The more, the better: Auditory threat conditioning using multiple conditioned and unconditioned stimuli over many trials

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Abstract

Threat conditioning research frequently relies on making inferences about associative learning mechanisms using a single item for each conditioned stimulus (CS+, CS-) and the unconditioned stimulus. However, this type of design does not allow for assessing the extent to which results generalise to populations of items and can lead to anticonservative inferences. In addition, employing a small number of trials when measuring noisy, psychophysiological responses may be problematic for establishing adequate signal-to-noise ratios. This study addresses these methodological issues and provides means for measuring associative learning and extinction mechanisms in a generalisable manner, using pupillometry as well as ratings of contingency awareness, valence, and arousal. To maximise the number of items and trials per condition, we employed an auditory blocked conditioning paradigm in which learning and extinction were established several times using different sets of conditioned (pure tones) and unconditioned stimuli (environmental sounds). We used cluster-based permutation to identify pupillary time windows of interest and linear-mixed effects modelling to account for by-subject and by-item variability. We demonstrate highly correlated early and late pupillary conditioned responses during acquisition, suggesting a common underlying learning mechanism. These responses habituated immediately during extinction. We also demonstrate (rating tasks) that conditioned valence responses are less susceptible to extinction and potentially index an additional learning mechanism. By contrast, subjective arousal ratings were less sensitive to associative learning. Finally, we demonstrate the potential risk of alpha inflation when using ‘traditional’ ANOVA-based analyses on our data.

1. Introduction

Classical conditioning is a well-established approach for studying fear-related associative memories and learning mechanisms (LeDoux, 2014). In this paradigm, *associative learning* is established by eliciting an association through pairing of a conditioned, typically neutral stimulus (CS+), and an aversive, unconditioned stimulus (UCS), (e.g. unpleasant odour, startling sound, electric shock), while another, *neutral* stimulus (CS-) is never paired with the UCS. Over a few CS+ - UCS pairings, the CS+ elicits an aversive reaction (a conditioned response) which occurs in an anticipation of the upcoming aversive stimulus. This conditioned response (CR) can then be reduced through *extinction*, in which CS+ is repeatedly presented without coupling it with the UCS. This process creates a new association in which the CS+ no longer signals threat, resulting in attenuation of the conditioned response.

The acquisition and extinction of threat-related CRs has been evidenced in a range of physiological outcome measures. The most commonly used CR readout (Lonsdorf et al., 2017) is the skin conductance response (SCR), which is an index of sympathetic nervous system activity and can be elicited through arousal (Jentsch et al., 2020; Tzovara et al., 2018). Studies have consistently demonstrated a larger SCR to CS+ than CS- during acquisition and a diminishing difference from early to late trials of extinction (Hopkins et al., 2015; Jentsch et al., 2020; Leuchs et al., 2019; Morriss et al., 2015; Reinhardt et al., 2010; Tzovara et al., 2018). Pupil size is a related, but in the present context less frequently employed autonomic measure of arousal, reflecting both sympathetic (dilation) and parasympathetic (constriction) activation (Lonsdorf et al., 2017; Ojala & Bach, 2020). Previous pupillometry studies have reported patterns of conditioned responding similar to those in SCR (García-Palacios et al., 2018; Hopkins et al., 2015; Jentsch et al., 2020; Kluge et al., 2011; Korn et al., 2017; Leuchs

et al., 2017, 2019; Reinhard & Lachnit, 2002; Tzovara et al., 2018; Visser et al., 2015, 2016, 2013). Specifically, they showed more dilated pupils in response to CS+ than CS- trials, and a reduction in this difference during extinction. However, there is a large variety in the methods used for quantifying the CR in both SCR and pupil measurements, relying, for instance, on calculating peak responses, mean responses or areas under the curve within pre-defined time windows (Jentsch et al., 2020; Korn et al., 2017). For trials with longer durations, it is also common to subdivide trials into first (FIR) and second (SIR) interval responses¹, using pre-defined time windows that, again, vary greatly across studies (Jentsch et al., 2020; Pineles et al., 2009). Inconsistencies in pre-defining time windows of interest can make comparisons between outcomes from different studies extremely difficult. A potential solution to this problem, which we will apply in our own analyses, is to select time windows of interest using a purely data-driven approach.

Along with online (neuro)physiological measures of conditioning, behavioural CR indices are also commonly employed. These are typically obtained offline following conditioning and after extinction and include, but are not limited to, subjective ratings of valence and arousal (Bröckelmann et al., 2011; Gawronski & Mitchell, 2014; Glotzbach et al., 2012; Junghöfer et al., 2015; Reinhardt et al., 2010; Sehlmeier et al., 2011; Steinberg et al., 2013; Wendt et al., 2020) as well as self-reported fear/anxiety (Abend et al., 2020; Glotzbach et al., 2012; Morriss et al., 2015). Following conditioning, such studies typically (but see, e.g., Bröckelmann et al., 2011) report that CS+ trials are perceived as more unpleasant, arousing or fear-inducing than CS- trials (Gawronski & Mitchell, 2014;

¹ Trial subdivisions into FIR and SIR have been motivated by early work suggesting that multiple CRs reflecting different underlying processes may be observable within a trial (Prokasy & Ebel, 1967). However, the empirical basis for such a distinction has been debated (Pineles et al., 2009).

Glotzbach et al., 2012; Sehlmeier et al., 2011; Morriss et al., 2015). This difference can diminish following extinction (Abend et al., 2020; Morriss et al., 2015). However, under certain conditions, cases of resistance to extinction have also been reported in both valence and arousal ratings (Gawronski & Mitchell, 2014; Sehlmeier et al., 2011; Winn et al., 2018).

This extensive body of conditioning research is accompanied by common methodological practices that may affect both the replicability of previous affective conditioning research and the inferences that can be drawn from it. In recent years, there has been a growing interest in improving the methodological consistency, replicability, and validity of inferences drawn from conditioning studies. Efforts have focused on increasing data and reporting transparency, reducing excessive ‘arbitrary’ data reduction (e.g., through statistical modelling that incorporates all available data), and using evidenced-based data exclusion criteria (Lonsdorf et al., 2019; Ney et al., 2018). In this paper, we focus on an additional set of inter-related experimental design and analytical decisions that can challenge the generalisability of findings both within and across studies.

First, many of the previous affective conditioning studies suffer from limitations in the generalisability of results across different items. This is because learning and extinction are typically investigated using only a single stimulus for CS+ and CS-, both of which are repeated over several trials (see Lonsdorf et al., 2017). Indeed, the use of several different CS-items within the same experiment is rather uncommon, because this may disrupt the acquisition of contingency awareness and thus delay, prevent, or reduce the CR of interest (Lonsdorf et al., 2017). However, the latter argument raises the question of whether results from studies with only one item per stimulus category are truly indicative of affective conditioning (which is thought of as an implicit process) or indeed of the potentially more strategic process of establishing contingency awareness.

More critically, experiments with such a limited number of items per condition cannot assess whether and to what extent results generalise to the population of items one could use for the same purposes (see Yarkoni, 2020 for a general discussion). This issue purely concerns the process of learning the initial association and is qualitatively different from the concept of generalisation of learning to related, unseen stimuli that have not previously been paired with an UCS (Dymond et al., 2015). Indeed, the point we are addressing here is that any conditioning effects observed between one CS+ (e.g., a circle) and one CS- (e.g., a triangle) are specific to those two items and any observed learning effects may not necessarily generalise to all shapes or even all triangles.

The problem with generalisability also applies to the UCS which in the context of threat conditioning is usually one unpleasant stimulus such as a mild electric shock or white noise² (Sperl et al., 2016). Indeed, there is limited research examining how different UCSs would affect the CR. The available evidence so far suggests that white noise leads to a more sustained CR compared to electric shocks, at least for studies that employ a very large number of trials (Sperl et al., 2016). In standard paradigms, it has been shown that a fearful scream may be less effective than an electric shock (Glenn et al., 2012), whereas an unpleasant metal scrape sound can be as effective as white noise and/or electric shock in eliciting CRs (Neumann & Waters, 2006). Nevertheless, these studies only used one item per condition, which cannot guarantee observing the same effect with another, similar item of the same category (e.g., female vs male scream).

