

Chronic stress reverses enhanced neophobia following an acute stressor in European starlings

Robert de Bruijn  | L. Michael Romero 

Department of Biology, Tufts University,
Medford, Massachusetts, USA

Correspondence

Robert de Bruijn, Department of Biological
Sciences, California State University
San Marcos, 333 S. Twin Oaks Valley Road,
San Marcos, CA 92096, USA.
Email: physiologistemail@gmail.com

Present address

Robert de Bruijn, Department of Biological
Sciences, California State University San
Marcos, 333 S. Twin Oaks Valley Road,
San Marcos, CA 92096, USA.

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Abstract

Neophobia is an animal's avoidance of novelty. Animals tend to respond to novel objects by increasing their latency to approach the objects, and they eventually habituate after repeated exposure by attenuating this increased approach latency. Interestingly, the physiological stress response does not appear to have a causal link to neophobia, although acute stress can prevent animals from habituating to novel objects, possibly through a permissive effect. Chronic stress can induce an anxiety-like state in animals, while often disrupting the ability to respond to acute stress. We thus hypothesized that chronic stress may increase neophobia and tested this by inducing chronic stress in wild-caught European starlings (*Sturnus vulgaris*). Four distinct anthropogenic stressors were administered daily for 30 min each in a randomized order for 21 days. We then evaluated whether exposure to chronic stress altered the latency to approach a novel object placed on or near a food dish presented after overnight fasting. Chronically stressed birds and nonstressed controls exhibited similar initial neophobic responses to novel objects and showed similar habituation in response to repeated exposure. However, when birds were exposed to 15 min of restraint before repeated exposure to the same object, habituation was eliminated in control birds (i.e., they continued to respond with neophobia), whereas chronically stressed birds continued to show habituation as measured by attenuated approach latencies. These results demonstrate that an acute stress response (restraint) has a different impact on neophobia depending upon whether the bird is or is not concurrently exposed to chronic stress.

KEYWORDS

acute stress, chronic stress, European starling, habituation, neophobia

1 | INTRODUCTION

Neophobia, an animal's avoidance of novelty (Barnett, 1954; Chitty & Shorten, 1946), can influence the success of species in new habitats by altering intake of new food sources or avoidance of potential threats (Crane et al., 2020; Duncan et al., 2003). This behavior can be readily measured in the laboratory and field by exposing animals to novel objects (Greenberg, 2003). There is large variability, both between species and within individuals, for this avoidance behavior

(Greenberg & Mettke-Hofmann, 2001). Relatively little is known about the physiological mechanisms underlying neophobia and the strength of the physiological response may depend on the severity of the novel stimulus. If a stimulus is perceived as a threat, the response involves a full range of physiological changes, including an increase in heart rate and glucocorticoid production (Zanette & Clinchy, 2017). Furthermore, a fear of novelty has been associated with glucocorticoid dynamics and early death in adult rats (*Rattus norvegicus*; Cavigelli & McClintock, 2003). In recent years, a lot of interest has

gone toward linking behavioral syndromes, such as the bold–shy continuum, and the physiological response to stress (Koolhaas et al., 1999, 2010), with conflicting results. For example, proactive roe deer (*Capreolus capreolus*) show a stronger behavioral and physiological response to stress than reactive animals (Monestier et al., 2016). However in contrast, glucocorticoids do not appear to play a role in the plasticity of boldness of rainbow trout (*Oncorhynchus mykiss*) in the context of hunger and predation (Thomson et al., 2012). In European starlings (*Sturnus vulgaris*), a previous study found that a stress response immediately preceding exposure to a novel object increases the frequency of neophobic responses in European starlings (de Bruijn & Romero, 2020). This suggests that stress exerts permissive actions on neophobia, in that it can alter how an individual responds to novelty (Sapolsky et al., 2000; Vera et al., 2017). There are many similarities. Here, we investigate how changes in stress physiology could affect this potential regulatory pathway to influence neophobia.

