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ORIGINAL ARTICLE

Postural freezing relates to startle potentiation in a human fear-conditioning paradigm

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Abstract

Freezing to impending threat is a core defensive response. It has been studied primarily using fear conditioning in non-human animals, thwarting advances in translational human anxiety research that has used other indices, such as skin conductance responses. Here we examine postural freezing as a human conditioning index for translational anxiety research. We employed a mixed cued/contextual fear-conditioning paradigm where one context signals the occurrence of the US upon the presentation of the CS, and another context signals that the CS is not followed by the US. Critically, during the following generalization phase, the CS is presented in a third and novel context. We show that human freezing is highly sensitive to fear conditioning, generalizes to ambiguous contexts, and amplifies with threat imminence. Intriguingly, stronger parasympathetically driven freezing under threat, but not sympathetically mediated skin conductance, predicts subsequent startle magnitude. These results demonstrate that humans show fear-conditioned animal-like freezing responses, known to aid in active preparation for unexpected attack, and that freezing captures real-life anxiety expression. Conditioned freezing offers a promising new, non-invasive, and continuous, readout for human fear conditioning, paving the way for future translational studies into human fear and anxiety.

KEYWORDS
anxiety, defensive responses, fear-conditioning, fear potentiated startle, freezing

1 | INTRODUCTION

In a constantly changing environment, it is imperative to learn and remember which cues signal threat. Subsequent exposure to such threat cues is known to elicit a distinct set of conditioned defensive responses, such as freezing in animals. The fear-conditioning model has helped to understand not only how defensive responses are learned, but also what situational factors can subsequently alter these responses. Fear conditioning has therefore evolved as the most compelling model for understanding the etiology and treatment of anxiety- and stressor-related disorders (Briscione et al., 2014). Because the model's neural underpinnings are highly preserved cross-species, and more and more studies take a translational approach for understanding psychopathology (Kozak & Cuthbert, 2016), the use of the fear-conditioning model is bourgeoning more than ever (Lonsdorf et al., 2017). However, the primary index of conditioned defensive responding in animals, defensive freezing, has not been studied in human conditioning studies, hampering true translational advances in human anxiety research.

In contrast to animal studies that predominantly employ freezing, human fear-conditioning studies most commonly employ skin conductance responses (SCR) and fear-potentiated startle (FPS) (Lonsdorf et al., 2017) to track learning of conditioned responding. This disparate use of readout measures is hampering reliable interpretation of human conditioning studies (Lonsdorf et al., 2017; Ney et al., 2018). The startle is currently the principal translational measure (Briscione et al., 2014), as it has been the only measure for which basic research has resulted in clinical applications ("bench to bedside," Fendt & Koch, 2013). The startle appears to index a basic, affective level of fear conditioning, and –reminiscent of non-rational anxiety—is less sensitive to modulation by higher order cognitive processes than SCR (Hamm & Weike, 2005). Indeed, alterations in conditioned startle relate to clinical symptoms of anxiety (e.g., Gazendam et al., 2012; Grillon et al., 2009).

In non-human animals, postural freezing is the prevailing conditioning readout (Fanselow & Poulos, 2005). Freezing to imminent threat is an imperative defensive response, involving strong suppression of body activity (Roelofs, 2017). Interestingly, stronger freezing reactions have been observed in anxious and traumatized rodents (Champagne et al., 2008). Despite this translational promise, only recently postural freezing was successfully assessed in humans, by indexing shifts in body posture (i.e., postural sway) to for instance unpleasant images (Roelofs et al., 2010). Like in animals, individual differences in freezing as a function of anxiety have been revealed (Hagenaars et al., 2014; Roelofs, 2017). Such studies also revealed that freezing is often accompanied by heart rate deceleration, bradycardia (Roelofs, 2017). In order to truly advance translational anxiety research it would be highly beneficial to examine postural freezing and bradycardia as potential conditioning readouts as these measures, just like the startle, can be directly linked to both basic animal studies and clinical anxiety (Kozak & Cuthbert, 2016).

Importantly, if freezing and bradycardia were to be appropriate alternatives to startle, it should also be assessed to what extent these relate to startle in comparison with other measures such as SCR. As freezing and bradycardia are considered anticipatory states preparing for effective coping with imminent threat (Gladwin et al., 2016), while the startle is elicited in response to a sudden sound or movement thought to facilitate fight or flight (Yeomans & Frankland, 1995), it can be fathomed that the intensity of such a preparatory state correspondingly amplifies the strength of an ensuing startle reflex. In line with this idea, freezing animals are easily startled (Fendt & Fanselow, 1999). More generally, an inverse relationship exists between postural mobility and the FPS: active rodents are harder to startle (Leaton & Borszcz, 1985; Walker et al., 1997; Wecker & Ison, 1986). Interestingly, in the advent of scientific interest in FPS, Leaton and Borszcz (1985) hypothesized that freezing was an essential premise to the initiation of FPS. Indeed, *within* individual animals the percentage of freezing was correlated with the magnitude of their startle amplitudes. This relationship was noticeably reduced when no direct threat was present (Leaton & Borszcz, 1985). Likewise, observations in other animal studies suggest that fear-conditioned bradycardia may facilitate subsequent FPS (Hunt et al., 1994; Whalen & Kapp, 1991). Insights regarding the mediating neural circuitry of these measures may provide further indications that the strength of preparatory freeze and bradycardia are closely related to the elicited startle magnitude. Sensory inputs containing information about a context and/or conditioned stimulus (CS) and unconditioned stimulus (US) terminate in the lateral amygdala (LA) where conditioning-induced plasticity represents the CS/

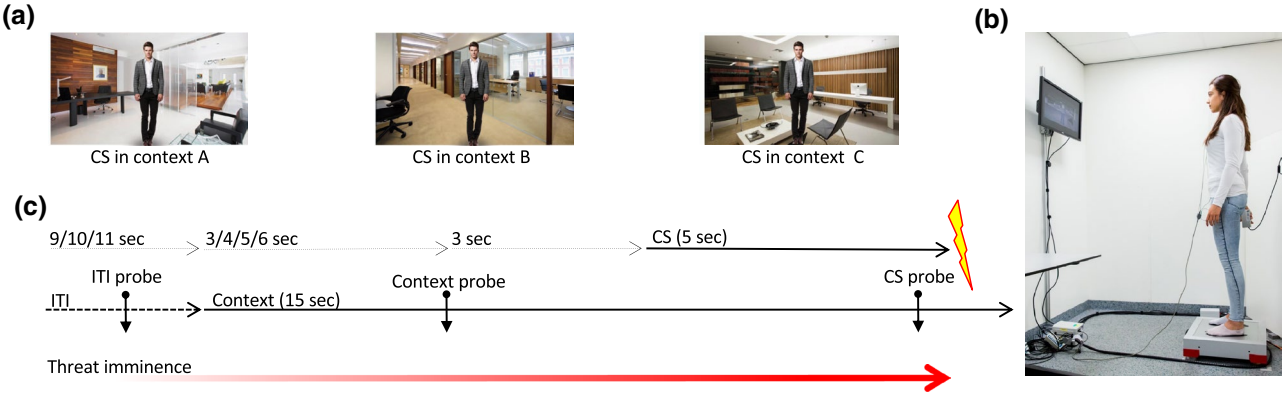
context-US link. But then, in response to a specific conditioned cue, *several* output pathways from the central medial nucleus of the amygdala (CEm) directly regulate distinct fear behaviors (Tovote et al., 2015). In humans (Kuhn et al., 2019) and rodents (Fendt, 1998) alike, FPS is mediated by the periaqueductal gray (PAG). Its ventrolateral part (vlPAG) has further been dubbed the "immobility center" driving freezing and bradycardia (Walker & Carrive, 2003). Specifically, the vlPAG mediates parasympathetic outflow directed to the heart, contributing to the expression of fear bradycardia (Koba et al., 2016), while simultaneously imposing postural immobility (Walker & Carrive, 2003). An adjacent PAG region, the lateral PAG (lPAG) can amplify the FPS (of which the motor reflex itself is initiated in the nucleus reticularis pontis caudalis [PnC]) (Fendt, 1998). Further, both freezing (Power & McGaugh, 2002) and startle (Greba et al., 2000; Winkler et al., 2000) are mediated by acetylcholine (ACh), the main neurotransmitter of the parasympathetic system. ACh injected into the vlPAG can indeed magnify freezing (Monassi et al., 1997), while its inhibition in the dorsolateral PAG (dlPAG) is associated with fight-or-flight-related actions (Burnstock, 1978). In contrast to parasympathetically mediated freezing, bradycardia, and startle, arousal measures such as SCR (Boucsein, 1992) and pupil dilation (Liu et al., 2017; Loewenfeld & Lowenstein, 1993) are mediated via other output regions from the CEm such as the locus coeruleus (Aston-Jones & Cohen, 2005), and these sympathetic measures are thought to reflect a different role in the defense cascade (Löw et al., 2015). Taken together, freeze, bradycardia, and startle may very well operate in synchrony during conditioned threat-anticipation, unlike SCR.

