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1 **Subtle short-term physiological costs of an experimental augmentation of fleas**
2 **in wild Columbian ground squirrels**

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9 Summary Statement: Parasite-host interactions were experimentally assessed in a wild
10 population of Columbian ground squirrels. Short-term physiological influences were minimal,
11 suggesting evolutionary cost minimization parasites and hosts.

12 Abstract:

13 Parasites affect many aspects of host physiology and behavior, and thus are generally thought
14 to negatively impact host fitness. However, changes in form of short-term parasite effects on
15 host physiological markers have generally been overlooked in favor of larger fitness measures.
16 Here we studied flea (*Oropsylla idahoensis* and *Oropsylla opisocroistis tuberculata*) parasitism
17 on a natural population of Columbian ground squirrels (*Urocitellus columbianus*; CGS) in Sheep
18 River Provincial Park, Alberta. Fleas were experimentally added to adult female CGS at
19 physiologically demanding times, including birth, lactation, and weaning of their young. The
20 body mass of adult females, as well as their oxidative stress and immunity were recorded
21 multiple times over the CGS active season under flea-augmented and control conditions. We
22 also measured the prevalence of an internal parasite (*Trypanosoma otospermophili*). Doubly
23 labelled water (DLW) was interperitoneally injected at peak lactation to examine energy
24 expenditure. Effects of parasites on oxidative stress were only observed after offspring were
25 weaned. There was no direct effect of experimentally heightened flea prevalence on energy
26 use. A short-term 24 h mass loss (-17 g) was detected briefly after parasite addition, likely due

27 to CGS preferentially allocating time for grooming. Our parasite augmentation did not strongly
28 affect hosts and suggested that short-term physiological effects were unlikely to culminate in
29 long term fitness consequences. Columbian ground squirrels appear to rapidly manage parasite
30 costs, probably through grooming.

31 **KEY WORDS: parasitism, immune cost, Columbian ground squirrel, flea, energy expenditure,**
32 **oxidative stress**

33

34 **Introduction**

35 The resources that parasites extract from their hosts are often thought to produce
36 negative effects on host fitness (Møller et al., 1994; Delahay et al., 1995; Careau et al., 2010).
37 Parasitism can induce direct costs through sapping resources from their hosts (Nelson et al.,
38 1975) and indirect costs through changes in behavioral activity (Giorgi et al., 2001; Scantlebury
39 et al., 2007), acting as pathogen vectors (Smith et al., 2005), or modifying physiological
40 tradeoffs (Bertrand et al., 2006b; Sorci et al., 2017). These host-parasite links are illustrated by
41 eastern grey kangaroos selectively foraging away from better quality, but faecally-
42 contaminated grass patches (Garnick et al., 2010) or male grey squirrels suffering from higher
43 flea parasitization in exchange for larger testes (Scantlebury et al., 2010). As a result, parasites,
44 when numerous, have the potential to generate a high resource toll on their hosts (Khokhlova
45 et al., 2002; Krasnov, 2008). For example, when feral rock dove had lice levels experimentally
46 increased, they steadily lost feather and body mass resulting in compromised integument
47 insulation (increased thermal conductance) and increased metabolic rate (Booth et al., 1993).

48 It is often difficult to distinguish direct resource loss to the parasite from the costs of
49 anti-parasite defense or environmental effects (Bonneaud et al., 2003), such as effects of
50 temperature extremes (Cohen et al., 2017) on energetics. Additionally, it is hard to discern
51 whether parasites are the cause of poor host health and body condition or the result of it
52 (Boonstra et al., 1980). Thus, it is useful to examine parasite effects through controlled
53 experimental manipulation (Keymer & Read, 1991) to directly address these questions. Such

54 research has been conducted in laboratory studies that often fail to account for natural host-
55 parasite dynamics such as the “80:20 rule”, an aggregated negative binomial distribution where
56 a few hosts (20%) harbor the majority of parasites (80%) in a population (Galvani, 2003; Poulin,
57 2004; Craig et al., 2007). This underlines the parasite preference for hosts in terms of age, sex,
58 and time of season (Dick & Patterson, 2007; Liberman et al., 2011), which are often overlooked
59 and indicate the value of further field studies.

60 These factors, coupled with experimental studies that frequently focus on long-term
61 fitness costs, may explain findings of muted parasite effects in wild (Khokhlova et al., 2002)
62 studies compared to laboratory tests. An alternative research design might quantify more
63 subtle short-term physiological modifications while preserving natural features of wild
64 conditions. Emphasis on short-term effects on physiological changes serves two purposes.
65 Firstly, short-term physiological shifts should be more detectable and directly quantifiable than
66 multi-faceted fitness outcomes. Secondly, collection of these chronically sustained short-term
67 effects may allow improved interpretation of potential long-term costs.

