Basic defensive reflexes elicited by exposure to fear-conditioned subliminal stimuli

Rianne Brouwer
0424420
Master Applied Cognitive Psychology, Universiteit Utrecht
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First Supervisors: Ivo Heitland & Sjoerd Stuit
Second Supervisor: Stella Donker

Summary

Exposure to potential dangers in the environment can evoke fear and anxiety, and lead to activation of the fear system. This system can be characterized by four central features; selectivity, automaticity, encapsulation and a specialized neural circuitry. Selectivity means that, rather than being open to any stimulus, the fear system can easily be activated by stimuli that have been associated with threatening situations in the evolutionary past. Automaticity of the fear system makes it possible for a stimulus to activate the fear system even by only rapid and preliminary perception of that stimulus. These two characteristics have been combined in several studies focusing on automatic responses to masked stimuli. Results of these studies indicate that emotional images, like faces and spiders, can elicit a conditioned fear response, even when stimuli are presented subconsciously. This study uses previously neutral images, horizontal and vertical gratings, as stimuli, which have been given emotional relevance through fear-conditioning. The goal of the current study is to determine if these unconsciously presented stimuli can still elicit a conditioned fear response. Using Continuous Flash Suppression to present stimuli subliminally, participants performed threshold measurements to determine two contrast levels; one at which stimuli would be invisible and one at which stimuli would be visible. Using these contrast levels, participants were then presented with subliminal stimuli and eye blink startle was measured by electromyographic (EMG) recordings from the orbicularis oculi beneath the right eye. The results indicated ineffective conditioning, and therefor provide no conclusive evidence that subliminal stimuli are sufficiently processed to elicit a conditioned response.

Introduction

The fast processing of threat signals is necessary to quickly respond to and cope with potential dangers in the environment (Singer, 2012). These potential dangers can evoke fear, which can be defined as an aversive emotional state elicited by external threatening cues that activate the defensive fear system of an organism (Hamm & Weike, 2005). The fear system then organizes a behavioral pattern to adjust to the threat, resulting in either defensive action or defensive immobility (freezing and increased vigilance) (Hamm & Weike, 2005; Lang, Bradley, & Cuthbert, 1997). The short-lived arousal that characterizes fear is caused by activation of the defensive fear system and dissipates quickly after the threat is removed or avoided (Sylvers, Lilienfeld, & LaPrairie, 2011). While fear is a response to a specific and imminent or acute threat, anxiety occurs when there is an ambiguous and uncertain threat (Sylvers et al., 2011). While fear is often elicited by a defined stimulus, anxiety is often anticipatory to threatening stimuli (Öhman, 2008). Characteristics of anxiety are hypervigilance and hyperarousal, which persist even when the threat is removed (Sylvers et al., 2011).

The fear system can be characterized by four central features; selectivity, automaticity, encapsulation and a specialized neural circuitry (Ohman & Mineka, 2001). Selectivity means that, rather than being open to any stimulus, the fear system can easily be activated by stimuli that have been associated with threatening situations in the evolutionary past (Ohman & Mineka, 2001). But the fear system also shows strong plasticity; previously harmless stimuli that have been associated with aversive outcomes can also activate the system (Hamm & Weike, 2005). Automaticity of the fear system makes it possible for a stimulus to activate the fear system even by only rapid and preliminary perception of that stimulus. Encapsulation entails that the fear system, once activated, can be resistant to conscious cognitive influences, like verbal instructions or awareness of stimuli (Hamm & Weike, 2005). The specialized neural circuitry associated with the fear system mediates the functional relationship between events in the environment and behavior. This neural circuitry is organized around the amygdala, which mediates inputs from cortical and thallic sites to hypothalamic and brain stem nuclei that control aspects of fear behavior (Hamm & Weike, 2005; Ohman & Mineka, 2001).

The plasticity of the fear system makes it possible to expand the range of stimuli that can activate the fear system (Hamm & Weike, 2005). This can be achieved through classical fear conditioning, during which a neutral stimulus is paired with a threatening or aversive stimulus, the unconditioned stimulus (US). After this pairing the previously harmless stimulus, now the conditioned stimulus (CS), becomes predictive of the US and will elicit responses that were previously associated with the aversive US (Critchley, Mathias, & Dolan, 2002). After aversive conditioning, presentation of the CS (now CS+) results in a conditioned fear response. To index basic defensive states, startle reflex methodology has become a popular psychophysiological measure (Grillon & Baas, 2003; Hamm & Vaitl, 1996). The startle reflex is an automatic response to abrupt and intense stimulation, and consists of a forward thrusting of the head and a descending flexor wave reaction extending to the knees (Grillon & Baas, 2003; Lang, Bradley, & Cuthbert, 1990). This muscle contraction can facilitate the flight response and/or protect the body from a sudden attack, by resulting in a protective posture with minimal exposure of sensitive areas (Grillon & Baas, 2003; Yeomans, Li, Scott, & Frankland, 2002). In humans the startle reflex can be measured by recording the eyeblink reflex, which is the most stable component of the startle reflex (Lang et al., 1990). The eyeblink, consisting of the rapid contraction of the orbicularis oculi muscle, can be elicited by short, intense stimuli of an auditory, visual or tactile nature, but the acoustic startle is most commonly used (Grillon & Baas, 2003). The eyeblink elicited by an acoustic startle has an onset latency ranging from 20 to 50 ms and can be detected by measuring the electrical activity associated with the contraction of the orbicularis oculi muscle, by placing two electrodes below one eye (electromyogram, EMG). The difference in startle amplitude between threat (CS+) and safe (CS-) conditions is the fear-potentiated startle and has become an important measure of conditioned fear responses in humans (Grillon & Baas, 2003). Advantages of the fear-potentiated startle as a measure of conditioned fear are that it measures aversive learning rather than non-specific arousal and is unrelated to contingency awareness (Grillon & Baas, 2003; Hamm & Weike, 2005).

Due to the automaticity of the fear activation, rapid and preliminary perception of a stimulus can be enough to activate the fear system. Stimuli can elicit conditioned fear responses, namely freezing, increased blood pressure, changes in heart rate and increased skin conductance response, without being processed in the primary visual cortex, or without conscious perception (Fanselow, 1994; Hamm & Weike, 2005; Williams et al., 2004). Non-conscious or subliminal perception has been an important field of study since the emergence of psychology. Perception is thought to be subliminal when a stimulus is demonstrated to be invisible while still influencing thoughts, feelings, actions, learning or memory (Kouider & Dehaene, 2007). To provide visual stimulation without conscious awareness several techniques have been developed. One of these techniques is backwards masking in which a stimulus is degraded by presenting it too briefly for reliable detection or by superimposing a mask on it. However, with backwards masking it is difficult to determine if the stimulus really falls outside conscious awareness (Kim & Blake, 2005). A more reliable method of influencing visual awareness is binocular rivalry. When presented with two different images to the two eyes, the two images rival for visibility, with conscious perception shifting between the two images (Tong, Meng, & Blake, 2006). Only one image is visible (or dominant) at any time, while the other is invisible (or suppressed). Unlike backward masking, wherein awareness is manipulated by changing visual stimulation, in binocular rivalry visual stimulation in unchanging; it is the observer's awareness that is constantly changing. This constantly changing perception is however a limitation of binocular rivalry; the switches in perception are unpredictable and the images are suppressed for a short time. Another method, continuous flash suppression (CFS), overcomes this limitation (Lin & He, 2009). By continuously flashing contour-rich, high-contrast patterns to one eye, the image presented to the other eye is suppressed. The period of suppression for CFS is about ten times that of binocular rivalry, which make CFS a more consistent and controllable way to manipulate awareness (Tsuchiya & Koch, 2005).