As the main aim of the current study was to investigate human freezing as a novel translational tool in human anxiety research, we reasoned that such a novel index should not only be sensitive to standard differential conditioning, it should also be responsive to fear generalization procedures, for this is considered a key symptom of clinical anxiety (Dunsmoor & Paz, 2015; Lissek et al., 2008). Specifically, given the central role contexts play in the interpretation of stimuli as being predictive of actual threat (e.g., a snake in a terrarium is harmless), it is believed that alterations in contextual processing may pose an important vulnerability for the development of anxiety (Maren et al., 2013), likely caused by alterations in hippocampal functioning contributing to exacerbated fear generalization (Kheirbek et al., 2012). In addition to context, another situational factor that is well-known to modulate the expression of (conditioned) fear responses—in animal models this has been freezing in particular—is threat imminence (Blanchard et al., 2011; Briscione et al., 2014; Fanselow, 1994). For this reason, we also aimed to test whether conditioned freezing indeed intensifies with increasing threat imminence. The employed experimental paradigm was designed to accommodate these requirements (see Figure 1), and we combined it with postural sway assessments in humans. Specifically, our paradigm is a conditional discrimination task based on context (e.g., Schmajuk & Buhusi, 1997), where one context (threat context) signals the occurrence of the US upon CS presentation (~80% reinforcement rate), and another context (safe context) signals that the CS is not followed by the US. In other words, the paradigm can be considered a mixed cued/contextual fear-conditioning paradigm. Critically, during the following generalization phase, the CS is presented without the US in a third novel and ambiguous context (the generalization context, Van Ast et al., 2012; Mühlberger et al., 2013; Sep et al., 2019). We hypothesized that postural freezing responses can be conditioned in humans, just as they can in other species. As part of a critical assessment for future translational anxiety-research, we additionally hypothesized that conditioned freeze would amplify along with threat proximity, as is described in threat-imminence and defense-cascade models (Fanselow, 1994; Lang & Bradley, 2010; Löw et al., 2015), and would generalize to new contexts, indicative of anxiety proneness (Maren et al., 2013). Further, given the role of the (vl)PAG in freeze, bradycardia, and startle, we hypothesized that the intensity of a given freeze or bradycardia response would predict the magnitude of a subsequent startle amplitude on a trial-by-trial base. In other words, within subjects, we tested whether larger freezing and/or bradycardia responses would be associated with stronger startle responses. For SCR, we expected no predictive value for startle magnitude (Hamm & Weike, 2005; Lang & Bradley, 2010).

2 | METHOD

2.1 | Participants

Because no other human studies have experimentally induced conditioned freezing responses, we based our a priori sample-size calculation on the following line of reasoning: the main aim of our study was to assess postural freeze as an alternative to FPS without the startle's shortcomings. For that reason, and as freezing is most closely connected to the startle in animals (Leaton & Borszcz, 1985), revealing statistically significant within-subject conditioning and generalization effects should require a similar amount of participants as earlier startle studies. Typically, differential conditioning (Gazendam et al., 2012; Kindt et al., 2009) and generalization (Lissek et al., 2008, 2010, 2014; Van Ast et al., 2012) in studies using the



(d)

	Probe habituation	Context habituation	Break	Acquisition part 1	Break	Acquisition part 2	Generalization	Break	Further extinction
Condition		2 × Cxt threat 2 × Cxt safe 2 × Cxt gener		6 × CS+ in Cxt threat 2 × CS- in Cxt threat 8 × CS- in Cxt safe		4 × CS+ in Cxt threat 4 × CS- in Cxt safe	4 × CS- in Cxt threat 4 × CS- in Cxt safe 4 × CS- in Cxt gener		4 × CS- in Cxt threat 4 × CS- in Cxt safe 4 × CS- in Cxt gener
Probe type	9 × NA	6 × Cxt probe 1 × NA		16 × CS probe 4 × Cxt threat probe 4 x Cxt safe probe 4 × NA		8 × CS probe 2 × Cxt threat probe 2 x Cxt safe probe 2 × NA	12 × CS probe 2 × Cxt threat probe 2 × Cxt safe probe 2 × Cxt gener probe 2 × NA		12 × CS probe 2 × Cxt threat probe 2 × Cxt safe probe 2 × Cxt gener probe 2 × NA

FIGURE 1 The fear-conditioning task. The pictures in (a) show images of the conditioned stimulus (CS, a person) against in total three background contexts (A, B, C) that represent the threat, safe, and generalization condition. In (b) the participant set-up can be seen, with the stabilometric platform that assessed postural sway and a screen at eye-height that presented the fear-conditioning task. A timeline of a typical trial can be observed in (c), with the timing of startle probes depicted. The red line represents increasing threat imminence over the course of a trial, starting from the inter-trial interval (ITI), through the context, to the CS. The fear-conditioning task consisted of several phases (d) that were intermitted with 1-min breaks off the platform. The amount of trials per condition are described as combinations of reinforced (CS+) or unreinforced (CS−) versions of the CS presented in the different contexts (Cxt). Further, the number of startle probes are described per condition: noise alone (NA) probes that are presented during habituation and the ITIs, Cxt probes that are presented during either of the three contexts, and the probes that are presented during the CS in any of these conditions

startle reveal medium to very strong effect sizes. Given risks of overestimation of effect size (Gelman & Carlin, 2014) we chose to be on the safe side and set our minimal effect of interest to medium ($f = 0.25$). To detect such a differential conditioned (i.e., main effect of Condition with two levels) freezing and startle effect with a power of 0.8 and a correlation among repeated measures of $r = 0.6$ (Van Ast et al., 2012), 28 participants would be sufficient. This is also well-above the minimal recommended group sample size for fear-conditioning studies (Ney et al., 2018). Anticipating some dropout, thirty students at the Radboud University Nijmegen participated in the study. This sample size is also sufficient to give reliable parameter estimates in a two-level multilevel model (Maas & Hox, 2005).

Participants were recruited through the online university recruitment system, and were rewarded by either course credit or €10. Eligibility was assessed by self-report, and conditional on being between 18 and 35 years of age, being sufficiently proficient in Dutch, having no current or past physical, psychological, or neurological disorder, and not having participated previously in a similar study. Participation of two participants was prematurely ended; one due to inability to keep standing on the force platform, and another due to a faulty shock electrode. Consequently, the final sample consisted of 28 participants (17 women), with a mean age of 22.9 years ($SD = 3.3$ years). Further, due to technical failure, ECG data of one participant was not recorded. The study was approved by the local ethics committee of the Radboud University Nijmegen, and all participants gave written informed consent prior to participation.

2.2 | Materials

2.2.1 | Experimental fear-conditioning task

The design of the current fear-conditioning task (Figure 1) capitalized on the idea that contexts serve to disambiguate the meaning of central cues. Given the chief role contexts play in the interpretation of a wide variety of stimuli surrounding us, it is believed that alterations in contextual

processing may pose an important vulnerability for the development of anxiety (Maren et al., 2013), likely caused by alterations in hippocampal functioning contributing to exacerbated fear generalization. The paradigm was based on previous studies using a similar design (Mühlberger et al., 2013; Schmajuk & Buhusi, 1997; Sep et al., 2019; Van Ast et al., 2012). For the *threat* condition, presentation of the CS in one specific context (background picture) predicted the occurrence of the unconditioned stimulus (US; shock). The reinforcement rate of the CS in the threat context was approximately 80% (2 trials out of 12 were not reinforced). Upon each context presentation, the CS was always presented, one single time. Timing of CS onset was variable relative to context onset. In another context, the same CS was not followed by the US (*safe* condition). During the unreinforced generalization phase, these two conditions were alternated with presentation of the CS in a new, and thereby ambiguous, context, enabling us to assess conditioned fear generalization across context (generalization condition). Modulation of defensive responses by threat imminence was assessed along a continuum commencing in the inter-trial-intervals, to the context, to the CS, up until startle probe presentation. Standard differential conditioning could be assessed by comparing defensive reactions during the CS in threat versus safe context.

More specifically, a total of three different background images (i.e., contexts, see Figure 1a) was used, all depicting offices. Assignment of these images to either the threat, safe, or generalization condition was counterbalanced across participants. In the experiment only one CS was used, a picture of a standing person in a casual-chic office suit. Any given trial during the experiment consisted of the same build-up (Figure 1c), starting with an inter-trial interval (ITI) that took a variable 10 ± 1 s. Then the context appeared, that was always presented for a total of 12 s. After a variable time-interval of 3, 4, 5, or 6 s, a context probe could be presented. The probe was followed by onset of the CS after three seconds. The CS was presented for 5 s and the according startle probe was always presented at 4.5 s. When applicable (i.e., when reinforced during the acquisition phase), the shock was presented at CS offset. Upon disappearance of the CS the context was visible for its remaining duration.

The paradigm consisted of several phases (for an overview, see Figure 1d). It started out with a *probe habituation phase* to the startle probes (noise alone, NA), in order to reduce possible initial reactivity of blink responding. In total 9 startle probes were presented, with an inter-probe interval of 9, 11, or 13 s. Then, the *context habituation phase* commenced, which was designed to familiarize participants with all contexts (presented without the CS) and to exclude the possibility that conditioned responses to the generalization context presented later in the test phase could be explained by mere novelty effects. Also, one NA probe was presented. After a first one-min break off the platform, the actual acquisition phase commenced. During this phase, a total of 12 threat trials and 12 safe trials was presented. The CS in the threat context was reinforced 10 times (reinforcement rate ~80%). The two unreinforced trials were fixed to the third and the seventh threat trial presentation to keep learning rates comparable across subjects. After 16 trials (i.e., at 2/3 of the phase) another break was implemented, followed by the remainder of acquisition. During the ensuing *generalization test*, again 4 threat and 4 safe trails were presented, intermixed with 4 generalization trials. None of these trials were reinforced. During the generalization trials, the same CS was presented but against a new background context. As such, the interpretation of the CS-context combination in terms of shock reinforcement was ambiguous. The generalization test phase always started with the generalization trial, followed by a safe and threat trial, in order to obtain a clean primary response on generalization that would be comparable across participants. After another short break off the platform, the experiment continued with the further *extinction test*, in fact just a repetition of the generalization test.

Presentation of all the stimuli in the experiment was semi-randomized. For acquisition, safe and threat trials were shuffled in blocks of two trials and in later phases the safe, threat and generalization trials were shuffled in blocks of three. Consequently, no more than two of the same trials could follow-up on each other. Startle probes were presented during all CS images. A context probe was presented randomly every two trials of each threat type. ITI probes were presented randomly every four trials of each threat type. In summary, every four trials (or six during the test phases) two safe and two threat trials, one context safe probe and one context threat probe, and one ITI probe was presented.

