Biological validation of fecal corticosterone metabolites as a non-invasive stress assessment in translocated California valley quail (*Callipepla californica*)

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Lay summary

This study biologically validates the use of fecal corticosterone metabolites as a non-invasive method for detecting stress in quail, demonstrates diurnal variation in quail stress levels, confirms that translocations elevate stress which likely impacts success, and establishes a new non-invasive tool for stress measurement in quail research, management, and conservation.

Abstract

U.S. quail species are vulnerable to population declines as a result climate change, habitat loss, and habitat fragmentation; all of which can induce physiological stress. Additionally, population restoration techniques (PRTs), like translocations, also induce stress. Traditional stress assessments include capturing and handling birds to extract blood, methods which are inherently stressful and can compound stress analyses. However, the stress hormone corticosterone is metabolized from the blood and excreted in feces as fecal corticosterone metabolites (FCMs). FCMs have been used as a non-invasive measurement of stress in a variety of species, but must be validated for each species. The objective of this study was to biologically validate the use of FCMs as a non-invasive measurement of stress-hormone levels in California valley quail (*Callipepla californica*). Reference and treatment quail were acclimated for 3 wks in an outdoor aviary. Subsequently, treatment quail were subjected to a simulated, 48-h translocation, a common and stressful PRT. Fecal samples were collected every 4 h and processed using an enzyme immunoassay. Mean FCM concentrations of treatment quail (41.50 ±16.13 ng/g) were higher than reference FCM concentrations (24.07 ±10.4 ng/g). These results biologically validate the use of FCMs as a non-invasive method to assess stress hormone levels in California valley quail, demonstrate diurnal variation in quail stress levels, and confirms that quail translocations are a stressful PRT. Ultimately, this research validates a new non-invasive tool for stress measurement to advance quail research, management, and conservation.

Keywords: biological validation, California valley quail, conservation, corticosterone, fecal corticosterone metabolites, physiology, population restoration, stress hormones, translocation

Introduction

Stress is a factor often overlooked in U.S. quail population declines and population restoration techniques. U.S. quail populations are vulnerable to decline as a result of climate change (Guthery *et al.*, 2000; Tanner *et al.*, 2017; Wilsey *et al.*, 2019), habitat loss, and habitat fragmentation (Brennan and Covington, 1994; Hernández *et al.*, 2013; Pope and Heekin, 2017); all of which are stressful to quail as indicated by increased levels of blood corticosterone, (CORT, 17-deoxycortisol), a steroid hormone produced in the cortex of the adrenal gland (Creel, 2001; Suorsa *et al.*, 2003; Tempel and Gutiérrez, 2004; Cockrem, 2007, 2013; Sheriff *et al.*, 2011; Wingfield *et al.*, 2017; Palme, 2019; van Vliet *et al.*, 2020). For example, elevated CORT levels have been observed in birds as a result of anthropogenic disturbances (Thiel *et al.*, 2011; Jankowski *et al.*, 2014; Arlettaz *et al.*, 2015) and extreme climatic events (Wingfield *et al.*, 2017), which are expected to increase in frequency and severity due to global climate change (Seneviratne *et al.*, 2012; IPCC, 2021). Stress from climate change can have synergistic effects, multiplying the deleterious impacts of other stressors (Felton *et al.*, 2009; Şekercioğlu *et al.*, 2012).

Elevated stress levels can directly influence bird populations by altering foraging, predator avoidance, fledging, and other behaviors (Cyr and Romero, 2007; Crespi *et al.*, 2013; Wingfield *et al.*, 2017). Elevated CORT levels in birds can also lead to increased predator encounters (Stephens *et al.*, 2004), which further increases stress levels (Clinchy *et al.*, 2013). Extreme stress can induce a type 1 allostatic overload, where birds decrease or eliminate behaviors (e.g., reproduction) that are not immediately necessary for survival (McEwen and Wingfield, 2003) by increasing gonadotropin-inhibitory hormone, reducing gonadotropin-

