^+ in comparison to the CS° , but this was not significant

(Wilcoxon: $p = 0.0532$, $N = 20$; Table 14, Figure 19B). With these different outcomes in responses between the vehicle and the fluoxetine group the question arose, whether the impaired learning is the effect of fluoxetine alone or in combination with the non-chronic defeat. Therefore, the third group of animals that received fluoxetine but experienced no defeats was examined. These animals scored low P_{resp} values to the CS° (median: 0.293) and high P_{resp} values to the CS^+ (median: 0.92) resulting in most individuals exhibiting a significantly higher response to the CS^+ compared to the CS° (Wilcoxon: $p = 0.0006$, $N = 20$; Table 14, Figure 19C). The resulting LIs show that the learning capacity of the vehicle treated animals with two defeats (median: 0.256; Table 14, Figure 19D) resembled the distribution of standard animals without defeat, as expected indicating no effect of the two defeats. The learning ability of the fluoxetine treated group with two defeats (median: -0.258) was strongly impaired compared to the vehicle control group (MWU: $p = 0.0002$; Table 14, Figure 19D). The LI of the fluoxetine treated animals that experienced no defeat on the other hand did not differ from the vehicle control (median: 0.247; MWU: $p = 0.7994$; Table 14, Figure 19D).

Table 14 | P_{resp} values and resultant learning indices for the effect of 5HT re-uptake inhibitor fluoxetine on learning after non-chronic defeat. Values for p are boldface where significant, W is from Wilcoxon signed rank tests, and U from Mann-Whitney U tests, for the latter, alpha is set to 0.025 due to two comparisons using the same data set. For each group median, interquartile range (IQR), 10%-90% percentiles and number of values (N) are given.

Group		Median	IQR	10%-90%	N	Comparison	p	W/U
<i>P_{resp}</i>								
vehicle, 2 def	CS°	0.038	0-0.5	0-0.924	20	CS° vs. CS^+	0.0042	W 148
	CS^+	0.682	0.239-0.935	0-0.992	20			
fluoxetine, 2 def	CS°	0.506	0.19-0.849	0.007-0.990	20	CS° vs. CS^+	0.0532	W -104
	CS^+	0.133	0-0.791	0-0.971	20			
fluoxetine, no def	CS°	0.293	0.006-0.756	0-0.993	20	CS° vs. CS^+	0.0006	W 172
	CS^+	0.920	0.449-0.971	0.004-0.999	20			
Learning index								
vehicle, 2 def	LI	0.256	-0.003-0.672	-0.027-0.974	20			
fluoxetine, 2 def	LI	-0.258	-0.449-(-0.027)	-0.812-0.880	20	vehicle vs. fluox, 2 def	0.0002	U 69
fluoxetine, no def	LI	0.247	0.008-0.705	-0.135-0.855	20	vehicle vs. fluox, no def	0.7994	U 190

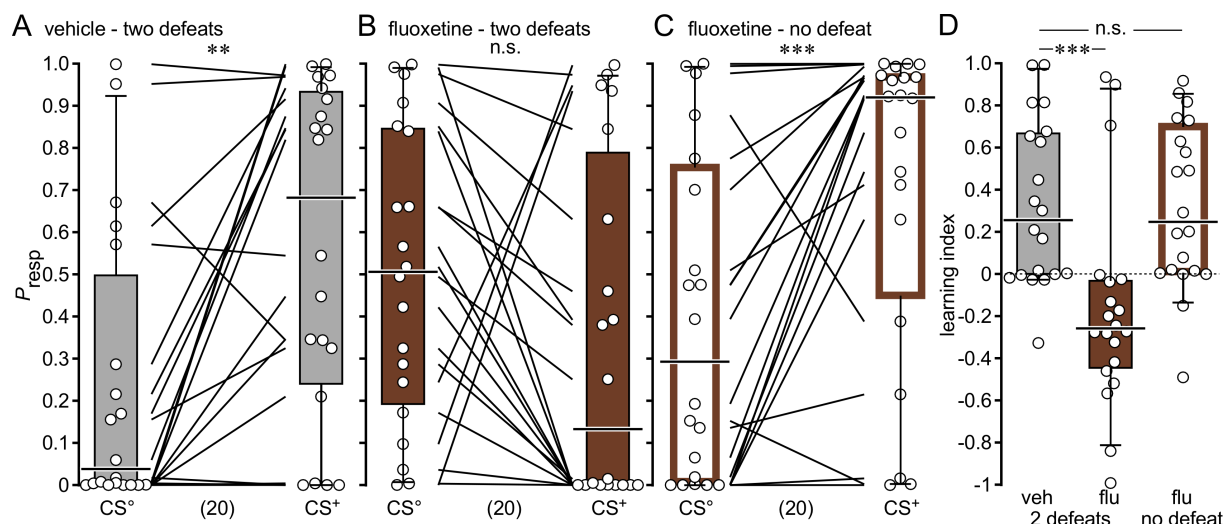


Figure 19 | Effect of 5HT re-uptake inhibitor fluoxetine on learning after non-chronic defeat. **A** Animals that were injected with vehicle (grey) and then subjected to two consecutive defeats. Boxplots giving the P_{resp} values for the non-rewarded and rewarded odour (CS^o, CS⁺). Bars: median; boxes: interquartile range; whiskers: 10th and 90th percentiles; circles: individual values; N = indicated in parenthesis under the graph. The interposing lines between plots indicate the difference in response for each individual. **B** As for A, but for animals that were injected with fluoxetine and experienced two defeats (brown). **C** As for A, but for animals that were injected with fluoxetine and experienced no defeats (brown outlines). **D** Learning indices for the animals that received vehicle (grey) or fluoxetine (brown) and two defeats, or fluoxetine and no defeat (brown outlines). Asterisks indicate significant differences between groups: A-C: Wilcoxon signed rank test; *** $p < 0.001$; ** $p < 0.01$; n.s., not significant. D: Mann-Whitney U-test, corrected for 2 comparisons: *** $p < 0.0005$; n.s., not significant.

3.3.3 Summary of learning capacities – chronic defeat and serotonin

To examine the differences in learning between the groups further and summarise them, the animals were sorted into the three learning categories: animals that score a positive ($LI > 0.05$), zero ($(-0.05) \leq LI \leq 0.05$) and negative LI ($LI < (-0.05)$). Generally, the impairment of learning seen in animals that received the vehicle control after chronic defeat is mainly due to the animals that had a negative LI (55%) and the animals that were not able to differentiate between the odours and exhibited a zero LI (25%). Only 20% had a positive LI (N = 20, Figure 20A). In the chronic defeat group treated with methiothepin the contingency for each learning type was almost distributed equally (positive $LI = 30\%$; zero $LI = 35\%$; negative $LI = 35\%$) and not significantly different from the vehicle group (Fisher: $p = 0.551$, N = 20; Figure 20A). In contrast to that, the contingency of the ketanserin treated animals that were subjected to chronic defeat differed significantly from the vehicle control (Fisher: $p = 0.007$, N = 20; Figure 20A) mainly due to less animals scoring a negative LI (10%) and more animals scoring a positive LI (60%), while 30% were not able to differentiate between the CS^o and CS⁺.

When animals were not subjected to chronic defeat, there was no difference in contingency of learning types (Figure 20B) between the vehicle (positive $LI = 70\%$; zero $LI = 15\%$; negative $LI = 15\%$, N = 20) and methiothepin treated animals (positive $LI = 75\%$; zero $LI = 10\%$;

negative $LI = 15\%$; Fisher: $p = 1.0$, $N = 20$; Figure 20B), or between the vehicle and ketanserin treated group (positive $LI = 65\%$; zero $LI = 20\%$; negative $LI = 15\%$; Fisher: $p = 1.0$, $N = 20$; Figure 20B). The biggest portion of the animals score positive LIs , additional to the animals that were not able to differentiate between the odour, in each group there are 15% of animals that score negative LIs . The control animals for the 5HT re-uptake inhibitor fluoxetine (Figure 20C), which received vehicle and 2 defeats, exhibited a normal frequency regarding the three learning types with the majority learning the odour (positive $LI = 60\%$; zero $LI = 35\%$; negative $LI = 5\%$, $N = 20$; Figure 20C). When the animals were treated with fluoxetine and subjected to non-chronic defeat, the percentage of animals scoring negative LIs was strongly increased (70%) Together with animals that were not able to differentiate between the odours and scored a zero LI (15%), they showed the impairment of learning of this group (only 15% score positive LIs) and differed significantly to the control (Fisher: $p < 0.001$, $N = 20$; Figure 20C). This was not true for the animals that were treated with fluoxetine and received no defeat. The contingency for this group did not differ from the vehicle control (positive $LI = 65\%$; zero $LI = 25\%$; negative $LI = 10\%$, $N = 20$; Fisher: $p = 0.791$; Figure 20C). All were corrected for two comparisons.

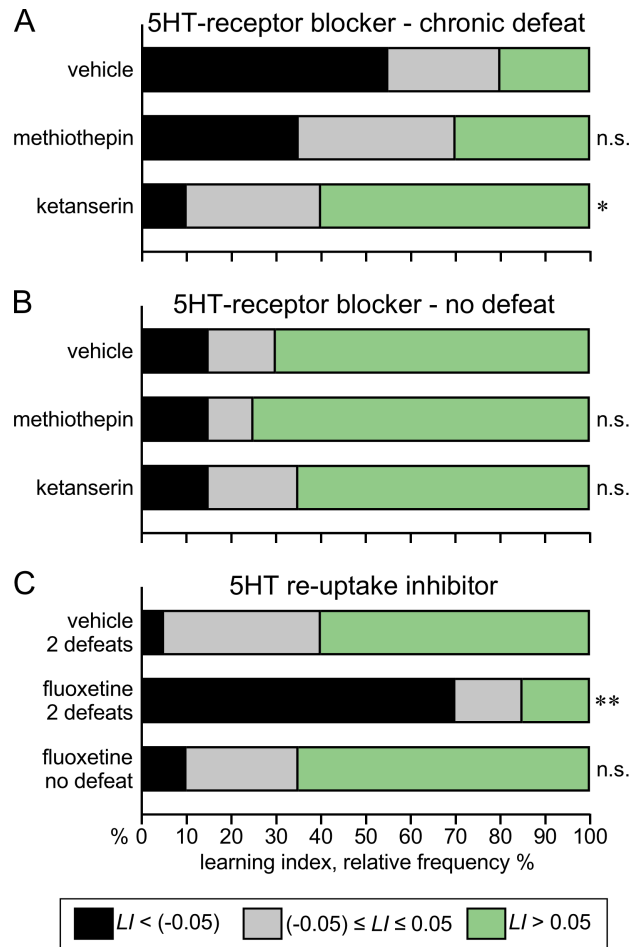


Figure 20 | Summary of the influence of serotonergic drugs on learning in relation to social experience. **A** Data for animals that experienced chronic defeat and were treated with vehicle, methiothepin or ketanserin. The bars give the percentages of individuals that exhibited either learning indices less than (-0.05) (black), no significant learning (LI between (-0.05) and 0.05, grey) or significant learning (LI greater than 0.05, green). **B** As for A, but for animals that experienced no defeat prior to drug treatment. **C** As for A, but for animals that received vehicle or fluoxetine and two defeats, or fluoxetine and no defeat. Asterisks indicate significant differences to control data (vehicle in A and B; vehicle 2 defeats in C) from Fisher's exact test with alpha correction for 2 comparisons: ** $p < 0.005$; * $p < 0.025$; n.s., not significant.