Combining the selectivity and the automaticity of the fear system, several studies have focused on automatic responses to masked stimuli. In many of these studies (Singer, Eapen, Grillon, Ungerleider, & Hendler, 2012; Williams et al., 2004; Yang, Zald, & Blake, 2007), the stimuli consisted of emotional faces, since the processing of these faces seems to be an automatic process. Williams et al. (2004) used backward-masking to present different facial expressions. Results of this study showed that fearful stimuli evoked faster skin-conductance response (SCR) rise times than neutral stimuli, but there were no significant differences in SCR amplitude between masked fearful and masked neutral stimuli. This indicates that conscious perception of fearful stimuli is necessary for autonomic arousal. Singer, Eapen, Grillon, Ungerleider and Hendler (2012) used a binocular rivalry procedure to present pictures of fearful or neutral expressions, which competed for conscious perception with pictures of a house. Participants consisted of social anxiety disorder patients, panic disorder patients and healthy controls, and were chosen based on the theory that the emergence and persistence of anxiety is caused by an exaggerated tendency to process threat signals. Both patient groups experienced a greater initial threat bias than healthy controls. This threat bias was determined by calculating the initial predominance of face (IPF) index to estimate whether the emotional expression of a face affects the incidence of its immediate selection into awareness (Singer et al., 2012). This increased initial threat bias could mean that increased anxiety can affect the visibility of stimuli, which would make it more difficult to consistently present them outside of conscious awareness. The most recently developed method of presenting stimuli outside of awareness, Continuous Flash Suppression (CFS), was used by Yang, Zald and Blake (2007) to present fearful, neutral and happy expressions to healthy participants. The participants had to indicate when (parts of) a face became visible. Fearful faces were identified faster than neutral or happy faces, which suggests that negatively charged facial expressions gain preferential access to awareness. This enables the rapid detection of potentially dangerous situations, even when stimuli are unconsciously perceived (Yang et al., 2007).

These studies demonstrate the fast unconscious processing of emotional faces, but studies have also been performed using different types of threatening stimuli. Öhman & Soares (1993, 1994) showed backwardly masked threatening stimuli (pictures of snakes and spiders) and neutral stimuli (pictures of flowers and mushrooms) to healthy subjects (1993) and phobic and normal subjects (1994). In healthy subjects the conditioned differential response to both the fear-relevant CS+ and the fear-relevant CS- remained during extinction, but with fear-irrelevant stimuli, masking was very effective in ruling out the conditioned SCRs (Ohman & Soares, 1993). Phobic subjects showed enhanced skin conductance responding specifically to their fearful stimulus, regardless of masking conditions. Masked stimuli were as effective in eliciting physiological fear responses as unmasked stimuli in phobic subjects (Ohman & Soares, 1994). However, this study had been criticized by Mayer, Merckelbach and de Jong (1999), mainly for their labeling of subjects as phobic, while they were fearful subjects; none were clinically diagnosed with a serious clinical phobia. Mayer, Merckelbach and de Jong have tried to apply the paradigm outlined by Öhman and Soares (1994) to patients suffering from phobias, but they were unable to replicate the results. Another study by Öhman and Soares (1998) focused on emotional conditioning to masked stimuli. The results showed that, in non-fearful participants, only the masked fear-relevant stimuli were able to elicit a differential response to the CS+ and the CS-, while the fear-irrelevant did not elicit a differential response (Ohman & Soares, 1998).

Aside from the unconscious processing of threatening stimuli, the study by Öhman and Soares (1993) also demonstrated that fear conditioning can occur independently of conscious awareness. When subjects are conditioned to an unseen visual stimulus, an association between the conditioned stimulus and threatening or harmful unconditioned stimulus is still formed (Critchley et al., 2002). This ability was further demonstrated in a recent study by Raio, Carmel, Carrasco, and Phelps (2012). Participants were presented with conditioned stimuli (fearful faces), which were suppressed using CFS for one group and not suppressed for the other group. One image co-terminated with a shock while the other was never presented with a shock. Fear learning was assessed by comparing the skin conductance responses (SCRs) between the CS+ and CS- conditions. Raio et al. (2012) found significantly greater SCRs to the CS+ then to the CS- in both the aware and unaware group. An important difference was the timing of fear learning; in the aware group learning increased over time, but the unaware group only showed significant learning during early acquisition (Raio et al., 2012). In a subsequent study by Lau, Carmel, Raio, and Schiller (2012) determined if fear reversal learning (modification of conditioned fear responses) is possible when stimuli are presented outside of awareness. Once again using CFS, during the fear learning phase two groups of participants (aware and unaware) were presented with two images of spiders; one was reinforced with a shock. Immediately after acquisition, fear reversal began; the CS+ became the CS- and vice versa. The results showed that reversal learning without awareness occurred quickly but was also quickly lost, while reversal learning without awareness was slower and lasted until the end of the experiment.

While masked fear-relevant stimuli like angry or fearful faces and pictures of snakes and spiders have been shown to elicit a fearful response, Alpers, Ruhleder, Walz, Mühlberger and Pauli (2005) used simple geometric patterns and induced emotional relevance in one of the two stimuli by fear conditioning. The horizontal and vertical gratings used as stimuli were presented, competing in binocular rivalry, which led to an alternating percept of the stimuli. One of the patterns was paired with an electric shock, and participants indicated their alternating perception by key presses. After conditioning, participants rated the, previously neutral, CS+ more negatively than the CS-. Participants showed an increasing, but weak, predominance of the CS+ with respect to the initial percept of each trial. The results of this study indicate that emotional relevance of visual stimuli can influence information processing (Alpers et al., 2005). Like Alpers et al. (2005) the current study uses neutral, simple geometric patterns (horizontal and vertical gratings) as stimuli, adding emotional relevance through fear-conditioning. Even though conditioning can be achieved when stimuli are presented subliminally (Critchley et al., 2002), to ensure effective learning of the CS-US pairings with little trials, the stimuli will be clearly presented to both eyes. Following conditioning, the stimuli will be presented using continuous flash suppression, which can render a stimulus invisible for a significantly longer period of time than binocular rivalry. The goal is to determine if these unconsciously presented stimuli can still elicit a conditioned fear response. This conditioned fear response will be measured by determining the eye-blink startle, which is one of the fear responses present even without conscious perception of the stimuli. Based on previous research, one would expect emotional stimuli to be processed regardless of conscious perception.

Methods

Participants

The research participants were 22 students at Utrecht University (15 women and 7 men, mean age = 23.6 years, sd = 3.0 years). Participants were recruited by posters and flyers distributed in University buildings. Only those with good stereoscopic vision were asked to respond. The posters and flyers also mentioned that participants would receive light electrical shocks. The study was presented as a study researching the link between visual perception and fear; they were naive to the specific goal of the experiment.

Of the 22 initial participants only 16 completed the experiment. One participant was excluded due to an inability to see any of the stimuli during the training sessions. One participant was excluded due to extreme dominance of the left eye. One participant was excluded for not following the instructions, this participant kept looking past the mirror stereoscope during several phases of the experiment, including instructed acquisition. One participant was excluded due to extremely low performance during the threshold measurements, making it impossible to present stimuli at a contrast where they would be visible. Two participants were excluded due to high performance during the threshold measurements, making it difficult to present stimuli at a contrast where they would be invisible to the participant.

The resulting 16 participants (10 women, 6 men) had a mean age of 24.2 years with a standard deviation of 2.9 years (see table 1).