Using a very limited set of items in the experimental design also extends to an analytical issue known as the *items-as-fixed-effect-fallacy* (Clark, 1973), i.e., failure to

² The differential effects of positive and negative UCSs on neutral CSs have also been studied in a form of classical conditioning often referred to as evaluative conditioning (De Houwer, 2007), however, there is little agreement on whether the two are qualitatively different.

appropriately account for stimulus variability in the statistical analysis when participants are presented with multiple items per condition. Specifically, analytical approaches that rely on aggregating data up to the participant level (e.g., within-subjects t-tests and ANOVA) prevent the assessment of by-item generalisability even in experiments where several items per condition are being used. This is because analyses on by-subject means conflate by-item variability with residual noise. As a consequence, such analyses can lead to anticonservative inferences that do not generalise to new sets of stimuli of the same type. Such tests are also likely to produce ‘too narrow’ confidence intervals or ‘too small’ p-values, respectively (Judd et al., 2012; Yarkoni, 2020). For example, inflation of test statistics in standard ‘by-subject only’ analyses has been estimated to reach 50% or more in a number of publicly available fMRI datasets (Westfall et al., 2017), and up to 60% in simulated datasets (Judd et al., 2012). Simultaneously modelling participants and items as random factors, as well as all dependencies with the experimental conditions is a necessary requirement for generalising findings to populations of stimuli and participants as well as for reducing the risk of a Type I error (Barr et al., 2013; Judd et al., 2017). This is particularly important when the aim is to gain insights into general learning mechanisms that may underlie certain anxiety disorders, intended to inform generally applicable interventions in this domain.

A final point concerns the number of trials used within experiments. Since psychophysiological measures (e.g., SCR) can exhibit amplitude reductions with repeated stimulus presentations (Leuchs et al., 2019; Lonsdorf et al., 2017; Ojala & Bach, 2020), studies usually employ a small number of trials (5-20 trials per condition, Lonsdorf et al., 2017) to prevent such habituation of the CR. At the same time, psychophysiological approaches are often noisy and therefore, require a large number of trials to achieve an acceptable signal-to-noise ratio (Ney et al., 2018; Steinberg et al., 2013; Tzovara et al., 2019). However, guidance for how to adequately consider the trade-off between signal-to-noise ratio

and CR habituation is limited. For SCR, piloting is recommended as a method for estimating the number of observations required for the detection of a CR (Lonsdorf et al., 2017), while for pupillometry, there are no systematic investigations examining the precise effect of number of trials required to detect an effect, not only in the context of emotion and fear processing but in psychophysiological research in general.

The necessity of using many trials to compensate for intrinsically noisy signals also extends to M/EEG measures, where detecting activity in subcortical structures (e.g., amygdala, thalamus, hippocampus) suggested to underlie fear and emotion processing (Duvarci & Pare, 2014; Fossati, 2012; Fullana et al., 2016) can require hundreds or even thousands of trials (Attal et al., 2007). For example, a simulation study by Quraan et al. (2011) demonstrated that while evoked hippocampal activity can be detected with 10 trials, the localisation accuracy is very poor (i.e., 18 mm away from the actual source for 10 trials and 10 mm for 50 trials). Therefore, it is unlikely that employing a traditional conditioning paradigm with a small number of trials would allow for an accurate detection of neural learning and extinction-related processes.

The goal of the present study was to test a conditioning paradigm that attempts to overcome many of the above methodological issues and provides a means for measuring associative learning and extinction mechanisms robustly, using multiple modalities. We used continuous pupil size recordings, as well as ratings of valence, arousal, and contingency awareness to examine threat conditioning and extinction. The procedure was an auditory blocked conditioning paradigm in which learning and extinction were established several times using different sets of conditioned (pure tones) and unconditioned stimuli (environmental sounds). Both CSs and UCSs were auditory rather than visual since sounds were expected to interfere less with the measurement of pupil size. To ensure that the CRs are driven by aversive anticipation rather than by valence-unspecific expectancy, we used both

pleasant and unpleasant UCSs. To maximise the distinction between the UCSs, positive and negative stimuli were presented at low and high intensity levels, respectively. Establishing learning and extinction several times in this blocked design can offer a potential solution to the issue of small numbers of items and trials in conditioning research. And while the present study does not employ M/EEG, the design can theoretically be applied to neurophysiological measures as well, and potentially offer enough trials to make investigations of learning effects in deep structures viable.

To make inferences about pupil size changes in response to conditioning and extinction, we used a data driven approach to identify significant time windows of interest for averaging over time. Unlike traditional methods, this approach does not rely on prior knowledge for time-window selection, allows for using all available data, and maximises detection of unpredicted effects (Huang & Zhang, 2017). At the same time, the method also provides a powerful control of both Type I and Type II error (Sassenhagen & Draschkow, 2019). We then used design-appropriate linear mixed effects modelling to examine differences between conditions in the most generalisable manner for both the behavioural and pupillometry data. Finally, we demonstrate the potential risk of alpha inflation when using statistical tests that fail to account for both item and subject variation, by comparing our mixed model findings to the results from conventional repeated-measures ANOVA analyses.

We expected that during acquisition, the mean pupil size in the time window of interest would be greater for CS trials paired with a negative UCS (CS Negative) than for those paired with a positive UCS (CS Positive). During extinction, we expected this difference to be reduced in magnitude or completely eliminated. We also expected to see more direct evidence for the gradual increase (acquisition) and decrease (extinction) of the CR over time, by descriptively comparing earlier with later trials during each experimental phase. For the behavioural ratings, we predicted lower valence and higher reported arousal

for CS Negative than CS Positive trials during acquisition. In analogy to the pupil size predictions, we expected these differences to diminish in magnitude during extinction. Finally, we collected self-reported data on measures of state and trait anxiety and emotion regulation. These were used for descriptive and exploratory purposes that were not critical to the main hypotheses being tested.

2. Method

2.1. Participants

We collected data from 30 participants, whose general demographic characteristics are shown in Table 1. All participants had normal or corrected-to-normal vision and normal hearing. Two participants completed only 3 out of 4 blocks (either because of technical problems or because they cut the experiment short) but their data were retained for all analyses. Five participants had to be excluded because of excessive measurement error in the pupil size data (more details further below). Hence, only 25 participants were included in the pupil size analyses, whereas all 30 participants were included in the behavioural analyses (valence and arousal ratings).

Table 1

Demographic information.

Sex	N (N Pupil*)	N Native Speakers	Mean Age	Age Range	Mean STAI-Trait (SD)	Mean STAI-State (SD)	ERQ- Reappraisal (SD)	ERQ- Suppression (SD)
Female	15 (12)	8	22.93	19 - 31	45.53 (9.8)	35.07 (10.2)	4.5 (0.65)	3.15 (1.63)
Male	15 (14)	7	22.67	19 - 29	39.93 (11.4)	32.13 (8.9)	4.88 (1.28)	3.5 (1.48)

*N Pupil: Number of participants in the pupil size dataset after exclusion

2.2. Psychological Assessment

Participants were asked to complete a basic demographic information questionnaire as well as the State Trait Anxiety Inventory (STAI) (Spielberger et al., 1983) and the Emotion Regulation Questionnaire (ERQ), (Gross & John, 2003). Descriptive, exploratory analyses of the relationship between psychological and conditioning measures can be seen in Supplementary Materials 6. Overall, correlations between conditioning and psychological measures were low to moderate, but none were statistically significant.

2.3. Stimuli

Conditioned stimuli (CSs) were 16 sine-wave tones of 4 second duration. Eight tones had low (200 – 400 Hz, 28.57 Hz steps) and 8 had high (600 – 800 Hz, 28.57 Hz steps) constant frequency. Stimuli were created in Matlab 2017a using Psychtoolbox (*makeBeep*). Unconditioned Stimuli (UCSs) were 4 positive (bongos, guitar, harp, and bird chirping) and 4 negative (metal squeak, knife scrape, drilling, and a female scream) sounds with a duration of 1 second. The latter were selected based on their valence ratings (high and low respectively) in a separate norming study (N=30, different from the participants in the main study, see <https://osf.io/dehxa/>). Positive sounds were selected based on the highest 25th percentile valence rated at 60 dB, whereas negative sounds were selected based on the lowest 75th percentile valence rated at 90 dB. To equalise the intensity of the CS and UCS stimuli, each audio file was mean-centred and then normalised to the same, maximum root mean square (RMS) amplitude, without inducing clipping, using a RMS equaliser (The Phonetics Lab, University of Washington, <https://depts.washington.edu/phonlab/resources/rmsLeveler.m>). The first and last 50 ms of the signal of all stimuli was gradually faded in and out in Audacity 2.1.2 (<https://www.audacityteam.org/>). The normalisation procedure was applied separately for CSs and UCSs. The resulting normalised CSs were presented at a maximum intensity of

approximately 50 dBA. Positive and Negative UCSs were presented at approximately 60 dBA and 90 dBA, respectively. Sound intensity was measured by Cadrim sound level meter. All stimuli used in the present study can be found at <https://osf.io/pnyrh/>.

