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ARTICLE

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Developmental stage-dependent effects of perceived predation risk on nestling tree swallows (*Tachycineta bicolor*)

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Abstract

The risk of predation directly affects the physiology, behavior, and fitness of wild birds. Strong social connections with conspecifics could help individuals recover from a stressful experience such as a predation event; however, competitive interactions also have the potential to exacerbate stress. Few studies have investigated the interaction between environmental stressors and the social landscape in wild bird populations. In 2 years of field studies, we experimentally simulated predation attempts on breeding female tree swallows (*Tachicyneta bicolor*). At the same time, we manipulated female breast plumage color, a key social signal. Simulated predation events on tree swallows early in the nestling period reduced young nestlings' mass by approximately 20% and shortened telomere lengths. Ultimately, only 31% of nestlings in the predation group fledged compared with 70% of control nestlings. However, the effects of experimental manipulations were timing dependent: the following year when we swapped the order of the experimental manipulations and simulated predation during incubation, there were no significant effects of predation on nestling condition or fledging success. Contrary to our expectations, manipulation of the social environment did not affect the response of tree swallows to simulated predation. However, manipulating female plumage during the nestling period did reduce nestling skeletal size and mass, although the effects depended on original plumage brightness. Our data demonstrate that transient stressors on female birds can have carry-over effects on their nestlings if they occur during critical periods in the breeding season.

KEYWORDS
bird, glucocorticoids, predation, stress, telomeres

Sabrina M. McNew and Conor C. Taff contributed equally.

INTRODUCTION

Predation is an important source of mortality for birds (Martin, 1993; Ricklefs, 1989). Nestlings are highly vulnerable while in the nest, and behaviors such as incubation and provisioning also expose parents to predation. Even in the absence of direct consumption, the perceived risk of predation impacts the behavior, physiology, and fitness of adults and their nestlings (Lima, 2009; Zanette et al., 2011). Predation therefore is not just an acute challenge that forces temporary changes in physiology and behavior; it also creates a "landscape of fear" that may affect individuals' life history and even population dynamics (Brown et al., 1999; Clinchy et al., 2013; Laundre et al., 2010).

Animals vary in their resilience to predation, that is, their ability to withstand this stressor and return to normal functioning following a confrontation with a predator (Davis et al., 2021). One potential factor influencing resilience is social connectivity. For example, baboons (*Papio hamadryas ursinus*) respond to the loss of a close family member to predation by increasing social grooming, which may help lower stress hormone levels back to baseline (Engh et al., 2006). Social position and connectedness have emerged as key mediators of the psychophysiological effects of stress (Charuvastra & Cloitre, 2008; Holt-Lunstad et al., 2010; Yang et al., 2016). However, the cognitive and behavioral costs of nonconsumptive predation are difficult to observe and measure in natural populations (Clinchy et al., 2013). In addition, studies of the relationship between social connectivity and stress resilience have been conducted primarily in primates (Creel et al., 2013), even though these effects have been observed in loosely gregarious and even solitary species (Hennessy et al., 2009; Martin et al., 2023).

The physiological mechanisms involved in the stress response are key to understanding stress resilience and the long-term consequences of stressors. A major component of the stress response is the hypothalamic–pituitary–adrenal (HPA) axis, which regulates glucocorticoid hormones (Sapolsky et al., 2000; Wingfield et al., 1998). The release of glucocorticoid hormones helps an organism maintain fitness by mobilizing energy stores and shifting resources away from reproduction, growth and maintenance and toward an "emergency" behavioral and physiological state focused on surviving the immediate threat (Wingfield et al., 1998). Although this physiological stress response is an important adaptation allowing animals to react rapidly to challenges, chronic exposure to glucocorticoids can have negative fitness consequences (Sapolsky et al., 2000). Particularly when facing repeated stressors, individual variation in the magnitude or duration (i.e., the strength of negative feedback) of the acute glucocorticoid stress response may predict its ultimate costs (Vitousek et al., 2018; Zimmer et al., 2019).

Glucocorticoids may connect stressors to fitness through effects on telomeres (Haussmann & Heidinger, 2015; Haussmann & Marchetto, 2010). Telomeres are repetitive sections of noncoding DNA that form the ends of chromosomes. They have important roles during meiosis and mitosis including helping to maintain chromosome integrity during replication (Monaghan & Haussmann, 2006). Telomeres generally degrade over the course of an animal's lifetime and telomere shortening is often associated with disease and senescence (Angelier et al., 2018; Asghar et al., 2015). Both genetic and environmental factors influence telomere length and so telomeres are used as a proxy for "long-term somatic state," an integrative measure of an individual's condition (Benowitz-Fredericks et al., 2022). Chronically high levels of glucocorticoids increase somatic damage from inflammation and oxidative stress, which are also linked to telomere loss (Angelier et al., 2018; Ridout et al., 2018). Early-life stress may have particularly strong effects on telomeres (Injaian et al., 2019; Ridout et al., 2018; van Lieshout et al., 2021). For instance, nestling European shags (*Phalacrocorax aristotelis*) exposed to simulated predation events experienced higher stress-induced corticosterone (the main glucocorticoid in birds) concentrations and increased telomere loss over the course of the experiment (Herborn et al., 2014). Telomere lengths in young birds can predict overall lifespan (Eastwood et al., 2023; Haussmann et al., 2005; Heidinger et al., 2012), thus even transient stressors early in life may shorten overall life expectancy. However, the relationship between stressors and telomere lengths can be complex. Nestling pied flycatchers (*Ficedula hypoleuca*) raised at sites with a higher threat of owl predation had longer telomeres than nestlings at control sites, potentially due to changes in parental provisioning behaviors under heightened predation risk (Kärkkäinen et al., 2019).

In this study we exposed breeding female tree swallows (*Tachycineta bicolor*) to two different experimental treatments: simulated predation and manipulation of the social environment.

Heightened predation risk is associated with changes in parental care of tree swallows (Wheelwright & Dorsey, 1991). Natural predators of tree swallows include other birds, mammals, and reptiles; common predators at our field site include mustelids and snakes (Winkler, 1992). Previous experiments that manipulated perceived predation risk in our population showed that breeding females that had a robust glucocorticoid response along with strong negative feedback to predator exposure were less likely to abandon nests during incubation (Zimmer et al., 2019). However, it is not clear whether the heightened risk of predation affects developing nestlings. The presence of predators may affect nestlings' telomere lengths and overall lifespan either directly (by nestlings mounting a stress response to a perceived attack) or indirectly (e.g., through changes to parental care).

In addition to simulating predation, we manipulated the social environment by dulling the white breast plumage of females. White plumage patches are widespread among birds; however, white pigmentation has received less attention as a signal compared with iridescent, melanin, and carotenoid-based pigmentation (Beck et al., 2015; Tickell, 2003). Brightness in white plumage is driven by feature structure and can be an honest signal of individual condition and/or quality (Laczi et al., 2021). Thus, brightness could be a sexually selected trait that both males and females use to identify high-quality mates. Intriguingly, the evolution of white plumage is associated with transitions to sociality, suggesting this cue has an important role in intraspecific signaling in birds beyond mate selection (Beauchamp & Heeb, 2001).

In tree swallows, naturally brighter white breast plumage is associated with greater immunity, reproductive success, and the frequency of social interactions at the nest (Beck et al., 2015; Taff et al., 2019). While the nature of these signal-mediated social interactions is not well understood, a previous experiment in our population found that manipulating female breast brightness—in the absence of a separate environmental stressor—changed the patterns of social interactions and led to higher reproductive success for dulled females, particularly when those females were initially bright (Taff et al., 2021). Thus, changes to this signal, in the absence of any changes to individual conditions, create feedback between the social environment, physiology, and fitness.

