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Food restriction and chronic stress alter energy use and affect immunity in an infrequent feeder

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Summary

1. Glucocorticoids are important mediators of energy utilization for key physiological processes, including immune function. Much work has focused on the effects of energy limitation and stress for key physiological processes such as reproduction and immunity. However, it is unclear how stress alters energy use across different energy states, and the physiological ramifications of such effects are even less clear.

2. In this study, we altered energy and stress states of an infrequent feeder, the terrestrial gartersnake (*Thamnophis elegans*), using fasting and repeated restraint stress (chronic stressors) to test how these challenges interacted to affect immune function, energy metabolites and glucocorticoid reactivity (a traditional indicator of stress state) to restraint stress, a standardized, acute stressor. After this acute stressor, the snakes which had received chronic stress had increased glucocorticoid reactivity, and both treatments altered energy metabolite use and storage. Evidence of interaction of food restriction and chronic stress treatments on innate immune function and energy metabolites (triglycerides and glycerol) suggests that stress alters energy use in a manner dependent on the energy state of the animal.

3. Snakes have a remarkable ability to maintain functionality of key physiological processes under stressful conditions but are still susceptible to multiple simultaneous stressors, a situation increasingly prevalent in our ever-changing environment.

Key-words: bactericidal ability, corticosterone, energy metabolites, food restriction, gluconeogenesis, reptiles, snakes, stress reactivity

Introduction

Most organisms have a finite amount of energy with which to fuel all of the processes of life (Nagy, Girard & Brown 1999). Most healthy individuals are able to maintain a 'dynamic equilibrium' in which energetic expenditure is roughly equal to energetic intake (McCue 2010). Yet often in an individual's life, external or internal perturbations, or stressors, result in a negative energy balance (Wingfield 2005). Energy limitation can cause changes in resource allocation among non-essential processes (French *et al.* 2007; Lucas & French 2012).

Stressors can include predictable changes (e.g. diel shifts and changing seasons) but also unpredictable challenges, such as a loss of food supply (Wingfield 2005, 2013). Prolonged food deprivation can be particularly detrimental to an animal for two primary reasons: (i) there is a decrease or cessation in energy input, and/or (ii) the physiological response to ongoing food deprivation, a type of chronic stressor, involves the long-term release of energy-mobilizing glucocorticoids (GCs) from the adrenal glands (Chowers, Einat & Feldman 1969; Kitaysky *et al.* 2001; Wingfield & Romero 2001). These hormones are beneficial during an acute stress response, but chronic exposure will eventually redirect all available energy from other energetically costly physiological processes, such as reproduction (Vitousek *et al.* 2010), immune function (Lochmiller & Deerenberg 2000), growth (Peterson, Walton & Bennett 1999) and towards protecting the central nervous system (Sapolsky, Romero & Munck 2000). If food deprivation occurs for an extended period of time, the organism will experience severe muscle wasting, increased susceptibility to infection and disease and ultimately death (Selye 1946; Wingfield 2005).

Because the release of GCs exacerbates the existing shortage of energy during starvation, the level of GCs can provide valuable insight as to how organisms cope with

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this severe stressor and how organisms shift their limited energy (Romero & Wikelski 2001; Angelier & Wingfield 2013). Glucocorticoids, such as corticosterone (CORT), can be used to assess the unpredictability of food availability in a natural environment (Fokidis *et al.* 2012). However, GC levels alone do not provide adequate representation of the overall health of an organism due to the variable and multifaceted nature of GC secretion (Breuner, Patterson & Hahn 2008; Dickens & Romero 2013). To address this complexity, hypothalamic–pituitary–adrenal axis activity must be examined in conjunction with other physiological processes that directly influence the condition and fitness of the organism, such as immunocompetence (Breuner, Patterson & Hahn 2008; Lucas & French 2012; Dickens & Romero 2013).

Immune system efficacy is one of the most important measures in determining the health of an organism (Lochmiller & Deerenberg 2000). It is also highly stress sensitive (Dhabhar *et al.* 2012). During an acute stress event, enhanced immune defence is typically activated by an increase in GCs (Dhabhar & McEwen 1997). The increase in immune components, such as cytokines, causes a further increase in GCs which then acts as negative feedback on the immune system and begins immunosuppression (Dobbs *et al.* 1996; Dhabhar *et al.* 2012). Innate immunity, such as bactericidal ability in the plasma, can provide information about a variety of immune components such as phagocytes, opsonizing proteins and natural antibodies (French & Neuman-Lee 2012). Measuring the circulating levels of immune cells (bactericidal ability) in conjunction with examining cutaneous wound healing can provide information on the action of the immune cells that have aggregated at the site of infection (Smith & Barker 1988; French, Matt & Moore 2006; Dhabhar *et al.* 2012; Neuman-Lee & French 2014). In the context of this study, the high energetic costs associated with immunity make it an ideal metric for energetic prioritization and energy shifts (Lochmiller & Deerenberg 2000).