2.4.Procedure

Prior to completing the main task, participants were asked to fill in the self-report measures (see Psychological Assessment). The main task comprised 4 blocks, each containing three experimental phases – Habituation, Acquisition, and Extinction (see Figure 1). Each block contained a different subset of 2 low and 2 high frequency sine tones, selected from the total set so that the minimum difference *between* high and low frequency tones was 286 Hz, and the minimum difference *within* low or high frequency tones was 114 Hz. For example, one block contained low frequency tones of 200 and 314 Hz, and high frequency tones of 600 and 714 Hz. Assignment of low and high frequency tones to the CS Negative condition was Latin square counterbalanced, resulting in 4 stimulus sets. Four additional sets were constructed by swapping the assignment of low and high frequency tones to the CS Positive condition, leading to a total of 8 different stimulus sets (see Supplementary Materials 1). Block order and assignment of the 4 UCS positive and 4 UCS negative sounds to each block were also Latin square counterbalanced.

Each CS was presented together with a black fixation cross, positioned at the centre of the screen for 4 seconds. The inter-trial interval (ITI) had a duration of 2.3 sec $\pm$ 300 ms and was accompanied by a black fixation cross. To minimise ocular artifacts, participants were asked to always maintain fixation at the centre of the screen. In each block and phase, each stimulus was presented 10 times, resulting in a total of 20 trials per condition per block (a total of 80 trials per condition across blocks). Trial order was randomised across participants with the restrictions that the first trial was always a CS Positive and no more than two trials of the same stimulus type (e.g., a CS Negative) could occur consecutively. To maintain

participants' attention during each block, they were asked to perform a tone judgement task. Participants were instructed that they will complete a 3-part task in which they will be presented with a series of tones that they have to listen to while maintaining fixation at the centre of the screen and indicate whether each tone is high or low in pitch (75% of trials) or hard or soft (25% of trials). They were informed that the only difference between the three parts would be that during part 2, the tones would be paired with positive and negative sounds. Participants were not informed about the contingency between CS and UCS. During Habituation and Extinction, the sine tones were presented on their own, and during Acquisition, CSs were paired with the UCSs, which occurred at CS offset for a duration of 1 second. At the end of each phase, participants completed a sound rating task where they were asked to rate each CS on valence ranging from unpleasant to pleasant and arousal ranging from boring to exciting using a slider scale ranging from -100 to 100. At the end of the Acquisition phase, participants also rated the UCS on valence and arousal (see Supplementary Materials 2). In order to determine participants' awareness of the relationship between CSs and UCSs, at the end of each block, participants completed a contingency awareness task in which they were asked to decide whether each pure tone was paired with a positive or a negative sound. Participants were also asked to provide a confidence rating on a slide scale ranging from "not at all confident" (-100) to "confident" (100).

Pupil size data were obtained using an EyeLink 1000 eye tracker and recorded continuously during each trial presentation, with initial sampling rate of 250 Hz.

Raw .edf data were exported into .txt format using DataViewer and imported into R. Pre-processing was performed separately for each participant and block using the *PupilPre* package in R and following the recommended guidelines provided by the package (see <https://bit.ly/3iBYCAR>). Raw, continuous data were transformed into time series with a trial length of -0.5 to 5.5 s relative to CS onset. The first trial in each block was removed, leaving 119 trials per block. This was necessary since the continuous pupil size recording began at the onset of the first trial which meant that no baseline window could be acquired for this trial.

Blink removal and artifact correction were performed in several steps. First, blinks identified by the EyeLink software were padded with 150 ms on either side. Within the padded blink window, data were examined in two stages, using the *clean_blink* function. First, data points within the padded window were removed if the difference between subsequent pupil size values was larger than 5 (package default). Next, any small runs of data points that were surrounded by missing values were identified and removed. Specifically, within the padded window, any 40 ms segment of data, surrounded by at least 2 missing values (default) on each side was removed.

Detected artifacts (extreme data points) were also removed using the *clean_artifact* function. Artifacts were detected using the defaults provided in the package vignette. First, to identify potential outliers, each trial was divided into bins of 100 ms within which the median absolute deviation (MAD) of the pupil size data was calculated. A bin was marked extreme if it had a MAD constant > 2 . The higher the constant the more extreme data point is required to be considered as an outlier. Each extreme bin included 200 ms of padding within which a multidimensional distributional distance (Mahalanobis distance) was calculated using the horizontal and vertical velocity and acceleration of the pupil. A pupil size was marked as extreme if the Mahalanobis distance exceeded 2 SD. Runs of data points surrounded by missing values were removed in 2 stages again, using the same procedure as for blink removal.

Finally, remaining small blinks and artifacts that were undetected by the automatic cleaning procedure were manually removed (*user_cleanup_app*). Following artifact removal, sparse trials were identified and removed. A trial was discarded if it contained less than 20% of baseline (at least 0.1 s) and less than 70% of post-baseline data (at least 3.85 s). Five participant data sets were discarded from the analyses altogether because more than 50% of their trials showed excessive blinks or movement artifacts and were removed during cleaning.

For the remaining 25 participants, the average data loss after cleaning was 12%. The missing values in the corresponding trials were replaced using spline interpolation (function *interpolate_nas* in *PupilPre*).

The cleaned and interpolated pupil size data were log10 transformed. Next, multiple linear regression was performed for each participant and block (log pupil size as outcome, and X and Y eye position as predictors) to remove small eye movement-related artifacts. From these regression analyses, the residual pupil sizes per trial and time point were extracted and used in subsequent analyses. Log10 pupil size change from baseline (mean pupil size between -0.5 and 0 s) was then calculated for each trial and time point using the *baseline* function ($change = pupil\ size - baseline$). The inverse of the baselined pupil was then calculated to obtain a measure of proportional change from baseline. Finally, data were down sampled to 10 Hz.

Results

All analyses were performed in R version 4.0.2. The code and data associated with these analyses can be found at <https://osf.io/pnyrh/>.

3.1 Pupil size

Figure 2 shows, for each phase (Habituation, Acquisition, and Extinction) the average proportional pupil size change from baseline over a 5.5 s time window including both CS presentation (0- 4 seconds) and UCS presentation (4 – 5 seconds). As seen in the figure, differences in mean pupil size between CS Positive and CS Negative trials during Acquisition become apparent from around 1 s post CS onset, reflecting the conditioned response (CR) and increase in magnitude following UCS onset (4 s), reflecting the unconditioned response (UR). Since any anticipatory processes should occur prior to the onset of the UCS, the confirmatory analysis focused on the CR only (0- 4 s). To quantify the effects of the

conditioned stimuli across the experimental phases, the analysis was performed in two stages. As explained in more detail below, we first employed a cluster-based permutation test to identify time windows of interest in a data-driven manner. Second, to account for subject and item-related random variation, a linear mixed effects (LME) model was built in which the mean pupil size across each time window of interest was used as the dependent variable. Since cluster-based permutation tests can presently only handle designs that are no more complex than 2×2 , the current 3×2 design was reduced to 2×2 by calculating pupil size changes from Habituation. For each participant, block and item, a mean pupil size during Habituation was calculated across time and trials which was then subtracted from each time point during Acquisition and Extinction (see Figure 2B). The LME modelling was also performed on Habituation-baselined data.

Figure 2. A) Changes in mean pupil size from baseline over time for each condition (green: CS Positive; orange: CS Negative) and in each of the three experimental phases (Habituation, Acquisition and Extinction). B) The same data for the Acquisition and Extinction phases after subtracting the Habituation phase baseline (reflecting the difference between Acquisition and Habituation and between Extinction and Habituation, respectively). Vertical, light-coloured bars indicate standard errors of the means. The vertical dashed lines indicate UCS onsets.

3.1.1. Identifying time window of interest using cluster-based permutation

The cluster-based permutation analysis was carried out using the R packages *clusterperm* and *exchangr* and involved the following steps. First, an *Experimental Phase* (Acquisition vs Extinction) * *Stimulus Type* (CS Positive vs CS Negative) within-subjects ANOVA (*aov_by_bin*) was performed for each time bin (0 – 4 s). Adjacent time bins with a $p < 0.05$ were combined into clusters, with the sum of the F values within each cluster serving as the cluster statistics for the two main effects and the interaction (*detect_clusters_by_effect*). Next, a Monte-Carlo test with 2000 permutations was performed (*cluster_nhds*) in which a cluster statistic was computed on trials that were randomly assigned to each condition within subjects, resulting in 2000 cluster statistics. For each of the main effects and the interaction, the permuted cluster statistics were compared against the observed statistic. Clusters were considered significant if they fell within the highest or lowest 2.5 % of the null distribution. The results from the cluster-permutation test can be seen in Table 3 and is visualised in Figure 3. The test revealed two significant time clusters for the *Experimental Phase* * *Stimulus Type* interaction: around 1.2 and 1.7 seconds and around 3 and 3.4 seconds, respectively. Since this test is only suggestive of where in time an effect may be observed (without providing clues about its generalisability across items and participants), the suggested interactions in these time clusters were further assessed inferentially using LME modelling (see below).