releasing hormone (GnRH), and displaying a reduced sensitivity to GnRH and luteinizing hormone (Sapolsky *et al.*, 2000; Wingfield and Sapolsky, 2003; Dickens and Bentley, 2014); though the mechanisms are not completely understood (Wingfield and Ramenofsky, 2011; Lattin *et al.*, 2016). For example, exposing zebra finches *(Taeniopygia guttata*) to stress stimuli increased CORT levels and decreased testosterone, resulting in reduced reproductive behavior (Lynn *et al.*, 2010). Capturing and holding wild European starlings (*Sturnus vulgaris*) blocked a natural shift in the hypothalamic-pituitary-adrenal (HPA) axis from early breeding (gonadal development) to active breeding activity, which inhibited breeding (Dickens and Bentley, 2014). The resultant reduction in reproduction in birds contributes to population declines.

Population restoration techniques (PRTs) can also increase CORT levels. U.S. quail species are economically important gamebirds (Johnson *et al.*, 2012; Wszola *et al.*, 2020) and part of a $3.7 billion per year upland gamebird hunting industry (Sportsmen's Alliance, 2021). For over 150 years, a variety of PRTs like translocations have been used in attempts to bolster populations (Hernández *et al.*, 2013; Gomez and Reyna, 2017; Whitt *et al.*, 2017). Quail Translocations in general are rarely successful (Perez *et al.*, 2002; Scott *et al.*, 2013; Whitt *et al.*, 2017), especially when used for reintroduction (Martin *et al.*, 2017). Although these PRTs have rarely resulted in long-term success, there is increasing interest in translocation of wild quail (Stephenson *et al.*, 2011; Martin *et al.*, 2017; Sisson *et al.*, 2017). Numerous hypotheses for the low success rate have been proposed (Sokos *et al.*, 2008; Martin *et al.*, 2017), but little is known about how stress influences translocation success.

Translocation consists of capturing, handling, holding, transporting, and releasing quail to a novel site, each of which can result in acute and chronic stress, independent of other processes (Buchanan, 2000; Fazio and Ferlazzo, 2003; Jones *et al.*, 2005; Dickens *et al.*, 2009, 2010;

90 Wingfield and Romero, 2011; Batson *et al.*, 2017; Martin *et al.*, 2017). For example, short
91 translocations may induce acute stress; however, the duration of a quail translocation is typically
92 1-3 days, which increases the occurrence of chronic stress (Dickens *et al.*, 2010).
93 Chronic stress can disrupt the fight or flight response as a result in downregulation of
94 hormonal responses to acute and chronic stressors, (Romero and Butler, 2007; Dickens *et al.*,
95 2009; Martin *et al.*, 2017). For example, European starlings caught in the wild and placed into
96 captivity lost the ability to produce a fight or flight response when presented with a stress
97 stimulus (Dickens and Romero, 2009). In addition, when the fight or flight response was
98 chronically stimulated it negatively impacted cardiovascular health, leading to hypertension due
99 to surplus catecholamine exposure (Rupp, 1999). Both chronic and acute stress responses can
100 inhibit translocated quail survival (Armstrong and Seddon, 2008; Dickens *et al.*, 2010; Breed *et*
101 *al.*, 2019), and lead to failure of the translocation by increasing the susceptibility to predation
102 (Dickens *et al.*, 2010; Martin *et al.*, 2017).
103 Weight loss has been observed when translocating wild birds to novel environments
104 (Rich and Romero, 2005; Dickens *et al.*, 2009; Fischer *et al.*, 2018), and is a symptom of chronic
105 stress. For example, European starlings lost 5–15% of their body weight after being exposed to a
106 14-d regime of multiple stressors during a translocation (Awerman and Romero, 2010).
107 California valley quail (*C. californica*) exhibited a mean weight loss of 14.3% when translocated
108 from Idaho to Texas (Reyna *et al.*, 2020). This is important because body mass can influence
109 survival, and the overall success of translocations. For example, Cirl buntings (*Emberiza cirlus*)
110 with a higher body weight at capture were more likely to survive in their new habitat than those
111 with lower body weights (Fountain *et al.*, 2017). Reducing the amount of stress during a
112 translocation could reduce weight loss and increase post-release survival (Warwick *et al.*, 2006).

