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CONNECTIVITY AND PLASTICITY OF PHYSIOLOGICAL MECHANIMS AND TEMPERMANT

A Dissertation in

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by

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ABSTRACT

Animal behaviour research has expanded over the past 20 years to study consistent individual differences in behaviour (i.e. temperament). These temperaments have been widely studied in relation to different ecological outcomes, such as fitness and survival. The underlying physiology of these traits may contribute to the relationships among fitness and temperament. My first chapter is a literature review that illustrates that there is limited information on causal relationships between physiology and temperament within individuals and hypothesizes physiological profiles for temperaments.

My dissertation addresses questions about the interactions between temperament and the underlying physiology associated with temperament. Specifically, I answer the questions: 1) how temperament is characterized, 2) how the underlying physiology is related to temperament and 3) how flexible is the physiological profile and in turn, the associated temperaments. I used Sprague-Dawley rats in a laboratory setting to answer these questions to prevent external factors from altering these results.

I first investigated how temperament is characterized. I conducted comprehensive and repeated behavior testing to compare two methodologies to identify temperaments that are individually-consistent across time and conditions. To measure single- and multi-behavior metrics of temperament, I measured rat behavior across five different arenas and repeated this at three time points. Certain single- and multi-behavior estimates of temperament were consistent across time and contexts than others; distance travelled/Activity along with Social Boldness, latency to interact with novelty and other Sociality- and Boldness-related behaviors were consistent across time and conditions, whereas Aggression and Exploration were less consistent. There was strong evidence that multiple behavioral estimates of temperament were more consistent over time or across

conditions than single behaviors.

Once I characterized different temperaments observed in Sprague-Dawley rats, I determined the underlying physiological profiles of each temperament. I measured HPA axis, innate immune system, adaptive immune system, gut microbiome and cardiac function and used correlation analysis to determine which physiological systems were associated with each temperament and used multiple linear regression to determine which physiological mechanisms predicted each temperament. Single-behavior metrics of temperament were more often correlated with physiological systems compared to multi-behavior metrics. Furthermore, HPA axis reactivity was predictive of multiple temperaments (both single- and multi-behavior metrics) while cardiac re/activity and HPA activity were significant predictors of some temperaments.

The final step was to determine the flexibility of physiological profiles and their associated temperaments. In this study, I measured Exploration and Social Boldness along with all physiological mechanisms from the prior study in Chapter 3 and manipulated basal glucocorticoid (GC) levels. Animals in the GC treatment group had significantly increased basal GCs and latency to interact with novelty (lower Exploration) from before to during GC exposure, and significantly decreased stress-induced GCs, cell-mediated immune activity, and stress-induced heart rate compared to control rats. The Social Boldness temperament trait did not significantly change with GC treatment.

This research provides the building blocks for future research to study the interactions between temperament and physiology. Once we understand how temperament and physiology interact, we can begin to understand how they influence individual differences in ecological outcomes.

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PREFACE

Chapters 1 and 2 of this dissertation were published or submitted to peer-reviewed journals for publication.

Chapter 1: This chapter is presented as previously published in the per-reviewed journal Proceedings of the Royal Society B: **McMahon, E. K.**, Youatt, E., & Cavigelli, S. A. (2022). A physiological profile approach to animal temperament: How to understand the functional significance of individual differences in behaviour. *Proceedings of the Royal Society B, 289*(1966), 20212379. EKM conducted literature searches, synthesized results of published studies, and drafted significant portions of the manuscript. EY identified, synthesized, and drafted text on human studies. SAC developed the idea for the manuscript, synthesized results of published studies, and drafted significant portions of the manuscript. All authors reviewed, provided feedback, edited the manuscript and gave final approval for publication.

Chapter 2: This chapter is currently under review in the peer-reviewed journal Animal Behaviour: **McMahon, E.K.**, Farhan, S., Cavigelli, S.A. 2021. How do we characterize temperament?: Broad testing of temperament across time and contexts in low variable conditions. EKM conducted the animal work, data collection, some of the data analysis and drafted a significant portion of the manuscript. SF conducted data analysis and statistical results and contributed to the manuscript. SAC conducted data analysis and drafted a significant portion of the manuscript. All co-authors edited the manuscript and approved it for submission.

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Chapter 1:

A physiological profile approach to animal temperament to understand the evolution and functional significance of individual differences in behaviour

Abstract

Animal behaviour research has experienced a renewed interest in consistent individual differences (i.e. animal personality or temperament). Recent ecological studies have identified environmental conditions that give rise to the development and evolution of temperaments and to fitness-related outcomes of temperament. Additional literature has also described relationships between temperaments and physiological regulation. However, one-to-one relationships between one behavioural trait and one physiological system do not account for co-selection of behavioural and physiological traits, nor the complex signalling among physiological systems. In the current paper, we review the literature on multiple physiological processes associated with temperament, propose temperament-specific physiological profiles, and focus on next steps to understand the functional significance, evolution, and maintenance of temperaments. We propose that to best understand causes and consequences of temperament we need to characterize integrative physiological profiles associated with different temperaments.

Keywords: animal personality, temperament, physiological profiles, behavioural consistency, behavioural syndromes, fitness

Introduction

In the past 20 years, the field of animal behaviour has experienced a renewed interest in individual differences, with a recent focus on ‘animal personalities’ or ‘temperaments’. This resurgence was spurred on by recognition that individually-distinct and consistent behavioural traits are not unique to humans, but are widespread across the animal kingdom [1–4]. Recent ecological studies have identified environmental

conditions that give rise to the development and evolution of temperaments as well as important fitness-related outcomes of these phenotypes [5–9]. In the current review, we focus on next steps to best understand the functional significance, evolution, and maintenance of temperament. Specifically, we propose that it is essential to characterize complex, underlying physiological profiles of temperament in order to understand associated causes and consequences. Doing so will provide a more nuanced, complex, and mechanistic understanding of how temperaments evolve and why certain temperaments thrive better in one environment vs. another. This information can be critical to advance evolutionary and ecological theory, but also for applied conservation efforts.

We begin with a brief background on temperament and discuss evidence and limitations related to temperament stability, covariance of different temperaments (i.e. behavioural syndromes), and the relationship between temperament and fitness. Then we review physiological processes that have been associated with temperaments, organized by widely-recognized categories, and propose a multi-system physiological framework to incorporate into future studies. This multi-systems physiological approach is key to understand proximate mechanisms that promote or limit behavioural rigidity/flexibility, covariance, and fitness.

Overview of Animal Temperament

Many terms are used to refer to reliable or consistent individual differences in behaviour. Recent literature tends to refer to ‘personality’ or ‘behavioural syndromes’ which are not synonymous. ‘Personality’ is often used to indicate consistent behavioural traits within an individual over time and across contexts (although this consistency is not

always verified [10], whereas ‘syndrome’ is used to refer to covarying behavioural responses within an individual (e.g. high aggression associated with elevated boldness behaviour). There has been controversy over the use of ‘personality’, partially because the definition is often loosely applied, and not necessarily in synchrony with the psychological literature from where the term originates, and because it can be teleological (e.g. [11]). Earlier literature often referred to consistent, individual differences in behaviour as ‘temperament,’ ‘alternative behavioural strategies,’ or ‘behavioural phenotypes’. In this review, we focus on basic individual differences in behaviour that are not specific to aspects of life history (e.g. not ‘alternative reproductive/mating strategies’), but rather behavioural traits that suggest innate and consistent differences in how individuals respond to all environmental conditions (i.e. ‘temperament’). We use the terms ‘behavioural trait’ or ‘temperament’ and use Reale and colleagues’ [12] temperament categories. Additionally, we use a strategy that has been used in human temperament research: a focus on standard temperament categories to identify physiological mechanisms that can impact health and interactions with the environment [13–17]. First, we briefly review three aspects of temperament that may be better understood with more comprehensive physiological information: within-individual temperament consistency, behavioural syndromes, and fitness/health outcomes associated with temperament.

Temperaments are individually-consistent: Early animal research included basic observations of behavioural variation among individuals [18,19]. In the late 1990s, it was hypothesized that the ‘Big-Five model’ of human personality could be used to characterize individual differences in animal behaviour [2,20], and most recently, Réale

and colleagues [12] identified five behavioural dimensions that are frequently studied across species (activity, exploration, boldness, sociability, and aggression). Important in the study of temperament, recent work has focused on whether temperaments represent stable traits, as had been documented in humans (e.g. [21,22]). In psychology, multiple behaviour tests or strategies are used to determine the relative consistency of individual temperament. This is not always the case in animal studies because of limited time, resources, and/or feasibility in field settings [23]. Additionally, there is no congruence on what defines consistency: is it over time, across conditions or both? However, when repeat testing is conducted with animals, results indicate behavioural consistency across time and across conditions that are on the same order as that for human personality traits (i.e. correlation coefficients of 0.2-0.7; [13]). These differences are thought to reflect systematic differences among individuals that are trait-like. These consistent behavioural traits may reflect consistent physiological underpinnings. By understanding the physiological profile of temperaments, and their relative flexibility, we can better understand biological mechanisms that allow a behavioural trait to persist or to be flexible within an individual.

Covariance of temperaments: Recent work shows that some behavioural traits covary, which is often referred to as 'behavioural syndromes' or 'behavioural profiles' [24,25]. These behavioural syndromes are not necessarily the same across species or environments. For example, exploration and boldness have been shown to be positively correlated in some species in certain environments, but not in others; and the same is true for exploration and sociability [12,26–30]. On the other hand, across several species, boldness and aggression have been shown to covary (funnel web spiders: [31] crabs: [32],

sticklebacks: [33]; song sparrows: [34]). Covarying traits have been characterized as 'coping strategies' (reactive vs. proactive) that include interrelated behavioural and physiological traits [35]. For example, proactive individuals in lab-based studies are aggressive, bold, and behaviourally-inflexible, with elevated sympathetic arousal and dampened hypothalamic-pituitary-adrenal (HPA) re/activity [12,24,35–40], whereas reactive individuals show the opposite suite of traits. It has been proposed that covarying behavioural traits emerge as a result of similar physiological processes that underlie different behavioural traits (e.g. elevated sympathetic reactivity promotes both aggression and activity [24]). Thus, a better understanding of the physiological processes that underlie these temperaments can provide the information necessary to determine why certain behaviour traits covary in some species or environments but not in others.

Associations with individual fitness: Before the early 2000s, studies on individual behavioural traits related to ecological fitness focused on alternative strategies [11,26,41–43]. Early studies focused on the relationship between certain behavioural traits and individual fitness, such as individual aggression predicting response to predators [24,44–48]. Several theoretical/review papers [2,3,49–51] encouraged ecologists to conduct more longitudinal studies to determine if and why behavioural consistency exists in naturalistic settings [5,12,52,53]. A variety of studies demonstrated that individual temperaments predicted ecological outcomes such as survival [4], dominance [54], offspring dispersal [27,55], offspring survival [56], reproductive success [57–59], and anti-predator responses (reviewed in [60]). Further, studies demonstrated that environmental conditions (e.g. predation pressure) can affect the relative numbers of different temperaments in a group and that the number of different temperaments in a group can influence and be

influenced by environmental conditions and can further influence group survival outcomes [28,45,61–65]. However, the mechanisms by which these temperaments affect survival, reproduction, and overall fitness are not yet clear.

Pursuing a mechanistic approach

The relative ease of measuring and characterizing temperaments has allowed for an abundance of research on the presence and fitness consequences of behavioural traits in natural populations. To better understand individual consistency of temperament, behavioural syndromes, and fitness consequences of temperament we need to pursue an advanced understanding of physiological mechanisms that underlie temperaments. In the current review, we take advantage of a recent growth in studies that analysed relationship between temperament and physiology in naturalistic conditions (i.e. not under necessarily experimental studies where physiology is manipulated to show cause influences on acute behaviours), and based on these data, we determine whether complex physiological profiles can be identified for different temperaments.

If slight biases in physiological regulation are relatively stable traits within an individual (e.g. [66–74]), then these may support or drive the relative stability, covariance, and fitness of behavioural traits [38,69]. To this end, there is already strong evidence that behavioural variance among individuals is systematically associated with neuroendocrine variance (reviewed below) [25,36,46,74]. Several studies have documented *causal* relationships between specific physiological processes and acute displays of certain behaviours. For example, acute injections of central corticotropin releasing hormone (CRH) and CRH receptor agonist decrease exploratory behaviour in

rodents and CRH antagonists increase exploration [75,76]. However, experimental evidence where physiological processes are manipulated in a chronic manner to stimulate trait-like physiological and behavioural regulation are rare and difficult to conduct. Thus, we focus on non-experimental/correlational studies where temperaments are compared to physiology.

Moreover, one-to-one relationships between a behavioural trait and a physiological system do not consider the co-selection of multiple traits nor the complex signalling that occurs among physiological systems. We propose that a more comprehensive understanding of a suite of key physiological processes associated with temperaments is necessary to determine mechanisms that support consistent temperaments, behavioural syndromes, and associated fitness and health consequences. Only by understanding multiple covarying physiological regulatory biases (e.g. elevated sympathetic reactivity, dampened adaptive immune responses, etc.) associated with temperaments can we determine why some temperaments are more fixed, why some temperaments covary, and why some temperaments survive better in some environments but not others. With these points in mind, in the current review we synthesize the research on different physiological mechanisms that have been associated with basic behavioural traits, and determine if specific temperaments are associated with specific physiological profiles (i.e. physiological biases among several systems).