The stress response in vertebrates modulates physiology and behavior so that an animal can properly cope with a stressor (Romero & Wingfield, 2016). The stress response is physiologically complex and characterized by the release of various hormones such as glucocorticoids, the catecholamines (including epinephrine and norepinephrine), and corticotropin-releasing factor (CRF), each of which has behavioral effects (Hauger et al., 2003; Romero & Butler, 2007; Romero & Wingfield, 2016; Sapolsky et al., 2000). Selective breeding for specific behaviors can lead to different stress reactivity profiles (Korte et al., 1997; Stöwe et al., 2010), suggesting that the stress response is involved in modulating particular behaviors. Previous research has focused on the effects of glucocorticoids on individual personality and behavior (e.g., Cockrem, 2007). However, the exact relationship between glucocorticoids and behavior is not clear, as some studies have found correlations between glucocorticoids and behavior (e.g., Kralj-Fišer et al., 2009; Wingfield & Silverin, 1986), while others have not (Garamszegi et al., 2012; Lendvai et al., 2011). The activation of the stress response (induced via restraint), but not circulating corticosterone concentrations, has been shown to alter neophobia to a novel object near the food dish in European starlings (de Bruijn & Romero, 2020). This suggests that a component of the stress response other than glucocorticoids may regulate this behavior, such as the release of epinephrine and norepinephrine (Hagemann et al., 1998) or CRF (Ohgushi et al., 2001), all of which can exhibit effects on feeding behavior.

Physiological changes can mediate behavioral responses to the environment (Ricklefs & Wikelski, 2002), such as the stress response enabling behavioral responses to appropriately cope with stressors (Wingfield et al., 1998). However, prolonged activation of the stress response can disrupt homeostasis and result in a state of chronic stress (Romero et al., 2009), a condition that is known to have deleterious effects on an animal's physiology, ranging from decreased body weight to compromised immune and reproductive systems (Sapolsky et al., 2000). In European starlings, chronic stress results in suppression of corticosterone release from the hypothalamic–pituitary–adrenal (HPA) axis (Awerman & Romero, 2010; Cyr & Romero, 2007;

Rich & Romero, 2005), altered neural glucocorticoid and mineralocorticoid receptor messenger RNA (mRNA) expression that may be indicative of an anxiety-like state (Dickens et al., 2009), and reduced heart rates (Cyr et al., 2009). Such effects appear to have negative fitness consequences in these birds, as evidenced by chronically stressed parents having reduced fledgling viability (Cyr & Romero, 2007), potentially driven by physiologically induced changes in behavior. Several consequences of chronic stress on behavior remain unclear, however, as previous studies have provided conflicting results. For example, while chronic stress affects spatial recognition in rats, it does not appear to reduce novelty-seeking behavior (Wright & Conrad, 2005). In contrast, markers of chronic stress have been shown to be associated with increased neophobia in captive roe deer (Monestier et al., 2017). Interestingly, short-term exposure to repeated stress over the course of 4 days appear to affect neophobia in house sparrows (*Passer domesticus*), however, this effect appears to be transient as it disappears upon recovery (Gormally et al., 2019) and it remains unclear if chronic stress would result in the same response.

This study, therefore, aimed to investigate the affect of chronic stress on neophobia and specifically on the the interaction between the acute stress response and neophobia. We predicted that chronic stress would result in increased neophobia, as measured by longer approach latencies toward novel objects. Additionally, while acute stress has been shown to have permissive effects on neophobia and prevent habituation (de Bruijn & Romero, 2020), we expected that this effect would be reduced in chronically stressed birds due to dysregulation of stress physiology.

2 | MATERIALS AND METHODS

2.1 | Animals

Sixteen adult European starlings (6 males, 10 females) were captured in Medford, MA throughout September and October 2012. Only birds that had fully completed molt, indicated by complete regrowth of the primary feathers and no evidence of body molt, were captured. Upon capture, animals were placed in individual cages (34.29 × 38.10 × 44.45 cm), each of which contained two perches, for at least 2 weeks before the start of the experiment. They were then randomly assigned to either a control group (three males, five females) or a chronic stress group (three males, five females). Each group was housed in separate rooms on an 11-h light cycle (11L:13D) with food (Purina Mills Start & Grow Sunfresh Recipe [18% protein]) and water available ad libitum, except where detailed below. The animals could hear and see each other throughout the experiment, except briefly during neophobia trials when dividers were placed between the cages to prevent the birds from seeing their neighbors interact with novel objects. Birds were weighed periodically throughout the study. This study was carried out under the *Guidelines to the Use of Wild Birds in Research* (Fair et al., 2010) and was approved by the Tufts Institutional Animal Care and Use committee.

2.2 | Chronic stress protocol

The chronic stress protocol was similar to the protocol first described by Rich and Romero (2005) and used in several other studies (Awerman & Romero, 2010; Cyr, Earle, et al., 2007; Cyr & Romero, 2007). Four stressors were administered daily in random order and at randomized time points, during the light hours of the light cycle, for 21 days. Stressors were designed to elicit mild psychological stress and induce an increase in plasma corticosterone (Rich & Romero, 2005). Each stressor lasted for 30 min and stressors were separated by at least 2 h. Each stressor was administered to the entire room, taking care to make sure each individual bird was exposed similarly across the entire experimental period. Stressors consisted of cage tapping (by running a spare perch along the front of a cage), music (played from a radio at a moderate volume), human presence and voice (reading at conversation level), and crowding (four individuals placed in a single cage).