Table 1. *Number of participants (male and female), mean and standard deviation of ages.*

	N	*M (age)*	*SD (age)*
Male	*6*	*25.33*	*4.03*
Female	*10*	*23.50*	*1.90*
Total	*16*	*24.20*	*2.90*

Apparatus & Materials

The stimuli were created on an Apple Mac Pro computer running OS X and Matlab 7.4 with the Psychophysics Toolbox extension (Brainard, 1997). Presentation of the stimuli occurred on a linearized LaCie III 22” display at 60 Hz. The stimuli were viewed through a mirror stereoscope, which ensured a standard optical path length of 57 cm from the eyes to the monitor.

Shock stimuli were delivered through a constant current simulator (Digitimer DS7A, Digitimer Ltd., Letchworth Garden City, United Kingdom) with tin cup electrodes placed over the inner wrist of the left arm. Startle stimuli, bursts of white noise with an instantaneous rise time, were presented through circumaural headphones (Sennheiser HD201). Eye blink startle was measured by electromyographic (EMG) recordings from the orbicularis oculi beneath the right eye; one electrode was centralized under the pupil and another approximately 15 mm lateral towards the outer cantus of the eye. CMS/DRL electrodes were used as isolated ground electrodes, placed behind each ear on the mastoid portion of the temporal bone.

Stimuli

The stimuli were presented against a background consisting of band-pass filtered noise in a black/gray pattern. The background was identical for both halves of the screen; fusion of this background leads to better rivalry between the target images (grating/noise and mask). Stimuli consisted of circular horizontal and vertical sine-wave gratings (1.91° of visual angle, 4 cpd) and a circular noise patch consisting of band-pass filtered noise. Band-pass filtered noise was also used as a mask (2.10° of visual angle). To create a flickering image necessary for continuous flash suppression, polarity of the mask was rapidly inverted (12 Hz). The edges of the images were smoothed with a cosine ramp of 0.29° of visual angle for the gratings and noise patch, and 0.42° of visual angle for the mask. The stimuli, noise patch and mask were presented inside a grey rectangle with a black outline of identical height and width as the mask. A fixation point was placed in the center of the stimuli. The gratings, noise patch and mask are shown in Figure 1. Total presentation time of the stimuli during all phases was four seconds. To avoid abrupt onset of the target images the contrast of these images was increased from 0% contrast to the desired contrast during the first half of the presentation time and then stayed constant at the desired contrast during the second half. The shock stimuli had a duration of 625 ms with an intensity determined individually. Startle stimuli were 105 db, 50 ms duration bursts of white noise.

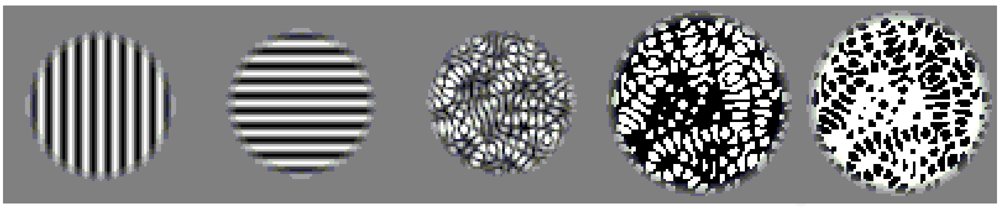

Figure 1. *Images used during the experiment. From left to right: Vertical gratings, horizontal gratings, noise patch, mask polarity A and mask polarity B.*

To determine a contrast at which the gratings would be invisible and a contrast at which the gratings would be visible to the participant, a Weibull distribution was fitted to the results of threshold measurements. From this distribution two contrast thresholds were extracted, one where the participant would have less than 50.5% correct and one where the participant would have more than 95% correct. When it was not possible to fit a Weibull distribution to the data or the thresholds could not be calculated, contrast thresholds were determined after visual inspection of the data (see Data Analysis for examples).

The orientation of the CS+ and CS- was counterbalanced based on participant number.

Procedure

After arriving in the laboratory, participants were asked several questions about their physical and mental health, focusing on anxiety disorders, epilepsy and claustrophobia. Male participants were also asked if they suffered from total or partial red-green color blindness. To determine if participants had any defects of binocular vision, they were asked to undergo part of the TNO test for stereoscopic vision (Plate I, II, III and V(1-3)) (TNO Test for Stereoscopic Vision, Richmond Products Inc., Albuquerque, USA). All participants were able to correctly identify the test figures on Plate V(2) at a retinal disparity of 60 seconds of arc. Following this test they signed a consent form and answered the questions pertaining to trait anxiety of a Dutch version of the Spielberger State-Trait Anxiety Inventory (STAI-T) (Defares, Ploeg, & Spielberger, 1980).

Training & Pre-test

After taking place behind the computer, participants were shown the gratings, masking images and noise patch twice, once without looking through the mirror stereoscope and once while looking through the mirror stereoscope. They were instructed to initiate presentation of the stimuli by pressing the space bar. After each trial participants used the arrow keys to indicate whether they were presented with gratings (horizontal or vertical) or a noise patch (2AFC discrimination task). Participants first completed two short training sessions, each consisting of 16 trials, during which they received verbal feedback from the experimenter on their performance. They were shown either vertical gratings or a noise patch to one eye and an alternating/flickering image to the other eye. The training was followed by the pre-test, consisting of 96 trials. They did not receive any feedback on their performance. This was followed by another two training sessions, this time participants were shown either horizontal gratings or a noise patch. They then performed another 2AFC discrimination task with horizontal gratings, also consisting of 96 trials.

During the two pre-tests, the contrasts of the gratings and the noise patch were varied using two independent, randomly interleaved staircases (QUEST, (Watson & Pelli, 1983)). Both staircases estimated the 75% correct threshold for discrimination between the two images. The gratings and noise patch were randomly presented to either the left or right eye.

Applying electrodes

After a short break, during which the contrast thresholds where determined for each individual participant (<50.5% correct & >95% correct), they returned to the laboratory and electrodes were applied.

Shock Work Up

Next, participants underwent a standardized shock workup procedure (Klumpers, Heitland, Oosting, Kenemans, & Baas, 2012). This procedure consisted of one extremely light electrical shock to test the set-up, followed by 5 sample shocks to set the shock intensity. Through this procedure the shock intensity was set individually for each participant at a level considered as "quite annoying" but not painful. The final intensity for all participants ranged from 0.5 mA to 2.2 mA (M = 1.4 mA, SD = 0.48 mA).

Startle Habituation

To adapt participants to startle stimuli, they underwent a short habituation procedure, during which the startle stimuli were presented 12 times. The first presentation occurred after 4 seconds, following presentation occurred after an inter-trial interval ranging from 19 to 23 seconds. During the procedure participants were asked to look at a fixation cross through the mirror stereoscope (presented to both eyes).

Instructed Acquisition

During the acquisition phase, participants were presented with a total of 12 CS+ and 12 CS- trials. Prior to the phase, instructions were displayed on screen indicating (both through text and example images) which of the gratings could be presented with a shock (threat) and which would never be paired with a shock (safe). The stimuli were presented unmasked at full intensity to both eyes; the participants viewed the stimuli through the mirror stereoscope. Participants were presented with eight CS+ and eight CS- trials in random order. The startle probe was presented in six out of 12 CS+ and six out of 12 CS- trials, with a random onset time between 2325 and 2825 ms. Six of the CS+ presentations were reinforced with a shock, with an onset time of 3375 ms. Total presentation time of the stimuli was 4 seconds. During the inter-trial interval (17-21 seconds) the fixation point was red. One second before the start of the next trial the fixation point turned green and stayed green during the trials.

