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SHORT COMMUNICATION

Plasticity of immunity in response to eating

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ABSTRACT

Following a meal, an animal can exhibit dramatic shifts in physiology and morphology, as well as a substantial increase in metabolic rate associated with the energetic costs of processing a meal (i.e. specific dynamic action, SDA). However, little is known about the effects of digestion on another important physiological and energetically costly trait: immune function. Thus, we tested two competing hypotheses. (1) Digesting animals up-regulate their immune systems (putatively in response to the increased microbial exposure associated with ingested food). (2) Digesting animals down-regulate their immune systems (presumably to allocate energy to the breakdown of food). We assayed innate immunity (lytic capacity and agglutination) in cornsnakes (*Pantherophis guttatus*) during and after meal digestion. Lytic capacity was higher in females, and (in support of our first hypothesis) agglutination was higher during absorption. Given its potential energetic cost, immune up-regulation may contribute to SDA.

KEY WORDS: Cornsnake, Hemoagglutination, Hemolysis, Immune function, Specific dynamic action, Tradeoff

INTRODUCTION

Although animals must consume and digest food to survive, they all incur energetic costs during digestion (i.e. specific dynamic action, SDA) (Secor, 2009). SDA is defined as the accumulated energy expended from the ingestion, digestion, absorption and assimilation of a meal, and it varies in magnitude (increase in metabolic rate of 25% to over 4000%) and duration (hours to weeks) depending on taxon and meal size (Secor, 2009). The SDA is caused by an up-regulation in absorptive processes, characterized as increases in gut size and enzymatic activity in addition to other morphological and physiological changes (McCue, 2006). However, other energetically costly physiological processes that are not directly associated with digestion or metabolism may be concurrently up-regulated and, thus, contribute to SDA.

The immune system is energetically costly to maintain and up-regulate (Martin et al., 2003), and it may be particularly important during increased exposure to foreign material in the gut (Barboza et al., 2010). Microbes are ubiquitous in and on food items (Barboza et al., 2010), and the immune system is critical for microbial defense (Ponton et al., 2013). Yet, although studies have examined the effects of nutrition (nutrient composition, Moret and Schmid-Hempel, 2000; Cotter et al., 2011; food limitation, Kristan, 2007; hydration, Moeller et al., 2013) on immunity, the effects of the digestive process itself are unknown.

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We used cornsnakes (*Pantherophis guttatus*), which have a large SDA response (Crocker-Buta and Secor, 2014), to examine the effects of absorptive state on two immune metrics (hemoagglutination and hemolysis) to test two competing hypotheses. First, digesting animals up-regulate their immune systems during meal processing, presumably to combat the increased microbial load associated with ingested food (Madsen et al., 2007; Conway, 1997). Under this hypothesis, increased immune activation would contribute to the SDA response. Second, animals down-regulate their immune systems during digestion, presumably to allocate more energy to the SDA response. Testing these hypotheses will elucidate interactions between two important and widespread physiological processes – digestion and immunity.

MATERIALS AND METHODS

Study species and husbandry

Pantherophis guttatus (Linnaeus 1766) are non-venomous, medium-sized colubrid snakes that are native to the southeastern USA (Gibbons and Dorcas, 2005). A captive sample of 32 *P. guttatus*, aged 14–16 months, was used to address our hypotheses. Snakes were the offspring (1st–3rd generation) of wild-caught individuals from Beaufort County, SC, USA. Snake husbandry has been described previously (Stahlschmidt et al., 2015). Briefly, snakes were kept individually in translucent plastic enclosures (27×41×15 cm) in a room with a 12 h:12 h light:dark cycle. Enclosures had subsurface heating elements that allowed snakes to thermoregulate along a gradient of temperatures from 24.5 to 33°C. This thermal gradient encompasses the preferred temperature range for *P. guttatus*, 26–29°C (Roark and Dorcas, 2000; Stahlschmidt et al., 2015). Snakes had *ad libitum* access to water. All snakes were well fed and in good body condition as they were offered food (frozen/thawed adult mice that were 15–20% of their body mass) every 2 weeks throughout the study.