2.3 | Blood sampling and hormonal analysis

To assess baseline and restraint-induced corticosterone levels, blood sampling occurred on Days 0, 4, 8, 12, and 15 of the chronic stress protocol, and always took place 2 h after behavioral trials with no object (see below), which was within 3 h after lights on. A similar protocol was used for the control group, except Day 12 sampling was omitted due to logistical reasons and to reduce the risk of inducing chronic stress due to repeated sampling. Baseline samples were taken within 3 min of entering the room (Romero & Reed, 2005). Birds were then restrained in a cloth bag for 30 min to induce an increase in circulating corticosterone levels, which is a standardized stressor (Wingfield et al., 1992). A second blood sample was taken at the end of this restraint period. Blood samples were taken via heparinized capillary tubes (60 μ l) from the brachial wing vein and immediately placed on ice until centrifuged.

Centrifugation occurred for 5 min at 400g, and plasma was then extracted and placed at -4°C . Corticosterone concentrations were analyzed from the plasma samples using radioimmunoassay techniques similar to Wingfield et al., (1992). In short, corticosterone labeled with radioactive tritium (^3H) was added to samples to allow for recovery to be calculated. Redistilled dichloromethane was added to samples to extract the corticosterone from the plasma. Samples were dried in a hot water bath under a nitrogen evaporator, followed by the addition of phosphate-buffered saline. Next, the samples were separated into duplicates and assayed with radioactively labeled corticosterone and corticosterone antibody (B3-163; Endocrine Sciences) along with a third aliquot to determine recovery. Charcoal was added to each duplicate sample to separate the bound from the unbound hormone, and the charcoal was then precipitated via centrifugation. Supernatants were counted for radioactivity. Radioactive counts in each sample were measured against a standard curve and adjusted for recovery percentage, to assess the original corticosterone concentrations. Intra- and interassay coefficients of variation were 2.7% and 2.1%, respectively.

2.4 | Novel objects, neophobia testing protocol, and behavioral analysis

Seven different novel objects were used throughout the study, which all have been shown to elicit neophobic responses in starlings (de Bruijn & Romero, 2020). Each object modified a normal white square plastic feeding dish (100 \times 70 \times 75 mm). The seven objects were a feeding dish painted red on the exterior (red dish), a red wrist coil keychain around the dish (ring), a blinking red light hung above the dish by a clip to the cage and directed at the front of the dish (light), a white plastic cover over the food dish (cover), a colored plastic egg placed in the middle of the dish (egg), an opened green or blue cocktail umbrella taped vertically to the side of the dish (umbrella), and two yellow pipe cleaners extending both horizontally and vertically around the dish, held in place by translucent red plastic clips (pipe cleaners). Note that because the goal of these studies was to explore the impact of various aspects of stress on neophobia per se, we used multiple objects for each experimental test. This allowed us to sample the “population” of stimuli as well as the population of subjects and thus determine the robust generalized neophobic responses rather than responses to a specific object. Using multiple stimuli is a standard and necessary technique in neophobia studies (Greggor et al., 2015). Our total of seven different novel objects allowed us to rotate the objects such that no object was used more than once on each individual animal throughout the three experiments, with the exception of the habituation experiments (see below).

Neophobia testing was designed to control for effects of attention and hunger between the animals by using an overnight fasting protocol before measuring behavior (de Bruijn & Romero, 2020). Food was removed 30 min before lights off, black poster board dividers were placed between each cage, and a video camera was placed on a tripod in the room. The dividers were necessary to prevent birds from being prematurely exposed to objects that other birds received, thereby preventing the effects of social learning (Campbell et al., 1999; Root-Bernstein, 2010; Stöwe et al., 2006). Food was returned the following morning, 30 min after lights on, either with or without the presence of a novel object at the food dish. Video recordings were taken for 10 min beginning after the experimenter left the room. At the end of the recording period, novel objects were removed, the normal food dish was replaced, and the video camera was removed from the tripod, which remained in the room. This food removal protocol was administered for 2 days before the beginning of the study, so the animals experienced the procedure twice before any data were recorded.

The entire 10 min of recording was analyzed. Approach latency was defined as the time from return of the food dish until the first time a bird perched on the food dish for at least 1 s, a method that has been previously used to measure neophobia (de Bruijn & Romero, 2020). A bird that did not approach the food dish throughout the observation period was assigned a ceiling latency of 600 s. The same person blind to treatment made all observations.