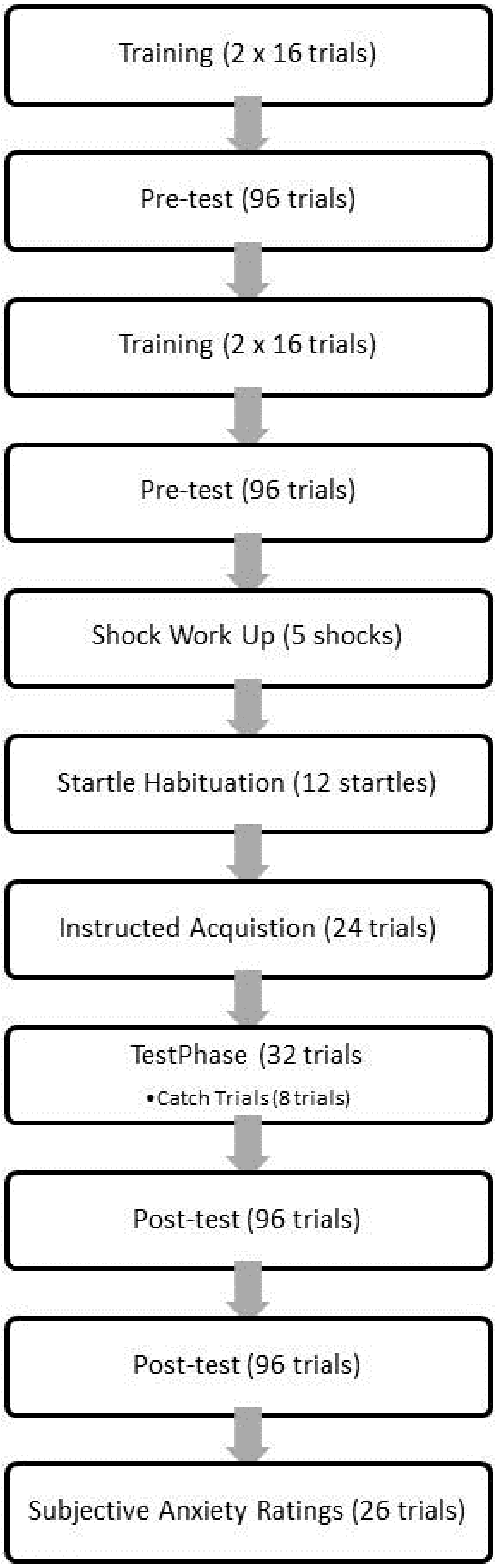

Figure 2. *The order of the different phases in the task and the number of trials per phase. The entire experiment lasted approximately two hours.*

TestPhase

Following acquisition, the participants were presented with 16 CS+ and 16 CS- trials. Both CS+ and CS- were presented at the low contrast at which the stimuli would be invisible to the participant (less than 50.5% correct), as determined after the threshold measurements. Of the 16 CS+ and 16 CS- trials, 12 of each (75%) were presented with the startle probe, which had a random onset time between 2325-2825 ms. Eight extra trials were presented randomly interspersed between the low contrast trials. During these trials (4 CS+ & 4 CS-) the stimuli where presented at higher contrast, also determined after the threshold measurements. At this higher contrast the stimuli should be visible to the participant. The startle probe was presented in three out of four trials; the third CS+ and third CS- trial were presented without a startle probe. Three out of four CS+ trials were reinforced with a shock; a shock was absent in the second CS+ trial. By presenting the stimuli at a contrast where the stimuli are visible and reinforcing the presentation of the CS+ with a shock, extinction is delayed/prevented.
The inter-trial interval had a random duration of 17 to 21 seconds. During this time the fixation point was red. The fixation point turned green one second before the start of the next trial and stayed green during the trials.

Post-test

Participants next performed the two 2AFC discrimination tasks again, in the same order as at the start of the experiment.

Subjective Anxiety Ratings

During the last part of the experiment participants were shown 26 trials consisting of six masked CS+ low contrast trials, six masked CS+ high contrast trials, six masked CS- low contrast trials and six masked CS- high contrast trials. During the last two trials participants were shown unmasked CS+ and CS- without looking through the mirror stereoscope. The trials were presented in random order. After each trial the participants were asked to rate their fear of receiving an electrical shock on a 10-point scale.

Data Analysis

Scores on the trait anxiety scale of a Dutch version of the Spielberger State-Trait Anxiety Inventory (STAI-T) (Defares et al., 1980) were calculated by inverting the responses to specific questions and adding up the scores to create a final score. Startle data were pre-processed and checked for artifacts according to previously published guidelines (Blumenthal et al., 2005) and procedures (Bocker, Baas, Kenemans, & Verbaten, 2004; Klumpers, Raemaekers, et al., 2010; Klumpers, van Gerven, et al., 2010). To analyze the results, the startle magnitudes were transformed to z-scores per subject. Z-scores were averaged according to phase and stimulus type (CS+/CS-, high/low contrast).

Contrast thresholds were determined by fitting a Weibull distribution to the results of the threshold measurements (see figure 3 for an example). In several cases this proved impossible (see figure 4 for an example), due to an extremely high or low percentage of correct answers or large differences between the two staircases.

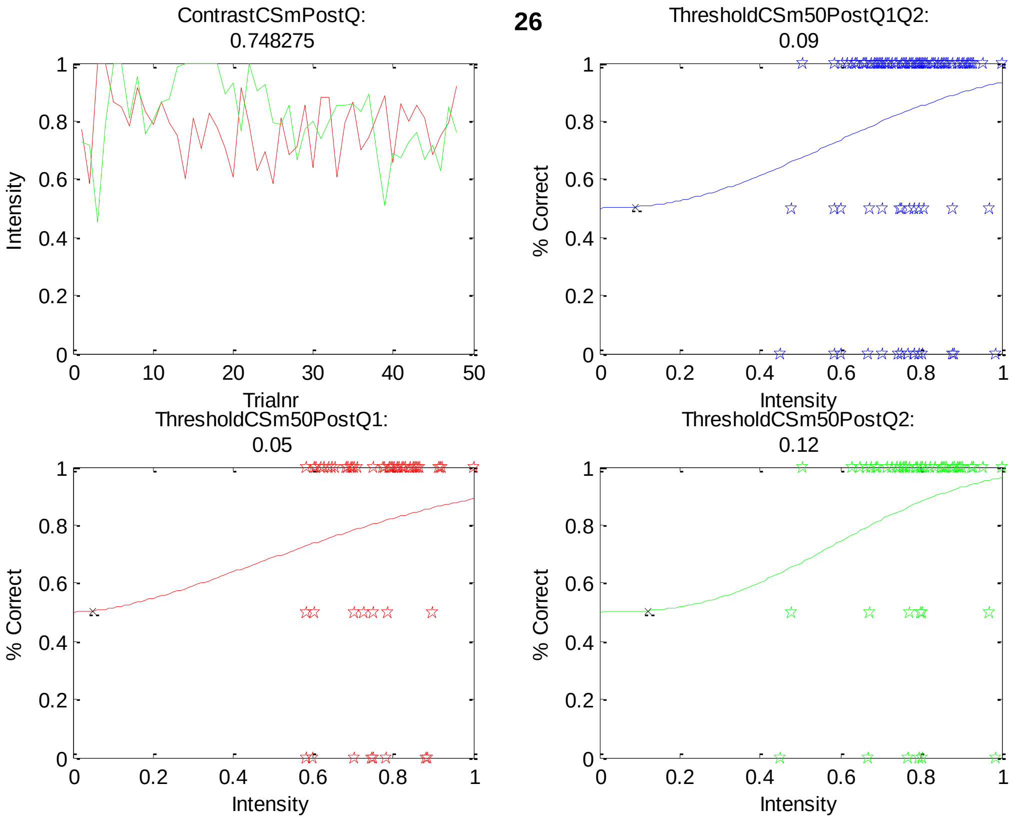

Figure 3. *Correctly fitted Weibull distribution to the results the post-test (CS-) of one participant. Top left panel: Changing contrast (intensity) of the target images during the post-test for both staircases (red/green lines) with 48 trials each. The value above the graph is the estimated 75% correct threshold. Top right panel: Weibull distribution fit to the results of both staircases, with the percentage of correct responses on the vertical axis and contrast on the horizontal axis. The value above the graph is the estimated <50.5% correct threshold. Bottom left panel: Weibull distribution fit to the results of a single staircases (red line in top left panel), with the percentage of correct responses on the vertical axis and contrast on the horizontal axis. The value above the graph is the estimated <50.5% correct threshold. Bottom right panel: Weibull distribution fitted to the results of a single staircases (green line in top*

left panel), with the percentage of correct responses on the vertical axis and contrast on the horizontal axis. The value above the graph is the estimated <50.5% correct threshold.

