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Effects of ectoparasite infestation during pregnancy on physiological stress and reproductive output in a rodent-flea system

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ABSTRACT

Biotic and abiotic stressors impose various fitness costs on individuals across a variety of taxa. In vertebrates, these stressors typically trigger complex neuroendocrine responses that stimulate glucocorticoid (GC) secretion from the adrenal cortex. Short-term elevation of GCs can be adaptive as it shifts energy toward physiological processes that cope with acute stressors; however, chronic increases in GC levels could have detrimental effects on fitness. Parasitism can be considered an important biotic stressor in nature and a possible cause of reproductive failure that could substantially affect an individual's fitness. Thus, we aimed to test the effects of parasitism and maternal stress, as measured by GCs, during pregnancy and the relationship between these variables and measures of reproductive output using a rodent-flea system. Female Egyptian spiny mice (*Acomys cahirinus*) were randomly assigned to flea (*Parapulex chephrenis)* infested or uninfested treatments before and during pregnancy. The offspring of these females were flea-free. Feces were collected at five time points during the experiment to determine maternal fecal glucocorticoid metabolite (FGCM) concentrations. Overall, infested females had lower FGCM levels during gestation but higher FGCM levels post-parturition and larger mass changes than uninfested females. Additionally, models related to pup quality and quantity often included some measure of maternal investment or body condition moderating relationships between infestation and stress. This suggests that flea parasitism or high GC levels alone might not significantly impact host reproduction but rather females can experience different effects depending on their level of investment, which could be limited by body condition and/or the number of pups present in a litter.

1. Introduction

The environmental conditions experienced by an individual organism can have wide-ranging effects on its fitness. Both abiotic stressors, such as drought or heat, and biotic stressors, such as interspecific competition or disease, impose fitness costs on individuals belonging to various taxa (e.g., Sapolsky, 1986; Svensson et al., 1998; Persons et al., 2002; Eccard and Ylonen, 2003; Meyer et al., 2003; Creel et al., 2009; Wingfield, 2013). In vertebrates, the classic physiological stress response is illustrated by a stressor activating the hypothalamic–pituitaryadrenal (HPA) axis and this, in turn, triggers a complex neuroendocrine response that stimulates the adrenal cortex to secrete glucocorticoids (GCs) (Munck et al., 1984). Depending on the species in question, either cortisol (e.g., primates, canids, felids) or corticosterone (e.g., birds, some rodent species) could be the major stress GC (Palme et al., 2005). Ultimately, short-term elevation of GCs is adaptive because it shifts energy from some physiological processes, such as digestion and reproduction, toward others that provide more immediate mechanisms to cope with acute stressors (Munck et al., 1984; Wingfield et al., 1998). However, prolonged increases in GC levels are correlated with higher mortality rates in the wild (Pride, 2005), while elevation of GC levels due to chronic stressors can impact immunity (e.g., McEwen, 1998; Marketon and Glaser, 2008; Tort, 2011) and reproduction (e.g., Dobson and Smith, 2000; Tilbrook et al., 2000; Whirledge and Cidlowski, 2010). Reproductive effects, including delay in the age of first reproduction (Crespi et al.,

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2013), reduction in the number of reproductive bouts (Hackländer et al., 2003), and decreases in offspring quantity (Hayward et al., 2011) and/or quality (Schreck et al., 2001), are significant fitness costs associated with chronic increases in GC levels.