68 In this study, we experimentally tested the effects of ectoparasitic fleas (*Oropsylla*
69 *idahoensis* and *Oropsylla opisocroistis tuberculata*) on a wild population of adult female
70 Columbian ground squirrels (*Urocitellus columbianus*; CGS). We subjected a group of Columbian
71 ground squirrels to an experimental increase in their natural flea loads and compared their
72 physiological responses to a group of ground squirrels where flea loads were left unchanged.
73 Columbian ground squirrels are hibernating sciurid rodents with a 3-4 month annual active
74 season, during which reproduction takes place (Dobson et al., 1992). Parental care is restricted
75 to the highly territorial mothers during the 24 days of gestation, 27 days of lactation and a short
76 period after weaning (Murie & Harris, 1982). This species is naturally parasitized by both ecto-
77 (ticks, mites, botflies) and endo-parasites (helminths, coccidia, trypanosomes). The most visible
78 of these are fleas (Raveh et al., 2011, 2015) that seem to follow the aggregated 80:20
79 distribution. Since fleas are often localized to individual hosts and burrows, natural parasite
80 dispersal is low, thus allowing enhanced isolation of parasite effects on hosts. As such, breeding
81 female CGS are an ideal model system to reveal parasite costs because of the lack of

82 confounding factors such as parasite transfer or dispersal (Krasnov et al., 2003b; Hawlena et al.,
83 2005).

84 Prior studies on parasite effects in CGS have resulted in variable outcomes (little to no
85 effect: Raveh et al., 2011, 2015; negative effect: Neuhaus, 2003). These studies applied
86 experimental reductions of fleas, in a species that naturally has relatively low levels of
87 infestation, to assess fitness consequences on individuals. Such detection of parasite effects
88 may have been limited by the natural parasite distribution when using the approach of parasite
89 removal in lightly infested populations. Parasite costs might only be relevant when present in
90 resource-deficient hosts. Co-evolution of host-parasite interactions might be favored by natural
91 selection when they minimize negative effects of the parasite on the host (Hinnebusch et al.,
92 2017). In these cases, lowering levels of parasites are unlikely to show strong effects on fitness.
93 Adding parasites to wild hosts provides an improvement over previous tests of ectoparasite
94 effects reported in the literature (Booth et al., 1993; Warburton et al., 2016), because treated
95 hosts should be more likely to reveal parasite consequences due to exacerbated costs.

96 Our approach to understanding host-parasite dynamics thus has two novel features:
97 augmentation of fleas that is more likely to reveal costs, and physiological measures that can
98 reveal such costs. Short-term parasite effects on physiological metrics were assessed during
99 energetically-constrained time-points, such as lactation (Rogowitz, 1998; Naya et al., 2008), to
100 augment visibility of costs through a higher energy budget (Metcalf et al., 2013). In particular,
101 we expected that CGS would employ behavioral and immune defenses against flea-induced
102 stress. Since fleas can serve as a vector for pathogens (Durden & Hinkle, 2019) such as the
103 blood parasite *Trypanosoma otospermophili* (Freedman, 1964; Lizundia et al., 2011),
104 trypanosome levels might also increase in the flea-treated group. We expected higher
105 trypanosome prevalence to lead to stimulation of nitric oxide (NO), which has been shown to
106 elevate in response to trypanosome infections (Vespa et al., 1994). By doing so, parasite
107 infestation would be positively correlated with energy use (Kam et al., 2011) and subsequently
108 enhanced oxidative stress due to a non-specific innate immune response (Plumel et al., 2016;
109 Bertrand et al., 2006a). A difference in the dynamics of mass, oxidative stress, immunity and

110 energetic demand of heavily infested individuals would provide evidence supporting a short-
111 term physiological consequence of parasites in CGS.

112

113 **Methods**

114 ***Ethics statement***

115 Animal care was carried out in accordance with Auburn University IACUC protocol
116 #2018-3227 (with additional approval by the University of Calgary). Authorization for
117 conducting research and collecting samples in Sheep River Provincial Park was obtained from
118 Alberta Environment and Parks (Research Permit #58954) and Alberta Tourism, Parks, and
119 Recreation (Research and Collection Permit #18-448).

120 ***Population monitoring***

121 Columbian ground squirrels (CGS) were followed in the Sheep River Provincial Park,
122 Alberta, Canada (Meadow B; 50°38'11.3" N, 114°39'56.7" W; 1550 m elevation) from April to
123 August 2018. The entire CGS population at Meadow B has been continuously monitored since
124 1992 (Wiggett & Boag, 1986; Viblanc et al., 2010; Rubach et al., 2016), from the onset of
125 emergence from hibernation in late April, to the end of offspring weaning in early July. Female
126 CGS have a short active season and a single reproductive period each year (Dobson et al.,
127 1992). Each squirrel in this population is permanently identified with unique numbered
128 fingerling eartags (#1-Monel metal; National Band and Tag Company, Newport, KY, USA), and is
129 given a unique hair dye marking (Clairol, Stamford, CT, USA) at the start of the season so it can
130 be identified from a distance during field observations. We followed all reproductive females
131 (n=31) to determine their mating day from behavioral observations and inspection of their
132 genitalia (Murie & Harris, 1982). CGS were trapped using Tomahawk live traps (13x13x40 cm;
133 Tomahawk, WI, USA) baited with a small amount of peanut butter. Some of the females (n=5)
134 either disappeared during the breeding season or were not re-captured and were thus excluded
135 from analyses.