Connecting Temperament to Physiology

We focus on correlational studies rather than causal experimental studies where a biological process was manipulated to determine impact on behaviour. We use this focus

because correlational studies are more abundant, and they often compare behavioural traits to basal physiological function. Basal physiological regulation is important in that it is more likely related to consistent/chronic behavioural patterns and associated fitness outcomes. Finally, we focus on physiological processes that have been frequently studied and that likely influence health and fitness (sympathetic, HPA, and immune regulation).

We conducted a literature review on physiological mechanisms associated with the five temperament categories identified by Réale and colleagues [12] and proactive/reactive coping styles, which have been identified as complex behavioural and physiological traits that may be akin to behavioural syndromes [36]. The search terms that we used are in *Supplementary Table 1.1*. We removed studies that did not focus on animal biology (i.e. chemistry, physics, etc.), this yielded a list of 14,723 papers. 1,702 of these papers were not primary source articles and were removed from the literature search. We then removed papers that did not include at least one of the above listed temperaments and at least one of the above physiological mechanisms, and removed human studies. Based on these criteria, we arrived at a final list of 145 papers.

Results of this search are summarized in **Table 1.1**. The table shows the number of papers (and total sample size) that indicated a positive, negative or no relationship between each temperament and physiological process. A 'positive' relationship indicates that individuals with a certain temperament showed evidence of an upregulation in the specific physiological process. These relationships were defined based on study results that identified a significant difference ($p<0.05$) in physiology associated with temperament. Specific information and references from each study that contributed to **Table 1.1** are in *Supplementary Table 1.1*. To indicate the relative power of relationships

between specific temperaments and specific physiological processes, we included total sample size across all studies (in parentheses) in each cell. *Supplementary Table 1.1* gives detailed information about sample size for each paper.

Table 1.1. Compilation of studies on associations between temperament and physiology. A 'Positive' association indicates that *greater* expression of the listed temperament (e.g. Exploration) was associated with *increased* activity of a certain physiological process (e.g. Sympathetic reactivity), whereas a 'Negative' association indicates that *greater* expression of a listed temperament was associated with *decreased* activity of a certain physiological process, and 'None' indicated no relationship between a certain temperament and a certain physiological process. Each cell indicates the number of published papers that showed each temperament-physiology association, and in parentheses the total number of individuals that contributed to all papers.

Temperament		Sympathetic reactivity	Basal HPA axis activity	HPA axis reactivity	Innate immune response	Cell-mediated immune response	Humoral immune response
Exploration	Positive	3 (146)	7 (545)	7 (1,633)	2 (107)	5 (220)	1 (10)
	Negative	-	7 (462)	23 (1,420)	4 (294)	1 (45)	3 (83)
	None	2 (43)	22 (2,716)	5 (217)	2 (73)	1 (49)	-
Boldness	Positive	1 (38)	1 (50)	6 (274)	3 (331)	2 (566)	1 (28)
	Negative	1 (108)	4 (109)	6 (289)	1 (23)	-	1 (159)
	None	1 (30)	7 (481)	9 (3,939)	1 (159)	-	1 (66)
Sociability	Positive	1 (16)	1 (77)	-	-	1 (7)	2 (6)
	Negative	-	4 (70)	2 (43)	1 (7)	-	-
	None	1 (170)	1 (58)	3 (279)	-	1 (26)	-
Aggression	Positive	1 (170)	7 (235)	7 (466)	3 (126)	2 (30)	-
	Negative	-	4 (259)	8 (284)	-	-	-
	None	-	1 (30)	2 (74)	-	-	2 (612)
Activity	Positive	2 (108)	3 (205)	3 (134)	1 (23)	-	-
	Negative	1 (42)	1 (125)	3 (90)	1 (18)	-	-
	None	1 (132)	5 (403)	6 (555)	1 (27)	-	1 (592)
Proactivity	Positive	2 (50)	1 (147)	3 (976)	1 (192)	1 (218)	1 (60)
	Negative	-	5 (1,109)	9 (1,306)	-	1 (50)	3 (302)
	None	-	3 (592)	2 (792)	2 (130)	-	1 (80)

Exploration

Exploration, defined as an individual's tendency to engage novel situations, is one of the most common temperaments studied. Highly exploratory individuals tend to disperse more [12,27,77,78] and thus will have more interactions with novel environments and conspecifics compared to less exploratory individuals.

Many studies have documented distinct physiological mechanisms associated with exploration (primarily in rodents and birds). Across species, highly-exploratory individuals tend to have elevated sympathetic reactivity [S1-S3; cf. S4,S5] and either dampened or heightened GC responses to stressors ([68],S1,S6,S7,S10,S11,S16,S17,S20,S22,S24-S26,S32,S34,S38-S45; cf. S2,S8,S9,S15,S18,S21,S43,S46-S50), with no relation to basal glucocorticoid (GC) concentrations [[68],S2,S5-S24; c.f. S25-S38]. Immune function in exploratory individuals tends to favour heightened cell-mediated responses, with minimum energy toward fast-acting non-specific innate immunity, or slower, longer-lasting humoral immunity [S17,S24,S25,S31,S44,S51-S56; reviewed in [79–81]; cf. S25,S29,S57]. Overall, exploratory individuals have been shown to have heightened sympathetic reactivity and cell-mediated immunity, dampened or heightened HPA reactivity, and lower innate and humoral immunity, with no relation to basal GC circulation.

Boldness

Boldness, defined as an individual's tendency to engage in risky situations, has been heavily studied in birds and fish. Bold individuals take more risks, expose themselves to novel antigens, and obtain more resources than shyer individuals [87; reviewed in 88]. The degree of an individual's boldness has been associated with learning

[89] and tends to predict social dominance within groups [1,36,82].

Physiological processes associated with boldness are less studied than for exploration. The most common physiological process related to boldness is circulating GCs: bolder individuals show either lower or no difference in baseline GCs [S12,S58,S59,S60; cf. S2,S8,S13,S30,S61-S64], and higher HPA reactivity to stressors, particularly in birds [S47,S65-S69; reviewed in [82]; cf. S2,S8,S45,S60,S61,S64,S70], but not in fish [S49,S59,S62,S63,S71,S72; cf. S73,S74]. Depending on taxa, bold individuals have elevated [S75], dampened [S76] or no difference in heart rate compared to non-bold individuals [S2]. Finally, bolder animals can have higher innate immune reactions [S54, S77, S78; cf. S79, S80]. Conflicting results are seen in adaptive immunity, with some studies showing a positive relationship between boldness and cell-mediated immune function [S81, S82] and others show no clear relationship between boldness and humoral immunity [S78, S80, S81]. Overall, the physiology of boldness is less clear than that of exploration, with a suggestion that boldness is associated with heightened innate and cell-mediated immune function, dampened sympathetic reactivity and humoral immunity, and no clear relationship between boldness and circulating GCs.

Sociability

Sociability is defined by an individual's tendency to interact with conspecifics – highly-sociable individuals interact more frequently with, or with a greater number of conspecifics. Highly sociable individuals tend to be more central in a group, function as leaders and may affect group dynamics like movement and size [83].

Sociability is frequently studied in primates. More sociable primates tend to have lower basal GCs [S34,S83-S85; cf. S86,S87] and heightened humoral immunity

compared to low-sociable individuals [S88-S89; reviewed in [84]]. Capitanio [85] and Sloan [S90] concluded that high-sociable primates had greater cell-mediated immune function but lower innate immune function compared to low-sociable individuals, and only one study contradicts this [S91]. The relationship between sociability and HPA reactivity is variable, with two studies showing a negative relationship [S34,S88] and three showing no relationship [S50,S91,S92]. Last, one study demonstrated that highly sociable goats had higher heart rate reactivity than less sociable goats [S4] while Kralj-Fišer and colleagues [S92] did not observe this relationship in birds. At present, our understanding of the physiological mechanisms that underlie sociability is limited, but there is a suggestion that sociable individuals have either decreased or increased basal HPA activity and have heightened humoral immune function.

Aggression

Aggressive individuals show more frequent or more intense agonistic reactions toward conspecifics. Aggressive behaviour is species-specific and includes different forms such as territorial aggression, dominance-related aggression, and maternal aggression [86]. The aggression trait has been frequently associated with increased exploration and boldness [[87]; cf. [88]], and aggressive individuals tend to locate at the group periphery, be involved in group defence, and have enhanced foraging compared to less aggressive individuals (e.g. [64,89,90]).

There are a limited number of studies on the relationship between aggression and physiological mechanisms in free-ranging animals. The most common measures are related to HPA function: more aggressive individuals, particularly in mammals, have higher basal GCs [S83,S93-S98; cf. S23,S64,S85,S99,S100], whereas the relationship to

HPA reactivity is less clear. Equal numbers of studies indicate that more aggressive individuals have dampened [S49,S86,S93-S94,S101-S104; reviewed in S58,[91]] or heightened HPA reactivity [S97,S105-S109] with two that showed no relationship [S64,S110]. Studies that showed a negative relationship between aggression and HPA reactivity were often conducted with males and primates while studies that showed a positive relationship included both sexes and a variety of taxa. Aggressive individuals have elevated sympathetic responses compared to less aggressive individuals [S92; reviewed in [40]]. Baboons that are more aggressive show faster wound healing [92], lower infection rates [93], and higher lymphocyte numbers suggesting an increased ability to fight off infection [S111]. Many studies show that aggressive individuals have heightened innate and cell-mediated immune responses and no relation to humoral immunity compared with less aggressive individuals [S100-S115]. Overall, there are few ecological studies of aggressive behaviour, likely because overt aggression is infrequent, and therefore difficult to quantify, in well-established social groups in naturalistic settings. Across studies and species, aggressive individuals tend to have greater HPA reactivity, either higher or lower basal circulating GC concentrations, increased innate and cell-mediated immune function, dampened humoral immunity, and greater sympathetic reactivity.

Activity

Activity is a metric of an individual's propensity to move through their landscape. Few studies directly measure activity as a temperament in ecological settings, let alone its connection to physiological mechanisms. Several studies show no relationship between activity and GC reactivity [S16,S34,S49,S59,S116,S117], and a few studies identified a

positive [S48,S118,S119] or negative association with GC reactivity [S45,S61,S120]. The same is true for basal GC levels and immunity, with the majority of studies showing no relationship between activity and basal GC levels [S13,S14,S59,S61,S121; cf. S16,S33,S34,S122] or immune function [S54,S115; cf. S31,S79], but a possible positive association with sympathetic reactivity [S76,S121; cf. S116,S123]. These studies are evenly split across a variety of taxa. The physiology of this basic temperament requires further elucidation, although a number of studies suggest is not reliably associated with physiology.

Proactivity

Proactive/reactive coping strategy is defined by coordinated behavioural responses to challenging situations. In particular, proactive individuals are more bold, aggressive and exploratory while reactive individuals are more shy and less aggressive and exploratory. Across studies, proactive-reactive strategies are quantified using different metrics but tend to focus on boldness or aggression. In less dynamic environments, proactive individuals tend to be more abundant while in changing environments reactive individuals tend to have higher fitness [24].

Individuals who are proactive tend to have dampened HPA basal activity and reactivity [S62,S124-S134; reviewed in [24,40]; cf. S129,S135-S137] but heightened sympathetic reactivity compared to reactive individuals [S123,S138; reviewed in [94]]. Proactive individuals demonstrate dampened humoral immune responses [S139-S141; cf. S142,S143; reviewed in [53]] and potentially enhanced innate and cell-mediated responses, depending on species [S138,S143-S145].

Summary

Overall, many studies have identified physiological processes associated with different temperaments. When compiled across species, some regular patterns emerge for certain behavioural traits. In particular, the physiological profile for exploratory, aggressive, and proactive individuals is more consistent than the profile for sociable, or active individuals. However, we note that across studies there is a good deal of variation, and that most studies to date only focus on one physiological system, with the majority of studies focused on HPA re/activity.

Considering Multi-System Physiological Profiles of Temperaments

Why we should consider more complex physiological profiles underlying temperaments

If temperament is complemented or supported by a unique regulatory bias in a physiological system (e.g. low HPA reactivity in highly exploratory individuals), then we should expect these complementary behavioural and physiological phenotypes to be co-selected [S48]. For example, high-exploration individuals that tend to expose themselves to more novel environments and antigens will be more likely to survive and reproduce if they have rapid sympathetic responses complemented by low-grade HPA reactivity to allow for a strong cell-mediated immune response. Within this natural selection framework, specific temperaments are likely co-selected along with regulatory biases in multiple physiological systems, leading to complex physiological profiles associated with each temperament. In addition, physiological systems signal to one another and are co-regulated thus we must consider complex multi-system physiological profiles.