Parasitism can be considered a biotic stressor; however, the relationship between parasitism and GCs becomes somewhat challenging to uncover with observational data because an individual's risk of becoming parasitized could either be a cause or a consequence of a chronically elevated stress response (e.g., Beldomenico et al., 2008). For example, inducing stress by isolating normally social large vesper mice (*Calomys callosus*) was associated with higher levels of *Trypanosoma cruzi* infection (Santos et al., 2008), while baseline circulating corticosterone concentration and nematode infestation were significantly positively related in male laboratory mice (Malisch et al., 2009). Similarly, territorial male Alpine chamois (*Rupicapra rupicapra*) had both higher fecal GC and lungworm burdens than non-territorial males (Corlatti et al., 2012). GCs are also positively correlated with species richness and/or abundance of intestinal helminths in various non-human primates (Muehlenbein et al., 2004; Martínez-Mota et al., 2016; Akinyi et al., 2019). This suggests that a prolonged increase in stress hormone levels could also increase host susceptibility to parasites. On the other hand, infection with nematodes (*Anguillicola novaezelandiae*) increased cortisol levels in European eels (*Anguilla anguilla*), suggesting that parasites can directly increase host physiological stress (Dangel et al., 2014). Indeed, St. Juliana et al. (2014) found that flea infestation increased GC levels in rodent hosts. In this case, the type of host exploitation used by different flea species, as well as the evolutionary history of association between host and flea species, contributed significantly to host fecal GC metabolite concentrations (St. Juliana et al., 2014). Thus, parasitism can be thought of as a biotic stressor that could affect an individual's fitness (e.g., Hare et al., 2010; Koop et al., 2013; Gonzalez-Warleta et al., 2014; Hurtado et al., 2016). However, it should be noted that relationships between parasitism and different types of stressors found in nature can be complex (Puehringer-Sturmayr et al., 2018) and responses might vary from species to species (Hammond et al., 2019). For example, helminthiasis has been found to negatively impact female baboon (*Papio anubis*) fertility with individuals hosting species-rich helminth communities exhibiting longer interbirth intervals than individuals hosting species-poor helminth communities (Akinyi et al., 2019). Although parasitism is ubiquitous in nature and has potentially significant effects on host reproduction, relatively few experimental studies have assessed the relationships between physiological stress responses, parasitism, and variables related to reproductive fitness in pregnant mammals.

Here, we used an experimental rodent-flea system to test the relationship between parasitism and maternal GC levels during pregnancy and the effects of these variables on measures of reproductive fitness (i.e. number, sex ratio, and growth of offspring). We used Egyptian spiny mice, *Acomys cahirinus*, and their characteristic fleas, *Parapulex chephrenis*, which are rarely found on rodents other than *Acomys* spp. in nature (Krasnov et al., 1999). We measured GCs via fecal glucocorticoid metabolite (FGCM) concentrations (Palme, 2019), in pregnant females from both infested and non-infested treatment groups. St. Juliana et al. (2014) demonstrated that parasitized females of this species exhibited higher GC levels than uninfested females. Additionally, high GCs during pregnancy have been linked to low litter size and low offspring quality, and skewed sex ratios with high maternal GCs during gestation correlated with male-biased litters in rodents (Ryan et al., 2014). Therefore, we predicted that females infested with fleas would have smaller litters and/or offspring with lower birth mass, weaning mass, or less mass gained before weaning than the offspring of uninfested females. Finally, we predicted that infested females would have litter sex ratios that favored male pups whereas litters from uninfested females would have similar numbers of male and female pups.

2. Materials and methods

2.1. Study animals

Rodents (*A. cahirinus*) and fleas (*P. chephrenis*) originated from our laboratory colonies, started with individuals collected from the field. Specific details regarding the routine maintenance of these colonies have been published previously (e.g. Krasnov et al., 2001, 2002, Khokhlova et al., 2009a, b). Prior to experiments, individual rodents were housed in plastic cages (28 cm × 20 cm × 13 cm) at 25 °C ± 1 °C air temperature and a 12:12 h dark:light regime. They were provided with wood shavings as bedding material, whole millet seeds ad libitum as a food source, and fresh alfalfa daily as a water source. In addition, animals received commercial cat chow (Nestlé Purina, Société des Produits Nestlé S.A., Switzerland) once each week as a protein source. For our experiments, we used nulliparous female *A. cahirinus* between five and 8 months of age. Although age has not been shown to have a significant effect on FGCM levels in this species (Nováková et al., 2008), we used animals that were considered young, sexually mature adults. In addition, none of these females had been previously exposed to flea infestation.

2.2. Experimental design

Prior to experiments, 36 females were randomly assigned to either infested (n = 18) or uninfested (i.e. control, n = 18) treatment groups and were placed in 33 cm × 26 cm × 16 cm plastic cages. Fifty fleas, representing a standardized infestation of approximately one flea per gram of rodent body mass, were selected at random from our laboratory colonies and released into each cage of females belonging to the infested group. Rodents were free to groom; therefore, we expected approximately 50% of fleas per week to be dislodged and killed by a host while the remainder would senesce shortly thereafter (Hawlena et al., 2007). Thus, every week 50 new fleas were added to each cage to keep flea pressure relatively consistent. Additionally, rodents were transferred to clean cages every 2 weeks to eliminate exposure to newly hatched fleas and weighed every day during the experimental period to track pregnancy.