When undergoing food deprivation, animals must utilize existing resources. Organisms undergoing food deprivation will typically utilize carbohydrates initially, followed by fat stores, and finally will begin to catabolize proteins and nucleic acids (Cherel, Stahl & Le Maho 1987; McCue 2010). Measuring energy metabolites is critical to elucidating the use of these energetic stores (Jenni *et al.* 2000; Seaman, Guglielmo & Williams 2005; McCue 2007b). Concentrations of plasma metabolites, such as triglycerides, glycerol, proteins and ketones, can indicate energy utilization (McCue 2010; Fokidis *et al.* 2011). These metabolites can indicate chronic stress but also can rapidly change, which allows for the detection of acute stressors (Fokidis *et al.* 2011). As an added benefit in the context of stress physiology, triglycerides are a measure of gluconeogenic activity and therefore can complement the more traditional measurement of glucocorticoid concentrations (Fokidis *et al.* 2011).

Up to now, studies on immune and endocrine consequences of food deprivation and starvation have focused primarily on avian and mammalian models that use entirely different energetic strategies than other vertebrates, such as squamate reptiles (Nakamura *et al.* 1990; Kitaysky *et al.* 2001; Wingfield 2005; Dhabhar *et al.* 2012). Little research has examined ectothermic organisms which regularly undergo energetic limitations and are uniquely adapted to fast for long periods of time, such as many species of snakes (Secor & Diamond 1998). Reptiles provide an alternative model to examine how stress and energetic state interact to influence an animal's endocrine and immune systems.

Gartersnakes (Genus *Thamnophis,* Reptilia: Colubridae) are an ideal organism for this type of study because of their feeding strategies. Most snakes eat large prey relative to their bodies, but infrequently (Secor & Diamond 1998). To facilitate this strategy, snakes can reduce their already low metabolic rate to accommodate energetic constraints (Hulbert & Else 1981; McCue 2007a). They accomplish this partly by downregulating the energetic costs of maintaining the digestive tract during periods of fasting (Secor & Diamond 2000) through a suite of physiological and morphological changes (Secor, Stein & Diamond 1994; Starck & Beese 2002), allowing snakes to maintain physiological processes even after prolonged periods of fasting (McCue 2007a,b). Even with these adaptations, snakes must still allocate limited resources among multiple costly physiological processes, potentially resulting in energetic trade-offs.

In this study, we examined the physiological effects of fasting, chronic restraint stress and their interaction on stress reactivity and innate immune function in terrestrial gartersnakes (*Thamnophis elegans*). To provide insight into resource utilization, we measured baseline metabolite concentrations (true triglycerides, glycerol, ketones and proteins) at the end of a 40-day fasting period (baseline) and then again following a 30-min acute stress challenge (post-acute challenge). We hypothesized that baseline levels would show differences in energetic metabolites with fasted snakes having lower levels of triglycerides, chronically stressed snakes having higher levels of glycerol and all groups having similar levels of ketones and proteins. We hypothesized the chronically stressed snakes would have higher levels of corticosterone, and snakes undergoing chronic stress, food restriction or both would have suppressed bactericidal ability and decreased per cent of wound healed. We hypothesized that, relative to baseline levels, post-acute stress (i) glycerol concentrations would decrease in chronically stressed snakes and increase in non-chronically stressed snakes, (ii) triglycerides and ketones would increase regardless of stress-state or food restriction, and (iii) protein concentrations would not change. We predicted that post-acute stress corticosterone levels would be higher in the chronically stressed snakes. Finally, we hypothesized an increase in bactericidal ability in all groups after the acute stress challenge.

Materials and Methods

ANIMAL MODEL

We collected 37 male terrestrial gartersnakes (*Thamnophis elegans*) from Cache Valley, Utah, in September 2011. Only males were used in this study to remove the potential variability of resource allocation to reproduction as females in this population do not reproduce every year (L. Neuman-Lee, unpublished data). Snakes were individually overwintered in dark temperature-controlled chambers (5 °C) until April 2012 to reduce negative or confounding effects of captivity and then removed from the chambers and housed individually in 37·8-L glass aquaria with newspaper substrate, a water dish and a plastic shelter filled with moist peat moss. Heat tape at one end of the aquarium provided a thermal gradient. The room was kept at 26 °C on a 12:12 on: off light cycle. All procedures and protocols were approved by the USU IACUC (protocol #2299).

EXPERIMENTAL DESIGN

We randomly assigned snakes to four treatment groups: no stress/food (NS/F, $n = 10$), no stress/no food (NS/NF, $n = 10$), Chronic stress/Food (CS/F, $n = 8$) and chronic stress/no food (CS/NF, $n = 9$). After randomization, we conducted an analysis of variance on snake body condition (computed as the residual of the regression of snout-vent length (SVL) on mass; Ujvari & Madsen 2006) comparing the four treatments and showed no difference between groups. There was additionally no differences in SVL or mass ($F_{(3,34)} = 0{\cdot}57$, $P = 0{\cdot}64$ and $F_{(3,34)} = 0{\cdot}61$, $P = 0{\cdot}61$, respectively). Snakes were offered thawed mice twice before food restriction began. Each snake consumed at least one meal prior to starting the food restriction regime. The food restriction protocol was implemented for 40 days. Because snakes being stressed daily (our chronic stress protocol) typically will not eat (L. Neuman-Lee, personal observation), chronic stress manipulations were not added until day 29 and continued through day 40 (Fig. 1).