113 The traditional method of measuring stress in birds requires capturing, handling, and
114 extracting blood samples; inherently stressful events (Arnold *et al.*, 2008), followed with
115 measuring blood CORT levels. It is assumed that if this process occurs within 3 min of initial
116 handling, CORT levels will be indicative of the physiological condition of the bird prior and will
117 not represent the elevated stress incurred during the procedure (Littin and Cockrem, 2001;
118 Cockrem and Silverin, 2002). However, CORT levels quickly rise during handling and peak
119 within 15–30 min. In addition, handling associated with blood extractions adds to the cumulative
120 stress experienced by the bird, and nearby birds, independent of the extraction.
121 Steroid hormones, like CORT, can also be measured from urine, feces, hair, and feather
122 samples (Bortolotti *et al.*, 2008; Sheriff *et al.*, 2011). One effective, non-invasive, and
123 increasingly popular method to evaluate stress is by measuring fecal corticosterone metabolites
124 (FCMs) (Romero and Remage-Healey, 2000; Tempel and Gutiérrez, 2004; Möstl *et al.*, 2005;
125 Dickens *et al.*, 2009; Sheriff *et al.*, 2010). Measuring FCMs as an assessment of stress has been
126 successful in a wide range of avian studies (Washburn *et al.*, 2003; Fletcher *et al.*, 2018; Sokół
127 and Koziatek-Sadłowska, 2020). Excretion of FCMs varies between species, as does the
128 suitability of different immunoassays to accurately detect CORT, therefore methods must be
129 validated for each species (Palme *et al.*, 1996; Wasser *et al.*, 2000; Palme, 2019). Physiological
130 validation of measuring FCM concentrations requires injecting a known quantity of CORT into
131 an animal (Touma and Palme, 2005), an invasive and stressful procedure (Palme, 2019;
132 Mohlman *et al.*, 2020). Biological validation of measuring FCM concentrations is a non-invasive
133 procedure where feces is collected before and after an event that increases HPA activity for an
134 extended time, resulting in increased CORT (Touma and Palme, 2005; Palme, 2019).

135 The goal of this study was to biologically validate the use of FCMs as a non-invasive
136 measurement of stress in California valley quail using a simulated translocation as the stressor.
137 This is the first biological validation of FCMs as a tool to evaluate stress in a new world quail
138 species. Mohlman et al. (2020) performed a physiological validation of FCMs with northern
139 bobwhite (*Colinus virginianus*), a close relative, but were unsuccessful at biological validation.

140 Materials and methods

141 Flight-ready captive-reared valley quail (*N* = 5 females, *N* = 8 males), 16–24 wks in age, were
142 acquired from a breeder (ZKD Game Birds, West Point, Texas, USA) in October, 2020. Quail
143 were housed and acclimated in an outdoor aviary (Quail Hotel, Fannin Fabrication, Bonham, TX,
144 USA) for 3 wks. Birds were provided with gamebird feed (Gamebird Starter and Grower, M-G
145 Inc., Weimar, TX, USA) and water *ad libitum,* and enrichments including social and
146 environmental stimuli (Hawkins et al. 2001). This mimicked the time and conditions in which
147 quail were held prior to the first shipment of trapped birds during a 2019 translocation (Reyna *et*
148 *al.*, 2020). Acclimation was assumed when birds exhibited typical behavior (e.g., eating,
149 drinking, roosting, grooming; Reyna and Newman, 2018).
150 To biologically validate FCMs in valley quail, reference (low stress) fecal samples were
151 obtained from acclimated valley quail. The floor of the aviary was lined with a plastic sheet and
152 fecal samples were collected daily. Once collected, individual fecal samples were placed into a
153 plastic storage bag (Ziploc freezer quart, S. C. Johnson & Son, Inc., Racine, WI) and labeled
154 with the date, time, and sex of the bird. Temperature, humidity, and presence of direct sunlight
155 were recorded on the plastic storage bag, since these factors can degrade hormone detection
156 probability (Shipley *et al.*, 2019). Immediately after collection and labeling, samples were stored

157 in a freezer at -20 °C until FCM analysis (Wasser *et al.*, 1988; Messmann *et al.*, 1999; Khan *et*
158 *al.*, 2002).

