The glucocorticoid response in a free-living bird predicts whether long-lasting memories fade or strengthen with time

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For decades, scientists have used threat conditioning (traditionally termed ‘fear conditioning’) to study the link between glucocorticoids and the consolidation of long-term memories (i.e. memories that last hours to weeks) in model organisms in the laboratory. We assessed this relationship in a free-living species, and examined a possible relationship between glucocorticoids and the retention of long-lasting memories (i.e. memories that last months to a lifetime). We developed a novel threat-conditioning protocol by which free-living Florida scrub-jays, *Aphelocoma coerulecens*, were either chased by a novel predator or exposed to a control. We measured flight initiation distance (FID) 48 h, 11 months and 2 years after conditioning or control exposures, and compared these measures to levels of stress-induced glucocorticoids. Conditioned subjects maintained significantly longer FIDs for at least 2 years. Furthermore, the long-term memory consolidation of conditioned subjects positively correlated with their stress-induced glucocorticoid response, similar to results from laboratory studies. Surprisingly, individuals with a moderate stress response exhibited an exaggerated defence response (i.e. FIDs increased) at 11 months and 2 years post-conditioning, whereas low and high stress responders exhibited memory decay or extinction (i.e. FIDs decreased). We speculate that the recently discovered processes of memory reconsolidation and system consolidation may help explain why some Florida scrub-jays exhibit more fearful-like behaviour with time.

Keywords:

antipredator

Aphelocoma coerulescens

consolidation

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Memories of emotionally arousing experiences tend to be better remembered than non-emotive memories (McGaugh & Roozendaal, 2002) in part due to the effects of stress-induced glucocorticoids (reviewed by Cahill & McGaugh, 1998; Rodrigues, LeDoux, & Sapolsky, 2009; Roozendaal, 2002). Glucocorticoids are adrenal cortex-derived steroid hormones that usually circulate at relatively low baseline concentrations but increase following activation of the hypothalamic–pituitary–adrenal (HPA) axis in response to a stressor, such as exposure to a live predator or a predator cue (e.g. Canoine, Hayden, Rowe, & Goymann, 2002; Cockrem & Silverin, 2002a; Jones, Smith, Bebus, & Schoech, 2016; Park, Zoladz, Conrad, Fleshner, & Diamond, 2008).

Memory consolidation is the process by which short-term memories become long-term memories (McGaugh, 2000), and it occurs within hours of initial learning (Clopath, 2012). The retention of consolidated memories can be diminished or prevented by memory decay (i.e. forgetting) or memory extinction (i.e. the masking of a previously learned association by a different, newly learned association; Myers & Davis, 2007; Pavlov, 1927; Quirk, 2002). So-called ‘fear’ conditioning is a form of Pavlovian conditioning that has been used extensively to evaluate the effects of acute stress on the consolidation of long-term memories in laboratory animals (e.g. McGaugh, 2015; Ng & Gibbs, 1991; Sandi & Pinelo-Nava, 2007; Sandi & Rose, 1994). Fear conditioning is a type of associative learning that is thought to have evolved because it allows animals to learn from past events to avoid future threats (Maren, 2001). Fear conditioning occurs when a stimulus that is innately threatening, known as an unconditioned stimulus (e.g. foot shock), is paired with a relatively benign stimulus (e.g. an audible tone). The benign stimulus then becomes what is known as a conditioned stimulus and is able to elicit a

conditioned response (e.g. freezing in laboratory rats) on its own. Although conditioned stimuli are often described as nonthreatening or neutral, rats that are not conditioned often exhibit some degree of freezing when presented with only a conditioned stimulus (e.g. Corley, Caruso, & Takahashi, 2012; Lee et al., 2011; Rau, DeCola, & Fanselow, 2005). ‘Fear’, as it is used in fear-conditioning research, is caused by exposure to a stimulus that is associated with a threat, which in turn triggers defensive behaviours and activates associated neuroendocrine circuits (Perusini & Fanselow, 2015). However, LeDoux (2014) advocated that the use of ‘fear’ should be reserved for the subjective state of feeling afraid and not the nonconscious neural systems that mediate defence behaviours. We agree, and hereafter use ‘threat conditioning’ in place of ‘fear conditioning’ and ‘defence response’ or ‘antipredator response’ instead of ‘fear response’.

The physiological stress response following a threatening stimulus is mediated by the amygdala, an area of the limbic system also known for its role in mediating emotional responses. In addition to activating an initial sympathetic nervous system response, the amygdala triggers the HPA axis, ultimately leading to a surge of glucocorticoids secreted from the adrenal cortex (Rodrigues et al., 2009). The physiological stress response is self-limiting, as negative feedback within the HPA axis, hippocampus and basolateral amygdala inhibit further release of glucocorticoids (Boyle, Kolber, Vogt, Wozniak, & Muglia, 2006; Herman & Cullinan, 1997; Herman, Dolgas, & Carlson, 1998). Additionally, the basolateral amygdala and hippocampus are key regulatory centres in the formation and retention of threat-conditioning memories (reviewed by Rodrigues et al., 2009) and have been implicated in the regulation of the overall magnitude of the glucocorticoid response following HPA axis activation (Herman, Ostrander, Mueller, & Figueiredo, 2005). Although our understanding of the neuroendocrine pathways of the

physiological stress response and learning and memory have been derived from rodent models, the relevant cerebral homologies between the avian and mammalian brain are similar in function and nomenclature (Jarvis et al., 2005).