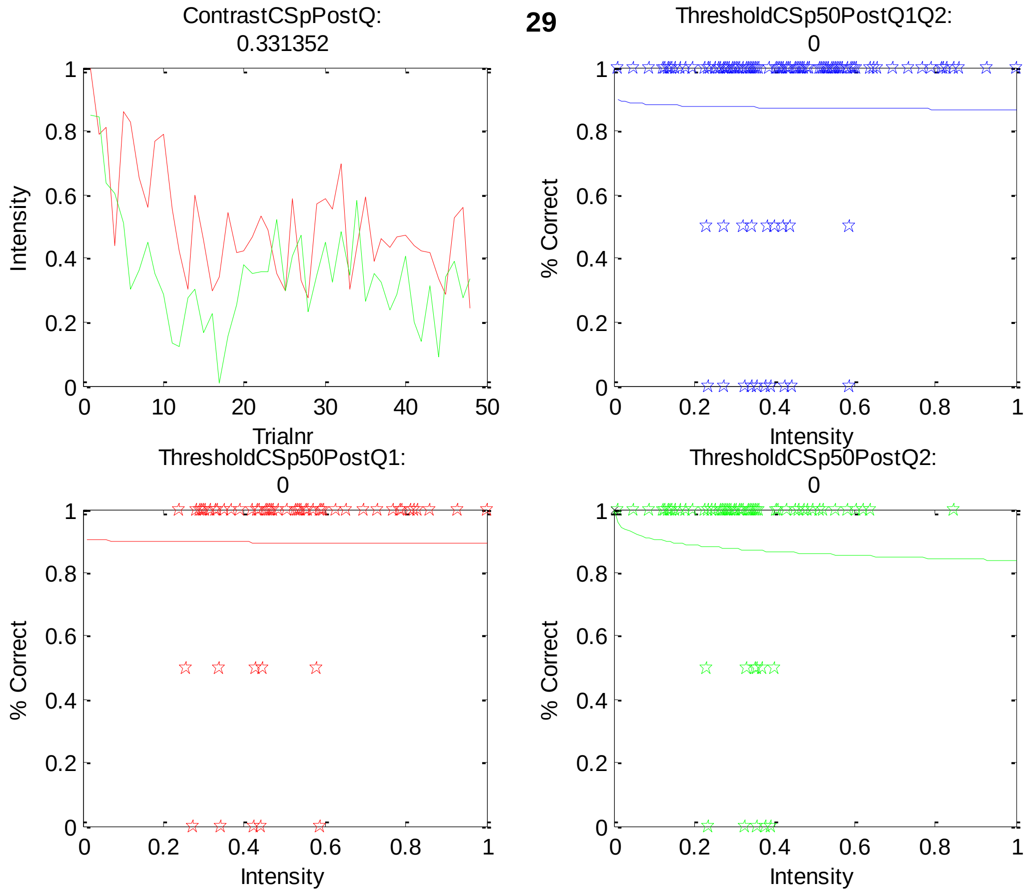

Figure 4. *Incorrectly fitted Weibull distribution to the results the post-test (CS+) of one participant. Top left panel: Changing contrast (intensity) of the target images during the post-test for both staircases (red/green lines) with 48 trials each. The value above the graph is the estimated 75% correct threshold. Top right panel: Weibull distribution fit to the results of both staircases, with the percentage of correct responses on the vertical axis and contrast on the horizontal axis. Bottom left panel: Weibull distribution fit to the results of a single staircases (red line in top left panel), with the percentage of correct responses on the vertical axis and contrast on the horizontal axis. Bottom right panel: Weibull distribution fitted to the results of a single staircases (green line in top left panel), with the percentage of correct responses on the vertical axis and contrast on the horizontal axis.*

All statistical analyses were carried out in SPSS Statistics 17.0 (SPSS, Chicago, Illinois). Mean startle amplitudes and contrast threshold differences were compared in repeated measures ANOVAs. State anxiety levels were compared in t-tests. Spearman's correlation was used for correlational analyses.

Results

Spielberger State-Trait Anxiety Inventory (STAI) scores: Prior to the experiment, all participants completed the part of the STAI pertaining to trait anxiety. Average scores and standard deviations are presented in table 2. The maximum score on the STAI-T is 80, corresponding to the highest level of trait anxiety.

Table 2. *STAI-T, average scores and standard deviations.*

	N	*M*	*SD*
Male	*6*	*31.50*	*4.23*
Female	*10*	*35.40*	*8.97*
Total	*16*	*33.94*	*7.62*

Startle data: All startle data were converted to standardized z-scores prior to the statistical analyses. For the sake of completeness the raw data are also displayed (figure 5). When comparing the standardized z-scores from the acquisition phase (see table 3), participants did not show a significantly higher startle amplitude when presented with the CS+ than when presented with the CS- ($F(1,15)= 3.962$, $p = 0.065$). After inspection of the data, one outlier was identified with a low standardized z-score for the CS+. Removal of this outlier from the data resulted in a lower p-value, but did not result in a significant difference ($F(1,14) = 4.372$, $p = 0.055$).

When comparing the standardized startle amplitudes from the test phase (table 3), there is no significant difference when participants are presented with the CS+ or the CS- ($F(1,15) = 0.321$, $p = 0.579$). The exclusion of the results from one outlier did not result in a significant difference ($F(1,14) = 2.319$, $p = 0.150$).

Participants also did not show a significant difference in startle amplitudes during the catch trials ($F(1, 15) = 0.594$, $p = 0.453$), when they were presented with clearly visible stimuli. Mean Z-scores and standard deviations for the catch trials can be found in table 3.

Standardized z-scores were also grouped (per 3 data points) to analyze the effect of habituation (figure 6 & 7). The differences between the four (grouped) mean z-scores from the habituation phase were significantly different ($F(2.122, 31.832) = 24.842$, $p < 0.0005$). The (grouped) mean z-scores from the acquisition phase showed a significant difference between the first and second half of the trials for both the CS+ ($F(1, 15) = 6.798$, $p = 0.02$)

and the CS- (F(1, 15) = 7.579, p = 0.015). The differences between the four (grouped) mean z-scores from the testphase for both the CS+ (F(2.165, 32.482) = 1.264, p = 0.298) and CS- (F(2.757, 41.355) = 1.246, p = 0.304) were not significant.