We tested for the effects of simulated predation and social manipulation on nestling size, physiology, and fledging success during one field season (2018). The following year (2019), we repeated the experiment, switching the order of the treatments between years to test how the timing of stressors in different developmental stages affected nestling outcomes. We predicted that elevated predation stress on mothers would lead to reduced parental care and overall poorer nestling outcomes. Second, we expected that there would be a trade-off between the stress response and telomeres: nestlings that responded to predation stress with elevated corticosterone levels would have shorter telomeres. Finally, because plumage brightness is correlated with social connectivity, we expected that experimentally dulled females would be less resilient to environmental stressors, and so we predicted that predation would have a more severe effect on the reproductive success of dulled females.

METHODS

We studied wild tree swallows breeding in nest boxes near Ithaca, New York, USA (42.503° N, 76.437° W) from May to July of 2018 (Year 1) and 2019 (Year 2). This population has been monitored continuously since 1986 using standardized field methods (Winkler et al., 2020). We conducted a separate experiment each year; however, general methods for monitoring reproductive behavior were the same each year except for where noted. Nest boxes were monitored every other day starting at the beginning of the breeding season and active nests were checked every day around the expected hatching date to determine the timing of clutch initiation, the onset of incubation (±1 day) and hatching (exact day, ~12 days after clutch completion).

Experimental manipulations

In Year 1 (2018) we carried out a 2 × 2 factorial experiment in which we first manipulated the signal coloration of breeding female swallows and then later imposed a simulated predation challenge (Figure 1). Plumage dulling began during day 6 of incubation and lasted the duration of the reproductive period. Between days 1 and 5 after hatching, we conducted three simulated "predation" events on female tree swallows while they were in the nest.

At ~day 6 of incubation (±1 day), we captured females and alternately assigned them to a "dulled" or control treatment. For this signal manipulation treatment, dulled females were colored across their entire white ventral surface with a light gray nontoxic marker (Faber-Castell PITT artist pen "big brush" warm gray III 272). We previously validated that this treatment maintains the spectral characteristics of the plumage patch while reducing overall brightness (Taff et al., 2021). As a control, we applied a colorless marker over the same plumage area for the same length of time (Prismacolor Premier Colorless Blender PB-121; Newell Brands, Oak Brook, IL, USA). The treatments were re-applied 1 day after hatching and again 6 days after hatching so that the signal manipulation lasted during most of their reproductive attempt. In this year, 20 females were experimentally dulled, and 22 females received the control treatment. We balanced treatment within age groups (birds born the previous year, i.e., "second year" vs. older birds, i.e., "after second year") because breeding phenology and reproductive success differ between these ages in tree swallows (Winkler et al., 2020).

The second part of the experiment simulated an attempted predation event. For nests in the "predation"

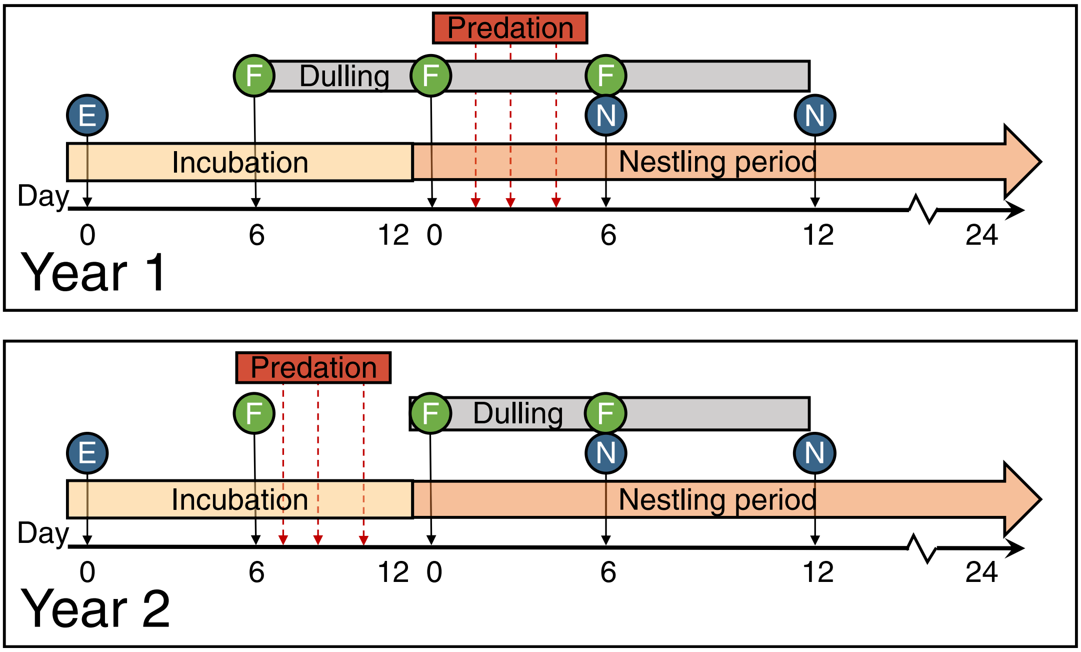

FIGURE 1 Schematic depicting the experimental design in Year 1 (2018) and Year 2 (2019) of the study. In each year, eggs were cross-fostered at day 0 of incubation (E), then nestlings were measured at days 6 and 12 (Ns) before fledgling on approximately day 24. Females were captured at day 6 of incubation, and days 1 and 6 post hatching (Fs). Females in the predation treatment received three additional "simulated predation" events either between days 1–6 post hatching (Year 1) or between days 6–12 of incubation (Year 2).

treatment, we simulated attempted predation on the female swallow by a mink (*Neovision vision*), which is a common predator of both adults and nestlings at our field sites. Females were trapped in the nest box and then gently pulled out of the box using a taxidermied mink wrapped around the researcher's hand. The bird was brought to the ground below the nest box and then allowed to escape. During this treatment, the researcher's face and body were covered with a camouflage suit and the female was held facing away from the researcher's body to make the predation experience seem as realistic as possible. Mink are stealth predators of birds (Jiménez et al., 2014) and likely provide few visual or auditory clues to their approach. We similarly sought to minimize noise when approaching the nest. Although female tree swallows typically do not vocalize when restrained, her mate and neighboring tree swallows make alarm calls when they detect predators, including the approach of humans to nest boxes (Winkler, 1994). Nestling tree swallows will quiet begging in response to alarm calls; however, this response is not developed until ~10 days of age, well after our predation treatments were applied (McIntyre et al., 2014). We do not know for sure how nestling tree swallows experience a real attempted predation event by a mink and acknowledge that there could be differences in the sensory cues present during predation. Nevertheless, we expect that our experimental manipulation was perceived by female tree swallows as an attempted predation event. For nestlings, they may have directly perceived changes in light, sound or smell during the attack, and/or received indirect signals on the predation event through hearing alarm calls or changes in female behavior following the attack.

The predation simulation was performed three times during days 2–5 after hatching (separately from the captures described above on day 6 of incubation (±1 day), day 1 and day 6 post-hatching). The control group received no additional treatment outside of the signal manipulation (dulling or control) described in the previous paragraph. As above, we alternately assigned the predation and control treatments, while balancing these treatments within age classes. In Year 1 (2018), 22 females were in the predation group and 20 females were in the control group. Sample sizes in each dulling × predation treatment combination ranged from 9 to 11 (Appendix S1: Table S1).