2.5 | Experiment 1: Test of neophobia throughout chronic stress

The presentation order of all objects was randomized for all birds, using a random number generator, and each object presented to each bird was novel, resulting in a pseudo-counterbalanced design. Before the initiation of the chronic stress protocol, behavior for a trial with no object and a trial with a novel object was recorded (analyzed together as Trial 1). Birds were then tested in trials with no object on Days 4, 8, and 12 after the initiation of chronic stress, to obtain a baseline measure of the response to overnight fasting. These same birds were tested in trials with novel objects on Days 5, 9, and 13, to assess neophobia throughout the chronic stress period. These no-object and object trials were analyzed together as Trials 2, 3, and 4. Birds in the control group were tested in parallel times to the chronic stress group.

2.6 | Experiment 2: Test of habituation during chronic stress

We tested the effects of chronic stress on habituation of the response to novel objects by repeatedly exposing birds to an initially novel object. Chronically stressed birds underwent a trial with no object on Day 12 after the initiation of chronic stress (Trial 4 from Experiment 1), to assess baseline behavior. Previously, habituation has been shown to occur in starlings within 3 days of repeated exposure to an object (de Bruijn & Romero, 2020). Birds received a randomly assigned novel object on Day 13 (Trial 4 from Experiment 1), which was also presented on Days 14 (Trial 5) and 15 (Trial 6), resulting in a total of three consecutive days of exposure to the same object. Control birds were tested in an identical manner, although testing occurred not in parallel, but a few days later due to logistical constraints.

2.7 | Experiment 3: Test of habituation following restraint during chronic stress

Previously, we have shown that restraint stress before presentation of a novel object eliminates habituation to repeated exposure to that novel object (de Bruijn & Romero, 2020). To examine how chronic stress affects the interplay between acute stress and neophobia, birds were restrained in a cloth bag for 15 min before the morning replacement of the food dish with or without a novel object (de Bruijn & Romero, 2020). This standardized restraint stressor elicits a robust stress response (Wingfield et al., 1992). Baseline approach latency to normal food dishes without restraint occurred on Day 16 (Trial 7). On Day 17, before replacement of the food dish, birds were restrained, and then normal food dishes were returned and behavior was measured (Trial 8). On Day 18, birds were restrained before

exposure to a randomly assigned novel object, and they experienced this same protocol and received the same object for Days 19 and 20, for a total of three consecutive trials with the same object (Trials 9, 10, 11). Control birds were tested in an identical manner, but as in Experiment 2, testing occurred a few days later due to logistical constraints. Control birds also received their objects for one additional day (Trial 12), resulting in four total days of exposure to the object, and concluded in a final trial with no restraint and no object (Trial 13).

2.8 | Statistical analysis

All statistical analyses on weight, baseline, and stress-induced corticosterone, and approach latencies (for a total of six analyses), were done using SAS (version 9.2; SAS Institute Inc.). Repeated measures analysis of variance (Proc Mixed SAS procedure) were used to test for main effects of treatment (control vs. chronic stress), time (throughout the length of the chronic stress period), and the interaction of treatment by time. A treatment effect indicates a difference between the control and chronic stress group, and a time effect signifies a difference across the chronic stress protocol. Simple effects tests were used to discern differences between treatments, where applicable (LSMEANS/SLICE option in SAS). Baseline and stress-induced corticosterone samples were tested separately, because we expected different aspects of the HPA axis to function independently, for example, because they are regulated through different receptors (Hodgson et al., 2007; Landys et al., 2006; Reul et al., 1987; Romero, 2006). We did not test the objects as a variable, nor the order of presentation, as previous research indicated that the different objects elicited similar responses (de Bruijn & Romero, 2020). We accepted significance at $p < .05$; total $n = 16$, with $n = 8$ for each group of birds.

3 | RESULTS

3.1 | Weights

There was no effect of treatment between the control and chronic stress groups ($F_{1,14} = 0.20$; $p = .70$), nor was there an interaction between treatment and time ($F_{4,63} = 1.03$; $p = .4$; Figure 1). There was a significant effect of time ($F_{5,63} = 12.90$; $p < .001$), with both groups showing approximately a 5% increase in weight after 20 days ($p < .001$ for both).

