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Elevated plasma corticosterone increases metabolic rate in a terrestrial salamander

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ABSTRACT

Plasma glucocorticoid hormones (GCs) increase intermediary metabolism, which may be reflected in whole-animal metabolic rate. Studies in fish, birds, and reptiles have shown that GCs may alter whole-animal energy expenditure, but results are conflicting and often involve GC levels that are not physiologically relevant. A previous study in red-legged salamanders found that male courtship pheromone increased plasma corticosterone (CORT; the primary GC in amphibians) concentrations in males, which could elevate metabolic processes to sustain courtship behaviors. To understand the possible metabolic effect of elevated plasma CORT, we measured the effects of male courtship pheromone and exogenous application of CORT on oxygen consumption in male red-legged salamanders (*Plethodon shermani*). Exogenous application of CORT elevated plasma CORT to physiologically relevant levels. Compared to treatment with male courtship pheromone and vehicle, treatment with CORT increased oxygen consumption rates for several hours after treatment, resulting in 12% more oxygen consumed (equivalent to 0.33 J) during our first 2 h sampling period. Contrary to our previous work, treatment with pheromone did not increase plasma CORT, perhaps because subjects used in this study were not in breeding condition. Pheromone application did not affect respiration rates. Our study is one of the few to evaluate the influence of physiologically relevant elevations in CORT on whole-animal metabolism in vertebrates, and the first to show that elevated plasma CORT increases metabolism in an amphibian.

1. Introduction

Glucocorticoid hormones (GCs) are released by the adrenal cortex during stimulation of the hypothalamic–pituitary–adrenal axis (Norris, 2006). Plasma concentrations of GCs may change diurnally, seasonally, and in response to exposure to acute and chronic stressors (e.g. Moore et al., 1991; Coe and Levine, 1995; Wingfield et al., 1998; Romero, 2002). Basal concentrations of GCs are believed to support basic processes, such as maintenance of vascular tone and blood sugar levels (Norris, 2006). Seasonal changes in GCs (e. g. Licht et al., 1983) may function to support behavioral and/or energetic changes associated with cyclic life history events, to suppress processes incompatible with a particular life history event (e.g., reproduction), and/or to prepare an organism for potentially stressful events (Romero, 2002). Finally, exposure to unpredictable threats or challenges (stressors) typically causes an elevation in plasma GCs. Stress-induced elevation of plasma GCs has been linked to behavioral, immunological, and metabolic responses to stressors (e.g. Kitaysky et al., 1999; Amaral et al., 2010; e.g. French et al., 2006; Martin, 2009; Ricciardella et al., 2010).

In temperate-zone amphibians, GCs tend to be elevated during the breeding season, and in response to social cues and stressful stimuli (Leboulenger et al., 1979; Licht et al., 1983; Pancak and Taylor, 1983; Zerani and Gobbetti, 1993; Homan et al., 2002). For example, plasma corticosterone (CORT; the primary GC in amphibians) concentrations were increased in male toads after mating with females compared to single males (Orchinik et al., 1988), in calling male anurans compared to non-calling anurans (Mendonca et al., 1985; Hopkins et al., 1997; Emerson and Hess, 2001; Leary et al., 2004, 2008), and in male red-legged salamanders exposed to pheromones (Schubert et al., 2009). Furthermore, exposure to stressors typically elevates plasma levels of GCs in amphibians (Herman, 1992). Confinement stress, food restriction, handling, and exposure to contaminants increased plasma CORT concentrations in amphibians (Moore and Zoeller, 1985; Hopkins et al., 1997; Glennemeier and Denver, 2002; Crespi and Denver, 2004).