Cross-signalling among physiological systems

Different physiological systems signal to one another, thus, at a functional level, a

bias in the regulation of one system can bias activity of another system. For example, chronically-elevated GC production can down-regulate certain aspects of immune function and neuronal signalling [95,96]. Cross-system signalling occurs among the sympathetic nervous system, the HPA axis, the immune system, and certain neurotransmitter systems (e.g. [97–99]). These systems have been well-studied, experimentally-manipulated, and implicated in behavioural and physiological responses to the environment, although, they have not been studied collectively with respect to temperament. Because physiological regulation involves a variety of feedback and feed-forward mechanisms within and among systems, networks of cross-signalling neurological, endocrine, and immune response systems may be relatively difficult to shift, which may explain why some temperaments are highly consistent, or even why some temperament traits regularly covary, and why temperament is associated with fitness in different environments.

Potential physiological profiles of temperament and adaptive aspects

Given abundant cross-signalling among physiological systems, and co-selection of traits (behavioural and physiological), it is incumbent on animal behaviourists that study proximate mechanisms to consider more complex physiological profiles. Based on evidence so far, and based on behavioural predispositions of each temperament, we can hypothesize on the structure of physiological profiles for each temperament (see next paragraph and **Figure 1.1**). A better understanding of the potential physiological profiles will help us understand advantages and disadvantages of specific temperaments in different environmental and social conditions.

Based on the current review, high-exploration is the most highly-studied temperament in relation to physiological mechanisms, and has been associated with heightened sympathetic activity and cell-mediated immunity, and attenuated innate and humoral immunity. Given an exploratory phenotype that frequently engages novel environments, this physiological profile is likely quite adaptive. Specifically, heightened sympathetic activity with attenuated HPA reactivity allows for rapid, sympathetic-driven responses to dynamic environmental conditions without exposing the exploratory organism to frequent high levels of glucocorticoids in circulation, which could dampen immunity. In addition, heightened sympathetic reactivity would support heightened cell-mediated immunity to maximize a relatively rapid and learned immune response to the frequent novel antigens that exploratory individuals expose themselves to when exploring novel habitats. On the other hand, bold and aggressive individuals that expose themselves to more risky conditions would be better supported with a physiological response that allows for more rapid innate immunity, in addition to cell-mediated reactivity in order to heal wounds in the face of danger and aggressive interactions. In this case, heightened sympathetic activity would support rapid behavioural and immune responses that are required in dangerous situations, and dampened basal glucocorticoid production would allow a heightened rapid immune response. Finally, we can hypothesize that more sociable individuals will benefit from heightened humoral immunity to respond to frequent re-exposure to antigens passed among social partners. In addition, strong social network and social group centrality that is often associated with sociability may require less sympathetic and HPA reactivity to respond to novelty or threats. **Figure 1.1** below displays hypothesized physiological profiles for each of four temperament types.

If we can test and determine potentially complex physiological profiles for different temperaments, then we are equipped to understand mechanisms that may explain temperament consistency vs. flexibility, covariation, and fitness in different environmental conditions. For example, a similar physiological profile underlying bold and aggressive temperaments (as depicted above and in **Figure 1.1**) may explain why boldness and aggression often covary within individuals, i.e. often make up a behavioural syndrome. The relative stability of different physiological profiles could help explain the relative stability of different temperaments. And finally, specific physiological profiles associated with a specific temperament can provide clues as to why certain temperaments thrive in some environmental conditions but not in others. For example, a bold or aggressive physiological profile which includes heightened innate and cell-mediated immunity and sympathetic activation may fair less well in environments that have low nutritional resources, but may do relatively well in environments with heavy predation. Additional understanding of any physiological profiles associated with temperament will provide a better understanding of selection pressures associated with each temperament, and may help explain relative behavioural flexibility.

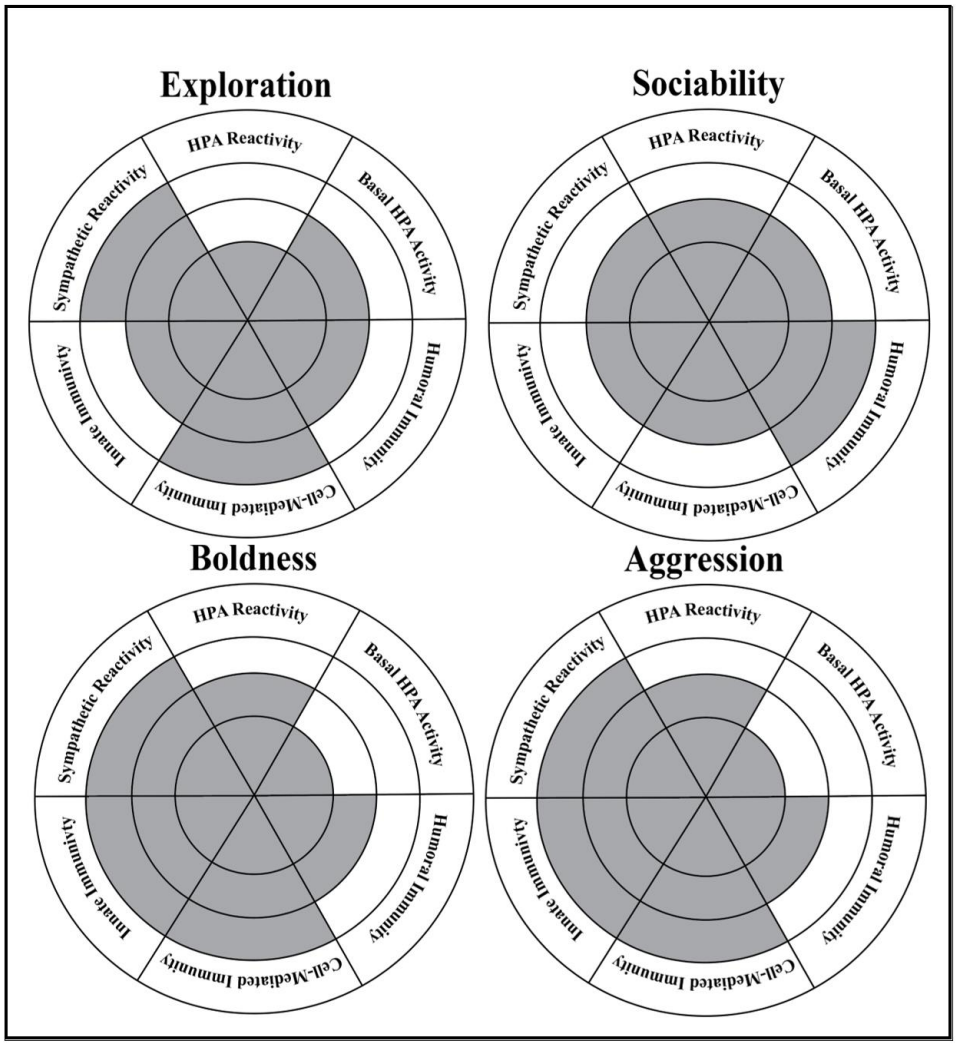


Figure 1.1. Expected physiological profiles for exploration, sociability, boldness and aggression. For each temperament, the grey shading indicates the relative regulation that we might expect of each physiological system (sympathetic reactivity, HPA reactivity, basal HPA activity, innate immunity, cell-mediate immunity, and humoral immunity). Grey shading, within any particular physiological system, that extends to the outer-most ring indicates up-regulation of that physiological system, while shading that only extends out one ring from the center indicates down-regulation of that system. Shading that reaches the middle ring indicates neither up- nor down-regulation of that physiological system. We present hypothetical physiological profiles for the four temperaments that have been most frequently studied.

Future Directions

There is limited information on naturally-occurring physiological regulatory profiles associated with specific temperaments. Few studies have investigated multiple

physiological traits as they relate to any one behavioural trait or temperament [40,100,101]. To understand how temperaments are maintained or change across time and conditions, and to determine long-term consequences of temperaments, we need additional information on the multiple, inter-related physiological systems underlying temperament. To accomplish more complex physiological profiling, we need (1) diverse data that include multiple behavioural and physiological measures, (2) studies conducted in both the natural habitat and controlled laboratory conditions, and (3) a prioritized list of physiological processes that can be quantified in field and laboratory settings.

A starting point to prioritize specific physiological systems to measure is to identify those processes that have important functional influence on behaviour and fitness. This can build on previous work that has focused heavily on HPA regulation. The neuroendocrine system mediates rapid and long-term behavioural changes and effects multiple physiological systems [reviewed in [102,103]]. In particular, given an interest in potential fitness and survival outcomes associated with temperament, a richer understanding of temperament-specific immune regulation biases should be a specific area of increased research (see specific immune measures and challenges are reviewed in [101]). In addition, to the neuroendocrine, immune, and sympathetic mechanisms that were reviewed here, an easily accessible and highly functional physiological process to include in temperament physiological profiles is gut microbiome diversity. Growing biomedical literature indicates that diversity in gut microbiome impacts temperament-related physiology, behaviour, and health [104,105]. Further, gut microbiome diversity can be measured from fecal samples, making it highly feasible in field research, and this non-invasive metric allows for repeat measurements across time and conditions in free-

ranging animals. Metabolic rate is another important physiological mechanism that has been related to behaviour in both endothermic and ectothermic organisms [69,106]. Although metabolic rate is not easily measured in free-ranging animals, prior research on this basic and systemic process suggests that it should be included in temperament-specific physiological profiles, even if limited to laboratory studies.

In addition to identifying other *a priori* physiological processes that are important for fitness, we propose that future work rely on '-omics' genetic expression methods (e.g. transcriptomics and bioinformatics) to identify novel physiological processes related to temperament and that may drive trait flexibility/consistency, covariation, and fitness outcomes. Large-scale transcriptomic analyses allow for global estimates of gene expression patterns that are related to temperament, and with the use of bioinformatics tools, researchers can begin to identify different physiological processes that may be associated with temperament [107,108]. Since temperaments are likely influenced by a multitude of physiological process driven by legions of genes, this work should involve whole-transcriptome or epigenome profiling [82,107,109]. As a first step, the most ideal cells for transcriptomic and bioinformatic analyses may include those present in circulation, given their relative accessibility and systemic function. This systems biology approach could be used in both field and laboratory studies.

Overall, a systems perspective on the physiological profiles associated with temperament will provide basic information necessary to understand the functional significance of temperament and to understand which temperaments are most suited for specific environmental conditions. This will require cross-disciplinary research with behavioural and physiological experts. Interdisciplinary work will lead to large-scale

breakthroughs on how and why individuals systematically behave differently from one another, how these differences are propagated over time, and how they lead to different survival, fitness, and health.

Authors' contributions statement: EKM conducted literature searches, synthesized results of published studies, and drafted significant portions of the manuscript. EY identified, synthesized, and drafted text on human studies. SAC developed the idea for the manuscript, synthesized results of published studies, and drafted significant portions of the manuscript. All authors reviewed, provided feedback, edited the manuscript and gave final approval for publication.

Chapter 2:

How do we characterize temperament?: Broad testing of temperament across time and contexts in low variable conditions

Abstract

Many animal species have been shown to maintain relatively consistent individual differences in behavior, indicative of temperament or personality. These stable individual differences are important for life history, survival, and health consequences, and the field has seen exponential growth in temperament (e.g. boldness, exploration, sociability, aggression, and activity) research. Quantification of animal temperament can be a difficult because it requires (a) repeated behavioral measures from the same individual (to gauge within-individual consistency across time and contexts), and (b) identification of the most appropriate behavior(s) to characterize temperament. In the current study, we conducted comprehensive and repeat behavior testing in a low-variable environment to compare methods to identify temperaments that are individually-consistent over time and across conditions. To control environmental effects on behavior, we conducted this work with individually-housed outbred male Sprague-Dawley rats in a laboratory. To determine behaviors that are individually-consistent, we measured each rat's behavioral response to five different arenas (different contexts) and repeated the five tests at three different time points. To identify potential composite, multi-behavior estimates of temperament, we used factor analyses to determine behaviors that regularly covaried on each test arena. To determine individual consistency across time and contexts, we conducted reliability/correlation analyses. Several behaviors consistently covaried on each test, and resulting factors tended to align with the five commonly-studied temperament categories, with some exceptions. Certain behaviors/factors were more consistent across time and contexts than others; distance travelled/activity were quite consistent along with sociality- and boldness-related behaviors, whereas aggression and exploration were less consistent. Last, we did not find strong evidence that multiple behavioral estimates of temperament were more consistent over time or across conditions than single behaviors. For those interested in first steps to quantify temperament in their study species, we propose similar comprehensive cross-time and cross-context behavior testing where possible.