159 For biological validation, quail were transported to the animal care facility at Texas
160 A&M University-Commerce to undergo a simulated 48-h translocation, a stressful event (Martin
161 et al. 2017). Each quail was given a unique leg band, and had their age, sex, and weight recorded.
162 Quail were held individually in research-approved breeding pens located in a temperature and
163 light-controlled room with a 12L:12D photoperiod. To ensure accuracy and reduce
164 contamination, fecal samples were collected from the underlying waste pan trays 4–5 times daily
165 (Millspaugh and Washburn, 2004; Mohlman *et al.*, 2020). The frequency of feces collection
166 ensured a representative sample, since FCMs may fluctuate diurnally (Breuner *et al.*, 1999).
167 Individual fecal samples were placed in a plastic storage bag and labeled with the bird ID
168 number, sex, time, and date of collection. Steroid hormone concentrations can be affected by
169 storage duration (Millspaugh and Washburn, 2004) and bacteria (Lexen *et al.*, 2008). Therefore,
170 high stress fecal samples were immediately stored in a freezer (-20 °C) until FCM analysis
171 (Wasser *et al.*, 1988; Messmann *et al.*, 1999).

172 FCM concentrations of reference and stressed quail fecal samples were measured using
173 an Enzyme Immunoassay (EIA) Kit (Arbor Assays, Ann Arbor, MI, USA). The kit was validated
174 for use on CORT extracted from dry fecal extracts on a multi-species design (Jackson, 2015;
175 Fletcher *et al.*, 2018), and included detailed instructions for extracting CORT from fecal samples
176 and analyzing FCM concentrations. To determine the assays suitability for valley quail, 10 fecal
177 extracts were pooled from the valley quail simulated translocation and a serial dilution ($N = 6$
178 dilutions) was performed. FCM concentrations from the pooled fecal extracts were parallel to the
179 slope of the standard curve, verifying suitability for valley quail. The sensitivity of the EIA was

180 determined to be 18.19 pg/ml. Optical density was calculated using a Synergy LX Multi-mode
181 microplate reader (Bio Tek Instruments, USA), and entered into MyAssay web
182 (https://www.myassay.com), to calculate FCM concentration in pg/ml. FCM concentrations
183 reported in pg/ml were converted to ng/g for comparison to other studies.