Elevations in CORT have been linked to changes in behavior and immune function in amphibians (Moore and Miller, 1984; Leary, et al. 2006; Martin, 2009), but the effect of CORT on whole animal metabolism is unknown in this class of vertebrates. CORT is directly or indirectly involved in a number of intermediate metabolic pathways

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that involve glucose, amino acid, and lipid metabolism (Mommsen et al., 1999; Norris, 2006). Through each of these pathways, CORT increases blood glucose concentrations in order to meet energetic demands. In the absence of compensatory mechanisms, changes in intermediary metabolism may be reflected in whole-animal metabolism. For example, both acute and chronic increases in CORT resulted in elevated whole-animal metabolic rates in lizards (DuRant et al., 2008; Preest and Cree, 2008). Thus, it is possible that increases in plasma CORT concentrations also influence whole-animal metabolism in amphibians.

Plethodontid salamanders are an excellent amphibian model for examining the function of acute increases in plasma CORT. They are abundant, available for study most of the year, and are amenable to both field and laboratory studies. Plasma levels of CORT increase in plethodontid salamanders in response to capture and/or handling (Schubert et al., 2009; Ricciardella et al., 2010; Woodley and Lacy, 2010), as well as social cues (Schubert et al., 2009). A previous study found that exposure to mental gland pheromones increased plasma levels of CORT in male red-legged salamanders (Schubert et al., 2009). Although mental gland pheromones are typically associated with courtship interactions between males and females (Houck and Verrell, 1993; Rollmann et al., 1999; Houck and Arnold, 2003), the CORT response elicited in males suggests that mental gland pheromones may have additional functions, including upregulation of metabolic processes important for supporting male–male interactions.

In the current experiment, we tested whether elevated plasma CORT might mediate metabolic changes in male red-legged salamanders (*Plethodon shermani*). We tested the hypothesis that courtship pheromones alter metabolic rate via an increase in CORT. We predicted the following: (1) courtship pheromones increase oxygen consumption, (2) courtship pheromones increase plasma CORT concentrations, and (3) elevation of plasma CORT increases oxygen consumption.

2. Materials and methods

2.1. Animal collection and husbandry

Adult male red-legged salamanders (*P. shermani*) were caught by hand in Macon County, North Carolina (83° 33′ 37″ N longitude; 35° 11′ 13″ W latitude) in August 2009, during their breeding season. *P. shermani* are in breeding condition from August to mid-October in the wild. However, their propensity to mate decreases as their time in captivity increases (personal observation). Animals were collected with appropriate permits from the North Carolina Wildlife Commission and US Forest Service. Throughout the experiments, animals were individually housed in 16×16×5 cm plastic boxes lined with moist paper towels, maintained on a 14:10 light:dark cycle at 16 °C, and fed wax worms.

2.2. Experimental design

The effects of male mental gland pheromone and exogenous CORT on oxygen consumption and plasma CORT were examined in two separate experiments in fall 2010. In the first experiment, oxygen consumption was measured using a closed-circuit respirometer at Virginia Tech. After measurement of oxygen consumption at Virginia Tech was completed, animals were transported to Duquesne University. Two weeks later, the effects of male pheromone and exogenous CORT on plasma CORT concentrations were measured in a second experiment. Methods were approved by Duquesne University's and Virginia Tech's Institutional Animal Care and Use Committees.

2.3. Experimental treatments

For each experiment, animals were randomly placed into one of three treatment groups: vehicle control (n = 16), pheromone (n = 16), or CORT (n = 15). Depending on the treatment group, pheromone or PBS vehicle was delivered to the nares of salamanders, and CORT or sesame oil vehicle was delivered transdermally via a minimally-invasive patch that was placed on the dorsum of the salamanders (Wack et al. 2010). Thus, animals in the control group had an oil patch placed on the dorsum and had vehicle (PBS) delivered to the nares, animals in the pheromone group had an oil patch placed on the dorsum and had pheromone delivered to the nares, and animals in the CORT group had a CORT patch placed on the dorsum and had vehicle (PBS) delivered to the nares. Treatments to the nares and the dorsum were delivered simultaneously and took approximately 45 min to apply.

