A TEST OF WHETHER HUMAN HANDLING CAUSES DEVELOPMENTAL OR HABITUATION EFFECTS ON THE HYPOTHALAMIC-PITUITARY-ADRENAL STRESS RESPONSE OF YOUNG AMERICAN KESTRELS (*FALCO SPARVERIUS*)

By

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The following individuals read and discussed the thesis submitted by student Erin Leigh Wonder, and they evaluated her presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

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DEDICATION

To my family

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ABSTRACT

Early exposure to stressors affects subsequent stress responses in both mammalian and avian species, with the likelihood for lasting effects depending, in part, on the magnitude of the stressor. However, it is unclear whether lasting effects are the result of developmental changes to the hypothalamic-pituitary-adrenal (HPA) axis or habituation to the stressor. I investigated the effects of human handling, a known stressor, in free-living American kestrel (*Falco sparverius*) nestlings to determine if this brief, non-invasive stressor causes lasting changes in the stress response of handled birds, and whether alterations in the stress response were the result of developmental changes (early-handled birds) or habituation (later-handled birds). Broods were randomly assigned to one of three treatment groups: 1) early-handled nestlings were held daily for 15 min for 7 days following hatching, 2) late-handled nestlings were held for the same time and duration beginning on the 18th day post-hatching, and 3) control birds were not handled before sampling for circulating corticosterone (CORT). When nestlings were 25 or 26 days old, they were exposed to either a handling restraint protocol (similar to the treatment of handling) or a novel noise stressor. The stress test not applied on the first day was conducted the next day. During the hour-long restraint protocol, I collected five 75 ul samples of blood through venipuncture of the alar vein. HPA response to a novel stressor was measured by sampling CORT immediately after exposing birds to 10 min of 96 dB(A) (±1 dB) white noise. I analyzed serum CORT levels using enzyme-linked immunoabsorbent assays (ELISA). There was no effect of handling treatment on CORT patterns sampled over the 1 hr restraint test ($P = 0.092$), though *a priori* contrasts showed a significant difference between CORT levels of late-handled and control birds at 60 min ($P = 0.01$). Late-handled birds had lower CORT compared to control birds. In addition, there was no significant differences among handling treatments on noise-induced CORT ($P = 0.94$). Taken together, these results suggest that the HPA axis was unaffected by human handling and that the changes in CORT at 60 min in late-handled birds is likely the result of habituation to human handling rather than to developmental changes. Further, results indicate that mild and brief stress associated with human handling is unlikely to have negative lasting effects on the stress physiology of young birds.

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Introduction

The vertebrate stress response, in which an organism reacts hormonally to a stressful event, is an essential component of survival. By releasing glucocorticoids (GCs), a class of steroid hormones, the hypothalamic-pituitary-adrenal (HPA) axis begins a cascade of events that initiates the stress response and later ends it via a negative feedback loop. The HPA response to stress is highly plastic and may be modulated by such factors as maternal care or reproductive status (Macri et al. 2008, Harris and Saltzman 2013). In addition, prior experiences with stressful stimuli, particularly stressors encountered during the postnatal developmental stage (Love and Williams 2008), affects both the magnitude and duration of the stress response.

Studies of both mammals and birds show that early postnatal stress leads to lasting changes in HPA responsiveness, with a wide variety of experimental stressors producing measurable changes in glucocorticoid levels. Rat (*Rattus norvegicus*) pups separated from their mothers for varying durations of time show increased stress-induced cortisol levels as adults (Biagini et al. 1998, Aisa et al. 2007), with longer periods of separation resulting in higher cortisol levels than shorter periods (Ladd et al. 2004). In addition, adolescent rats that experience stress in the form of social isolation or forced interaction with a novel individual also have increased cortisol levels several weeks later

(McCormick et al. 2006); this indicates that psychological stress alone is capable of inducing changes in the HPA response.

The results of similar studies focused on birds had variable results. Mountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*) nestlings that hatch near roads, a putative source of stress, have increased basal and stress-induced corticosterone (CORT) levels (Crino et al. 2011). Conversely, Whitman et al. (2011) found that handling American kestrel nestlings daily from hatching through fledging results in lower stress-induced CORT levels. Two-week-old Japanese quail (*Coturnix japonica*) exposed to various unpredictable stressors for one week experience no alteration in CORT levels compared to control birds (Calandreau et al. 2011). The lack of consistency in the type, magnitude, and timing of the stressors used in these studies has made it difficult to draw general conclusions regarding the effects of early postnatal stress on avian species.