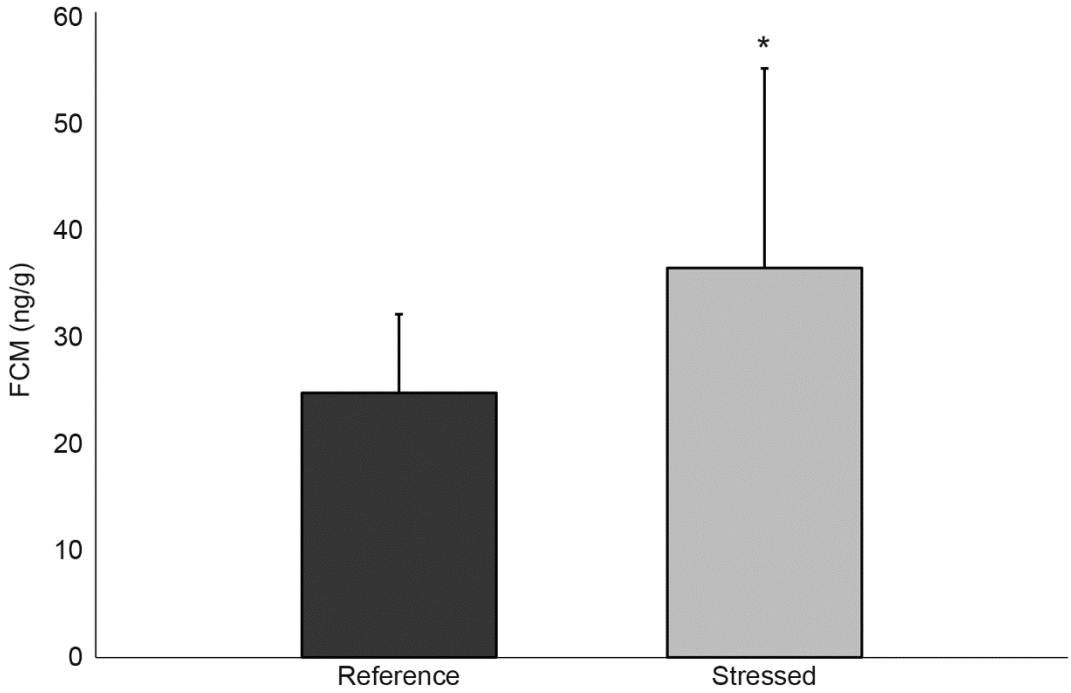
184 All animals were handled in accordance with procedures outlined in the Guide for the
185 Care and Use of Laboratory Animals (National Research Council, 2010), and Texas A&M
186 University-Commerce Animal Use Protocol P20-013.

187 Statistical Analysis

188 All statistical analyses were conducted in R (version 4.0.2, R Foundation, Austria). Shapiro-
189 Wilk's statistical test was used to check for normality (Zar, 1996). Welch's 2-sample t-test was
190 used to calculate the p-value, mean, and 95% confidence interval between 2 variables with
191 normal distributions. Mann-Whitney *U*-test was used to compare 2 variables with non-normal
192 distributions. Kruskal-Wallis test was used to compare multiple variables with non-normal
193 distributions, followed by Dunn's test with Bonferroni correction for pairwise comparisons.
194 Correlations were tested using Pearson's product-moment correlation with Bonferroni correction
195 (Zar, 1996). All weight data are presented as mean ± SD. Results were significant at alpha <0.05.

196 RESULTS

197 Mean ±SD FCM concentrations of valley quail during the simulated translocation were
198 36.21 ±18.56 ng/g, and higher than reference FCM concentrations (24.07 ±10.4 ng/g; $N = 13$
199 quail; Mann-Whitney U, $Z = 4.20$, $P < 0.001$; Fig. 1). Mean 48-hour weight loss was 17.8 ±6.3 g,
200 or 12 ±4.4% of initial body mass.



201

202 **Fig 1. Biological validation of using fecal corticosterone metabolites in translocated**

203 **California valley quail.** Fecal corticosterone metabolites (FCM) concentrations (mean ±SD)

204 were extracted from scat collected from acclimated quail (*N*=13) prior to (Reference) and during

205 a 48-hr simulated translocation (Stressed). FCM concentrations from stressed birds were higher

206 than reference FCM concentrations (Mann-Whitney U, $Z = 4.20$, $P < 0.001$). Asterisk indicates

207 statistical difference.