3.2 | Corticosterone

For baseline corticosterone (Figure 2a), we observed no effect of treatment ($F_{1,14} = 1.03$; $p = .33$), time ($F_{4,46} = 1.95$; $p = .12$), or an

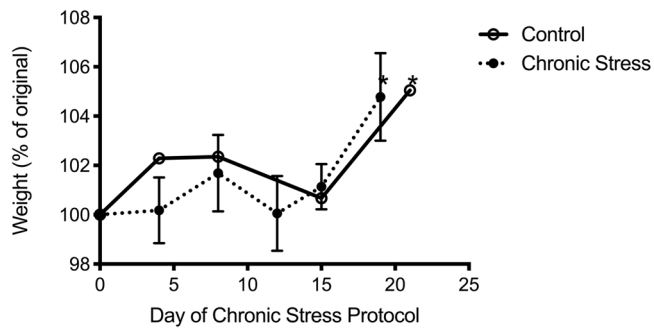


FIGURE 1 Average weights of adult European starlings, \pm SEM. Weights are displayed as a percentage of original weight measured on Day 0, the day before the chronic stress protocol began. The protocol continued for the duration of days is represented in the figure. Control birds were weighed at parallel times but were not exposed to chronic stress. In each group, $n = 8$; * $p < .05$

interaction between treatment and time ($F_{3,46} = 0.38$; $p = .77$). Similarly, for restraint-induced corticosterone (Figure 2b), we found no effect of treatment ($F_{1,14} = 0.49$; $p = .50$), time ($F_{4,38} = 0.53$; $p = .71$), or an interaction ($F_{3,38} = 1.28$; $p = .29$).

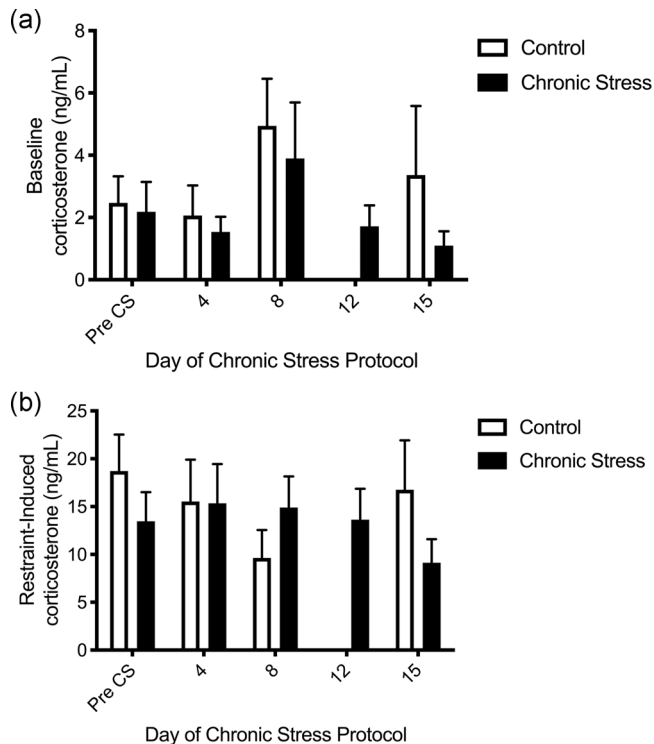


FIGURE 2 Baseline (a) and restraint-induced (b) corticosterone levels for adult European starlings, \pm SEM. Samples were taken before the chronic stress (CS) protocol (Day 0) and at specific intervals throughout. Following baseline sampling, birds were restrained in a cloth bag for 30 min, and then the restraint-induced samples were collected. Sampling occurred 2 h after birds had received their food following an overnight fasting period. In each group, $n = 8$