Table 3*Cluster-based permutation results.*

Effect	B0	B1	Sign	Cms	P-value
Experimental Phase	0	100	-1	11.29	1
Experimental Phase	800	900	-1	11.908	0.583
Stimulus Type	1500	2100	1	39.324	0.065
Experimental Phase X Stimulus Type	0	0	1	4.301	0.143
Experimental Phase X Stimulus Type	1200	1700	1	31.855	0.024*
Experimental Phase X Stimulus Type	2300	2400	1	8.819	0.09
Experimental Phase X Stimulus Type	3000	3400	1	27.68	0.028*
Experimental Phase X Stimulus Type	3900	3900	1	4.146	0.151

Note. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 3. Significant Experimental Phase X Stimulus Type time cluster derived from the cluster-based permutation test. The vertical, light-coloured lines indicate the standard error of the mean.

3.1.2. Linear mixed effects modelling of data within the identified time clusters

To examine the development and extinction of anticipatory learning, an LME model (R package *lme4*) was built for each of the two significant time clusters identified in the cluster-based permutation analysis. From this point onward, the effect in the time window between 1.2 and 1.7 s post CS onset would be referred to as the first interval response (FIR) and that between 3 and 3.4 s post CS onset as the second interval response (SIR). For each subject, block and trial, a mean pupil size was calculated for the FIR (see Figure 4A) and SIR (see Figure 5 A). These served as the dependent variables in the two models. Each model consisted of a 2 *Experimental Phase* (Acquisition vs Extinction) by 2 *Stimulus Type* (CS Positive vs CS Negative) fixed effects design. The models included mean-centred contrasts (deviation coding) for the two categorical fixed-effect predictors. Following Barr et al. (2013), models with design-appropriate *maximal* random effects structure were fitted. Specifically, *Subjects* and *CS Items* were added as random intercepts. Since experimental manipulations were both within-subjects and within-items, the model also included by-subject and by-item random slopes for each main effect as well as for the interaction (see Supplementary Materials 3 for random effects summary). Since each item was presented multiple times, an interaction between the *Subjects* and *CS Items* random intercepts was also included, coupled with the main effects and the interaction of the by-subject and by-item random slopes. Random variability due to *UCS Item* variability was not modelled since UCSs were only present during Acquisition and not during Extinction. However, separate models, accounting for UCS variability were conducted on the Acquisition phase only (see Supplementary Materials 4). P-values for the fixed effects were determined via Type III Wald Chi-square tests. Table 4 shows the fixed-effects results and Figure 4C shows the model estimates, with associated 95% CIs. As shown, the interaction between *Experimental Phase* and *Stimulus Type* in the FIR model was significant. Simple effect analysis of the interaction

was performed using estimated marginal mean simple contrasts (package *emmeans*) with Satterthwaite method for degrees of freedom approximation. Consistent with the descriptive data, these contrasts confirmed a reliably larger mean pupil size for CS Negative than CS Positive trials during Acquisition ($t(56.6) = 3.2, p = 0.003$), but no clear simple effect of *Stimulus Type* during Extinction ($t(22.2) = -0.3, p = 0.79$), (see Supplementary Materials 3 and Figure 4B). In addition, a larger mean pupil size was also observed for CS Positive trials during Acquisition compared to CS Positive trials during Extinction ($t(22.6) = -2.4, p = 0.024$). Similarly, the interaction between *Experimental Phase* and *Stimulus Type* in the SIR model was also significant. Again, the simple contrasts revealed a larger pupil size for CS Negative than CS Positive trials during Acquisition ($t(23.3) = 2.42, p = 0.023$), but no significant simple effects during Extinction ($t(15.9) = -0.3, p = 0.75$), (see Supplementary Materials 3 and Figure 5B).

Table 4

Type III Wald Chi-square tests and R-squared values for the complete pupil models and each of the fixed effects for the first and second interval responses.

	Chisq	Df	P-value	R ² Fixed (CI)
FIR				
Full Model (Fixed)				0.003 (0.001 – 0.006)
Experimental Phase	2.29	1.000	0.13	0.001 (0-0.002)
Stimulus Type	2.10	1.000	0.15	0.001 (0-0.003)
Experimental Phase X Stimulus Type	6.08	1.000	0.01*	0.001 (0-0.004)
SIR				
Full Model (Fixed)				0.002 (0 – 0.005)
Experimental Phase	0.79	1.000	0.37	0.000 (0-0.002)
Stimulus Type	1.44	1.000	0.23	0.001 (0-0.002)
Experimental Phase X Stimulus Type	5.51	1.000	0.02*	0.001 (0-0.003)

Note. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 4. Summary of pupil size fixed effects for the first interval response (FIR). A) Distribution of mean pupil size between 1.2 and 1.7 s post CS onset of Habituation-baselined data. B) Estimated marginal means per condition derived from the mixed effects model of pupil size (error bars represent 95% CIs for the means conditioned on the random effects). C) Fixed effect estimates (labelled dots) derived from the mixed effects model of pupil size; bars represent 95% CIs for the estimates.

Figure 5. Summary of pupil size fixed effects for the second interval response (SIR). A) Distribution of mean pupil size between 3 and 4 s post CS onset of Habituation-baselined data. B) Estimated marginal means per condition derived from the mixed effects model of pupil size (error bars represent 95% CIs for the means conditioned on the random effects). C) Fixed effect estimates (labelled dots) derived from the mixed effects model of pupil size; bars represent 95% CIs for the estimates.

3.2. Valence and arousal ratings

For comparability with the pupil size analysis, the rating-data analysis was performed on Habituation-baselined valence and arousal data (see Figure 6B and 6D). Since each item was rated once per block, the baseline-adjustment was performed by subtracting a given item's rating during the Habituation phase from the same item's rating during the Acquisition and Extinction phases, respectively. As seen in Figure 6A and B, mean valence ratings were slightly lower for CS Negative than CS Positive trials during both Acquisition and Extinction, although the distributions overlapped to a substantial degree. The difference in the means between the two stimulus types was even more pronounced when the valence data were put in relation to the Habituation-phase baseline. Mean arousal ratings (Figure 6C and D) were

slightly higher for CS Negative than CS Positive trials, but only after adjusting for the Habituation-phase baseline (Figure 6D).

In analogy to the pupil size analyses, we fitted two LME models - one predicting the valence ratings and the other one predicting the arousal ratings after subtracting the Habituation-phase baseline ratings. As with the pupil size model, each of the two rating models employed 2 *Experimental Phase* (Acquisition vs Extinction) by 2 *Stimulus Type* (CS Positive vs CS Negative) fixed effects design, using mean-centred contrasts for the two categorical fixed effect predictors. By-subject and by-item random intercepts were added, together with by-subject and by-item random slopes for both main effects and the interaction (see Supplementary Materials 3 for random effects summary).

For valence ratings, a Type III Wald Chi-square test (see Table 5 and Figure 7A for model estimates) revealed a significant *Stimulus Type* main effect. Since the interaction was non-significant, the estimated marginal means contrasts were computed by averaging over the levels of *Experimental Phase*. These revealed (see Supplementary Materials 3 for contrasts table) that overall, CS Negative trials had lower valence than CS Positive trials ($t(18.03) = 8.5, p = 0.0215$). In terms of arousal ratings, no significant main effects or interactions were observed (see Table 5 and Figure 7B).

Table 5

Type III Wald Chi-square tests and R-squared values for the complete valence and arousal models and each of the fixed effects.

	Chisq	Df	P-value	R ² Fixed (CI)
Valence				
Full Model				0.014 (0.004 – 0.03)
Experimental Phase	0.27	1.000	0.6	0 (0 – 0.006)
Stimulus Type	6.46	1.000	0.01**	0 (0 – 0.007)
Experimental Phase X Stimulus Type	0.21	1.000	0.64	0.013 (0.003 - 0.032)
Arousal				
Full Model				0.005 (0.001-0.02)
Experimental Phase	0.029	1.000	0.866	0 (0 – 0.005)
Stimulus Type	1.519	1.000	0.218	0 (0 – 0.006)
Experimental Phase X Stimulus Type	0.095	1.000	0.757	0.005 (0 – 0.017)

Note. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 6. Mean valence and arousal ratings. A) and C) Mean valence and arousal ratings across Habituation, Acquisition and Extinction phases. B) and D) Habituation-baselined mean valence and arousal ratings.