Experimental design

Over the course of the 8 week study, blood from each snake (N=32: 18 females and 14 males) was sampled at two time points: 1 and 7 days post-feeding (dpf). These time points were chosen because the metabolic rate of *P. guttatus* peaks at 1 dpf (absorptive) and declines back to pre-feeding levels at approximately 4 dpf (non-absorptive) when fed 15–20% of their body mass (Crocker-Buta and Secor, 2014). Snakes do not exhibit starvation stress until >112 days without food (colubrids: >150 days) (McCue, 2008); thus, at 7 dpf, snakes in our study were non-absorptive but not starving. It is highly unlikely that non-absorptive snakes were resource-limited; colubrid snakes store excess energy in abdominal fat bodies (Weatherhead and Brown, 1996; Bonnet et al., 1998), lipid can account for over 30% of dry body mass in colubrids (McCue, 2008), and prolonged fasting (40 days) does not significantly influence either body mass or immune function in colubrids (Neuman-Lee et al., 2015). Thus, regardless of absorptive state, the snakes in our study had abundant resources available for physiological processes.

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Sampling order was randomized (i.e. 16 of the 32 snakes were first sampled during the absorptive state), and samples were separated by at least one full meal. To control for effects of date, snakes that were 1 and 7 dpf were sampled (intracardiac blood draws of 0.3 ml) during each sampling period. Samples were placed on ice prior to centrifugation at 2350 ***g*** for 5 min. Plasma was removed, and an aliquot of 35 µl was stored at −80°C prior to immune assays (see below). All procedures were approved by the Institutional Animal Care and Use Committee at Georgia Southern University (protocol no. I14004).

Assays of innate immunity

Innate immunity is the first line of defense against foreign microbes in the body (Matson et al., 2005), and most vertebrates rely upon it more heavily than adaptive immunity (Sandmeier and Tracy, 2014; Pap et al., 2015). Hemoagglutination is mediated by natural antibodies (NAbs; poly-reactive immunoglobulins), which can opsonize foreign microbes. Because of their structure, NAbs can also promote agglutination and initiate the complement enzyme cascade (Matson et al., 2005). Complement activation can reduce the integrity of cellular membranes, resulting in hemolysis of foreign erythrocytes (Trouw and Daha, 2011). Hemoagglutination and hemolysis assays were performed at an incubation temperature of 26.5°C (preferred body temperature of study animals: Stahlschmidt et al., 2015) using previously described methods (Butler et al., 2013). Assays were scored blind to treatment independently by R.L.L. and Z.R.S. Because scores were highly correlated (Pearson correlation: R=0.7, P<0.001), the mean value of the scores was used to perform statistical analyses (see 'Statistical analyses', below).

Total protein assays

To validate that animals were in either the absorptive or non-absorptive state at sampling, we quantified plasma protein concentration using the Coomassie Plus (Bradford) Assay (no. 23236, Thermo Scientific, IL, USA) by combining 10 µl of a sample that was diluted 1:100 with ddH_2O with 300 µl reagent, incubating at room temperature for 10 min, and then measuring the absorbance at 595 nm. Plasma protein concentrations (in µg ml^{-1}) were calculated relative to a standard curve using bovine serum albumin (no. 23209, Thermo Scientific).

Statistical analyses

Linear mixed models were performed in SPSS (v.22, IBM Corp., Armonk, NY, USA), and two-tailed significance was determined at α<0.05. Absorptive state (1 versus 7 dpf), sex and absorptive state×sex were included as fixed effects. Animal ID was included as a random effect. To determine relationships between hemoagglutination and hemolysis during each stage of absorption, Pearson correlation analyses were performed.

RESULTS AND DISCUSSION

Results

Being in the absorptive state increased hemoagglutination (1 dpf>7 dpf; $F_{1,29}$=11, P=0.003) by approximately 50% based on the serial dilution nature of the assay (e.g. a difference of 1 in agglutination titer is equivalent to a 100%, or twofold, difference in agglutination capacity; Fig. 1A). Hemoagglutination was not affected by sex ($F_{1,30}$=1.1, P=0.30) or an absorptive state×sex interaction ($F_{1,29}$=0.039, P=0.84). Hemolysis differed by sex (females>males; $F_{1,30}$=7.7, P=0.007; Fig. 1B), but not by absorptive state ($F_{1,30}$=1.1, P=0.31) or an absorptive state×sex interaction ($F_{1,29}$=0.045, P=0.83). The amount of protein in plasma was affected by absorptive state (19.8% higher at 1 dpf; $F_{1,23}$=65.84, P<0.001) but not by sex or an absorptive state×sex interaction (both $F_{1,24}$<0.92, both P>0.35). Hemoagglutination and hemolysis were statistically correlated at 1 dpf (N=30, r=0.69, P<0.001) and at 7 dpf (N=29, r=0.64, P<0.001; Fig. 2).