2.2.2 | Physiological and postural measures

All data were sampled at a rate of 3000 Hz using a BrainAMP ambulatory device (EXG MR 16 channel and EXG AUX Box) and recorded using BrainVision Recorder software (Brain Products GmbH, Munich, Germany).

Postural sway

Following the procedure of previous studies in the same lab (Gladwin et al., 2016; Roelofs et al., 2010), participants' task-induced changes in postural sway were assessed by having them stand on a custom-made 50 × 50 cm strain-gauge force platform (Figure 1b). Four pressure sensors, one at each corner, allowed for recording a time series of

changes in resistance due to dynamics in body posture of a participant during the experiment. Prior to each test-session, the platform was calibrated using a 20 kg weight.

Fear-potentiated startle

FPS reflexes were probed by 104 dB, 40 ms bursts of white noise with a near instant rise time. Probes were delivered binaurally through headphones. Prior to each test session sound pressure and dB level of the startle probes were measured and if needed (re)calibrated using a sound level meter (Rion, NA-27, Japan). Three 2.5 mm Ag/AgCl electrodes filled with a conductive gel (Signa, Parker) were used to measure electromyography (EMG) of the left orbicularis oculi muscle. Two of these electrodes were placed approximately 1 cm under the pupil and 1 cm below the lateral canthus (outer corner of the eye; Fridlund & Cacioppo, 1986). Another electrode was used as reference, and placed on the forehead (Blumenthal et al., 2005), 1 cm below the hairline while taking care not to compromise the participant's vision with the help of some tape.

Heart rate

Electrocardiograms (ECG) were collected using three Ag/AgCl electrodes containing adhesive patches (3 M Red Dot Electrode). One electrode was placed below the right clavicle and one on the left side of the chest, just below the sixth rib. The ground electrode was attached under the left clavicle.

Skin conductance response

Skin conductance was registered by placing two Ag/AgCl electrodes that were attached to the medial phalanges of the first and third fingers of the left hand.

Electrical stimulation

Electrical shocks in the fear-conditioning task were delivered to the outside of the participants' wrist of the non-preferred hand by a 9V battery-operated Tens Elpha 2000 device (Danmeter, Odense, Denmark) using standard Ag/AgCl electrodes filled with electrode gel. Shocks were delivered using a MAXTENS 2000 (Bio-Protech) device. Shock duration was 200 ms at 150 Hz, and intensity varied in 10 intensity steps between 0 and 80 mA.

2.2.3 | Study procedure

Upon arrival participants were explained the upcoming procedures by means of an information brochure, and informed consent was obtained. Next a short medical interview was taken, and participants filled out some questionnaires to assess baseline self-reported mood states (not further analysed). After electrode attachment for heart rate and startle and a small check of their proper functioning, the experimenter attached the electrodes for the electrical stimulation. The participant was instructed on the procedure of the upcoming shock intensity calibration. According to a standardized procedure (Klumpers et al., 2010) during which participants received and rated 5 consecutive shocks, intensity of the stimulation was set to a level that the participant experienced as being uncomfortable but not painful. With regard to the main task, the participant was instructed to learn to predict the occurrence of the electric stimulation on the basis of the combination of foreground and background pictures. With regard to the force platform, participants were instructed to equally distribute their weight over both legs, while adapting a comfortable posture with their arms relaxed along their torso and their feet slightly separated as indicated by two pictures of black footprints that were stickered to the platform. After taking their shoes of and stepping on the force platform, their posture was corrected if necessary and headphones were placed on the participant's head. The computer monitor was adjusted to the eye-height of the participant, at a viewing distance of 50 cm. During the several breaks in the task -standing on the platform is fatiguing- participants sat on a chair. After the main task, all electrodes were removed, and the participant filled out again some mood questionnaires and a post-experimental questionnaire.

2.3 | Data reduction

2.3.1 | Postural sway

Posturographic analyses were conducted in MATLAB (MathWorks, Natick, Massachusetts, USA). To reduce the total amount of data, the data set was down-sampled from 3000 to 600 Hz. Data were analyzed in accord with previous studies from the lab (Gladwin et al., 2016; Niermann et al., 2015). First, data were filtered using a 10 Hz low-pass and a 0.1 Hz high-pass filter. Next, for each participant, the mean position of the center of pressure (COP) in the anterior-posterior (AP) was calculated per sample point. Then, variability in raw sway per 500 ms was computed as the standard deviation from the COP, while adjusting for the individual's weight. Finally, for data per individual, segments containing outliers (defined as $Z > 3$), were replaced by taking the mean of the closest two ensuing data points (computed per threat type and per phase). In total, this procedure resulted in 1.7% of data that were replaced. Previous studies have shown that emotion does not -or to a lesser extend- modulate postural sway in the medial-lateral (ML) direction. This is related to the fact

that bipedal stance leaves more leverage to move in the AP-direction as compared to the ML-direction (Gladwin et al., 2016; Hashemi et al., 2019; Niermann et al., 2015; Roelofs et al., 2010). For this reason, and following a multitude of previous studies, we focused on the AP-data. Nevertheless, for completeness, we exploratorily analyzed the ML data as well, and report on these data in the supplement. Note that lower postural sway scores demarcate decreased body mobility, and thus, increases in postural freeze.

2.3.2 | Fear-potentiated startle

The startle data were initially processed with Vision Analyzer software (Version 2, Brain Products, brainproducts.com). To maximize signal-to-noise ratio, raw EMG data were conditioned to a band-pass between 28-Hz, 12- dB/oct high-pass and a 400-Hz, 24-dB/oct low-pass and a 50-Hz notch filter in line with recommendations (Blumenthal et al., 2005). Then, using a custom-made MATLAB (MathWorks, Natick, Massachusetts, USA) pipeline used in previous published work (Klumpers et al., 2010), data were locked to the startle probes starting 50 ms before onset and ending 200 ms after onset and then segmented into epochs. Then, the signal was baseline corrected, rectified, and a low-pass filter (12 Hz, 12 dB/oct) was applied for smoothing. From this baseline-corrected signal, blink response-amplitudes were derived by searching for the first peak in a latency window of 25–100 ms. Trials that had activity in a window of 30 ms preceding the marker and 20 ms after the marker that was greater than 2 standard deviations from the mean baseline activity were considered an artefact and consequently, rejected. Null responses were defined as trials in which the standard deviation of the signal increased with less than 55% from baseline (Klumpers et al., 2010). Then, for data per individual, trials containing outliers (defined as $Z > 3$) or artefacts, were replaced by linear trend at point (computed per threat type and per phase) (Van Ast et al., 2012). In total, this procedure resulted in 5.1% of replaced missing data.

2.3.3 | Heart rate

The electrocardiogram (ECG) data were initially processed with Vision Analyzer software (Version 2, Brain Products, brainproducts.com). R-peak detections were visually inspected, wherever necessary manually corrected, and then extracted to calculate inter-beat intervals (IBI, the interval between two successive R-spikes). Just like the postural sway, average beats/minute were calculated for each bin of 500 ms.

2.3.4 | Skin conductance response

Skin conductance data were analyzed using an in-house analysis program written in MATLAB (MathWorks, Natick, Massachusetts, USA), as implemented in VSRRP (developed by the Technical Support Group Psychology at the University of Amsterdam). Like for heart rate and sway, averages were calculated for each bin of 500 ms. Responses were defined by calculating peak differences versus preceding baseline, as further described in the data analysis section.

2.4 | Data analysis

Is freezing sensitive to fear conditioning, context generalization, and threat imminence?

For the first set of analyses, the main focus was to assess whether human postural sway can be conditioned, can be modulated by threat imminence, and shows generalization across contexts. For comparative purposes, besides the primary postural sway measure, SCR, HR and startle were analyzed in a similar vein as well (see Figures 2–5, and Supplementary Results in the Supporting information). Because we are introducing a new measure of conditioned fear responses, we aimed to stay close to the traditional analytical approach in the fear-conditioning field (Ney et al., 2018), to ensure that results are maximally comparable to earlier studies. Therefore, these analyses were conducted using repeated measures ANOVAs.

2.4.1 | Fear conditioning and generalization

For an initial fine-grained analysis of dynamic changes in the continuous measures such as postural sway we ran an analysis for the entire duration of the CS (until startle probe onset) with a Condition (safe and threat for acquisition, generalization was added for the generalization phase) × Time (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 s) repeated measures ANOVA. To do so, HR and SCR change scores (per segment of 0.5 s) were computed relative to a 0.5-s baseline preceding the CS (Van Ast et al., 2012), and then averaged per 0.5 s segment across the respective phase. We expected a main effect of Condition. Then, to assess fear conditioning in a more traditional way, we ran another analysis that included only aggregated responses during the CSs with a Condition (safe and threat for acquisition, generalization was added for the generalization phase) × Trial number (1–12 for acquisition, 1–4 for generalization) repeated-measures ANOVA. For sway, depending on the presence or absence of an interaction with the factor Time in the previous analysis (i.e., indicating that responses change over time during the CS-presentation), responses were