2.3.1. Preparation and application of pheromone

Pheromone consisted of extract from male mental glands (Wirsig-Wiechmann et al., 2002; Schubert et al., 2006). To obtain mental gland extract, a separate group of males was captured and mental glands were removed (for surgical details see Wirsig-Wiechmann et al., 2002; procedure approved by Oregon State University ACUP to Dr. Lynne Houck). Mental glands from multiple males were pooled and pheromone was extracted with acetylcholine-chloride. A pheromone concentration of 1 μg/μL was used because it elevated plasma CORT concentrations (Schubert et al., 2009) and activated vomeronasal sensory neurons in male *P. shermani* (Wirsig-Wiechmann et al., 2002; Schubert et al., 2006). A volume of either 5 μL of pheromone or PBS vehicle was pipetted onto the nares every 5 min for 45 min for a total of 10 applications, following procedures of Schubert et al. (2009).

2.3.2. Application of CORT via dermal patches

We used dermal patches containing CORT to elevate plasma CORT. Patches consisted of a 1.5×3 mm rectangle cut from filter paper (Cat No. 1820–070 from Whatman) that was placed onto the dorsum between the two front legs of the salamander with forceps. Using a pipette, 0.625 μg of CORT was applied to the patch in a volume of 1.25 μL (Cat. no. Q1550-000, Steraloids Incorp.; 0.5 mg/mL). This amount of CORT was half of a quantity previously shown to elevate plasma CORT to high physiological concentrations in *P. shermani* (Wack et al., 2010). Controls received vehicle pipetted onto the patch. Patches were removed with forceps approximately 45 min after initial application.

2.4. Plasma CORT concentrations

To determine the effects of treatments on plasma CORT, treatments were delivered between 1515 and 1545, and trunk blood was collected from animals at 2, 4, or 10 h after patch removal. Blood was centrifuged and the plasma portion was frozen at −20 °C until assayed. CORT concentrations were measured by the Endocrine Services Laboratory at the Oregon National Primate Research Center. Briefly, a double ether extraction was performed on approximately 3 μL of plasma, and CORT concentrations were then determined by standard radioimmunoassay procedures (Resko et al., 1980; Gruenewald et al., 1992). The intra-assay coefficient of variation was 8.5%.

2.5. Oxygen consumption

To determine the effects of treatments on whole-animal metabolism, a computer-controlled, closed circuit respirometer measured the rate (mL/h) of oxygen consumption (hereafter V_{O_2}). Oxygen consumption was measured in salamanders that were individually placed in glass respiratory chambers (150 mL) lined with a Kimwipe moistened with ddH_2O. Incurrent air passed through columns of Drierite® to absorb water before passing into individual respirometry chambers. Air leaving respirometry chambers was dried again using magnesium perchlorate before passing into the oxygen sensor containing an

electrochemical fuel cell. We simultaneously determined CO_2 production rates in each chamber, and oxygen consumption values were corrected for CO_2 concentrations using the MicroOxymax software. Further details of the respirometry system are discussed elsewhere (Hopkins et al., 1999, 2004).

Measurements were made while animals were maintained at 16 °C in constant darkness. Testing was done at least 2 weeks after the last feeding to ensure that animals were post-absorptive (Roe et al., 2005).

Respiration of each animal was measured both before and after treatments. Respiratory measurements began between 0900 and 0930. Prior to treatment with vehicle, pheromone, or CORT, V_{O_2} was measured every 42 min for 6.3 h. At 6.3 h (i.e., between 1515 and 1545), salamanders were removed from their chambers and treated with pheromone, CORT, or vehicle as described above. Immediately after treatments were completed, animals were returned to their chambers and V_{O_2} was measured every 42 min for 15 h. Air in each animal's respiratory chamber was partially refreshed with ambient air at every sample point. After respirometry measurements were completed, each salamander was weighed.