It is well established that glucocorticoids enhance memory consolidation (reviewed by McGaugh, 2000, 2015; Roozendaal, 2000, 2002; Sandi & Pinelo-Nava, 2007). However, studies have typically examined ‘long-term’ memories only hours to days beyond learning (e.g. Akirav et al., 2004; Roozendaal, Carmi, & McGaugh, 1996; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006; Salehi, Cordero, & Sandi, 2010), or at most 1 week after learning (e.g. Cordero & Sandi, 1998; Flood et al., 1978; Frost, Castellucci, Hawkins, & Kandel, 1985; Shors, Weiss, & Thompson, 1992). This is surprising given that many animals can retain memories for years (Balda & Kamil, 1992; Bednekoff, Balda, Kamil, & Hile, 1997; Vaughan & Greene, 1984) or even decades (Beran, Pate, Richardson, & Rumbaugh, 2000). Studies of long-term memory have often used ‘long-lasting’ to describe memories that persist for days to weeks (e.g. Marin, Hupbach, Maheu, Nader, & Lupien, 2011; Quirk, 2002; Zoladz, Fleshner, & Diamond, 2012). However, we use the term ‘long-lasting memory’ to refer to memory that is retained for months to throughout the lifetime of an individual (see McGaugh, 2000). To the best of our knowledge, the influence of stress physiology on long-lasting memory is unexplored.

The mediation of memory by stress physiology has been well studied in model organisms in the laboratory, but few have studied this relationship in captive wild animals (Mateo, 2008; Pravosudov, 2003; Thaker, Vanak, Lima, & Hews, 2010), or free-living animals (Croston et al., 2016; Thaker et al., 2010). Investigating the effects of stress on memory in free-living animals is

valuable for at least two reasons. First, the behaviour and physiology of free-living animals frequently differ markedly from that of their domesticated and laboratory counterparts (e.g. Calisi & Bentley, 2009; Fleming, Agustsson, Finstad, Johnsson, & Bjornsson, 2002; Künzl, Kaiser, Meier, & Sachser, 2003; Stryjek, Modlinska, Turlejski, & Pisula, 2013). Second, wild-caught animals kept in captivity can suffer from chronic stress, which can alter HPA axis function (Dickens, Delehanty, & Romero, 2010; Dickens, Earle, & Romero, 2009; Morgan & Tromborg, 2007; Terio, Marker, & Munson, 2004), and thus confound experiments designed to evaluate the effects of acute stress on memory.

We developed a protocol, based on the threat conditioning (traditionally termed ‘fear conditioning’) paradigm developed in the laboratory (reviewed by Maren, 2001), to assess the effects of glucocorticoids on the consolidation and retention of long-lasting memory in a free-living species, the Florida scrub-jay, *Aphelocoma coerulescens*. We hypothesized that exposure to glucocorticoids during a threatening event would mediate the consolidation and long-lasting memory of that event. Specifically, we predicted that the amount of endogenous glucocorticoids an individual produced in response to a standardized stressor, which is repeatable within an individual for years (Small & Schoech, 2015), would positively correlate with the consolidation and retention of long-lasting memory of a novel predator attack.

METHODS

Study System

The Florida scrub-jay is a nonmigratory, socially and genetically monogamous, cooperatively breeding avian species endemic to the fire-dependent Florida scrub ecosystem (Quinn, Woolfenden, Fitzpatrick, & White, 1999; Townsend, Bowman, Fitzpatrick, Dent, & Lovette, 2011; Woolfenden & Fitzpatrick, 1984). We studied a population of ca. 200 individuals at Archbold Biological Station, Highlands County, Florida (27°19'N, 81°21'W, elevation 38–68 m). We used the Florida scrub-jay as a model to study long-lasting memory and stress physiology in a free-living animal because individuals are easily located within year-round territories and are readily identified by unique combinations of colour bands. Also, the population at Archbold Biological Station readily eats and caches peanuts, which we use to bait traps and motivate individuals to participate in behavioural tests (Schoech et al., 2007; Schoech, Mumme, & Moore, 1991). As caching species, *Aphelocoma* jays have well-developed cognitive and memory capabilities (e.g., Bebus, Small, Jones, Elderbrock, & Schoech, 2016; Bednekoff et al., 1997; Brown, 1997; Clayton, Dally, & Emery, 2007), making them an ideal model to study long-lasting memory in a free-living animal. Additionally, Florida scrub-jays have baseline and stress-induced levels of corticosterone (CORT; the primary glucocorticoid in a number of vertebrate taxa, including birds) that are repeatable within individuals across life history stages (Rensel & Schoech, 2011) and multiple years of adulthood (Small & Schoech, 2015).

Threat Conditioning

We developed a novel threat-conditioning protocol to be used in free-living birds to study memory because threatening experiences tend to be better remembered (McGaugh & Roozendaal, 2002) and can stimulate glucocorticoid secretion (Canoine et al., 2002; Cockrem &

Silverin, 2002a; Jones et al., 2016). We conditioned randomly selected jays to avoid novel ‘predators’ (an ‘umbrella’ in late March through April 2012, and a ‘puppet’ in April 2013; Fig. 1) by simulating an attack; we chased subjects with the predators for less than 5 s from within 4 m. Many animals learn the dangers of predators via threat conditioning, which is the mechanism by which innately threatening stimuli (unconditioned stimuli; e.g. an attack, an alarm call) become associated with the predators themselves (conditioned stimuli; Griffin, Blumstein, & Evans, 2000; Griffin, 2004).