In Year 2 (2019) we repeated these experiments, but we altered the timing of the two treatments, applying simulated predation first, during the incubation stage, and then subsequently dulling females' plumage during the nestling stage (Figure 1). Females were assigned to either predation or control treatments at day 6 (±1 day) of incubation and females in the predation treatment received two additional simulated predation attempts between days 8 and 12 of incubation. We included 29 females in the predation group and 33 in the control group. Then, on days 12–13 of incubation females were alternately assigned to either a plumage dulling or control signal manipulation treatment. Signal manipulation treatments were applied exactly as described for 2018, with coloring re-applied at the third capture on day 6 after hatching. In 2019, 31 females were experimentally dulled, and 31 females received the control treatment (Appendix S1: Table S1). Sample sizes for each dulling × predation treatment combination ranged from 14 to 17 (Appendix S1: Table S1). The results of these experiments on adult behavior and physiology have been published separately (Taff et al., 2023).

Nestling cross-fostering and measurements

Differences in initial female quality are known to have a large effect on reproductive performance in tree swallows (Winkler et al., 2020). While our randomly assigned experimental treatments should account for these differences, we also sought to separate the effects of our treatments from any pretreatment maternal effects by cross-fostering eggs at each nest in the study. Nests were paired by breeding stage and on the fourth day of the egg-laying stage we swapped half of the eggs from each nest and marked the bottom of all eggs with a pencil. For half of the nest pairs, we swapped an additional unmarked egg on the next day. This scheme ensured that egg-laying order was not associated with cross-fostering status. In a few cases, we modified the swapping scheme to include three nests when appropriately timed matches were not available and a few later-season nests were not cross-fostered. For all nests, the ultimate clutch size remained the same after cross-fostering.

Nestling size and physiology at each nest were monitored as follows: On day 6 after hatching, we took a "total brood mass" with all nestlings counted and weighed together (nearest 0.5 g). This total brood mass was divided by the number of nestlings to calculate a mean nestling mass at day 6. On day 12 after hatching, we banded nestlings with a USGS aluminum band and measured each nestling individually. We measured head + bill length (to the nearest 0.1 mm), flat wing length (to the nearest 0.5 mm), and mass (to the nearest 0.25 g). Structural measures (head + bill length and wing length) are measurements of nestling development while mass is a relative indicator of condition. All blood samples were collected by brachial venipuncture and placed into a heparinized micro-hematocrit tube. A baseline sample ($<70\ \mu L$) was collected within 3 min of capture to measure circulating levels of corticosterone before the onset of the challenge. A stress-induced sample ($<30\ \mu L$) was then collected after 30 min of restraint to measure peak circulating corticosterone levels in response to a standardized acute stressor. Immediately after the stress-induced sample was taken, we injected birds intramuscularly into the pectoral muscle with $4.5\ \mu L\ g^{-1}$ of dexamethasone (Mylan $4\ mg\ mL^{-1}$ dexamethasone sodium phosphate, product no. NDC 67457-422-00). Dexamethasone binds to the same receptors as corticosterone, inducing negative feedback and reducing the secretion of corticosterone; thus, this protocol, which has previously been validated in tree swallows, measures individual variation in the efficacy of negative feedback (Zimmer et al., 2019). A third blood sample was collected 30 min after dexamethasone injection.

All blood samples were stored on ice in the field for <3 h and then red blood cells and plasma were separated by centrifugation. Red blood cells were divided: part of the sample was stored in Longmire lysis buffer at room temperature for genotyping (Longmire et al., 1997). The other part was stored in NBS buffer (90% newborn calf serum and 10% DMSO) for telomere analysis. Samples for telomeres were kept at −80°C until analysis. Plasma was stored at −30°C until processing. We measured corticosterone with enzyme immunoassay kits (DetectX Corticosterone, Arbor Assays: K014-H5) that were previously validated for tree swallows in this population (see Appendix S1 for details on extractions and hormone measurements; Taff et al., 2019).

We used blood samples from nestlings to determine the nest of origin using a previously validated set of nine microsatellite markers (Hallinger et al., 2019; Makarewich et al., 2009). For the purposes of this study, we were only interested in assigning nestlings to their correct mother from two or three possible females. Females were considered good matches if they matched nestlings at eight of nine loci and we were able to assign maternal origin to 374 of 386 nestlings sampled. Details on the laboratory procedure and criteria for assigning a nest of origin can be found in Appendix S1.

Telomere quantification

We quantified relative nestling telomere length using a quantitative real-time PCR protocol following methods described in (Taff & Freeman-Gallant, 2017) and using current best practices (Morinha et al., 2020). Briefly, we extracted DNA from erythrocytes preserved in NBS buffer using Qiagen DNeasy Blood and Tissue Kits (Catalog No. 69504, Valencia, CA). We used a QuickDrop spectrophotometer (Molecular Devices, San Jose, CA) to assess the DNA concentration and purity. The mean A260/280 absorbance ratio was 1.96 and the mean A260/230 absorbance ratio was 1.58. Because the A260/230 ratios tended to be lower than the mean recommended value (~1.8; Morinha et al., 2020) we tested for a correlation between sample

A260/230 and the T/S ratio. We found no indication that the absorbance ratio was influencing our estimate of telomere length. We verified DNA integrity by running a subset of samples (~25%) on a 2% agarose gel; in all cases the DNA formed a single bold band with high molecular mass.

qPCR reactions were run on 384 well plates in reaction volumes of 13.5 μL. Each reaction contained 7 μL of PerfeCTa SYBR Green SuperMix, Low ROX (Quantabio, Beverly, MA), 2.8 picomoles of each primer and 14 ng of sample DNA. We amplified telomeres using qPCR with the primers Tel1b (5′-CGGTTTGTTTGGGTTTGGGTTTG GGTTTGGGTTTGGGTT-3′) and Tel2b (5′-GGCTTGCCT TACCCTTACCCTTACCCTTACCCTTACCCT-3′), which had been optimized for birds (Criscuolo et al., 2009) We amplified a single copy control gene (GAPDH: glyceraldehyde-3-phosphate dehydrogenase) using the primers GAPDH-F (5′-TTGACCACTGTCCATGCCATC AC-3′) and GAPDH-R (5′-TCCAGACGGCAGGTCAGG TC-3′). Both GAPDH and telomere reactions were run on a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). The telomere thermocycling conditions were as follows: 95°C for 10 min then 28 amplification cycles (95°C for 15 s, 58°C for 30 s, 72°C for 30 s), followed by a melt curve (95°C for 15 s, 60°C for 60 s, 95°C for 15 s). The GAPDH thermocycling conditions were as follows: 95°C for 10 s, then 40 amplification cycles (95°C for 30 s, 60°C for 30 s), followed by a melt curve (95°C for 15 s, 60°C for 60 s, 95°C for 15 s). Samples were run in triplicate for each reaction (telomere or GAPDH). Each plate also included three negative controls, a calibrator or "golden" sample (run on each plate to control for interplate variation) and five serial dilutions of a single high-quality sample. We calculated the repeatability of our qPCR reactions following best practices for telomere length estimation (Morinha et al., 2020) by calculating the intraclass correlation coefficient (ICC) using the R package *rptR*. We calculated the ICC with 1000 bootstraps for two sets of samples: our standards, which were run on every plate, and our standards plus a haphazard subset of samples that were run 2+ times. The repeatability of our standards in the telomere reaction was 0.93 ($\mathrm{SE} = 0.21$; $N = 13$). The repeatability of all samples run 2+ times in the telomere reaction was 0.90 ($\mathrm{SE} = 0.05$; $N = 44$). The repeatability of our standards in the GAPDH reaction was also 0.93 ($\mathrm{SE} = 0.21$, $N = 15$). The repeatability of all samples run 2+ times in the GAPDH reaction was 0.72 ($\mathrm{SE} = 0.\ 06$; $N = 126$).