The snakes in the food treatment (NS/F and CS/F) groups were given 20% of their body weight in thawed, previously frozen mice weekly while the snakes in the no food treatment (NS/NF and CS/NF) groups were fasted (Fig. 1). Mice that were not eaten were removed after 24 h and recorded. Each snake in the chronic stress treatment (CS) groups was removed from its cage daily and placed into an opaque, breathable bag for 30 min, after which the snake was replaced in its cage. All snakes in the NS group remained in their aquaria, which were covered while the researchers were present to ensure that human presence did not cause a stress response. All snakes were weighed weekly when cages were cleaned.

ACUTE STRESS CHALLENGE

To obtain baseline samples of (i) free glycerol, (ii) total triglycerides, (iii) the ketone, β-hydroxybutyrate, (iv) total protein (v) CORT and (vi) bactericidal ability, all snakes were removed from their cages on the final day of the experiment (day 40), and within a minute, a blood sample was taken from the caudal vein (Romero & Reed 2005). The snake was then placed in a separate, opaque, breathable bag for 30 min after which the snakes were removed and a second blood sample, post-acute stress sample, was collected (Fig. 1). All blood sampling was completed between 0900 and 1100. The samples were kept on ice until all samples were collected and then centrifuged at 2200 rpm for 10 min. The plasma was separated and frozen at −20 °C until further analysis.

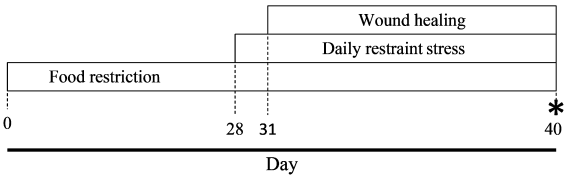

Fig 1. Timeline of experimental procedures testing the effects of food restriction and chronic stress on stress reactivity, immune function and plasma metabolite levels in male gartersnakes (*Thamnophis elegans*). The asterisk on day 40 indicates the stress reactivity test (baseline and post-acute stress samples).

METABOLITE ASSAYS

To assess how stress and food deprivation alter the usage of energy fuels from different physiological stores, we measured plasma levels of the four metabolites listed above in both baseline and post-acute stress samples: (i) free glycerol, indicating the breakdown of liver and adipose triglyceride stores; (ii) total triglycerides, the circulating levels of both free glycerol and triglycerides; (iii) the ketone, β-hydroxybutyrate, which correlates with the oxidation of fatty acids (a component of triglycerides) and (iv) total protein, a measure of overall circulating albumin and globulins, indicative of starvation and muscle wasting.

Free glycerol was measured using a spectrometric endpoint assay, and then, on the same assay, for the same samples, total triglycerides were measured (for details, see Guglielmo *et al*. 2002; Fokidis *et al*. 2011, 2012). The 'true' triglyceride concentration was defined as the difference between the total triglycerides and free glycerol concentrations and indicates deposition into adipose tissue. Plasma concentrations of ketones and proteins were determined using a commercial colorimetric assay (#700190, Cayman Chemical Co., Ann Arbor, MI, USA) and a Bradford assay modified for plasma (see Bradford 1976; Okutucu *et al*. 2007), respectively. Assay sensitivities were 0·16 mM (glycerol and triglycerides), 0·01 mM (ketones) and 0·93 g/dL (proteins). The mean intra- and interassay coefficients of variation were as follows: 5·9 and 10·1% for glycerol; 6·7 and 9·8% for triglycerides; 7·8 and 16·4% for ketones; and 3·8 and 6·9% for proteins. Validations included tests for parallelism between a standard curve and serially diluted samples derived from pooled gartersnake plasma (all $P > 0{\cdot}069$) and recovery of spiked samples (79 ± 11·4%, 82% ± 16·1, 88% ± 7·4 and 94% ± 10·7 for glycerol, triglycerides, ketones and proteins, respectively).

RADIOIMMUNOASSAY FOR CORTICOSTERONE

Circulating corticosterone concentrations in both baseline and post-acute stress samples were determined using a radioimmunoassay protocol modified from French, Fokidis & Moore (2008). Samples were extracted using a solution of 30% ethyl acetate: isooctane in duplicate for CORT (MP Biomedicals, Lot #3R3PB-19E). Final concentrations were calculated by averaging the duplicate samples and adjusted using individual recoveries (average 76·1%). Intra-assay variation was 11·3% and precision was 90·9%.

BACTERICIDAL ASSAY

We performed the bactericidal assay in baseline and post-acute stress samples following the protocol outlined in French & Neuman-Lee (2012). Briefly, we combined a 1:5 plasma dilution with CO_2-independent media plus 4 nM L-glutamine, 10^4 colony-producing units *Escheria coli* (EPowerTM Microorganisms #483-237-1, ATCC 8739, MicroBioLogics, St. Cloud, MN, USA)

and agar broth on a 96-well microplate. We incubated the plate for 12 h and calculated absorbance using a microplate reader (300 nm, Bio-Rad Benchmark, Hercules, CA, USA).