136 ***Experimental manipulation of fleas***

137 We experimentally increased ectoparasite loads on 16 females (Treatment group, T) and
138 compared them to 15 control females (Control group, C, see below). At the start of the
139 experiment we ascertained CGS body condition and then randomly assigned females of similar
140 condition and ages to both C- and T-groups. Body condition was estimated by regressing
141 individual body mass on zygomatic arch breadth (an index of structural size; Dobson, 1992).
142 Fleas were collected from squirrels at a neighboring meadow less than 400 m away from the
143 study site (50°38'19.7" N, 114°39'47.1" W) by brushing individuals with a fine-tooth flea comb
144 (Four Paws, Hauppauge, NY, USA) into an aerated plastic container and transferring the fleas on
145 the same day to experimental subjects. Due to the need for the same-day transfer to new
146 hosts, fleas were not identified to species or sex, and were assumed to belong to one of the
147 two common species found in prior studies (Hubbard, 1947; Hilton & Mahrt, 1971). Prior to flea
148 addition, each squirrel was carefully combed on all sides of the body including the head to
149 assess initial natural flea numbers. After counting, all initially present fleas were returned to
150 their host.

151 An average of 20 fleas were added to each experimental subject at each time-point, in
152 addition to their inherent number of parasites (see Results). Fleas were added at 3 separate
153 time-points during the season: gestation (T1), at lactation onset (T2) and at peak lactation (T3)
154 prior to weaning. These time-points were chosen because they represented important
155 transitions in the breeding cycle and likely exhibited elevated physiological demands.
156 Additionally, they coincided with other manipulations of the long-term study, hence reducing
157 animal handling and stress. We re-captured all non-hibernating females at a 4th time-point (T4;
158 roughly a week before the onset of hibernation), but did not re-infest any animals, as a negative
159 control. Fleas were deposited on the ventral side of the restrained animal and rubbed into the
160 fur. We insured all fleas had entered the animal's pelage before releasing it. The control group
161 had their pelage rubbed in a similar manner to simulate flea addition, but with no change in
162 number of natural fleas.

163 ***Trypanosomes***

164 We assayed presence of *Trypanosoma otospermophili* through collection of 100 μ l of
165 whole blood in capillary tubes. After collection, we centrifuged those capillary tubes at 5,000 g
166 for 10 min to apply quantitative buffy coat methodology. Centrifugation of whole blood serves
167 to concentrate trypanosomes in the buffy coat of the solute and enhance parasite detection
168 (Sato et al., 2007). Five μ l of the buffy coat was spread on a glass slide into a thin smear and
169 Wright-Giesma stained (Shandon Kwik-Diff stain, Thermo Fisher Scientific, Waltham, MA, USA),
170 followed by count estimates of trypanosomes.

171 ***Behavior***

172 After flea addition, we released squirrels at the place of initial capture. We then visually
173 observed control and treated squirrel behavior for 15 minutes to gauge how differentially
174 parasitized squirrels allocated their time-budget to body maintenance. We recorded the
175 number of seconds spent self-grooming.

176 ***Oxidative status and innate immunity***

177 Individual oxidative stress levels and innate immunity were estimated during T1, T2, T3
178 and T4. Blood (0.5 mL) was collected from the saphenous vein using a 27-G needle fitted to a 1
179 mL heparinized syringe. We kept blood on ice packs in a cooler box while in the field. After
180 centrifugation (5,000 g for 10 min) within 1–2 hrs of collection, plasma was separated and kept
181 frozen at -20°C until the end of the field season, before transportation on dry ice and
182 subsequent frozen storage at -80°C until laboratory analyses.

183 We assessed female oxidative status in plasma by global measures of oxidative damage
184 (d-ROMs test; 8 μ l of plasma) and antioxidant defenses (OXY-absorbent test; 5 μ l of 1:100
185 diluted plasma) (Diacron International, Grosseto, Italy) (see Costantini, 2011; Costantini, 2016;
186 Viblanc et al. 2018 for details). In addition, we measured nitric oxide (NO) in plasma
187 (Diazotization assay; 10 μ l plasma; see Bourgeon et al. 2007 for details) as a reflection of
188 macrophage activation by intracellular pathogens (Playfair & Bancroft, 2004). ROM and OXY
189 sample measurements were run in duplicate and NO was run once per sample. Intra-plate
190 variation was 5.15% for ROMs, 12.1% for OXY. Inter-plate variation based on a standard sample
191 repeated over plates was 2.74% for ROMs, 8.61% for OXY, and 1.51% for NO.