Figure 7. Summary of valence and arousal fixed effects. Fixed effect estimates (labelled dots) derived from the mixed effects model of valence (A) and arousal (C) ratings; bars represent 95% CIs for the estimates. Estimated marginal means per condition derived from the mixed effects model of valence (B) and arousal (D) ratings (error bars represent 95% CIs for the means conditioned on the random effects).

3.4. Contingency awareness

Detailed descriptions of the contingency awareness data, and related analyses can be seen in Supplementary Materials 5. Overall, we found that participants were clearly aware of the CS-UCS contingency. Importantly, however, there was no clear evidence for a relationship between contingency awareness and the conditioned response in any of our outcome measures.

3.5. Conventional Analysis approaches

For comparison, Table 6 shows the results from by-subject repeated-measures ANOVAs performed on pupil size, valence ratings, and arousal ratings as dependent variables. As seen in the table, the general pattern of results is consistent with that of the mixed effects modelling. However, in most instances the strength of association (standardized ‘effect size’) is larger, and associated p-values much smaller in the ANOVA outputs than in the mixed effects model analyses. These differences are particularly noticeable for the interaction effects, where the p-value for the pupil FIR and SIR interactions have decreased from 0.01 and 0.02 in the LME to 0.007 and 0.005 in the ANOVAs, respectively. The p-value for the main effect of valence rating has also decreased from 0.01 to 0.008. As seen in Figure 10, this is also reflected in the confidence intervals (CIs), whereas across outcome measures, the CIs derived from the mixed models tend to be wider. While the consistent patterns are reassuring, there is a suggestion of more anticonservativity in the ANOVA analyses, potentially because the latter only take by-subject but not by-item variability of effects into account (unlike our LME analyses which considered both simultaneously).

Table 6*Repeated measures ANOVA and Generalised eta-squared for each effect.*

Effect	DFn	DFd	MSE	F	p	ges
Pupil Size FIR						
Experimental Phase	1	24	0.001	3.3	0.08	0.03
Stimulus Type	1	24	0.001	2.4	0.14	0.03
Experimental Phase X Stimulus Type	1	24	0.002	8.5	0.008**	0.05
Pupil Size SIR						
Experimental Phase	1	25	0.002	1.52	0.3	0.01
Stimulus Type	1	25	0.001	1.98	0.2	0.02
Experimental Phase X Stimulus Type	1	25	0.001	9.8	0.005**	0.04
Valence						
Experimental Phase	1	29	79.4	0.769	0.388	0.003
Stimulus Type	1	29	244.9	8.251	0.008**	0.1
Experimental Phase X Stimulus Type	1	29	89.7	0.392	0.536	0.002
Arousal						
Experimental Phase	1	29	88.4	0.039	0.845	0
Stimulus Type	1	29	294.3	2.021	0.166	0.027
Experimental Phase X Stimulus Type	1	29	86.9	0.158	0.694	0.001

Note. The measure of effect size is generalised eta squared (ges).* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 10. Estimated marginal means and 95 % confidence intervals derived from the repeated-measures ANOVAs and LME models, computed on the pairwise stimulus type contrasts for each experimental phase.

3. Discussion

The aim of this study was to assess the feasibility of a conditioning paradigm that uses multiple conditioned and unconditioned stimuli and many trials to examine learning and extinction mechanisms. A related objective was to determine the generalisability of results across both participants and items, thus going beyond previous studies in this area, which primarily only considered by-participant variation in their designs and analyses. Our paradigm demonstrated variable success in the detection of associative learning and extinction effects, depending on the outcome measure.

4.1. Pupil Data

During Acquisition, the task elicited a clearly measurable conditioned response, as manifested in significantly more dilated pupils for CS Negative than CS Positive stimuli before UCS onset. Our results corroborate previous findings (based on standard conditioning paradigms) whereby the CS+ elicits more pupil dilation than the CS- (Jentsch et al., 2020; Kluge et al., 2011; Korn et al., 2017; Leuchs et al., 2019). It has been argued that pupillary responses during threat conditioning reflect a valence-*unspecific* anticipation of the UCS, as pupil dilation-correlates with anterior cingulate activity have been independently observed during both threat and reward learning tasks (Leuchs et al, 2019). However, by showing larger pupil responses to CSs paired with unpleasant rather than pleasant UCSs, the present study provides direct, within-task evidence for valence-*specific* pupillary anticipation effects.

Importantly, instead of taking an average (or peak) pupil size over the entire trial period, or within pre-defined time bins (García-Palacios et al., 2018; Jentsch et al., 2020; Koenig et al., 2017; Leuchs et al., 2017), the differential pupil CRs we observed held true for time windows that were derived from the data themselves.

The cluster-based permutation results identified two temporal clusters of interest, a first interval (FIR) occurring between around 1.2 and 1.7 seconds, and a second interval (SIR) observed around 3 to 3.4 seconds. These findings were corroborated when mixed effects modelling was performed on the average pupil size within these windows, showing reliably increased pupil dilation for CS Negative than CS Positive trials during Acquisition in both the FIR and SIR. These results partly contrast with previous findings (using pre-defined time windows) which suggested only a *single* CR in pupillometry data, but a dual (FIR, SIR) pattern in skin conductance responses (Jentsch et al., 2020) - with the latter being more comparable to the present findings. It seems plausible that data-driven time window selection yields greater power for detecting multiple CRs in pupillometry data. At the same time, it is unclear to what extent *early* and *late* responses are qualitatively different, and how they compare across physiological measures. In the context of SCR, it has previously been suggested that early CRs (FIR) may reflect novelty responses as well as associative processes, whereas late CRs (SIR) could reflect the acquisition of the CS-UCS contingency and temporal prediction of UCS occurrence (Jentsch et al., 2020). Yet, despite SCR studies consistently reporting multiple CRs within pre-defined time windows, there is little or mixed empirical evidence to support a qualitative distinction between early and late responses. While Jentsch et al. (2020) provided some evidence for distinct underlying processes involved in FIR and SIR (i.e., only the early SCR was susceptible to extinction), they did not directly examine the relationship between the two CRs. In contrast, Pineles et al. (2009) showed that FIR and SIR derived from SCRs are correlated (Pineles et al., 2009). Similarly, in the present study we observed a high correlation ($r=0.8$) between the pupillary FIR and SIR (see Supplementary materials 6), suggesting that at least in the context of pupillometry, these responses may not necessarily reflect independent processes.

Similar to Leuchs et al. (2019), we found no significant differences between CS Positive and CS Negative trials during the Extinction phase, indicating that the aversive association established during Acquisition was successfully extinguished. Studies attempting to trace the development of extinction commonly either compare the first and last extinction trials (Dunsmoor et al., 2019; Morriss et al., 2015; Sperl et al., 2018) or arbitrarily bin trials into small groups (Jentsch et al., 2020; Reinhard & Lachnit, 2002). For comparative purposes with previous research, the current dataset was examined descriptively by splitting trials into blocks of 5 (see Supplementary Materials 7), where we found no clear evidence of ‘extinction development’ in the form of gradually fading conditioned responses. However, there was a clear pattern in the development of the conditioned response during Acquisition, which appeared to be strongest from the 6th until the 15th repetition, following which the response appeared to diminish. We conjecture that the pupillary CR had already habituated by the time extinction was assessed, and that a slightly lower number of stimulus repetitions during acquisition may be preventative of such habituation.

It is also plausible that the extinction of the CR, as well as its acquisition, were influenced by type of instruction. In the present study, participants were told that the conditioned stimuli will only be paired with positive and negative sounds during the learning phase without receiving information about the CS-UCS contingency. As a result, participants expected positive and negative UCS delivery during learning but were aware that no UCS would be administered during extinction. Recent studies have demonstrated the differential impact of instruction type on cognitive and affective systems during extinction. In particular, lack of explicit instructions about the removal of the UCS results in a typical pattern of gradual reduction in the conditioned UCS expectancy, SCR and startle over time (Sevenster et al., 2012). However, the conditioned SCR and UCS expectancy diminish immediately if participants are instructed that the UCS will no longer be presented even when the device

delivering the UCS (i.e., an electrode) is not removed. In contrast, the startle response diminishes at a slower rate compared to other measures (Sevenster et al., 2012), but physical removal of the UCS electrode facilitates its extinction (Wendt et al., 2020). According to Sevenster et al. (2012), these observations fit within the dual-process framework of fear learning and suggest the involvement of separate cognitive and affective systems. Within this context, the SCR may reflect anticipatory responses driven by the established CS-UCS contingency, while the startle response may be a more automatic, affective response linked to the valence of the CS, elicited through the UCS. (Sevenster et al., 2012). Assuming that the pupillary responses detected in the present study are anticipatory in nature (comparable to the SCR), such an “extinction by instruction” account may explain why we were unable to detect any sign of gradual attenuation of the pupillary conditioned response during the Extinction phase.