Our simulated novel predators were collapsible, easily concealed by the operator and quickly sprang open. Randomly selected controls were not exposed to the predator, but were approached and briefly observed by a field biologist in typical field garb. We subsequently quantified conditioned responses by slowly approaching (ca. 1 m/s) subjects with the novel predator from a starting distance of 40 m and measured their flight initiation distances (FID; the distance at which an individual flees from an approaching threat). FID has been used extensively to test the economic risk-sensitivity hypothesis (Cooper & Frederick, 2007; Ydenberg & Dill, 1986), which predicts that an organism will flee from an approaching predator when the costs of staying outweigh the costs of fleeing. Various factors can influence perception of predation risk, including previous experience with predators (Bateman, Fleming, Jones, & Rothermel, 2014; Bateman & Fleming, 2014; Cooper & Frederick, 2007; Stankowich, 2009). Based on the observations that threat-conditioned rats freeze for a longer duration compared to controls when presented with a conditioned stimulus, we predicted that, when slowly approached by a novel predator, birds previously chased by the predator would flee sooner (i.e. have longer FIDs) compared to controls not previously exposed to the predator. FID trials were conducted on open sandy firebreaks, and we used peanuts to attract scrub-jays to focal locations 40 m from a blind

from which the predator emerged. To avoid the possible confounds of different levels of appetite among subjects, we conducted FID trials after all individuals had stopped eating and had begun to collect peanuts to cache. The approaching researcher operating the predator (Fig. 1) dropped weighted flagging as each bird fled. The distance from the dropped flagging to each bird's departure point was measured as that individual's FID. A second researcher confirmed the total number of birds present and recorded each fleeing bird's unique colour band combination and departure point with binoculars. To measure memory consolidation, we measured FIDs ca. 48 h (mean $\pm$ SD: umbrella: 48.54 ± 1.60 h, range 45.16–52.00 h; puppet: 48.44 ± 1.47 h, range 45.12–50.94 h) after conditioning. To assess long-lasting memory, we measured FIDs 11 months (umbrella: 10.89 ± 0.51 months, range 9.10–12.83 months; puppet: 11.03 ± 0.53 months, range 10.23–12.00 months) and 2 years (umbrella: 1.93 ± 0.05 years, range 1.83–2.08 years) after conditioning. We conducted all conditioning and control exposures and FID trials between 1200 and 1730 hours.

Florida scrub-jays live in social groups. As a result, 86% of the 358 individual FID measures were collected in groups of two or more (mean $\pm$ SD = 2.55 ± 1.28 individuals). Individuals of a social group maintained close proximity to each other (ca. 4 m or less) at the initiation of conditioning treatments and FID trials, and no other scrub-jays were seen or heard by the biologists during conditioning or FID trials. We address the potential violation of independence in the statistical analysis below.

Stress-induced CORT

To investigate the relationship between CORT and memory, we measured individuals'

responses to a standardized capture-and-restraint protocol (Wingfield, Vleck, & Moore, 1992) as a proxy for the stress response experienced during the conditioning trials. We used this standardized measure for two reasons. First, quantifying the dynamics of a stress response following a conditioning treatment would have required multiple blood samples over a period of time, a process that elicits a CORT response of its own (Romero & Romero, 2002; Wingfield et al., 1992), and thus would confound measures of CORT released in response to being chased by a predator. Second, stress-induced measures of CORT are repeatable within individual Florida scrub-jays (Rensel & Schoech, 2011; Small & Schoech, 2015). To obtain standardized stress-induced measures, we captured individuals in peanut-baited Potter traps from February to early March 2012 before umbrella conditioning and control exposures, and again from February to March 2013 before puppet conditioning and control exposures. We obtained baseline blood samples from individuals within 3 min of trapping (Romero & Romero, 2002, but see Romero & Reed, 2005; Small et al., n.d.) and collected stress-induced samples at 5, 15 and 30 min post-capture. Between blood samples, jays were held in a loosely woven cloth bag placed in the shade. At each time point we collected ≤ 120 μ l of blood in heparinized microhematocrit tubes following puncture of the brachial vein with a 26-gauge needle. Volumes were well within recommended limits (Fair et al., 2010). Samples were held on ice for 1–4 h, after which plasma was separated by centrifugation, drawn off and stored at -20 °C until analysis. We used an enzyme-linked immunoassay (Cayman Chemical 500655; detection limit of 30 pg/ml) to quantify nonextracted total plasma CORT, which we previously validated in Florida scrub-jays (Small & Schoech, 2015). Breuner, Delehanty, and Boonstra (2013) argued that measures of corticosteroid-binding globulin (CBG) and circulating ‘free’ CORT are essential for assessing levels of biologically available CORT. However, we report total CORT and argue that it is the

more appropriate and interpretable measure (Schoech, Romero, Moore, & Bonier, 2013). In addition to the arguments discussed by Schoech et al. (2013), more recent studies further support the idea that ‘free’ CORT cannot circulate in an aqueous medium, such as plasma, because the lipophilic steroid requires transport by CBG to be made available to target tissues that mediate immunological, neurological and behavioural processes (Moisan et al., 2014; Nenke et al., 2015). Prior to CORT assay, we diluted plasma samples (1:20 for baseline and control samples, and 1:100 for stress-induced samples) to ensure values fell on the linear part of the standard curve. Samples were assayed in duplicate on a total of 27 plates with intra-assay coefficients of variation (CVs) ranging from 1.7 to 4.4% (mean = 2.9%) and an interassay CV of 13.4%. To quantify stress-induced CORT responses, we calculated the area under the response curve for each individual (i.e. integrated CORT response; Cockrem & Silverin, 2002b). We did not use corrected integrated CORT, which only includes CORT secretion above basal levels, because CORT receptors in target tissues are unlikely to differentiate between baseline and stress-induced CORT, and the statistical significance of our results did not differ between integrated and corrected integrated CORT.

