Individual differences in female aggression: Does peripheral metabolism maintain variation?

Background: Consistent individual differences in behavior – or "personalities" – are ubiquitous in animals and have long captivated biologists[1,2]. Individual differences are a prerequisite for natural selection, and many evolutionary biologists explore how variation in behavior predicts survival and reproduction. Meanwhile, neuroendocrinologists experimentally alter an animal's internal state to change behavior. Only recently have these disciplines been integrated to begin evaluating the mechanisms that maintain natural *individual* differences in adaptive behaviors in wild animals[3]. So far, this work has advanced our understanding of the neural mechanisms of social behavior, but remarkably, has found few patterns linking brain gene expression to individual behavioral differences[4,5]. This suggests that top-down processes are missing key determinants of individual variation in behavior. Certainly, behavior requires more than motivation; it also requires that both brain and body are properly fueled. Thus, I propose that peripheral metabolic processes may be the fundamental force driving consistent individual differences in behavior.

The liver is the primary driver of metabolism and is under high demands to power the brain and muscle to execute energetically expensive behaviors (Figure). In times of endurance, the liver undergoes ketogenesis to secrete ketone bodies into the blood for organs to use as energy[6]. This metabolic pathway is associated with behavioral variation: Migrating birds use ketogenesis to maintain energy homeostasis[7], and racehorses have higher ketone levels during long-distance compared to short-distance races[8]. Given supplemental ketones, bees behave more aggressively[9], and human athletes improve exercise efficiency relative to controls[10]. These observations suggest that variation in ketogenesis is critical for performing energetically expensive behaviors, but this has never been assessed at the individual level. Consequently, there is uncertainty about how natural selection maintains animal personalities. **I hypothesize that natural *individual* differences in behavior stem from variation in the ability to mobilize energy.**

I will test my hypothesis with two specific aims, focusing on social aggression in free-living female birds. First, I will assess how natural differences in aggression correlate with: (a) hepatic **HMGCS2**, the rate-limiting enzyme in ketogenesis, and (b) beta-hydroxybutyrate (**BHB**), the main ketone body produced[6]. Second, I will manipulate circulating BHB and test effects on individual aggressiveness in a repeated-measures design. Both aims build off preliminary data I generated in my first year as a PhD student.

Study system: Tree swallows (*Tachycineta bicolor*; TRES) are obligate secondary cavity-nesters; they cannot excavate a nesting site and must fiercely compete for a pre-made cavity to reproduce. Females readily take to artificial cavities (i.e. nestboxes), and they are more aggressive than males. Social aggression requires endurance, as females engage in extended aerial chases and intense physical attacks during competition for nestboxes. High aggression individuals have better body condition[11] and are more likely to breed than low aggression females, showing that aggression is adaptive[12]. Such strong natural selection should erode this trait variation, and yet substantial individual differences in aggression persist.

Preliminary data: Last spring, I conducted 5-minute simulated territorial intrusions (STIs) on free-living female TRES. I measured aggression (e.g. time spent hovering, diving, pecking) towards a conspecific decoy placed at the nestbox. Individual aggression was repeatable in consecutive STIs ($R=0.90$; $p<0.001$). 2-7 days after the last STI, I collected 10 high and 10 low aggression females and conducted a genome-wide analysis of their brains (i.e. RNAseq in hypothalamus and amygdala). Despite a well-powered design, I found very few differentially expressed genes between high and low aggression birds, indicating that substantial behavioral variation cannot be explained by differences in baseline neural gene activity. These results further support my hypothesis that behavioral differences emerge beyond the brain.

AIM 1: To what degree do individual differences reflect variation in ketogenesis? From these same high and low aggression birds, I will extract RNA from the liver, where I confirmed HMGCS2 is highly expressed based on TRES transcriptomic data[13]. Using established lab protocols, I will design HMGCS2 primers and perform qPCR to measure HMGCS2 gene expression, running samples in triplicate for HMGCS2 plus two endogenous control genes. I will also quantify BHB concentration in 10µl of blood from these individuals, using test strips read by a handheld ketone meter that is already validated in wild

birds[14]. I will employ linear models to examine the degree to which natural variation in aggression is predicted by HMGCS2 expression, BHB concentration, or a combination of the two.