Key words: behavioral phenotype, temperament, behavioral consistency, repeatability

Introduction:

Animals often display individually-distinct behavioral traits that are consistent over time and across conditions. These are frequently referred to as temperament traits [12] or personality. The study of temperament in animals has grown rapidly over the past 20 years with a variety of studies demonstrating that traits like boldness, exploration, activity, sociability, and aggression, predict ecological outcomes like survival, dispersal, reproductive success, and offspring growth, dispersal, and survival [4,27,55,56,60]. Further, the relative consistency of these traits (across time and across conditions) has been shown to predict these adaptive or maladaptive aspects [4,26,68,110–114]. However, often times the relative consistency (or repeatability) of temperament traits within a study population is not considered or measured (as would be suggested by [10]). In the current study, we used a laboratory animal model to minimize environmental variability among individuals in order to address several basic questions about animal temperament: (1) How do we best quantify temperament?, (2) What behaviors/temperaments are individually-consistent across time?, and (3) What behaviors/temperaments are individually-consistent across contexts?

In 2007, a comprehensive review of temperament studies [12], stimulated a dramatic increase in ecological studies on individual differences across many species and environments [9]; e.g. free-ranging lizards:[115] ; fish:[116] ; urban animals:[117] . Réale and colleagues [12] reviewed five commonly-studied temperaments: (1) Exploration – response to novelty, (2) Boldness – willingness to take risks, (3) Activity –

relative movement/sedentary nature, (4) Sociability – frequency or intensity of interactions with conspecifics, and (5) Aggression – frequency or intensity of antagonistic behavior toward conspecifics. While this is not a comprehensive list of possible temperament traits, it is comparable to the five basic dimensions of human personality identified from data-driven analyses [118]. Thus, the five temperament categories identified by Réale and colleagues [12] provide a good basis from which to characterize temperaments in animals.

To quantify temperament, it is common to measure one specific behavior. This method is well-established in the literature and is practical for field studies. For example, to quantify Boldness, main metrics include latency to move into, or time spent in the center of an arena or a risky area [62,119–121]. Or to measure Exploration, it is common to track latency to interact with, and/or the amount of activity near, novel objects or novel social partners (Wood warblers: [122] ; Zebra finch: [4,26,30,110,123,124]. These metrics are often chosen because they are easy to observe and are thought to reflect specific and persistent underlying perceptual biases or interpretations of environmental stimuli (e.g. novelty is dangerous vs. novelty is interesting).

There are just as many studies that have measured and combined multiple behaviors to characterize one temperament. For example, Boldness has been measured using a combination of behaviors that includes distance from novel objects, latency to interact in risky environments after a stressor, and time spent in a risky area [61,125]. Exploration has been measured using multiple behaviors on a single test (e.g. latency and frequency to approach novel items; Webster and Lefebvre 2000) or latency measured on multiple different tests (Birds: [126,127]; Rodent: [128]). Aggression has been measured

with a combination of multiple behavior metrics such as latency to attack, number of aggressive contacts and posture [26,56,61]. And Sociability has been measured with multiple behaviors such as number of interactions, time spent socializing, and centrality within a social network (Primate: [129,130]; Reptile: [131,132]; Bird: [133,134]. Activity is not frequently quantified with multiple behaviors, although Dingemanse and colleagues [61] determined activity scores for sticklebacks using number of squares visited, distance travelled, and number of square changes. These multi-metric estimates can be traced back to psychology tests developed to quantify basic personality traits in humans using factor analyses (e.g. Eysenck three dimensions of personality, Cattell sixteen factors of personality). In the above animal study examples, multiple metrics are collected, and animals are given an overall temperament score using multivariate statistics like principal component analysis. Given their more comprehensive nature, the use of multiple behaviors or composite measures to quantify temperament may be more accurate estimates of individually-consistent underlying traits. In the current paper, we address this issue of whether single vs. composite estimates of temperament are more consistent across time and contexts.

In wild and captive studies, the use of just one behavioral measure is often used to capture individual consistency (reviewed in [110]), which may suggest that one consistent behavior may provide a key component of a temperament. However, in reality, it is likely that temperament (e.g. Boldness, or Exploration, etc.) is expressed across multiple behaviors. In the current study, to compare relative quality of simple (single-behavior) vs. composite (multi-behavior) estimates of temperament, we compared the relative consistency of single- vs. multiple-behavior metrics. The objectives were to: (1)

identify behaviors that covary within specific behavioral tests and whether covarying behaviors are related to temperaments reviewed by [12], (2) determine relative temporal consistency of individual vs. multi-behavior measures of temperament within the same context (i.e. same test), and (3) determine the relative consistency of individual vs. multi-behavior estimates of temperament measured across different conditions (i.e. different behavior tests). We tested this with an outbred, genetically- and behaviorally-variable laboratory rodent. To identify temperaments that are consistent across time and across conditions and to provide a template for future studies with other species, we repeatedly tested animals on five different behavior tests (to assess the five temperaments reviewed by [12]). We recorded multiple behaviors in each test, and repeated each test three times. With different and repeated behavior tests, we are able to characterize which behaviors covary in any one condition (test), compare these covarying behavior profiles to frequently-used temperament categories, and determine whether single- or multi-behavior estimates of temperament are more consistent across time and across conditions.

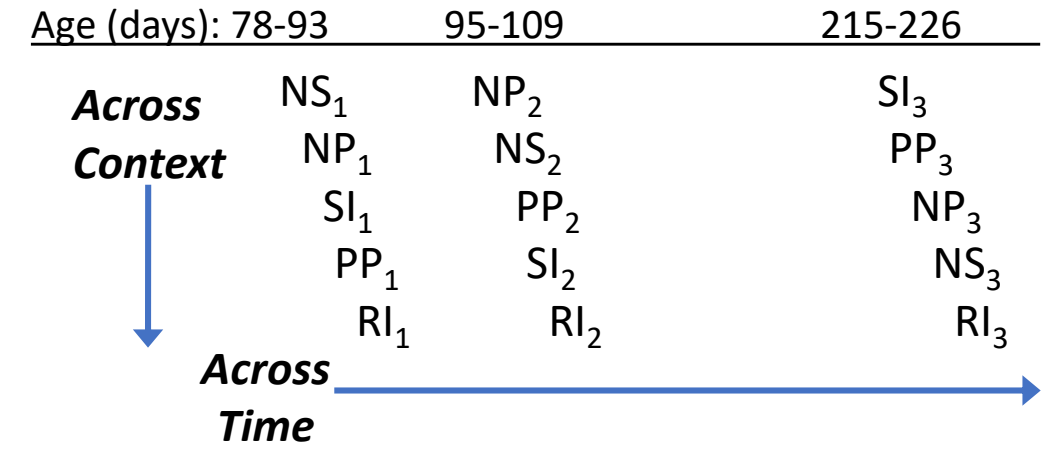
Methods:

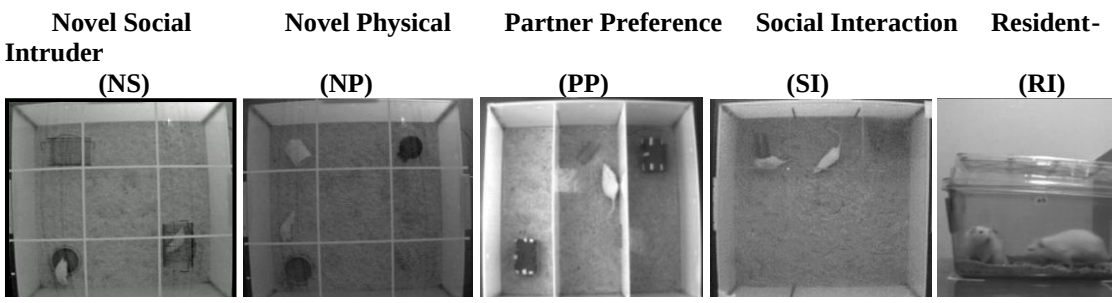
General:

To characterize temperament in lab rats, we used several established behavioral tests to assess five broad traits summarized in Réale et al. [12]: Boldness, Exploration, Sociability, Aggression, and Activity. We selected tests that were designed to assess more than one of the above temperaments, and we used at least two tests to estimate each temperament category. Within each test we measured multiple behaviors, and to establish multi-behavior estimates of temperament we used principal component and factor analyses to identify behaviors that regularly covaried in each test. Based on factor

analysis, latent factor scores were assigned to each rat for each factor in each test (i.e. possible multi-behavior estimates of temperament). To test for behavioral consistency across time, each rat completed each test three times and we calculated repeatability estimates for each behavior and each factor score on the same test across time. To determine behavioral consistency across conditions, we calculated mean correlation coefficients between different behaviors and factor scores across the different tests administered at any point in time. See **Figure 2.1** for a visual of overall study design and methods.

Figure 2.1. Overall Study Design: Five different behavior tests were conducted to measure different aspects of temperament: NS – Novel Social, NP – Novel Physical, PP – Partner Preference, SI – Social Interaction, and RI – Resident Intruder. To measure behavioral consistency across contexts and across time, each test was conducted at each of three time points (~85, 100, 220 days of age; with ~3 days between each test at each time point).





Animals:

Adult male Sprague-Dawley rats (N= 57, 60 days of age Charles River Laboratories, Raleigh, NC) were individually-housed in plastic cages (43.5 × 23.5 × 20.5 cm), and maintained on a 12L:12D light schedule (lights off at 10:00 h and on at 22:00 h EST). The colony room was sustained at 22°C with an average of 50% humidity. To provide enrichment, home cages contained a large red tube and a wooden chew stick. Food and water were available *ad libitum*. Rats were allowed to acclimate to laboratory housing and handled daily for 2 weeks prior to testing. All methods in this study were approved by the Pennsylvania State University Institute for Animal Care and Use Committee (PROTO201800433), and adhered to the guidelines developed by ASAB/ABS for use of animal in behavioural research, and those outlined by the National Research Council (Guide for the Care and Use of Laboratory Animals).

Behavior Tests:

We used five behavioral tests, each conducted at three different times. The first and second round of testing were separated by three weeks, and the third round was conducted 4 months later. (Rats were approximately 85, 100, and 220 days of age at each round.) Between the second and third round of testing, all rats were exposed to the same battery of physiological tests that are not included in this study. To randomize test order effects, the order of the five tests within each round of testing was changed, and the order in which animals were tested within each test was randomized. All tests were performed during the active/dark phase (13:00-15:00 h) in a room separate from the colony room. For all but the resident intruder test, rats were transported to the test arena by being carried in the red enrichment tube from their home cage. Each test lasted 5 minutes, after

which time rats were immediately returned to their home cage and feces removed from the test arena. Recording began immediately after placement of the rat into the arena. For the Novel Social, Novel Physical and Partner Preference Tests, behaviors were analyzed using AnyMaze (Stoelting Company, Wood Dale, IL). For the Social Interaction and Resident Intruder Tests, hand coding was conducted independently by two researchers. Behaviors measured in each of the five tests are listed and defined (and given acronyms) in **Table 2.1**. Behaviors measured in each test were based on prior studies in which boldness was quantified with time spent in the center [62,119–121] exploration quantified with latency to interact with novelty [4,26,30,110,124,128] aggression quantified by latency to attack [126,135,136]; activity quantified by measuring total distance travelled [30,137], and sociability quantified using latency to interact with a familiar partner, or tendency to join a group [138–140].

Novel Social Test: This test was used to measure Exploration, Sociability, Activity, and Boldness [68]. The dimensions of the arena were 120cm x 120cm with 46cm walls. The arena floor was coated with fresh sawdust bedding. The arena contained two cages in diagonal corners from each other - one empty and the other with a novel same-sex rat of similar size and age as the test rat. Each study rat, within its enrichment tube, was placed into one of the free corners of the arena.

Novel Physical Test: This test was used to measure Exploration, Activity, and Boldness [68]. The test arena was the same size and height as the Novel Social Arena, but instead of two cages, there were three rat-sized objects placed in three of the four corners of the arena. Study rats, within the enrichment tube from their home cage, were placed in the free corner of the arena.

Partner Preference Test: This test was used to assess Sociability and Activity [141]. The Partner Preference Test arena was the same size as for the Novel Social Test arena with two additional walls that divided the arena into three equal sections of 40cm x 120cm. The internal walls each had a 5-inch square cutout at the bottom to allow for passage between arena sections. In one section a familiar rat was placed in a cage and in the other side section an unfamiliar rat was placed in a cage. (The familiar stimulus rat was the one used in the previous Novel Social Test whereas the study rat had never interacted with the unfamiliar stimulus rat.) The center section was left empty and the study rat, along with its red enrichment tube, was placed into this section of the arena.

Social Interaction Test: This test was used a priori to assess individual rat Sociability and Aggression [142,143] The Social Interaction Test arena was the same dimensions as the Novel Social Test arena. A study rat and an untethered, unfamiliar stimulus rat were placed in the arena simultaneously. Three different stimulus rats were used in total for each study animal for this test.

Resident Intruder Test: This test was used to assess Aggression [144,145]. An unfamiliar stimulus rat was placed in the study rat's (the resident's) home cage. After testing, the stimulus rat was returned to its home cage. Three different stimulus rats were used in total for each study animal for this test.

Table 2.1. Behaviors documented in each test. Behaviors are divided based on Behavior Test. Acronyms used for each behavior recorded are comprised of the Behavior Test followed by the specific behavior recorded.