Two weeks after initial infestation, one male was added to every female's cage. For infested treatment groups, 100 fleas were added so that there would be a sufficient number of fleas to infest both males and females. Males and females were housed together for 2 weeks to allow successful copulation. One week after introducing a male, another set of 100 fleas was added to each cage for the infested treatment. After 2 weeks, male rodents were removed and we collected all fleas from their bodies via brushing their pelage with a toothbrush over a white plastic pan. Then, males were returned to their respective laboratory colony while females were placed in new cages and again infested with 50 fleas weekly until shortly before parturition (approximately the thirty-fifth day of pregnancy as determined by the pattern of female mass gain; (Nováková et al., 2010). Fleas were removed from females using a toothbrush as described above and females were transferred to individual, flea-free cages before giving birth. Thus, females were not infested during lactation and no pups were ever infested during the experiment. All animals belonging to uninfested or infested treatment groups experienced the same handling procedures (i.e. either sham cleaning or cleaning, respectively).

2.3. Analysis of FGCMs

Collection of feces for analysis of FGCMs followed the protocol of Frynta et al. (2009). In short, females were placed into individual fecal collection cages at specific time points in the experimental schedule (Supplementary Table S1) that corresponded to time points within the study such as baseline (T_0), pre-parturition (T_1, T_2, T_3), and post-parturition (T_4, T_5) measurements. FGCM measurements represent an approximately 24 h timescale of GC secretion in this species (Nováková et al., 2008). The bottoms of these collection cages were lined with paper and three wire screens were placed over the paper liner to separate the animal from the feces. Animals moved freely around the collection cage and were allowed to defecate for 2 h. As FGCM concentrations in rodents can vary depending on the time of day (Sipari et al., 2017), all fecal collections occurred within the same 3 h window (i.e., 13:00–16:00), with the exception of fecal collection after birth (T_4) as the collection time depended on when pups were born and parturition does not follow a set schedule. Fecal pellets were obtained from adult females between approximately two and 4 h after birth (T_4). Fecal pellets were also obtained from adult females within a 2 h window immediately after weaning (T_5). After fecal collection, females were removed from feces collection cages and either placed in their experimental cages (T_0-T_2), cleaned and moved to flea-free cages (T_3), returned to clean cages (T_4), or returned to the main rodent colony (T_5).

After collection, fecal pellets were placed in 1.5 ml SafeLoc microcentrifuge tubes (Eppendorf, Hamburg, Germany), following the sample preparation procedures outlined in Warburton et al. (2020). In short, moisture was removed from feces by placing tubes in a 60 °C drying oven overnight and then tubes were stored in a freezer at −20 °C until the end of the experimental period. Then FGCMs were extracted using procedures of Touma et al. (2003, 2004) with a few slight modifications outlined in Warburton et al. (2020). This method produced fecal residue containing FGCMs that lined the interior of the microcentrifuge tubes and these tubes could then be safely stored at room temperature until they were used for FGCM analysis.

We followed the methods of Touma et al. (2003, 2004) for FGCM analysis, in which fecal residue was reconstituted by adding 500 µl of 80% methanol to each tube, vortexed for 1 min, and then aliquots of reconstituted supernatant were diluted 1:10 with assay buffer (Tris/HCl 20 mM, pH 7.5) in new titer tubes and frozen at −20 °C until analyzed in an established group-specific enzyme immunoassay (5α-pregnane-3β,11β,21-triol-20-one EIA). This EIA measured metabolites with a 5α-3β,11β-diol structure and its details, as well as cross-reactions with different steroids, and can be found in Touma et al. (2003). The intra- and interassay coefficients of variation were below 10.0% and 12.0%, respectively. Cortisol, not corticosterone, as in laboratory rodents, is the major stress hormone for the genus *Acomys* and an appropriate adrenocorticotropic hormone challenge test was used to validate the above protocol for *A. cahirinus* (Nováková et al., 2008). All experimental protocols met the requirements of the 1994 Law for the Prevention of Cruelty to Animals (Experiments on Animals) of the State of Israel and were approved by the Ben Gurion University (Israel) Committee for the Ethical Care and Use of Animals in Experiments (Permits IL-72-10-2012 and IL-36-07-2017).

2.4. Statistical analyses

First, we determined if FGCM levels differed significantly between treatment groups at different time points by using a generalized linear mixed model with the dependent variable of FGCM, predictor variables of time point and treatment group, and animal identity as a random effect using the function "lmer" in the package "lme4" (Bates et al., 2015) of R (R Core Team, 2019, R Foundation for Statistical Computing, Vienna, Austria) with corresponding ANOVA and multiple comparisons. If this was the case, then we analyzed the effects of treatment group and female FGCM levels on litter size, litter sex ratio (number of males divided by total litter size; (Wilson and Hardy, 2002)), pup birth mass, pup weaning mass, and pup mass gain from birth to weaning (=difference between body mass at weaning and body mass at birth) at that time point using general linear models via the function glm within the base "stat" package of R. See Supplementary Table S2 for a summary of variables and the rationale for their inclusion in maternal and offspring models.