To determine whether the threat-conditioning treatment elicited an endogenous CORT response, we randomly selected 18 jays that were not used in the main study, and measured circulating CORT following umbrella conditioning ($N = 7$) or control exposure ($N = 5$) as described in the main experiment above. We also included a less threatening conditioning treatment (‘mild threat’; $N = 6$) in which the operator slowly opened the umbrella predator from ca. 15 m away and then approached the birds at a moderate rate of ca. 2 m/s. Unlike the conditioning used in the main experiment, this mild threat did not use an element of surprise and the predator did not pursue individuals once they fled. We then captured individuals in peanut-

baited Potter traps 11–32 min after treatments (mean $\pm$ SD = 19.41 ± 7.25 min, range 10.53–31.56 min). We chose this time frame because stress-induced CORT is typically at or near peak values between 15 and 30 min in Florida scrub-jays (Rensel, Boughton, & Schoech, 2010; Schoech, Mumme, & Wingfield, 1997). We then obtained a single blood sample from each individual within 2.5 min of initial trapping to ensure circulating plasma CORT levels were not affected by the trapping event (Romero & Reed, 2005; Romero & Romero, 2002). We collected, processed, stored and analysed blood as described above using an enzyme-linked immunoassay (Cayman Chemical 501320; detection limit of 30 pg/ml). Samples were assayed in duplicate on a single plate with an intra-assay CV of 3.6%.

All protocols were completed under permits from the U.S. Fish and Wildlife Service (TE-117769) and the U.S. Geological Survey Bird Banding Laboratory (23098) and were approved by the University of Memphis Institutional Animal Care and Use Committee (0667) and Archbold Biological Station Institutional Animal Care and Use Committee (AUP-013-R). Capturing, handling and blood sampling has had no deleterious effects on the survival or reproduction of individuals in the population of Florida scrub-jays at Archbold Biological Station (Schoech, n.d.).

Statistical Analyses

We conducted all statistical analyses in R (R version 3.2.3; R Core Team, 2015), and used an a priori confidence level of 95% ($\alpha = 0.05$) to determine statistical significance for all tests. Model assumptions of normality, collinearity, linearity and homoscedasticity were confirmed, unless otherwise stated.

To test whether our threat-conditioning protocol induced a conditioned response in scrub-jays, we evaluated the effect of treatment (i.e. previously chased or unexposed control) and time since treatment on FID using a linear mixed-effects model with the 'lme4' package in R (Bates, Maechler, Bolker, & Walker, 2015). We included five additional fixed effects: sex, predator type (i.e. umbrella or puppet), age, total birds present (i.e. total number of individuals present during FID trials as determined by a second biologist with binoculars) and time since last fire (i.e. elapsed time since the surrounding vegetation had been burned). Time since last fire is a strong predictor of vegetation diversity, cover and height in Florida scrub habitat (Menges & Hawkes, 1998; Menges, 2007; Schmalzer, 2003), and we included it in our model because habitat structure, particularly proximity to adequate refuge, can influence an individual's perception of risk and thus their decision to flee an approaching threat (Stankowich & Blumstein, 2005). Homogeneity of regression coefficients between treatment groups is a key assumption for the inclusion of covariates in linear models and is easily tested by assessing the statistical significance of interactions between the response variable and covariates (Engqvist, 2005). We thus determined the statistical significance of first-order interactions between treatment and each of the following: sex, age, time since treatment, time since last fire, total number of birds present and type of novel predator. We removed all nonsignificant interactions from the final model, as their inclusion precludes the ability to make accurate estimates of differences between groups (Engqvist, 2005). We estimated variances separately for control and conditioned treatments to account for heteroscedasticity between treatments (Pinheiro & Bates, 2000). We included random intercepts and slopes for individuals and random intercepts for the geographical location of FID trials and each unique FID trial event, as most scrub-jays were tested in groups. We calculated significance using the Kenward–Roger approximation (Kenward & Roger, 1997) from

the ‘pbkrtest’ package in R (Halekoh & Højsgaard, 2014) and estimated effect sizes (Cohen's *d*) for treatment effects and novel predator type (i.e. umbrella or puppet).