192 ***Estimation of total daily energy expenditure (DEE):***

193 *Field protocol:* Daily energy expenditure for treated and control females was determined
194 only during peak lactation (day 25; T3), when reproductive demands on females were expected
195 to be the highest. DEE was estimated using the doubly labeled water (DLW) technique, as
196 extensively described elsewhere (Kenagy et al., 1990; Rimbach et al., 2018), including in CGS
197 (Skibieli et al., 2013). Briefly, females were weighed (to the nearest 5 g using a spring scale; 1 kg,
198 Pesola Ag, Baar, Switzerland) and a first blood draw (100 μ L) was collected from the saphenous
199 vein using a 30-G needle in two 100 μ L non-heparinized capillary tubes to establish background
200 levels of ^{18}O and ^2H . Capillaries were immediately sealed with a micro-jet flame and stored at
201 room temperature until analyses (within 3 months). Squirrels were then injected intra-
202 peritoneally with a premixed 5 g/kg dose of DLW (10% H_2^{18}O and 99.9% $^2\text{H}_2\text{O}$, Cambridge
203 Isotopic Laboratories, Cambridge, MA, USA). After injection, females ($n = 26$) were held in traps
204 in a quiet, shaded location and covered with a dark cotton pillowcase for 75 min to allow for
205 isotope equilibration (Król & Speakman, 1999; Simmen et al., 2010; Skibieli et al., 2013).
206 Following the equilibration period, another blood sample was drawn ($n=26$), fleas were added
207 to the experimental animals, and the subjects were released. As part of the DLW test, a
208 subsequent blood sample and weight measurement was taken at 24 h and 72 h post-
209 enrichment ($n=26$) to estimate isotope elimination rates (Speakman & Racey, 1987). During the
210 DLW experiment, we recorded the average ambient temperature (T_A) experienced by
211 individuals to control for potential thermoregulatory effects on metabolic rate. We used
212 thermo-logging iButtons (DS1921G, Maxim Integrated, San Jose, CA, USA), which recorded T_A
213 with 15 min intervals over the course of the experiment. iButtons were centrally located in the
214 colony, attached to the bases of elevated observation benches, with the iButtons one meter
215 above ground level.

216 *Isotope analyses:* Sealed capillary tubes were vacuum distilled for 10 mins and the
217 resulting water distillate analyzed by a continuous flow isotope ratio mass spectrometer (IRMS;
218 IRMS-DELTA V PLUS, Thermo, Bremen, Germany) as described previously (Chery et al., 2015;
219 Mahlert et al., 2018). Distillates were pyrolyzed at 1400°C into H_2 and CO_2 gases in a glassy
220 carbon tube under pure He flow at 90 mL min^{-1} . H_2 and CO_2 were separated at 110°C on a

221 molecular sieve GC column before sequential analysis in IRMS. Results were normalized using
222 the VSMOW2/SLAP2 international scale. In addition, memory-effect and drift-corrections were
223 applied as needed. All analyses were performed in quadruplet and samples were re-analyzed if
224 SD exceeded 2% for ^2H or 0.2% for ^{18}O in more than three out of the four analyses. We
225 calculated the total body water (TBW) from the ^{18}O dilution space divided by 1.007 to correct
226 for *in vivo* isotopic exchange (Racette et al., 1994). The average isotope dilution space ratio was
227 1.029 ± 0.016 (mean \pm SD). We calculated the CO_2 production rate from the single pool model
228 as recommended for the body size of CGS (Speakman, 1993; Speakman & Hambly, 2016). We
229 converted CO_2 production into DEE using a modification of Weir's equation and an assumed
230 food quotient of 0.85 based on the prior literature involving CGS and DLW (Skibieli et al., 2013;
231 Speakman, J. R., University of Aberdeen, *personal communication*). For 6 animals, we observed
232 either capillary leakage or incomplete DLW equilibration occurring within the standardized
233 equilibration period, thus prompting their removal from subsequent analyses.

234 ***Statistical Analysis***

235 All statistics were done in R 3.5.1 (R Core Team, 2018; <https://www.R-project.org>). We
236 proceeded in a 3-step analysis. First, we assessed the efficiency of our treatment by comparing
237 the initial and final parasite loads of our control and treated individuals at each time-point of
238 infestation. For this, we used either linear or generalized linear mixed effects model (LMM and
239 GLMM; lme4 package in R; Bates et al., 2015) with initial or final (initial + additional fleas)
240 parasite counts entered as the dependent variable, time-point (T_1 to T_4), treatment (C or T) and
241 their interaction entered as independent variables, and female ID as a random factor to
242 account for longitudinal data collection. For initial flea levels, we used a Poisson distribution as
243 is appropriate when working with count data and given the distribution of initial flea loads. For
244 final flea loads, the addition of ca. 20 fleas per squirrel in the treated group normalized the
245 distribution of residuals in our model. Second, we investigated changes in body mass, oxidative
246 status (ROM and OXY) and innate immunity (NO and trypanosomes) over the season using a
247 similar procedure. Mass, ROM, OXY, NO or trypanosome levels were entered as dependent
248 variables in separate LMM and we tested for the fixed effects of time-point, treatment and
249 their interaction. In those models, we added female ID and age as random factors to account