CUTANEOUS BIOPSY

On day 31, 3 days after initiation of chronic stress treatment, each snake was given a cutaneous biopsy on its dorsal surface using a 3·5-mm biopsy punch (Miltex Instruments, York, PA, USA). Based on a previously validated protocol (French, Matt & Moore 2006), we waited 3 days to ensure that the snakes were in the experimental stress state, given that the initial healing period is so critical. The punch was lightly twisted, and the circular portion of skin was removed using forceps. All wounds were photographed immediately after being administered. After securing the snake with Velcro restraints, a ruler was placed in the same plane as the wound for scale, and the wound was photographed with a digital camera. At the termination of the experiment (day 40), another image was taken in the same manner. Previous studies in reptiles found that 9 days were adequate to detect stress-related differences in wound healing (French, Matt & Moore 2006; Neuman-Lee & French 2014). Images were analysed using ImageJ v. 1.48 (National Institute of Mental Health, Bethesda, MD, USA) to estimate wound area. Wound measurements were blinded such that the investigator did not know treatment assignments.

STATISTICAL ANALYSES

To assess the effects of chronic stress and fasting treatments on change in body mass over the course of the experiment, we used a two-way factorial in a completely randomized design with repeated measures in a generalized linear mixed model. The effects of chronic stress and fasting treatments on baseline values of each plasma measure, bactericidal ability and wound healing were assessed using a two-way factorial in a completely randomized design, both with and without initial body condition incorporated as a covariate, using a generalized linear model. Pairwise comparisons of means among the four treatment combinations were adjusted for inflated type I error using the Tukey–Kramer method. To evaluate the impact of the acute stress challenge on each plasma measure and bactericidal ability, we used a two-way factorial in a completely randomized design with repeated measures, both with and without body condition incorporated as a covariate, using a generalized linear mixed model. For each response, we omitted snakes for which we did not have both baseline and post-acute stress data. Tests of acute stress challenge effect (i.e. the difference between post-stress and baseline values) for all fasting and stress treatment combinations were adjusted using the step-down Bonferroni method. Pairwise comparisons of acute stress challenge effects among the four treatment combinations also were adjusted using the stepdown Bonferroni method. Bactericidal ability and wound healing (measured as the proportion of initial wound area minus final wound area to initial wound diameter) were analysed using a beta-distribution with a logit link; protein, free glycerides, ketone, true triglycerides and corticosterone were analysed using a normal distribution following transformation ($\log_e$ for mass, protein and glycerol; square root for ketone, triglyceride and corticosterone) to better meet assumptions of normality and homogeneity of variance. Two baseline observations were omitted from the analysis of protein due to unrealistic values that could not be resolved. Body condition was not a

Table 1. Analysis of results at baseline and following acute stress challenge. Significant findings are denoted with an asterisk

	Degrees of freedom	Food	Stress	Food* Stress	Time	Food*Time	Stress*Time	Food*Stress*Time
Baseline								
Triglycerides	$F_{(1, 33)}$	15·30	13·59	48·27	–	–	–	–
	P	<0·001*	<0·001*	<0·001*				
Glycerol	$F_{(1, 33)}$	32·76	19·29	1·44	–	–	–	–
	P	<0·001*	<0·001*	0·239				
Ketones	$F_{(1, 33)}$	1·52	0·67	2·47	–	–	–	–
	P	0·226	0·419	0·125				
Protein	$F_{(1, 31)}$	0·86	0·71	0·59	–	–	–	–
	P	0·361	0·406	0·447				
Corticosterone	$F_{(1, 31)}$	3·33	0·01	0·35	–	–	–	–
	P	0·078	0·926	0·557				
Bactericidal Ability	$F_{(1, 33)}$	1·50	1·38	0·00	–	–	–	–
	P	0·229	0·249	0·949				
Wound Healing	$F_{(1, 33)}$	1·76	4·94	0·01				
	P	0·194	0·033*	0·938				
Acute Stress Challenge								
Triglycerides	$F_{(1, 33)}$	38·64	17·71	24·75	3·11	4·14	0·05	17·87
	P	<0·001	<0·001	<0·001	0·087	0·050*	0·831	<0·001*
Glycerol	$F_{(1, 33)}$	64·05	4·63	0·00	1·53	1·60	13·23	3·02
	P	<0·001	0·039	0·956	0·224	0·215	<0·001*	0·092
Ketones	$F_{(1, 33)}$	5·55	0·39	0·67	31·30	4·18	0·20	2·50
	P	0·025	0·538	0·417	<0·001	0·049*	0·657	0·123
Protein	$F_{(1, 31)}$	0·24	0·44	0·28	0·65	1·15	0·43	5·22
	P	0·628	0·514	0·602	0·427	0·291	0·518	0·029*
Corticosterone	$F_{(1, 26)}$	0·14	6·93	0·11	143·99	1·72	8·55	1·41
	P	0·714	0·014	0·747	< 0·001	0·201	0·007*	0·245
Bactericidal Ability	$F_{(1, 30)}$	3·43	3·05	3·18	2·31	0·38	0·08	6·02
	P	0·074	0·091	0·085	0·139	0·541	0·784	0·020*
Mass	$F_{(1, 33)}$	2·16	0·69	0·36	15·55	26·16	5·21	3·20
	P	0·151	0·413	0·552	<0·001	<0·001	0·029	0·083