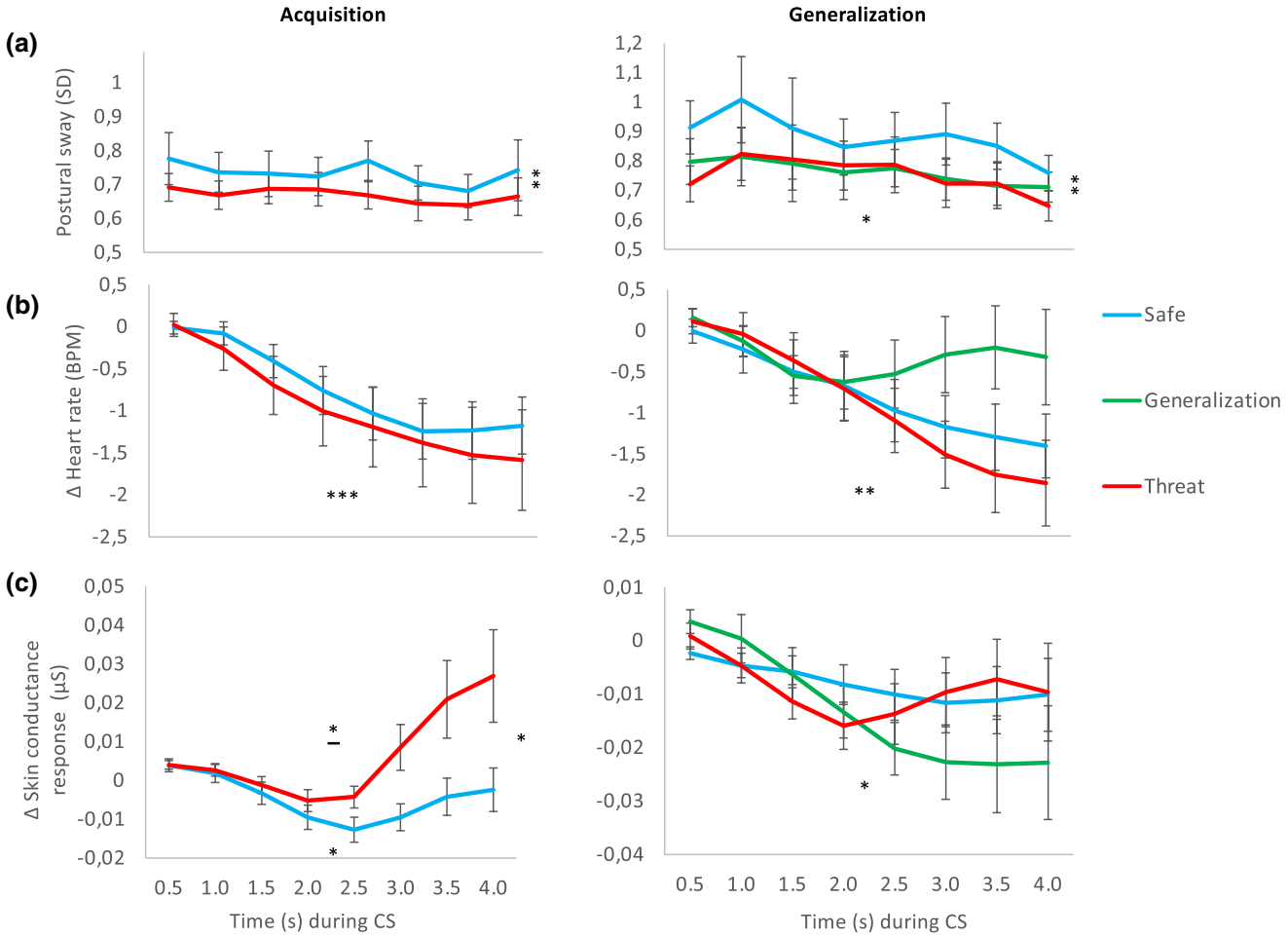


FIGURE 2 Dynamic physiological responses during the time window of the CS averaged across all trials per phase. Data for all continuous measures (i.e., postural sway (a), heart rate (b), skin conductance response (c)) sampled from the time-window from CS-onset until startle probe presentation, averaged in bins of half seconds for the acquisition phase and generalization phase, as a function of condition (safe, generalization, and threat). Data in (b) and (c) are presented relative to a half-second average preceding the CS. Error bars represent standard errors of the mean. Significant main effects of Condition are depicted right of the respective graph, main effects of Time below, and Condition × Time are underscored and depicted above the graph. Obtained *p*-values are indicated by: ***$p < .001$, **$p < .01$, *$p < .05$

obtained by either averaging across the entire CS-duration, or defining a peak-response based on the 0.5 s segments, respectively. Again, we expected a main effect of Condition.

2.4.2 | Threat imminence

For the threat-imminence analysis, segments of continuous data from the ITIs (3-s before potential probe onset), early context (first 3-s), late context (2-s before CS onset), and CS (4-s) were averaged. The choice of data-segments was such that timing of these segments was as comparable as possible to the startle probes, while at the same time minimizing interference by preceding startle probe presentation. For HR and SCR change scores were then computed relative to a 0.5-s baseline preceding the ITI-segment (Van Ast et al., 2012). The omnibus repeated measures ANOVA contained the within-factors Condition (safe and threat for acquisition, generalization was added for the generalization phase) and Imminence (ITI, context early, context late, CS). We expected a linear decrease in postural body sway for Imminence, most pronouncedly so for the threat Condition (indicated by a linear Imminence × Condition interaction contrast). As the freeze data indicated extinction at the end of the generalization phase, the extinction phase data were not further analyzed. For FPS, there were 3 Imminence data points (ITI, context, CS), instead of 4. All analyses were performed using SPSS 25.0 (IBM SPSS Statistics, IBM Corporation, Armonk, NY). We set $p < .05$ for all statistical tests. To compensate for skewed distributions, data were square-root transformed prior to analysis. Greenhouse-Geisser corrections of degrees of freedom were applied whenever necessary. Effect sizes are reported as partial eta-squared.

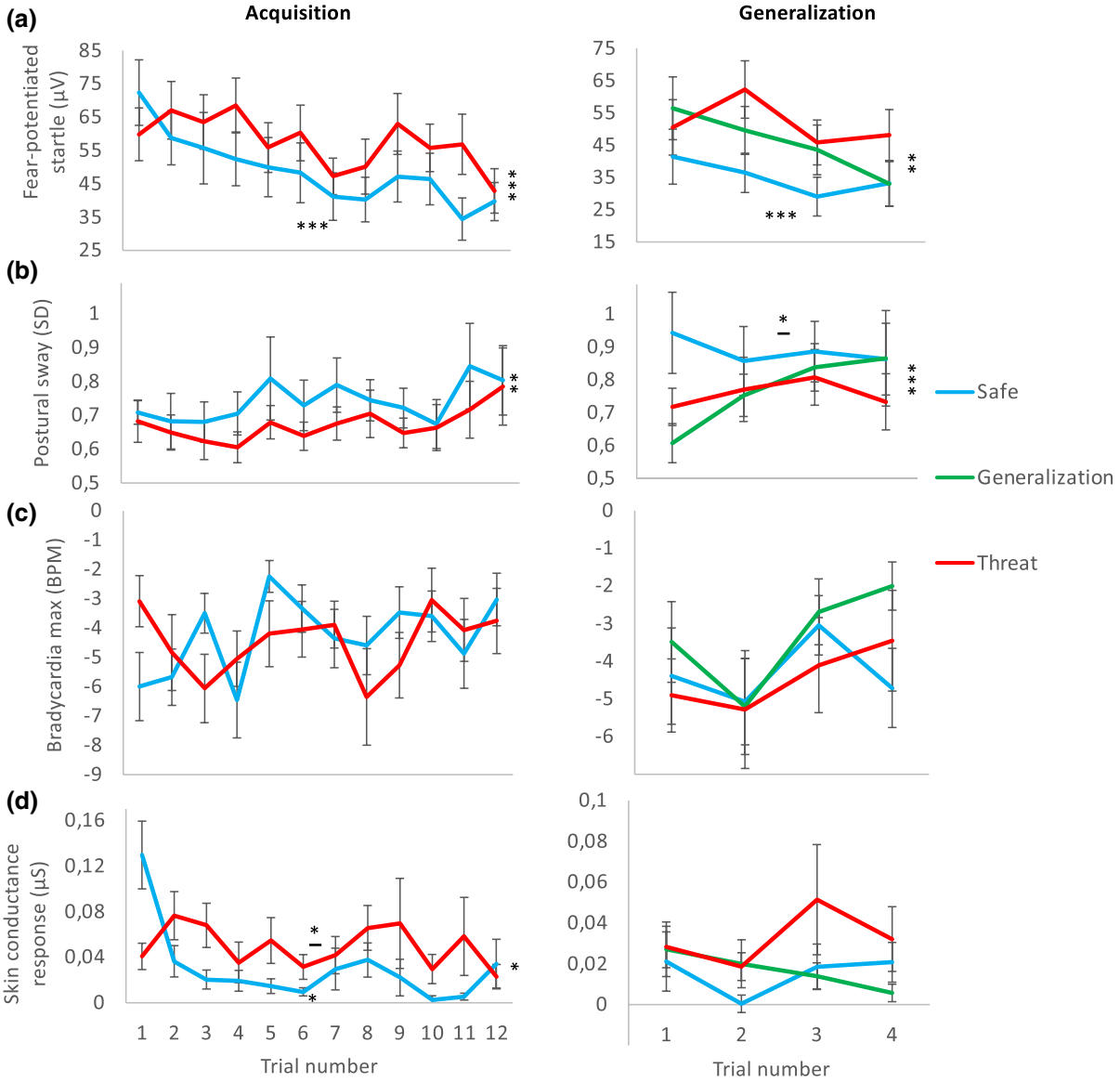


FIGURE 3 Conditioned responses during the CS presentations over the course of acquisition and generalization for each of the physiological measures. Fear-potentiated startle (a), postural sway (b), bradycardia (c), and skin conductance response (d) depicted per trial and per phase (acquisition and generalization) as a function of condition (safe, threat, and generalization). Data for the postural sway (b) are means calculated of the data sampled during each CS until startle probe presentation, while SCR represents the maximum response relative to the preceding baseline in that window, and bradycardia represents the maximum deceleration relative to the preceding baseline in that same window. Error bars represent standard errors of the mean. Significant main effects of Condition are depicted right of the respective graph, main effects of Trial number below, and Condition × Trial number are underscored and depicted above the graph. Obtained *p*-values are indicated by: ***$p < .001$, **$p < .01$, *$p < .05$

2.4.3 | Multilevel modelling

Do preparatory postural freeze and bradycardia modulate ensuing intensity of a startle response?