250 for repeated observations and potential age effects on physiological variables. The number of
251 observations (n) and corresponding number of individuals (N) are indicated for each model.
252 Because of repeated observations on individuals $n > N$. Finally, we analyzed the effects of our
253 treatment on female energy expenditure during peak lactation, the period of highest
254 reproductive demand. We compared treatment and control groups in terms of body mass, DEE,
255 oxidative status and immunity using linear models (LMs). We included female age, litter mass at
256 weaning (reproductive investment) and T_A (average temperature between release of the
257 individual after DLW injection and collection of last blood sample), as covariates in the model to
258 test for their effects on DEE. For all analyses we visually inspected the distribution of model
259 residuals using QQ-plots to insure a reasonable fit to normality. Mean differences between
260 groups (time-points or treatment) were assessed using post-hoc Tukey's HSD test ('glht'
261 package in R; Hothorn et al., 2008). For each model, we calculated effect sizes (Cohen's d) and
262 their 95% confidence intervals ('effsize' package in R; Torchiano, 2018). We used benchmarks d
263 = 0.2, 0.5, 0.8 to indicate small, medium and large effect sizes respectively (Nakagawa & Cuthill,
264 2007). The alpha-level was set at 0.05 for all statistical analyses and results are presented as
265 averages \pm 1 standard error (s.e.m.).

266 **Results**

267 ***Changes in parasite loads and individual condition over the experiment***

268 *Fleas:* Over the course of the experiment, treated individuals were infested at 3
269 different time-points with an average of 19.59 ± 0.71 fleas/animal on top of their originally
270 present 0.93 ± 0.24 fleas. In contrast, control animals had on average 0.61 ± 0.16 fleas/animal
271 at each time-point (Fig. 1B). Thus after treatment, individuals averaged 33.63 times the flea
272 load of the control individuals (LMM; $t = 22.94$, $P < 2e^{-16}$, $n = 90$ observations, $N = 31$
273 individuals). Treated individuals had returned to a level near their initial flea levels by the next
274 experimental infestation (Fig. 1A).

275 *Mass:* We did not observe differential mass changes between groups (Table 1) from T1
276 to T4. During the DLW experiment, at T3, initial mass was not significantly different between
277 control and treatment groups prior to flea application (Table 1): treated CGS experienced a

278 short-term (24 h) mass loss compared to control animals. This disparity was negligible at 72
279 hours post-flea application. Due to incomplete DLW equilibration, some individuals ($n = 5$) were
280 removed from mass analyses.

281 *Behavior:* After controlling for age, treated CGS responded to flea infestation by
282 increasing their self-grooming behavior by 53.64% compared to controls (Fig. 2; T1-T3;
283 treatment = 20.45 ± 3.41 s, control = 11.8 ± 2.18 s; LMM; $t = 2.22$, $P = 0.03$, $n = 89$, $N = 31$).
284 Grooming decreased for both groups at peak lactation.

285 *Trypanosomes:* Trypanosome prevalence steadily decreased over the season in both
286 treatment and control groups (Fig. 3). At T4, we observed a statistically insignificant increase in
287 average count of trypanosomes (per 5 μ l of buffy coat) in the control group (329.82 ± 272.43 ;
288 LM; $t = 1.789$, $P = 0.09$, $n = 23$, $N = 23$) compared to the treatment group (25.46 ± 19.14). Upon
289 removal of an extreme outlier, there was no appreciable difference between treatment and
290 control groups (Fig. 5; control = 62.8 ± 59.72 ; LM; $t = -0.66$, $P = 0.52$, $n = 22$, $N = 22$).

291 ***Total daily energy expenditure at peak lactation (T3):***

292 During peak lactation, treated individuals did not show significantly higher DEE than
293 controls (Fig. 4; LM; $t = 0.31$, $P = 0.76$, $n = 20$, $N = 20$), even when we accounted for differences
294 in fat free mass. Energy expenditure (DEE) significantly increased with age (Fig. 3; LM; $t = 2.48$, P
295 $= 0.02$, $n = 20$, $N = 20$). Older breeders did not have larger litter masses at weaning (LM; $t = 0.84$,
296 $P = 0.41$, $n = 28$, $N = 28$), nor did litter mass differ substantially between treated and control
297 individuals (Table 1; LM; $t = 0.3$, $P = 0.77$, $n = 28$, $N = 28$). We did not observe a relationship
298 between DEE and ROM or NO levels (Fig. 4; LM; $t = 0.64$, $P = 0.54$, $n = 20$, $N = 20$; LM; $t = 0.19$, P
299 $= 0.85$, $n = 20$, $N = 20$; respectively). A similar analysis on antioxidant defenses (OXY) revealed a
300 relationship of decreased OXY levels (LM; $t = -2.26$, $P = 0.04$, $n = 20$, $N = 20$) with increased
301 energy expenditure in treated CGS. Upon removal of an outlier, this relationship disappeared
302 (LM; $t = -0.61$, $P = 0.55$, $n = 19$, $N = 19$).