Table 3. *Average Z-scores and standard deviations from both the acquisition phase, test phase and catch trials for all participants.*

Phase	Stimulus	Mean Z-Score	Standard Deviation	F	*p*
Acquistion	CS+	.499	.330	F(1,15) = 3.962	0.065
	CS-	.160	.590		
Testphase	CS+	-.195	.287	F(1,15) = 0.321	0.579
	CS-	-.150	.313		
Catch Trials	CS+	-.339	.499	F(1,15) = 0.594	0.453
	CS-	-.219	.414		

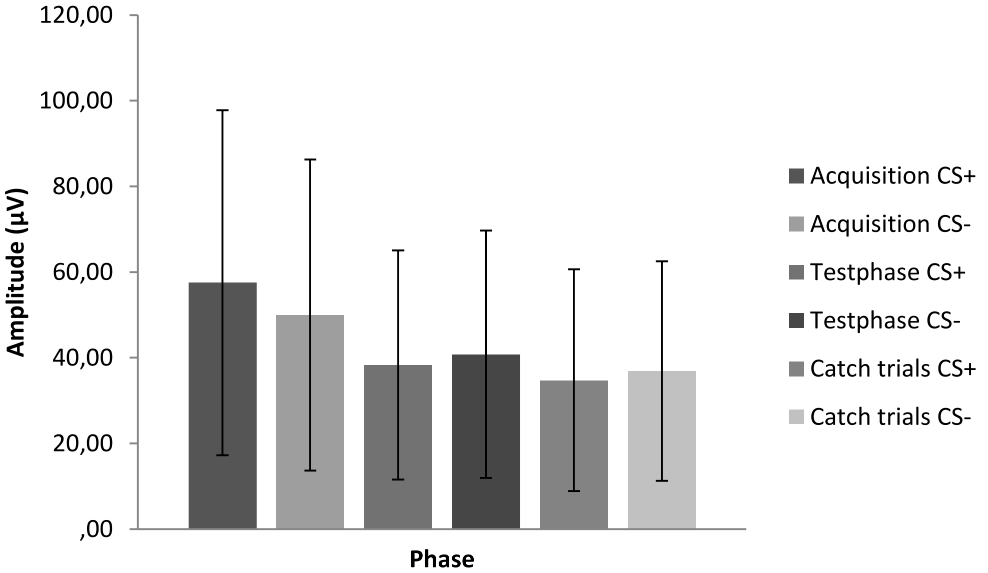

Figure 5. *Mean startle amplitudes (µV) from the acquisition phase, test phase and catch trials for both CS+ and CS-. The error bars represent the standard deviation for each phase per stimulus type. These raw startle amplitudes show no large differences between stimuli for each of the phases.*

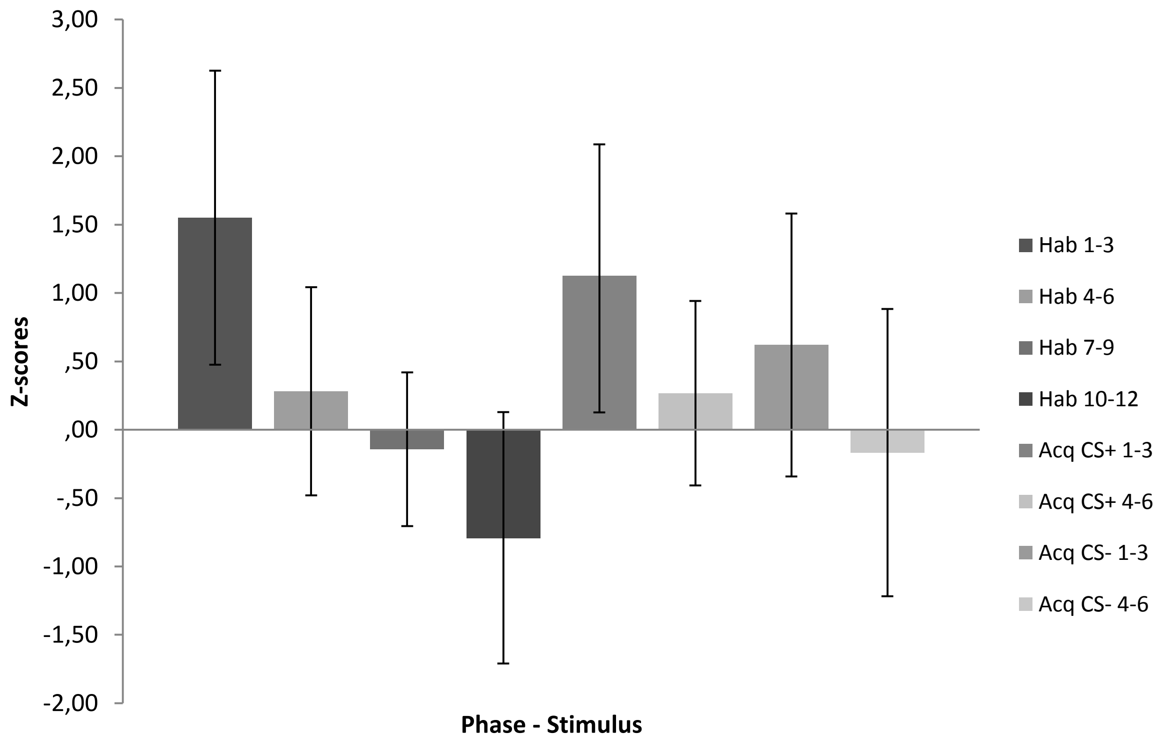

Figure 6. *Mean standardized startle amplitudes (z-scores) from the habituation and acquisition phase, averaged per three subsequent data points. For the acquisition phase, mean standardized startle amplitudes are displayed for both CS+ and CS-. Error bars represent the standard deviation for each of the mean z-scores. Differences between the four grouped z-scores from the habituation phase were significant, as were the differences between the first and second half of the trials during acquisition for both CS+ and CS-.*

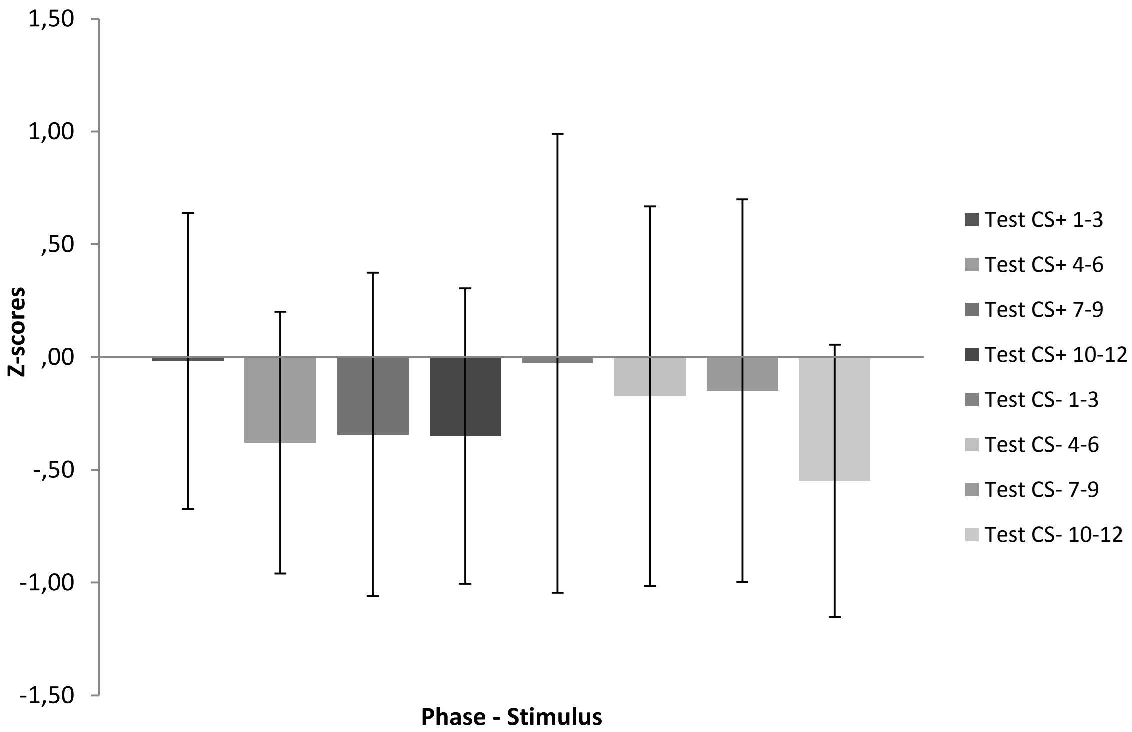

Figure 7. *Mean standardized startle amplitudes (z-scores) from the test phase, averaged per three subsequent data points. Mean standardized startle amplitudes are displayed for both CS+ and CS-. Error bars represent the standard deviation for each of the mean z-scores. The differences between the four (grouped) z-scores for both the CS+ and CS- were not significant.*

Mean fear-potentiated startle (FPS) was determined for the acquisition phase, testphase and catch trials (figure 8). Participants showed different fear-potentiated startles during the three phases, but these differences were not significant.