Nearly all of the species used in both mammalian and avian studies on postnatal stress are altricial and experience most neural growth and organization after birth or hatching, making the early postnatal days a developmentally sensitive period. Both rats (Sapolsky and Meaney 1986) and American kestrels (Sockman and Schwabl 2001) show little to no HPA responsiveness in the days immediately following birth and hatching, respectively. Sapolsky and Meaney (1986) termed this the “stress non-responsive period” and hypothesized that it functions to protect the neonate from the catabolic action of glucocorticoids during a sensitive growth period. Sockman and Schwabl (2001) found that while 5-day-old American kestrel nestlings do not experience significant CORT increases in response to gentle handling, 10- and 15-day-old nestlings do experience this increase. This suggests that 5-day-old nestlings are still undergoing HPA development and are therefore unable to respond to stressors to the same extent as more well-developed 10-day-old nestlings. Other altricial avian species also have CORT levels that increase with age throughout the nestling period and into adulthood (Schwabl 1999, Sims and Holberton 2000). However, rat pups that experience maternal separation during this hypo-responsive period display elevated cortisol levels later in life, indicating the possibility that lasting alterations can be made to the HPA response even without significant increases in glucocorticoids during the critical developmental stage.

Many field studies on birds involve investigators handling nestlings to obtain morphometric data, apply bands, or conduct experimental treatments despite the uncertain long-term consequences of stress during development. Although nestlings can experience stress-induced increase in CORT levels simply as the result of being held (Sockman and Schwabl 2001), little is known about the extent to which human contact may affect the development of the HPA axis and subsequent stress responses in birds. In a study by Love et al. (2003), American kestrel nestlings held in a box for 1 hr and removed periodically for blood sampling have progressively lower CORT levels upon repetition of the stressor. Love et al. (2003) attributed this effect to habituation to the handling and bleeding experience, with each exposure to the stressor eliciting a lower CORT response. However, this handling protocol is likely to be unrepresentative of the more brief and less invasive handling that nestlings may experience as part of a typical field study that includes just measuring and banding nestlings at a nest. Whitman et al. (2011) applied a shorter, non-invasive handling stressor by holding American kestrel nestlings for 15 min once a day during the entirety of the nestling period and found that handling caused a depressed CORT response to a 1-hr period of restraint. However, in contrast to Love et al.'s conclusion, Whitman et al. attributed this decrease in HPA responsiveness to changes in the HPA axis brought on by increased CORT during development.

This distinction—habituation versus developmental change—is critical for understanding the effect of early exposure to stress on birds. If nestlings are prone to stress-induced developmental changes in the HPA axis, then care should be taken not to disrupt them during their periods of peak sensitivity because long-term effects of altered HPA responsiveness are not well-understood. However, if the effect of early handling is habituation to a specific stressor, then there may be no long-term negative effects of non-invasive handling of nestlings.

My objective was to determine if brief, non-invasive handling causes lasting changes in the stress response of birds, and whether alterations in the stress response were the result of developmental changes (early handled birds) or habituation (later-handled birds). I predicted that, if long-term changes to the HPA response are caused by developmental effects, then repeatedly handling birds in the days immediately after hatching would result in altered stress-induced CORT levels, regardless of whether the subsequently encountered stressor was familiar or novel. In contrast, I predicted that, if habituation caused lasting changes to the HPA response, then nestlings handled during the days immediately prior to stress tests would have altered stress induced CORT levels, compared to controls, after human handling, but that there would be no difference between treatment groups to a novel stressor.

Methods

The Boise State University Institutional Animal Care and Use Committee approved all methods used in this study under protocol 006-AC11-07.

I conducted my study on the effects of early human handling on stress responses of free-living American kestrel nestlings in nest boxes located in southwestern Idaho within Ada, Canyon, and Camas Counties in 2011 and 2012. Previously installed wooden nest boxes were located on utility poles (with permission from Idaho Power) along secondary roads and were attached at a height of 2-3 m.

Beginning in March of each year, nest boxes were monitored for the presence of breeding adult kestrel pairs and evidence of clutch initiation. When one or more eggs were discovered in a nest box, the date that the first egg was laid was estimated based on the assumption that kestrels lay one egg every other day. The nest box was then left undisturbed until 2-3 days prior to the predicted hatching date (after approximately 30 days of incubation, which commences with the laying of the penultimate egg) and checked every other day until eggs successfully hatched. Once hatched, I randomly assigned each brood to one of three groups: early handling, late handling, or no handling (control group).