More recently, it has been shown that the CS+/CS- discrimination during learning is largest when explicit instructions about the CS-UCS contingency are provided, with the magnitude of the difference being smallest when no explicit contingency instruction is used (Mertens et al., 2020). Since our protocol combined “uninstructed acquisition” with “instructed extinction”, the relatively small effects we observed may not be overly surprising. Nevertheless, it is also worth noting that because we accounted for by-participant and by-item variability of effects simultaneously (see crossed random effects in our mixed effects models) our significance tests were arguably less anticonservative than the more traditional within-subjects ANOVA approach used in previous research. In particular, the latter approach had been shown to increase the risk of false positives when data are from populations in which effects vary not only across participants *but also across stimuli*, which is a very plausible general assumption (see, e.g., Barr et al., 2013; Clark, 1973; Yarkoni, 2020). We will return to this point further below.

Finally, to ensure that repetitively eliciting associative learning and extinction several times does not hamper the conditioning task, we examined the effect of block number on the elicitation of the conditioned response (see Supplementary Materials 7). We found no evidence of block-related learning effects during either acquisition or extinction which suggests that the blocked design of the study does not have a negative impact on the development of associative memories.

4.2. Rating Data

Indirect evidence for successful conditioning at the behavioural level was obtained from the contingency awareness task, suggesting that participants were likely aware of the relationship between conditioned and unconditioned stimuli. On the other hand, we found limited evidence in support of a relationship between the different measures of conditioned responding and contingency awareness (see Supplementary Materials 5). Even though a small proportion of participants failed to develop contingency awareness, all data were retained in the analyses to avoid introducing selection bias through exclusions based on arbitrary criteria (Lonsdorf & Merz, 2017).

In terms of evaluative signatures of conditioning and extinction, we found that arousal ratings did not significantly differ across CS Positive and CS Negative trials during either learning or extinction. This is not surprising, since the arousal measurement (‘boring’ to ‘exciting’) was valence-unspecific and therefore related to positive and negative stimuli in equal measures. This is consistent with the well-established U-shaped relationship between valence and arousal, in which arousal is high for extremely positive and negative stimuli but remains low for neutral-valence stimuli (Bradley et al., 1992; Bradley & Lang, 2000). In contrast, we observed a main effect in the valence ratings, with CS Negative trials being rated as more unpleasant than CS Positive trials regardless of experimental phase. This suggests that the valence differences elicited during learning did not (or at least not fully) extinguish

during extinction training. This observation supports previous studies (Luck & Lipp, 2015a, 2015b; Wendt et al., 2020) which suggested the presence of independent processing systems where valence may reflect an evaluative process that is more difficult to extinguish, even when explicit extinction-supporting instructions are provided. Specifically, online conditioned valence ratings show resistance to extinction at the beginning of extinction training regardless of instruction type and even following removal of physical threat (Luck & Lipp, 2015a, 2015b). When ratings are obtained offline and regardless of instruction type, CS+ trials are still perceived as less pleasant, although there is some evidence to suggest that this resistance may be more prominent when no instructions are delivered (Wendt et al., 2020).

Across the literature, mixed findings have been reported when using subjective valence and arousal as measures of the conditioned response. Standard paradigms assessing only one CS+ and one CS-, typically demonstrated a reduction in valence and an increase in arousal for CS+ trials during learning (Gawronski & Mitchell, 2014; Reinhardt et al., 2010; Sehlmeier et al., 2011). In contrast, Multi-CS conditioning paradigms in which many different stimuli are used, elicit much smaller and less consistent effects (Bröckelmann et al., 2011; Junghöfer et al., 2015; Rehbein et al., 2014; Steinberg et al., 2013) which is more consistent with the findings of the present study. Indeed, inferences about associative memory mechanisms may not be very robust and generalisable when based on only two stimuli. On the other hand, when many different stimuli must be evaluated and/or contingency awareness is poor, subjective metacognitive judgements of affect may not be sufficiently powerful to detect the presence of learning and extinction.

Like the pupil size data, the valence and arousal effects observed in the present study were small, but comparable to previous findings based on larger trial numbers and varying stimuli. In particular, a reanalysis of the valence ratings from a study by Rehbein et al.

(2014), who used a Multi-CS conditioning task to elicit learning, revealed an effect size for the *Experimental Phase X Stimulus Type* interaction that was comparable to that in the present study, even when a traditional repeated-measures ANOVA on aggregated data was performed (see Supplementary Materials 8).

4.3. Methodological considerations and conclusions

The present study offers a more generalisable alternative to the hitherto available associative learning paradigms in the literature. By using a blocked design in which learning and extinction are established several, consecutive times, we demonstrate that it is possible to use a greater range of conditioned and unconditioned stimuli while still allowing for the development of a contingency awareness that seems crucial for the development of a conditioned response (Mertens & Engelhard, 2020). Since there was no evidence that the blocked nature of the paradigm would negatively influence the development of a conditioned response, the task can theoretically also be applied to other noisy measurement modalities (such as M/EEG) and expanded to include a greater range of stimuli and number of trials.

Consistent with other investigations highlighting the necessity of modelling random variation across both subjects and items (Barr et al., 2013; Judd et al., 2012, 2017; Westfall et al., 2017; Yarkoni, 2020), the present study also demonstrated that conventional analysis methods, when applied to our design, can potentially inflate the test statistic at the expense of truly generalisable conclusions. Furthermore, while the effects reported here appear weaker than those typically found in the threat conditioning literature, the present study offers a much greater degree of generalisability based on (a) data-driven identification of time windows of interest and (b) statistical modelling that takes both by-participant and by-item variation of effects into account.

We show that both early and late pupillary conditioned responses can be detected during acquisition, and that these vanish almost immediately during extinction. We show that early vs. late interval responses may not be reflecting independent processes as they are highly correlated. While our descriptive analysis suggests that habituation may have already developed during learning, it is possible that the type of instructions delivered to participants for the extinction phase also contributed to the quick onset of CR habituation. Future work should focus on establishing precisely how instructed versus uninstructed protocols affect the acquisition and extinction of conditioning using many conditioned and unconditioned stimuli. In addition, the paradigm may potentially benefit from a slight reduction in the number of stimulus repetitions, to avoid habituation of the CR prior to extinction. Finally, we show that our subjective measure of arousal was not sensitive enough to detect associative learning processes while subjective valence may potentially be less susceptible to extinction. Overall, our findings confirm previous suggestions (Lonsdorf et al., 2017; Sevenster et al., 2012) that different outcome measures may tap onto different processes related to associative learning and extinction.

4. References

- Abend, R., Gold, A. L., Britton, J. C., Michalska, K. J., Shechner, T., Sachs, J. F., Winkler, A. M., Leibenluft, E., Averbeck, B. B., & Pine, D. S. (2020). Anticipatory Threat Responding: Associations With Anxiety, Development, and Brain Structure. *Biological Psychiatry*, 87(10), 916–925. <https://doi.org/10.1016/j.biopsych.2019.11.006>
- Attal, Y., Bhattacharjee, M., Yelnik, J., Cottureau, B., Lefèvre, J., Okada, Y., Bardinet, E., Chupin, M., & Baillet, S. (2007). Modeling and detecting deep brain activity with MEG & EEG. *Annual International Conference of the IEEE Engineering in Medicine and Biology - Proceedings*, 4937–4940. <https://doi.org/10.1109/IEMBS.2007.4353448>
- Barr, D. J., Levy, R., Scheepers, C., & Tily, H. J. (2013). Random effects structure for confirmatory hypothesis testing: Keep it maximal. *Journal of Memory and Language*, 68(3), 255–278. <https://doi.org/10.1016/j.jml.2012.11.001>
- Bradley, M. M., Greenwald, M. K., Petry, M. C., & Lang, P. J. (1992). Remembering Pictures: Pleasure and Arousal in Memory. *Journal of Experimental Psychology: Learning, Memory, and Cognition*, 18(2), 379–390. <https://doi.org/10.1037/0278-7393.18.2.379>
- Bradley, M. M., & Lang, P. J. (2000). Affective reactions to acoustic stimuli. *Psychophysiology*, 37(2), 204–215. <http://www.ncbi.nlm.nih.gov/pubmed/10731770>
- Bröckelmann, A., Steinberg, C., Elling, L., Zwanzger, P., & Pantev, C. (2011). *Emotion-Associated Tones Attract Enhanced Attention at Early Auditory Processing: Magnetoencephalographic Correlates*. 31(21), 7801–7810. <https://doi.org/10.1523/JNEUROSCI.6236-10.2011>
- Clark, H. H. (1973). The language-as-fixed-effect fallacy: A critique of language statistics in psychological research. *Journal of Verbal Learning and Verbal Behavior*, 12(4), 335–359. [https://doi.org/10.1016/S0022-5371\(73\)80014-3](https://doi.org/10.1016/S0022-5371(73)80014-3)
- De Houwer, J. (2007). A conceptual and theoretical analysis of evaluative conditioning. In *Spanish Journal of Psychology* (Vol. 10, Issue 2, pp. 230–241). Cambridge University Press. <https://doi.org/10.1017/S1138741600006491>
- Dunsmoor, J. E., Kroes, M. C. W., Li, J., Daw, N. D., Simpson, H. B., & Phelps, E. A.

