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

We validated the use of corticosterone metabolites in feces as a non-invasive method to quantify stress in California valley quail using a simulated translocation. Quail were more stressed during the simulated translocation, and stress levels showed a diurnal pattern in which they were higher during the day, lower at night.

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 and indirect 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 validate the use of FCMs as a non-invasive method to assess stress hormone levels in California valley quail, and demonstrate the stressful nature of translocations. This methodology and results can be used to test stress mitigation methods and improve PRTs, while informing future investigations of stress in birds.

Keywords: biological validation, California quail, corticosterone, ELISA, fecal corticosterone metabolites, population restoration techniques, stress, 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).

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

Materials and methods

Biological Validation of FCM in Valley Quail

Flight-ready captive-reared valley quail (N = 5 females, N = 8 males), 16–24 wks in age, were acquired from a breeder (ZKD Game Birds, West Point, Texas, USA) in October, 2020. Quail were housed and acclimated in an outdoor aviary (Quail Hotel, Fannin Fabrication, Bonham, TX, USA) for 3 wks. Birds were provided with gamebird feed (Gamebird Starter and Grower, M-G Inc., Weimar, TX, USA) and water *ad libitum,* and enrichments including social and environmental stimuli (Hawkins et al. 2001). This mimicked the time and conditions in which quail were held prior to the first shipment of trapped birds during a 2019 translocation (Reyna *et al.*, 2020). Acclimation was assumed when birds exhibited typical behavior (e.g., eating, drinking, roosting, grooming; Reyna and Newman, 2018).

To biologically validate FCMs in valley quail, reference (low stress) fecal samples were obtained from acclimated valley quail. The floor of the aviary was lined with a plastic sheet and fecal samples were collected daily. Once collected, individual fecal samples were placed into a plastic storage bag (Ziploc freezer quart, S. C. Johnson & Son, Inc., Racine, WI) and labeled with the date, time, and sex of the bird. Temperature, humidity, and presence of direct sunlight

156 were recorded on the plastic storage bag, since these factors can degrade hormone detection
157 probability (Shipley *et al.*, 2019). Immediately after collection and labeling, samples were stored
158 in a freezer at -20 °C until FCM analysis (Wasser *et al.*, 1988; Messmann *et al.*, 1999; Khan *et*
159 *al.*, 2002).

160 For biological validation, quail were transported to the animal care facility at Texas
161 A&M University-Commerce to undergo a simulated 48-h translocation, a stressful event [41].
162 Each quail was given a unique leg band, and had their age, sex, and weight recorded. Quail were
163 held individually in research-approved breeding pens located in a temperature and light-
164 controlled room with a 12L:12D photoperiod. To ensure accuracy and reduce contamination,
165 fecal samples were collected from the underlying waste pan trays 4–5 times daily (Millspaugh
166 and Washburn, 2004; Mohlman *et al.*, 2020). The frequency of feces collection ensured a
167 representative sample, since FCMs may fluctuate diurnally (Breuner *et al.*, 1999). Individual
168 fecal samples were placed in a plastic storage bag and labeled with the bird ID number, sex,
169 time, and date of collection. Steroid hormone concentrations can be affected by storage duration
170 (Millspaugh and Washburn, 2004) and bacteria (Lexen *et al.*, 2008). Therefore, high stress fecal
171 samples were immediately stored in a freezer (-20 °C) until FCM analysis (Wasser *et al.*, 1988;
172 Messmann *et al.*, 1999).

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

179 dilutions) was performed. FCM concentrations from the pooled fecal extracts were parallel to the
180 slope of the standard curve, verifying suitability for valley quail. The sensitivity of the EIA was
181 determined to be 18.19 pg/ml. Optical density was calculated using a Synergy LX Multi-mode
182 microplate reader (Bio Tek Instruments, USA), and entered into MyAssay web
183 (https://www.myassay.com), to calculate FCM concentration in pg/ml. FCM concentrations
184 reported in pg/ml were converted to ng/g for comparison to other studies.
185 All animals were handled in accordance with procedures outlined in the Guide for the
186 Care and Use of Laboratory Animals (National Research Council, 2010), and Texas A&M
187 University-Commerce AUP P20-013.