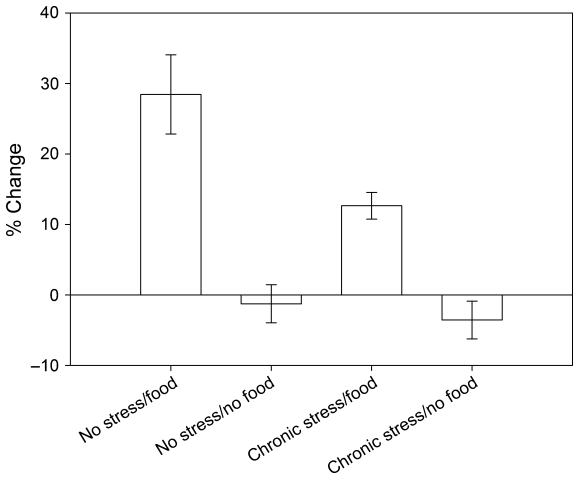

Fig 2. Mass change in male gartersnakes (*Thamnophis elegans*) as a consequence of treatment with food restriction, chronic stress, neither or both. Bars indicate mean ± 1 standard error computed as descriptive statistics based on the raw data.

significant factor in any analysis ($P > 0{\cdot}139$); thus, we reported here models without body condition. The baseline bactericidal ability between the NS/F and CS/NF group was analysed using a Kruskal–Wallis test.

All analyses were performed using the GLIMMIX procedure in SAS/STAT 13.2 in the SAS System for Windows 9.4 (SAS Institute Inc., Raleigh, NC, USA), with the exception of the initial body mass and SVL analyses and the Kruskal–Wallis test, which was conducted on JMP 11.0 (SAS Institute Inc., Raleigh, NC, USA).

Results

BODY MASS AND ENERGY METABOLITES

Fed (F) snakes gained more mass than non-fed (NF) snakes, and CS snakes gained less mass than NS snakes (Table 1; Fig. 2). There is some evidence that the effect of food on mass increase was enhanced in NS snakes.

Both fasting and chronic stress increased glycerol baseline concentrations. Relative to baseline concentrations, post-acute stress concentrations decreased for snakes experiencing chronic stress and increased for snakes experiencing no chronic stress (Tables 1 and 2, Fig. 3a).

The true triglyceride baseline concentration was influenced by the interaction of the fasting and chronic stress treatments (Table 1). The NS/NF group had lower concentrations of true triglycerides than the other three groups, the CS/F had intermediate concentrations, and the NS/F and CS/NF groups had highest concentrations (Table 2, Fig. 3b). The difference in concentrations between baseline and post-acute stress samples was affected by the interaction of fasting and stress treatments (Table 1). Relative to the baseline, the post-acute stress concentrations increased for the NS/NF and CS/F groups, decreased for the CS/NF group and had no apparent change for the NS/F group (Fig. 3b).

Table 2. Metabolite concentrations in baseline and post-acute stress plasma samples taken from male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Delta values are the mean differences between baseline and post-acute stress measurements. Values are means ± 1 standard error computed as descriptive statistics based on the raw data

	Baseline				Post-acute stress				Delta			
Treatment	Glycerol (mM)	True triglyceride (mM)	Ketone (mM)	Protein (g/dL)	Glycerol (mM)	True triglyceride (mM)	Ketone (mM)	Protein (g/dL)	Glycerol (mM)	True Triglyceride (mM)	Ketone (mM)	Protein (g/dL)
No stress/food (NS/F) $n = 10$	0·41 ± 0·04	1·41 ± 0·11	0·14 ± 0·01	1·10 ± 0·03	0·55 ± 0·07	1·39 ± 0·13	0·31 ± 0·06	1·11 ± 0·02	0·14 ± 0·09	0·0 ± 0·18	0·17 ± 0·05	0·004 ± 0·02
No stress/no food (NS/NF) $n = 10$	0·91 ± 0·05	0·28 ± 0·08	0·15 ± 0·01	1·11 ± 0·04	1·07 ± 0·07	0·45 ± 0·07	0·19 ± 0·02	1·16 ± 0·04	0·16 ± 0·07	0·17 ± 0·10	0·04 ± 0·02	0·05 ± 0·05
Chronic stress/food (CS/F) $n = 8$	0·80 ± 0·09	0·95 ± 0·09	0·24 ± 0·07	1·11 ± 0·02	0·46 ± 0·09	1·77 ± 0·25	0·32 ± 0·05	1·17 ± 0·01	−0·3 ± 0·15	0·82 ± 0·25	0·08 ± 0·04	0·06 ± 0·02
Chronic stress/no food (CS/NF) $n = 9$	1·43 ± 0·19	1·39 ± 0·18	0·13 ± 0·01	1·18 ± 0·06 ($n = 7$)	1·17 ± 0·13	0·96 ± 0·13	0·20 ± 0·03	1·11 ± 0·03 ($n = 7$)	−0·26 ± 0·11	−0·4 ± 0·12	0·07 ± 0·03	−0·07 ± 0·06 ($n = 7$)