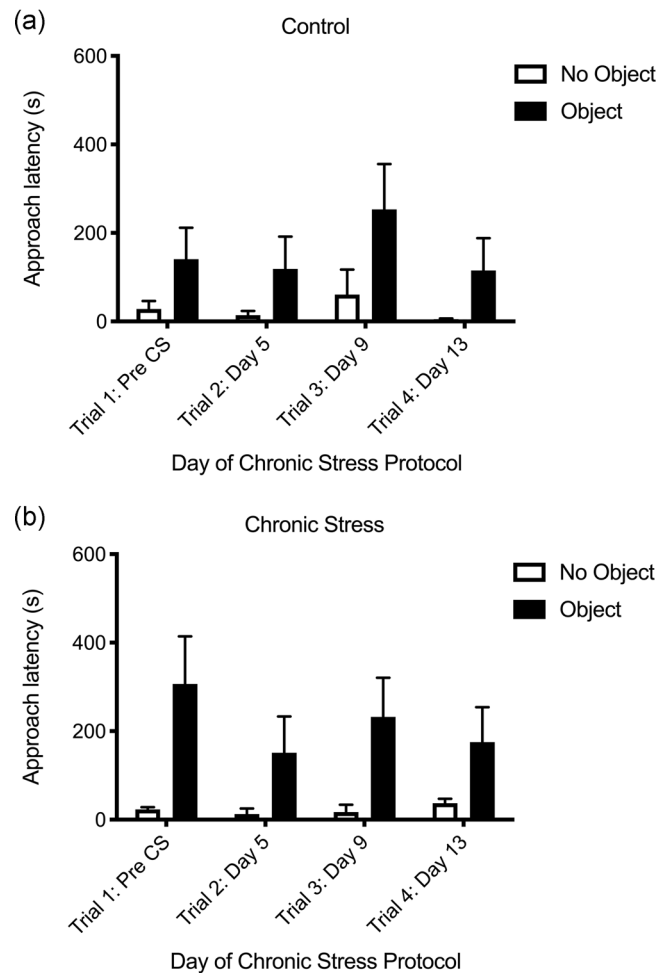


FIGURE 3 Approach latency toward the food dish for European starlings in a control group (a) and a chronic stress group (b), \pm SEM. Behavior was recorded for 10 min on video following an overnight fast. Trials are represented sequentially throughout the chronic stress protocol. Baseline measurements without an object took place the day before trials with an object. Each trial was with a new, randomly assigned object. In each group, $n = 8$

3.3 | Neophobia trials (Experiment 1)

For the baseline approach latency response to overnight fasting, without exposure to novel objects, we found no effect of treatment ($F_{1,14} = 0.05$; $p = .82$), time ($F_{3,42} = 0.65$; $p = .59$), or an interaction ($F_{3,42} = 1.36$; $p = .27$) for either group. For approach latency in response to a novel object, we found no effect of treatment ($F_{1,14} = 0.62$; $p = .44$), time ($F_{3,42} = 1.24$; $p = .31$), or an interaction ($F_{3,42} = 0.65$; $p = .59$) for either group (Figures 3a and 3b).

3.4 | Habituation (Experiment 2)

For approach latency in response to repeated exposure to the same object, we found no effect of treatment ($F_{1,14} = 0.99$; $p = .34$) or an interaction between time and treatment ($F_{3,42} = 1.38$; $p = .26$). There

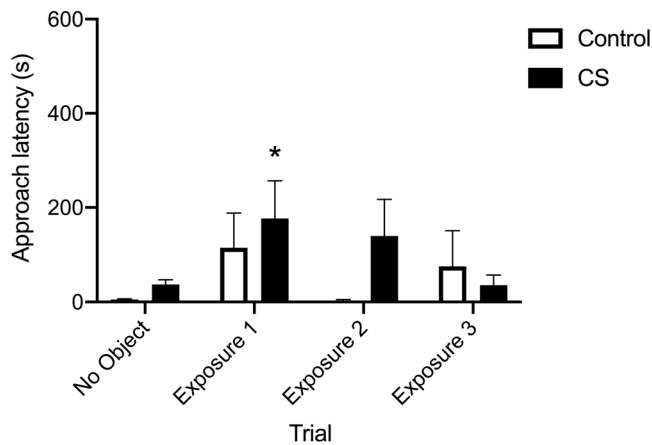


FIGURE 4 Approach latency toward the food dish for European starlings in a control group (white bars) and a chronic stress (CS) group (black bars), \pm SEM. Behavior was recorded for 10 min on video following an overnight fast. Each bird saw a randomly assigned object on the first exposure, and then received this same object for consecutive days. In each group, $n = 8$; * $p < .05$

was an effect of time ($F_{3,42} = 2.84$; $p = .049$; Figure 4). A slice time by treatment test revealed that the chronic stress group exhibited increased approach latency on the first day of exposure to a novel object, but that this response eliminated after repeated exposure ($p = .061$; Figure 4). There was no slice time by treatment effect in the control group ($p = .21$), suggesting that for the control group the neophobic response did not change throughout the duration of the experiment (Figure 4). However, there was an increase in latency on the first exposure, and this increase disappeared on the second exposure, but the latency then only appears to increase on the third exposure due to a maximally high value of 600 s from one individual. Latencies for all other individuals were at or near 0 s on the third exposure (Figure 4).