Telomere data processing

We exported raw fluorescence data from the Thermo Fisher Scientific Design and Analysis Software (v. 2.4.3) and then used LinRegPCR (v. 1.5.3) (Untergasser et al., 2021) to analyze amplification curves and calculate per-well efficiency and the quantification cycle (C_q; the cycle number at which fluorescence rises above the threshold). Per-well reaction efficiencies ranged from 1.76 to 1.86 for the telomere reactions and from 1.87 to 2.03 for the GAPDH reaction (an efficiency value of two indicates the amount of the amplicon doubled each cycle). We examined the C_q values of triplicates to ensure precision in our estimation of telomere length. We averaged C_q values for each sample, including only replicates whose C_q values were within 0.25 standard deviations of one another. If we did not have at least two replicates within 0.25 standard deviations, we excluded or re-ran the sample.

We calculated the relative telomere length (RTL) for each sample using the following equation:

$$\mathrm{RTL} = \left(E_{\mathrm{TEL}}^{\left(C_q\mathrm{TEL}[\mathrm{Calibrator}] - C_q\mathrm{TEL}[\mathrm{Sample}] \right)} \right) \Big/ \left(E_{\mathrm{GAP}}^{\left(C_q\mathrm{GAP}[\mathrm{Calibrator}] - C_q\mathrm{GAP}[\mathrm{Sample}] \right)} \right),$$

where E is the mean reaction efficiency across all samples on a given plate; C_q[Calibrator] is the mean C_q across the calibrator samples on the plate, and C_q[Sample] is the mean C_q of a given sample (Reichert et al., 2017). Because the average efficiency for the telomere reaction was lower than expected, we investigated whether per-sample efficiency was correlated with our per-sample estimation of RTL. However, the correlation coefficient between per-sample efficiency and RTL was 0.001, suggesting that variation in efficiency was not driving estimation in RTL.

Analysis

Analyses were run in R (version 4.3.2) using the packages *lme4*, *lmerTest*, *coxme*, *survminer*, *survival*, and *scales*. We used linear mixed effect models to test for effects of the coloration and predation treatments on nestling size and physiological measurements, including mass, skeletal measurements, corticosterone, and RTL. Each response variable was modeled separately as a function of the fixed effects of color treatment, predation treatment, and their interaction. When the interaction term was not significant (in all models), we removed it and reported the results of the additive model. Models also included the covariate of female brightness before manipulation (numeric, centered, and scaled). We included female brightness because the effect of dulling depended on how bright the female was at pretreatment. Previous work in this system also showed that the effects of experimental dulling depend on pretreatment brightness (Taff et al., 2021). We tested first for an interaction between initial brightness and color treatment; when the interaction effect was not significant, we removed the interaction. We also included female age (second year, SY or after second year, ASY) because female age often affects reproductive success in our population (Winkler et al., 2020). We also initially included a covariate of whether the nestling was raised in its natal nest or was cross-fostered (binary), but this effect was never significant, so we did not include it in any final model. Finally, models included two random effects: social nest box (i.e., the nest where the nestling was raised) and genetic mother. In some models the random effect of genetic mother did not explain any residual variance and caused a singular fit warning, indicating that the random effects structure was overfit. In those cases, we removed the random effect of the genetic mother, leaving only the random effect of the social nest box. In R notation the full model structure is as follows: response_variable ~ predator_treatment + color_treatment × female_brightness + female_age + (1|social_nest_id) + (1|genetic_mom). Because of differences in experimental design each year, we ran separate models for 2018 and 2019. To test for differences in reproductive success between years, we ran logistic generalized linear mixed effect models predicting per-nest hatching success and per-nest fledging success with year as a fixed factor, and nest id as a random effect.

We used mixed effects Cox proportional hazards models to test for differences in nestling survival between treatment groups. We included the fixed effects of color treatment and predation treatment and nest as a random effect. We also tested for differences in overall fledging success using a logistic regression predicting nestling fate (fledged or died) based on color treatment and predation treatment, with female brightness and female age as fixed effects, and nest as a random effect.

RESULTS

Nestling size and physiology

In 2018, when perceived predation risk was manipulated during the nestling period, the predation treatment had a negative effect on some measures of nestling size. At 12 days of age, nestling wing length was significantly shorter in the predation group compared with controls ($b = -7.55$, CI = −13.56 to −1.54, $p = 0.014$; Appendix S1: Table S2; Figure 2). Nestling head + bill length was also smaller on average for predation nestlings compared with controls; however, the difference was not significant ($b = -0.84$, CI = −1.79 to 0.11, $p = 0.084$; Appendix S1: Table S3). Nestling mass at day 6 was significantly lower in the predation treatment compared with controls ($b = -2.20$, CI = −3.74 to 0.66, $p = 0.007$; Appendix S1: Table S4). However, by day 12 mass was not significantly different between predation nestlings and control nestlings ($b = -1.66$, CI = −4.26 to 0.94, $p = 0.210$; Appendix S1: Table S5).

In contrast, the dulling treatment (which occurred during incubation and the nestling period) in 2018 had only minor effects on nestling size and effects depended on female brightness at the start of the season. There was no significant effect of dulling treatment on 12-day-old nestling wing length, head + bill length, or mass (Figures 2 and 3; Appendix S1: Tables S2, S3, and S5). There was a significant interaction between initial female brightness and the dulling treatment on 6-day-old nestling mass (Appendix S1: Table S4): females in the experimentally dulled treatment had a positive relationship between pretreatment brightness and nestling mass, whereas for females in the control treatment there was not a significant relationship between brightness and nestling mass (Appendix S1: Figure S1).

In 2019, the predation treatment (which occurred during incubation) did not have a significant effect on nestling mass, wing length, or head + bill length (Figures 2 and 3; Appendix S1: Tables S2–S5). However, the dulling treatment (which occurred during the nestling period) did have effects on nestling size, which were mediated by initial female brightness. There was a significant interaction between initial female brightness and the dulling treatment on nestling wing length ($b = 6.37$, CI = 2.11–10.63, $p = 0.004$), head + bill length ($b = 0.96$, CI = 0.35–1.57, $p = 0.002$) and 12-day nestling mass ($b = 2.83$, CI = 1.30–4.36, $p < 0.001$). For experimentally dulled females, nestling size was slightly positively correlated with initial female brightness. However, for control females, nestling size was negatively correlated with female brightness (Appendix S1: Figures S1 and S2).

We tested for the effects of the predation and dulling treatments on nestling corticosterone. Each year we quantified baseline, stress-induced, and post-dexamethasone corticosterone in 12-day old nestlings. There were no significant differences either between predator treatments or between dulling treatments in any corticosterone measurement (Figure 4; Appendix S1: Tables S6 and S7). In contrast with models of nestling size, there was no effect of initial female brightness on nestling corticosterone in either year.