Behavior Test	Behaviors Recorded	Behavior Acronyms
Novel Social	Total distance travelled	NSDist
	Novel Social Cage:	
	Approaches (n)	NSsApp
	Time in proximity (s)	NSsTime
	Latency to approach (s)	NSsLat
	Empty Cage:	
	Approaches (n)	NSeApp
	Time in proximity (s)	NSeTime
	Latency to approach (s)	NSeLat
	Center:	
	Distance travelled	NScDist
	Entries (n)	NScEnt
	Time spent (s)	NScTime
	Latency to enter (s)	NScLat
	Periphery:	
	Distance travelled	NSpDist
	Entries (n)	NSpEnt
	Time spent (s)	NSpTime
	Latency to exit (s)	NSpLat
Novel Physical	Total distance travelled	NPDist
	All Objects:	
	Approaches (n)	NPoApp
	Time in proximity (s)	NPoTime
	Latency to approach first object (s)	NPoLat
	Center:	
	Distance travelled	NPcDist
	Entries (n)	NPcEnt
	Time (s)	NPcTime
	Latency to enter (s)	NPcLat
	Periphery:	
	Distance travelled	NPpDist
	Entries (n)	NPpEnt
	Time (s)	NPpTime
	Latency to exit (s)	NPpLat
Partner Preference	Total distance travelled	PPDist
	Familiar Rat Cage:	
	Approaches (n)	PPfcApp
	Time in proximity (s)	PPfcTime
	Latency to approach (s)	PPfcLat
	Center:	
	Entries (n)	PPcEnt

	Time (s) Latency to exit (s)	PPcTime PPcLat
	Unfamiliar Rat Cage: Approaches (n) Time in proximity (s) Latency to approach (s)	 PPucApp PPucTime PPucLat
Social Interaction	Latency to interact (s)	SIsLat
	Time together (s)	SIsTT
	Nose-to-Nose (n)	SIsNN
	Anogenital sniff (n)	SIsAS
	Follow (n)	SIsF
	Run Away (n)	SIsRA
	Mount (n)	SIsMount
	Rear (n)	SIsRear
	Fight (n)	SIsFight
	Pin (n)	SIsPinn
	Total distance travelled (lines crossed on a 3x3 grid)	SIsDist
Resident Intruder	Latency to attack (s)	RILat
	Rear (n)	RIRear
	Move toward stimulus rat (n)	RIMoveTo
	Attack (n)	RIAttack
	Be attacked (n)	RIBeAtt
	Chase (n)	RIChase
	Be chased (n)	RIBeChase
	Lay on back (n)	RILay
	Pin stimulus rat (n)	RIPin
	Sniff (n)	RISniff
	Ano-genital sniff (n)	RIAgSniff
	Freeze (n)	RIFreeze

Data Analyses:

To address objective 1 (identify behaviors that covary on a given test), principal component analysis (PCA) was conducted to reduce dimensionality of the dataset and to determine which behavior variables regularly accounted for the least amount of total variance. This was used to identify variables to remove prior to exploratory factor analysis. A correlation matrix of behavioral measures was used to perform PCA in R

4.0.2. Variables were standardized to account for different scales. The factoextra package was used to construct bar plots to indicate relative variable contributions to the first two principal components (PC1 and PC2). A correlation map was used to observe the direction and magnitude of each variable's contribution to PC1 and PC2. PCA was used to explore the data, but not used as an analytical technique because in previous studies it has been shown to produce poor models due to a low percent variance explained by the first few components (e.g. [146–149].

To determine behaviors that regularly covaried on each test at each time point, we used exploratory factor analysis (FA), and then for each rat calculated a factor score (based on variable loadings) for the first three most significant factors. FA explores the underlying "latent" structure of the factors and is preferable to PCA when the goal is to account for the correlations among variables [150]. We used Budaev's [150] suggested methods for behavioral data sets. Exploratory FA was conducted in IBM SPSS 26.0 using a correlation matrix and the principal axis factoring method. Parallel analyses tests were run in R to determine the number of factors to be extracted from the model [151]. A scree plot (paran package) and non-graphical solutions (nFactors package) were compared for accuracy. The factor structure was subject to Varimax rotation with Kaiser Normalization due to the relatively small sample size, and factor scores were calculated for each individual using the regression method. Variables with low communality scores that prevented model convergence at all three timepoints (R1, R2, R3) were further removed. Kaiser–Meyer–Olkin measure of sampling adequacy (KMO) and Bartlett's Test of sphericity (KMO > 0.5, Bartlett's significance < 0.05) were examined to determine relative validity of the factor structure for each behavior test. To interpret factors, we only

considered those behaviors that had loadings of greater than 0.30.

To address objectives 2 (determine temperament consistency across time), we determined if individual rats displayed behavioral consistency on the same test administered across time by testing the repeatability of individual behavior and factor scores across timepoints (R1, R2, and R3) using intraclass correlations (ICC). This was conducted independently for each behavior test. We also tested repeatability/consistency of individual behaviors on each test across time using ICC. For all ICC analyses, we used two-way mixed model, with single measure, and consistency type to calculate r [152–155]. Repeatability signifies the percentage of total phenotypic variance explained by within-individual variance [149,156]

To address objective 3 (determine temperament consistency across contexts), we conducted Pearson correlations among all behaviors and among all factors on all five tests within each timepoint.

Results:

Objectives 1 and 2: Behaviors that covary on each test arena and Consistency of behaviors/factors across time

Novel Social Test

Across the three timepoints, the cumulative proportion of variance explained by the first, second, third, and fourth components of PCA were 28-31%, 43-51%, 55-65%, and 63-77% respectively. The four latency variables (i.e., latency to approach the empty & novel social cages, latency to enter center of arena, and latency to leave the periphery of arena) consistently did not make significant contributions to the first two components at any timepoint; these variables were excluded from the factor analysis. These same

variables regularly had low communality values when included in factor analyses. Factor analyses were conducted with data from each time point using the remaining untransformed behavioral variables. PA and MAP indicated that 3 factors were optimal. With 10-variables and a three-factor model, KMO at each timepoint ranged from 0.622-0.672, and Bartlett's approximate $\chi 2$ from 216-324 (df=45). Variable loadings on each factor are shown in *Supplementary Table 2.1*. The first factor accounted for 30-40% of the variance at each timepoint, and variables that consistently loaded on this factor at all three time points were related to sociality and boldness: social cage approaches, time near social cage, time spent in periphery (negative loading), and distance travelled in center. We termed this factor Social Boldness. Other bold behaviors, like time in center, and center entries, loaded on this factor at timepoints 2 and 3, providing further support for this interpretation. A second factor that accounted for 16-25% of the variance at each timepoint included consistent loading of the following periphery-related behaviors: Periphery entries, Time in periphery, and Distance travelled in periphery. We termed this factor Safe Activity. A third factor accounted for 15-18% of the variance with physical exploratory behaviors consistently loading at each time point: Empty cage approaches, and Time near empty cage. We termed this final factor Physical Exploration.

An individual's factor scores for Social Boldness and Safe Activity were relatively consistent over time, and more consistent than the majority of specific behaviors measured on the novel social test (**Table 2.2**). The one behavior that was particularly more consistent than the factor scores across time was latency to social cage, a behavior which was not included in the factor analyses. An individual's factor scores for Physical Exploration were not consistent over time (**Table 2.2**).

Table 2.2. Across time consistency – Novel Social Test: Relative consistency/reliability of individual factor and scores and behaviors across time. Intraclass correlation coefficient (single measure ICC, *r*), 95% confidence interval (CI), and *p*-value, based on two-way mixed model consistency type ICC analysis (k=3, n=54), are reported. Bold font indicates statistically significant ICC.

New Factors & Original Metrics	NS1 Mean (SD)	NS2 Mean (SD)	NS3 Mean (SD)	Single Measures ICC (*r*)	95% CI	*p*
F1 - Social Boldness	0 (0.95)	0 (0.98)	0 (0.94)	**.266**	.099-.445	.001
F2 - Safe Activity	0 (0.94)	0 (0.93)	0 (0.87)	**.342**	.173-.514	.000
F3 - Physical Exploration	0 (0.89)	0 (0.94)	0 (0.97)	.120	-.035-.302	.068
Total Distance Traveled	21.4 (7.8)	28.6 (10.6)	22.8 (7.7)	**.352**	.184-.523	.000
Social cage approaches	15.1 (7.7)	29.30 (15.0)	18.3 (10.4)	**.216**	.051-.397	.004
Time near social cage	41.1 (16.6)	61.2 (28.0)	27.8 (15.6)	**.156**	-.004-.338	.028
Latency to social cage	35.0 (35.1)	27.2 (29.0)	14.8 (13.7)	**.408**	.241-.572	.000
Empty cage approaches	12.0 (4.7)	16.8 (6.2)	14.5 (6.7)	**.154**	-.006-.336	.030
Time near empty cage	48.3 (20.0)	28.9 (11.6)	19.7 (10.8)	.058	-.089-.237	.228
Latency to empty cage	47.0 (37.9)	17.6 (19.0)	17.2 (41.1)	**.163**	.003-.345	.023
Periphery entries	25.3 (8.0)	29.8 (9.7)	38.6 (11.9)	**.359**	.191-.529	.000
Time in periphery	94.9 (35.4)	77.3 (33.4)	108.8 (41.3)	.087	-.065-.267	.138
Latency to leave periphery	11.0 (9.2)	16.3 (17.8)	9.3 (12.9)	.031	-.112-.208	.339
Distance traveled in periphery	7.0 (3.6)	5.4 (2.5)	7.6 (4.0)	**.210**	.046-.392	.005
Center entries	51.4 (13.2)	74.9 (20.8)	9.3 (5.3)	**.296**	.127-.472	.000
Time in center	8.3 (4.8)	13.5 (10.6)	18.6 (12.0)	-.012	-.148-.160	.550
Latency to enter center	26.0 (14.1)	26.4 (15.7)	39.9 (49.9)	.046	-.100-.224	.277
Distance traveled in center	12.5 (5.1)	16.9 (6.4)	2.2 (1.2)	**.358**	.190-.528	.000

Novel Physical Test

Across the three test ages, cumulative proportion of variance explained by the first, second, third, and fourth components of PCA were 28-44%, 45-63%, 55-77%, and 61-88% respectively. Based on PCA with 11 variables at each time point, the one behavior that consistently contributed less than 10% to the first two components at each time was latency to approach object. This variable was excluded and factor analyses conducted with the remaining variables. PA and MAP indicated that 3 factors were optimal. With 10-variables and a three-factor model, KMO at each timepoint ranged from 0.551-0.735, and Bartlett's approximate $\chi 2$ from 199-318 (df=45). Variable loadings on each factor are shown in *Supplementary Table 2.2*. At time point 3, a 3- or 4-factor model did not converge, so a 2-factor model was used. Across all time points, the first factor accounted for 31-46% of the variance and consistently included loading of the following center-oriented variables: center entries, time in center, latency to center (negative loading), distance travelled in center. We termed this factor Boldness since the center of the arena is considered a vulnerable location. The second factor accounted for 18-21% of the variance at each time point, and had consistent loading of periphery-oriented variables: periphery entries, and distance travelled in periphery. We termed this factor Safe Activity. The third factor accounted for 11-15% of the variance at time points 1 and 2, with only one variable that consistently loaded: novel object approaches. We termed this final factor Physical Exploration. On the Novel Physical Test, individual Boldness factor scores were moderately consistent over time, and were more consistent than about half of the individual behaviors. On the other hand, the Safe Activity and Physical Exploration factor scores were not consistent across time (**Table 2.3**).

Table 2.3. Across time consistency – Novel Physical Test: Relative consistency of individual factor and scores and behaviors across time. Intraclass correlation coefficient (single measure ICC, *r*), 95% confidence interval (CI), and *p*-value, based on two-way mixed model consistency type ICC analysis (k=3, n=54), are reported. Bold font indicates statistically significant ICC.