4 DISCUSSION

Winning or losing aggressive encounters between conspecifics can have severe effects on the future life of an animal. From studies on aggression in crickets it is clear that winning a fight can higher the chances of winning a subsequent fight and *vice versa* (Alexander, 1961; Hsu et al.; 2006; Rillich and Stevenson, 2011; Stevenson and Rillich, 2012). Furthermore, it is known that multiple experiences of losing aggressive interactions can impair future aggression severely (Rose et al., 2017b) and most importantly induce depression-like symptoms in mammals (Hollis and Kabbaj et al., 2014). Defeat stress can affect various parts of animals' lives, of mammals (Yu et al., 2016, Der-Avikan et al., 2017) and invertebrates (Rillich and Stevenson, 2018; Balsam and Stevenson, 2020, 2021) alike. Consistent inter-individual differences in crickets are known to be determined by agonistic experiences (Rose et al., 2017a; Jäger et al., 2019; Balsam and Stevenson, 2021). Due to the findings that multiple agonistic experiences affecting learning and memory in mammals (Der-Avikan et al., 2017; Hanson et al., 2017), the importance of considering individuals in classical conditioning in honey bees (Pamir et al., 2011) and support of individuality in learning in cockroaches (Arıcan et al., 2020) this study hypothesised that chronic social defeat stress influences the learning capacity of male crickets and the aggressive state of an individual gathered from early life social experiences is an underlying cause of inter-individual differences in learning. Therefore, a new semi-automated system for measuring conditioned responses and ultimately learning in individually assayed *Gryllus bimaculatus* was established and validated by employing video tracking and binary logistic regression modelling. With this tool at hand, crickets were subjected to defeat stress and their learning capacity was analysed afterwards. Additionally, the influence of multiple winning experiences was tested. It is known that aggression is regulated *via* the biogenic amines octopamine, dopamine and serotonin (Stevenson and Rillich, 2012, 2019) of which all are also known to be involved in learning and memory (Selcho et al., 2009; Perry and Barron, 2013; Huser et al., 2017; Sitaraman et al., 2017; Verlinden, 2018). Serotonin is known to maintain the reduced aggression after chronic social defeat (Rillich and Stevenson, 2018) and was therefore investigated using several serotonergic drugs for its role in learning after chronic social defeat stress.

4.1 The semi-automated measurement of olfactory learning in individually assayed crickets

In order to investigate effects on learning in individuals it was important to have an adequate tool to precisely measure the learning capacity and to be able to capture changes in behaviour. In crickets so far, associative learning has mainly been measured by comparing pre- and post-preferences in an appetitive-aversive olfactory paradigm by allowing the animals to freely walk around and visit two odour sources, whereas an initially rather unattractive odour was rewarded and another more attractive odour punished, and the time spent at each odour source measured (Matsumoto and Mizunami, 2000, 2002). Since it got clear from pilot studies, that the conditioned response of crickets to a previously trained odour comprised of distinct behaviours, for example the bobbing of the head and typical searching behaviour (Figure 5) the mere observation of the described response could have led to a simple measure of learning that assessed whether an animal exhibited a conditioned response to an odour or not (Matsumoto et al., 2015). Besides this being a subjective evaluation done by the experimenter, individual variances in learning capability would be overlooked by this approach (Pamir et al., 2011). Therefore, the goal for measuring learning in individual crickets was to establish a method that can detect and quantify this response in individual animals, automatically distinguish between animals that exhibit a conditioned response and those that do not and give a probability of an animal exhibiting a conditioned response. Studies in locusts show that it is possible to employ tracking technology to differentiate between gregarious and solitary locusts using various monitored behavioural traits in binary logistic regression modelling (Roessingh et al., 1993; Gray et al., 2009) and the application enabled the researchers to measure external influences on locust phase behaviour (Anstey et al., 2009; Cullen et al., 2010).

First, to build a binary logistic regression model that could describe a conditioned odour response it was essential to generate two groups that represent the dependent variable that is coded binary for model building (0 and 1). In the learning context the dependent variable is *conditioning*, 1 represents the 50 animals that were trained to an odour paired with a sucrose reward (CS+US-paired; Figure 2) in absolute conditioning and that form associative memory as has been shown in honey bees (Menzel et al., 2001). They therefore can exhibit a conditioned response to the subsequent presentation of an odour, whereas 0 represents the 50 animals that received the reward only (US-only; Figure 2) and therefore are not able to show a conditioned response to the presented odour in the test. In order to have these groups differ as far as possible in their behaviour and response (see Roessingh et al., 1993 on gregarious and solitarious locusts) it was opted for six training repetitions. The proboscis extension reflex (PER) is

exhibited by 80% of honey bees after 30 min with an inter-training interval of 3 min, in an comparable conditioning regime, but what also becomes is, that not all animals in a group are capable of exhibiting the conditioned response (Menzel et al., 2001). It should not have been opted for more trainings as the concept of the prediction error sets a limit to the training repetitions. If the animal is trained in an absolute conditioning paradigm, in the first odour presentations the animal is not predicting the reward, and this results in a pleasant surprise. The more the odour is associated to the reward, the smaller the prediction error gets until no learning occurs anymore (Rescorla and Wagner, 1972; Schleyer et al., 2018). Additionally, too much repetitions can increase satiation and therefore lead to reduced US-related motivation (Menzel et al., 2001; Krashes and Waddell, 2008; Mizunami et al., 2019). This acts as one of the reasons that for the group representing the unconditioned response, US-only trained animals were analysed for model building. In comparison to animals that were only presented with the odour six times (CS-only) the animals of the CS+US-paired and US-only groups had balanced satiation levels. Additionally, the energy intake from the sucrose reward is the same in those two groups, therefore for example an increased motility from this could be ruled out. Also, too few repetitions could have led to a decreased number of animals being able to learn (Matsumoto and Mizunami, 2002). CS-only trained animals could have shown habituation effects (Carew et al., 1972; Jones and Agrawal, 2017) to the odour, whereas CS+US-unpaired trained individuals (US applied 2.5 min after CS) were approached twice as much during the training period by the pipette and the odour application syringe (Figure 1 and 2). This would lead to more handling, but these disturbances should be at a minimum and the same level between the groups (Gouveia and Hurst, 2017). The goal to minimise effects due to handling and changed surroundings is also the reason that the animals were trained and tested in the same arena, contrary to other studies (Matsumoto and Mizunami, 2002; Watanabe et al., 2003). Whereas many other paradigms require the animal to locate an odour source, or visual stimulus in a Y-maze, or similar (e.g. Matsumoto and Mizunami, 2000; Watanabe et al., 2003; Giurfa, 2004; Dupuy et al., 2006) with often large distances to walk to even reach it, the application of the odour directly over the antennae and the video-tracking of each animals' immediate response (Figure 3) had the advantage that every movement of the animal is taken into account over the following 2 min.

From video-tracked conditioned and unconditioned odour responses of 100 crickets 13 behavioural variables were extracted. Fitting univariate models (Hosmer and Lemeshow, 2000; Stoltzfus, 2011; Sperandei, 2014) revealed the eight independent variables, namely *time to move*, *initial distance*, *total distance*, *total time moving*, *time moving in odour zone*, *time moving*

in odour area, velocity and *bobbing*, that significantly contributed to each model describing the dependent variable and therefore held the potential to characterise the distinct conditioned response (Table 2). When these were compared between the US-only and CS+US-paired trained animals, seven differed significantly. CS+US-paired trained crickets started to move earlier, moved further and spent more time moving. They moved slightly faster and longer in the vicinity of the odour stimulus. While the US-only trained crickets were seldom exhibiting bobbing behaviour, the CS+US-paired animals were engaging for a longer period in head-bobbing movements. The distance they walked directly after odour application did not differ significantly between the two groups (Table 3, Figure 7).

Binary logistic regression analysis yielded a variety of mathematical models that implemented various combinations of the eight behavioural variables to discriminate between the two binary groups. As applied in other studies (Gray et al., 2009; Cullen et al., 2012; Stettin, 2014), the models were evaluated and compared by the Hosmer-Lemeshow goodness-of-fit test (Hosmer and Lemeshow, 2000), the overall discrimination accuracy, the Akaike information criteria score (Akaike, 1974) and the area under the receiver operating characteristic curve (AUC under ROC, Hosmer and Lemeshow, 2000; Austin and Steyerberg, 2012). Additionally, the implemented variables for each model were tested for multicollinearities using the variance inflation factor (VIF) which is also an important tool to evaluate the correctness of a model and identify possible errors (Thompson et al., 2017). Unfortunately, there is no consensus which VIF thresholds to use, but as they are often given with 10 (Thompson et al., 2017). The values in the selected model are small indicating no influence of multicollinearities. It is important to have an adequate number of samples, measured as events per variable (EPV) in a regression analysis for a good model reliability (Ott, 2018). Here, all models have a larger EPV than 10 which is often advised (Peduzzi et al., 1996) but suggested to be loosened (van Smeden et al., 2016). From these analyses, the most parsimonious and accurate model based on the variables *time to move, total time moving* and *bobbing* (*Model-2*) was selected over the other models calculated (Table 4). The overall discrimination accuracy of 93% (US-only 96 %, CS+US-paired 90%) of the final model compares favourably with the averages of 89.5 - 90.7% given by several models with a differing count of variables included (three, four, or more) that were applied to discriminate between gregarious and solitary locusts (Cullen et al., 2012 for references). Even though the binary groups could be distinguished, in all models calculated, the accuracy of prediction for the CS+US-paired group was always lower than that for the US-only group. This was most likely due to the fact that in a randomly selected group of animals not 100% are able to associate the odour to the reward and also show a conditioned response, which

can also be observed in honey bees (Menzel, 2001). This acts as a limitation of a behavioural model, not all animals are behaving 100% according to their respective group.

With *Model-2* determined to be used for future analyses, the actual P_{resp} values could be calculated precisely for all animals used to build the model. These were clearly differentiated between the US-only group with the animals mostly around zero and the CS+US-paired group in which most of the animals were near 1.0 (Table 5, Figure 8). In order to test if the sucrose reward alone had an effect on the odour response, the data for the US-only group presented with the odour experimentally naive was analysed. This revealed similarly low values for the US-only group before training compared to the test responses and, therefore, no effect. A models' accuracy can also be assessed by calculating the P_{resp} values for animals with known training history that were not used for model construction (Hosmer and Lemeshow, 2000; Cullen et al., 2010) and doing this confirmed the use of *Model-2* (avg. 90%, US-only 92 %, CS+US-paired 88%). Additionally, CS-only trained animals scored low P_{resp} values, which confirms and controls that multiple presentations of odour alone do not lead to high scores (Matsumoto and Mizunami, 2002). The P_{resp} values of the CS+US-paired group representing associative memory could be tested by comparing these to the values of animals that received CS+US-unpaired during training and had correspondingly low P_{resp} scores, confirming that the association of odour and reward could be measured (Table 5, Figure 6 and 8).