- (2019). Role of human ventromedial prefrontal cortex in learning and recall of enhanced extinction. *Journal of Neuroscience*, 39(17), 3264–3276.
<https://doi.org/10.1523/JNEUROSCI.2713-18.2019>
- Duvarci, S., & Pare, D. (2014). Amygdala microcircuits controlling learned fear. *Neuron*, 82(5), 966–980. <https://doi.org/10.1016/j.neuron.2014.04.042>
- Dymond, S., Dunsmoor, J. E., Vervliet, B., Roche, B., & Hermans, D. (2015). Fear Generalization in Humans: Systematic Review and Implications for Anxiety Disorder Research. *Behavior Therapy*, 46, 561–582. <https://doi.org/10.1016/j.beth.2014.10.001>
- Fossati, P. (2012). Neural correlates of emotion processing: From emotional to social brain. *European Neuropsychopharmacology*, 22(SUPPL3), S487–S491.
<https://doi.org/10.1016/j.euroneuro.2012.07.008>
- Fullana, M. A., Harrison, B. J., Soriano-Mas, C., Vervliet, B., Cardoner, N., Àvila-Parcet, A., & Radua, J. (2016). Neural signatures of human fear conditioning: an updated and extended meta-analysis of fMRI studies. *Molecular Psychiatry*, 21(4), 500–508.
<https://doi.org/10.1038/mp.2015.88>
- García-Palacios, A., Costa, A., Castilla, D., Del Río, E., Casaponsa, A., & Duñabeitia, J. A. (2018). The effect of foreign language in fear acquisition. *Scientific Reports*, 8(1), 1–8.
<https://doi.org/10.1038/s41598-018-19352-8>
- Gawronski, B., & Mitchell, D. G. V. (2014). Simultaneous conditioning of valence and arousal. *Cognition and Emotion*, 28(4), 577–595.
<https://doi.org/10.1080/02699931.2013.843506>
- Glenn, C. R., Lieberman, L., & Hajcak, G. (2012). Comparing electric shock and a fearful screaming face as unconditioned stimuli for fear learning. *International Journal of Psychophysiology*, 86(3), 214–219. <https://doi.org/10.1016/j.ijpsycho.2012.09.006>
- Glotzbach, E., Ewald, H., Andreatta, M., Pauli, P., & Mühlberger, A. (2012). Contextual fear conditioning predicts subsequent avoidance behaviour in a virtual reality environment. *Cognition & Emotion*, 26(7), 1256–1272.
<https://doi.org/10.1080/02699931.2012.656581>
- Gross, J. J., & John, O. P. (2003). Emotion Regulation Questionnaire. *NeuroImage*, 48(10), 9–9. <https://doi.org/10.1037/0022-3514.85.2.348>

- Hopkins, L. S., Schultz, D. H., Hannula, D. E., & Helmstetter, F. J. (2015). Eye movements index implicit memory expression in fear conditioning. *PLoS ONE*, *10*(11), 1–19. <https://doi.org/10.1371/journal.pone.0141949>
- Huang, G., & Zhang, Z. (2017). Improving sensitivity of cluster-based permutation test for EEG/MEG data. *International IEEE/EMBS Conference on Neural Engineering, NER*, 9–12. <https://doi.org/10.1109/NER.2017.8008279>
- Jentsch, V. L., Wolf, O. T., & Merz, C. J. (2020). Temporal dynamics of conditioned skin conductance and pupillary responses during fear acquisition and extinction. *International Journal of Psychophysiology*, *147*(November 2019), 93–99. <https://doi.org/10.1016/j.ijpsycho.2019.11.006>
- Judd, C. M., Westfall, J., & Kenny, D. A. (2012). Treating stimuli as a random factor in social psychology: A new and comprehensive solution to a pervasive but largely ignored problem. *Journal of Personality and Social Psychology*, *103*(1), 54–69. <https://doi.org/10.1037/a0028347>
- Judd, C. M., Westfall, J., & Kenny, D. A. (2017). Experiments with More Than One Random Factor: Designs, Analytic Models, and Statistical Power. *Annual Review of Psychology*, *68*(1), 601–625. <https://doi.org/10.1146/annurev-psych-122414-033702>
- Junghöfer, M., Bröckelmann, A. K., Küppers, K., Ohrmann, P., & Pedersen, A. (2015). Abnormal, affect-specific modulatory effects on early auditory processing in schizophrenia: Magnetoencephalographic evidence. *Schizophrenia Research*, *161*(2–3), 308–313. <https://doi.org/10.1016/j.schres.2014.11.025>
- Kluge, C., Bauer, M., Leff, A. P., Heinze, H.-J., Dolan, R. J., & Driver, J. (2011). Plasticity of human auditory-evoked fields induced by shock conditioning and contingency reversal. *Proceedings of the National Academy of Sciences*, *108*(30), 12545–12550. <https://doi.org/10.1073/pnas.1016124108>
- Koenig, S., Nauroth, P., Lucke, S., Lachnit, H., Gollwitzer, M., & Uengoer, M. (2017). Fear acquisition and liking of out-group and in-group members: Learning bias or attention? *Biological Psychology*, *129*, 195–206. <https://doi.org/10.1016/j.biopsycho.2017.08.060>
- Korn, C. W., Staib, M., Tzovara, A., Castegnetti, G., & Bach, D. R. (2017). A pupil size response model to assess fear learning. *Psychophysiology*, *54*(3), 330–343.

<https://doi.org/10.1111/psyp.12801>

LeDoux, J. E. (2014). Coming to terms with fear. *Proceedings of the National Academy of Sciences*, 111(8), 2871–2878. <https://doi.org/10.1073/pnas.1400335111>

Leuchs, L., Schneider, M., Czisch, M., & Spoormaker, V. I. (2017). Neural correlates of pupil dilation during human fear learning. *NeuroImage*, 147, 186–197. <https://doi.org/10.1016/j.neuroimage.2016.11.072>

Leuchs, L., Schneider, M., & Spoormaker, V. I. (2019). Measuring the conditioned response: A comparison of pupillometry, skin conductance, and startle electromyography. *Psychophysiology*, 56(1), 1–16. <https://doi.org/10.1111/psyp.13283>

Lonsdorf, T. B., Klingelhöfer-Jens, M., Andreatta, M., Beckers, T., Chalkia, A., Gerlicher, A., Jentsch, V. L., Drexler, S. M., Mertens, G., Richter, J., Sjouwerman, R., Wendt, J., & Merz, C. J. (2019). Navigating the garden of forking paths for data exclusions in fear conditioning research. *ELife*, 8. <https://doi.org/10.7554/eLife.52465>

Lonsdorf, T. B., Menz, M. M., Andreatta, M., Fullana, M. A., Golkar, A., Haaker, J., Heitland, I., Hermann, A., Kuhn, M., Kruse, O., Meir Drexler, S., Meulders, A., Nees, F., Pittig, A., Richter, J., Römer, S., Shiban, Y., Schmitz, A., Straube, B., ... Merz, C. J. (2017). Don't fear 'fear conditioning': Methodological considerations for the design and analysis of studies on human fear acquisition, extinction, and return of fear. *Neuroscience and Biobehavioral Reviews*, 77, 247–285. <https://doi.org/10.1016/j.neubiorev.2017.02.026>

Lonsdorf, T. B., & Merz, C. J. (2017). More than just noise: Inter-individual differences in fear acquisition, extinction and return of fear in humans - Biological, experiential, temperamental factors, and methodological pitfalls. *Neuroscience and Biobehavioral Reviews*, 80(April), 703–728. <https://doi.org/10.1016/j.neubiorev.2017.07.007>

Luck, C. C., & Lipp, O. V. (2015a). A potential pathway to the relapse of fear? Conditioned negative stimulus evaluation (but not physiological responding) resists instructed extinction. *Behaviour Research and Therapy*, 66, 18–31. <https://doi.org/10.1016/j.brat.2015.01.001>

Luck, C. C., & Lipp, O. V. (2015b). To remove or not to remove? Removal of the unconditional stimulus electrode does not mediate instructed extinction effects.