2.6. Analysis

Plasma CORT, V_{O_2}, total oxygen consumption, and mass data were log-transformed to meet assumptions of normality (Kolmogorov–Smirnov, $P<0.05$) and homogeneity of variance (Levene's test, $P<0.05$) for the models described below. Significant ($P\leq0.05$) main and interaction effects were followed by Tukey's post hoc pairwise comparisons. All interaction terms were included in all analyses. Interactions that were not statistically significant are not reported in the results. Because metabolism increases with body mass (Andrews and Pough, 1985), plots present V_{O_2} and total oxygen consumption after having controlled for body mass (least squares means). All data were analyzed with SAS 9.2 (SAS Institute, Carey, NC, USA). Unless otherwise noted, data are presented as mean or LS mean ± 1 SE.

2.6.1. Plasma corticosterone data

Plasma CORT concentrations were analyzed with an ANOVA with treatment and time as main effects (PROC GLM). Mass was initially included in the model as a covariate but was dropped from the final model, because it did not significantly vary with plasma CORT concentrations ($P=0.60$).

2.6.2. Pretreatment respirometry data

To describe the relationship between mass and respiration, we averaged pre-treatment V_{O_2} for each individual and then regressed this against body mass (PROC REG). To determine if treatment groups differed before treatments were applied, V_{O_2} was analyzed with a repeated measures ANCOVA with treatment group as the between subjects factor, and mass as a covariate (PROC GLM). In all V_{O_2} analyses, mass was a significant covariate. Since there were multiple measurements of V_{O_2} during the pretreatment period, time was used as a repeated variable.

2.6.3. Post-treatment respirometry data

To analyze the effects of treatment on respiration, post-treatment measurements were divided into three intervals: 2 to 4, 4 to 10, and 10 to 15 h post-treatment (PROC GLM). Measurements from 0 to 2 h could not be obtained due to the time required to initiate the respirometry trial and obtain initial measurements. The intervals were based on previous observations of how plasma CORT changed after exposure to CORT patches and pheromones (Schubert et al., 2009; Wack et al., 2010). Based on these previous studies, animals treated with CORT patches or pheromone typically had elevated plasma CORT concentrations during the first 4 h (first interval). During the second interval, plasma CORT concentrations in animals treated with CORT patches were typically declining in these prior studies. By the third interval, plasma CORT concentrations had returned to baseline (Schubert et al., 2009; Wack et al., 2010).

Post-treatment respiration data are presented as V_{O_2} (mL/h) and total oxygen consumed (mL). We present the V_{O_2} data to demonstrate the temporal dynamics of how V_{O_2} changed over the post-treatment period. In contrast, total oxygen consumption was calculated as the integral under the respiratory curve for each time period. These data can be directly converted to energy equivalents (assuming 1 mL O_2 = 19.8 J; Secor and Diamond, 1995) over the time intervals described above.

The effect of treatment on V_{O_2} for each interval was analyzed with a repeated measures ANCOVA (PROC GLM), with treatment as the between subjects factor, and mass as a covariate. Each time interval was analyzed separately. Since there were multiple measurements of V_{O_2} within each time interval, time was used as the repeated variable.

The effect of treatments on total oxygen consumption for each interval was analyzed with an ANCOVA with treatment as the between subjects factor and mass as the covariate. Each interval was analyzed separately.

3. Results

3.1. Plasma CORT concentrations

Overall, there was a significant effect of treatment on plasma CORT concentrations (Fig. 1; $F_{2,64}=3.43$, $P=0.038$). Treatment with CORT, but not pheromone, elevated plasma CORT relative to treatment with vehicle control (post hoc comparisons: CORT versus control, $P=0.05$; pheromone versus control, $P=0.87$). The effects of CORT treatment were most evident at 4 h after treatments (Fig. 1; $F_{2,23}=7.65$, $P=0.003$) and males with CORT patches had elevated plasma CORT concentrations compared to males treated with pheromone and vehicle controls (CORT versus pheromone, $P=0.003$; CORT versus control, Tukey's $P=0.025$). In contrast, plasma CORT concentrations were similar among treatment groups at 2 h (Fig. 1; $F_{2,22}=2.41$, $P=0.11$) and 10 h (Fig. 1; $F_{2,21}=0.03$, $P=0.98$) after treatment application.