303 ***Changes in innate immunity and oxidative status***

304 *Innate immunity (Nitric Oxide levels):* Level of inflammation, assayed through NO
305 concentration, was similar in both groups and over time (Fig. 5, LMM with Tukey's post-hoc; $t =$
306 $0.25, P = 0.63, n = 69, N = 29$). NO concentration was not associated with ROM (LMM; $t = 0.07, P$
307 $= 0.95, n = 69, N = 29$), OXY (LMM; $t = 1.17, P = 0.25$) or trypanosome (LMM; $t = 0.893, P = 0.38$)
308 levels.

309 *Oxidative stress:* There was a significant effect of time period on ROM and OXY levels
310 (Fig. 6; ROM $z = -5.08, P < 0.001, n = 107, N = 31$; OXY $z = -3.26, P < 0.001, n = 108, N = 31$). ROM
311 levels decreased from T1 to T3, but slightly rebounded at T4 in the treated group (LMM;
312 Tukey's post hoc test; $t = -2.5, P < 0.02, n = 54, N = 15$) compared to the control group (LMM;
313 Tukey's post hoc test; $t = -5.1, P < 0.01, n = 53, N = 16$). OXY levels steadily decreased in both
314 groups as the season progressed. Like in other metrics, ROM (LM; $t = -0.42, P = 0.68, n = 69, n =$
315 $24, N = 24$) and OXY (LM; $t = -0.36, P = 0.72$) values were not significantly different between the
316 treatment and control groups at peak lactation (T3).

317 **Discussion**

318 Our experimental transformation of an aggregated parasite distribution to a bimodal
319 distribution by adding fleas to some ground squirrels was an attempt to discern short-term
320 parasite effects. However, like other CGS studies that removed parasites, our results indicated
321 that these fleas did not significantly impact their hosts over the short-term in regards to any of
322 our physiological measures.

323 ***Flea augmentation and grooming***

324 By experimentally adding fleas to CGS, we expected a multitude of downstream
325 physiological responses. This was partially fulfilled, at least in terms of the treatment of fleas
326 temporarily enforcing a large short-term increase of parasites. This level of parasites was at the
327 extreme high end of what is normally seen in adult female CGS at this specific field site, but not
328 outside the natural range of variation. CGS, especially males and juveniles, have the capacity to
329 harbor and maintain numbers of fleas equivalent or in excess of the treatment level under
330 natural conditions (Raveh et al., 2015; JDR, FSD, VAV *personal observations*). Post-flea addition,

331 treated CGS allocated almost double the time of their non-parasitized counterparts to
332 grooming. More importantly, they allocated energy that would normally be devoted to
333 acquiring resources into maintenance of low flea levels at energetically and nutritionally
334 demanding times. Within 24 hours (*personal observations of recaptured animals*), almost all of
335 the added fleas were removed, as evidenced by the equilibration of initial flea levels at each
336 time-point. This grooming timeframe coincides with the 24-hour mass shifts seen only in the
337 treated group at the lactation peak time-point. However, in the context of an acute high
338 infestation, this statistically significant mass loss is hardly biologically costly due to the return to
339 normal mass within 72 h. Given that this population behaviorally enforces low parasite levels, a
340 situation of prolonged high parasitization is improbable and thus not of high consequence for
341 most host individuals.

342 Surprisingly, even during peak lactation, a critical period of the year where female
343 energy demands are typically highest in mammals (Oftedal et al., 1984; Speakman &
344 McQueenie, 1996), we did not observe an effect of our treatment on female energy
345 expenditure. This result indicates that these parasites do not have a high energetic cost on their
346 host or that the cost of managing parasites (*i.e.* grooming) is compensated through other
347 pathways. Indeed, the time invested in flea removal likely accounts for the loss in body mass
348 through changes in potential energy intake. With both species of fleas being ground squirrel
349 specialists (Hubbard, 1947; Hilton & Mahrt, 1971) and a lack of alternative hosts in the area, it
350 makes sense that the fleas have muted effects on CGS. If parasites have co-evolved to specific
351 hosts, they are more likely to deliver less irritable bites and introduce saliva that does not elicit
352 an immune or behavioral response from the host (Dick & Patterson, 2007). Unfortunately, our
353 observations do not account for a sex-biased effect whereupon fleas of a particular sex
354 differentially induce stress (Hawlena et al., 2005; Krasnov et al., 2008). Since fleas were
355 collected and assigned randomly to treated individuals, we can assume that any sex-bias in the
356 parasites averaged out when the treated and control groups are compared. However, whether
357 potential sex ratio biases in flea populations may differently affect individuals remains to be
358 tested in the future. Given larger blood meals consumed by female fleas (Krasnov et al., 2003a),

359 one might expect female-biased populations to have larger impacts on hosts than male-biased
360 populations.