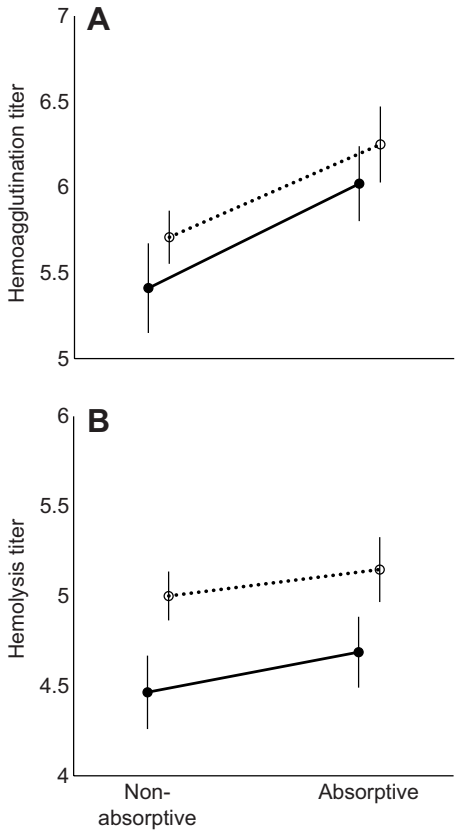

Fig. 1. Effects of non-absorptive and absorptive states on hemoagglutination and hemolysis in corn snakes, *Pantherophis guttatus*. (A) Hemoagglutination is higher during absorption (linear mixed model: P=0.003). (B) Hemolysis titer is higher in females (linear mixed model: P=0.007). Values for males (filled symbols) and females (open symbols) are presented as means±1 s.e.m., N=32.

Discussion

We found that hemoagglutination was significantly higher during the absorptive state (Fig. 1A), supporting our hypothesis that an animal's innate immune system is up-regulated during digestion. Food is covered with potentially pathogenic microbes and live prey can bite snakes, facilitating the transmission of pathogens. The potential pathogenicity of food can be an important predictor of immune function. Scavenging vultures ingest more microbes than eagles, and they have evolved specialized genetic responses to enhance gastrointestinal immune capacity, including positive selection on genes that promote complement activity, natural killer cell-mediated cytotoxicity, and leukocyte migration (Chung et al., 2015). Thus, an allocation toward immune function – particularly an up-regulation of NAbs in snakes – may be an adaptive response to eating (Conway, 1997; Barboza et al., 2010). Most NAbs are of the pentameric immunoglobulin M (IgM) isotype (reviewed in Matson et al., 2005), and IgMs can respond rapidly to microbial components (reviewed in Nguyen et al., 2015). Yet, the cells that produce natural IgM antibodies (B-1 cells) also facilitate oral tolerance (lymphocyte suppression upon repeated exposure to

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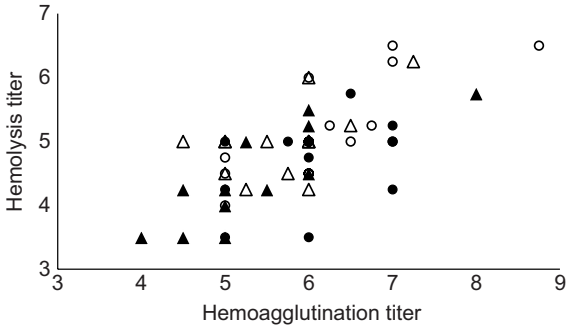

Fig. 2. Significant relationships between hemoagglutination and hemolysis during the absorptive and non-absorptive state. Males, filled symbols; females, open symbols; absorptive state, circles; non-absorptive state, triangles (Pearson correlation: all $P<0.001$, $N=32$).

an orally administered antigen) (De-Gennaro et al., 2009). Clearly, future work is required to better understand the role of NAbs in acute (e.g. individual feeding events) and chronic (e.g. tolerization) immunological responses to eating.

We further demonstrate that regulatory shifts in innate immunity can happen very quickly (i.e. agglutination increased within 24 h of the onset of absorption), which is in accordance with previous work (e.g. hemolysis and hemoagglutination are reduced after a flight lasting ≤4 h in European starlings, *Sturnus vulgaris*: Nebel et al., 2012). These findings suggest a temporally dynamic innate immune system (*sensu* Zylberberg, 2015) that responds to food consumption. Fine-scale resolution of the speed with which the peak increase occurs and the time until returning to baseline levels will further elucidate the relative importance that eating-induced immune activation has on the energetic budget for consuming food (SDA). In some endothermic vertebrates (e.g. mammals), rapid eating-induced shifts in immune function are likely regulated by the relative activity of each branch of the autonomic nervous system (e.g. parasympathetic control of digestion, Rogers et al., 1996; sympathetic activation of immune activities, Bellinger and Lorton, 2014), but in ectothermic vertebrates, eating-induced shifts in physiology (heart rate) are regulated by both autonomic and non-autonomic factors (snakes: Wang et al., 2001; frogs: Claësson et al., 2015). Thus, we advocate for further inquiry into the interactions among nervous, immune and digestive systems across taxa.