3.2. Pre-treatment oxygen consumption

Masses of salamanders ranged from 2.19 to 4.09 g ($\bar{x}=3.06$ g ± 0.07 SEM). There was a significant positive relationship between log mass and logV_{O_2} (Fig. 2; $y=0.66x-1.38$; $r^2=0.21$, $P<0.001$). The level of oxygen consumption in red-legged salamanders was similar to those reported in a related species of salamander, *P. cinereus* (Gatten et al.,

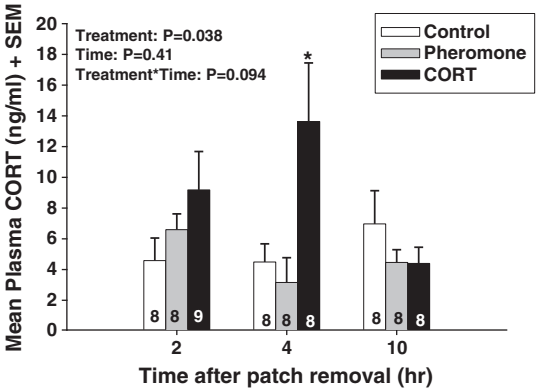

Fig. 1. Mean (±1SE) plasma CORT concentrations in male *P. shermani* exposed to male courtship pheromone, CORT patches, or vehicle controls at 2, 4, and 10 h after last treatment application. Sample sizes are listed within each bar. P-values of main and interaction effects from ANOVA are listed in the panel. Asterisk denotes difference from other treatment groups at 4 h.

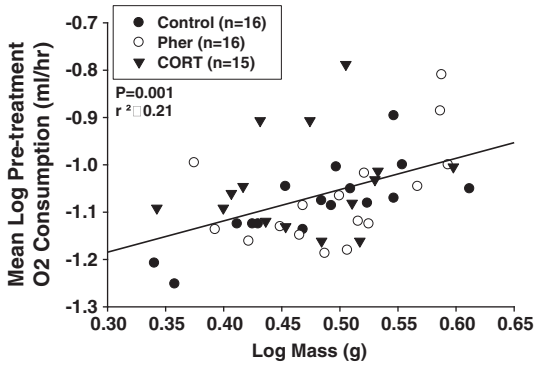

Fig. 2. Relationship between log body mass (g) and log rate (mL/h) of oxygen consumption (V_{O_2}) measured in male *P. shermani* prior to experimental treatments. Corresponding P and r^2 values are listed in the box. Sample sizes are listed within legend.

1992; Homyack et al., 2010). Masses of individuals allocated to the three treatment groups did not differ ($F_{2,44} = 0.69$, $P = 0.51$). There also was no difference in respiration among animals allocated to the different groups during the pre-treatment trial (Fig. 2; $F_{2,43} = 0.32$, $P = 0.73$).

3.3. Post-treatment oxygen consumption

V_{O_2} measured 2–4 h after treatments was higher in animals treated with CORT patches in comparison to animals treated with pheromone or vehicle (Fig. 3; $F_{2,43} = 7.12$, $P = 0.0021$). There was no effect of treatment on the V_{O_2} of salamanders measured 4–10 h (Fig. 3; $F_{2,43} = 0.49$, $P = 0.62$) or 10–14 h (Fig. 3; $F_{2,43} = 0.97$, $P = 0.97$) after treatment. Additionally, the total amount of oxygen consumed (mL) from 2 to 4 h was highest in animals treated with CORT relative to animals treated with pheromone or vehicle (Fig. 4A; $F_{2,43} = 7.07$, $P = 0.0022$). There was no effect of treatment on the total oxygen consumed 4–10 h (Fig. 4B; $F_{2,43} = 0.29$, $P = 0.75$) or 10–14 h after treatment (Fig. 4C; $F_{2,43} = 0.04$, $P = 0.96$). When converted to energy equivalents, salamanders consumed 0.25 ± 0.011 J, 0.25 ± 0.013 J, or 0.28 ± 0.0095 J of energy when treated with control, pheromone, or CORT, respectively, during the first interval.