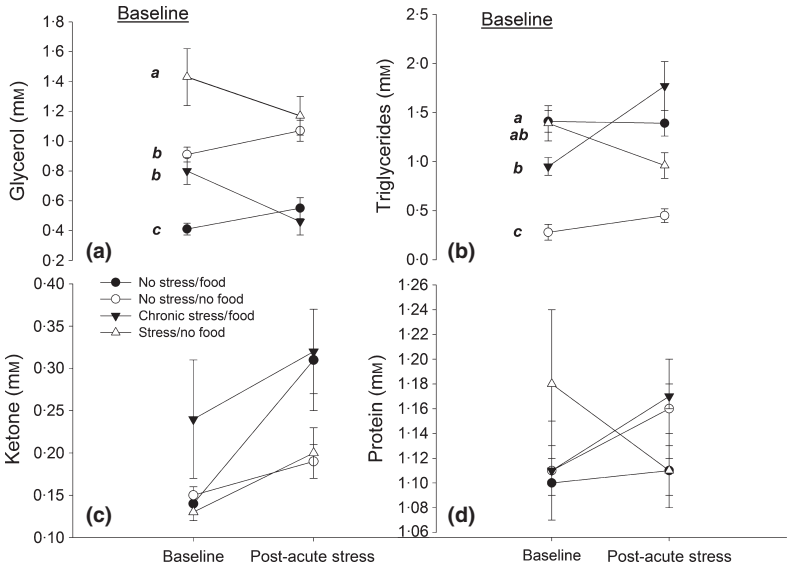

Fig 3. Mean (±1 standard error) metabolite levels at baseline and following acute stress in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both: (a) glycerol, (b) true triglycerides, (c) ketone and (d) protein. Values are computed as descriptive statistics based on the raw data. In panels a and b, different letters (lower case) indicate significant differences at baseline.

There was no evidence of a fasting or chronic stress effect on baseline concentrations of ketone (Tables 1 and 2, Fig. 3c). Relative to baseline concentrations, post-acute stress concentrations increased in fed snakes.

Protein baseline concentrations were not significantly affected by fasting or chronic stress (Tables 1 and 2, Fig. 3d). Although analysis suggested that the response to the acute stress challenge was affected by the interaction of fasting and chronic stress, post hoc analyses indicated that this result merely reflected noise in the data and was not biologically meaningful.

STRESS REACTIVITY

Corticosterone baseline concentrations were slightly higher in fed snakes, and there was no evidence of any effect of chronic stress (Tables 1 and 3, Fig. 4). Relative to baseline concentrations, post-acute stress concentrations increased, and the increase was greater for snakes that were chronically stressed.

IMMUNE FUNCTION

Baseline bactericidal ability did not differ among fasting or chronic stress regimes; however, the analysis between the NS/F and CS/NF revealed a higher bactericidal ability in the NS/F group (Tables 1 and 3; Fig. 5). The response to the acute stress challenge was affected by the interaction of fasting and chronic stress. There was no evidence of a change from baseline to post-acute stress for any treatment group other than CS/NF, for which bactericidal ability increased. Relative to baseline concentrations, the increase in bactericidal ability in unfed snakes under chronic stress was shown to be different than the change in fed snakes under chronic stress (Tables 1 and 3; Fig. 6).

The proportion of wound healed was greater for non-stressed snakes (Table 1, Fig. 7).

Discussion

Overall, the results of our study indicate that chronic stress and food restriction play important roles in how an organism responds to acute stress. Even in snakes, which are infrequent feeders, food restriction had important physiological consequences. The metabolite levels in snakes in the food restricted groups indicated that they were not undergoing severe energetic limitation because they had not started to utilize protein stores. NF snakes also had not lost substantial body mass, but F snakes had significantly higher increases in body mass. Because snakes can reduce the amount of energy they invest in maintaining their gut (Secor, Stein & Diamond 1994), it is likely that these individuals had enough energy to shift to other processes, such as their immune system. However, snakes that were chronically stressed were likely secreting more CORT continuously over the treatment period (each time they were removed from their enclosure and stressed) and were therefore more energetically compromised and could not invest as much in their immune function, resulting in suppressed immunity. The dramatic increase in baseline glycerol in chronically stressed snakes compared to the non-stressed, fed snakes provides strong evidence of gluconeogenesis.

Glycerol, which is indicative of the utilization of adipose stores, is a key substrate for gluconeogenesis (Guglielmo, Cerasale & Eldermire 2005). Snakes that were chronically stressed and fasted had the highest levels of baseline and post-acute challenge glycerol, which corroborates our hypothesis that more energy was mobilized in snakes that were likely experiencing higher levels of corticosterone on a consistent basis. It also demonstrates that snakes were energetically impacted by the 40-day food restriction, even if there was not a dramatic decrease in body mass. The chronically stressed snakes had decreased total glycerol after the acute stress, while the non-chronically stressed snakes had increased total glycerol. An increase in glycerol after acute

Table 3. Physiological measurements in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Values are means ± 1 standard error computed as descriptive statistics based on the raw data

Treatment	CORT (ng/mL) Baseline	Bactericidal ability (%)	CORT (ng/mL) Post-acute Stress	Bactericidal ability (%)	CORT (ng/mL) Delta	Bactericidal ability	% Wound healed
No stress/food (NS/F) $n = 10$	8·54 ± 1·2 ($n = 9$)	93·40 ± 6·6	27·77 ± 3·5 ($n = 9$)	100 ± 0 ($n = 9$)	19·23 ± 3·4 ($n = 9$)	7·34 ± 7·3 ($n = 9$)	51·74 ± 4·0
No stress/no food (NS/NF) $n = 10$	11·99 ± 2·4	74·18 ± 11·2	30·65 ± 6·5 ($n = 9$)	62·44 ± 13·0 ($n = 9$)	19·65 ± 6·1 ($n = 9$)	−8·86 ± 4·4 ($n = 9$)	44·45 ± 4·0
Chronic stress/food (CS/F) $n = 8$	8·08 ± 2·5 ($n = 7$)	74·97 ± 13·3	53·0 ± 5·46 ($n = 5$)	67·38 ± 15·4 ($n = 7$)	43·95 ± 5·2 ($n = 5$)	−4·02 ± 12·5 ($n = 7$)	39·42 ± 6·1
Chronic stress/no food (CS/NF) $n = 9$	12·32 ± 1·7	55·42 ± 14·2	46·25 ± 9·1 ($n = 7$)	81·79 ± 9·84	33·62 ± 8·0 ($n = 7$)	26·38 ± 17·8	36·91 ± 6·6