The second analysis approach served to assess the extent to which freeze, bradycardia, and SCR were interrelated with startle responses. We predicted that the intensity of a given freezing and related bradycardia response would predict the magnitude of a subsequent startle reflex on a given trial, on a trial-by-trial base. In the current fear-conditioning data-set, due to the repeated-measures design defensive responses are highly correlated within participants, and the strength of possible relationships between preparatory states and subsequent startle magnitudes may vary per subject. Multilevel modelling therefore is an appropriate approach, as it effectively deals with nested data and can assess whether relationships vary across subjects. It further allows for

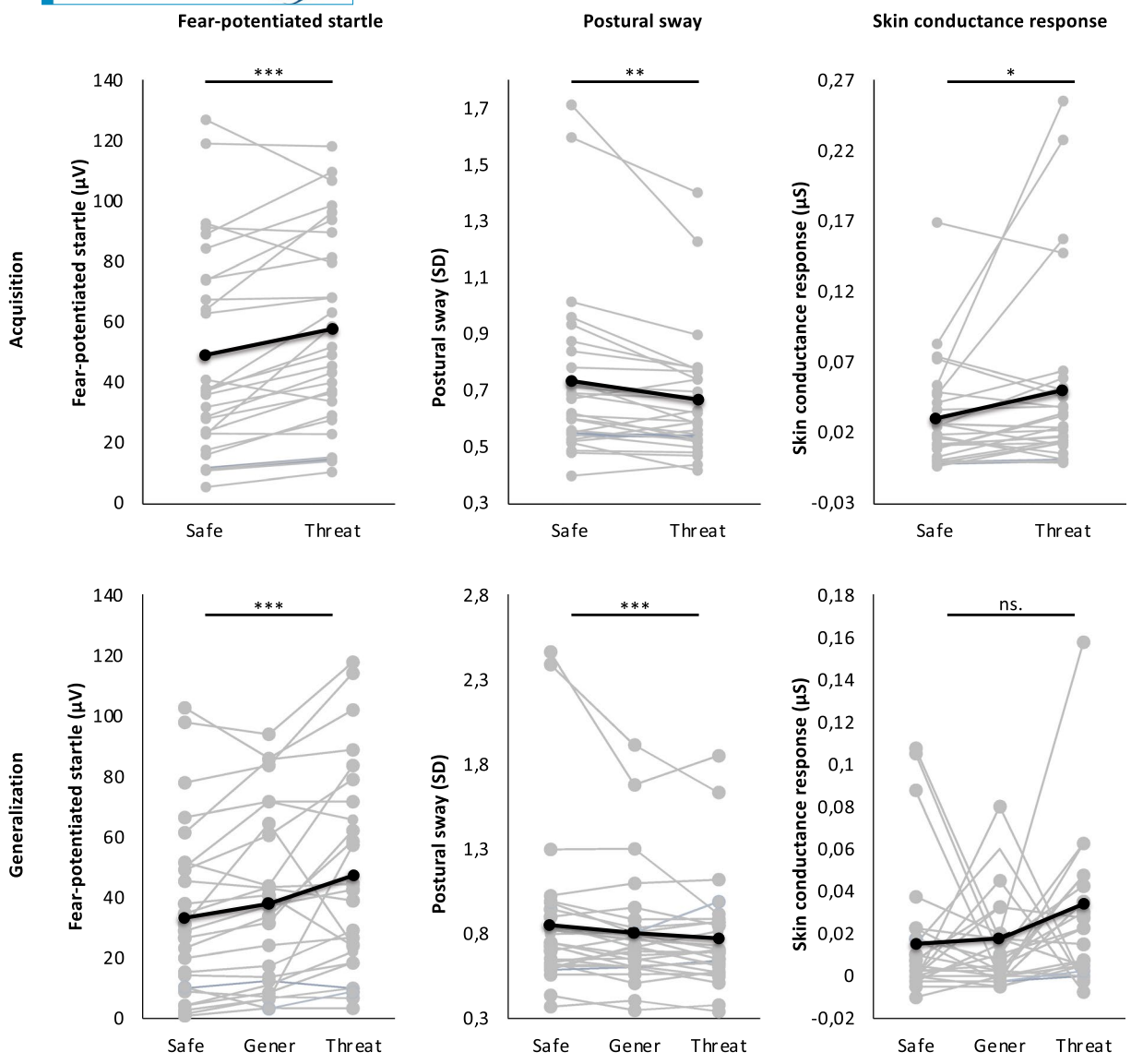


FIGURE 4 Individual conditioned responses during the CS presentation in the different contexts for the acquisition and generalization phase, for the three psychophysiological variables that revealed significant conditioning effects. The grey lines represent individual participants, while the black lines represent the group means for the different conditions. The depicted *p*-values represent significant main effects of Condition. Obtained *p*-values are indicated by: ***$p < .001$, **$p < .01$, *$p = .05$

reliable inference at different hierarchies of the data, enabling the assessment of relationships between physiological measures at the trial level (i.e., within-subjects) and across subjects (i.e., as individual differences). The analyses were performed in RStudio version R version 3.5 (R Core Team, 2019). The lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2017) packages were used to fit and test the linear effect mixed models using restricted maximum likelihood estimation. We chose to test our hypothesis only on data segments sampled during the CSs, as conditioning effects were most strongly present there (i.e., as an effect of threat imminence), making it most likely to reveal a relationship between anticipatory defense mechanisms and startle potentiation, if present. We ran one model for the acquisition phase, and one model for the generalization phase, the latter also serving as a (within-subject) replication of physiological interrelationships in the acquisition phase. In all analyses, the three anticipatory defensive responses (i.e., Bradycardia, Freeze, and SCR) and Trial number (1–12 for acquisition, 1–4 for generalization) were included as trial level within-subjects (Level 1) variables. Threat type (threat and safe, generalization was added for the generalization phase) was included at the participant level (Level 2) variables. Startle served as the outcome variable. To maintain individual differences in absolute startle value, we did not normalize or standardize these in any way. Skewness of the data was best treated by a square root transformation. The three anticipatory defensive responses were each mean-centered within subject and within condition. Also,

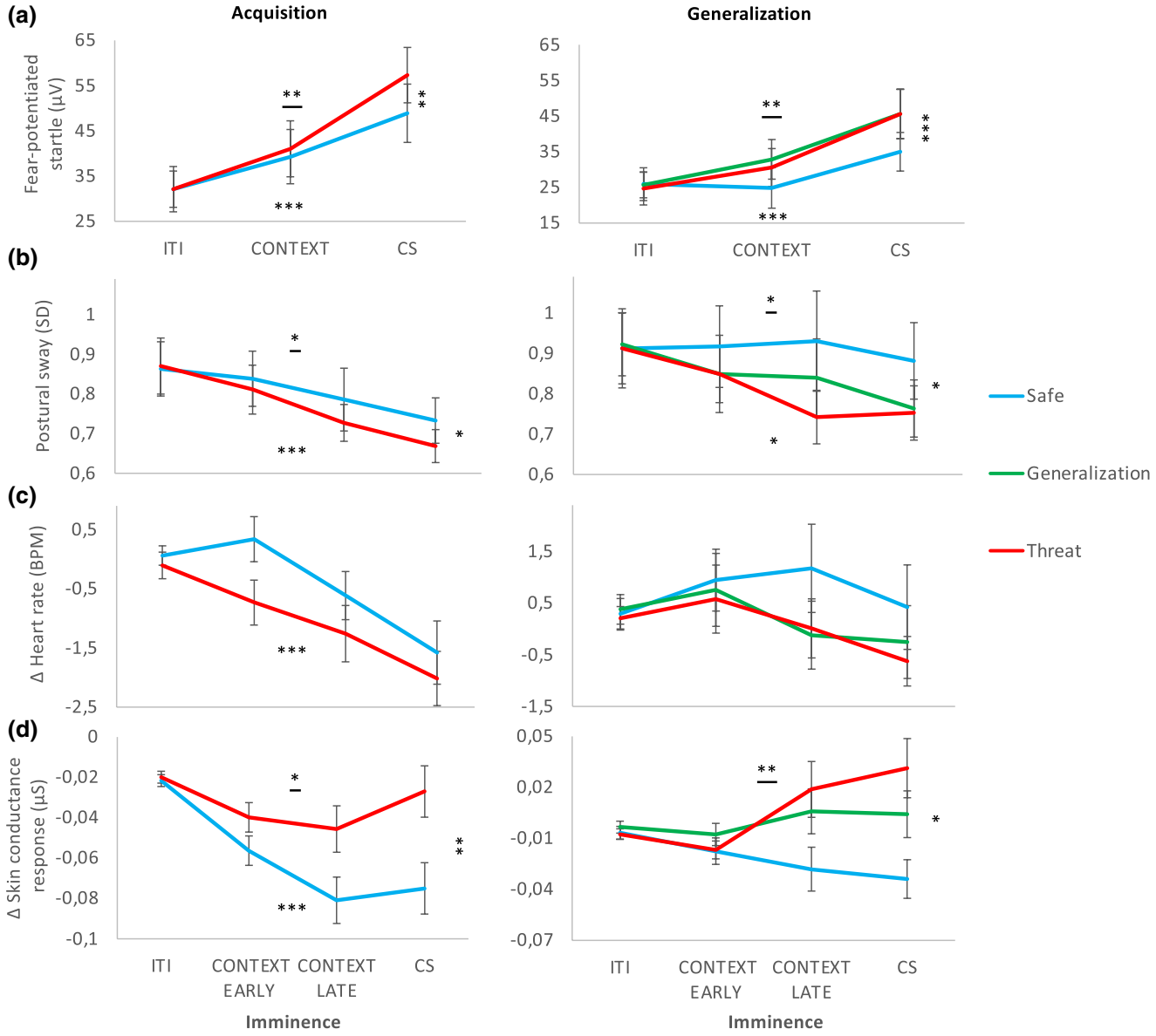


FIGURE 5 Threat imminence for all physiological measures, for the acquisition and generalization phases. Mean responses for the different dependent variables as a function of imminence (inter-trial interval (ITI)), early context, late context, and conditioned stimulus (CS) and condition (safe and threat during acquisition, safe, threat and generalization during the generalization phase). In the different panels the fear-potentiated startle (a), postural sway (b), baseline-corrected heart rate (c), and baseline-corrected skin conductance response (d) can be seen for the acquisition and generalization phase. Error bars represent standard errors of the mean. Significant main effects of Condition are depicted right of the respective graph, main effects of Imminence below, and Condition × Imminence are underscored and depicted above the graph. Obtained *p*-values are indicated by: ***$p < .001$, **$p < .01$, *$p < .05$