3.5 | Habituation with prior restraint (Experiment 3)

For approach latency in response to repeated exposure to an object following restraint, there was no effect of treatment ($F_{1,14} = 1.25$; $p = .28$), although there were effects of time ($F_{5,63} = 17.82$; $p < .001$) and an interaction of treatment by time ($F_{4,63} = 3.89$; $p = .0069$; Figure 5). A slice time by treatment test revealed significance for both the control group ($p < .001$; Figure 5) and chronic stress group ($p < .001$; Figure 5). The control group responded with increased approach latency for every exposure to the novel object following restraint. The chronic stress group showed an increase in approach latency on the first instance of exposure to novel object preceded by a restraint stressor, but this response was reduced on subsequent days.

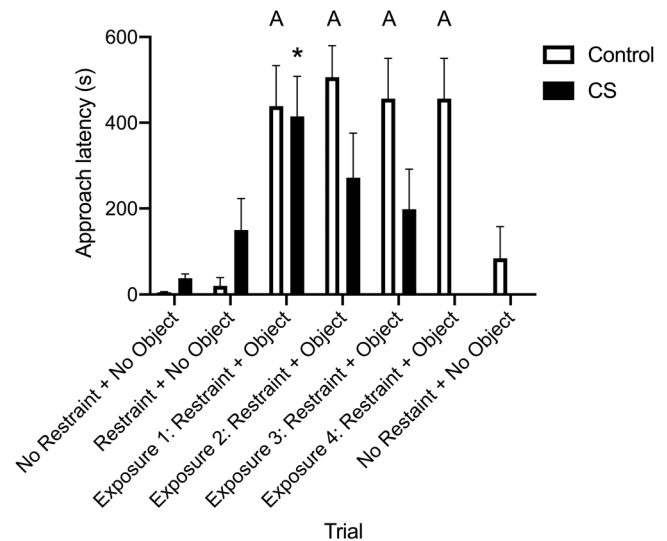


FIGURE 5 Approach latency toward the food dish for European starlings in a control group (white bars) and a chronic stress (CS) group (black bars), \pm SEM. Behavior was recorded for 10 min on video following an overnight fast. Before exposure to the novel object, each bird experienced a 15-min period of a restraint stressor. Each bird saw a randomly assigned object on the first exposure, and then received this same object for consecutive days. Trials are represented sequentially. In each group, $n = 8$. *and A denote $p < .05$

4 | DISCUSSION

In this study, we sought to investigate the interaction between neophobic behavior and stress physiology during a period of chronic stress. Previous research has shown that acute stress exerts permissive actions on neophobia by increasing the frequency of increased approach latency and preventing habituation to novel objects in starlings (de Bruijn & Romero, 2020). Additionally, chronic exposure to stress can cause significant physiological changes in starlings (Awerman & Romero, 2010; Cyr, Earle, et al., 2007; Cyr & Romero, 2007; Rich & Romero, 2005), which suggests that chronic stress may alter the interaction between neophobia and acute stress. We found that chronic stress did not change the initial behavioral response to novel objects. However, behavior following activation of the stress response (induced by restraint) showed marked differences between the control group and chronic stress group. The permissive actions of acute stress on neophobia appear diminished during chronic stress.

We predicted that the chronically stressed animals would have heightened neophobia because chronically stressed animals have an alteration of glucocorticoid and mineralocorticoid mRNA receptor expression in the hippocampus that may be indicative of an anxiety-like state (Dickens et al., 2009). Furthermore, short-term exposure to repeated stress affects neophobia in house sparrows (Gormally et al., 2019) and hematological indicators of stress in roe deer are associated with neophobia (Monestier et al., 2017). In contrast to our

prediction, chronically stressed starlings showed neophobic responses to novel objects that were similar to the responses of the control group. The magnitude of these neophobic responses has also been seen in other studies (An et al., 2011; de Bruijn & Romero, 2020; Feenders et al., 2011; Martin et al., 2005). A similar response was found in chronically stressed rats, where novelty-seeking behavior was not affected (Wright & Conrad, 2005). Therefore, it appears that chronic stress does not affect the initial neophobia response, which suggests that the behavioral response to neophobia is regulated separately from the hormonal responses to chronic stress. Previous work has shown differential regulation of the physiological, hormonal, and behavioral components of the stress response in response to an acute stressor (Nephew et al., 2003), supporting the idea that neophobia is regulated independently from physiology.

The chronically stressed animals also showed habituation in response to repeated exposure to the same object. This habituation rate after one exposure is typical for starlings (de Bruijn & Romero, 2020), but there is considerable species variation. For example, yellow-eyed penguins (*Megadyptes antipodes*) generally habituate to humans after 5 days (Ellenberg et al., 2009), and house sparrows (*Passer domesticus*) continue to show further reductions in approach latencies to novel objects after the second and third exposures (Ensminger & Westneat, 2012). Thus, chronic stress appeared to have no effect on either the initial behavioral response to a novel object or on habituation to repeated exposure to an object. The control group did not seemingly show habituation, but this was clearly due to the behavior of one bird that responded with greatly increased approach latency on the third day of exposure. For all other animals, approach latency was highest on initial exposure and reduced on the second and third exposure. Individual variability largely explains the observed lack of habituation.