Fig 4. Mean (±1 standard error) corticosterone concentrations at baseline and following acute stress in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Values are computed as descriptive statistics based on the raw data. The asterisk indicates a significant difference.

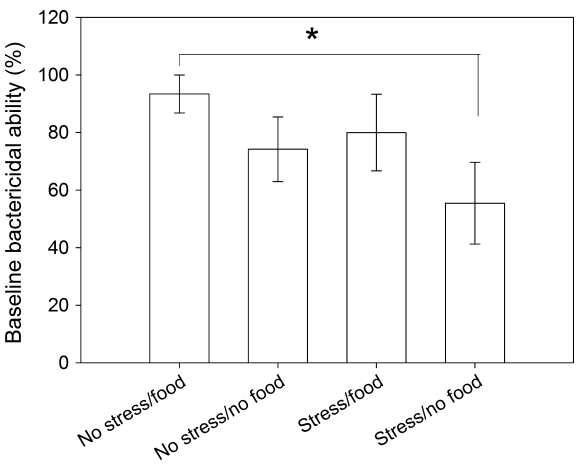

Fig 5. Mean (±1 standard error) bactericidal ability at baseline in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Values are computed as descriptive statistics based on the raw data. Asterisk indicates that the two groups are significantly different ($P = 0{\cdot}026$).

stress also has been observed in mammals (Ricart-Jan *et al.* 2002) due to the fact that catecholamines (e.g. norepinephrine) stimulate glycerol release (Lin 1977). However, animals that are chronically stressed show decreased turnover of catecholamines (Roth, Mefford & Barchas 1982; Konarska, Stewart & McCarty 1989) and plasma glycerol concentrations (Fokidis *et al.* 2011). In our study, the chronically stressed snakes may have had a dampened sympathetic-adrenal medullary response, which caused less mobilization of adipose stores, thus decreasing total glycerol concentration.

Triglyceride levels, which represent the amount of glucose being processed by the liver to be stored as adipose (Fokidis *et al.* 2011), were lower in the fasted, non-stressed snakes at baseline and were reduced in the post-acute stress sample in chronically stressed, non-fed snakes. This reduction in triglyceride levels was likely due to the substantial

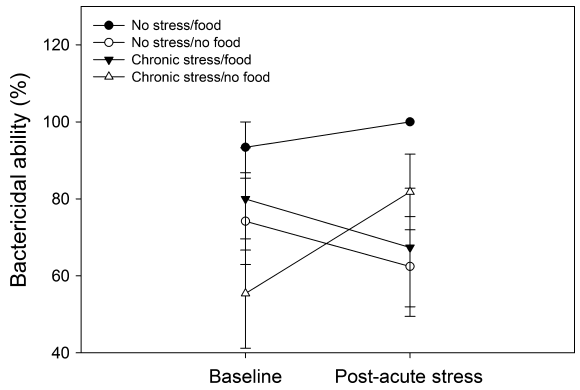

Fig 6. Mean (±1 standard error) bactericidal ability at baseline and following acute stress in male gartersnakes (*Thamnophis elegans*) under food restriction, chronic stress, neither or both. Values are computed as descriptive statistics based on the raw data.

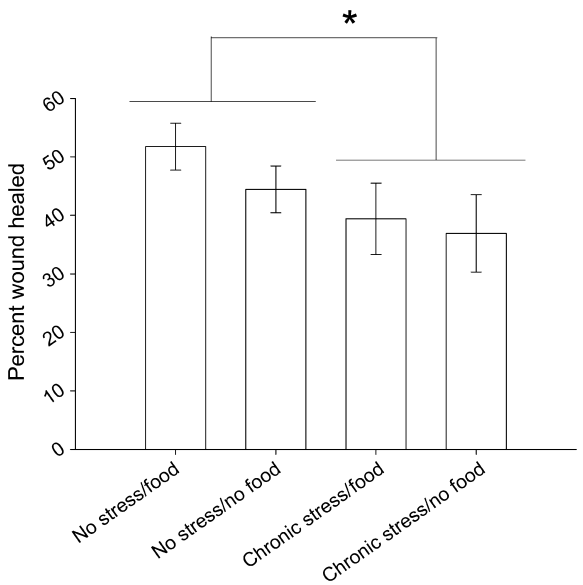

Fig 7. Mean (±1 standard error) per cent area of the cutaneous biopsy healed after 9 days in gartersnake (*Thamnophis elegans*) males under food restriction, chronic stress, neither or both. The asterisk indicates a significant difference between the two stress groups. Values are computed as descriptive statistics based on the raw data.

increase in gluconeogenesis during chronic stress over the 12-day period added to the lack of new energetic resources to mobilize for glucose storage. Conversely, the CS/F snakes increased triglyceride levels significantly after the acute stress challenge. NS/NF snakes had slightly higher levels. An increase in triglycerides after acute stress has been documented (Stoney *et al.* 1988) but contradicts other studies showing that acute stress, regardless of previous stress state, causes a decrease in triglyceride levels (Ricart-Jan *et al.* 2002; Teague *et al.* 2007).