Telomere length

Nestlings in 2018 had significantly shorter RTLs in the predation group compared with the control group ($b = -0.07$, CI = −0.11 to −0.03, $p = 0.002$; Figure 5;

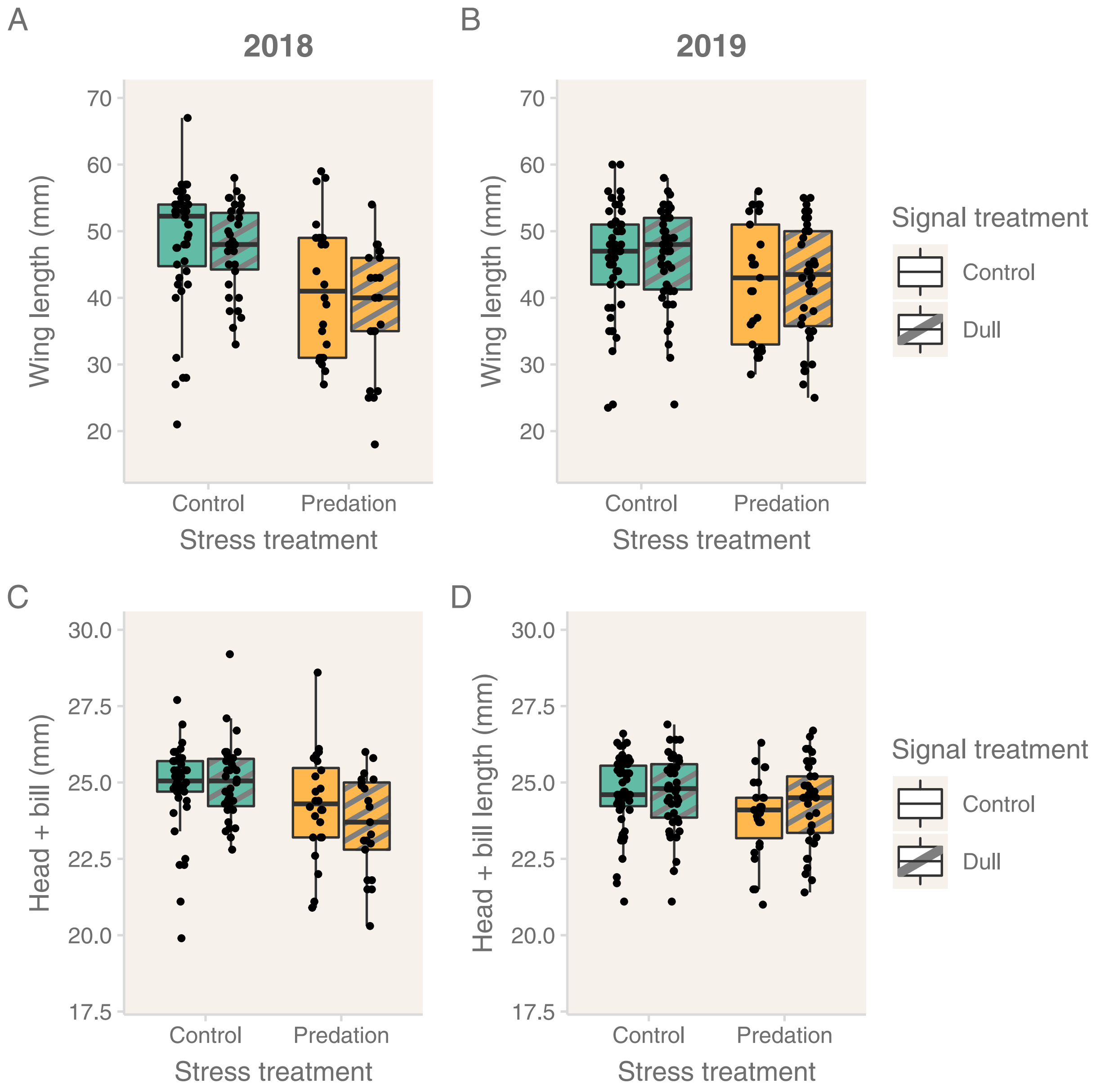

FIGURE 2 Nestling skeletal size at 12 days of age in predation and dulling treatments. Upper row: wing length in (A) 2018 and (B) 2019. Lower row: head + bill length in (C) 2018 and (D) 2019. Nestlings in the predation group (yellow) had significantly smaller wings at day 12 than nestlings in the control group (teal) in 2018. See results for full statistical comparisons.

Appendix S1: Table S8). There was no significant difference between dulling and control treatments ($b = 0.04$, CI $= -0.01$ to 0.08, $p = 0.086$). In contrast, nestlings in 2019 had no significant difference in telomere lengths between predation and control groups ($b = 0.04$, CI $= -0.03$ to 0.11, $p = 0.212$). Again, there was no difference between dulling and control treatments ($b = 0.01$, CI $= -0.06$ to 0.07, $p = 0.869$; Figure 5; Appendix S1: Table S8). We tested whether RTL was correlated with measurements of nestling size. There was no relationship between telomere length and mass or wing length of 12-day-old nestlings in either study year (Appendix S1: Tables S9 and S10). Telomere length also was not a significant predictor of fledging success in either year (Appendix S1: Table S11); however, this analysis only included those nestlings that survived to 12 days of age (the date of blood sampling). There was no relationship between any of the three measurements of corticosterone at 12 days of age and RTL (Appendix S1: Table S12).

Reproductive success

There was no effect of the dulling treatment on hatching success in 2018 or of the predation treatment on hatching success in 2018 (Appendix S1: Table S13). We