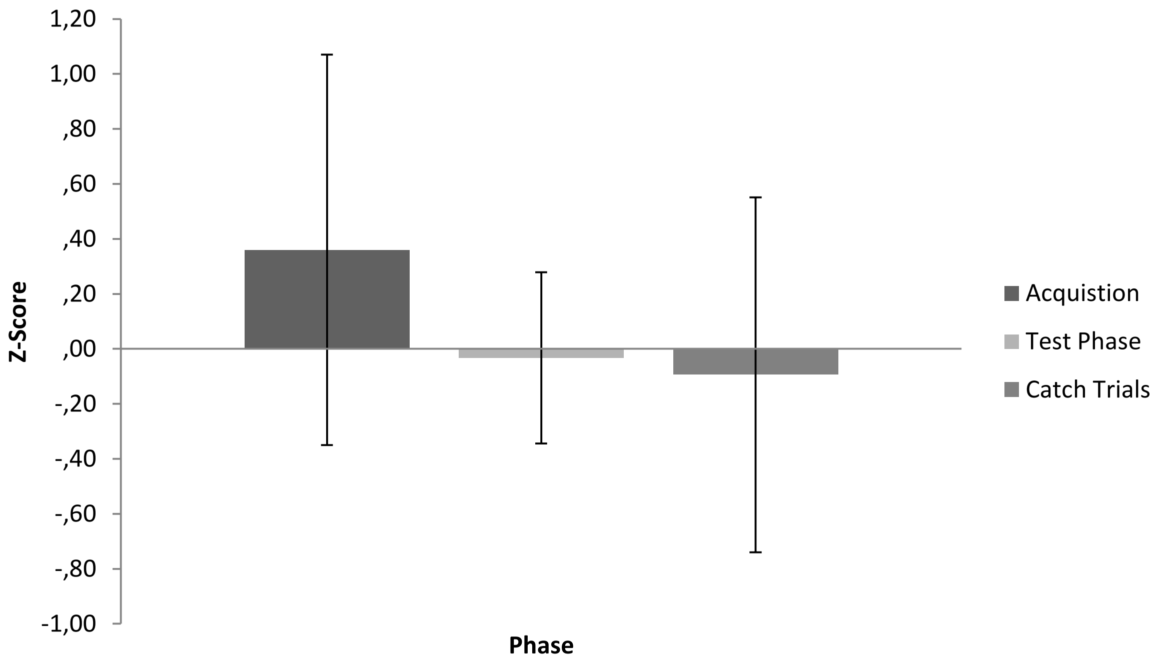

Figure 8. *Mean fear-potentiated startle (startle CS+ - startle CS-) for three phases of the experiment. Error bars indicate the standard deviation for each of the means. The small differences between the z-scores for each phased were not significant.*

Contrast thresholds: As described in the Methods sections, many of the contrast thresholds were determined based on visual inspection of the results of the pre- and post-tests. To be able to compare these thresholds, values were estimated again after all participants had completed the experiment. The average of the two estimated contrast thresholds were used for further analysis. To eliminate individual differences between participants, all mean contrast thresholds were converted to standardized z-scores. The mean z-scores are displayed in figure 9.

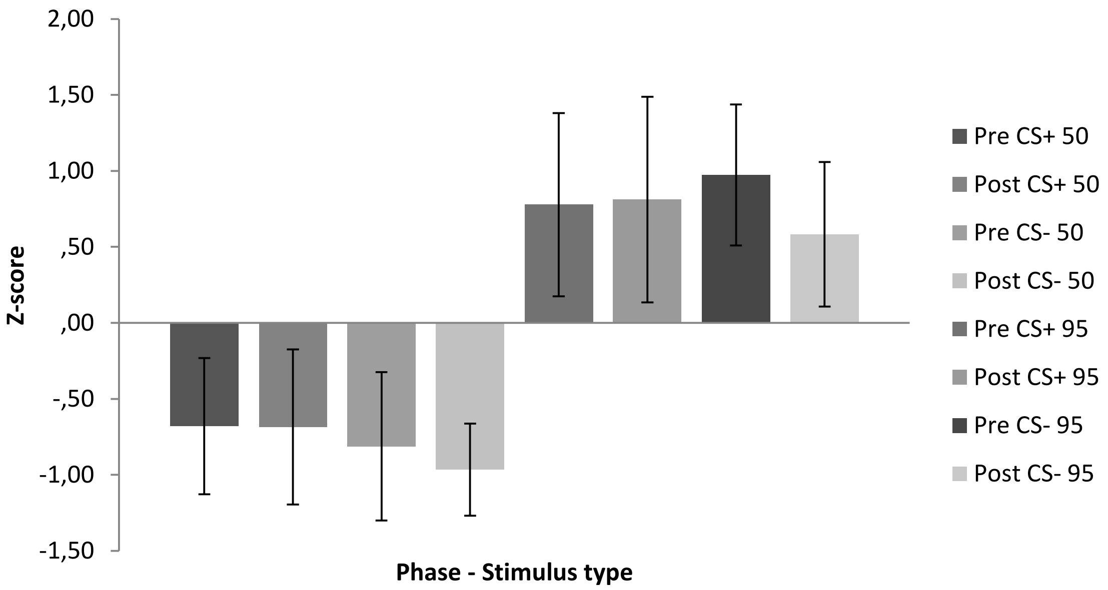

Figure 9. *Mean z-scores of the contrast thresholds per test (pre- and post-test), stimulus type (CS+ and CS-) and percentage of correct responses (<50.5% and >95%). Error bars indicate the standard deviation for each of the mean scores.*

There were no significant differences between the standardized contrast thresholds as determined with the pre- and post-tests (table 4).

Table 4. *Significance of the differences for each stimulus/percentage correct between pre- and post-test (dfM = 1, dfR = 15).*

Contrast Threshold	F value	*p*
CS+ 50 Pre/Post	.001	.979
CS- 50 Pre/Post	1.035	.325
CS+ 95 Pre/Post	.013	.910
CS- 95 Pre/Post	3.720	.073

To test for an effect of conditioning on the (in)visibility of the stimuli, the difference scores of the standardized contrast thresholds (CS+ - CS-) were compared. There were no significant differences between the difference scores at low contrast ($F(1,15) = 0.632$, $p = 0.439$) or at high contrast ($F(1,15) = 2.233$, $p = 0.156$).

State anxiety: State anxiety was measured through the final anxiety questionnaire. Participants reported significantly higher levels of anxiety/fear of receiving a shock when presented with the CS+ than the CS- at low contrast (t(15) = 2.67, p = 0.018) and at high contrast (t(15) = 4.062, p = 0.001), on a 6 to 60 scale. Average scores and standard deviations are presented in table 5. Participants also reported a significantly higher level anxiety/fear when the CS+ was presented without suppression than when the CS- was presented (t(15) = 4.070, p = 0.001).