We analyzed the effect of parasitism on deviation of maternal FGCM from baseline during gestation for time points where FGCM significantly differed between treatment groups (e.g. FGCM concentration at T_x – FGCM concentration at T_0), litter size, sex ratio, and total female mass change (a proxy for female investment during gestation and lactation) over the course of the experiment (i.e. body mass at T_5 – body mass at T_0) via generalized linear models using the function "lmer" implemented in the R package "lme4" (Bates et al., 2015). For the dependent variables of litter size and sex ratio (each considered separately), explanatory variables were female mass at pairing (proxy for female body condition at fertilization; Trivers and Willard, 1973; (Huck et al., 1988)), deviation of maternal FGCMs from baseline at T_x, and treatment group.

For offspring, we tested the effects of treatment on birth mass, weaning mass, and mass gain. We applied linear mixed-effects models using the function "lmer" in package "lme4". Each model included litter size as a fixed effect. Because *A. cahirinus* that lose body mass during flea infestation have higher FGCM levels than those that do not (St. Juliana et al., 2014), and this change in body mass could impact a female's ability to provision for her offspring, mass change was also included. Given that pups could originate from the same litter, maternal identity was included as a random factor for all pup-related models. Sexual dimorphism of both body size and growth rate are well-documented in these rodents (Koffler, 1972; Nováková et al., 2010), therefore, we held the effects of sex constant in all pup-related models. Additionally, birth mass was held constant in models of weaning mass.

Visual inspection of residual plots did not show deviations from homoscedasticity or normality. Initially, we constructed a model with all possible terms and interactions for each dependent variable. Then, we selected the best model using Akaike Information Criterion corrected for sample size with the function "dredge" of the R package "MuMIn" (Barton, 2016). If the best-fit model for a dependent variable was an intercept-only model, we pursued no further analysis of that dependent variable. If best-fit models included interactions between terms, we then we used the SPSS macro PROCESS (Hayes, 2018) to perform tests of moderation to investigate these interactions. In short, these tests determine if the significance of the relationship between predictor and outcome variables changes depending on the value of a given moderator variable. For example, very low values of a moderator might not have a significant effect on the relationship between the predictor and outcome variables; however, very high values might have a significant effect. In addition, the effects of more than one moderator can be tested, if necessary (see Supplementary Fig. S1 for a conceptual diagram of possible models for tests of moderation). Additionally, we used a logit transformation for proportional data such as sex ratios (Wharton and Hui, 2011).

3. Results

3.1. Contribution of infestation to maternal variables

In total, 33 of 36 females in the experiment gave birth (17 of 18 females in the infested treatment group and 16 of 18 females in the

control group). We found that uninfested and infested mothers had similar mean litter sizes and mean numbers of female and male pups in a litter (Fig. 1). However, FGCM levels differed between infested and uninfested mothers in relation to experimental time point (Supplementary Table S3). Although females from each treatment group had similar FGCM concentrations at T_0 (baseline), T_1 (early pregnancy), T_3 (late pregnancy), and T_5 (weaning), they differed at T_2 (mid-pregnancy) and T_4 (post-parturition) (Fig. 2). Thus, we included deviation from baseline at T_2 in models related to pre-parturition reproductive output (i.e. litter size, sex ratio, birth mass) and T_4 in models related to pre-parturition reproductive output (i.e. weaning mass, mass gain).

Infested mothers had a greater mean mass change (-6.202 g ± 0.943) compared with uninfested mothers (-2.388 g ± 1.149), which was significant over the course of their pregnancies according to a t-test (t = 2.517, df = 55, P = 0.015). When comparing the top-ranked models (Supplementary Table S4), the best-fit model for litter size was the intercept-only model, which essentially functioned as the null model. However, we found that the best-fitting model of litter sex ratio included predictor variables of treatment group, female mass, FGCM levels at T_2, and interactions between these variables (Table 1). Results from tests of moderation indicated that these interactions were significant and terms relating to female mass significantly influenced the effect of treatment group and FGCMs on litter sex ratio (Table 2). For those females with pairing mass that was either 1 S.D. above or below the mean value, treatment group had a significant effect on sex ratio while those at the mean did not experience this effect. In addition, in females whose mass change was either 1 S.D. above the mean value or at the mean value, FGCMs had a significant effect on sex ratio, whereas those females whose mass change was 1 S.D. below the mean did not experience this effect. Tests of moderation for these terms indicated that when pairing mass was lower than average, it interacted with treatment group to produce litters with significantly male-biased sex ratios and when pairing mass was higher than average, it interacted with treatment group to produce litters that were significantly female-biased. In addition, when mass change was highly negative, it interacted with large FGCM values to produce litters that were significantly male-biased.