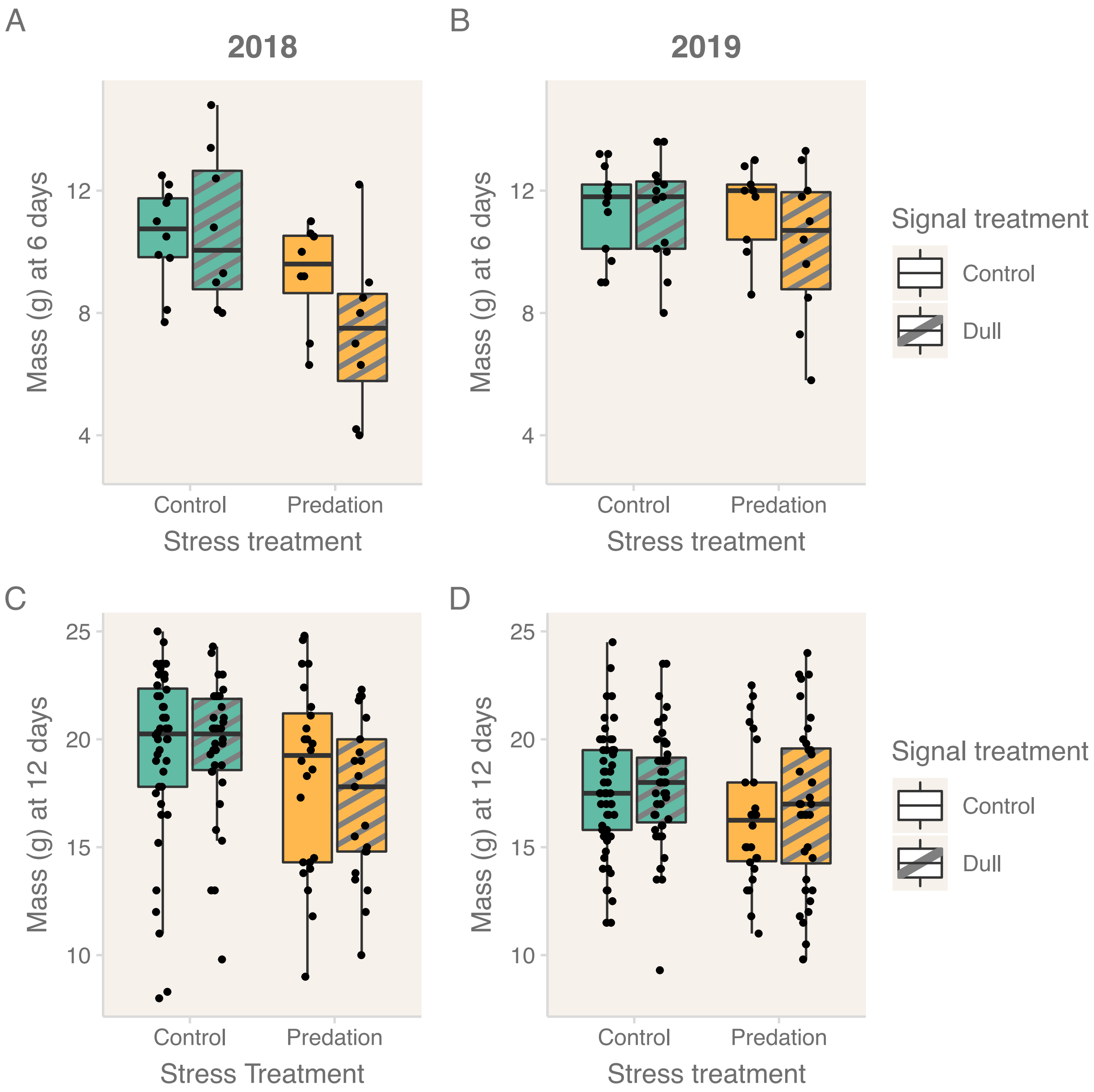

FIGURE 3 (A) Mean mass at 6 days of age in 2018 and (B) 2019. (C) Nestling mass at 12 days in 2018 and (D) 2019. At 6 days of age all nestlings in the brood were massed together and then an average mass was calculated by dividing the total by the number of nestlings. At 12 days of age each nestling was individually massed. Each point in (A, B) is a mean for a nest, each point in (C, D) is an individual nestling. Nestlings in the predation treatment (yellow) were significantly smaller than controls (teal) at 6 days of age in 2018. No other differences between treatment groups were significant.

compared reproductive success (hatching success and fledging success) between our two study years to investigate whether environmental differences between years could have contributed to our results. Hatching success did not differ between years. In 2018, 81% of eggs hatched and in 2019, 87% of eggs hatched (Odds ratio = 1.55, CI = 0.55–4.39, $p = 0.407$). Once nestlings hatched, fledging success did not differ overall between years. In 2018, 51% of nestlings fledged and in 2019 52% of nestlings fledged (Odds ratio = 0.94, CI = 0.24–3.66, $p = 0.932$).

We used a Cox proportional hazards model to test for differences between treatments in nestling survival from hatching (day 0) to fledging (~day 23). In 2018, nestlings in the predator treatment had a higher risk of death (hazard ratio = 3.16, CI = 1.26–7.91, $p = 0.014$, Figure 6). In 2019, however, survival did not differ between predator and control treatments (hazard ratio = 1.88, CI = 0.65–5.46, $p = 0.245$, Figure 6). There was no significant difference in either year between dulling treatments (Appendix S1: Table S14). Fledging success was lower for nestlings in the

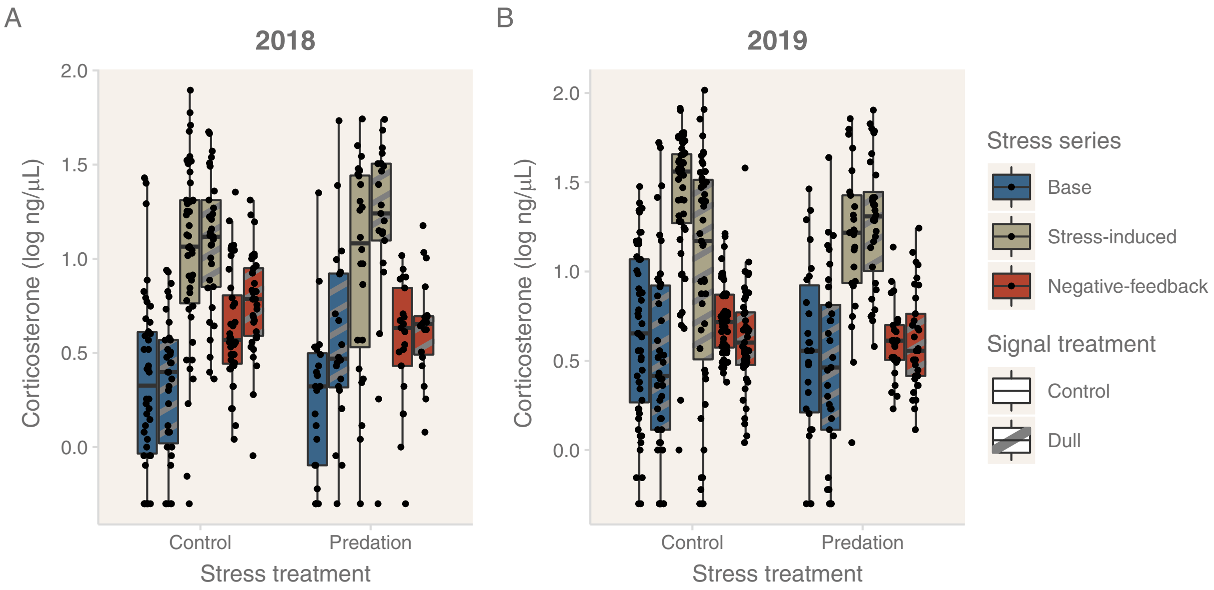

FIGURE 4 (A, B) Corticosterone (cort) concentrations for nestlings at 12 days of age. We conducted a stress series for each nestling quantifying baseline (within 3 min of disturbance), stress-induced (after 30 min) and negative-feedback cort levels (30 min after injection with dexamethasone, see *Methods*). There was no significant effect of either predation or signal manipulation in either year.

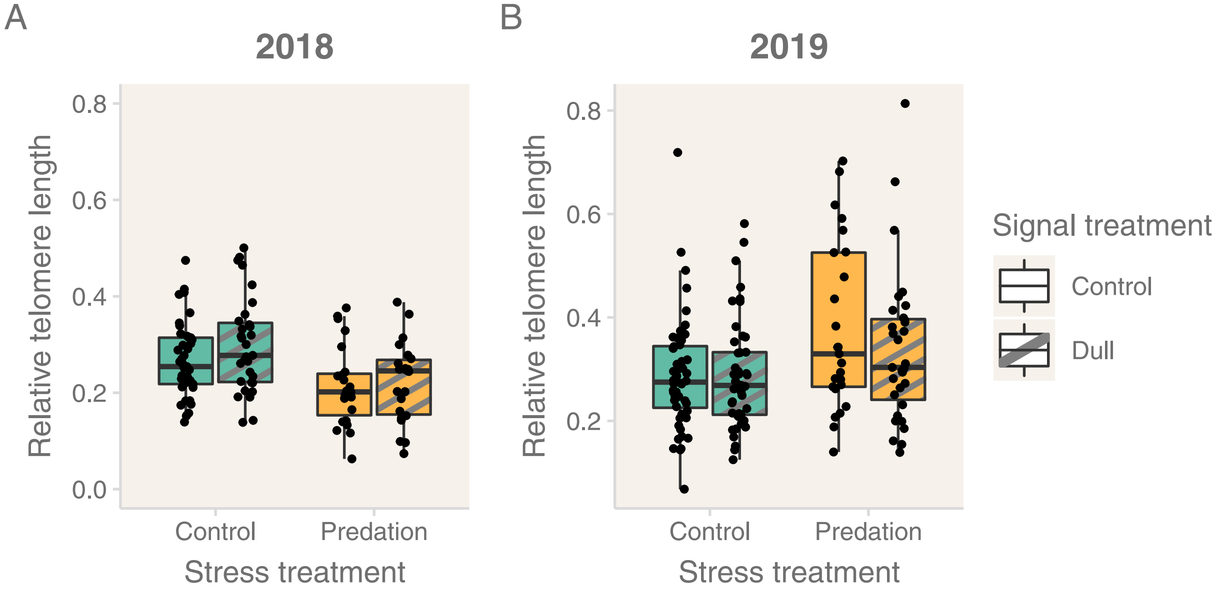

FIGURE 5 Relative telomere length in 2018 (A) and 2019 (B). Telomeres were significantly shorter in the predation group (yellow) compared with controls (teal) in 2018. There was no significant difference between predation and control groups in 2019 or between signal treatment groups in either year.

predator treatment compared with the control treatment in 2018; however, it was not significantly different between predator and control treatments in 2019 (Appendix S1: Table S15). There was no significant difference in fledging success between dulling and control treatments in either year (Appendix S1: Table S15).