4. Discussion

Elevation of plasma CORT resulted in an elevation in oxygen consumption in male red-legged salamanders relative to vehicle controls. The plasma CORT concentrations achieved by the CORT patches were physiologically relevant, being of similar magnitude to levels measured after handling or treatment with pheromones (Schubert et al., 2009). In contrast, neither oxygen consumption nor plasma CORT concentrations were altered by pheromone administration. Our study is one of the few to evaluate the influence of physiologically relevant elevations in CORT on whole-animal metabolism in wildlife, and the first to show that elevated plasma CORT increases metabolism in an amphibian.

Compared to subjects treated with vehicle or pheromone, treatment with exogenous CORT elevated plasma CORT. Differences among treatments were most evident 4 h after treatment. By 10 h after CORT treatment, plasma CORT levels were no longer elevated compared to the other treatment groups. The temporal dynamics of CORT delivery by the patches was similar to previous studies in plethodontid salamanders, where plasma CORT was elevated for several hours following treatment with CORT patches, but had returned to baseline by 8 h after removal of CORT patches (Wack et al., 2010).

In contrast to an earlier study (Schubert et al., 2009), application of male courtship pheromone did not elevate plasma CORT concentrations. The lack of a CORT response to pheromone treatment in

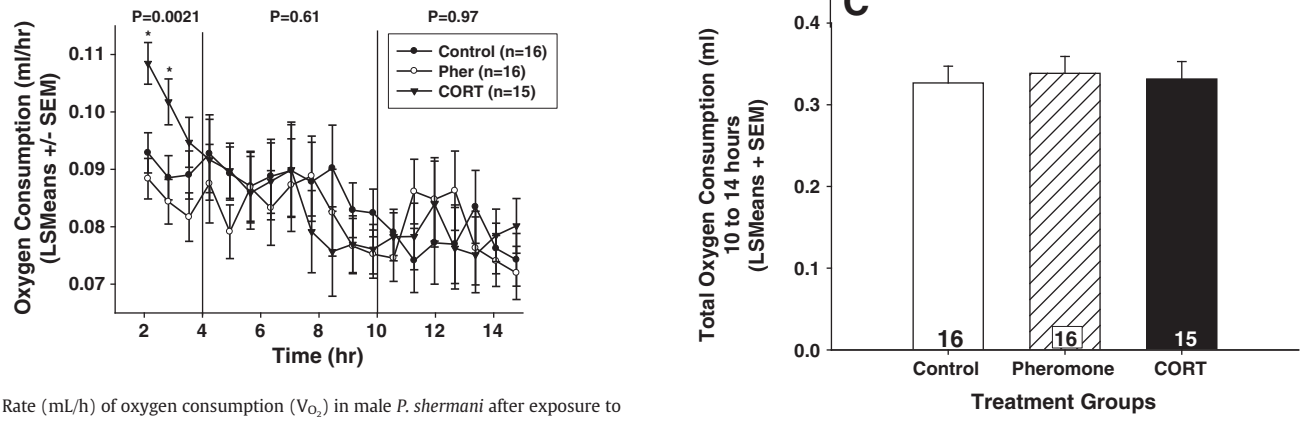

Fig. 3. Rate (mL/h) of oxygen consumption (V_{O_2}) in male *P. shermani* after exposure to CORT patches, male courtship pheromone, or vehicle controls. Heavy vertical lines indicate the three time intervals that were analyzed. P-values from analyses for each interval are listed. Data are presented as least squares means (±1SE) in order to correct for mass. Asterisks indicate significant differences from other treatment groups based on Tukey post-hoc analyses. Sample sizes are listed in the box.

