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

Abstract:

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

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

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

## Introduction

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

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

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

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

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

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

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

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

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

## Methods

### *Ethics statement*

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

### *Population monitoring*

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

### *Experimental manipulation of fleas*

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

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

***Trypanosomes***

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

***Behavior***

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

***Oxidative status and innate immunity***

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

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

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

*Field protocol:* Daily energy expenditure for treated and control females was determined only during peak lactation (day 25; T3), when reproductive demands on females were expected to be the highest. DEE was estimated using the doubly labeled water (DLW) technique, as extensively described elsewhere (Kenagy et al., 1990; Rimbach et al., 2018), including in CGS (Skibiel et al., 2013). Briefly, females were weighed (to the nearest 5 g using a spring scale; 1 kg, Pesola Ag, Baar, Switzerland) and a first blood draw (100 µL) was collected from the saphenous vein using a 30-G needle in two 100 µl non-heparinized capillary tubes to establish background levels of $^{18}O$ and $^{2}H$. Capillaries were immediately sealed with a micro-jet flame and stored at room temperature until analyses (within 3 months). Squirrels were then injected intra-peritoneally with a premixed 5 g/kg dose of DLW (10% $H2^{18}O$ and 99.9% $^{2}H_2O$, Cambridge Isotopic Laboratories, Cambridge, MA, USA). After injection, females (n = 26) were held in traps in a quiet, shaded location and covered with a dark cotton pillowcase for 75 min to allow for isotope equilibration (Król & Speakman, 1999; Simmen et al., 2010; Skibiel et al., 2013). Following the equilibration period, another blood sample was drawn (n=26), fleas were added to the experimental animals, and the subjects were released. As part of the DLW test, a subsequent blood sample and weight measurement was taken at 24 h and 72 h post-enrichment (n=26) to estimate isotope elimination rates (Speakman & Racey, 1987). During the DLW experiment, we recorded the average ambient temperature ($T_A$) experienced by individuals to control for potential thermoregulatory effects on metabolic rate. We used thermo-loggering iButtons (DS1921G, Maxim Integrated, San Jose, CA, USA), which recorded $T_A$ with 15 min intervals over the course of the experiment. iButtons were centrally located in the colony, attached to the bases of elevated observation benches, with the iButtons one meter above ground level.

*Isotope analyses:* Sealed capillary tubes were vacuum distilled for 10 mins and the resulting water distillate analyzed by a continuous flow isotope ratio mass spectrometer (IRMS; IRMS-DELTA V PLUS, Thermo, Bremen, Germany) as described previously (Chery et al., 2015; Mahlert et al., 2018). Distillates were pyrolyzed at 1400°C into $H_2$ and $CO_2$ gases in a glassy carbon tube under pure He flow at 90 mL $min^{-1}$. $H^2$ and $CO_2$ were separated at 110°C on a

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

***Statistical Analysis***

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

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

## Results

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

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

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

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

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

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

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

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

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

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

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

## Discussion

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

### *Flea augmentation and grooming*

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

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

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

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

***Conclusion***

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

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

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## Conflicts of interest

The authors declare no competing or financial interests.

## Author contributions

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

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**Figure Legends:**

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

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

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

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

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

**Fig. 6: (A) ROM concentration (mg $H_2O_2$ $dl^{-1}$) and (B) OXY concentration (µ mol HCl $ml^{-1}$) in Columbian ground squirrels between April and August 2018.** T1 = Gestation (16c, 15t), T2 = 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 *d* |
|---|---|---|---|
| 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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