To test for the influence of glucocorticoids on the consolidation of threat conditioning, we assessed the relationship between the integrated CORT response and FID measured 48 h after conditioning with a generalized additive mixed-effects model (GAMM) with a penalized spline link function (i.e. smoothing spline) that is optimized via generalized cross-validation with the ‘gamm4’ package in R (Wood & Scheipl, 2014; Wood, 2006, 2011). We used a GAMM because the relationship between CORT and learning and memory is often described as having an inverted U-shape (e.g. Salehi et al., 2010; Schilling et al., 2013) and initial exploration of our data revealed a skewed distribution and a nonlinear relationship between FID and integrated CORT. GAMMs offer a greater degree of flexibility compared to traditional regression techniques because they can use nonparametric means to optimize the smoothing of nonlinear regression spline functions and accommodate data that are not normally distributed (Hastie & Tibshirani, 1990; Wood, 2006). We analysed the relationship between FID and integrated CORT for control and conditioned birds within the same GAMM. We also included the type of predator as a fixed covariate, as it had a near-statistically significant effect on FID in the mixed-effects model described above, and included sex as a covariate, as Florida scrub-jays have sex-specific differences in baseline and stress-induced CORT (Small & Schoech, 2015). Additionally, we included a random intercept for each individual, territory and unique testing event.

Similarly, to test for the influence of glucocorticoids on the retention of long-lasting memory, we assessed the relationship between integrated CORT and the change in FID (Δ FID) from 48 h to 11 months and from 48 h to 2 years after conditioning using a GAMM model similar to that described above. We analysed the relationship between Δ FID and integrated

CORT for control and conditioned birds within the same GAMM. We once again included predator type and sex as fixed effects, as well as time since treatment.

The vast majority (86%) of the 358 individual FID measures were collected in groups of two or more (mean $\pm$ SD = 2.55 ± 1.28 individuals), and thus individual FIDs may not be independent (i.e. pseudoreplication within groups; Hurlbert, 1984). To test whether FIDs of individuals were influenced by the FIDs of others, we compared individuals that were in ‘monotypic’ groups (i.e. groups that consisted of only control birds or only conditioned birds during FID trials) to individuals that were in ‘mixed’ groups (i.e. groups with conditioned and control birds tested together during FID trials). Mixed groups resulted from dispersal of young, the occasional ‘divorce’ and the tendency for neighbouring family groups to socialize. If the behaviour of others strongly influenced the FID of an individual, then control birds in mixed groups would have had longer FIDs than control birds in monotypic groups. Similarly, we predicted that conditioned birds in mixed groups would have had shorter FIDs compared to the FIDs of conditioned birds in monotypic groups. We used a linear mixed-effects model with the ‘lme4’ package in R (Bates et al., 2015) in which we included group, treatment and predator type as fixed effects and the interaction between treatment and group type. We estimated variances of control and conditioned treatments separately to account for heteroscedasticity between the treatments (Pinheiro & Bates, 2000). We also included individuals, the spatial location of FID trails and each unique FID trial as random effects. We calculated significance using the Kenward–Roger approximation (Kenward & Roger, 1997) from the ‘pbkrtest’ package in R (Halekoh & Højsgaard, 2014) and estimated effect sizes (Cohen's *d*) for the effect due to group composition (i.e. mixed or monotypic).

We used a general linear model to assess whether the threat-conditioning protocol triggered

an endogenous CORT response. We included treatment type (i.e. conditioned, control, mild threat) as a fixed effect, time post-treatment as a covariate, and their interaction. A statistically significant interaction between the main effect and covariate was present (see Results), so we centred the covariate around the mean to calculate conditional effect estimates (i.e. ‘simple slopes’) among the three treatments (Bauer & Curran, 2005). We report Tukey’s adjusted pairwise comparisons and Cohen’s d effect size estimates.

We tested whether scrub-jays generalized their conditioned response beyond the conditioned stimulus (e.g. acted fearful of the umbrella when they had been chased only by the puppet). We compared individuals that were either a control for both predators or a control for one predator, but chased by the other predator. If jays generalized their conditioned response, then individuals that had been chased by the umbrella, for example, would have had greater FIDs in response to the puppet than individuals that had not been chased by either predator. We used a linear mixed-effects model using the ‘lme4’ package in R (Bates et al., 2015) with a random intercept for individuals. We report Tukey’s adjusted pairwise comparisons among the four exposure types.

RESULTS

We recorded 358 FIDs of 154 individuals over 3 years. Birds previously chased by the novel predator had markedly longer FIDs compared to controls (all comparisons hereafter are expressed as least squares mean difference $\pm$ SE; 13.71 ± 0.85 m; $F_{1,166.77} = 216.57$, $P < 0.0001$; Cohen’s $d = 2.00$; Fig. 2).

The difference between FIDs in response to the umbrella versus the puppet was near statistical significance ($F_{1,144.68} = 3.06$, $P = 0.083$), but the effect was negligible (-1.62 ± 0.81 m; Cohen's $d = 0.16$). We found little evidence for first-order interactions between treatment and sex ($F_{1,203.83} = 0.33$, $P = 0.569$), age ($F_{1,198.64} = 0.48$, $P = 0.491$), predator type ($F_{1,191.03} = 0.28$, $P = 0.597$), time since last fire ($F_{1,193.49} = 1.59$, $P = 0.209$), total birds present ($F_{1,248.81} = 0.06$, $P = 0.815$) or time since treatment ($F_{2,90.07} = 0.21$, $P = 0.815$). Thus, we assumed homogeneity of slopes and excluded these interactions from the final model (Engqvist, 2005). FIDs did not vary by time since treatment ($F_{2,72.69} = 0.72$, $P = 0.490$). Additionally, we found no evidence that sex ($F_{1,106.71} = 0.61$, $P = 0.437$), age ($F_{1,119.94} = 0.23$, $P = 0.631$), time since fire ($F_{1,60.69} = 0.45$, $P = 0.507$) or total birds present ($F_{1,195.03} = 0.34$, $P = 0.562$) influenced the FIDs of jays. The fixed effects in our final linear mixed-effects model (predominantly treatment effects) explained roughly 50% of the variation in FID measurements (marginal $R^2 = 0.49$; Nakagawa & Schielzeth, 2013).