3.2. Contribution of infestation to offspring variables

In total, 68 pups resulted from this experiment. We found that pups from uninfested and infested mothers had similar birth mass, weaning mass, and mass gain (Fig. 3). When comparing the top-ranked models (Supplementary Table S4), the best-fit model for birth mass included only litter size, with larger litters having pups with lower birth mass. However, we found that the best-fit model of weaning mass included litter size, mass change, and the interaction between them. In addition, the best-fit model for mass gain included treatment group, mass change, litter size, and two interaction terms (litter size*treatment group and mass change*litter size). Maternal FGCM at T_4 was not present in the best-fit models (Table 1). Results from tests of moderation indicated that these interactions were significant and terms relating to female mass strongly influenced the effect of treatment group and litter size on pup mass (Table 3). For those pups from litters where mass change that was either 1 S.D. above the mean value or at the mean value, litter size had a significant effect on weaning mass while those at 1 S.D. below the mean did not experience this effect (Table 3). Therefore, the effect of litter size on weaning mass occurred in offspring from mothers that experienced either relatively high mass gain or loss. This effect did not occur in those pups whose mothers had little mass loss over the experiment. Similarly, for pups from litters where mass change was either 1 S.D. above or below the mean value, treatment group had a significant effect on mass gain while those at the mean did not experience this effect (Table 3). Pups from parasitized mothers had lower mass gain if their mass change was low and higher mass gain if mass change was high. Thus, maternal treatment group only had an effect on mass gain when mothers had rather large mass change during the experiment. Finally, for pups from litters where mass change was either 1 S.D above the mean value or at the mean value, litter size had a significant effect on mass gain while those at 1 S.D. below the mean did not experience this effect (Table 3) and mothers with high mass change and small litter size had pups with high mass gain.

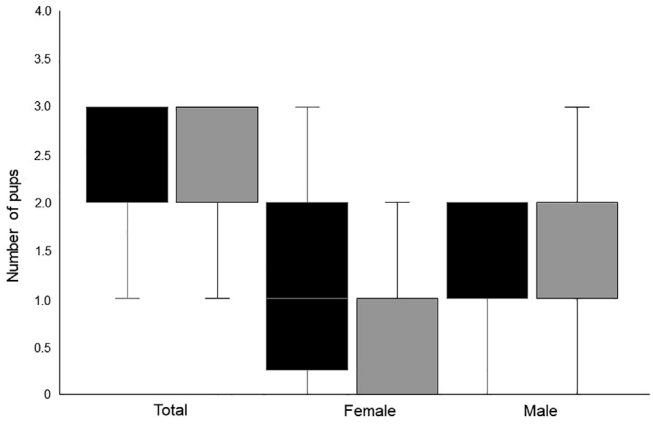

Fig. 1. Boxplot of mean number of total, female, and male pups produced by infested (light bars) and uninfested (dark) female Egyptian spiny mice (*Acomys cahirinus*) in the experiment. Error bars denote standard errors.

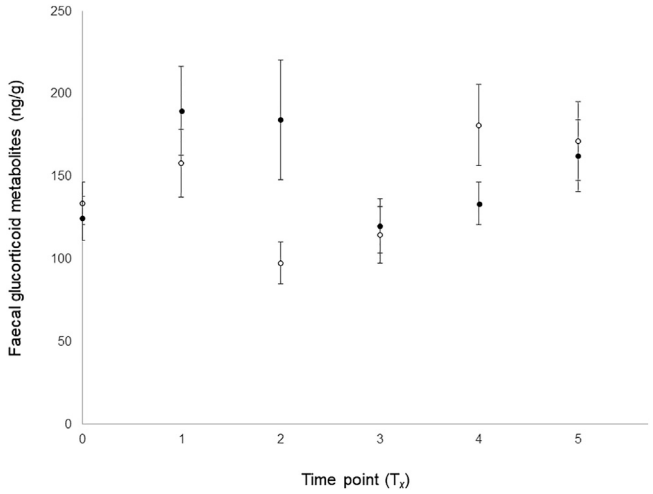

Fig. 2. Mean level of fecal glucocorticoid metabolites found in uninfested (black circles, n = 18) and infested (white circles, n = 18) Egyptian spiny mice (*Acomys cahirinus*) mothers at each experimental time point. Error bars denote standard errors.