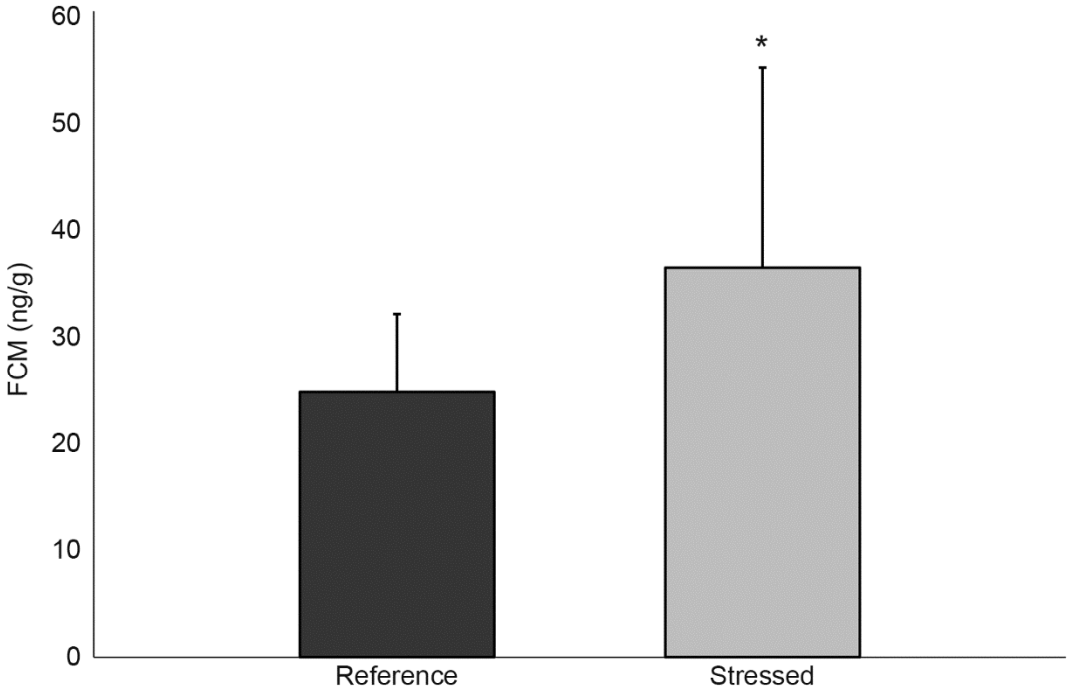
188 **Statistical Analysis**

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

198 # RESULTS

199 ## Biological validation of FCMs in valley quail

200 Mean ±SD FCM concentrations of valley quail during the simulated translocation were
201 36.21 ±18.56 ng/g, and higher than reference FCM concentrations (24.07 ±10.4 ng/g; $N = 13$
202 quail; Mann-Whitney U, $Z = 4.20$, $P < 0.001$; Fig. 1). Mean 48-hour weight loss was 17.8 ±6.3 g,
203 or 12 ±4.4% of initial body mass.



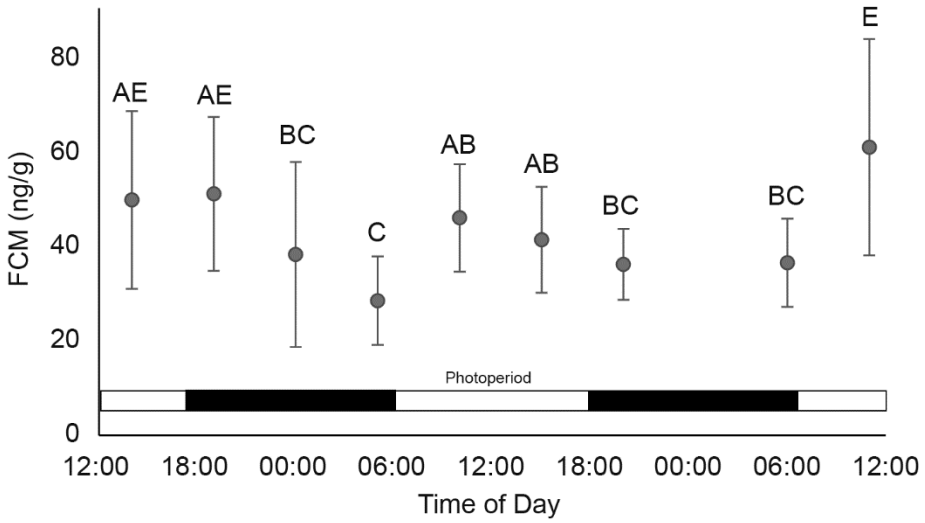
204

205 **Fig 1. Biological validation of using fecal corticosterone metabolites in translocated**
206 **California valley quail.** Fecal corticosterone metabolites (FCM) concentrations (mean ±SD)
207 were extracted from scat collected from acclimated quail (N=13) prior to (Reference) and during
208 a 48-hr simulated translocation (Stressed). FCM concentrations from stressed birds were higher

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

211 Valley quail with higher initial body mass prior to the simulated translocation
212 experienced lower mean FCM concentrations ($r^2 = 0.14$, $P < 0.001$), and lower percentage of
213 initial mass lost ($r^2 = 0.13$, $P < 0.001$) during the simulated translocation. FCM concentrations
214 positively correlated with percent of initial body mass loss ($r^2 = 0.27$, $P < 0.001$). No difference
215 in FCM concentrations (Mann-Whitney U, $Z = 0.15$, $P = 0.44$), total mass lost (Welch's t-test, P
216 $= 0.58$), or percentage of initial mass lost (Welch's t-test, $P = 0.29$) was observed based on sex.