The relationship between integrated CORT and FID 48 h after treatment depended on the type of treatment that an individual received. We found little evidence for a relationship between FID and integrated CORT of controls ($F_{1,104.57} = 0.01$, $P = 0.909$, $R^2_{\text{adj}} = -0.02$; Fig. 3b). However, there was a statistically significant positive correlation between FID and integrated CORT in conditioned birds ($F_{3,44,104.57} = 4.08$, $P = 0.004$, $R^2_{\text{adj}} = 0.2$; Fig. 3a). The effects of the type of predator ($t_{104.57} = -0.64$, $P = 0.521$) and sex ($t_{104.57} = 1.334$, $P = 0.185$) were not statistically significant.

The relationship between the integrated CORT response and Δ FID depended on the type of treatment that an individual received. We did not find evidence for a relationship between integrated CORT and Δ FID in controls ($F_{1,99.69} = 0.0002$, $P = 0.989$, $R^2_{\text{adj}} = -0.01$; Fig. 4b), but

we found evidence for an inverted U-shaped relationship between an individual's integrated CORT response and their Δ FID among conditioned birds ($F_{4,31,99,69} = 23.00$, $P < 0.0001$, $R^2_{\text{adj}} = 0.55$; Fig. 4a). Specifically, the FIDs of low and high stress responders decreased from 48 h to 11 months and remained shorter at 2 years post-conditioning, while the FIDs of moderate responders increased over the same time periods. Neither time since treatment ($t_{99,69} = 0.09$, $P = 0.929$), predator type ($t_{99,69} = 0.44$, $P = 0.662$), nor sex ($t_{99,69} = 0.99$, $P = 0.325$) had a significant influence on Δ FID.

We found little evidence that the composition of an individual's social group during FID trials (i.e. mixed or monotypic groups) affected FID. We did not find a statistically significant interaction between treatment and group composition ($F_{1,119,05} = 0.23$, $P = 0.629$), and thus removed the interaction from the final model. Group composition was not statistically significant ($F_{1,89,91} = 1.87$, $P = 0.175$). In fact, the subtle differences in the means of FIDs between individuals of different group compositions for control and conditioned birds were in the opposite direction of what we predicted assuming that individuals were influenced by the behaviour of their neighbours (Fig. 5).

Our model that tested whether threat conditioning triggered an endogenous CORT response indicated an interaction between exposure type (i.e. control, mild threat, threat conditioned) and time caught post-exposure ($F_{2,12} = 6.59$, $P = 0.012$). Therefore, we assessed differences between exposure groups with a mean-centred covariate, in which we compared CORT levels among the three exposure types adjusted as if all samples had been taken at the mean time post-exposure (mean = 19.41 min). CORT levels differed significantly between individuals chased by the umbrella and unexposed controls (25.46 ± 1.58 ng/ml; $t_{12} = 16.11$, $P < 0.0001$; Cohen's $d = 4.57$, Fig. 6). Likewise, CORT levels differed significantly between chased individuals and individuals

that were exposed to a less threatening display of the umbrella predator (25.85 ± 1.53 ng/ml; $t_{12} = 16.92$, $P < 0.0001$, Cohen's $d = 4.89$). CORT values of controls and less threatened individuals were not statistically different (0.39 ± 1.66 ng/ml; $t_{12} = 0.24$, $P = 0.970$, Cohen's $d = 0.23$).

We found no evidence that individuals had generalized their conditioned response beyond the specific predator used during threat conditioning. The mean FIDs of controls in response to the puppet did not differ based on their exposure to the umbrella (i.e. control or threat-conditioned; -1.76 ± 2.18 m; $t_{30.56} = -0.809$, $P = 0.850$; Fig. 7). Likewise, the mean FIDs of controls in response to the umbrella did not differ based on their exposure to the puppet (1.75 ± 1.97 m; $t_{34.83} = 0.891$, $P = 0.810$; Fig. 7). Additionally, individuals that were controls for both predators responded similarly to both predators (3.99 ± 2.01 m; $t_{35.80} = 0.215$, $P = 0.810$; Fig. 7).

DISCUSSION

Our study demonstrated that Florida scrub-jays are able to learn to avoid a novel predator after a single threatening encounter of brief duration, and that individuals can remember this association for at least 2 years (Fig. 2). Additionally, stress-induced levels of glucocorticoids were positively correlated with consolidation (Fig. 3) and had an inverted U-shaped relationship with long-lasting memory retention (Fig. 4). Specifically, individuals with a moderate stress response exaggerated their defence response at 11 months and 2 years relative to their response 48 h after threat conditioning, while low and high stress responders decreased their conditioned defence response with time (Fig. 4a). We also demonstrated that our novel predator threat-conditioning protocol is a unique yet feasible method to assess the effects of stress physiology on learning and memory in Florida scrub-jays (and likely other species), as (1) the protocol elicited

an HPA axis response during conditioning (Fig. 6), (2) individuals did not generalize their conditioned responses (Fig. 7) and (3) individuals were not significantly influenced by their social environment during FID trials (Fig. 5).