Broods assigned to the early handling were handled every day for 7 d after hatching; as American kestrel eggs typically hatch within 1-4 days of each other, this resulted in the handling of each nestling beginning when they were 0-2 days old. After the nest box entrance was blocked with a PVC pipe plugged and wrapped in foam, the adult female (if present) was removed and the nestlings were placed in a small basket; after all of the nestlings were collected, the adult was placed back in the nest box for the duration of the handling. The nestlings were then held in hand for 15 min before being returned to the nest box. Nestlings assigned to the late handling group were handled from approximately 18 to 24 days of age. Nestlings were removed from the nest box and held in hand for 15 min before being returned to the nest box. Broods assigned to the control group were not disturbed after eggs in these nests hatched.

Just prior to fledging, when the nestlings were 25 or 26 days old, a pair of stress tests was applied to nestlings from each brood. A capture and handling restraint test was conducted to assess baseline CORT levels and stress-induced CORT levels, and the second test assessed CORT levels after exposure to a novel auditory stressor, as follows. From each brood, two nestlings were randomly chosen for CORT sampling: one bird following the restraint test and one bird following the noise stressor. If only one nestling was present in the nest at the time of testing, or if the second nestling fledged before the second day of testing, then only one nestling was tested at that box. One test was conducted each day, for 2 consecutive days, and the order of the tests was randomly assigned for each nest.

For the handling restraint stress test, a nestling was removed from the nest box and a baseline blood sample (time point 0) was collected within 3 min of opening the box. Additional blood samples were collected 5, 15, 30, and 60 min after the nestling was removed from the box, with the nestling kept in a closed cloth bag between time points. Blood sample collection occurred between 0800 and 1500 hours. Blood samples were collected through venipuncture of the alar vein with a 26 gauge needle. Approximately 75 ul of whole blood was collected into micro-capillary tubes and stored on ice until returned to the laboratory. No samples were kept on ice longer than 4 h. In the laboratory, samples were immediately centrifuged, and plasma was removed and stored in micro-centrifuge tubes at -80° C until assayed. After the 60-min blood sample was collected, morphological measurements (weight, wing and tail length) were taken and the bird was banded with a single USGS aluminum leg band (size 3B) and returned to the nest.

For the noise stress test, a speaker was placed against the circular hole (7.6 cm diameter) on the front of the nest box. As soon as the speaker was placed, a continuous sample of white noise (SimplyNoise's Signature White Noise) was played at 96.2 dB(A). In this experiment, the white noise acted as a novel stressor to which the nestlings had no prior exposure. After 10 min of noise, the speaker was turned off, a nestling was immediately removed from the box, and a blood sample was taken within 3 min of removal using the same technique as described above. White noise is a sound that contains many frequencies played at equal intensity; at 100 dB, it acts as a stressor on avian species (Campo et al. 2005, Chloupek et al. 2009). The decibel level was measured using a Larson-Davis System 824 sound level meter with an integration time of 3 min and was verified every 3 weeks. The decibel meter was placed at the bottom of the nest box interior, 20 cm below the speaker, in the approximate position occupied by nestlings.

Nestling mass was determined to the nearest gram using a mechanical spring scale and the bird was banded with a USGS aluminum leg band prior to being returned to the nest box. Mass was standardized by sex by dividing each nestling's mass by the average mass of nestlings of the same sex. Nestling sex was determined via plumage differences (Pyle 2008).

CORT Analysis

Total CORT concentrations were measured using enzyme-linked immunoabsorbent assays (ELISA, Caymen Chemicals; Ann Arbor, MI, USA). Samples were run in duplicate. CORT was twice extracted from 10-50 ul of thawed plasma with 5 ml of diethyl ether. The lipophilic supernatant was poured off and placed under a gentle stream of nitrogen gas in a warm water bath to dry. Extracted samples were reconstituted with 100 ul of EIA buffer and vortexed; 50-ul aliquots were then added to 96-well plates coated with mouse monoclonal antibody. Corticosterone-specific acetylcholinesterase tracer and rabbit corticosterone antiserum were then added and plates were placed on an orbital shaker for 2 h. Plates were developed and read at 405 nm with a Biotek EL800 plate reader. CORT concentrations were calculated by comparing results to a standard curve and adjusting for extraction efficiency and plasma sample size. Extraction efficiency was determined by analyzing a standard CORT sample and inter-assay variation was calculated from repeated values of a pooled sample. All values were corrected for assay extraction efficiency (mean ± SEM) 81.4 ± 8.3%. Inter-assay variation averaged 5.0% and average intra-assay variation was 3.7%.