AIM 2: How does experimentally manipulating BHB alter individual aggressiveness? I will expose incubating females to a commercially available BHB cream (BPI Keto Cream) applied to a fake egg in the nest for 12 hours. As the female incubates overnight, BHB will be absorbed via the brood patch, a featherless area of vascularized skin on the belly. Control females will receive a fake egg with a vehicle cream. Past work has used this noninvasive approach to manipulate hormones in TRES[15]. Here, it will allow manipulation of ketone levels independent of handling-induced stress. In a within-subjects design, I will use 30-minute (prolonged) STIs to measure intensity and duration of aggression the morning before and after BHB (or control) treatment, analyzing results with a repeated-measures ANOVA. Blood BHB will be quantified in both groups after the second STI using the ketone meter described in Aim 1. I will also separately validate that BHB treatment elevates BHB blood concentration in a subset of birds.

Predictions, Alternatives, & Next Steps: I predict that individual variation in aggression will positively correlate with both HMGCS2 and BHB, with greater levels in high vs. low aggression individuals (Aim 1). Likewise, I predict that individuals will increase aggressiveness in response to supplemental BHB (Aim 2). Support for my hypothesis in Aim 1, but not Aim 2, would suggest that birds must engage in prolonged competition to promote ketogenesis, considering that females in Aim 1 were unprovoked at the time of collection. In this case, a future step would be to assess ketogenesis during sustained competition by manipulating nestbox availability, which is shown to increase aggression and metabolically challenge the brain[16]. I am also well-positioned to explore additional tissues from these same birds, such as the pectoral muscle where BHB is converted into usable fuel[6]. Nearly all TRES fighting occurs in flight, suggesting the pectoral muscle is a promising tissue to connect energetic constraints to social behavior (Figure).

Intellectual Merit: Individual differences serve as the raw material for evolutionary change, leading to the diversity of behaviors seen in nature. However, we have limited insight into the origin of this variation. My work explores the potentially critical role of peripheral energetics in shaping natural individual differences in the wild. Recent work reveals other routes by which the periphery influences brain and behavior (e.g. gut-brain axis, microbiome), a view that my research extends. In the long-term, my proposal will not only build the foundation of my dissertation, but it will also serve as a springboard for applying energetic perspectives more broadly, to understand how metabolism accounts for diverse behavioral differences within and among species. Ultimately, my work will examine how both evolutionary and proximate mechanisms work together to build an aggressive female, an overlooked perspective in a field that, since Darwin, often assumes that females do not compete or that their aggression is just like that of males.

Broader Impacts: As a first-generation, biracial graduate student, I strive to diversify STEM by helping historically underrepresented undergraduates overcome institutionalized barriers, thereby demystifying academia's hidden curriculum. My efforts include the creation of a "how to" guide for applying to graduate school, which I disseminated to local and national groups. As a co-facilitator of an anti-racism group at Indiana University (IU), I developed action plans to hire and support diverse undergraduate researchers in my lab. These efforts set the foundation for my goals as a graduate student and future faculty member to improve recruitment and retention in STEM. Mentorship is central to this plan. I honed these skills with mentorship training while working with an undergraduate mentee in IU's Center for the Integrative Study of Animal Behavior NSF REU summer program. Moving forward, I will work with the Jim Holland Summer Enrichment Program, which provides research experience for high-achieving minority high school students and helps them transition into an IU STEM major, extending my efforts to broaden inclusion.

References: [1]Koolhaas et al. *Front Neuroendocrinol* (2010). [2]Sih et al. *TREE* (2004). [3]Hofmann et al. *TREE* (2014). [4]Bell et al. *Behaviour* (2016). [5]Benowitz et al. *Behav Ecol* (2019). [6]Grabacka et al. *IJMS* (2016). [7]Frias-Soler et al. *Biol Lett* (2021). [8]Volek et al. *Metabolism* (2016). [9]Rittschof et al. *J Exp Biol* (2018). [10]Dearlove et al. *Med Sci Sports Exerc* (2021). [11]Rosvall. *J Avian Bio* (2011). [12]Rosvall. *An Behav* (2008). [13]Bentz et al. *Sci Rep* (2019). [14]Sommers et al. *J Field Ornithol* (2017). [15]Vitousek et al. *Proc B* (2018). [16]Bentz et al. *PNAS* (2021).