Our experiment differed considerably from traditional threat-conditioning studies. It is one among only a few to examine the effects of stress physiology on memory in free-living, nonhuman animals. Also, to the best of our knowledge, we are the first to report the effects of glucocorticoids on the retention of long-lasting memory (i.e. memory lasting months to years). Furthermore, we assessed memory performance among individuals based on naturally occurring variation in stress-induced CORT release, whereas most laboratory studies have compared groups that differ by intensity of stressful stimuli exposure (e.g. Salehi et al., 2010), or administration of glucocorticoid receptor agonists or antagonist (e.g. Pugh, Tremblay, Fleshner, & Rudy, 1997). Despite these differences, our finding that glucocorticoids enhanced consolidation mirrors decades of findings from laboratory studies (reviewed by McGaugh, 2000, 2015; Roozendaal, 2000, 2002; Sandi & Pinelo-Nava, 2007).

Individuals that responded to the standardized capture-and-restraint protocol with low or high levels of integrated CORT showed a decrease in their conditioned response over time, presumably because of memory decay or memory extinction. In contrast, subjects with a moderate stress response increased their conditioned response (i.e. had longer FIDs) over time. We are perplexed by this finding as conditioned responses typically remain the same or weaken with time. We contend that our results are difficult to interpret within the context of contemporary theories of long-term memory, which are largely based on laboratory experiments conducted over relatively short durations (i.e. hours to weeks). Below, we offer possible explanations for the observed heightened defence response in scrub-jays within the context of

‘reconsolidation’ and ‘system consolidation’, and further discuss the possible ecological significance of our unexpected results.

Traditionally, consolidated memories were considered more or less stable, and resist amnesic effects (Davis & Squire, 1984; Duncan, 1949; McGaugh, 1966). Recently however, Nader, Schafe, and LeDoux (2000a) demonstrated that consolidated memories become labile once reactivated and that they are vulnerable to amnesic effects of various pharmacological manipulation before ‘reconsolidation’ is complete (reviewed by Dudai, 2012; Nader, Schafe, & LeDoux, 2000b; Nader, 2015). Inactivation of glucocorticoid receptors in the basolateral amygdala and hippocampus disrupts reconsolidation (Jin, Lu, Yang, Ma, & Li, 2007; Nikzad, Vafaei, Rashidy-Pour, & Haghighi, 2011; Tronel & Alberini, 2011), and administration of glucocorticoids at stress-induced levels in the basolateral amygdala has the same disruptive effect (reviewed by Schwabe, Joëls, Roozendaal, Wolf, & Oitzl, 2011). These disruptions suggest an inverted U-shaped relationship between glucocorticoids and reconsolidation. Indeed, stress can affect memory function in an inverted U-shaped manner (Yerkes-Dodson Law; Yerkes & Dodson, 1908). However, the impact of glucocorticoids on a specific memory processes is typically linear, and the direction of the correlation depends on the specific memory process (e.g. acquisition, consolidation, reactivation; Roozendaal & McGaugh, 2011; Sandi & Pinelo-Nava, 2007; Conrad, Lupien, & McEwen, 1999; Pugh et al., 1997).

Some have suggested that long-term memories may be enhanced or strengthened during the short mutable window between reactivation and reconsolidation (Dudai, 2012; Sara, 2000). Indeed, enhancement of previously consolidated memories has been achieved in rats via activation of amygdalar protein kinase A (Tronson, Wiseman, Olausson, & Taylor, 2006), enhanced noradrenergic activity in the amygdala (Dębiec, Bush, & LeDoux, 2011) and inhibition

of histone deacetylase (Bredy & Barad, 2008). However, to the best of our knowledge, whether such enhancement is mediated by glucocorticoids is unknown. Conceivably, the memory of being chased by the umbrella or puppet was modified during reconsolidation following its activation during the FID trial 48 h post-conditioning. If glucocorticoids mediate reconsolidation in an inverted-U shaped manner, moderate stress responders may have reconsolidated an exaggerated memory of being chased by the novel predator, resulting in the more fearful-like responses observed at 11 months and 2 years. However, this assumes that the FID trial 48 h after conditioning triggered a glucocorticoid response, which is currently unknown. However, the sight of a predator can activate the HPA axis and subsequent glucocorticoid response in a number of avian species (Canoine et al., 2002; Cockrem & Silverin, 2002a; Jones et al., 2016; Silverin & Wingfield, 1998). Therefore, it is not unreasonable to speculate that sight of the umbrella or puppet after being chased by it had a similar result.

Forty-eight hours is enough time for short-term memory to consolidate into long-term memory via what is termed ‘synaptic consolidation’ (Clopath, 2012; Dudai, 2012), and it is within the recommended duration to ensure consolidation occurs (Roozendaal, 2002). However, in contrast to synaptic consolidation, ‘system’ or ‘trace-transfer’ consolidation is the process by which memories are reorganized or shifted from one region of the brain to another, over the course of days to years (Knowlton & Fanselow, 1998; McClelland, McNaughton, & O’Reilly, 1995; Smith & Squire, 2009; Squire, 1992). System consolidation has only recently been discovered to play an important role in threat conditioning (Sacco & Sacchetti, 2010). ‘Remote’ threat-conditioned memories (i.e. those that have lasted 30 days) rely on secondary cortices and the hippocampus and amygdala for normal memory recall, whereas disruption of higher sensory cortical structures has no effect on ‘recent’ memories (i.e. 24 h or shorter; Cambiaghi et al.,

2016; Do-Monte, Quiñones-Laracuate, & Quirk, 2015; Sacco & Sacchetti, 2010; Zelikowsky, Bissiere, & Fanselow, 2012). Although glucocorticoids are well known for their ability to modulate memory via synaptic consolidation (Cahill & McGaugh, 1996; Roozendaal & McGaugh, 2011), their effects on system consolidation is unknown. We speculate that dysregulation of system consolidation may have contributed to the intensified defence responses over time in scrub-jays that had moderate physiological stress responses.