DISCUSSION

The presence of predators affects bird physiology, behavior, and reproductive investment (Allen et al., 2022). Here, we tested for the effects of increased perceived predation risk on tree swallow reproductive success and sought to identify the physiological mechanisms involved

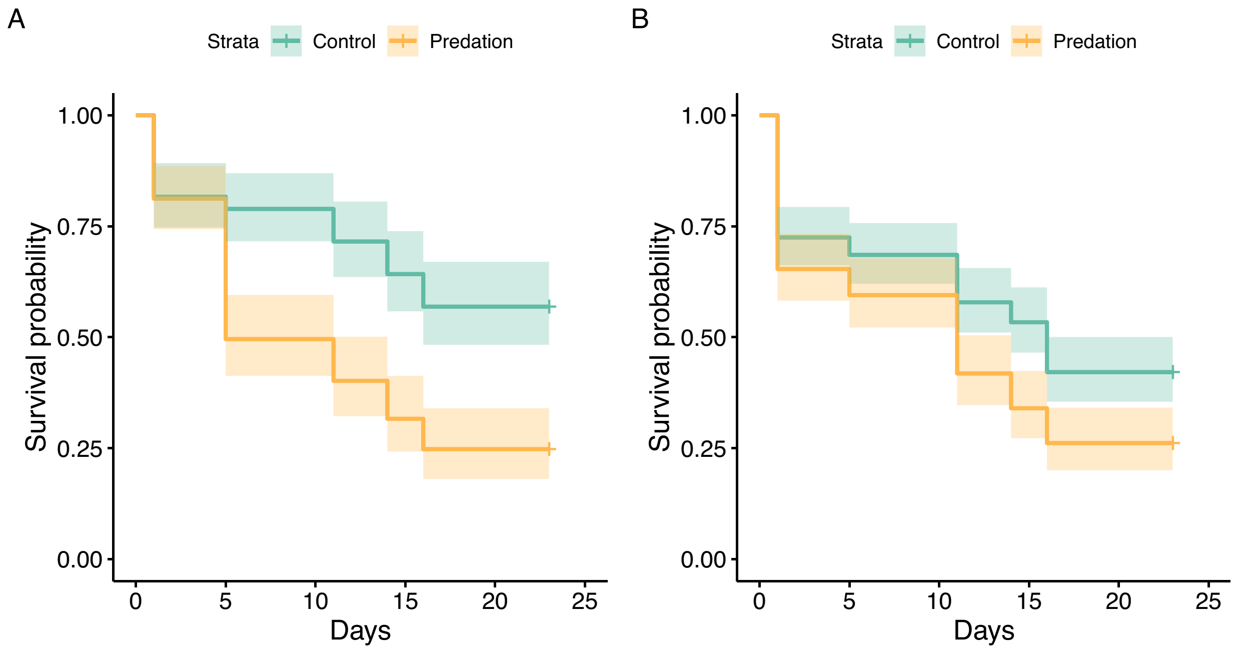

FIGURE 6 Daily survival probability of nestlings in the predator and control groups in 2018 (A) and 2019 (B). Shaded lines represent confidence intervals. Nests were checked at hatching (0 days), 6 days, 12 days, 15 days, and fledging (~23 days). In 2018 (A) simulated attempted predation occurred between days 1 and 5 of the nestling period. In 2018 (B) simulated attempted predation occurred before hatching (Figure 1).

in nestling stress resilience. We found that simulated predation attempts negatively affected tree swallows; however, the effects differed between experimental years. During the first year of the study, when breeding females experienced three simulated predation attempts during the early post-natal period, their nestlings suffered lower fledging success and those that survived had reduced size and shorter telomeres. In contrast, in the second year of the study, when predation attempts occurred during the prenatal period, there were no effects on nestling survival, size, or physiology. Contrary to our predictions, signal manipulation (i.e., plumage dulling) did not mediate resilience to predation stress. The plumage dulling treatment did have minor effects on nestling size, but only when it occurred during the nestling period and only after considering initial female brightness.

In 2018, the largest proportion (42%) of nestling deaths in the predation group occurred during the first 6 days of life, that is, during or shortly after the experimental manipulation (Figure 6). Tree swallow nestlings are unable to thermoregulate on their own until they are approximately 10 days old (Dunn, 1979); thus, reduced parental brooding to avoid predation may have caused nestling mortality. In addition to the immediate effects of predation stress on nestling survival, we observed secondary effects on nestling size. At 6 days of age (shortly after predation events), nestlings in the predation group were significantly smaller in mass than those in the control group (Figure 3). By 12 days of age there was no difference in mass between treatments, suggesting that nestlings may have been able to accelerate growth and recover from a temporary reduction in parental care. However, wing length at 12 days of age was still shorter in the predation group, indicating that not all aspects of development recovered. Previous studies that have manipulated the growth rate of nestlings have found that an acceleration in growth rate during development can have long-term metabolic consequences for adults (Alonso-Alvarez et al., 2007; Criscuolo et al., 2008). Thus, even if swallows only temporarily reduce parental care in response to predators in the environment, the effects on the growth and physiology of their offspring may be lasting. Measures of size (mass and wing length) in our population are correlated with post-fledging survival (McCarty, 2001), therefore we expect that the negative effect of simulated predation on nestling size could ultimately result in negative fitness consequences even for individuals that successfully fledge.

In 2019, we reversed the order of the signal and predation manipulations and simulated predation before nestlings hatched. Previous studies have found that reduced incubation by females in response to predation or environmental changes can affect nestlings' subsequent condition and immune function (Ardia et al., 2010; Basso & Richner, 2015). Developing birds can directly also perceive their environment and the threat of predation even before they hatch (Kostoglou et al., 2021; Noguera & Velando, 2019a; Warkentin, 2011). Thus, we predicted that an increased risk of predation during incubation could have negative effects on tree swallow reproductive success, either due to changes in female incubation and/or effects on the embryos themselves. However, we found that simulated predation during incubation had no significant effect on subsequent nestling conditions or survival. We investigated whether increased perceived predation risk could have caused increased nest abandonment, which may be high during incubation, when parental investment in reproduction is still relatively low (LaManna & Martin, 2016). However, there was no significant difference in hatching success between predator and control groups. Thus, our data indicate that breeding tree swallows are more resilient to simulated predation stress during the incubation period compared with the nestling period. Behavioral changes in females during the incubation period may be less consequential than similar changes during the first days of the nestling period because of the heightened care that young nestlings require (both provisioning and brooding).