It was very important to test the viability of the binary logistic regression model for scoring learning in individual crickets. Therefore, a differential appetitive olfactory conditioning paradigm was applied. For this, the P_{resp} to one odour that was previously rewarded (CS^+) was compared to a different odour that was not rewarded during training (CS°). In this paradigm, it was opted for three training repetitions (each odour 3 times, alternating) which has proven to be adequate to form associative memory in crickets (Matsumoto and Mizunami, 2002) and other insects (Sandoz et al., 1995; Watanabe et al., 2003; Gerber et al., 2013). For future experiments it was not important to achieve the highest learning scores possible but to be able to detect improvements and impairment of learning capacity, therefore the number of training repetitions was not increased further. Applying the model in a differential conditioning paradigm (Table 1) revealed that the majority of crickets showed a higher P_{resp} to CS^+ than to CS° and, therefore, 69% differentiated between the two odours at the recall test 30 min after training (Figure 9). Comparing the P_{resp} scores for CS-only obtained from absolute conditioning (CS-only, Figure 8B) and the CS+US-unpaired trained group to those from the CS° group revealed that both seemed to be lower. In the CS-only, the CS+US-unpaired trained group (Figure 2 and 8) and in the differential conditioning paradigm odour is presented six times,

therefore there is no difference in sensory experience. But there was a difference in odour identity. For the CS^o there were fewer training repetitions in the differential compared to the absolute paradigm (3 vs. 6), and because the task requiring the animals to differentiate between two odours that are equally attractive to experimentally naive crickets it was more complex. Generalisation of the unrewarded odour, especially for structurally similar odours can be observed in the first trials of differential conditioning in honey bees (Smith and Menzel, 1989; Guerrieri et al., 2005; Lehmann et al., 2011).

Since each individual crickets' odour responses were measured as a probability (P_{resp}) to both the CS⁺ and CS^o, an appetitive learning index (LI) could be calculated directly from the difference of the P_{resp} to the CS⁺ and the P_{resp} to the CS^o. In invertebrates, comparable measures are rarely calculated for single animals (*Drosophila* larvae: Scherer et al., 2003; honey bees: Scheiner et al., 2020). Contrary to this, a “preference”-, “performance”-, “discrimination”-, or “learning”-index is typically estimated from the net preference of a group of animals or as percentage of animals exhibiting a conditioned response (Selcho et al., 2009; Lehmann et al., 2011; Apostolopoulou et al., 2013; Gerber et al., 2013; Nishijima and Maruyama, 2017). Additionally, it is sometimes calculated by comparing pre- and post-training preferences (Matsumoto and Mizunami, 2002). When performing a new conditioning paradigm, it is important to check for effects related to the identity and sequence of the odour which is regularly done in other studies (Scherer et al., 2003). Therefore, in the differential conditioning paradigm eight systematic permutations of odour identity and reward sequence were checked, but no differences were found, and the data pooled (Table 1, Table 6). Additionally, the odours (amyl acetate and 1-octanol) and their concentrations (1:10 in paraffin oil and pure, respectively) that were adapted from different established conditioning procedures (Scherer et al., 2003; Gerber et al., 2013) were controlled for differences in the initial response to the presentation. Here also no odour preferences could be detected. Significant learning was defined as $LI > 0.05$ indicating that the animals exhibit the conditioned response to the CS⁺ and therefore associate the reward to the response, and in this paradigm 69% of the crickets showed significant learning (Figure 9). Direct comparisons to other studies are difficult due to methodological differences, but under reasonable comparable conditions, after six training repetitions and a pause of one day, 80% of crickets exhibit the maxillary palpi extension response (MER) to the paired, and only 20% to the unpaired odour (Matsumoto et al., 2015). After six repetitions and 30 min pause 78% to 83% of honey bees exhibit the PER to the CS⁺ and 19% to 32% to the CS⁻ (Lehmann et al., 2011).

Regarding the fact that a single behavioural metric is mostly used for evaluating the behavioural response to a conditioned stimulus, like for example the PER, MER and MLR, (e.g. Bitterman et al., 1983; Matsumoto et al., 2015; Arican et al., 2020) it was important to analyse whether the multi-variable analysis using the model is superior. Head-bobbing was selected representing a single behavioural metric since it nearly always occurs towards the CS⁺, is often an immediate response to a conditioned odour and with the highest coefficient, is the most weighted variable in the model. Bobbing-behaviour has not previously been described in crickets, but parts of it like the palpal extension while raising the head in response to water on the antennae or a conditioned odour had been defined as part of the MER in crickets (Matsumoto et al., 2015). The additional component, antennal waving or fencing, had been observed in crickets during courtship (Adamo and Hoy, 1994) and aggression (Rillich and Stevenson, 2015) or in response to mechanical stimulation (Balsam and Stevenson, 2020). Even though head-bobbing is a good indicator of a conditioned response, it is not entirely specific. There are several instances where it can occur, for example when a sugar water drop is removed from the animal before satiation or, but rarely, to an attractive odour. This can happen in experiments regarding most single behavioural measures of a conditioned response, such as the MER (Matsumoto et al., 2015), and the PER (Bitterman et al., 1983). This is why individuals that respond spontaneously to the odour stimulus in the first training trial are often discarded from learning experiments (e.g. Behrends and Schreiner, 2012). For this work the goal was to analyse the entire spectrum of individual learning that an unbiased selection of laboratory animals exhibits and for this reason no individuals were discarded. It also has to be mentioned that spontaneous conditioned response-like behaviour to a novel odour happened very rarely.

In order to achieve a reasonable comparison between the P_{resp} scores and *bobbing*, the values of the latter were normalised. This revealed that the multifactorial analysis with the model is superior to the single metric *bobbing* for analysing learning (Table 7, Figure 9). If the *bobbing-LI* were equally suitable to measure learning in crickets, there would be no difference between *model-LI* and *bobbing-LI*. This is not the case, as *model-LI* scores were significantly greater than the *bobbing-LI* (median: 0.395 and 0.054, respectively). There are several reasons for this. When regarding the responses to CS^o, the lower normalised *bobbing* values compared to the P_{resp} fit better to a theoretical outcome of the conditioning protocol, which is that animals would not respond to the unrewarded odour (Table 7; Figure 9A and B). In contrast to the model data, the normalised *bobbing* scores to the CS⁺ are also comparatively low. The large difference between the *model-LI* and the *bobbing-LI* (Table 7, Figure 9C) is mainly due to a large number of animals with zero or small *bobbing* scores (-0.05 to 0.05), which is also shown by the mode

at zero in the histogram with a skewed distribution (Figure 9D). Problematic is that due to normalisation outliers tamp down lower scores. Additionally, the normalisation is specific for each dataset and could not be used in a new group of experimental animals. In a differential, olfactory appetitive conditioning paradigm, in which the times spent visiting a rewarded and non-rewarded odour source are evaluated, and that were tested 2 h after 2 training trials, the crickets score a mean performance index of around 30 (Figure 3B, white bar in Matsumoto and Mizunami, 2002). Although this is not entirely comparable the value would correspond to a mean *LI* of 0.3. The *bobbing-LI* is at a mean of 0.14, whereas the *model-LI* is at a mean of 0.42. Also, the question arises if it is appropriate to use the limit value of *bobbing-LI* > 0.05 to define learning. As bobbing is such an exclusive behaviour exhibited to the CS⁺ this might not be appropriate. An alternative could be to use 0 as the limit value. What cannot be denied, however, is that *bobbing* is a key predictive variable in the model. This becomes clear when regarding the P_{resp} once more. Here eight of ten individuals scoring a maximum P_{resp} of > 0.95 to CS^o also show bobbing, whereas the majority individuals with a low P_{resp} towards CS⁺ (< 0.5) do not show bobbing (12 of 14 animals). Nonetheless, the impact of the additional variables is one of the main advantages of the model. This is that animals that exhibit no bobbing can regardless be captured as learners which confirms the complexity of a conditioned response (Onodera et al., 2019). This is supported by *Model-4*, which explicitly excludes *bobbing* (Table 4), with an average predictive power of 87%. This reveals that a conditioned response can be measured by general movement variables. In general, these findings reveal the advantage of using binary regression analysis, which also encompasses interactions among the behavioural variables in relation to the P_{resp} outcome (Cullen et al., 2012). Nonetheless, a potential disadvantage is that the influence of any environmental, genetic or other factors with antagonistic effects on different model components may be underestimated, but this could partly be accounted for by always comparing responses to CS^o and CS⁺ and proper control groups. If needed, the variables could be analysed separately.

The distribution of data points was assessed using histograms (Figure 9D) and sorting of the values in ascending order (Figure 10) to compare the *model-* and *bobbing-LIs*. The coefficient of bimodality (Pfister et al., 2013) revealed a significant bimodality which is depicted by two modes at 0 and 1.0. Contrasting this, the histogram of the *bobbing-LI* shows a skewed distribution. It could be argued that the bimodal distribution is simply an outcome of binary regression analysis, which is restricted to the maximal possible extreme values of 0 and 1. When the CS^o and CS⁺ scores were pooled and arranged in ascending order the data form a typical sigmoid curve (Figure 10A) with most of the CS⁺ data in the upper half of the graph and the

CS^o data in the lower half. But there is no separation between CS^o and CS⁺ values and a lot of intermediate scores even mixing in their classification. Such a distribution is not evident when the *bobbing* values are considered, here the data resemble an exponential function (Figure 10B). A correction of the *bobbing* values using logarithmic calculus could have been considered, but this would strongly inflate small *bobbing* values which would be unfavourable for calculating the *bobbing-LI*. In contrast to the sorted *bobbing-LI* (Figure 10B), the plot for the sorted *model-LI* yields a more linear distribution, with relatively evenly distributed values (Figure 10A). This illustrates that the model, in contrast to the single metric *bobbing*, can precisely cover measures of learning and has the power to effectively capture inter-individual differences in learning. The bimodal distribution of the learning data of a randomly selected group of crickets suggests a broad spectrum of learning capacity of individuals, with animals that do not differentiate between the two odours and are considered very poor learners contrasting to the excellent learners that strongly react to the CS⁺, but not the CS^o. However, the cause of these inter-individual differences had not been identified here. In classical conditioning of honey bees it had been shown that a population comprises two types of animals, those that have acquired a conditioned response and those that have not (Pamir et al., 2011) and also fast and slow learners had been identified (Tait and Naug, 2020). Consistent inter-individual differences in crickets have already been investigated regarding multiple behavioural traits and these are linked to agonistic experiences (Rose et al., 2017a, Balsam and Stevenson, 2020, 2021).