terms were entered at the second level in the equation for the intercept representing each participant's mean defensive response (per condition) centered around the respective grand mean. By doing so, these terms can be interpreted as variation across individuals (i.e., individual differences), and the model's intercept can be interpreted as the grand startle mean (Hamaker & Grasman, 2015). For both the acquisition and generalization model, we always included a random participant intercept, as recommended by Twisk (2006), and we included a diagonal covariance matrix. A model selection procedure in comparison with this more restricted model was adopted to decide whether or not to allow for additional random slopes, for all combinations of the first-level variables. It turned out that inclusion of the random slope variance parameter(s) for any (combination) of the anticipatory defense predictors did not significantly improve model fit (neither in the acquisition model nor for the generalization phase model), as evidenced by non-significant reductions in AIC-values. In two instances, addition of the

random slope(s) yielded zero variance estimates, causing the model to fail to converge. Thus, we arrived at the following model for both the acquisition and generalization phases, where the FPS response of participant i in condition j for trial number k is:

Trial level (level 1)

$$\mathrm{FPS}_{ijk} = \alpha_{ij} + \beta_{1,ij}\,(\mathrm{Sway})^{c}_{ijk} + \beta_{2,ij}\,(\mathrm{Bradycardia})^{c}_{ijk} + \beta_{3,ij}\,(\mathrm{SCR})^{c}_{ijk} + \beta_{4,ij}k_{ij} + \varepsilon_{ijk}$$

Participant (level 2)

$$\alpha_{ij} = \gamma_{00} + \gamma_{01}(\mathrm{Condition})_{ij} + \gamma_{02}\left(\overline{\mathrm{Sway}}\right)_{ij} + \gamma_{03}\left(\overline{\mathrm{Bradycardia}}\right)_{ij} + \gamma_{04}\left(\overline{\mathrm{SCR}}\right)_{ij} + U_{0,i}$$

$$\beta_{m,i,j} = \gamma_{10} + \gamma_{m1}(\mathrm{Condition})_{ij} \qquad m = 1, 2, 3$$

Here, $(X)^c$ indicates mean-centered within subject and within condition defensive response X (Sway, Bradycardia, or SCR), and $\overline{X}$ the grand-mean centered participant's mean defensive response. The residual is represented by (ε_{ijk}). The Level 1 equation further consists of trial number ($\beta_{4,ij}$), participant- and condition-specific intercepts α_{ij}, and linear effects of X ($\beta_{m,ij}$). The latter two are further modelled at level 2. Finally, $U_{0,i}$, refers to the random error component, indicating deviation from the intercept of a participant from the overall intercept. In the equation random deviation of a participant's slope from the overall slopes for Sway, Bradycardia, or SCR ($U_{m,i}$) are not depicted since random slope variance did not significantly improve model fit.

With this obtained model, we were able to test whether the intensity of participants' postural sway, bradycardia and/or SCR responses were predictive of ensuing startle response magnitudes, and whether any of these possible relationships were significantly stronger under higher threat conditions. Significance of the model's parameter values and general analysis of variance effects were tested using Satterthwaite's method (Kuznetsova et al., 2017). For all predictors in the acquisition and generalization model, the variance inflation factor was 2.7 or lower, indicating that multicollinearity was not at play in the multilevel model.

3 | RESULTS

Is freezing sensitive to fear conditioning, generalization across context, and threat imminence?

In line with expectations and previous human fear-conditioning observations, analysis of the FPS and SCR data confirmed successful fear conditioning, generalization across contexts, and clear effects of threat imminence (see below and Figures 2–5; for details see Supplementary Results in the Supporting information). Only for HR no such effects were revealed. These analyses overall confirm that the paradigm was successful, and can be reliably used to test our hypotheses with regard to postural freezing.

3.1 | Fear conditioning and generalization

To characterize fear conditioning on the short presentation of the CSs specifically, we started out with an analysis that included sway data sampled from the time-window from CS-onset until startle probe presentation, with a Condition (safe, threat) × Time (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 s) repeated-measures ANOVA (see Figure 2a). A significant main effect of Condition ($F_{1,27} = 11.12$, $p = .002$, $\eta_p^2 = 0.292$) confirmed that overall, participants displayed less postural sway during the threat CS as compared to the CS indicating safety. There was no evidence that sway decreased during the small time-window of the CS, as indicated by an absence of a significant main effect of Time ($F_{7,189} = 1.64$, $p = .126$) or Condition × Time interaction ($F_{7,189} = 0.40$, $p = .902$).

In line with the observations in the acquisition phase, the analyses of the generalization data (see Figure 2a) revealed a significant main effect of Condition ($F_{2,54} = 5.28$, $p = .008$, $\eta_p^2 = 0.164$), qualifying conditioned fear generalization effects. This time, a main effect of Time ($F_{3.19,85.00} = 2.86$, $p = .038$, $\eta_p^2 = 0.096$) suggested that sway did somewhat decrease over the course of the CS, but this was not modulated by threat Condition ($F_{9.35,252.51} = 0.99$, $p = .46$).

As no consistent time-dependent sway-dynamics emerged within the short CS window, we calculated averages over the 4-s time interval of sway for all CSs. To assess — now in a more traditional way, collapsing over the factor Time from the previous analysis — whether freezing indeed can be conditioned, we ran a Condition (safe, threat) × Trial number (1–12) repeated measures ANOVA (see Figure 3b). For a depiction of individual means, see Figure 4. Again, sway was notably reduced during threat ($F_{1,27} = 11.98$, $p = .002$, $\eta_p^2 = 0.307$). Conditioned responses seemed to be learned quickly (absence of an interaction effect with Trial number, $F_{6.31,170.40} = 0.225$, $p = .996$) and, as opposed to the traditional fear-conditioning measures (see Figure 3 & supplementary results), there was no evidence that freezing generally habituated over the course of acquisition ($F_{5.5,149.37} = 1.24$, $p = .257$). In order to estimate to what extent the observation of differential conditioning generalizes across participants, we calculated the

percentage of participants that displayed the same sign of effect size for the threat versus safe comparison as compared to the group effect size (Cohens' *d*, Bach & Melinscak, 2020). This indicates whether the freezing measure adequately classifies the threat versus safe condition for a given subject (Xia et al., 2020). The results reveal that for 82% of participants, freezing correctly classified the threat and the safe condition (for startle this number was 79%).

For the generalization phase (see Figure 3b), again a significant main effect of Condition ($F_{2,54} = 9.71$, $p < .001$, $\eta_p^2 = 0.265$) was found. Planned contrasts indicated that freezing was significantly stronger on both the threat ($p = .001$, 95% CI [0.027, 0.094]) and generalization CS ($p = .001$, 95% CI [−0.029, −0.101]) as compared to the safe CS, but the threat and generalization CS did not seem to differ from each other ($p = .816$, 95% CI [−0.028, 0.035]). Interestingly, a marginally significant Condition × Trial number interaction ($F_{6,162} = 2.12$, $p = .053$, $\eta_p^2 = 0.073$) suggested that conditioned freezing responses to CSs of the different conditions changed over the course of generalization. Indeed, in line with gradual extinction of the responses to the threat and generalization CSs, planned contrasts indicated that on the first trial participants froze more during the threat CS ($p = .020$, 95% CI [−0.187, −0.17]) and the generalization CS ($p < .001$, 95% CI [−0.240, −0.039]) as compared to the safe CS. Freezing was even marginally stronger on the generalization trials in comparison with the threat trial ($p = .056$, 95% CI [−0.002, 0.131]). On all further trials however, no such differences emerged (all $p > .129$). For the generalization phase, classification of the threat versus safe condition was 71% (startle 75%), classification of the generalization context from the safe context was 54% (startle 75%).

Together, these observations provide important evidence that freezing as assessed by postural sway can reveal fear-conditioned responses in humans. Interestingly, notable reductions in postural sway were observed on the generalization condition, indicating that conditioned freeze responses generalized from an unmistakably threatening context to a more ambiguous context.

3.2 | Threat imminence

A repeated measures ANOVA on the complete acquisition postural sway data (see Figure 5b) containing the within-factors Condition (safe, threat), and Imminence (ITI, early context, late context, CS) revealed a main effect of Condition ($F_{1,27} = 5.91$, $p = .022$, $\eta_p^2 = 0.180$), indicating that overall, participants' postural sway was considerably reduced when anticipating the US, as compared to the safe condition that was not followed by an adverse outcome at the end of the trial. A main effect of Imminence ($F_{2.03,54.81} = 18.77$, $p < 0.001$, $\eta_p^2 = 0.410$) suggested that regardless of Condition, sway was reduced with increasing imminence towards the end of the trial. The Condition × Imminence interaction did not reach significance ($F_{2.03,54.95} = 1.77$, $p = .179$), but the more sensitive planned linear Condition × Imminence contrast revealed a significant effect ($F_{1,27} = 6.16$, $p = .020$, $\eta_p^2 = 0.186$), indicating stronger sway reduction for threat cues with more imminent threat.