208

209 Valley quail with higher initial body mass prior to the simulated translocation

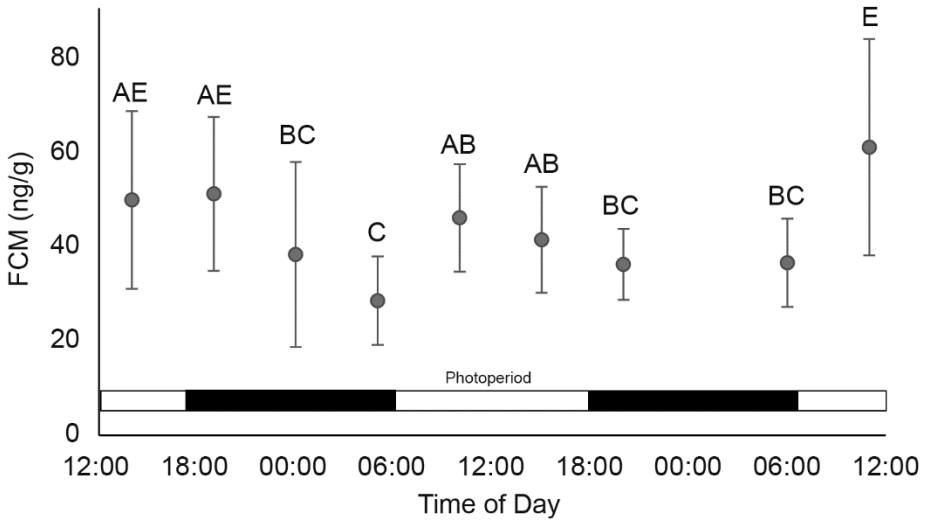
210 experienced lower mean FCM concentrations ($r^2 = 0.14$, $P < 0.001$), and lower percentage of

211 initial mass lost ($r^2 = 0.13$, $P < 0.001$) during the simulated translocation. FCM concentrations

212 positively correlated with percent of initial body mass loss ($r^2 = 0.27$, $P < 0.001$). No difference

213 in FCM concentrations (Mann-Whitney U, $Z = 0.15$, $P = 0.44$), total mass lost (Welch's t-test, P
214 $= 0.58$), or percentage of initial mass lost (Welch's t-test, $P = 0.29$) was observed based on sex.

215 Valley quail FCMs varied diurnally during the simulated translocation (Fig. 2), with
216 higher concentrations from 10:00–18:00 and lower concentrations from 20:00–06:00 (Kruskal-
217 Wallis, H = 62.3, $P < 0.001$). Mean FCM concentrations were not different between the first 24 h
218 and last 24 h (Mann-Whitney U, $Z = 1.86$, $P = 0.32$).



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220 **Fig 2. Diurnal variation in fecal corticosterone metabolite (FCM) concentrations**
221 **from California valley quail during a simulated 48-h translocation.** FCM concentrations
222 correlated with photoperiod (12L:12D) and quail circadian rhythm. Letters indicate statistical
223 groupings.

224 Discussion

225 This study was the first biological validation of fecal corticosterone metabolites as a non-
226 invasive assessment of stress for a new world quail species. When exposed to a simulated

227 translocation, valley quail experienced a 72.4 % increase in FCMs compared to reference
228 concentrations, indicating that this protocol successfully detected an increase in FCMs during a
229 stressful event. These results are comparable to the 73% increase in FCMs recorded in northern
230 bobwhites during a physiological validation (Mohlman *et al.*, 2020). However, the increase in
231 FCM levels is lower than that recorded in many other avian species. This isn't surprising because
232 excretions of metabolites can vary between species (Palme *et al.*, 1996; Wasser *et al.*, 2000;
233 Palme, 2019). For example. experimentally stressed European starlings showed a ~100%
234 increase in FCMs (Cyr and Romero, 2008). Following capture and holding for a veterinary
235 examination, African penguins (*Spheniscus demersus*) showed a 155–349% increase in FCMs.
236 Wild Dickcissels (*Spiza americana*) experienced a 1,700% increase in FCM concentrations
237 within 24 h of having leg harness transmitters attached (Suedkamp Wells *et al.*, 2003). Valley
238 quail and northern bobwhite quail are both new world quail and their similar stress response
239 during validation, and differences from other taxa were expected.

240 The correlation of weight loss with FCM levels further supports the association of
241 chronic stress with weight loss in birds (Cyr and Romero, 2007; Dickens *et al.*, 2009; Awerman
242 and Romero, 2010). Quail in our study lost ~12% of body weight which is consistent with weight
243 loss in translocated starlings (5-15%) and wild valley quail (14.3 %) in an actual translocation of
244 similar duration [60], indicating that translocation weight loss may primarily be a result of stress.
245 Similar to our study, Reyna et al. (2020) reported that larger birds lost less weight during
246 translocation. Larger birds may be healthier overall and future studies may benefit from
247 considering a minimum weight for translocation.