4. Discussion

In all, our predictions were partly supported. We predicted that parasitized females would have higher FGCM levels than unparasitized females, and thus, when compared with the control group, females infested with fleas would have: (i) litter sex ratios that favored male pups, (ii) smaller litters, and/or (iii) offspring with lower birth mass, weaning mass, or less mass gained before weaning. Generally, we found that infested females had lower mean FGCM concentrations mid-pregnancy but higher mean FGCM con-

Table 1
Best fit models for fitness-related response variables in Egyptian spiny mice (*Acomys cahirinus*) under infested or flea-free conditions. Intercept-only models are considered the null model. Maternal response (T_2) variables include litter size (LS), change in fecal glucocorticoid metabolite concentration from baseline over the course of gestation (FGCM), and litter sex ratio (SR). Offspring response (T_4) variables include pup mass at birth (PBM), pup mass at weaning (PWM), and pup mass gained before weaning (PMG). Maternal mass change during the course of the experiment (MC), maternal treatment group (TG), and maternal mass at pairing with males (PM) are included with their corresponding models. Effects of pup sex were held constant in all offspring models. Only those model terms included in the highest-ranked model are given. See Supplementary Table S5 for top-ranked models of all response variables.

Response Variable	Model terms	$F_{(df,df)}$	P	R^2_{adj}
Maternal				
LS	Intercept only	$1.252_{(1,26)}$	0.309	0.117
SR	TG + PM + MC + FGCM + PM*TG + MC*FGCM	$6.658_{(1,26)}$	0.0002	0.515
Offspring				
PBM	LS	$26.317_{(1,64)}$	0.0001	0.371
PWM	MC + LS + MC*LS	$19.187_{(1,64)}$	0.0001	0.665
PMG	TG + MC + LS + LS*TG + MC*LS	$2.905_{(1,64)}$	0.041	0.346

Table 2
Results of tests of moderation in Egyptian spiny mice (*Acomys cahirinus*) under infested or flea-free conditions for interactions between the moderator variable of female pairing mass (PM) and the predictor variable of treatment group (TG) and interactions between female mass change over the course of the experiment (MC) and maximum change of fecal glucocorticoid metabolite concentration from baseline over the course of gestation (FGCM) in the best-fit model of litter sex ratio (SR). These results show the significance of the effect of the predictor variable on SR at moderator values at one standard deviation below the mean (-S.D.), the mean, and one standard deviation above the mean (+S.D.). Table includes standard error of the mean (S.E.M.).

Outcome	Predictor	Moderator	Moderator Level	Centered Value	S.E.M.	t	P
SR	TG	PM	−S.D.	−7.763	0.443	2.360	0.026
			Mean	0	0.309	0.166	0.870
			+S.D.	7.763	0.437	−2.156	0.040
SR	FGCM	MC	−S.D.	−6.274	0.002	−2.363	0.025
			Mean	0	0.001	−0.859	0.367
			+S.D.	6.274	0.002	1.035	0.309

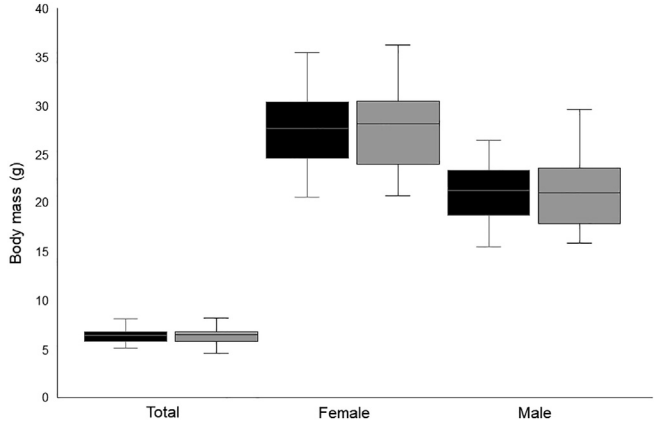

Fig. 3. Boxplot of mean amount of mass of pups from uninfested (dark bars, n = 18) and infested (light bars, n = 18) Egyptian spiny mice (*Acomys cahirinus*) mothers in the experiment at birth and weaning as well as the amount of mass gained during lactation. Error bars denote standard errors.