361 A multitude of studies in other systems have demonstrated that the complexity of
362 parasite and host energetics obscure the quantification of parasite effects (Hicks et al., 2018;
363 Careau et al., 2010). For example, cape ground squirrels' (*Xerus inauris*) DEE is similarly
364 unaffected when parasite levels were manipulated (Scantlebury et al., 2007). Rather than
365 parasites, increasing age stimulated slightly higher DEE. Many attributes associated with body
366 composition such as larger litters or heavier young (Adams, 2005) would logically result in
367 increased maternal investment and thus higher DEE. For example, older female CGS appear to
368 undergo reproductive senescence and may require extra energy to succeed at breeding
369 (Broussard et al., 2003, 2005). In addition, younger breeding females may exhibit reproductive
370 inefficiencies when breeding for the first time (Broussard et al., 2008; Rubach et al., 2016),
371 perhaps resulting in younger animals having lower reproduction-associated DEE than older
372 animals (e.g., through reduced milk production). As such, instead of parasites, age-related body
373 composition and activity levels largely influence DEE (Klausen et al., 1997).

374 ***Oxidative stress and immunity***

375 Given their relationship, it is natural that a lack of parasite effects on energy use
376 culminated in a similar lack of treatment consequences on immunity and oxidative stress.
377 However, given that NO is a key immune factor involved in the oxidative killing machinery of
378 macrophages (Playfair & Bancroft 2004) and been implicated in the fighting trypanosome
379 infections (Magez et al., 2006; Gobert et al., 2000), it is somewhat surprising that nitric oxide
380 concentration did not mirror trypanosome levels in our study. Trypanosome levels declined in
381 both groups over the season, but nitric oxide concentration equally fluctuated in both groups.
382 One likely explanation of the lack of treatment effect is that this species of flea is simply an
383 inefficient trypanosome vector (Eisen et al., 2009). This putative inefficiency, in addition to the
384 rapid grooming response, affords only a short transmission window and subsequent lack of
385 nitric oxide response to trypanosomes. This absence indicates that, at least in CGS,
386 trypanosomes are of little consequence or are at least not managed by NO in macrophages.

387 In contrast, oxidative stress patterns were more responsive to change over time in that
388 they mirrored prior studies (Viblanco et al., 2018), likely due to the establishment of an oxidative
389 shield early on during lactation (Blount et al., 2016; Vitikainen et al., 2016) that allowed costs to
390 be offset. The oxidative shielding model proposes that mothers increase antioxidant defenses
391 early in reproduction to prevent the transfer of damaged molecules to their offspring, which
392 may occur as maternal oxidative stress increases through gestation and lactation (i.e. the
393 oxidative cost of breeding). In our study, effects of parasites appeared to manifest in the post-
394 shielding period (T4), with treated individuals experiencing larger increases in ROM levels. This
395 may reflect a poorer capacity of parasitized females to buffer reproduction-associated oxidative
396 increases or a potential delayed effect of parasites. Generally, similar flea infestation studies
397 have largely found no results of parasites on oxidative stress (Devevey et al., 2008; Maronde et
398 al., 2018; Wegmann et al., 2015). That said, our finding of an interaction between parasitism
399 and oxidative stress where others have not is not surprising given the multifaceted and non-
400 linear relationship between the two (Costantini and Møller, 2009).

401

402 **Conclusion**

403 We attempted to quantify the previously variable costs of parasitism in CGS by
404 discriminating between short and long-term effects (Asghar et al., 2015). However, it became
405 clear that the cognizance of CGS to prioritize immediate grooming of parasites was likely the
406 reason for the initial low level of fleas (Raveh et al., 2011, 2015) and the lack of seasonal effects
407 of our experimental parasite manipulation. This unexpectedly strong grooming response
408 coupled with oxidative shielding likely resulted in the dampening of any physiologically
409 detectable parasite effects, even during the energetically demanding reproductive period.
410 Some subtle short-term effects do manifest but are unlikely to culminate in detrimental long-
411 term fitness consequences unless parasitemia is chronically sustained. As such, fleas, even
412 when experimentally augmented to increase their impact, do not strongly affect these hosts.
413 This result suggests that while hosts in poor condition may exhibit high flea loads, flea
414 infestation is unlikely to debilitate hosts. Seasonal variance in parasite levels over many species
415 coupled with larger processes (i.e. oxidative shielding T1-T3) temporarily masking costs may

416 result in studies overlooking parasite effects due to a short detection timeframe. Given the
417 current direction of climate change, it is eminently possible for parasite prevalence to increase
418 (Cohen et al., 2017), and with it, the manifestation of these subtle costs. As such, short-term
419 physiological measurements may be a better approach than long-term fitness estimates to
420 detect parasite costs in wild populations.