Statistical Analysis

CORT concentrations from the restraint stress test were evaluated using a linear mixed model with Treatment Group, Time, and an interaction between Treatment Group and Time as fixed effects. Year, sex, and standardized mass score were used as covariates, and individual bird was fit as a random effect. *A priori* linear contrasts were conducted on CORT levels collected at 0 (baseline), 30 (maximum), and 60 min because previous studies show these to be significantly affected by stressors, and they represent biologically significant minimum, maximum, and recovery CORT levels (Crino et al. 2011, Whitman et al. 2011). Results from the noise stress test were evaluated by ANOVA with Treatment Group as the independent variable, CORT levels as the response variable, and year, sex, and standardized mass score as covariates. Prior to analysis, the data were examined for normality, variance, and sphericity and there were no departures from assumptions. Analyses were performed in R (R Development Core Team 2012). Results are reported as mean ± standard deviation, and I considered differences significant when $p < 0.05$.

Results

I measured the stress response of American kestrel nestlings from 27 different broods (2011: n = 13; 2012: n = 14). Ten broods were considered controls, eight broods were handled early, and nine broods were handled late. Brood sizes ranged from two to six nestlings. At the time when I collected blood from each, female nestlings weighed 131.9 ± 9.3 g (n = 27) and males weighed 125.09 ± 6.19 g (n = 11).

The results of the restraint test showed no significant interaction between time and treatment group, CORT increased significantly over time for all treatment groups (Table 1, Fig. 1), and there were no significant effects of covariates on CORT (Table 1). *A priori* contrasts of baseline and maximum (30 min) showed no significant differences between treatment groups, but at 60 min the late-handled group had significantly lower CORT compared to control birds (Table 2).

Handling treatment had no significant effect on CORT levels of American kestrel nestlings exposed to a noise stressor ($F_{2,15} = 0.049$, $P = 0.952$, Fig. 2), and there were no significant effects of any covariates on CORT (Table 3). Overall, CORT concentrations sampled at the end of 10 min of noise exposure was 810.6 ± 286.6 ul (n = 21).

Discussion

The overall pattern of hormonal response did not differ between early-handled, late-handled, and control birds, although late-handled birds did show lowered CORT levels 60 min into the restraint protocol when compared to control birds. There was no difference in CORT levels between any handling groups after exposure to a novel stressor. These results indicate that altered hormonal responses were likely the result of habituation to handling and not long-term developmental effects. If handling had persistent effects on the developing HPA axis, then nestlings handled early would experience stress-induced developmental changes that would have resulted in altered CORT levels during both the restraint and auditory stress tests. Conversely, older nestlings, having already undergone the majority of their neural development prior to handling, would experience no developmental changes but would be more likely to show habituation to handling, resulting in normal CORT levels during the auditory stress test but altered levels during the restraint stress test. Kestrels showed no differences in responses to the novel auditory stress in both early- and late-handled birds, indicating no developmental changes in either group. However, handling during the immediate pre-fledging period resulted in a lower 60 min CORT level during the restraint test, indicating that late-handled birds experienced habituation to human handling that affected their response to the stressor.

Although there was no difference between handling groups in the response to a novel stressor, the timing of blood sampling during this test (10 min after the initial exposure to the stressor) may have been inadequate for observing the effects of developmental changes. For example, it may be that early-handled birds do react differently to a novel stressor but that differences in CORT levels do not appear until 30 or 60 min after the stressor is encountered. If this were the case, such differences may have appeared during the restraint stress test. However, the results did not indicate significant differences between early-handled and control birds at any time point.

Results from my study contradict conclusions in Whitman et al. (2011). Whitman et al. (2011) handled kestrel nestlings for 15 min every day throughout the nestling period, from hatching to fledging, and found that handled birds had significantly lower CORT levels than non-handled birds at 15, 30, and 60 min into the restraint stress test. This effect was interpreted as a result of developmental changes to the HPA axis induced by the daily handling of the nestlings. Whitman et al. (2011) applied a longer handling stressor to the nestlings (25 days), and it is possible that the accumulated time spent experiencing handling stress throughout the nestling stage had a stronger developmental effect than the 7 days of handling in my study. Alternatively, birds may have become habituated to handling during the 25 days of handling that Whitman et al. (2011) applied.