The same ANOVA on the generalization phase data (see Figure 5b) again revealed that overall, postural sway was significantly modulated depending on Condition ($F_{1.94,53.07} = 7.51$, $p = .001$, $\eta_p^2 = 0.218$). Also, with increasing imminence to threat, sway generally decreased ($F_{1.87,50.41} = 4.12$, $p = .024$, $\eta_p^2 = 0.132$). More informatively, sway responses to the different threat types were modulated by imminence, as evidenced by a marginally significant Condition ×Imminence interaction ($F_{4.018,108.49} = 2.73$, $p = .066$, $\eta_p^2 = 0.078$), and the more sensitive planned linear Condition × Imminence contrast indeed revealed a significant effect ($F_{1,27} = 16.42$, $p < .001$, $\eta_p^2 = 0.378$). During late context, freezing was already stronger on threat in comparison to the safe contexts ($p = .001$, 95% CI [−0.136, −0.037]), but only during CS presentations sway was significantly reduced on both threat ($p = .001$, 95% CI [−0.099, −0.031]) and generalization trials ($p = .001$, 95% CI [−0.093, −0.027]) as compared to the safe trials. Generalization and threat trials did not seem to differ from each other ($p = .737$ 95% CI [−0.037, 0.027]).

In conclusion, defensive conditioned freezing is amplified by threat imminence, such that freezing started to develop over the course of the context, but was strongest toward the end of the trial in presence of the CS.

Do preparatory postural freeze and bradycardia modulate ensuing intensity of a startle response?

After having established the fear-conditioning effects with respect to postural sway, SCR, and FPS (see supplementary results in the Supporting information for the latter), we assessed whether the intensity of a given freezing or bradycardia response would predict the magnitude of a subsequent startle reflex on a trial-by-trial base (i.e., within-subject) using multilevel modelling.

Parameter estimates obtained from the acquisition multilevel model (see Figure 6) confirmed strong effects of condition ($\beta = 0.59$, $t(635.92) = 3.36$, $p < .001$) and trial number ($\beta = -0.13$, $t(610.60) = -5.60$, $p < .001$) on startle, the latter indicating general habituation over the course of acquisition. Notably, in line with our hypothesis, the interaction term between condition and postural sway contributed significantly to the model, indicating that depending on the amount of threat, decreases in sway during the CS

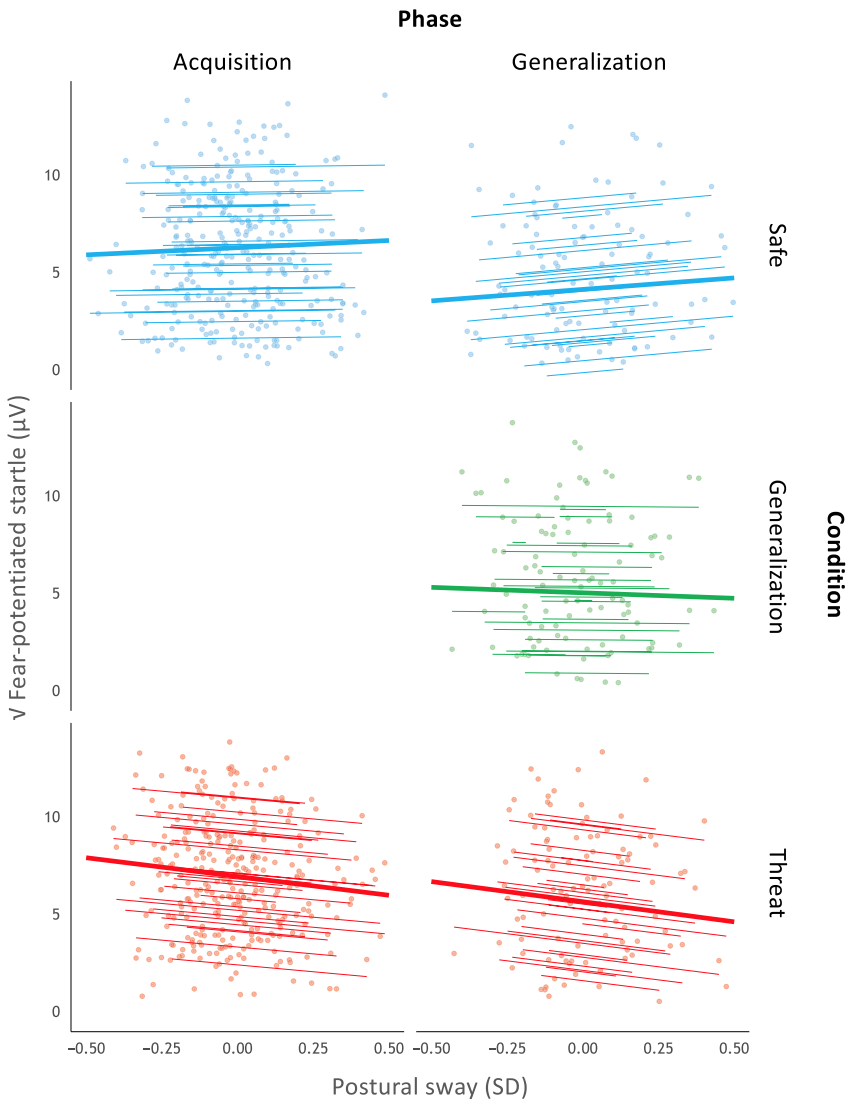


FIGURE 6 Relationships between postural sway and fear-potentiated startle. The multilevel analysis revealed that for both experimental phases stronger reductions in preparatory postural sway predicted larger magnitude of the ensuing startle response, during the threat condition. A similar significant relationship was revealed during the generalization condition. The image depicts the optimal fitting model (i.e., random intercept only). Postural data are raw data (calculated based on the means sampled during each CS until startle probe presentation) corrected for the random intercepts. The thin lines represent the model's estimates per participant, while the thick lines represent the model's predictions

preceded ensuing startle responses ($\chi^2(1) = 7.99$, $p = .005$), but none of the other interaction terms were significant (all $\chi^2 < 0.4$, all $p > 0.5$). Further parameter estimates confirmed a significant negative relationship during threat between postural sway reduction and larger magnitude of startle responses ($\beta = -1.41$, $t(610.6) = -3.20$, $p = .001$), that was significantly stronger as compared to the safe condition ($\beta = -1.61$, $t(610.60) = -2.81$, $p = .005$). In contrast, no evidence for a relationship between sway and startle emerged for trials in the safe condition ($\beta = 0.2$, $t(635.20) = 0.54$, $p = .586$). Together these observations suggest that for a given participant, if freezing on a given threat trial is particularly strong, the ensuing startle response is likewise more likely to be amplified. If on the other hand freezing on another threat trial is less strong, the ensuing startle response would be less large as well. Notably, our model selection procedure indicated that adding random participant slopes for the sway-startle relationship did not significantly improve model fit. This suggests that variation in strength of the within-subject sway-startle relationship is negligible, and thereby indicates that a negative sway-startle relationship is consistently present across participants. A comparable sway-startle relationship was also observed when looking at the means of postural sway per condition at the participant level, that predicted startle responses as well ($\beta = -2.39$, $t(106.31) = -2.11$, $p = .037$), suggesting that general lower absolute postural sway levels across participants also related to overall larger startle reactivity. Notably, none of the other defensive indices predicted startle responses (see for all parameter estimates Table 1).

Parameter estimates obtained from the generalization multilevel model yielded a highly comparable pattern of results, with again a predicting effect of trial number ($\beta = -0.3$, $t(282.22) = -2.97$, $p = .003$) and a significant condition term ($\chi^2(2) = 23.64$, <0.001). Crucially, the term representing the interaction between condition and postural sway was again significant ($\chi^2(2) = 2.86$, $p = .013$).

TABLE 1 Parameter estimates and significance levels for the multilevel models of the acquisition and generalization phases predicting startle magnitudes

	Acquisition	Generalization
Predictor	B **(SE)**	B **(SE)**
(Intercept)	**7.06 (0.48)*****	**5.79 (0.59)*****
Generalization	–	**0.91 (0.29)****
Threat	**0.59 (0.17)*****	**1.39 (0.29)*****
Postural sway	0.2 (0.37)	1.39 (0.84)
Skin conductance response	0.99 (1.52)	−7.53 (4.17)
Bradycardia	0 (0.02)	−0.03 (0.04)
Postural sway (participant)	**−2.4 (1.14)***	−1.27 (0.79)
Skin conductance response (participant)	4.25 (3.09)	−0.67 (3.94)
Bradycardia (participant)	−0.02 (0.06)	**−0.1 (0.05)***
Trial number	**−0.13 (0.02)*****	**−0.3 (0.1)****
Postural sway: Generalization	–	−1.51 (1.06)
Postural sway: Threat	**−1.61 (0.57)****	**−3.35 (1.17)****
Skin conductance response: Generalization	–	1.57 (5.91)
Skin conductance response: Threat	−1.2 (1.91)	6.78 (4.85)
Bradycardia: Generalization	–	−0.03 (0.06)
Bradycardia: Threat	0 (0.03)	−0.02 (0.05)

Note: Effect estimates in bold represent significant effects, *p*-values are indicated by: ***$p < .001$, **$p < .01$, *$p < .05$. Standard errors are shown in parentheses. The models were run on a sample of $n = 27$. In the estimation of the beta values, the safe condition served as the reference condition. For acquisition, trial number was 1–12, for the generalization phase it was 1–4. Acquisition comprised two conditions, threat and safe, the generalization phase included an additional generalization condition. "Participant" stands for the grand-mean centered participant's mean response, while the other predictors involve within-subject relationships.