New Factors & Original Metrics	NP1 Mean (SD)	NP2 Mean (SD)	NP3 Mean (SD)	Single Measures ICC (*r*)	95% CI	*p*
F1 - Boldness	0 (0.96)	0 (0.96)	0 (0.96)	**.194**	.031-.376	.009
F2 - Safe Activity	0 (0.87)	0 (0.95)	0 (0.99)	.129	-.028-.311	.055
F3 - Physical Exploration	0 (0.86)	0 (0.87)	--	.057	-.212-.318	.340
Total distance traveled	26.4 (7.0)	24.1 (8.2)	24.5 (7.1)	.132	-.025-.314	.052
Object approaches	29.9 (6.9)	31.6 (7.8)	29.7 (8.7)	**.268**	.101-.447	.001
Time near object	66.7 (18.9)	70.9 (17.2)	66.6 (21.7)	**.266**	.098-.444	.001
Latency to object	10.8 (12.5)	9.1 (5.3)	5.3 (5.5)	.128	-.029-.310	.057
Periphery entries	31.0 (7.5)	24.8 (7.8)	32.6 (8.3)	**.145**	-.013-.328	.037
Time in periphery	111.6 (19.1)	104.9 (29.1)	117.6 (38.3)	.062	-.086-.241	.215
Latency to leave periphery	9.5 (14.3)	13.5 (21.1)	5.2 (5.9)	-.045	-.175-.123	.709
Distance traveled in periphery	9.5 (3.1)	8.6 (3.5)	9.3 (3.3)	**.355**	.186-.525	.000
Center entries	4.9 (2.5)	5.6 (3.0)	7.7 (4.1)	**.201**	.037-.383	.007
Time in center	4.7 (2.7)	5.3 (3.6)	11.2 (7.5)	.112	-.043-.294	.082
Latency to center	91.5 (66.9)	49.0 (53.4)	41.4 (57.5)	**.270**	.102-.448	.001
Distance traveled in center	1.0 (0.6)	1.2 (0.7)	2.2 (1.2)	**.183**	.021-.365	.013

Partner Preference Test

Across time, cumulative variance explained by the first, second, third, and fourth components of PCA were 43-51%, 58-74%, 71-88%, and 76-94% respectively. No variable repeatedly showed low contribution to the components so we used all 10 variables for factor analyses. PA and MAP indicated that 3 factors were optimal. With 10-variables and a three-factor model, KMO at each timepoint ranged from 0.635-0.770, and Bartlett's approximate $\chi 2$ from 236-378 (df=45). Variable loadings on each factor are shown in *Supplementary Table 2.3*. We termed the first factor Familiar Sociality which accounted for 36-51% of variance across time, with the following variables consistently loading: familiar social cage approaches, time near familiar social cage, latency to approach familiar social cage (negative loading), and time spent in center (negative loading). The next factor, which accounted for 13-23% of the variance at each timepoint, was labeled as Social Boldness because the following variables consistently loaded: unfamiliar social cage approaches, time near unfamiliar social cage, and time in center (negative loading). (In this test, the center of the arena consists of the middle third of the arena where the rat is first placed at the beginning of testing, thus less time spent in this center third likely reflects increased risky behavior, as opposed to time spent in the center of the Novel Social or Novel Physical arenas.) The last factor was termed Activity/Non-Social Boldness, and accounted for 12-16% of the variance at each timepoint, with the only consistently loading variable being center entries. At different time points, variables that loaded on this factor also included total distance travelled and latency to leave center (negatively). In the Partner Preference test, individual animal factor scores for Social Boldness and Activity/Non-Social Boldness were relatively consistent across time, and

were more consistent than most individual behaviors. However, total distance travelled was more consistent than these two factors (**Table 2.4**). Individual scores on the Familiar Sociality factor, which accounted for the greatest amount of variance at each time point, were not consistent across time (**Table 2.4**).

Table 2.4. Across time consistency – Partner Preference Test: Relative consistency/reliability of individual factor scores and behaviors across time. The table reports intraclass correlation coefficient (single measure ICC, *r*), 95% confidence interval (CI), and *p*-value, based on two-way mixed model consistency type ICC analysis (k=3, n=54). Bold font indicates statistically significant ICC.

New Factors & Original Metrics	PP1 Mean (SD)	PP2 Mean (SD)	PP3 Mean (SD)	Single Measures ICC (*r*)	95% CI	*p*
F1 - Familiar Sociality	0 (0.92)	0 (0.96)	0 (0.95)	.115	-.040-.297	.077
F2 - Social Boldness	0 (0.94)	0 (0.99)	0 (0.93)	**.271**	.103-.449	.001
F3 – Activity/Non-Social Boldness	0 (0.89)	0 (0.96)	0 (0.94)	**.382**	.214-.549	.000
Total distance traveled	27.9 (9.9)	28.8 (11.3)	20.3 (8.1)	**.448**	.284-.606	.000
Familiar social cage approaches	16.8 (8.6)	17.7 (9.9)	5.4 (4.5)	.093	-.059-.274	.121
Time spent near familiar social cage	32.8 (18.1)	24.0 (16.0)	7.0 (6.8)	.108	.046-.290	.089
Latency to approach familiar social cage	49.1 (56.6)	71.4 (80.8)	136.2 (104.5)	.092	-.060-.272	.126
Unfamiliar social cage approaches	14.6 (7.6)	16.7 (12.7)	13.3 (7.4)	**.307**	.138-.482	.000
Time spent near unfamiliar social cage	33.6 (17.0)	23.7 (18.3)	16.7 (9.4)	**.262**	.095-.441	.001
Latency to approach unfamiliar social cage	92.8 (68.7)	69.7 (79.8)	52.0 (65.4)	**.321**	.153-.496	.000
Center entries	9.9 (3.4)	10.5 (3.9)	9.4 (4.0)	**.258**	.091-.437	.001
Time in center	101.1 (31.5)	115.1 (48.0)	170.3 (47.7)	**.278**	.110-.456	.000
Latency to center	34.4 (31.1)	18.0 (22.3)	25.8 (58.9)	.083	-.068-.263	.149

Social Interaction Test

Across time, cumulative variance explained by the first, second, third, and fourth components of PCA were 24-36%, 43-52%, 58-64%, and 69-73%. When all 11 behavioral variables were entered into PCA, latency to approach, nose-to-nose, distance travelled, and run away were relatively low contributors to the first two components at every timepoint. To avoid losing a significant number of variables, we retained these variables in factor analyses, which thus included all 11 variables. PA and MAP tests revealed that 3 factors were adequate to extract for the Social Interaction test. At timepoint 3, a 3-factor model did not resolve, and thus we used a 4-factor model, and to use similar factor models across time, we used a 4-factor model at time point 1, but this did not converge for time point 2, where we used a 3-factor model. With 11-variables and a three- or four-factor model, KMOs ranged from 0.515-0.720, and Bartlett's approximate $\chi 2$ ranged from 176-242 (df=55) across timepoints. Variable loadings on each factor are shown in *Supplementary Table 2.4*. The first factor accounted for 24-35% of the variance across time points, and included the following consistently heavily loading variables: fight, rear toward partner, pin, time together, and at time points 2 and 3, mount also loaded on this factor. We interpreted this factor as an index of Aggression. The second factor accounted for 13-19% of the variance across time with the following variables loading: follow time together, and at time points 2 and 3 anogenital sniff positively loaded. This factor was interpreted as an index of Sociality (and was the third factor at timepoint 2.) The third factor accounted for 11-16% of the variance across time points, with total distance travelled, and run away loading positively at all timepoints and time together loading positively at timepoints 1 and 3. We interpreted this factor as an

index of Activity.

This Social Interaction test was used to assess sociability and aggression toward an unfamiliar partner. Three factors emerged that represented Aggression, Sociality, and Activity. The Aggression and Sociality factors were weakly consistent within individuals over time, but less consistent than certain behaviors: total distance travelled, rear toward partner, and fight (**Table 2.5**). These three behaviors contributed to the Activity and Aggression factors. Thus, in the Social Interaction test, raw measures of Rear and Fight frequency likely provide a more accurate estimate of an aggressive behavioral trait rather than a composite factor score based on multiple aggressive behaviors. The same may be true when measuring an Activity trait; distance travelled is likely a better estimate rather than a complex factor estimate that includes multiple behavioral variables.

Table 2.5. Across time consistency – Social Interaction Test: Relative consistency of individual rat factor scores and behaviors across time. The table reports intraclass correlation coefficient (single measure ICC, *r*), 95% confidence interval (CI), and *p*-value, based on two-way mixed model consistency type ICC analysis (k=3, n=54).

New Factors & Original Metrics	RI1 Mean (SD)	RI2 Mean (SD)	RI3 Mean (SD)	Single Measures ICC (*r*)	95% CI	*p*
F1 - Aggression	0 (0.92)	0 (0.97)	0 (0.93)	**.154**	-.005-.337	.029
F2 – Sociality	0 (0.93)	0 (0.87)	0 (0.95)	**.143**	-.015-.326	.039
F3 – Activity	0 (0.83)	0 (0.99)	0 (0.84)	.085	-.066-.266	.142
Total distance traveled	78.9 (21.0)	82.4 (26.6)	50.0 (18.4)	**.326**	.158-.500	.000
Run away	10.6 (6.1)	6.5 (5.7)	1.0 (2.0)	.092	-.060-.273	.123
Latency to approach social partner	5.0 (5.8)	2.9 (2.1)	4.6 (6.2)	.033	-.111-.210	.332
Follow social partner	11.9 (6.5)	10.0 (6.5)	7.5 (5.3)	.056	-.091-.235	.236
Nose-to-nose	6.2 (3.5)	5.5 (4.2)	8.9 (4.0)	.084	-.067-.265	.145
Anogenital sniff	13.1 (5.1)	9.9 (5.8)	11.0 (5.9)	.070	-.079-.250	.188
Time together	167.8 (31.6)	127.6 (46.8)	63.8 (28.4)	.076	-.074-.256	.170
Rear	6.5 (4.0)	8.7 (5.6)	3.0 (2.8)	**.218**	.054-.400	.004
Fight	10.6 (5.7)	12.7 (8.8)	1.1 (2.2)	**.202**	.038-.383	.007
Pin	3.3 (3.0)	5.0 (4.5)	0.7 (1.3)	.035	-.109-.212	.324
Mount	1.8 (6.1)	1.4 (1.6)	0.8 (1.5)	.056	-.091-.235	.235

Resident Intruder Test

The cumulative variance explained by the first, second, third, and fourth components of PCA were 23-34%, 43-50%, 55-64%, and 65-74% respectively. When all 12 behavioral variables were entered into PCA, being chased and chase variables were low contributors to the first two components at every timepoint, and both variables accounted for little variance. These two variables were excluded, and factor analyses were performed with the remaining 10 variables. The resulting factor structure revealed low communality scores (<0.4) for pin intruder and rear toward intruder, so we removed these variables from the model to yield a final 8-variable model. PA and MAP tests revealed that 3 factors were adequate to extract for the resident intruder test. However, at timepoints 2 and 3, a 3-factor model did not converge, so a 2-factor model was used. With 8-variables and a two-factor model, KMO at each timepoint ranged from 0.515-0.623, and Bartlett's approximate $\chi 2$ from 106-182 (df=28). Variable loadings on each factor are shown in *Supplementary Table 2.5*. The first factor accounted for 30-40% of the variance across all time points, and variables that consistently loaded included move toward intruder (negative loading), lay on back, freeze, and be attacked, and thus this first factor was interpreted as an index of Submission. The second factor accounted for 18-24% of the variance at each timepoint with latency to attack (negative loading), attacks, and ano-genital sniffs consistently loading. We interpreted this factor as an index of Aggression.

The Resident-Intruder test was primarily used to assess aggressive behavior toward an unfamiliar partner. Two factors representing Aggression and Submission emerged. Unlike the Novel Social, Novel Physical and Partner Preference tests, resident behavior in the Resident-Intruder test was not individually-consistent over time – there

were no significant ICC reliabilities values for any of the behaviors or factor scores across time (**Table 2.6**, all r values less than 0.13).

Table 2.6. Across time consistency – Resident Intruder Test: Relative consistency of individual rat factor scores and behaviors across time. The table reports intraclass correlation coefficient (single measure ICC, *r*), 95% confidence interval (CI), and *p*-value, based on two-way mixed model consistency type ICC analysis (k=3, n=54).

New Factors & Original Metrics	RI1 Mean (SD)	RI2 Mean (SD)	RI3 Mean (SD)	Single Measures ICC (*r*)	95% CI	*p*
F1 - Submission	0 (0.87)	0 (0.96)	0 (0.97)	.044	-.101-.222	.284
F2 - Aggression	0 (0.99)	0 (0.93)	0 (0.97)	.031	-.112-.208	.338
Latency to attack intruder	68.9 (102.0)	113.1 (124.7)	271.5 (62.5)	.062	-.086-.241	.214
Attack intruder	3.9 (3.9)	3.8 (4.8)	0.3 (0.7)	.042	-.103-.220	.292
Move toward intruder	4.0 (4.2)	4.4 (3.9)	2.8 (2.8)	-.098	-.216-.061	.895
Sniff intruder - body	7.2 (5.0)	7.7 (4.7)	7.9 (3.0)	-.153	-.259-.006	.979
Sniff intruder - anogenital	4.7 (4.4)	7.3 (5.6)	2.5 (2.2)	.121	-.035-.303	.067
Rear toward intruder	8.7 (5.4)	7.4 (5.1)	15.72 (6.9)	-.059	-.186-.106	.768
Pin intruder	3.0 (3.2)	2.0 (2.5)	0.3 (0.7)	-.062	-.188-.103	.781
Be attacked	7.1 (5.5)	5.0 (5.4)	0.3 (0.7)	.036	-.108-.214	.316
Lay on back	4.3 (4.4)	3.9 (5.2)	0.3 (0.7)	-.050	-.178-.117	.730
Freeze	7.0 (6.1)	5.7 (6.4)	0.8 (1.3)	-.055	-.182-.112	.750

Objective 3: Consistency of behaviors and factors across conditions

Individual Social Boldness factor scores calculated from behavior in the Novel Social and Partner Preference tests were consistently positively correlated at all three time points (**Table 2.7a**). Activity factor scores on the Novel Social test were positively correlated with Activity scores on the Novel Physical, Social Interaction, and Partner Preference tests. Whereas Activity scores on the Novel Physical, Partner Preference, and Social Interaction tests were not strongly correlated with each other. Individual factor scores for Physical Exploration in the Novel Social and Novel Physical tests were positively related. Aggression scores in the Resident Intruder and Social Interaction tests were not strongly related to one another, and the two Sociality scores in the Partner Preference and Social Interaction tests were not strongly related. Non-social Boldness in the Novel Physical and Partner Preference tests were not strongly related to one another.