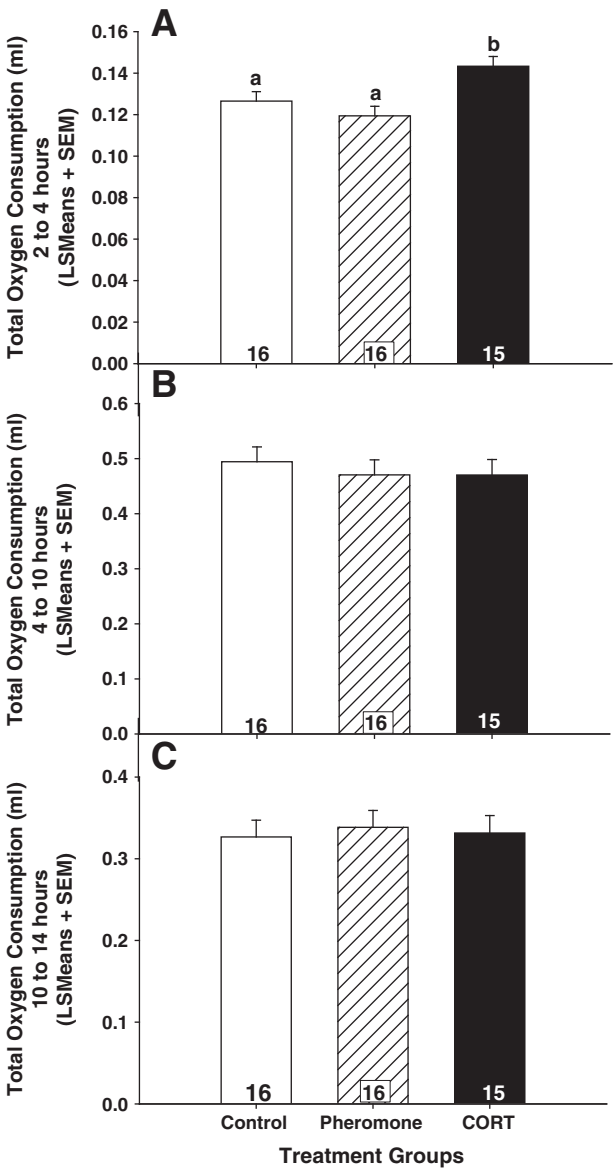

Fig. 4. Total oxygen consumption (mL) of male *P. shermani* from (A) 2 to 4 h, (B) 4 to 10 h, and (C) 10 to15 h. Data are presented as least squares means (±1SE) of total oxygen consumption corrected for body mass. Letters denote significant differences after Tukey post hoc tests. Sample sizes are listed within each bar.

the current study may be due to the use of males that were no longer in breeding condition, whereas males from the Schubert et al. (2009) study were recently collected from the field and tested during the breeding season (August to early October). Unfortunately, unforeseen logistical constraints precluded using males in breeding condition in the current study. However, consistent with the lack of increase in CORT in response to pheromone treatment, pheromone treatment had no effect on oxygen consumption. This finding suggests that pheromone alone is insufficient to elevate metabolism in male *P. shermani*, but future studies will be needed to see if physiological responsiveness differs seasonally.