My study suggests that brief early handling during the nestling stage did not cause persistent developmental changes but did result in habituation to human handling. However, several studies have demonstrated that nestlings exposed to other types of stressors experience changes in CORT levels that cannot be attributed to habituation. Nutritional stress during the nestling stage, for example, increases stress-induced CORT levels in red-legged kittiwakes (*Rissa brevirostris*) (Kitaysky et al. 2001). Additionally, the noise and disturbance associated with nests built near busy roads raises basal and stress-induced CORT levels in white-crowned sparrows (Crino et al. 2011). Moreover, exogenous CORT applications to nestling birds of a variety of species alters behavior (Spencer and Verhulst 2007, Wada and Breuner 2008), lowers immune function (Butler et al. 2010), reduces growth (Spencer and Verhulst 2007, Wada and Breuner 2008, Müller et al., 2009), increases stress-induced CORT levels later in life (Spencer et al. 2009), and even reduces lifespan (Monaghan et al. 2011). The circulating CORT levels obtained through exogenous application are likely much higher than the levels induced through the handling that nestlings experienced in my study, but more invasive or prolonged handling of nestlings could conceivably result in long-term results similar to those seen in exogenous CORT studies.

My results from American kestrels show that very young nestlings may be handled without significant or long-term alterations to their stress responsiveness as long as the experience is brief and non-invasive. Ideally, handling protocols should be designed with this in mind. However, studies examining the CORT levels of both nestling and adult birds should be mindful of the potential for habituation as even brief prior handling may affect a bird’s responsiveness to a similar stressor.

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APPENDIX

Tables and Figures

Table A.1 A comparison between different handling treatments on restraint-induced CORT levels

All terms were non-significant at an $\alpha = 0.05$.

Fixed Variable	χ^2	**df**	***P***
Handling Condition	1.012	2	0.603
Time	188.708	4	<0.001
Sex	0.805	1	0.370
Year	2.207	1	0.137
Mass Score	0.001	1	0.992
Handling Condition*Time	13.635	8	0.092