Up-regulation of innate immunity appears to be a component of the integrated response to digestion. Many organs in the gastrointestinal, pulmonary and cardiovascular systems undergo dramatic shifts in size and function due to the demands of digestion (McCue, 2006). Our results indicate that SDA-associated energy may also be allocated toward at least one component of immune function, putatively because immune activation is important for fitness and survival (Martin et al., 2003; Lochmiller and Deerenberg, 2000; Graham et al., 2011). While the energetic costs of NAb up-regulation are unknown, the costs of antibody production by vertebrates, in general, are high (Sandmeier and Tracy, 2014). Additionally, we examined just two facets of immunity, and energy devoted to other components of the immune system (e.g. increasing protein catabolism to provision hyper-metabolic macrophages: Lochmiller and Deerenberg, 2000) could substantially affect an individual's energy budget. Thus, to fully appreciate the amount of SDA devoted to immune activation, we advocate research into how eating influences other aspects of the immune system (a) that may be even more energetically demanding than NAb up-regulation (e.g. cell-mediated and adaptive immunity: Sandmeier and Tracy, 2014), and (b) in taxa capable of substantially increasing energy expenditure during innate immune activation (e.g. immune-challenged ducks can increase metabolic rate by over 33%: Marais et al., 2011).

Our results add to the growing literature on the interplay between food and immunity. Previous work has shown that caloric restriction alone or in combination with chronic stress decreases immune function and bactericidal activity in insects and reptiles (Siva-Jothy and Thompson, 2002; Ayres and Schneider, 2009; Chambers and Schneider, 2012; Neuman-Lee et al., 2015), while diet composition (e.g. proportion of protein or carbohydrate) can also affect immune function in insects (Cotter et al., 2011). Here, we show that the mere act of eating a meal can, within 24 h, significantly increase immunity by approximately 50%. This up-regulation may be directly related to ingestion-dependent changes in circulating levels of hormones or nutrients, the bacterial load of the meal, or changes in the microbiota that may subsequently affect an organism's immunity. Post-prandial immune regulation may also be influenced by the contents of the meal. For example, the magnitude of immune up-regulation may match the size of the meal (*sensu* SDA: Secor, 2009) or the microbial load on/in the meal.

Hemoagglutination and hemolysis were correlated at each absorptive state (Fig. 2), likely due to the linked mechanism of complement activation by NAbs via the classical pathway (Matson et al., 2005). Despite this statistical correlation, there were still notable differences between hemoagglutination and hemolysis. For example, only hemolysis was affected by sex (potentially due to the immunosuppressive effects of testosterone: Schuurs and Verheul, 1990; Folstad and Karter, 1992) (Fig. 1B), and eating affected only hemoagglutination (likely due to the high responsivity of NAbs to microbes: Madsen et al., 2007). Differences between hemoagglutination and hemolysis in response to treatments have also been detected in other experimental contexts, including temperature (Butler et al., 2013) and reproductive status (Stahlschmidt et al., 2013). These differences between lysis and agglutination may be due to lysis activation that is independent of NAbs. For example, our study animals may also use lectin or alternative pathways to activate complement (Trouw and Daha, 2011); thus, increased NAbs would not always obligate increased lysis.

Our study represents an important first step to better understand digestion–immunity interactions because (to our knowledge) we are the first to demonstrate that the act of eating directly affects immunity. Our results indicate that some SDA-associated energy is likely devoted to up-regulating the immune system, potentially to combat increased foreign microbial exposure. This allocation of energy provides evidence that the definition of SDA may need to be expanded to include other physiological responses associated with eating (e.g. antioxidant defenses may need to be up-regulated to offset oxidative damage associated with meal processing, as discussed in the companion paper: Butler et al., 2016).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.L.L. and Z.R.S. were responsible for the conception and design of the experiment. R.L.L., M.W.B. and Z.R.S. performed the experiment and prepared the manuscript. Z.R.S. carried out statistical analyses.

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