4.2 The influence of multiple agonistic experiences on learning

With the method to measure learning employing a semi-automated multivariable analysis in crickets established, the possibility to investigate relations of aggression and learning arose. From various studies it became clear that inter-individual differences regarding multiple behavioural traits, like aggression (Rose et al., 2017b) or the decision approach or avoid a novel stimulus resulting in two ethotypes comprising of aggressive-proactive and submissive-reactive individuals (Balsam and Stevenson, 2020, 2021), are a result of previous experiences in the life of a cricket. Chronic social defeat stress as an intensive form of social agonistic experiences which induces depression-like symptoms in crickets (Rose et al., 2017b; Rillich and Stevenson, 2018) and is also considered as a model for depression in mammals (Hollis and Kabbaj et al., 2014). This study provides evidence that the experience of social chronic defeat stress impairs learning and memory in crickets.

The animals were subjected to chronic social defeat by matching them multiple times against a hyper-aggressive male (Stevenson and Rillich, 2015; Rillich and Stevenson, 2018), and the

aggressive potential was measured at the first encounter and the test match after conditioning (Figure 11A). From studies in short term (STI) and long term (LTI) isolated animals it became clear that STI, that experienced numerous agonistic experiences with conspecifics previously in their life, are more susceptible to chronic defeat stress in comparison to LTI animals that did not experience aggressive interactions before and are more resilient (Rose et al., 2017b). Earlier studies revealed that the effect of reduced aggressiveness after 6 defeats in 1 h intervals lasts longer than that of only 3 defeats (Iwasaki et al., 2006; Rose et al., 2017b). In this work additionally to being subjected to chronic defeat the animals were trained and tested in a differential conditioning paradigm and therefore it was opted for nine defeats over two days rather than six defeats on one day (Rose et al., 2017b). Since the influence of chronic defeat in the form of long-term depression of aggression was previously mostly looked at as a group effect (Rose et al., 2017b; Rillich and Stevenson, 2018) regardless of the individual resilience and the specific resulting aggressiveness and seldom distinguished further (Iwasaki et al., 2006), it was interesting to separately analyse animals that were aggressive (level of aggression ≥ 4 ; Figure 4) and those that were submissive (level of aggression ≤ 2) after chronic defeat. Generally, it can be confirmed that in the nine-defeat paradigm, chronic defeat also has long term effects on aggression, because in the test match against a hyper-aggressive male after conditioning the majority of the STI animals behave submissively (20 of 30; Figure 11B), whereas ten animals behaved aggressively. The loser effect of reduced aggressiveness after a single defeat should have vanished after 0.5 h to 3 h (Adamo and Hoy, 1995; Iwasaki et al., 2006; Rillich and Stevenson, 2014). In this paradigm, at least 25 h after the six defeats on the first day and at least 4.5 h after nine defeats, effects of reduced aggressiveness are still present. For the LTI, in order to include a comparable number of 20 submissive individuals in the experiments, 40 animals had to participate in the experiment (Figure 11C). This reflects the differences in susceptibility to chronic defeat between STI and LTI animals described earlier (Rose et al., 2017b). From previous experiments in crickets it is not clear how many of the animals subjected to chronic defeat stress were really exhibiting a suppressed aggressiveness. It had been shown that 3 h after 3 losses 90% of the crickets show avoidance behaviour (Iwasaki et al., 2006). It was surprising that a lot of animals were aggressive again and this raised the question whether the experimental design, mainly the energy intake from the sugar reward or the conditioning procedure, influences this result. It is known that physical exertion, like flying can increase aggressiveness (Hofmann and Stevenson, 2000) and possibly the training in the conditioning paradigm could also have an effect. Very interesting in the chronic defeat paradigm is the retrospective analysis of the first match after isolation compared between STI

and LTI. STI already differ in their aggressiveness at the first match, meaning that animals that behaved aggressively after chronic defeat had also been more aggressive in the first match, submissive animals less aggressive, respectively (Figure 11B). These behavioural differences had also been shown for the approach and avoidance of a stimulus directed at an antenna (Balsam and Stevenson, 2021). Retrospectively, the aggressive and submissive LTI animals do not fight differently in the first match regarding level of aggression and duration of the fight (Figure 11C). This was to be expected from the two isolation protocols and the differing previous agonistic experiences and their influences on the behaviour of STI and LTI animals (Rose et al. 2017b; Balsam and Stevenson, 2020, 2021) resulting in experimentally naive animals representing an inhomogeneous (STI) and a homogeneous (LTI) group regarding their aggression.

The animals that were exposed to chronic social subjugation were trained in the differential learning paradigm and their learning capacity was measured. Control animals that were not subjected to defeat scored mostly positive *LI* scores and exhibited a median learning score of 0.247 (Table 8, Figure 12Di). As observed previously, the crickets show a broad spectrum of *LIs* emphasising the individuality that can be observed in learning abilities (Pamir et al., 2011; Arican et al., 2020). Most interesting is, that in the group of animals that were subjected to chronic social defeat the median *LI* (-0.006) of the submissive animals was strongly impaired in comparison to the control group (Table 8, Figure 12 Di). This effect was not only present in STI crickets, but could also be observed in LTI animals (Table 9, Figure 12Dii) confirming that this is not a random, one-time result. It is very striking that the submissive animals not only show *LIs* of 0 which would mean that they do not differentiate between the odours, but a lot of animals even exhibit negative *LIs*, meaning that they show a stronger response to the CS[°] compared to the CS⁺ (Figure 12Bi and Bii). Astonishingly, this was to the extent that some of the individual responses to the unrewarded odour were resembling a normal conditioned response even with the very distinct *bobbing* behaviour. This also becomes clear from the contingency of positive ($LI > 0.05$), negative ($LI < (-0.05)$) and zero *LIs* ($(-0.05) \leq LI \leq 0.05$; Figure 14A and B). In comparison to the no defeat control group, the main differences lie in the reduction of the number of animals exhibiting positive *LIs* and the increased number of animals with negative *LIs* (Figure 14A and B). One explanation for this could be that they cannot sense the difference between the odours anymore because sensation is impaired. If this would be true, a more random distribution of P_{resp} values to the CS[°] and the CS⁺ would be expected. But what becomes clear (Figure 12Bi and Bii) is that the P_{resp} values to the CS[°] are generally higher and the P_{resp} to the CS⁺ lower than in undefeated individuals (Figure 12Ai and

Aii) with the CS^+ values impaired and not random. An inability to retrieve memory due to satiation (Brüner et al., 2020) is unlikely because the control groups being starved and fed in the same manner as the submissive and aggressive group and show normal learning (Table 8 and 9, Figure 12Ai and Aii). Furthermore, it is known that the valence of punishment and reward is not always perceived as aversive and appetitive, respectively. Timing dependent valence reversal describes the effect, that the termination of punishment is perceived as relief and that the termination of a reward induces an aversive effect (Vogt et al., 2015; Gerber et al., 2019). If feeding the animal would be perceived as aversive, because it means a touch sensation to the mouthparts that may be critical because of the negative fighting experience, the absence of the reward would be perceived as a positive experience. This could maybe be part of the conditioned like behaviour the animals exhibit to the CS^0 and would also explain the low P_{resp} values they show to the CS^+ . But this definitely needs to be looked further into. In cockroaches it had been shown that the social context can invert the valence of an odour (Günzel et al., 2021). Additionally, for aggression it had been shown that when the experience of losing is coupled to an odour, this odour is perceived as aversive (Kim et al., 2018). And if this is true for crickets the anticipation of chronic defeat and therefore punishment could maybe inhibit sensory modalities like the feeding response to sucrose (Pirger et al., 2021). Another possible explanation, because the rewarded odour seems to be strongly affected, is that the value of the reward is reduced. In mice it had been shown that chronic social stress leads to a reduced reward salience (Kúkel'ová et al., 2018) and a depressive-like phenotype with reduced sucrose preference (Macedo et al., 2018). An endophenotype identified in research of the major depressive disorder in humans is anhedonia, which is known as non-reactivity to pleasurable stimuli (Pizzagalli, 2014) and is assumed to reflect the dysregulation in reward processing (Heshmati and Russo, 2015).

In general, what becomes clear in this first experiments is that chronic defeat induced stress impairs learning in crickets, regardless of the isolation protocol. In other invertebrate systems, different stressors were found to alter and impair memory retrieval (Bees: Piiroinen and Goulson, 2016; *Lymnea stagnalis*: Swinton et al., 2020). Also, in rats it had been shown that social defeat impairs memory and memory retrieval (de Quervain et al., 1998; Novic et al., 2013; Patki et al., 2014; Der-Avakian, 2017) and in mice social stress can impair neurogenesis (Mouri et al., 2018). For humans it had been shown that the time point of experiencing stress is affecting the memory conversely, but when it is happening long before encoding impairs memory formation (Vogel and Schwabe 2016; Bierbrauer et al., 2021). Flight in crickets can restore aggressiveness after defeat (Hofmann and Stevenson, 2000) and in rats the social defeat-

induced memory impairment can be rescued through treadmill exercise (Patki et al., 2014), therefore examining the effect of flight on the impairment of learning would be interesting. In the group of animals that experienced chronic defeat some were not as susceptible to chronic defeat and therefore behaved aggressively after chronic defeat and conditioning (Figure 11B and C). Surprisingly, the impairment of learning is not coupled to this (Table 8 and 9, Figure 12Ci and Cii). The group of aggressive animals have significantly lower *LIs* compared to the control, but when the contingency of the animals is concerned, meaning distribution of animals with positive, zero and negative *LI* (Figure 14A and B), the difference is not significant ($p = 0.0651$) in the LTI animals, but for the STI animals it is ($p = 0.002$). The finding that the impairment of learning is decoupled from the aggressive state of the animals at the test match raises the question if the subjugation to chronic defeat is the real trigger for this or if agonistic contact to a conspecific is enough to alter learning for example through the increased stress, energy loss or pheromones on the antennae (Iwasaki and Katagiri, 2008). For bees it had been shown that preexposure to certain pheromones modulated the appetitive motivation in olfactory conditioning (Baracchi et al., 2020).

To test this, a new group of animals experienced nine fights, but they won every encounter, but the physical and experimental experiences were similar to the chronic defeat subjected animals (Figure 13A). As the LTI and STI did not differ in their general effects of chronic defeat to learning, all following experiments were conducted with STI animals. Normally, after winning two consecutive fights the animals are more aggressive for less than 20 min and the best results were achieved with a fight interval of 5 min (Rillich and Stevenson, 2011) in the present paradigm the interval was 1 h. Interestingly, when they won nine fights on two consecutive days a winner effect could be observed for at least 4.5 h after the last win (Figure 13B). The animals fought almost exclusively at level 6 and significantly higher than the control. However, this effect was not present in the animals' persistence.