Table 2.7a. Across context consistency: Correlation coefficients (*r*) that represent the strength of linear relationships between comparable factor scores (temperaments) measured in different contexts (i.e. behavior tests) at each of 3 testing time points. **indicates statistical significance of $p<0.01$,*indicates statistical significance value of $p<0.05$, and + indicates statistical significance value of $p<0.1$.

Specific Factor Scores	**1**	**2**	**3**
Social Boldness			
Novel Social vs Partner Preference tests	0.38**	0.36**	0.25^{+}
Sociality			
Partner Preference (Familiar) vs Social Interaction	0.17	-0.11	-0.06
Partner Preference (Unfamiliar) vs Social Interaction	0.32*	-0.09	-0.08
Novel Social-Social Boldness vs Partner Preference-Familiar Sociability	0.29*	0.32*	0.21
Novel Social-Social Boldness vs Social Interaction	0.28*	-0.19	0.14
Activity			
Novel Social vs Novel Physical tests	0.54**	0.31*	0.55**
Novel Social vs Partner Preference tests	0.26^{+}	0.27*	0.31*
Novel Social vs Social Interaction tests	0.30*	0.28*	0.28*
Novel Physical vs Partner Preference tests	0.24^{+}	0.14	0.35*
Novel Physical vs Social Interaction tests	0.04	0.22	0.02
Partner Preference vs Social Interaction tests	0.11	0.13	0.27*
Physical Exploration			
Novel Social vs Novel Physical tests	0.31*	0.39**	N/A
Aggression			
Resident Intruder vs Social Interaction tests	0.17	0.24^{+}	0.18
Non-social Boldness			
Novel Physical vs Partner Preference tests	0.13	0.13	0.49**

Some behaviors that were similar across tests were correlated across different tests (**Table 2.7b**). For example, time interacting with novelty (social) was modestly correlated across the Novel Social and Partner Preference tests, but time interacting with novelty (when it involved social novelty in one test vs. physical novelty in another test) were not related. However, latency to interact with novelty (social vs. physical) and approaching novelty (physical) were modestly correlated across the Novel Physical and Partner Preference tests, although not among other tests. Last, the number of times an individual entered the periphery (periphery entries) and the distance travelled were highly

consistent behaviors across all tests in which these behaviors were measured, whereas Rear and Attack in the Resident Intruder and Social Interaction tests were not correlated at any time point. Importantly, similar behaviors that were measured in different contexts (tests) (e.g. center entries, latency to interact and approach novelty) became more correlated with time - i.e. many behaviors were consistent across contexts at time point 3 but not at time points 1 or 2.

Table 2.7b. Across context consistency: Correlation coefficients (*r*) that represent the strength of linear relationships between comparable specific behaviors measured in different contexts (i.e. behavior tests) at each of 3 testing time points. **indicates statistical significance of $p<0.01$, *indicates statistical significance value of $p<0.05$.

Specific Behaviors	**1**	**2**	**3**
Time interacting with Novelty			
Novel Social vs Novel Physical (rat vs object)	0.05	0.11	0.47**
Novel Social vs. Novel Physical (empty cage vs. object)	0.20	-0.26	0.41**
Novel Social vs Partner Preference (rat vs rat)	0.32*	0.32*	0.22
Novel Physical vs Partner Preference (object vs rat)	0.02	0.15	0.15
Latency to interact with Novelty			
Novel Social vs Novel Physical (rat vs object)	-0.06	0.20	0.30*
Novel Social vs Novel Physical (empty cage vs object)	0.20	0.03	0.69**
Novel Social vs Partner Preference (sociality)	0.14	0.19	0.31*
Novel Physical vs Partner Preference (object vs rat)	0.27*	0.13	0.65**
Approach Novelty			
Novel Social vs Novel Physical (rat vs. object)	0.20	0.20	0.45**
Novel Social vs Novel Physical (empty cage vs. object)	0.18	0.20	0.45**
Novel Social vs Partner Preference (rat vs rat)	0.36**	0.52*	0.28*
Novel Physical vs. Partner Preference (object vs. rat)	0.16	0.25	0.22
Time in the Center			
Novel Social vs Novel Physical	0.10	0.03	0.41**
Novel Social vs Partner Preference	-0.01	0.05	-0.18
Novel Physical vs Partner Preference	-0.18	-0.11	-0.17
Latency to enter/exit Center			
Novel Social vs Novel Physical (enter vs enter)	0.11	0.41**	0.76**
Novel Social vs Partner Preference (enter vs exit)	0.16	0.33*	0.79**
Novel Physical vs Partner Preference (enter vs exit)	0.17	0.42**	0.78**
Center Entries			
Novel Social vs Novel Physical	-0.03	0.21	0.47**
Novel Social vs Partner Preference	0.19	0.61**	0.40**
Novel Physical vs Partner Preference	0.19	0.24	0.40**
Periphery Measures			
Entries: Novel Social vs Novel Physical	0.36**	0.47**	0.40**
Time: Novel Social vs Novel Physical	0.21	0.18	0.60**
Latency to exit: Novel Social vs Novel Physical	-0.84	0.38**	0.29*
Rear			
Resident Intruder vs Social Interaction	0.13	0.02	-0.15
Attack			
Resident Intruder vs Social Interaction	-0.15	0.08	-0.03
Distance travelled			
Center: Novel Social vs Novel Physical	0.38**	0.31*	0.57**
Periphery: Novel Social vs Novel Physical	0.54**	0.48**	0.66**
Total:			
Social Interaction vs Partner Preference	0.27*	0.54**	0.57**
Novel Social vs Novel Physical	-0.06	0.57**	0.78**
Novel Social vs Partner Preference	0.31*	0.82**	0.82**
Novel Social vs Social Interaction	0.07	0.60**	0.54**
Novel Physical vs Partner Preference	0.22	0.60**	0.66**
Novel Physical vs Social Interaction	0.18	0.33*	0.44**

Discussion:

We used five different behavior tests each repeated three times, to identify behaviors or suites of behaviors that are individually-consistent across time and contexts in an outbred rat model. Based on factor analyses and correlational analyses, we observed several trait-like factors that were not singly-aligned with those listed by Réale and colleagues [12]. For example, a Social Boldness factor extracted from the current data suggests that bold individuals tend to engage both physical and social risk/novelty. We also found that some factors, but not all, were highly consistent across both time and conditions, suggestive of trait-like temperaments (e.g. Social Boldness and Activity), and that certain behaviors, like distance travelled, and engagement with the center of arenas and with novel social partners were consistent across both time and across contexts. For some tests, multi-behavior estimates of temperament were more consistent over time than single behavioral measures, but this was not true across all tests. Finally, we found that behavioral consistency across contexts increased with age.

Objective 1: Behaviors that covary in a specific context and whether these relate to Réale et al. [12]temperament categories.

Réale and colleagues [12] identified five distinct traits (boldness, exploration, sociality, aggression, activity) that are frequently quantified in behavioral temperament studies. In the current study, we used multiple behavior tests to assess each of these five traits in a group of 57 outbred Sprague-Dawley male rats in laboratory conditions. Behaviors quantified on each test were based on previous studies (Novel Social and Novel Physical: [68]; Social Interaction: [143]; Partner Preference: [157–159]; Resident Intruder: [145], and factors were extracted from the multiple behaviors in each test. In the

simplified laboratory environment, we found that behaviors that consistently covaried with one another (i.e. factors) across time and contexts tended to align with temperament categories that were reviewed by Réale et al. [12]. For example, in the Novel Physical test, the first factor that repeatedly emerge at each time point involved Boldness behaviors focused on the center of the arena (center entries, time spent in the center, latency to enter center, and distance travelled in center), and in the Resident Intruder and Social Interaction tests, the same Aggression behaviors (latency to attack, number of attacks, pin, fight, rear) covaried at each time point. This was expected given the behavior testing regime that we used, although it is good to verify that the similar behaviors covaried with one another (i.e. similar factor structure across time and conditions), and that these factors were related to the five temperaments previously identified in Réale et al.[12].

In addition to the expected covarying behaviors that reflect the five temperaments, we found that behaviors previously associated with one temperament regularly covaried with behaviors previously associated with a different temperament. For example, in the Novel Social and Partner Preference tests, behaviors that are normally-associated with boldness (time in the less-protected center of an arena) covaried with behaviors associated with willingness to engage a novel social partner (time, latency, and frequency to approach novel social partner); thus we labeled this factor Social Boldness. Also, in the Novel Social and Novel Physical tests, a factor emerged (that we labeled Safe Activity) that included behaviors specific to the arena periphery - such as distance travelled in, entries into, and time in the periphery. This suggests an Activity trait that is associated with low bold thigmotaxis in addition to Activity broadly defined by Réale et al.[12].

Overall, many of the covarying behaviors (factors) identified in each test aligned with temperaments identified in Réale et al. [12], and factors that were not neatly aligned with the five temperaments suggest that behavioral syndromes [160] exist in this model. Further, broad behavior testing may be important to identify a range of behavior categories that are consistent across time and contexts and that contribute to more than five broad categories.

Objective 2: Consistency of single- vs multi-behavior estimates of temperament over time

Behavioral factor scores that were particularly consistent across time were Activity and Safe Activity (in the Novel Social and Partner Preference tests; ICC ~0.34-0.38), and Social Boldness (in the Novel Social and Partner Preference tests; ICC ~0.27). Other factors that were modestly consistent across time were Boldness, Aggression, and Sociality (in the Novel Physical and Social Interaction tests; ICC ~0.14-0.19). These results differ from results in Bell et al.'s [10] meta-analysis where Activity was one of the least repeatable behaviors based on data from multiple studies and species. At the time of the meta-analysis, Activity was less frequently studied than other temperament-related behaviors and may have been more variably-defined across studies. Interestingly Physical Exploration (in the Novel Social and Novel Physical tests) was not consistent across time, and none of the factors or specific behaviors in the Resident Intruder test were consistent across time. This latter result was unexpected because the Resident Intruder test is frequently used to measure aggression [12,145,161,162]. The current results suggests that the Resident Intruder test provides a method to measure acute aggressive reactions but that it may not be the best method to measure trait-like temporally-consistent individual

differences in aggressive behavior. Alternatively, it is possible that the number of aggressive interactions displayed by male Sprague-Dawley rats in this test (an average of 2.7 aggressive attacks/5 minute test, with a range of 0-14 attacks) was too few to provide a reliable estimate of aggressive temperament that is consistent across time. This frequency of aggression in the Resident Intruder was half of that in the Social Interaction test where Aggression factor scores were more consistent over time. Thus, for repeatability estimates, it is important to identify behaviors or tests that will elicit temperament-related behaviors frequently enough.

ICC results in the current study indicated that composite factor scores did not necessarily provide better estimates of consistent behavioral traits over time compared to a single behavior. Many of the behaviors that contributed to consistent factors were themselves individually consistent over time, and in some cases more consistent than composite factor scores. For example, latency to, number of visits, and time near a novel social partner (ICC range ~0.16-0.40), entries to center of arena (ICC ~0.0.20-0.29) and distance travelled (ICC range ~0.13-0.44) were behaviors that were particularly consistent within an individual over time. Interestingly, some of these consistent single behaviors (e.g. latency to approach novel partner/objects), which are often used to quantify temperament in the Novel Social, Novel Physical and Partner Preference tests [61,125,128,163] did not reliably contribute to any factors. In the current study, we removed latency measures from final factor analyses. This is in contrast with other studies that have used multiple behaviors to assess exploration [163] and suggests that we may not have had enough power to reliably quantify traits like Physical Exploration. Overall, some single behaviors that are individually consistent may provide good

estimates of temperament, and contribute little to composite estimates of temperament. Another behavior – distance travelled – was highly consistent over time in three of the four tests (Novel Social, Novel Physical and Partner Preference), although this metric is not frequently used to estimate temperament in natural populations. Given the results of the current study, which indicate that distance travelled is one of the most individually-consistent behaviors in a laboratory setting, this metric should be considered to measure Exploration or Activity in the natural environment, particularly with the advent of newer technologies that can make it feasible to track animal movement in natural settings. Thus, overall, in the current study, we did not find strong evidence that complex estimates of behavior are more individually-consistent over time than simple metrics. In particular, we found that certain behaviors were particularly consistent within individuals over time and that some of these did not contribute to consistent composite behavioral factors, and therefore should still be used to quantify temperament.