421

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432 **Conflicts of interest**

433 The authors declare no competing or financial interests.

434 **Author contributions**

435 JDR, FSD and VAV designed the study; JDR, PU, FSD and FC collected the data; JDR, AZ, and FC
436 did the laboratory work; AB and AZ provided expertise on DLW; JDR and VAV analyzed the data;
437 JDR wrote the manuscript. All authors gave feedback and final approval for publication.

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446

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653 **Figure Legends:**

654 **Fig. 1: (A) Initial and (B) Final flea numbers in Columbian ground squirrels between April and**
655 **August 2018.** T1 = Gestation (16c, 15t), T2 = Early lactation (14c, 14t), T3 = Peak lactation
656 (14c, 15t), T4 = Prior to hibernation immergence (11c, 13t). Data are medians±s.e.m. (*, **,
657 ***) indicate significant differences between groups (≤ 0.05 , ≤ 0.01 , ≤ 0.001 , respectively).

658 **Fig. 2: Time spent Grooming (s) in Columbian ground squirrels in Columbian ground squirrels**
659 **between April and August 2018.** T1 = Gestation (16c, 15t), T2 = Early lactation (14c, 14t),
660 T3 = Peak lactation (14c, 15t). Data are means±s.e.m. Tukey letters above boxes detail if
661 there are significant differences between time-points.

662 **Fig. 3: Regression of Age (y) on Daily Energy Expenditure (kJ d^{-1}).** Effects of age on DEE are
663 plotted with a light grey 95% confidence interval.

664 **Fig. 4: Impact of Daily Energy Expenditure (kJ d^{-1}) on (A) OXY and (B) ROM concentrations in**
665 **plasma.** Effects of DEE on oxidative stress are plotted with a light grey 95% confidence
666 interval.

667 **Fig. 5: (A) Log Nitric Oxide (μM) and (B) Trypanosome Count in Columbian ground squirrels**
668 **between April and August 2018.** T1 = Gestation (16c, 15t), T2 = Early lactation (14c, 14t),
669 T3 = Peak lactation (14c, 15t), T4 = Prior to hibernation immergence (11c, 13t). Data are
670 medians±s.e.m. Trypanosome count is per 5 μl of buffy coat.

671 **Fig. 6: (A) ROM concentration ($\text{mg H}_2\text{O}_2 \text{ dl}^{-1}$) and (B) OXY concentration ($\mu\text{mol HCl ml}^{-1}$) in**
672 **Columbian ground squirrels between April and August 2018.** T1 = Gestation (16c, 15t), T2
673 = Early lactation (14c, 14t), T3 = Peak lactation (14c, 15t), T4 = Prior to hibernation

674 immergence (11c, 13t). Data are medians±s.e.m. Tukey letters above boxes detail presence
 675 of significant differences between groups.

676 **Table 1: Seasonal data.** T1 = Gestation (16c, 15t), T2 = Early lactation (14c, 14t), T3 = Peak
 677 lactation (14c, 15t), T4 = Prior to hibernation immergence (11c, 13t). Data are means±95%
 678 CI. Cohen's *d* is given, with 0.2 being a small effect size, 0.5 being a medium effect size, and
 679 0.8 being a large effect size.

Variable	Control	Treatment	Cohen's <i>d</i>
Age (y)	4.07 ± 0.40	4.13 ± 0.43	0.03 (-0.35, 0.4)
Littersize	1.714 ± 0.37	1.64 ± 0.33	-0.04 (-0.41, 0.34)
Litterweight	179.21 ± 36.99	195.87 ± 40.96	-0.13 (-0.5, 0.24)
Temperature (°C)	13.94 ± 0.29	13.67 ± 0.28	0.27 (-0.54, 1.08)
(T3) Mass change 24H (g)	4.5 ± 6.6	-17 ± 6.67	1.02 (0.02, 2.02)
(T3) Mass change 72H (g)	3.5 ± 8.5	-2.22 ± 8.38	0.22 (-0.75, 1.19)
Mass			
T1 Mass (g)	552.81 ± 11.36	563.33 ± 8.36	-0.27 (-1.00, 0.47)
T2 Mass (g)	538.21 ± 15.61	540 ± 16.28	-0.03 (-0.81, 0.75)
T3 Mass (g)	529.64 ± 14.21	546.13 ± 19.23	-0.28 (-1.05, 0.48)
T4 Mass (g)	545.45 ± 19.93	550.38 ± 19.61	-0.07 (-0.92, 0.78)

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