centrations just after birth compared with uninfested females. Additionally, infested females had larger mean mass changes over the course of pregnancy than uninfested females. Therefore, models of sex ratio, pup quantity, and pup quality, that are themselves related to elements of host reproductive output, generally included interactions between these terms and some measure of maternal investment or maternal body condition at conception often moderating the relationship between predictor and outcome variables. This suggests that flea parasitism or high GC levels alone might not significantly impact reproductive fitness in their hosts but rather females can experience different effects depending on their level of maternal investment, which can be limited by their body condition and/or the number of pups present in a litter. Additionally, females in poorer condition could have a lower capacity to mount an appropriate stress response after perceiving a stressor, which is also considered deleterious. For example, horses (*Equus caballus*) in poor condition and exhibiting depressive-like behaviors had lower fecal and plasma GC concentrations, likely because exposure to chronic stressors can change physiology within the body and brain so that it no longer responds appropriately to these stressors after a prolonged period of time (Pawluski et al., 2017).

A similar study comparing offspring quality and quantity between infested and uninfested jirds (*Meriones crassus*) and *A. cahirinus*, found that infested *M. crassus* mothers invested more in male offspring growth than uninfested mothers when litters were small; however, there was no significant difference between *A. cahirinus* pups (Warburton et al., 2017). The authors posited that perhaps *A. cahirinus* might not exhibit these differences because: (i) *A. cahirinus* females have a much longer gestation period and shorter lactation period than *M. crassus* and therefore they could have fewer opportunities for postnatal investment than *M. crassus*, or (ii) flea infestation might not be as stressful in *A. cahirinus*, leaving them less likely to provision offspring for a risky environment. Here we see, however, that flea infestation causes some physiological stress responses in *A. cahirinus* mothers and some sort of pre- or post-natal maternal investment takes place; however, this investment is heavily mediated by maternal condition. Additionally, Warburton et al. (2017) did not note any differences between sex ratios of litters from infested and uninfested mothers. However, here we have evidence that sex ratio was impacted by maternal FGCM levels and treatment group, again with these relationships moderated by maternal condition. Warburton et al. (2017) did not measure FGCM levels and thus, did not include it as a possible predictor of litter sex ratio. However, as our results suggest, including this measure could be useful in future studies of the effects of parasites on sex allocation.

Indeed, using FGCM, measures of maternal condition and parasite infestation, as well as the interaction between them, as predictors of host litter sex ratio could also provide novel insight into how two possible mechanisms behind offspring sex allocation operate. Skewed sex ratios with high maternal GCs during gesta-

Table 3
Results of tests of moderation for two offspring models in Egyptian spiny mice (*Acomys cahirinus*) under infested or flea-free conditions. In the best-fit model of pup mass at weaning (PWM) the interaction between the moderator variable of maternal mass change over the course of the experiment (MC) and the predictor variable of litter size (LS) was tested. Interactions between maternal treatment group (TG) and LS as well as MC and LS in the best-fit model of litter sex ratio (SR) were also tested. These results show the significance of the effect of the predictor variable on SR at moderator values at one standard deviation below the mean (-S.D.), the mean, and one standard deviation above the mean (+S.D.). Table includes standard error of the mean (S.E.M.).

Outcome	Predictor	Moderator	Moderator Level	Centered Value	S.E.M.	*t*	*P*
PWM	LS	MC	-S.D.	−6.274	2.429	1.145	0.262
			Mean	0	1.823	−2.469	0.020
			+S.D.	6.274	2.477	−4.753	0.001
PMG	TG	MC	-S.D.	−4.218	1.249	1.863	0.067
			Mean	0	0.878	−0.300	0.765
			+S.D.	4.218	1.244	−2.294	0.025
PMG	LS	MC	-S.D.	−4.218	0.686	0.346	0.731
			Mean	0	0.544	−4.253	0.001
			+S.D.	4.218	0.780	−6.244	0.0001

tion correlated with male-biased litters in rodents (Ryan et al., 2014); however, according to the Trivers and Willard (1973) hypothesis of sex allocation, mothers in poorer condition should have female-biased offspring sex ratios. Our results indicate that depending of the level of female body condition at conception (i.e. pairing mass in the present experiment) and the level of maternal investment over pregnancy and lactation (i.e. maternal mass change in the present experiment) sharply influence the effect of GC levels (i.e. FGCM concentrations in the present experiment). Therefore, part of the reason why support for the Trivers-Willard (1973) hypothesis of sex ratio adjustment is inconsistent both outside (Cameron, 2004) and within studies involving parasites (Kankova et al., 2007a,b; Fisher, 1999; Dama et al., 2016) could be because useful predictor variables are not being considered. Additionally, as stated in Warburton et al. (2017), host-parasite systems do not often meet the very specific set of assumptions of the Trivers-Willard hypothesis (Trivers and Willard, 1973) and thus might not provide good tests of it.