Regarding the learning data, both the control group and the multiple winners showed significantly lower P_{resp} values to the CS° in compared to high P_{resp} values to the CS^+ (Table 10, Figure 13C and D). Additionally, the *LIs* between the group did not differ either (Table 10, Figure 13E). There was no impairment of learning present in the multiple winner, and even no animals with a $LI < (-0.5)$ regarding the contingency (Figure 14C), which is remarkable because the absence of negative *LIs* in a group was not observed in any group throughout this whole work. Therefore, it could be ruled out that the experience of nine encounters of and contact to a conspecific, handling or physical exhaustion was the reason for the learning impairment in defeated animals. In *Drosophila*, winning was found to be perceived as appetitive (Kim et al.,

2018), but the possibility of winning enhancing learning capacity could not be detected in this paradigm. It could be possible that a reduction of the interval between the last fight and the conditioning paradigm could reveal an influence.

With aggression and learning assessed, the intriguing question of a correlation between the aggressive potential of an animal and its ability to learn could be addressed. The learning indices from evaluation of the model for learning were distributed bimodally (Figure 9C and D) and given that STI animals form an inhomogeneous group regarding their aggression and other behaviours (Rose et al., 2017a, 2017b; Balsam and Stevenson, 2021), this possible coupling of variations in cognitive abilities and other traits (Sih and Del Giudice, 2012) needed to be investigated even if aggressive animals also showed impaired learning after chronic defeat. In Figure 9 C and D it is visible that there are always animals that are good learners and animals that are not able to learn the task (see also Pamir et al., 2011). Although submissive animals score low *LIs* and aggressive animals after multiple wins show no negative *LIs*, there was no correlation between level of aggression or duration of the fight in the first or test fight with the *LI* for neither the control animals, the chronic defeat subjected animals (LTI and STI) or the multiple winners (Table 11). For bees it had been shown that cognitive phenotypes with multiple covarying cognitive traits also correlate with some other behavioural traits like nursing behaviour, but not with all traits investigated (Tait and Naug, 2020). A second reason that hints to a non-existing correlation is the distribution of individual *LIs* in the STI and LTI control groups (Figure 12 Di and Dii). The broad distribution of *LIs* for the STI animals was to be expected from previous experiments (Figure 9). But if the lack of agonistic encounters and the resulting homogeneity in aggression in the LTI animals (compare first fights in STI and LTI animals Figures 11B and C) would also have the same influence on learning, the distribution of individual *LIs* would be also more homogeneous and not as widespread. Even if individuality in learning is present in insects (Pamir et al., 2011; Arican et al., 2020) and the rearing environment can have influences on the formation of long-term memory (in *Lymnea stagnalis*: Rothwell et al., 2018), the aggressive state does not seem to influence individual learning. It appears to be the direct effect of concentrated, highly stressful agonistic experience that strongly influences learning in individual animals. What is observed here could be the natural range of good and bad learners and learning is not influenced by previous interactions (< 48 h) or learning defects that are caused from agonistic experiences are erased after the isolation time, which was also thought of for aggression and other related behaviours (Stevenson and Rillich, 2013), but new evidence show that 48 h isolated animals still differ in aspects of their behaviour

(Rose et al., 2017b; Balsam and Stevenson, 2021). It needs to be investigated if animals taken directly from the breeding colony show a correlation between aggression and learning indices. On a side note, the overall examination of all groups sorted into animals with negative *LIs*, zero *LIs* and positive *LIs* reveals the benefit and the general quality of the use of the semi-automated analysis of a conditioned response using a binary logistic regression model in a differential appetitive paradigm (Figure 14). There is a consistency in the data for the controls for all of the animals achieved that can be detected by the model. STI, LTI animals with no defeat (3 groups; Figure 14) and no defeat with vehicle treatment groups (1 group; Figure 20B) show consistent distributions of individuals in the $LI < (-0.05)$ group: 5-15%; the $(-0.05) \leq LI \leq 0.05$ group: 15-25% and the $LI > 0.05$ group: 65-75%.

4.3 The role of serotonin in chronic social defeat influenced learning

Serotonin is known to be an important neuromodulator involved in the regulation of aggression in crickets and maintaining reduced aggressiveness after defeat (Rillich and Stevenson, 2018; Stevenson and Rillich, 2019). It is also known that serotonin plays important roles in multiple behavioural functions including learning and memory (Review: Bacqué-Cazenave et al., 2020) and it was therefore hypothesised that the impairment of learning capacity in crickets after chronic social defeat is influenced by serotonin. From earlier studies it became clear that in crickets the effect on aggression is mediated *via* 5HT₂ receptors (Rillich and Stevenson, 2018). Here the serotonergic drugs methiothepin, ketanserin and fluoxetine were used to confirm the impact on aggression and investigate the influence of serotonin on impaired learning after chronic defeat.

Therefore, the animals were subjected to chronic defeat before injecting methiothepin, ketanserin or the vehicle, conditioning and measuring of their aggressive potential (Figure 15A). The animals did not differ in level of aggression and duration at the first match (Figure 15B), but after being subjected to chronic defeat, animals of the vehicle as well as the methiothepin treated group showed a significantly reduced aggression (Figure 15C). However, the animals treated with ketanserin recovered and fought at a significantly higher level and with a significantly higher persistence in comparison to the vehicle group (Figure 15C). The same was observed in a previous study analysing the role of serotonin in defeat stress (Rillich and Stevenson, 2018). This difference in the effect of both 5HT receptor blockers is proposed to be based on the differing affinities to various subtypes of 5HT receptors. In insects, multiple subtypes of 5HT receptors were identified (Vleugels et al., 2015) and in crickets two 5HT₁, two 5HT₂ and a 5HT₇ receptor are known (Watanabe et al., 2011; Watanabe and Aonuma, 2012).

Methiothepin is considered as an unspecific 5HT receptor blocker with differing affinities to the subtypes (Vleugels et al., 2015) and ketanserin is known to block 5HT₂ receptors in *Drosophila* (Johnson et al., 2011). In honey bees methiothepin has no inhibitory effect on 5HT_{2β} receptors and an inhibitory effect on 5HT_{2α} receptors, whereas it is *vice versa* for ketanserin (Thamm et al., 2013). Therefore, for crickets it is assumed that methiothepin blocks all 5HT receptor subtypes except 5HT₂ and ketanserin only blocks 5HT₂ receptors (Rillich and Stevenson, 2018). Since fluoxetine, a 5HT reuptake inhibitor (Corey et al., 1994), is blocking the removal of serotonin after its release through defeat it can additionally reveal the effects of serotonin on aggression (Rillich and Stevenson, 2018). This was tested by injecting the animals with fluoxetine and then subjecting them to non-chronic defeat stress (2 defeats) before conditioning and evaluating the aggression afterwards in a test match (Figure 18A). In the first match after drug injection level and persistence of vehicle and fluoxetine injected animals did not differ (Figure 18B), but confirming previous data (Rillich and Stevenson, 2018) at the test match after just two defeats, a pause of 24 h and conditioning, the fluoxetine treated animals behave submissively with a significantly lower level of aggression and match duration in comparison to the control animals (Figure 18C). For the control group the loser effect of reduced aggression after non-chronic defeat does not last 24 h and the animals are aggressive again.

Chronic defeat subjected and vehicle injected animals also showed impaired learning (Figure 16), comparable to the submissive animals in Figure 12, confirming that the vehicle has no influence on learning. Corresponding to the results regarding the aggression (Figure 15), the methiothepin injected animals are also impacted by chronic defeat stress and their learning is impaired. Both groups showed low *LIs* (vehicle: 0.077, methiothepin: 0; Table 12, Figure 16D). Interestingly, the effect of ketanserin on the aggressiveness after chronic defeat can also be observed in the learning data. The group of animals injected with ketanserin after chronic defeat stress exhibited significantly higher *LIs* compared to the vehicle group ($p = 0.0013$; Table 12, Figure 16D), especially the responses to the CS⁺ are high (Figure 16C). This means that not only the maintenance of reduced aggressiveness after defeat, especially chronic defeat stress, is mediated by serotonin via 5HT₂ receptors (Rillich and Stevenson, 2018), but also the associated impairment of learning is influenced thereby. The influence of serotonin on the impairment of learning was also confirmed by the administration of fluoxetine with remarkable results. The injection of the vehicle followed by non-chronic defeat basically resulted in low P_{resp} values to the CS[°] and high P_{resp} values to the CS⁺ with mainly positive *LIs* (median: 0.256) and did not influence the learning ability of the animals (Table 14, Figure 19). When fluoxetine was

injected, the animals subjected to non-chronic defeat were severely impaired in their ability to learn the odours. The maximum of 70% of the crickets scored a negative *LI* with the lowest individual value at *LI* -0.99 (Table 14, Figure 19 and 20). This confirms the effect serotonin has on learning after chronic defeat. To exclude any influences on learning of the drugs alone, multiple groups of animals were not subjected to chronic defeat and then injected with the respective drug (methiothepin, ketanserin, fluoxetine). All of these groups show a normal learning performance and distribution of individual *LIs* and no difference to vehicle (Table 13 and 14, Figure 17 and 19). Therefore, it could be ruled out that the drugs alone alter learning capacity, which had also been reported for aggression (Rillich and Stevenson, 2018). The findings of this thesis lead to the result that serotonin is significantly involved in crickets being not able to perform in a differential conditioning paradigm after being subjected to chronic social defeat stress.

This study provides evidence that serotonin is impairing learning in chronic defeat stress. In honey bees the treatment with 5HT impairs visual learning and retention (Mancini et al., 2018) and leads to decreased response levels in classical olfactory conditioning (Menzel et al., 1999). In *Drosophila*, multiple serotonin receptors have been shown to be involved in olfactory learning (Johnson et al., 2011) and the importance for serotonin in learning was revealed (Sitaraman et al., 2008; Sitaraman et al., 2017; Ganguly et al., 2020). In general serotonin is shown to be mainly important for aversive olfactory learning and especially salt learning (Huser et al., 2017). Interestingly, a pair of serotonergic neurons is thought to act as a switch for long-term memory formation (Scheunemann et al., 2018). But because of the different receptor subtypes (Vleugels et al., 2015), it is difficult to compare the effects of serotonin between animals. It had also been shown that activation of subsets of serotonergic neurons differs to a global stimulation (Xu et al., 2016; Kasture et al., 2018).

In vertebrates, serotonin is known to mediate the impact of stress on multiple behaviours (Natarajan et al., 2017; de Abreu et al., 2020), but it had been shown that deficits in learning after stress are cause of a reduced impact of serotonin (Lapiz-Bluhm et al., 2009; Natarajan et al., 2017). Serotonin is also linked to learning in mice (Grossman et al., 2021), but seems to have a learning enhancing effect (Iigaya et al., 2018), whereas it is mainly thought to be involved in modulating aversive signals (Cools et al., 2007). Even prenatal stress can induce brain dysfunction which can be partially restored by a selective serotonin reuptake inhibitor (Ishiwata et al., 2005). But generally speaking, the modulatory effects of serotonin on various behaviours are not always clear as they differ between species, receptor subtypes and the behaviours considered with often conflictive influences in learning and memory (Štrac et al.,

2016). Therefore, comparisons to other animals are very difficult. It is important to further study the influence of serotonin specifically in crickets and also consider that other neuromodulators that definitely play an important role in the aggression of crickets, like octopamine, dopamine and nitric oxide (Stevenson and Rillich, 2019) could potentially add to influences on learning of crickets after chronic defeat stress. For example, NO in *Manduca sexta* is known to affect short-term memory (Gage et al., 2013) and in crickets can rescue age-related memory impairment (Matsumoto et al., 2016). Dopamine and octopamine are known to mediate aversive and appetitive learning (Unoki et al., 2005; Matsumoto et al., 2015; Mizunami and Matsumoto, 2010, 2017; Sabandal et al., 2020).