Our results clearly demonstrate that application of exogenous CORT elevated metabolic rate of salamanders, but other studies in vertebrates examining the effects of CORT on oxygen consumption have produced variable results. Exogenous CORT increased metabolic rates in titmice and gulls (Hissa and Palokangas, 1970; Palokangas and Hissa, 1971), decreased nocturnal metabolic expenditures in pine siskins and white-crowned sparrows (Buttemer et al., 1991; Astheimer et al., 1992), and had no effect in pigeons (Hissa et al., 1980). CORT increased oxygen consumption in geckos and fence lizards (DuRant et al., 2008; Preest and Cree, 2008), but decreased metabolic rates in side-blotched lizards (Miles et al., 2007). Additionally, exogenous CORT increased oxygen consumption in salmon and trout (Chan and Woo, 1978; Morgan and Iwama, 1996; DeBoeck et al., 2001), but decreased oxygen consumption in Coho salmon (Davis and Schreck, 1977). Comparison of these studies suggests that one of the key factors that may have contributed to contradictory results was differences in plasma CORT concentrations. Plasma CORT concentrations were in the pharmacological range in some studies (Hissa and Palokangas, 1970; Palokangas and Hissa, 1971), but were at physiological concentrations in others (Davis and Schreck, 1977; Hissa et al., 1980; Buttemer et al., 1991; Morgan and Iwama, 1996; DuRant et al., 2008). A number of other factors including species-specific and seasonal responsiveness, differences in CORT delivery (i.e., acute versus chronic administration), and respiratory methods (i.e., a single measure of gas exchange versus a temporal profile following CORT administration) may also explain the differences observed among various studies. Our study is an important contribution to the field because it is one of the few studies that measured the effects of physiologically relevant elevations of CORT on oxygen consumption over the time course when plasma CORT was elevated.

A number of important life history events are associated with elevated metabolic rates in amphibians, including being gravid (carrying oocytes/eggs) and nest-building (Bucher et al., 1982; Finkler, 2006). A classic example of an energetically demanding behavior is male anuran advertisement calling (Taigen and Wells, 1985). Male advertisement calling is associated with elevated plasma CORT (Mendonca et al., 1985; Hopkins et al., 1997; Emerson and Hess, 2001; Leary et al., 2004, 2008). It is hypothesized that energetic demands of male vocalization drives the observed elevation of plasma CORT which consequently modifies endocrine and behavioral endpoints, such as switching among male reproductive tactics (Emerson, 2001). Our study in red-legged salamanders showed that increased CORT can also produce an increase in whole animal metabolic rate in amphibians, which may be the result of upregulated intermediary metabolism important for meeting the needs of energetically demanding activities.

The metabolic consequences of the acute physiological elevation in plasma CORT were significant and most likely occurred in the absence of behavioral-induced increases in metabolism. In red-legged salamanders, oxygen consumption from 2 to 4 h after treatment with CORT was 12% greater than oxygen consumption in vehicle controls (equivalent to an increase of 0.33 J). For comparison, courtship increased oxygen consumption rate by 38% and male–male agonistic interactions increased oxygen consumption rate by 27% in the plethodontid salamander, *Desmognathus ochrophaeus* (Bennett and Houck, 1983). The CORT-induced increase in metabolic rate was probably not a secondary consequence of CORT-induced behavioral changes for the following reasons. First, treatment of amphibians with exogenous CORT tends to suppress, not activate, behaviors (Moore and Miller, 1984; Leary et al., 2006). Second, application of CORT patches did not alter activity in dusky salamanders or red-legged salamanders (Ricciardella et al., 2010; Wack, unpublished data). Thus, a brief increase in plasma CORT induced by exposure to stressors or social cues may have significant energetic costs, independent of behavioral changes.

In conclusion, elevation of plasma CORT to physiological levels increased metabolic rates in red-legged salamanders. Our study is one of the few to evaluate the influence of physiologically relevant elevations in CORT on whole-animal metabolism in wildlife, and the first to show that elevated plasma CORT increases metabolism in an amphibian. Our study was limited to acute elevations in plasma CORT, but future studies should determine whether seasonal patterns of sustained plasma CORT concentrations (e.g., Ricciardella et al., 2010) cause seasonal patterns of whole-animal metabolism, independent of other environmental factors (e.g., temperature). If so, seasonal patterns of CORT may be involved in preparing other systems to meet energetic challenges associated with breeding (Romero, 2002).

Acknowledgments

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