- Psychophysiology*, 52(9), 1248–1256. <https://doi.org/10.1111/psyp.12452>
- Mertens, G., Boddez, Y., KryptosIris, A.-M., & Engelhard, I. (2020). *PsyArXiv Preprints / Human fear conditioning depends on stimulus contingency instructions*. <https://psyarxiv.com/by964/>
- Mertens, G., & Engelhard, I. M. (2020). A systematic review and meta-analysis of the evidence for unaware fear conditioning. In *Neuroscience and Biobehavioral Reviews* (Vol. 108, pp. 254–268). Elsevier Ltd. <https://doi.org/10.1016/j.neubiorev.2019.11.012>
- Morriss, J., Christakou, A., & van Reekum, C. M. (2015). Intolerance of uncertainty predicts fear extinction in amygdala-ventromedial prefrontal cortical circuitry. *Biology of Mood and Anxiety Disorders*, 5(1), 4. <https://doi.org/10.1186/s13587-015-0019-8>
- Neumann, D. L., & Waters, A. M. (2006). The use of an unpleasant sound as an unconditional stimulus in a human aversive Pavlovian conditioning procedure. *Biological Psychology*, 73(2), 175–185. <https://doi.org/10.1016/j.biopsycho.2006.03.004>
- Ney, L. J., Wade, M., Reynolds, A., Zuj, D. V., Dymond, S., Matthews, A., & Felmingham, K. L. (2018). Critical evaluation of current data analysis strategies for psychophysiological measures of fear conditioning and extinction in humans. In *International Journal of Psychophysiology* (Vol. 134, pp. 95–107). Elsevier B.V. <https://doi.org/10.1016/j.ijpsycho.2018.10.010>
- Ojala, K. E., & Bach, D. R. (2020). Measuring learning in human classical threat conditioning: Translational, cognitive and methodological considerations. In *Neuroscience and Biobehavioral Reviews* (Vol. 114, pp. 96–112). Elsevier Ltd. <https://doi.org/10.1016/j.neubiorev.2020.04.019>
- Pineles, S. L., Orr, M. R., & Orr, S. P. (2009). An alternative scoring method for skin conductance responding in a differential fear conditioning paradigm with a long-duration conditioned stimulus. *Psychophysiology*, 46(5), 984–995. <https://doi.org/10.1111/j.1469-8986.2009.00852.x>
- Prokasy, W. F., & Ebel, H. C. (1967). Three components of the classically conditioned GSR in human subjects. *Journal of Experimental Psychology*, 73(2), 247–256. <https://doi.org/10.1037/h0024108>
- Rehbein, M. A., Steinberg, C., Wessing, I., Pastor, M. C., Zwitterlood, P., Keuper, K., &

- Junghöfer, M. (2014). Rapid plasticity in the prefrontal cortex during affective associative learning. *PLoS ONE*, 9(10). <https://doi.org/10.1371/journal.pone.0110720>
- Reinhard, G., & Lachnit, H. (2002). Differential conditioning of anticipatory pupillary dilation responses in humans. *Biological Psychology*, 60(1), 51–68. [https://doi.org/10.1016/S0301-0511\(02\)00011-X](https://doi.org/10.1016/S0301-0511(02)00011-X)
- Reinhardt, I., Jansen, A., Kellermann, T., Schüppen, A., Kohn, N., Gerlach, A. L., & Kircher, T. (2010). Neural correlates of aversive conditioning: Development of a functional imaging paradigm for the investigation of anxiety disorders. *European Archives of Psychiatry and Clinical Neuroscience*, 260(6), 443–453. <https://doi.org/10.1007/s00406-010-0099-9>
- Sassenhagen, J., & Draschkow, D. (2019). Cluster-based permutation tests of MEG/EEG data do not establish significance of effect latency or location. *Psychophysiology*, 56(6), e13335. <https://doi.org/10.1111/psyp.13335>
- Sehlmeyer, C., Dannlowski, U., Schöning, S., Kugel, H., Pyka, M., Pfleiderer, B., Zwitterlood, P., Schiffbauer, H., Heindel, W., Arolt, V., & Konrad, C. (2011). Neural correlates of trait anxiety in fear extinction. *Psychological Medicine*, 41(4), 789–798. <https://doi.org/10.1017/S0033291710001248>
- Sevenster, D., Beckers, T., & Kindt, M. (2012). Instructed extinction differentially affects the emotional and cognitive expression of associative fear memory. *Psychophysiology*, 49(10), 1426–1435. <https://doi.org/10.1111/j.1469-8986.2012.01450.x>
- Sperl, M. F. J., Panitz, C., & Hermann, C. (2016). *A pragmatic comparison of noise burst and electric shock unconditioned stimuli for fear conditioning research with many trials*. 53, 1352–1365. <https://doi.org/10.1111/psyp.12677>
- Sperl, M. F. J., Panitz, C., Rosso, I. M., Dillon, D. G., Kumar, P., Hermann, A., Whitton, A. E., Hermann, C., Pizzagalli, D. A., & Mueller, E. M. (2018). Fear Extinction Recall Modulates Human Frontomedial Theta and Amygdala Activity. *Cerebral Cortex*, April, 1–15. <https://doi.org/10.1093/cercor/bhx353>
- Spielberger, C. D., Gorsuch, R. L., Lushene, R. E., Vagg, P. R., & Jacobs, G. A. (1983). *Manual for the State-Trait Anxiety Inventory*.
- Steinberg, C., Bröckelmann, A., Rehbein, M., Dobel, C., & Junghöfer, M. (2013). Rapid and

- highly resolving associative affective learning : Convergent electro- and magnetoencephalographic evidence from vision and audition. *Biological Psychology*, 92(3), 526–540. <https://doi.org/10.1016/j.biopsycho.2012.02.009>
- Tzovara, A., Korn, C. W., & Bach, D. R. (2018). Human Pavlovian fear conditioning conforms to probabilistic learning. *PLOS Computational Biology*, 14(8), e1006243. <https://doi.org/10.1371/journal.pcbi.1006243>
- Tzovara, A., Meyer, S. S., Bonaiuto, J. J., Abivardi, A., Dolan, R. J., Barnes, G. R., & Bach, D. R. (2019). High-precision magnetoencephalography for reconstructing amygdalar and hippocampal oscillations during prediction of safety and threat. *Human Brain Mapping*, 40(14), 4114–4129. <https://doi.org/10.1002/hbm.24689>
- Visser, R. M., de Haan, M. I. C., Beemsterboer, T., Haver, P., Kindt, M., & Scholte, H. S. (2016). Quantifying learning-dependent changes in the brain: Single-trial multivoxel pattern analysis requires slow event-related fMRI. *Psychophysiology*, 53(8), 1117–1127. <https://doi.org/10.1111/psyp.12665>
- Visser, R. M., Kunze, A. E., Westhoff, B., Scholte, H. S., & Kindt, M. (2015). Representational similarity analysis offers a preview of the noradrenergic modulation of long-term fear memory at the time of encoding. *Psychoneuroendocrinology*, 55, 8–20. <https://doi.org/10.1016/j.psyneuen.2015.01.021>
- Visser, R. M., Scholte, H. S., Beemsterboer, T., & Kindt, M. (2013). Neural pattern similarity predicts long-term fear memory. *Nature Neuroscience*, 16(4), 388–390. <https://doi.org/10.1038/nn.3345>
- Wendt, J., Hufenbach, M. C., König, J., & Hamm, A. O. (2020). Effects of verbal instructions and physical threat removal prior to extinction training on the return of conditioned fear. *Scientific Reports*, 10(1), 1–14. <https://doi.org/10.1038/s41598-020-57934-7>
- Westfall, J., Nichols, T. E., & Yarkoni, T. (2017). Fixing the stimulus-as-fixed-effect fallacy in task fMRI. *Wellcome Open Research*, 1, 23. <https://doi.org/10.12688/wellcomeopenres.10298.2>
- Winn, M. B., Wendt, D., Koelewijn, T., & Kuchinsky, S. E. (2018). Best Practices and Advice for Using Pupillometry to Measure Listening Effort: An Introduction for Those Who Want to Get Started. In *Trends in Hearing* (Vol. 22). SAGE Publications Inc.

<https://doi.org/10.1177/2331216518800869>

Yarkoni, T. (2020). *PsyArXiv Preprints / The Generalizability Crisis*.

<https://psyarxiv.com/jqw35>