4.4 Overall conclusion and outlook

In this thesis learning in individual crickets is successfully quantified by using video tracking of the conditioned response and binary logistic regression modelling. Besides multiple models tested, the resulting model, which is based on three behavioural variables, could be successfully tested, validated and proved to consistently measure learning in comparable groups of animals. Therefore, a new method to quantified individual learning scores based on a multi-variable semi-automated analysis of conditioned responses in a differential appetitive olfactory paradigm in crickets is now available. Its application reveals, besides the general learning capacity of crickets, that an untreated STI group of animals shows a bimodal distribution representing animals that learned to discriminate between the odours and those that did not. A question that remains to be answered is if these differences are consistent over time and multiple different learning experiments indicating individuality in learning.

The application of the model revealed that chronic social defeat, which is influencing multiple aspects of a cricket's behaviour (Rose et al., 2017b; Balsam and Stevenson, 2020, 2021) and is a model for depression in mammals (Hollis and Kabbaj et al., 2014), in crickets also strongly impairs the animals' ability to learn in a differential olfactory conditioning paradigm for both LTI and STI. Despite the hypothesis that because of differences in homogeneity regarding the initial aggression of STI and LTI and the bimodal distribution of an untreated animal group in learning capacity, aggressive potential and learning index are correlated, the results suggest that the impairment of learning after chronic social defeat rather is a concrete consequence of this experience than being correlated to the general aggressive state of the individuals. Even if learning is impaired in both groups in this work, it is advised to differentiate between animals less and more susceptible to social subjugation when regarding aggression data to not oversee differing effects in additionally examined behaviours. For future experiments it would be very

interesting to examine how long the impairment of learning is present after chronic defeat and if the animals will be able to recover and if this is influenced by the aggressive state of the animals. It is also known that flight restores the aggressiveness of crickets after defeat (Hofmann and Stevenson, 2000) and the effect of flight on the impairment of learning could be tested. Furthermore, this work shows that the impairment of learning is modulated by serotonin with an involvement of 5HT₂ receptors. But the general effect of serotonin on learning and memory without chronic defeat stress has to be investigated still. As the serotonergic effects between animals and receptor subtypes are often contradicting, additional serotonergic drugs have to be tested. It is also not clear if several other neuromodulators, like octopamine, nitric oxide and dopamine, that are known to be involved in aggression and also play a role in invertebrate learning are influencing learning after chronic social defeat.

5 SUMMARY

The ability to learn, which involves changing future behaviour based on past experiences, is crucial for the survival and life of animals. Through the years, insects have been proven to have extraordinary abilities in this field ranging from simple associative learning to complex cognitive abilities (review: Giurfa, 2013) and because of an easily accessible nervous system they have become important for understanding the neuronal basis of behaviour. Research has shown that animals exhibit consistent inter-individual differences in behaviour with correlations of specific traits that can be defined as a behavioural syndrome (review: Sih and Bel, 2008; Wilson et al., 2019). In honey bees the learning behaviour of individuals differs from that of the group (Pamir et al., 2011) and two cognitive phenotypes, fast and slow bees that differ in associative learning and other traits, have been found (Tait and Naug, 2020). In crickets it became evident, that multiple agonistic experiences between conspecifics in the form of chronic social defeat forge inter-individual differences and influence future aggression (Rose et al., 2017b; Rillich and Stevenson, 2018; Balsam and Stevenson, 2021). This social subjugation in mammals is proposed as a model for depression (Hollis and Kabbaj et al., 2014) and induces depression-like symptoms (Carnevali et al., 2012) to the extent that it disrupts reward learning (Der-Avikan et al., 2017). Crickets act as a model system for both, mechanisms of aggression and the underlying neuromodulator systems (review: Stevenson and Rillich, 2019), as well as learning (Mizunami et al., 2013; Mizunami and Matsumoto, 2017). This thesis hypothesised that chronic social defeat stress influences learning and that the aggressive state of an individual gathered from early life agonistic experiences is an underlying cause of inter-individual differences in learning abilities in *Gryllus bimaculatus*.

In order to be able to investigate this, a method to measure the learning capacity of individual crickets, which is suitable for future application in social subjugation experiments, had to be established. In earlier studies on crickets, learning was measured in form of a preference index which was calculated from the time spent at each of two odour sites in an appetitive-aversive paradigm (Matsumoto and Mizunami, 2002), or based on the percentage of animals that exhibit the maxillary palpi extension response in response to conditioned odours (Matsumoto et al., 2015). Observations of individual crickets that were trained in an appetitive classical conditioning paradigm to associate an odour to a sugar water reward, revealed that they exhibit a complex behaviour as conditioned response to an odour involving searching behaviour in proximity to the odour, antennal waving and characteristic bobbing movements of the head. The goal for measuring learning was to build a binary logistic regression model that can identify

and quantify this response. Comparing individual video tracks of the odour response of 50 animals that were conditioned to an odour by rewarding it with sugar water in the training with tracks of 50 animals that were presented with the odour in the recall test after only being rewarded (without odour) in the training revealed eight variables that potentially specifically could describe the conditioned response deriving from paired conditioning. In comparison to the animals that were only given the reward, crickets that were conditioned to the odour start to move earlier and faster, walk a greater distance and a longer period of time. They move in closer vicinity to the odour application zone and area and exhibit bobbing behaviour for a longer period of time. The initial distance the animals walk does not differ between the two groups. With these variables multiple models containing different combinations of three or four of the variables could be calculated using binary logistic regression analysis. Analysing and comparing the models employing multiple statistical tests favoured one. The most parsimonious model includes the variables *time to move* (s), *total time moving* (s) and *bobbing* (s) and can discriminate between animals that were only presented with the reward and the odour plus reward conditioned animals with a 93% accuracy, whereas the other models were rejected. The test of the model using out-model animals with known training history (four training groups: odour only; reward only; odour and reward without temporal proximity; odour and reward temporally paired) confirmed it to be suitable to also describe unknown data. After extracting the behavioural variables from video tracks and application of the binary logistic regression, the probability of an individual exhibiting a conditioned response can be determined (P_{resp}). A P_{resp} of 0 means that the animal did not respond to the odour and an allocation to the reward only group, whereas a P_{resp} of 1 means that the animal exhibited a conditioned response. The cut-off value for the classification is 0.5.

In order to test the model for further application, individuals were trained in a differential appetitive conditioning paradigm, in which one of two odours is rewarded in the training, the other not. This reveals that the animals generally exhibit low P_{resp} values to the unrewarded odour and high P_{resp} values to the rewarded odour. From this a learning index (*LI*) for each individual could be calculated from the difference of both values, that revealed that this group of animals showed learning with a high median *LI* (0.395). Furthermore, sequential effects of odour and reward presentation were controlled for and are not present in this paradigm. The examination of the distribution of individual *LIs* on the other hand revealed that together with the animals scoring very high *LIs* the same percentage of animals (20%) score *LIs* around zero which resembles a binary distribution with these two modes representing animals that have and have not learned to discriminate between the odours. Applying the model for measuring

learning proves superior to a single metric of a conditioned response (in this case *bobbing*), as is often used in learning experiments of bees or other insects (Bitterman et al., 1983; Arican et al., 2020). With this, the calculation of individual learning scores in crickets based on a multi-variable, semi-automated analysis of a conditioned response is established for use in further experiments.

Learning in insects is known to be influenced by various factors, like for example age (Matsumoto et al., 2016) and pollution (Leonard et al., 2019). Crickets are known to exhibit a well-studied stereotypic fighting behaviour which can be assessed using the level of aggression (1-6, increasingly aggressive) and the duration of the fight (Hofmann and Stevenson, 2000; Stevenson et al., 2000) and a long-term depression of aggression is the result of chronic social defeat stress (Rose et al., 2017b). To test the hypothesis that multiple agonistic experiences affect learning abilities in crickets and additionally influence crickets raised under variable conditions differently, short term isolated (STI, socially isolated for 48 h) and long term isolated (LTI, isolated as last nymphal instar) animals were subjected to chronic social defeat stress. Therefore, they were repeatedly defeated by a hyper-aggressive opponent, then trained in the differential olfactory conditioning paradigm and their learning capacity and aggressiveness assessed afterwards. This showed that not all animals are affected by social subjugation the same way, as some are aggressive, and some are submissive at the test match after applying the experimental paradigm. As known from previous experiments LTI are not as susceptible to chronic defeat (Rose et al., 2017b) and therefore 50% of the animals are submissive whereas in the STI group two third of the animals were submissive. Interestingly, sorting them retrospectively into the respective groups for the first fight of the chronic defeat paradigm revealed, that STI animals already differ in their aggressiveness, meaning that animals that are submissive in the test fight are already more submissive in the first fight and the aggressive animals more aggressive, respectively. LTI crickets do not differ in the aggression in the first fight. This resembles the influence of differing agonistic experiences with conspecifics the animals had (Balsam and Stevenson, 2021).

The analysis of the learning data reveals that STI animals that were submissive after chronic defeat are significantly impaired in their learning ability compared to animals that did not experience defeat. They do not differentiate between the rewarded and the unrewarded odour (45%), or score higher P_{resp} values to the unrewarded odour, which leads to negative *LIs* (45%). This is partly to the extent that they show a conditioned-like response to the unrewarded odour, even with bobbing behaviour. It can be observed that the animals that are aggressive in the test match are also impaired in their learning ability. Interestingly, for the LTI animals these results

are also true as both aggressive and submissive animals exhibit significantly lower *LIs* compared to animals that did not experience chronic defeat. To further confirm that this effect is caused by the loss of multiple fights and not by the experience of fighting or contacting a conspecific or handling within the experimental procedure alone, a new group of animals that won multiple fights was tested in the paradigm. This revealed that multiple winners show normal learning and no differences in *LIs* compared to the control animals that did not experience wins. Moreover, none of the multiple winners scored a negative *LI*, which 5 – 10 % of animals without experiences did. Furthermore, multiple winners show a winner effect in aggression, as they fight at a significantly higher level in the test match 4.5 h after the last win although the known effects of increased aggressiveness after winning are only lasting less than 20 min (Rillich and Stevenson, 2011). To test if inter-individual differences in learning abilities are correlated to the aggressive state of an individual, the level of aggression and the duration of the match (first match; test match) of animals that did not experience defeat and those that experienced chronic defeat (aggressive and submissive) were correlated with the *LIs*. This revealed no significant correlations and contrary to the aggression hints to no measurable influence of agonistic experiences prior to the experiment on learning.