Table 5. *Mean scores and standard deviation on reported levels of anxiety/fear.*

Stimulus - Contrast	Mean Score	Standard Deviation	t	*p*
CS+ low	21.66	9.33	t(15) = 2.67	0.018
CS- low	17.56	8.26		
CS+ high	29.81	12.85	t(15) = 4.062	0.001
CS- high	15.88	9.41		

STAI & FPS: A significant correlation (Spearman) was found between the STAI score and the FPS of the acquisition phase, r = -0.706, p = 0.002 (figure 10). No significant correlations were found between the STAI score and FPS during the test-phase (r = -0.080, p = 0.769) or catch trials (r = 0.028, p = 0.918).

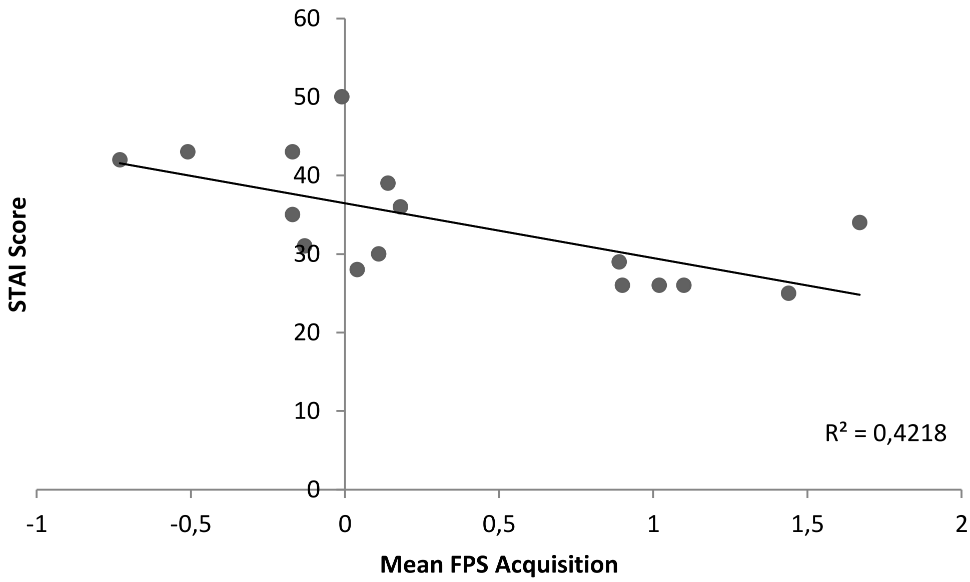

Figure 10. *Correlational plot of the STAI scores and fear-potentiated startle (FPS) during the acquisition phase of all participants. A significant negative correlation was found between the two variables.*

STAI & Contrast Threshold Differences: No significant correlations were found between the STAI score and the differences in contrast thresholds between pre- and post-test.

Discussion & Conclusion

The goal of this study was to determine if fear-potentiation is possible when stimuli are presented subliminally. Participants first completed two pre-tests, during which the contrast thresholds of the stimuli were determined where each participant would have less than 50.5% correct. Using this contrast level to present the stimuli with continuous flash suppression should render the stimuli invisible. Following the acquisition phase, stimuli were presented subliminally and eye-blink startle was determined as an index of acquired fear. Based on previous studies (Alpers et al., 2005; Ohman & Soares, 1993, 1994, 1998; Raio et al., 2012; Yang et al., 2007), the expectation was that even unconsciously perceived stimuli would elicit a fear-potentiated startle response.

Participants showed no significant difference in their response when presented with the CS+ than when presented with the CS- during the acquisition phase, test phase or during the catch trials. There were significant differences between the blocked standardized results from the habituation phase and acquisition phase. The mean fear-potentiated startle, an important measure of conditioned fear, did not differ significantly between the three phases of the experiment (acquisition, testphase and catch trials). A comparison of the contrast thresholds determined with the pre- and post-test did not show any significant differences. This study did find a significant negative correlation between the STAI-T score and the fear-potentiated startle during the acquisition phase, indicating that in this study individuals reporting higher levels of trait anxiety showed less difference between their responses to threat and safe conditions.

Surprisingly, the results of the acquisition phase indicate that conditioning was not effective. Participants did not show significantly higher startle amplitudes when presented with the CS+ than when presented with the CS-. But participants were aware of the contingencies, as demonstrated by the significantly different scores on state anxiety reported in the final stage of the experiment. Hamm and Vaitl (1996) stated that instructions specifying which stimulus would be paired with an electric shock or an acoustic startle would lead to consistent enhancement of the startle eyeblink magnitude. Solely based on this statement, the ineffective conditioning could have been caused by the participants not paying attention to the instructions or the trials, but other studies have indicated that conscious awareness or attention is not necessary to achieve fear conditioning (Critchley et al., 2002; Lau et al., 2012; Raio et al., 2012). Since the stimuli consisted of horizontal and vertical gratings, participants could have found it difficult to recognize the two different stimuli, even though they were shown clear examples during the instructions. However, the use of horizontal and vertical gratings as opposed to gratings at an oblique angle should make it easier to correctly identify the stimuli (Li, Peterson, & Freeman, 2003). Another explanation could be that the shock stimulus was not strong enough to be very aversive. The shock work-up was performed with the experimenter in the room, which meant that participants could more easily pick up on the fact that their responses influenced the strength of the electric shock.

Since the participants did not demonstrate effective conditioning, this makes it difficult to interpret the results of the test-phase and catch trials. The small sample size could be contributing to these results; only 16 participants completed the experiment showing large variation (high SDs) in startle amplitudes. The participants did not show any significant differences in standardized startle amplitudes between CS+ and CS- in both phases. However, it is impossible to determine if the same results would be found if conditioning was effective. Based on previous studies of unconscious perception of emotional faces, threatening images and neutral images given emotional valence through aversive conditioning, one would expect that the results would show a larger startle amplitude when participants are presented with the CS+ than the CS-.

During the startle habituation and conditioning phases, participants did show a steady decline in average startle amplitude per three trials (blocked). This indicates that over time, participants reacted less to the acoustic startle probes presented during startle habituation. This steady decline was not present during the test-phase, which would indicate extinction. The main reason for the inclusion of the enforced catch trials during the test-phase was to prevent extinction, which appears to have had the desired effect.

An important aspect of this study was the unconscious presentation of stimuli. High and low contrast thresholds were determined prior to conditioning. Since it was not always possible to fit a Weibull distribution to the results of the pre-test, some of the thresholds were estimated based on visual inspection of the data. Estimates were conservative for the low contrast thresholds to ensure invisibility and liberal for the high contrast thresholds to ensure visibility of the stimuli. However, it is difficult to state that stimuli were indeed presented subliminally throughout the entire experiment. This could be overcome by asking participants to identify the stimulus after each trial and rate their confidence. Raio et al. (2012) used this method to verify successful manipulation of awareness; objective unawareness would be indicated by performance at chance level in identifying the stimulus and subjective awareness would be indicated by low confidence in the identification.

In conclusion, the results of this study provide no conclusive evidence that subliminal stimuli are sufficiently processed to elicit a conditioned response. Main problems were the ineffective conditioning, even though state anxiety scores did indicate contingency awareness, and the uncertainty of continuous subliminal presentation of the stimuli. Other, easily overcome, issues are the low number of participants and the duration of the task; participants had to stay focused for approximately two hours. For a follow-up study, one would first have to achieve effective conditioning, perhaps through the use of more easily recognized stimuli.

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