248 By collecting fecal samples 4–5 times daily during the simulated translocation, we were
249 able to capture diurnal variation in FCM concentrations. While not previously observed in new

250 world quail (Mohlman *et al.*, 2020), diurnal variation in corticosterone metabolites has been
251 recorded in a variety of species (Florant and Weitzman, 1980; de Jong *et al.*, 2001; Touma *et al.*,
252 2004; Bosson *et al.*, 2009; Sheriff *et al.*, 2009), generally corresponding to the circadian rhythm
253 of the animal. The diurnal variation in valley quail FCM concentrations was similar to variations
254 observed in broiler chickens (de Jong *et al.*, 2001), with lower concentrations at night and
255 elevated concentrations during the day. This trend was expected since valley quail are active in
256 the day and inactive during the night (Leopold, 1977).

257 This study verifies that translocations are stressful events for quail that result in
258 significant weight loss; results that suggest stress influences translocation success. Chronic stress
259 is associated with increased mortality (Cyr and Romero, 2007; Dickens *et al.*, 2010), and animal
260 weight at release is a predictor of survival (Fountain *et al.*, 2017). Further, stress is a major driver
261 of reproduction, which is one major indicator of translocation success (Griffith *et al.*, 1989;
262 Dickens *et al.*, 2010). Future studies could benefit by focusing efforts on stress mitigation to
263 increase long-term translocation success.

264 The use of FCM as a non-invasive measurement in new world quail could be extended
265 beyond translocations to benefit quail conservation. There is considerable environmental and
266 economic interest in conserving and restoring populations of quail and other gamebird species.
267 Increased plasma CORT can be indicative of environmental disturbances and habitat-related
268 metabolic challenges (Homyack, 2010; Shipley *et al.*, 2022). For example, FCMs have been used
269 as an indicator of declining habitat quality in greater sage-grouse (*Centrocercus urophasianus*;
270 Rabon *et al*., 2021). FCMs have also been used to detect elevated CORT levels associated with
271 extreme weather events, noise related to fossil fuel extraction (Cinto Mejia *et al.*, 2019), and
272 other anthropogenic disturbances (Thiel *et al.*, 2008, 2011; Blickley *et al.*, 2012; Arlettaz *et al.*,

2015; Formenti *et al.*, 2015). Through the use of innovative field collection methods (Shipley *et al.*, 2019), FCMs may be used to assess chronic stress levels in wild quail populations, evaluate responses to multiple stressors (e.g., extreme drought), and map stress levels across a landscape to identify focus areas for conservation (Rabon *et al.*, 2021). Ultimately, the use of FCMs could alert quail biologists to the presence of environmental stressors before population demographics are impacted (Ellis *et al.*, 2012). Clearly, FCMs have enormous potential as a non-invasive indicator of stress in quail and as a tool for improving quail research, restoration, management, and conservation.

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Author Contributions

All authors contributed equally to this project and writing of this manuscript.

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290 # Data Availability

291 The data underlying this article will be shared on reasonable requests to the corresponding

292 author.

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Fig 1. Biological validation of using fecal corticosterone metabolites in translocated California valley quail. Fecal corticosterone metabolites (FCM) concentrations (mean ±SD) were extracted from scat collected from acclimated quail (N=13) prior to (Reference) and during a 48-hr simulated translocation (Stressed). FCM concentrations from stressed birds were higher than reference FCM concentrations (Mann-Whitney U, $Z = 4.20$, $P < 0.001$). Asterisk indicates statistical difference.

Fig 2. Diurnal variation in fecal corticosterone metabolite (FCM) concentrations from California valley quail during a simulated 48-h translocation. FCM concentrations correlated with photoperiod (12L:12D) and quail circadian rhythm. Letters indicate statistical groupings.