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



221

222 **Fig 2. Diurnal variation in fecal corticosterone metabolite (FCM) concentrations**
223 **from California valley quail during a simulated 48-h translocation.** FCM concentrations

224 correlated with photoperiod (12L:12D) and quail circadian rhythm. Letters indicate statistical
225 groupings.
226

227 # Discussion

228 This study was the first biological validation of fecal corticosterone metabolites as a non-
229 invasive assessment of stress for a new world quail species. When exposed to a simulated
230 translocation, valley quail experienced a 72.4 % increase in FCMs compared to reference
231 concentrations, indicating that this protocol successfully detected an increase in FCMs during a
232 stressful event. These results are comparable to the 73% increase in FCMs recorded in northern
233 bobwhites during a physiological validation (Mohlman *et al.*, 2020). However, the increase in
234 FCM levels is lower than that recorded in many other avian species. This isn't surprising because
235 excretions of metabolites can vary between species (Palme *et al.*, 1996; Wasser *et al.*, 2000;
236 Palme, 2019). For example. experimentally stressed European starlings showed a ~100%
237 increase in FCMs (Cyr and Romero, 2008). Following capture and holding for a veterinary
238 examination, African penguins (*Spheniscus demersus*) showed a 155–349% increase in FCMs.
239 Wild Dickcissels (*Spiza americana*) experienced a 1,700% increase in FCM concentrations
240 within 24 h of having leg harness transmitters attached (Suedkamp Wells *et al.*, 2003). Valley
241 quail and northern bobwhite quail are both new world quail and their similar stress response
242 during validation, and differences from other taxa were expected.
243 The correlation of weight loss with FCM levels further supports the association of
244 chronic stress with weight loss in birds (Cyr and Romero, 2007; Dickens *et al.*, 2009; Awerman
245 and Romero, 2010). Quail in our study lost ~12% of body weight which is consistent with weight

246 loss in translocated starlings (5-15%) and wild valley quail (14.3 %) in an actual translocation of
247 similar duration [60], indicating that translocation weight loss may primarily be a result of stress.
248 Similar to our study, Reyna et al. (2020) reported that larger birds lost less weight during
249 translocation. Larger birds may be healthier overall and future studies may benefit from
250 considering a minimum weight for translocation.

251 By collecting fecal samples 4–5 times daily during the simulated translocation, we were
252 able to capture diurnal variation in FCM concentrations. While not previously observed in new
253 world quail (Mohlman *et al.*, 2020), diurnal variation in corticosterone metabolites has been
254 recorded in a variety of species (Florant and Weitzman, 1980; de Jong *et al.*, 2001; Touma *et al.*,
255 2004; Bosson *et al.*, 2009; Sheriff *et al.*, 2009), generally corresponding to the circadian rhythm
256 of the animal. The diurnal variation in valley quail FCM concentrations was similar to variations
257 observed in broiler chickens (de Jong *et al.*, 2001), with lower concentrations at night and
258 elevated concentrations during the day. This trend was expected since valley quail are active in
259 the day and inactive during the night (Leopold, 1977).

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

267 The use of FCM as a non-invasive measurement in new world quail could be extended
268 beyond translocations to benefit quail conservation. There is considerable environmental and

269 economic interest in conserving and restoring populations of quail and other gamebird species.
270 Increased plasma CORT can be indicative of environmental disturbances and habitat-related
271 metabolic challenges (Homyack, 2010; Shipley *et al.*, 2022). For example, FCMs have been used
272 as an indicator of declining habitat quality in greater sage-grouse (*Centrocercus urophasianus*;
273 Rabon *et al*., 2021). FCMs have also been used to detect elevated CORT levels associated with
274 extreme weather events, noise related to fossil fuel extraction (Cinto Mejia *et al.*, 2019), and
275 other anthropogenic disturbances (Thiel *et al.*, 2008, 2011; Blickley *et al.*, 2012; Arlettaz *et al.*,
276 2015; Formenti *et al.*, 2015). Through the use of innovative field collection methods (Shipley *et*
277 *al.*, 2019), FCMs may be used to assess chronic stress levels in wild quail populations, evaluate
278 responses to multiple stressors (e.g., extreme drought), and map stress levels across a landscape
279 to identify focus areas for conservation (Rabon *et al.*, 2021). Ultimately, the use of FCMs could
280 alert quail biologists to the presence of environmental stressors before population demographics
281 are impacted (Ellis *et al.*, 2012). Clearly, FCMs have enormous potential as a non-invasive
282 indicator of stress in quail and as a tool for improving quail research, restoration, management,
283 and conservation.

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