Together this shows that the acute experience of chronic social defeat stress has a major influence on the learning abilities. The cause of general inter-individual differences in learning abilities without the influence of chronic defeat has to be investigated further in future studies. It should be mentioned that the advantage of using the binary logistic regression model for measuring learning in crickets is evident in these applied experiments. For instance, the contingency of animals scoring negative, zero and positive *LIs* is very constant for the three control groups that experienced no defeat.

Various neuromodulators are influencing aggression and learning (Mizunami and Matsumoto, 2017; Stevenson and Rillich, 2019), especially serotonin (5HT) is known to be released after the loss of a fight and mediates the maintenance of depressed aggressiveness after chronic social defeat mainly via 5HT₂ receptors (Rillich and Stevenson, 2018; Stevenson and Rillich, 2019). Therefore, it was hypothesised that the detrimental effects of chronic social defeat on learning are influenced by serotonin. First, the effects of the serotonergic drugs methiothepin, an unselective 5HT receptor blocker, ketanserin, a 5HT₂ receptor blocker (Vleugels et al., 2015) and fluoxetine, a 5HT re-uptake inhibitor, on aggression in the chronic defeat paradigm could be confirmed (Rillich and Stevenson, 2018). The injection of methiothepin 1 h after chronic defeat has no effect on the reduced aggressiveness, whereas after the injection of ketanserin the animals are aggressive again. The injection of fluoxetine reveals that non-chronic defeat, i.e.

two defeats, also induces reduced aggressiveness. The analysis of the corresponding learning data revealed the injection of methiothepin does not influence the chronic defeat induced impairment of learning, as the animals still show no learning or negative *LIs* with no difference to the vehicle control. Ketanserin treated animals on the other hand show significantly higher learning scores compared to the vehicle control, resembling a normal learning capacity. From an additional experiment in which animals were not subjected to chronic defeat and injected afterwards with methiothepin or ketanserin it became evident, that the used drugs alone do not influence the learning abilities. The role of serotonin could be further confirmed by injecting the animals with fluoxetine prior to non-chronic defeat. In this experiment only two defeats induce a strong impairment in learning compared to the vehicle control group that showed normal learning scores. The injection of fluoxetine alone does not have an effect on learning. Together this indicates a strong influence of serotonin on the learning in crickets after chronic social defeat mainly via the 5HT₂ receptors, which manifests itself by an impairment of learning abilities. Future studies need to investigate the effects of other neuromodulators known to be involved in aggression and learning and memory in crickets, like octopamine, dopamine and nitric oxide, that may also take part in orchestrating the effects of chronic social defeat stress on learning.

6 ZUSAMMENFASSUNG

Die Fähigkeit zu lernen, d. h. künftiges Verhalten auf der Grundlage früherer Erfahrungen anzupassen, ist entscheidend für das Leben und Überleben von Tieren. Im Laufe der Jahre hat sich gezeigt, dass Insekten in diesem Bereich über außergewöhnliche Fähigkeiten verfügen, die von einfachem assoziativen Lernen bis hin zu komplexen kognitiven Fähigkeiten reichen (Giurfa, 2013), und auf Grund ihres leicht zugänglichen Nervensystems sind sie für das Verständnis der neuronalen Grundlagen des Verhaltens wichtig geworden. Die Forschung hat gezeigt, dass Tiere konsistente interindividuelle Verhaltensunterschiede mit Korrelationen bestimmter Merkmale aufweisen, die als Verhaltenssyndrom definiert werden können (Sih und Bel, 2008; Wilson et al., 2019). Bei Honigbienen wurde gezeigt, dass sich das Lernverhalten von Individuen von dem der Gruppe unterscheidet (Pamir et al., 2011) und es konnten zwei kognitive Phänotypen, schnelle und langsame Bienen, nachgewiesen werden, die im assoziativen Lernen und anderen Merkmalen differieren (Tait und Naug, 2020). Bei Grillen hat sich gezeigt, dass mehrfache agonistische Erfahrungen zwischen Artgenossen in Form von chronischen sozialen Niederlagen interindividuelle Unterschiede begründen und die zukünftige Aggression der Tiere beeinflussen (Rose et al., 2017b; Rillich und Stevenson, 2018; Balsam und Stevenson, 2021). Diese soziale Unterwerfung wird bei Säugetieren als Modell für die Erforschung von Depressionen vorgeschlagen (Hollis und Kabbaj et al., 2014) und induziert depressionsähnliche Symptome (Carnevali et al., 2012) in dem Maße, dass das Belohnungslernen gestört werden kann (Der-Avikan et al., 2017). Grillen dienen als Modellsystem sowohl für die Mechanismen der Aggression und den zugrundeliegenden Neuromodulatorsystemen (Stevenson und Rillich, 2019) als auch für das Lernen (Mizunami et al., 2013; Mizunami und Matsumoto, 2017). In dieser Arbeit wurde die Hypothese aufgestellt, dass chronisches Verlieren gegen einen Artgenossen das Lernen beeinflusst und dass die Aggressivität eines Individuums, welche sich aus früheren agonistischen Erfahrungen ergibt, eine Ursache für interindividuelle Unterschiede in der Lernfähigkeit von *Gryllus bimaculatus* ist.

Um dies untersuchen zu können, musste eine Methode zur Messung der Lernfähigkeit einzelner Grillen etabliert werden, die für eine zukünftige Anwendung in Kampfexperimenten geeignet ist. Die Lernfähigkeit von Grillen wurde in früheren Studien in Form eines Präferenzindex gemessen, der aus der Zeit berechnet wurde, die die Tiere an jedem der Standpunkte zweier Düfte in einem appetitiv-aversiven Paradigma verbracht haben (Matsumoto und Mizunami, 2002), oder auf der Grundlage des Anteils der Tiere einer Gruppe, die als Reaktion auf einen

konditionierten Duft die Maxillarpalpen ausstrecken (Matsumoto et al., 2015). Beobachtungen einzelner Grillen, die in der klassischen appetitiven Konditionierung darauf trainiert wurden, einen Duft mit einer Belohnung in Form von Zuckerwasser zu assoziieren, ergaben, dass sie als konditionierte Reaktion auf einen Duft ein komplexes Verhalten zeigen. Dieses umfasst Suchverhalten in der Nähe des Duftes, Wedeln mit den Antennen und die charakteristische Auf- und Ab-Bewegungen des Kopfes (bobbing). Das Ziel zum Erfassen des Lernens war ein binäres logistisches Regressionsmodell zu berechnen, mit dem diese Reaktion identifiziert und quantifiziert werden kann. Der Vergleich einzelner *Video-Tracking* Analysen der Reaktion auf den Duft von 50 Tieren die im Training konditioniert wurden, indem sie nach der Präsentation des Duftes mit Zuckerwasser belohnt wurden, mit den Spuren von 50 Tieren, denen der Duft im Test präsentiert wurde, nachdem sie im Training nur belohnt worden waren (ohne Duft), ergab acht Variablen, die potenziell spezifisch die konditionierte Reaktion beschreiben. Im Vergleich zu den Tieren, die nur belohnt wurden, starten die konditionierten Grillen früher mit der Bewegung, bewegen sich schneller, laufen über eine größere Strecke und eine längere Zeitspanne. Sie bewegen sich mehr im Bereich, in dem der Duft appliziert wurde und zeigen über einen längeren Zeitraum bobbing-Verhalten. Die Distanz, die die Tiere am Anfang der Aufnahme zurücklegen, unterscheidet sich nicht zwischen den beiden Gruppen. Mit diesen Variablen konnten mit Hilfe der binären logistischen Regressionsanalyse mehrere Modelle, welche verschiedenen Kombinationen von drei oder vier der Variablen einschließen, berechnet werden. Ein Modell wurde nach der Analyse und dem Vergleich der Modelle durch mehrere statistische Tests bevorzugt. Das bevorzugte, einfachste Modell enthält die Variablen *Zeit bis die Bewegung startet* (s), *gesamte Bewegungszeit* (s) und *bobbing-Verhalten* (s) und kann mit einer Genauigkeit von 93 % zwischen Tieren, denen nur die Belohnung präsentiert wurde, und Tieren, die mit Duft und Belohnung konditioniert wurden, unterscheiden. Die anderen Modelle wurden verworfen. Der Test des Modells anhand von Tieren deren Daten nicht zur Berechnung dessen genutzt wurden und welche einen bekannten Trainingsablauf hatten (vier Trainingsgruppen: nur Duft; nur Belohnung; Duft und Belohnung, präsentiert ohne zeitliche Nähe; Duft und Belohnung zeitlich gepaart) bestätigt, dass das Modell geeignet ist, auch unbekannte Daten zu beschreiben. Nach Extraktion der Verhaltensvariablen aus den Videos und Anwendung des binären logistischen Regressionsmodells kann die Wahrscheinlichkeit bestimmt werden, dass ein Individuum eine konditionierte Reaktion zeigt (P_{resp}). Eine P_{resp} von 0 bedeutet, dass das Tier nicht auf den Duft reagiert hat und der Gruppe zugeordnet wird, die nur die Belohnung präsentiert bekommen hatte, während ein P_{resp} von 1 bedeutet, dass das Tier eine konditionierte Reaktion gezeigt hat. Der Grenzwert für diese Klassifizierung ist 0,5.

Um das Modell für weitere Anwendungen zu testen, wurden die Tiere in einem differenziellen appetitiven Konditionierungsparadigma trainiert, bei dem einer von zwei Düften im Training belohnt wurde, der andere nicht. Dabei zeigte sich, dass die Tiere im Allgemeinen niedrige P_{resp} -Werte für den unbelohnten und hohe P_{resp} -Werte für den belohnten Duft aufweisen. Aus der Differenz dieser Werte konnte ein Lernindex (LI) für jedes Individuum berechnet werden, welcher aufzeigte, dass diese Gruppe von Tieren mit einem hohen medianen LI (0,395) lernfähig ist. Darüber hinaus wurden mögliche sequentielle Effekte der Duft- und Belohnungspräsentation kontrolliert und es wurde gezeigt, dass diese in diesem Paradigma nicht vorhanden sind. Die Analyse der Verteilung der individuellen LIs zeigte andererseits, dass zusätzlich zu den Tieren, die sehr hohe LIs erzielen, derselbe Prozentsatz (20%) LIs um Null erzielt, was insgesamt einer binären Verteilung ähnelt. Dabei repräsentieren diese beiden Modi die Tiere, die gelernt haben zwischen den Gerüchen zu unterscheiden, und die, die nicht gelernt haben. Die Nutzung des Modells zur Messung von Lernen erweist sich als besser als ein einzelnes Merkmal einer konditionierten Reaktion (in diesem Fall *bobbing*-Verhalten), wie es häufig in Lernexperimenten bei Bienen oder anderen Insekten verwendet wird (Bitterman et al., 1983; Arican et al., 2020). Somit konnte die Berechnung individueller Messwerte des Lernens für Grillen auf der Grundlage einer multivariablen, halbautomatischen Analyse einer konditionierten Reaktion zur Verwendung in weiterführenden Experimenten etabliert werden. Es ist bekannt, dass die Lernfähigkeit von Insekten durch verschiedene Faktoren beeinflusst werden kann, wie z.B. Alter (Matsumoto et al., 2016) und Luftverschmutzung (Leonard et al., 2019). Grillen zeigen ein gut untersuchtes, stereotypes Kampfverhalten, welches anhand von Aggressionsstufen (1-6, zunehmend aggressiv) und der Dauer des Kampfes quantifiziert werden kann (Hofmann und Stevenson, 2000; Stevenson et al., 2000). Eine langfristige Reduktion der Aggression ist das Ergebnis von chronischem Stress durch mehrfache Niederlagen (Rose et al., 2017b). Um die Hypothese zu testen, dass sich mehrfache agonistische Erfahrungen auf die Lernfähigkeit von Grillen auswirken und darüber hinaus Tiere die unter variablen Bedingungen aufgezogen wurden unterschiedlich beeinflussen, wurden kurzzeit-isolierte (STI, sozial isoliert für 48 h) und langzeit-isolierte (LTI, isoliert während des letzten Nymphenstadiums) Tiere Stress durch chronische Niederlagen ausgesetzt. Dazu wurden sie wiederholt von einem hyperaggressiven Gegner besiegt, dann in dem differenziellen olfaktorischen Konditionierungsparadigma trainiert und anschließend ihre Lernfähigkeit und Aggressivität analysiert. Dabei zeigte sich, dass nicht alle Tiere in gleicher Weise von der Unterwerfung betroffen sind, da einige nach der Durchführung des Experiments aggressiv und andere submissiv sind. Wie aus früheren Experimenten bekannt ist, sind LTI nicht so