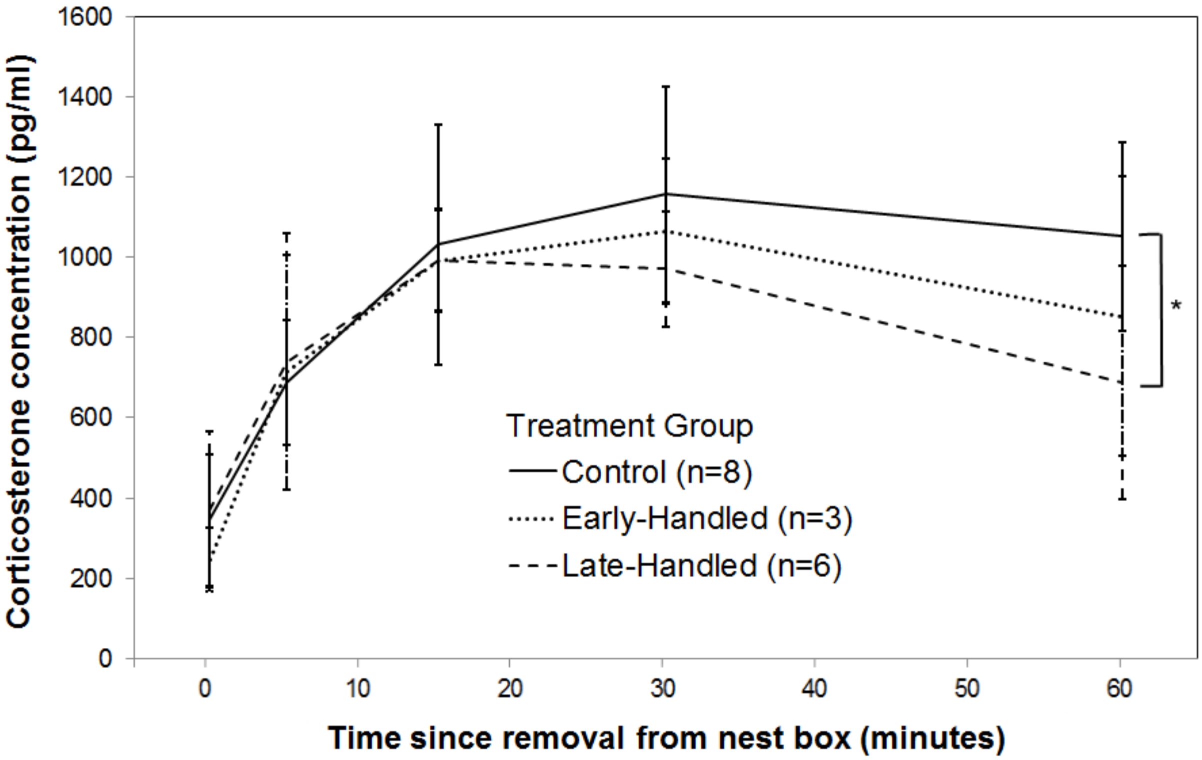

Figure A.1 A comparison between different handling treatments on restraint-induced CORT levels. Symbols represent mean plasma corticosterone (± SD). CORT increased significantly over time, and then began to decline in the 60 min sampling period. There was no significant difference in overall CORT patterns between treatment groups, but late-handled birds had significantly lowered 60 min samples compared to control birds.

Table A.2 Contrasts of restraint-induced CORT levels of handling treatment groups at 0, 30, and 60 minutes.

Time Point	**Contrast**	***T***	***P***
0 min	Control vs. Early	0.393	0.697
	Early vs. Late	-0.520	0.607
	Control vs. Late	-0.177	0.861
30 min	Control vs. Early	0.349	0.729
	Early vs. Late	0.759	0.454
	Control vs. Late	1.391	0.175
60 min	Control vs. Early	1.005	0.323
	Early vs. Late	1.172	0.251
	Control vs. Late	2.719	0.011

Table A.3 A comparison between different handling treatments on noise-induced CORT levels. All terms were not significant α > 0.05.

Fixed Variable	**df**	***F***	***P***
Handling Condition	2	0.049	0.952
Year	1	2.676	0.123
Sex	1	0.184	0.674
Mass Score	1	0.464	0.506

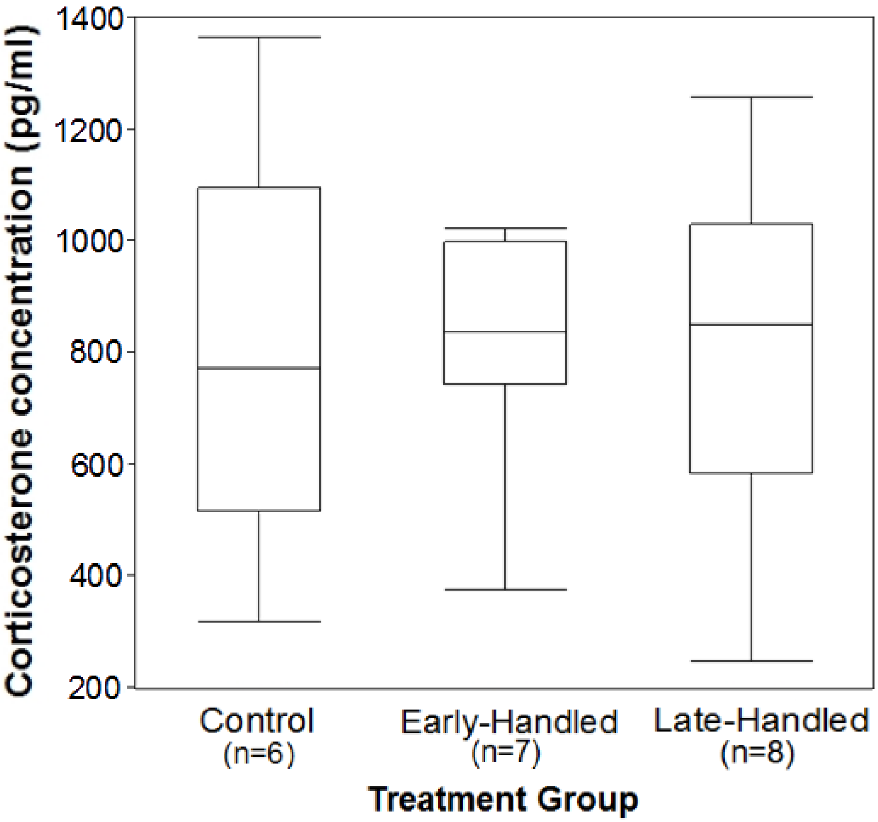

Figure A.2 A comparison between different handling treatments on noise-induced CORT levels. There was no significant difference in CORT levels between the groups (see text). Plots show the median, interquartile range, and maximum and minimum values for each handling group.