In contrast, when exposure to a novel object was combined with 15 min of prior restraint in a cloth bag, the chronically stressed group responded differently compared with the control group. The initial exposure to a novel object following restraint elicited similar increases in approach latency in both groups, but when control birds were exposed to the same object with prior restraint on subsequent days, they failed to habituate. Conversely, the chronic stress group continued to exhibit habituation to the object. Chronic stress, therefore, appeared to reverse the lack of habituation induced by restraint before exposure to the object in control birds. Currently, the mechanism underlying the effect of stress on habituation is not known. Injected exogenous corticosterone before exposure to a novel object, thereby mimicking the corticosterone aspect of the stress response, did not elicit a lack of habituation (de Bruijn & Romero, 2020). It thus seems unlikely that corticosterone itself plays a major role in this lack of habituation. Other aspects of the stress response could be involved in mediating neophobia, such as the release of catecholamines or CRF (Romero & Wingfield, 2016; Sapolsky et al., 2000). Chronic stress also affects other aspects of physiology that were not measured in this study, such as heart rate and heart rate variability (Cyr et al., 2009). For example, a previous study did

not find that increased heart rate returns to baseline slower following exposure to a novel object (Fischer et al., 2016). Although this is not evidence of relationship between neophobia and heart rate, it does not necessarily rule out a potential effect of chronic stress in this regard. Future experiments should examine other components of the stress response to clarify which elements contribute to the observed behavioral differences.

The physiological responses to chronic stress in this experiment were unexpected. We predicted a decrease in weight and in baseline and restraint-induced corticosterone of the chronic stress group. However, there was an increase in weight and no change in corticosterone, and this weight increase was also seen in the control group. Previous studies on European starlings employing a similar chronic stress protocol showed decreases in weight (Awerman & Romero, 2010; Cyr, Earle, et al., 2007; Cyr & Romero, 2007; Rich & Romero, 2005) and robust decreases in basal and restraint-induced levels of corticosterone (Cyr, Earle, et al., 2007; Cyr & Romero, 2007; Rich & Romero, 2005). While the chronic stress protocol for this study was identical to the previous studies, one major difference is that the birds here experienced daily overnight fasting. In one study that altered food availability in European starlings every day for 3 weeks, similar increases in weight and no changes in corticosterone were found (Bauer et al., 2011). This suggests that daily food removal may affect physiological responses and could be responsible for the lack of changes in HPA axis functioning in our study. Additionally, corticosterone at baseline levels acts as a metabolic regulator and is responsible for various aspects of energy storage (Blas, 2015; Sapolsky et al., 2000). Food removal can alter the physiology of an animal, as fasting over an extended period of time increases corticosterone levels and decreases corticosteroid-binding protein capacity in zebra finches (*Taeniopygia guttata*; Lynn et al., 2010).

Prior research has examined the link between hormonal and behavioral phenotypes, and to what extent physiology drives behavior (Greggor et al., 2017; Malisch et al., 2007; Schoech et al., 2011, 2012). For example, Florida scrub jays (*Aphelocoma coerulescens*) that have lower corticosterone levels are the boldest individuals (Schoech et al., 2009). In addition, dark-eyed juncos (*Junco hyemalis*) from an urbanized population have lower baseline and restraint-induced corticosterone levels, compared with a native population, and they also exhibit greater exploratory behavior (Atwell et al., 2012). While these studies indicate a correlation between corticosterone levels and behavioral phenotypes, the present results indicate that corticosterone may not be the proximate factor underlying this relationship. It may be that some other aspect of stress physiology, such as upstream processes, are responsible for these behavioral associations.

In summary, starlings that experienced a sustained period of chronic stress showed no differences in initial neophobia, and they habituated quickly after repeated exposure to novel objects. In contrast, the augmenting effect of acute stress on the neophobic response, which normally prevents habituation, was reduced, as the chronic stress group habituated to repeated exposure to an object preceded with restraint stress. These results demonstrate that the

acute stress response interacts with and augments behavior associated with neophobia by strengthening neophobia and its persistence over time, but this effect disappears with chronic stress.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Robert de Bruijn  <https://orcid.org/0000-0001-6945-5642>

L. Michael Romero  <http://orcid.org/0000-0002-8854-8884>

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