Predation pressure influences behavioural and morphological phenotypes via behavioural flexibility (Cooper & Frederick, 2007; Griffin, 2004; Lima & Dill, 1990; Stankowich & Blumstein, 2005), selection (Bell & Sih, 2007; Dingemanse et al., 2009; Pruitt & Goodnight, 2014) and developmental plasticity (Relyea, 2001; Schalk, Forbes, & Weatherhead, 2002). Conceivably, predation influences learning and memory capacities as well. Florida scrub-jays can remember a novel predator for multiple years after a single threatening encounter. One might predict such an ability given that failure to learn the dangers of a novel predator after its first attacks may lead to death during a second attack. However, our results indicate wide-ranging variation of the long-lasting memory of antipredator behaviour over time. Specifically, many jays exhibited decreased FIDs with time, suggesting memory decay, while others maintained or increased their FID in response to subsequent encounters with the novel predator. On the surface, forgetting the dangers of a potential predator seems maladaptive, but it may be beneficial under certain contexts. For example, jays that forget rarely encountered predators may free valuable neuronal real estate to retain information more essential for survival, such as those of frequently encountered predators or the location of food caches. Conversely, the ability to mount a robust antipredator response may be adaptive when predators are infrequent yet efficient. The existence of such wide variation in the ability to retain long-lasting memories of

predators may have resulted from selection or developmental plasticity (or both) in a dynamically shifting landscape of predation pressures, which in turn would affect the adaptive value of retaining long-lasting memories of rarely encountered predators.

Conclusion

Our study demonstrated that free-living Florida scrub-jays retained the memory of a single, brief encounter with a novel ‘predator’ for at least 2 years. The consolidation of this threat-conditioned memory was positively correlated with an individual’s glucocorticoid response to a standardized stressor. Surprisingly, moderate integrated CORT responders developed an enhanced defence response with time, whereas jays with high and low integrated CORT responses exhibited an attenuated response, suggesting an inverted U-shaped relationship between glucocorticoids and long-lasting memory. Our study suggests numerous avenues for further exploration, particularly in more ecologically relevant contexts and time frames.

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Figure 1. Novel predators ('umbrella' and 'puppet') used to threat-condition Florida scrub-jays.

We designed novel predators to have textures, patterns, colours and shapes uniquely different from those of known Florida scrub-jay predators (e.g. snakes, raptors) and from each other.

Figure 2. Flight initiation distance (FID; $N = 358$; start distance = 40 m) of Florida scrub-jays ($N = 154$) measured ca. 48 h, 11 months and 2 years after threat conditioning (grey plots) or control treatments (white plots). Results represented as Tukey box plots.

Figure 3. Correlation between flight initiation distance (FID) in response to a novel 'predator' and stress-induced corticosterone (CORT), as measured by integrated CORT, in response to a standardized capture-and-restraint protocol. Florida scrub-jays were either (a) previously chased by the novel predator (threat-conditioned), or (b) unexposed to the novel predator (control). FIDs were measured 48 h after threat conditioning or control treatments. Grey areas represent the 99% confidence interval of the smoothing spline regression of the generalized additive mixed model.

Figure 4. Correlation between change in flight initiation distance (Δ FID) in response to a novel 'predator' and stress-induced corticosterone (CORT), as measured by integrated CORT, in response to a standardized capture-and-restraint protocol. Florida scrub-jays were either (a) previously chased by the novel predator (threat-conditioned), or (b) unexposed to the novel predator (control). Δ FID was measured as FID 11 months after treatment (i.e. threat conditioning or control) minus FID 48 h after treatment (open circles), and FID 2 years after treatment minus

FIDs 48 h after treatment (solid circles). Positive Δ FID values indicate FIDs that became longer over time, whereas negative values indicate FIDs that became shorter over time. Grey areas represent the 99% confidence interval.

Figure 5. Flight initiation distance (FID) of Florida scrub-jays previously chased by a novel predator (threat-conditioned) or unexposed to the novel predator (control). Groups tested during FID trials were either ‘mixed’ (consisted of both control and threat-conditioned individuals, grey plots) or ‘monotypic’ (consisted of either all control or all threat-conditioned individuals, white plots). Results represented as Tukey box plots.

Figure 6. Total plasma corticosterone measured from Florida scrub-jays ca. 20 min (mean $\pm$ SE = 19.41 min, range 10.53–31.56 min) following one of the three experimental treatments. All samples were collected within 2.5 min of initial trapping. See Methods for a description of the experimental treatments. Results represented as Tukey box plots.

Figure 7. Flight initiation distance (FID) of Florida scrub-jays previously unexposed to a novel predator (umbrella controls and puppet controls). Individuals were further subsetted by the treatment received from the other novel predator; either threat-conditioned (grey plots) or control (white plots). Results represented as Tukey box plots.