More specific parameter estimates again revealed no convincing evidence for a relationship between sway and startle during safe trials ($\beta = 1.4$, $t(282.22) = 1.66$, $p = .098$), but a strong within-subject relationship between sway reduction and amplified startle responses in the threat condition ($\beta = -1.96$, $t(282.22) = -2.41$, $p = .016$), that was significantly stronger as compared with the safe condition ($\beta = -3.35$, $t(282.22) = -2.87$, $p = .004$). Like for the acquisition data, random slopes for the sway-startle relationship did not significantly improve model fit, which again indicates that the sway-startle relationship is equivalent across participants. In the generalization condition no evidence for a within-subject relationship with the startle emerged ($\beta = -0.12$, $t(282.22) = -0.19$, $p = .848$). At the participant level, a significant relationship between bradycardia and startle emerged, suggesting stronger mean participant bradycardia related to increased overall startle reactivity ($\beta = -0.1$, $t(300.63) = -1.99$, $p = .048$). None of the other interaction terms or parameter estimates were predictive of startle response magnitudes (see for all parameter estimates Table 1). Also, the analyses of the acquisition and generalization phases yielded highly comparable results when bradycardia and SCR were baseline corrected with their preceding ITI level.

Together, in line with our predictions, these results suggest that especially under conditions of high threat, stronger preparatory postural freeze responses predict larger FPS responses. Interestingly, even though SCR were highly affected by threat, this sympathetic index was not predictive of ensuing startle responses on a trial-by-trial base like parasympathetically mediated freeze was.

4 | DISCUSSION

The present study reveals that human postural freezing is highly responsive to several key fear-conditioning manipulations. Specifically, freezing increased with threat imminence, conditioned on threat versus safe stimuli, and generalized to ambiguous situations. Interestingly, stronger within-subject freezing predicted amplified startle responses on a trial-by-trial basis, most pronouncedly so under conditions of high threat. This relationship suggests that these two measures are closely interrelated. In

contrast, skin conductance did not relate to startle intensity, despite clear responsivity to most experimental manipulations. These results have several implications. Conceptually, we observe that humans, like animals, show conditioned postural freezing. Theoretically, our findings confirm synchronicity in psychophysiological readouts in parasympathetically mediated anticipatory threat-responses which supports the role of freezing in human action preparation. Methodologically, these findings reveal a novel and promising translational alternative for the startle. Together, these findings pave the way to study freezing as a translational marker of vulnerability for anxiety.

4.1 | Human freezing as promising translational fear-conditioning index to study anxiety

In line with an overwhelming amount of animal studies, the current work is the first to reveal that human defensive freezing responses can be conditioned. Further, in accord with influential theoretical models on threat imminence (Fanselow, 1994; Lang & Bradley, 2010; Löw et al., 2015), freezing did increase over the course of a trial, but most pronouncedly so for the threat condition. Conditioned freezing responses also generalized to an ambiguous situation. The experimental observation that conditioned freezing was sensitive to generalization in healthy humans is essential to bridge basic research in animals and clinical human studies (Grillon et al., 2019) rendering freezing a promising translational tool to study vulnerability for anxiety. This conclusion is underscored by studies revealing that freezing to threatening (i.e., unconditioned) stimuli is amplified with a history of unsecure attachment (Niermann et al., 2015) and genetic vulnerability (Schipper et al., 2019). Perhaps exacerbated freezing responses to threat may also pose a stress vulnerability factor, a question that is only beginning to be addressed (Koch et al., 2017).

In line with a host of previous studies, the startle data showed not only reliable conditioned responses, but also revealed high sensitivity to threat imminence (Löw et al., 2015) and generalization (Lissek et al., 2008). The FPS has been considered the perfect translational conditioning index (Briscione et al., 2014), but it knows some shortcomings: The need to present aversive sounds limits the investigation of quick dynamic changes in time, may induce general background anxiety, and may delay the development of other conditioned responses (Sjouwerman et al., 2016). It further habituates substantially (Leuchs et al., 2018). The multilevel analysis revealed that startle and freeze are closely interconnected, suggesting that these measures could possibly be used interchangeably. Thus, also pragmatically, conditioned freezing can be a promising alternative to startle, as it can be assessed continuously, is less invasive, does not habituate, and is unlikely to interfere with other measures.

4.2 | Freeze for startle: Freezing as preparation to sudden attack

Intriguingly, the intensity of individual startle responses was predicted by variability in preceding defensive freezing, both during acquisition and (unreinforced) generalization phases. The strength of this within-subject relationship was further proportional to the amount of threat, with consistent relationships during threat. The freeze-startle relationship seemed also to be present in differences across individuals, but only during acquisition. These observations suggest a close relationship between preparatory freezing and defensive startle responses. Since SCR was also sensitive to the conditioning procedures, but was not related to FPS, it is not likely that it was another, latent, state such as fear itself that would be driving these two measures. But why would these measures act in such close synchronicity? Orchestration of defensive responses is heavily dependent on the possibility of active coping (Gladwin et al., 2016; Löw et al., 2015). Even though fear conditioning may seem a passive manipulation, conditioned responses likely represent defensive preparations to actively cope with impending threat, as soon as the situation allows it. It is thought that the vlPAG inhibits phasic motor functions to optimally prepare for action the moment the "brake" releases (Roelofs, 2017), characterized by parasympathetic dominance over sympathetic activation (Walker & Carrive, 2003). A startle probe may very well initiate such release, and propel an animal in action thought to protect against sudden attack (Yeomans & Frankland, 1995). Indeed, animal work has revealed a behavioral shift from freezing to active startle behavior (Reimer et al., 2012), while a human study has revealed that startle probes elicited reflexive movements (in the anterior-posterior direction) that were correlated with FPS intensity (Hillman et al., 2005). Because in our study, the intensity of each freezing response directly related to subsequent startle reactivity, it is suggested that "freezing is not just immobility" (Walker & Carrive, 2003), it actively prepares for sudden attack. Conversely, intensity of these preparations may directly modulate the intensity of a subsequent behavioral response to such sudden attack.

It has been suggested that bradycardia also modulates motor reflexes (Hunt et al., 1994). However, even though heart rate generally declined with threat imminence,

conditioning here did not result in differential bradycardia responses, and only a relationship with startle on the individual level emerged. Using a high amount of conditioning trials, conditioned bradycardia responses in humans have been observed (here, heart period was used, Castegnetti et al., 2016) others revealed bradycardia to the CS+ in a subset of participants who also displayed hypoventilation in anticipation of threat (Van Diest et al., 2009). A recent large-scale fear-conditioning fMRI study revealed that conditioned bradycardia linked to PAG activity most notably for stress-sensitive individuals (Schipper et al., 2019). Such observations suggest that large individual differences exist in the extent that bradycardia is being observed.

An interesting question concerns how freezing under threat relates to expressions of nervousness such as fidgeting or hair-pulling. Freezing and the associated parasympathetic dominance may be most frequently observed in situations when being confronted with concrete threats that require a timely response. Nervousness might be associated with sympathetic dominance reflecting a more tonic stress level in situations without concrete threats (for example awaiting a job interview), and fidgeting could then help with tension-relief (Mohiyeddini & Semple, 2013). We could speculate that the way fear is being expressed depends on the ratio of sympathetic versus parasympathetic activation (Hamm & Vaitl, 1996; Van Diest et al., 2009), with sympathetic dominance resulting in more agitated behaviors. To investigate such predictions, extensive and carefully designed future studies are required.

Some limitations regarding the present study deserve attention. Even though an important motivation for the current study was to overcome issues of translational generalizability and replicability due to the use of disparate fear-conditioning indices, variation in data pre-processing, analyses, and experimental designs can likewise hamper generalizability and replicability (Bach et al., 2018; Haaker et al., 2019; Lonsdorf et al., 2017; Ney et al., 2018; Ojala & Bach, 2020). The current paradigm is less commonly employed in human fear-conditioning studies, and generalization of our findings to for instance delay-conditioning procedures may not be as straightforward as could be. Likewise, some decisions in our data analysis procedure were data-driven, and can therefore be considered exploratory. For instance, it was unclear in advance whether freezing would continue to intensify even during the short duration of the CS. For this reason we made the quantification method of the conditioned freezing response (i.e., average versus peak-response) contingent on an interaction with time. Finally, even though most analyses involve within-subject tests that overcome the problem of between-subject variance (Lonsdorf et al., 2017), the current sample size is relatively modest, especially given the considerable amount of statistical tests performed (Cramer et al., 2015). Instead of anticipating medium effect sizes based on earlier startle studies, a conservative approach would have been to estimate small effects. For these reasons, the current observations should be replicated with other fear-conditioning paradigms, while using a larger sample size.

5 | TO CONCLUDE

Freezing to impending threat is a core defensive response that has been extensively researched using fear conditioning in non-human animals. Here, we show that freezing in humans is subject to conditioning as well. Conditioned freezing is not just another human fear-conditioning readout, as it holds translational promise similar to FPS but without its shortcomings. Freezing may thereby prove an excellent tool for studying the vulnerability, development, or even treatment of psychopathology that is characterized by aberrant emotional learning and memory. The divergence and convergence of the different physiological systems observed in the present study further emphasizes that the interpretation of conditioned responses is by no means straightforward, and should be carefully considered (Ney et al., 2018). These observations however also raise intriguing new questions: How do different defensive response systems interact to execute situation-specific behaviors, pose vulnerability for dysfunctional anxiety, or relate to other symptoms of anxiety such as jitteriness or fidgeting? The present observations may be an inspiration for future exciting scientific explorations.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest with respect to the authorship or the publication of this article.

AUTHOR CONTRIBUTIONS

Vanessa van Ast: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Validation; Visualization; Writing—original draft; Writing—review & editing. **Floris Klumpers:** Conceptualization; Formal analysis; Methodology; Resources; Writing—review & editing. **Raoul Grasman:** Formal analysis; Methodology; Validation; Visualization; Writing—review & editing.

Angelos Krypotos: Formal analysis; Methodology; Validation; Writing—review & editing. **Karin Roelofs:** Conceptualization; Funding acquisition; Supervision; Writing—review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.
Supplementary Material

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