empfindlich für die Auswirkungen chronischer Niederlagen (Rose et al., 2017b) und daher sind in dieser Gruppe 50% der Tiere submissiv, während in der STI Gruppe zwei Drittel der Tiere submissiv sind. Interessanterweise zeigt die nachträgliche Einteilung der Daten für den ersten Kampf der multiplen Niederlagen in die jeweiligen Gruppen, dass sich die STI Tiere bereits zu diesem Zeitpunkt in ihrer Aggressivität unterscheiden, d.h. Tiere, die im Testkampf submissiv sind, sind bereits im ersten Kampf unterwürfiger und die aggressiven Tiere aggressiver. LTI Grillen unterscheiden sich im ersten Kampf nicht in ihrer Aggression. Dies ist das Ergebnis des Einflusses unterschiedlicher früherer agonistischer Erfahrungen der Tiere mit Artgenossen (Balsam und Stevenson, 2021).

Die Analyse der Lerndaten zeigt, dass STI Tiere, die nach chronischen Niederlagen submissiv sind, im Vergleich zu Tieren die keine Niederlage erlitten haben, in ihrer Lernfähigkeit erheblich beeinträchtigt sind. Sie können nicht zwischen dem belohnten und dem unbelohnten Duft unterscheiden (45%) oder zeigen höhere P_{resp} -Werte für den unbelohnten Duft, was in negativen LIs resultiert (45%). Das liegt zum Teil daran, dass sie ein Verhalten zum unbelohnten Duft zeigen, das der konditionierten Reaktion ähnelt, inklusive bobbing-Verhalten. Zusätzlich konnte beobachtet werden, dass die im Testkampf aggressiven Tiere auch in ihrer Lernfähigkeit beeinträchtigt sind. Interessanterweise treffen diese Ergebnisse auch auf die LTI Tiere zu, da sowohl aggressive als auch submissive Tiere im Vergleich zu Tieren, die keine chronischen Niederlagen erlitten haben, deutlich niedrigere LIs aufweisen. Um weiterhin zu bestätigen, dass dieser Effekt durch die Erfahrung des Verlierens mehrerer Kämpfe und nicht durch die Erfahrung des Kampfes an sich, durch den Kontakt mit einem Artgenossen oder der Handhabung innerhalb des Experiments verursacht wird, wurde eine neue Gruppe von Tieren in dem Paradigma getestet welche mehrere Kämpfe gewonnen hatten. Dabei zeigte sich, dass mehrfache Gewinner normal lernen und die LIs im Vergleich zu den Kontrolltieren nicht unterschiedlich sind. Darüber hinaus erzielte keiner der mehrfachen Gewinner einen negativen LI , was normalerweise bei 5 - 10 % der Tiere ohne antagonistische Erfahrungen der Fall war. Mehrfache Gewinner zeigen allerdings einen Gewinner-Effekt in der Aggression, da sie 4,5 Stunden nach dem letzten Sieg im Testkampf auf einem signifikant höheren Level kämpfen, obwohl die bekannten Effekte der gesteigerten Aggressivität nach einem Sieg nur weniger als 20 Minuten anhalten (Rillich und Stevenson, 2011). Um zu testen, ob interindividuelle Unterschiede in der Lernfähigkeit mit der Aggressivität eines Individuums korreliert sind, wurden das Aggressionslevel und die Dauer des Kampfes (erster Kampf; Testkampf) von Tieren, die keine Niederlage erlebt hatten und denen die eine chronische Niederlage erlebt hatten (aggressiv und submissiv) mit den LIs korreliert. Es ergaben sich keine signifikanten

Korrelationen und im Gegensatz zur Aggression kein messbarer Einfluss agonistischer Erfahrungen vor dem Experiment auf die Lernfähigkeit.

Insgesamt zeigt dies, dass die akute Erfahrung von chronischem Stress durch multiple Niederlagen einen großen Einfluss auf die Lernfähigkeit hat. Die Ursache für generelle interindividuelle Unterschiede in der Lernfähigkeit ohne den Einfluss der chronischen Niederlage muss in zukünftigen Studien weiter untersucht werden. Es sollte erwähnt werden, dass auch in der weiteren Anwendung der Vorteil des binären logistischen Regressionsmodells zur Messung des Lernens bei Grillen offenbart wird. Zum Beispiel ist die Kontingenz der Tiere, die negative, positive und *LIs* um null erzielen für die drei Kontrollgruppen, die keine Niederlage erfahren haben, sehr konstant.

Die Aggression und das Lernen können durch verschiedene Neuromodulatoren beeinflusst werden (Mizunami und Matsumoto, 2017; Stevenson und Rillich, 2019). Es ist bekannt, dass insbesondere Serotonin (5HT) nach der Erfahrung des Verlierens eines Kampfes freigesetzt wird und die Aufrechterhaltung der Reduktion der Aggressivität nach chronischen Niederlagen hauptsächlich über 5HT₂-Rezeptoren vermittelt (Rillich und Stevenson, 2018; Stevenson und Rillich, 2019). Daher wurde die Hypothese aufgestellt, dass die nachteiligen Auswirkungen chronischer Niederlagen auf das Lernen durch Serotonin beeinflusst wird. Zunächst konnte die Wirkung der serotonergen Wirkstoffe Methiothepin, ein unselektiver 5HT-Rezeptorblocker, Ketanserin, ein 5HT₂-Rezeptorblocker (Vleugels et al., 2015) und Fluoxetin, ein 5HT-Wiederaufnahmehemmer, auf die Aggression nach chronischen Niederlagen bestätigt werden (Rillich und Stevenson, 2018). Die Injektion von Methiothepin eine Stunde nach den mehrfachen Niederlagen hat keinen Einfluss auf die Reduktion der Aggressivität, während hingegen die Tiere nach der Injektion von Ketanserin wieder aggressiv sind. Die Injektion von Fluoxetin zeigt, dass auch zwei aufeinanderfolgende nicht-chronische Niederlagen die Aggressivität reduzieren. Die Analyse der entsprechenden Lerndaten ergab, dass die Injektion von Methiothepin keinen Einfluss auf die Beeinträchtigung des Lernvermögens hat welche durch chronische Niederlagen induzierte wurde, da die Tiere nach wie vor kein Lernen oder negative *LIs* zeigen und sich nicht von der Kontrolle (Vehikel) unterscheiden. Mit Ketanserin behandelte Tiere hingegen zeigen im Vergleich zur Kontrolle signifikant höhere *LIs*, was auf eine normale Lernfähigkeit hindeutet. Durch ein zusätzliches Experiment, bei dem die Tiere keiner Kampferfahrung ausgesetzt wurden und anschließend Methiothepin oder Ketanserin injiziert bekamen, geht hervor, dass die verwendeten Wirkstoffe allein keinen Einfluss auf die Lernfähigkeit haben. Die Rolle von Serotonin konnte bestätigt werden, indem den Tieren vor zwei Niederlagen (nicht chronisch) Fluoxetin injiziert wurde. In diesem Experiment führen nur

diese zwei Niederlagen zu einer starken Beeinträchtigung der Lernfähigkeit im Vergleich zur Kontrollgruppe, die normale Lernwerte zeigt. Die Injektion von Fluoxetin allein hat allerdings keinen Einfluss auf das Lernen. Insgesamt deutet dies auf einen starken Einfluss von Serotonin auf das Lernen bei Grillen nach chronischen sozialen Niederlagen hin, welcher sich in der Beeinträchtigung der Lernfähigkeit manifestiert und hauptsächlich über die 5HT₂-Rezeptoren vermittelt wird. Künftige Untersuchungen müssen die Auswirkungen anderer Neuromodulatoren untersuchen, wie z. B. Octopamin, Dopamin und Stickstoffoxid, welche an der Funktion von Aggression, Lernen und Gedächtnis bei Grillen und möglicherweise ebenfalls an der Steuerung der Auswirkungen von chronischem Stress durch mehrfache Niederlagen auf die Lernfähigkeit beteiligt sind.

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8 APPENDIX

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8.2 Publications and published abstracts

Publication

Borstel, K. J., and Stevenson, P. A. (2021). Individual scores for associative learning in a differential appetitive olfactory paradigm using binary logistic regression analysis. *Front. Behav. Neurosci.* 15: 741439. doi:10.3389/fnbeh.2021.741439.

Short communications (posters with published abstracts)

1. Borstel, K. J., and Stevenson, P. A. (2019). Influences of dominance and social subjugation on learning and memory in crickets. At 15th Research Festival for Life Sciences, January 18, Leipzig.

2. Borstel, K. J., and Stevenson, P. A. (2019). Learning and memory in crickets: Influences of social experiences. At Entomologentagung 2019, March 11-14, Halle (Saale).

3. Borstel, K. J., and Stevenson, P. A. (2019). Influences of aggression on learning in crickets. At 13th Göttingen Meeting of the German Neuroscience Society, March 20-23, Göttingen.

4. Borstel, K. J., and Stevenson, P. A. (2020). Social experience influences learning and memory in crickets. At 16th Research Festival for Life Sciences, January 30, Leipzig.

5. Borstel, K. J., and Stevenson, P. A. (2021). Chronic social stress impairs learning and memory in crickets. At 14th Göttingen Meeting of the German Neuroscience Society “Virtual Meeting”, March 22-30, virtual meeting.

8.3 Curriculum vitae

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