We investigated the physiological mechanisms linking simulated predation to swallow fledging success. Direct contact with a predator can prompt a rise in nestling corticosterone (Herborn et al., 2014). The presence of predators may also indirectly provoke a hormonal response in nestlings through changes in parental care, e.g., reductions in provisioning or brooding (Crino et al., 2020; DuRant et al., 2010; Rensel et al., 2010; van Oers et al., 2015). Although we expected that the effect would be most evident in nestlings, developing embryos also have physiological responses to predator risk. For example, yellow-legged gull eggs exposed to increased alarm calls hatched nestlings with elevated corticosterone levels and shorter telomeres (Noguera & Velando, 2019b). Despite our predictions, we found no difference in corticosterone levels between predation and control groups in either year. There are several potential explanations for the lack of effect: First, the treatment may have only induced an acute response during the "predation" event or treatment period, and the response may have subsided by the time nestlings were sampled. Second, the HPA axis develops over the nestling period in passerines, and stressors early in development may not induce a detectible glucocorticoid response (Wada et al., 2009). Finally, corticosterone is just one of a number of hormones involved in the physiological response to a stressor, and corticosterone has physiological roles beyond the stress response (Bonier et al., 2009; MacDougall-Shackleton et al., 2019). Indeed, the glucocorticoid response is not always predictable; other studies have similarly found no effect or unexpected effects of early-life stressors on glucocorticoids in nestling birds (Ibáñez-Álamo et al., 2011; Wada et al., 2015). Correspondingly, there are increasing calls for a broader approach to characterizing the stress phenotype, including measures of oxidative stress, telomeres and other physiological traits (Bateson, 2016; MacDougall-Shackleton et al., 2019; Whitham et al., 2020).

In contrast with corticosterone, we found that simulated predation negatively affected the telomere length of nestlings when predation events occurred early in the nestling period. In the first year of the study, nestlings in the predation treatment had shorter telomeres relative to controls (Figure 5). Although telomere lengths were not associated with corticosterone concentrations, our results are consistent with previous studies showing that stressful conditions shorten telomeres, potentially through the negative effects of oxidative stress, and/or inflammation (Haussmann & Heidinger, 2015; Monaghan, 2014). Nestlings were not the direct targets of the simulated predation events; however, the stressor still had lasting negative effects on their skeletal size and telomere lengths. An earlier study at our field site found that telomere length in 1-year-old tree swallows predicts survival over the next 3 years (Haussmann et al., 2005). Thus, the indirect effects of predation stress on nestling tree swallows have the potential to affect long-term survival, even for those birds that did fledge successfully.

We observed different effects of simulated predation stress in the 2 years of our study. While the most striking difference between our two field seasons was the timing of the experimental manipulations (pre- or post-hatching), it is possible that other factors may have contributed to the contrasting results. Tree swallow reproductive success is closely tied to environmental conditions and adverse weather events such as cold snaps reduce food availability and fledging success (Shipley et al., 2020). The average daily temperature during the breeding season (May to July) was similar each year (2018 = 18.1°C; 2019 17.2°C; Northeast Regional Climate Center). However, 2019 had more "cold snap" days than 2018 (i.e., days where the maximum temperature was <18.5°C; 2018 = 10; 2019 = 18). It is possible that colder days early in the breeding season in 2019 may have reduced the reproductive success of all females and reduced the effect of the predation treatment. However, there was no significant difference in overall reproductive success between years, so it is not clear how much environmental variation between years affected our results. Additional replication of this experiment would be necessary to confirm that predation risk has different effects in the prenatal versus post-natal period.

Despite the differences between experimental years, our results emphasize the importance of predation in shaping wild birds' fitness. Our experiment showed that a heightened risk of predation—even in the absence of any direct consumption—can have significant negative effects on the reproductive success of wild tree swallows. These results add to a growing body of literature showing that the fear of predators can have population-level consequences (Allen et al., 2022; Clinchy et al., 2013; Zanette et al., 2011). A recent study that manipulated the threat of predation by broadcasting predator vocalizations found that song sparrows (*Melospiza melodia*) in the high-predation group produced 53% fewer recruits in the subsequent season compared with controls (Allen et al., 2022). An analysis of this effect on population growth rates suggested that a heightened risk of predation could reduce population size by 50% over 5 years (Allen et al., 2022). In our study in 2018, parents in the predation treatment had a 58% reduction in fledging success compared with parents in the control group. Although we did not measure recruitment (the number of nestlings that returned to the breeding population a year after the experiment), such as Allen et al. (2022), our results suggest that fear of predation could be a factor capable of driving population trends in tree swallows. However, we caution that the simulated predation events we applied were not calibrated to real rates of encounters with mink. Future work is needed to quantify rates of predation and predation attempts in different populations to understand the relative importance of predation risk in regulating bird populations.

The effects of the dulling treatment on tree swallow reproductive success were minor and did not affect the response to predation stress. Like the predation manipulation, plumage dulling had a stronger effect during the nestling period compared with the incubation period. In the second year of the study (2019), we found a significant effect of plumage dulling; however, the effect depended on initial female brightness. Earlier studies in our population and other populations of tree swallows found that females with brighter breast plumage are more resilient to environmental challenges and have higher reproductive success (Beck et al., 2015; Taff et al., 2019). However, manipulation of this social signal sometimes leads to unexpected results. A previous study that experimentally dulled female plumage, as we did here, found that dulled females invested more in reproduction and had higher reproductive success (Taff et al., 2021). Manipulation of the breast plumage has the potential to create a "mismatch" between the social signal and the true quality of a female. Brighter females receive more aggressive interactions from conspecifics, and therefore may be forced to defend their territories more often (Coady & Dawson, 2013). In our study, we saw a positive relationship between initial brightness and nestling size in the dulled females (Appendix S1: Figure S1). For females who were initially bright, dulling had a positive effect on the size of their nestlings. It is possible that bright females experienced the advantages of bright plumage earlier in the breeding season while they were securing territories and mates but avoided negative conspecific interactions after they were dulled during the nestling period. Conversely, for females that were initially duller than average, experimental dulling had a negative effect on the size of their nestlings on day 12 (Appendix S1: Figure S2). Experimental dulling of the lowest-quality females may have exacerbated any negative social position they had, further reducing their reproductive success.

Contrary to our initial predictions, plumage manipulation did not mediate resilience to predator stress. Interaction with the predator manipulation could have led to a "canceling-out" effect of the dulling treatment. For instance, brighter females may have naturally been of higher quality that increased their resilience to predation stress. However, experimentally dulled females may have been able to avoid aggressive interactions with other tree swallows, allowing them to invest more in reproduction and maintain fitness even under heightened predation stress. However, this theory does not explain why we saw no differences between dulling and control treatments in the absence of predation stress. Our sample sizes were slightly smaller than in Taff et al., 2021 ($N = 34$–36 per group in the previous study, $N = 20$–34 here) and so it is possible we did not have the power to detect small effects of the dulling treatment. Finally, it is also likely that the effects of the social environment are context-dependent and vary over the years with environmental conditions, population density, and age/breeding conditions of the females. The social environment of species such as tree swallows likely interacts with HPA axis function and has the potential to mediate resilience to environmental stressors (Creel et al., 2013). However, isolating and manipulating these complex processes is difficult in wild populations and requires future investigation in our system.

CONCLUSIONS

We found that the perceived risk of predation alters tree swallow reproductive success. Simulated predation events early in the nestling period resulted in increased nestling mortality, reduced size, and shorter telomeres. The effects of heightened perceived predation risk on nestling telomeres are especially notable because telomere length is linked to overall lifespan. Thus, our results add to a growing body of evidence demonstrating that transient, early-life stressors may have lasting effects, and that telomeres may link early-life conditions to later health and survival. Although previous studies have shown that the social environment may mediate how animals respond to stressors, here we did not find an interaction between our manipulation of a key social signal and the response of swallows to simulated predation. Birds live in dynamic environments involving challenges from intra- and interspecific interactions. More work is needed in avian systems to understand the role of the social environment in a complex and changing world.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data and code (McNew, 2024) are available in Zenodo at https://doi.org/10.5281/zenodo.10815579.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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