Baseline ketone levels did not differ among treatment groups. We expected to see higher ketone levels in the fasted snakes (Akram 2013); however, given snakes' ability to fast for long periods of time, a 6-week treatment period may not have been long enough. For all treatments, ketone levels increased after the acute stress challenge. This increase after an acute stress is consistent with previous studies conducted with rats (Ricart-Jan *et al.* 2002; Teague *et al.* 2007).

At baseline, protein levels were not different among the treatment groups, nor was there any consistent evidence of effects of fasting or chronic stress on response to acute stress. This provides evidence that snakes, like most animals, preferentially preserve protein for energy during later stages of starvation, which was not reached (Cherel, Stahl & Le Maho 1987; McCue 2010).

In this study, the total CORT released after the acute stress challenge was higher in snakes which had been chronically stressed. While this increase in CORT levels after a stress challenge is consistent (though not universal) with previous reptile studies (reviewed in Moore & Jessop 2003), it also is consistent with findings that the amount of change can vary widely across species, populations and individuals depending upon context (Dickens & Romero 2013). Clearly, an animal will respond differently dependent upon recent stressful events and whether or not it has eaten. Therefore, it is critical to evaluate the CORT response in context of the organism's natural history.

Despite the effects on stress reactivity, there was no evidence that chronic stress affected baseline CORT, and only little evidence that food restriction elevated CORT, which is a traditional stress indicator. Our results demonstrate the need to exercise caution when interpreting baseline CORT levels, because chronic stress treatment did not influence baseline CORT as predicted. Much work has correlated fitness or health of a population to baseline CORT levels across many taxa (Hopkins, Mendonça & Congdon 1997; Marra & Holberton 1998; Romero & Wikelski 2001; Jaatinen *et al.* 2013), although there is strong evidence that fitness and CORT do not necessarily have a predictable relationship (Breuner, Patterson & Hahn 2008; Bonier *et al.* 2009). Our work reinforces the need to examine stress-responsiveness in addition to other fitness-related metrics to more fully determine stress status and its consequences for the organism (Breuner, Delehanty & Boonstra 2013).

There is not a clear consensus in the literature as to what constitutes an 'optimal stress response' (Dickens & Romero 2013), requiring additional measures (Rich & Romero 2005; Lattin *et al.* 2012; Brooks & Mateo 2013; Jessop, Woodford & Symonds 2013). Corticosterone is a mediator for a wide variety of physiological processes, including the immune response (Dhabhar *et al.* 1995, 1996). This relationship between CORT and immune activity can allow us to link the stress response with a functional measure of energetic investment in self-maintenance. For example, acute stress typically corresponds with an increase in immune function when energy is available (Dhabhar 2009), and the individuals in the NS/F treatment in the present study had the most effective bactericidal ability that was further augmented after the post-acute stress increase in CORT, which is consistent with previous

studies (Dhabhar & McEwen 1997; Dhabhar 2002). Chronically elevated levels of CORT typically correspond with a suppression of immune processes (Dhabhar & McEwen 1997). Here, we find that animals with both chronic stress and food restriction have suppressed bacterial killing response relative to the NS/F animals (akin to controls). Additionally, we believe the fact that all but one NS/F snakes had 100% bactericidal killing relative to the assay control for baseline, and then, all NS/F snakes had 100% killing after the acute stress challenge is biologically significant. In further support of this theory, there was a significant interaction of food, stress and time, with snakes experiencing either food restriction, chronic stress or the combination of responding differently via bactericidal ability following an acute stress challenge.

We found consistent results with our other measure of immunity, wound healing. In the present study, snakes undergoing chronic stress had reduced healing ability relative to the non-stressed snakes. Wound healing has been shown to be negatively affected by chronic stress due to the suppression of pro-inflammatory cytokines (Christian *et al*. 2007) and increased CORT levels (Padgett, Marucha & Sheridan 1998; French, Matt & Moore 2006).

Conclusions

This study demonstrates that energetic state, in combination with energy-mobilizing GCs, can affect vital processes for health and survival. Thus, this evidence suggests that stress alters energy use and immunity in a manner dependent on the energy state of the animal. Although an acute stress challenge altered stress reactivity, chronic stress treatment increased reactivity and did not affect baseline CORT concentrations. Baseline glycerol, an indicator of gluconeogenesis, was increased in all snakes, even in the absence of altered baseline CORT. This suggests that hormonal indicators may not detect all important consequences of stress. This work also demonstrates the resilience of snakes in dealing with both stressors and infrequent food supply. These snakes have a remarkable ability to maintain functionality of key physiological processes under stressful conditions but are still susceptible to multiple simultaneous stressors. The combined effects of fasting and repeated stress are probably not far removed from the myriad stressors these snakes encounter under natural conditions.

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Data accessibility

Data deposited in the Dryad Digital Repository: doi:10.5061/dryad.dk166 (Neuman-Lee *et al*. 2015).

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