Changes in stress hormone levels during pregnancy are well documented in many mammals (Edwards and Boonstra, 2018) including rodents (Robinson et al., 1989; Atkinson and Waddell, 1995). Gestation itself is considered an important physiological stressor (Foley et al., 2001; Rolland et al., 2005) with stress hormone levels typically increasing steadily during pregnancy and then dropping sharply after parturition (de Weerth and Buitelaar, 2005). Proximally, high glucocorticoid concentrations are likely due to physiological changes induced by the placenta, chorion, and amnion serving to help meet metabolic demands during gestation (Foley et al., 2001). All three tissues produce corticotropin-releasing hormone (CRH) which modulates maternal and fetal pituitary-adrenal function (de Weerth and Buitelaar, 2005). Placental CRH likely stimulates maternal secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland which, in turn, leads to increases in glucocorticoid levels throughout gestation (Goland et al., 1994; Wadhwa et al., 1997; de Weerth and Buitelaar, 2005). From an evolutionary perspective, high maternal glucocorticoid levels have several other benefits. Corticosteroids can promote fetal brain and lung development during late pregnancy (Crowley et al., 1990) and influence the start of mechanisms behind the process of labor (Valenzuela-Molina et al., 2018). Higher prenatal glucocorticoid levels in pregnant females have also been linked to more attentive maternal responses after birth (Bardi et al., 2004). Thus, high GC levels during pregnancy might not be necessarily deleterious. However, the ways in which mothers can cope with increased physiological stress during pregnancy might be limited if they are in poor body condition, as the present study suggests.

Given that increased GC concentrations during pregnancy are linked to the metabolic demands of gestation (Foley et al., 2001; Soma-Pillay et al., 2016), the relationship between maternal body condition and GCs during pregnancy, and possible tradeoffs between the two, are logical (e.g. Butte and King 2005). It was previously found that, in the *A. cahirinus* – flea system, GC levels changed during pregnancy and birth, albeit not significantly (Warburton et al., 2020). Indeed, FCCM concentrations rose as pregnancy progressed, then fell after females gave birth, yet infested females did not exhibit higher GC levels than their uninfested counterparts. Our results supported a significant interaction between the stressors of parasitism and social contact; however, flea infestation alone did not have a significant effect on female FGCM levels (Warburton et al., 2020). This suggests that the single potential stressor of flea infestation might be mitigated by an individual mother's physiological state and maternal condition is a reasonable preliminary candidate for mediating this relationship. However, more studies are necessary to better tease apart these interactions.

It is also worth noting that in this experiment we did not test various combinations of infestation with other stressors such as limited resource availability. Indeed, multiple stressors are found in nature and including them could lead to different outcomes. For example, *A. cahirinus* that decrease in mass over the course of flea infestation have higher FGCM concentrations than those individuals whose mass remains more or less constant (St Juliana et al., 2014). Animals were fed ad libitum in our experiments; therefore, their reproductive response to GCs and flea parasitism could differ from food-restricted individuals. Further, field results suggest that elevated GC levels alone might not have a significant impact on measures of reproductive output, such as litter survival, in nature (e.g. van Kesteren et al., 2019). Additionally, we did not examine infestation lasting longer than 8 weeks and a chronic infestation that lasts for years could produce results that are more pronounced.

In conclusion, we found that parasitized females had lower mean GC levels during gestation than unparasitized females; however, they also had higher GC levels post-parturition and larger mean mass changes over the course of the experiment than unparasitized females. Additionally, models of sex ratio, pup quantity, and pup quality included interactions between these terms, with some measure of maternal condition often moderating the relationship between predictor and outcome variables. This suggests that females might be able to somewhat mitigate the effects of flea parasitism on host reproductive output in their hosts, depending on their level of maternal investment. However, if maternal investment does provide some mitigating effect, previous results suggest that there could be an upper ceiling to this investment delineated by female body condition and/or the number of pups present in a litter (Warburton et al., 2017). Thus, expanding this line of research to include multiple potential stressors, including parasitism, which

animals commonly experience in the environment during their lifetime would further elucidate the relative roles that parasites and other stressors play in the sub-lethal fitness effects hosts experience in nature.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2020.12.005.

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