Last, in the current study, estimates of behavioral consistency across time were relatively low. There are several possible reasons for this low temporal consistency. Many previous studies conducted consistency testing at only two time points and within 3-4 weeks of each other [128,164–166]. In the current study, we tested behavior at three time points, with intervals of 3 weeks and 3 months between consecutive time points (in a relatively short-lived rodent). Increased time intervals between test and retest have been shown to decrease repeatability [10]. Also, between the last two time points, we conducted a battery of physiological measures on the study animals, which could have influenced behavior at the final time point. Last, the tests that we conducted were relatively short, and were conducted with laboratory-housed animals – two conditions

that have been associated with lower test-retest reliability [10]. Any of these aspects of the current study may have lead to relatively low, although statistically significant, repeatability across time.

Objective 3: Consistency across contexts of single- vs multi-behavior estimates of temperament

Social Boldness, Activity, and Physical Exploration were the only factor scores that were consistent across different behavior tests (r ~0.25-0.38, 0.02-0.55, and 0.31-0.39 respectively). Aggression, Sociality, and Non-Social Boldness measured on different tests arenas were not regularly consistent (r ~0.17-0.24, -0.19-0.32, 0.13-0.49). Several behaviors were consistent across both tests and time (e.g. distance travelled and periphery entries), while other behaviors were only consistent across different tests but not consistent across time in the same test (e.g. time interacting with novelty in the Novel Social and Partner Preference test). This suggests that certain behaviors are consistent across contexts, but that these behaviors are not consistent across time. Importantly, as rats aged in the current study, many behaviors became more consistent across different test contexts. For example, correlation coefficients for approaching and interacting with novelty, entries and time in center, and distance travelled across different tests were often stronger at the third vs. first or second test ages. Thus, age or repeat exposure to the same stimuli may lead animals to be more behaviorally-consistent across contexts. These results are reminiscent of prior human studies that showed that multiple personality traits are more individually-consistent in middle age as compared to during development [167–171]. This has also been narrowly studied in animal populations showing behavior traits are more stable in adults than in juveniles [172–175], but a few studies show the opposite

effect [10,176–179]. This age-related variation in repeatability may depend on the behaviors measured, the variability of the conditions and the species being studied [180]. As has been elegantly suggested by others, future animal studies should incorporate analyses of trait consistency across ages to understand how temperaments become more or less fixed over time [6].

Conclusion: Diverse and repeat behavior tests to characterize temperament, and single- vs. multi-behavior estimates of temperament

In the current paper, we used multiple behavioral tests repeated over time, along with factor analysis, to identify behaviors that regularly covaried to characterize temperament traits. We then determined the relative consistency of single- vs multi-behavior (factor score) estimates of temperament. Our results indicate that while some behavioral traits are highly consistent across both time and conditions, others are not, suggesting that certain behaviors are more fixed than others (e.g. Activity and Social Boldness are more fixed than Physical Exploration or Aggression). This basic descriptive work for any given study species or population provides necessary background information on behavioral traits and their relative flexibility/rigidity, which is key to understanding functional or mechanistic aspects of temperament.

To identify and characterize temperament for the first time in a study species, and to determine the relative flexibility or rigidity of each trait, we propose similar test batteries that include measures of behavior in multiple environmental contexts (e.g. social situations, novel situations, territorial defense contexts, etc.), and repeated observations in each context. Statistical methods of dimensionality reduction provide insight about complex traits, however, future studies should continue to use specific behaviors that

have been used in previous studies to determine consistency across time and contexts.

Acknowledgements

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Chapter 3:

Physiological profiles of temperament

Abstract

Temperaments can be relatively consistent within an individual over time and across situations, and can predict individual fitness and survival. Several temperaments have been identified within ecological settings, for example, some individuals are reliably more Exploratory, Bold, Active, or Social than others. While much research has characterized these temperaments in different species, the physiology that may drive their consistency is not well understood. The objectives of this study were to determine physiological profiles associated with specific temperaments, and 2) determine which physiological systems are the best predictors of each temperament. To measure temperament, we used 5 different behavioral tests (Novel Social, Novel Physical, Partner Preference, Social Interaction and Resident Intruder), each conducted three times with 54 Sprague-Dawley rats. To assess physiological processes, we measured glucocorticoid (GC) responses to acute restraint, innate immune responses to lipopolysaccharide, adaptive immune responses to keyhole limpet hemocyanin, cardiac function in response to acute stress and gut microbiome alpha diversity. We found that temperaments that were consistent across time and contexts, (Social Boldness and Activity), had more significant physiological system associations compared to temperaments that were not consistent. Additionally, single-behavior metrics of temperament had more significant associations with physiological systems than multi-behavior metrics of temperament. HPA reactivity was a modest predictor of two multi-behavior metrics and a relatively strong predictor of one of the single-behavior metrics of temperament (Activity, Social Boldness, *latency to interact with novelty*). On the other hand, cardiac activity and reactivity were also significant predictors of temperament (Activity, *latency to interact with novelty*, *time spent with unfamiliar partner* and *average distance travelled*) while HPA activity was a modest predictor of single-metric temperaments (*latency to interact with novelty*, *average familiar time*). Once we better understand the connections between physiological mechanisms and temperament, we can start to understand evolutionary adaptations of these traits and why we see variability in ecological outcomes within the same population.

Introduction

Variability within populations has been quantified according to individual behavior and/or physiology. Often times, these behavioral and physiological traits are associated and complementary. Individual behavior and physiology work in tandem to determine actions and reactions to different environments and situations. Consistent individual differences in behavior are defined as temperament traits and include categories such as Exploration, Boldness, Activity, Sociability and Aggression [12]. Temperament has been used to articulate individual differences in ecological and health outcomes such as fitness, survival and reproductive success [4,27,55,56,60,181,182]. To have a comprehensive grasp on the drivers of individual differences in temperament and associated ecological outcomes within populations, it is imperative to understand underlying physiological mechanisms associated with temperament or behavioral variance.

Physiological systems independently have been linked to ecological and health outcomes (reviewed in [101,183]), with many studies that have focused on the Hypothalamic Pituitary Adrenal (HPA) axis, identifying glucocorticoids as a major influence on fitness and survival [184–186]. Other physiological systems have been associated with ecological outcomes, including immune function. Over the past decade, predominantly studies in birds have shown both positive and negative tradeoffs between immune function and reproductive success [187–189], longevity [190], and overall health and survival [191,192]. Additionally, while the gut microbiome literature is still new, trends in the literature show it has a significant role in vertebrate evolution and is potentially linked to individual fitness [193,194].

The relationship between different temperament traits and physiological mechanisms has been widely studied over the past decade. For example, across several studies, individuals that are slow to explore novel situations (i.e. Low Explorers) have been shown to have heightened glucocorticoid levels at baseline and in responses to acute stressors, dampened responses to familiar antigens (adaptive immunity), and delayed responses to novel antigens (innate immunity) when compared to higher exploring individuals [79,128,148,195–204] . However, most ecological studies focus on one physiological system at a time and few have quantified multiple physiological systems that form a profile and underlie any specific temperament [197]. This limits the interpretation of results and does not account for the co-selection of multiple physiological traits along with different temperaments, or the interactive co-regulatory signaling that occurs among physiological systems. By understanding how temperament is associated with slight biases in multiple physiological systems we can create physiological profiles of temperaments to better understand biological mechanisms that drive behavior and result in temperament-specific ecological outcomes. In the current paper, we measured multiple specific physiological systems that have been shown to cross-signal [97,183,205,206], that have been associated with at least one temperament [24,197], and that have been associated with ecological outcomes [186,192,193].

To better understand individual differences, we propose a more comprehensive study of the individual by measuring multiple temperaments and multiple physiological systems. In this study, our objectives were to: 1) determine physiological profiles associated with specific temperaments, and 2) determine which physiological systems are the best predictors of each temperament. To control for environmental variance, we

completed these objectives using an outbred, genetically and behaviorally variable lab rodent, Sprague-Dawley rats. To identify temperament traits, we used five behavior tests previously described [207]. Methods to identify temperament traits vary from using multiple behavioral estimates of a temperament to just single behavioral estimates of temperament [207]. Within this study, we analyze both multi-behavior and single-behavior estimates of temperament. We measured a variety of physiological processes with tests that quantify both baseline activity and reactivity to specific stimuli. The physiological systems included: glucocorticoid secretion (circulating corticosterone), innate and adaptive immune function (pro-/anti-inflammatory cytokine response to antigen, and cell-mediated delayed type hypersensitivity response), fecal gut microbiome (alpha diversity), and cardiac function (heart rate).

Methods

Animal Model:

Adult male Sprague-Dawley rats (N= 57, 60 days of age Charles River Laboratories, Raleigh, NC) were individually-housed in plastic cages (43.5 × 23.5 × 20.5 cm), and maintained on a 12L:12D light schedule (lights off at 10:00 h and on at 22:00 h EST). The colony room was sustained at 22°C and an average 50% humidity. Home cages contained a large red tube and a wooden chew stick for enrichment. Food and water were available *ad libitum*. Rats were allowed to acclimate to laboratory housing and handled daily for 2 weeks prior to testing. All methods in this study were approved by the Pennsylvania State University Institute for Animal Care and Use Committee (PROTO201800433).

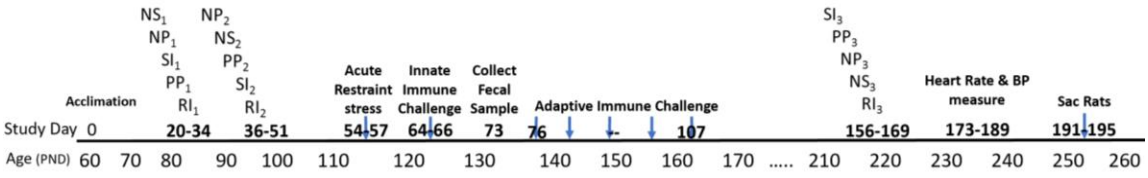


Figure 3.1. Timeline depicting when each behavioral and physiological test was conducted. Numbers above the line indicate study day and numbers below the line indicate rat age (post-natal date). Blue arrows indicate when blood samples were collected.

Behavior Testing:

Behavior testing is described in [207]. **Figure 3.1** depicts the timeline for this study. Briefly, we used five behavioral tests, each conducted at three different times. The first and second round of testing were separated by three weeks, and the third round was conducted 4 months later. (Rats were approximately 85, 100, and 220 days of age at each round.) Animal test order was randomized for each test and test order was changed in each round. All tests were performed in the middle of the active/dark phase (13:00-15:00 h) in a room separate from the colony room. Each test lasted 5 minutes, after which time rats were immediately returned to their home cage. AnyMaze software (Stoelting Company, Wood Dale, IL) was used to code behavior in the Novel Social, Novel Physical, and Partner Preference Tests, and hand coding by two independent researchers was used for the Social Interaction and Resident Intruder Tests.

Novel Social Test: This test was used to measure exploration, sociability, activity, and boldness [68]. The dimensions of the arena were 120cm x 120cm with 46cm walls. The arena floor was coated with fresh sawdust bedding. The arena contained two cages in diagonal corners from each other - one empty and the other with a novel same-sex rat of similar size and age as the test rat. Each study rat, within its enrichment tube, was placed into one of the free corners of the arena.

Novel Physical Test: This test was used to measure exploration, activity, and boldness [68]. The test arena was the same size and height as the Novel Social Arena, but instead of two cages, there were three rat-sized objects placed in three of the four corners of the arena. Study rats, within the enrichment tube from their home cage, were placed in the free corner of the arena.

Partner Preference Test: This test was used to assess sociability and activity [141]. The Partner Preference Test arena was the same size as for the Novel Social Test arena with two additional walls that divided the arena into three equal sections of 40cm x 120cm. The internal walls each had a 5-inch square cutout at the bottom to allow for passage between arena sections. In one section a familiar rat was placed in a cage and in the other side section an unfamiliar rat was placed in a cage. (The familiar stimulus rat was the one used in the previous Novel Social Test whereas the study rat had never interacted with the unfamiliar stimulus rat.) The center section was left empty and the study rat, along with its red enrichment tube, was placed into this section of the arena.

Social Interaction Test: This test was used *a priori* to assess individual rat sociability and aggression [142,143]. The Social Interaction Test arena was the same dimensions as the Novel Social Test arena. A study rat and an untethered, unfamiliar stimulus rat were placed in the arena simultaneously. Three different stimulus rats were used in total for each study animal for this test.

Resident Intruder Test: This test was used to assess aggression [144,145]. An unfamiliar stimulus rat was placed in the study rat's (the resident's) home cage. After testing, the stimulus rat was returned to its home cage. Three different stimulus rats were used in total for each study animal for this test.

Physiological Testing:

Between the second and third round of behavior testing, all rats were exposed to the same battery of physiological tests. The timeline for these tests is in **Figure 3.1**.

Overall physiology procedures: All physiological tests were conducted during the active/dark phase (13:00-15:00 h) except for the Innate Immune challenge which extended from 08:00 to 16:00 h. All blood was collected initially via tail tip amputation and then removing the scab of the wound site for further blood collection throughout the study.

Glucocorticoid Response to Acute Restraint: To measure circulating glucocorticoid (corticosterone) response to a physical acute stress, rats were placed in a restraint tube for 30 minutes. Blood samples to measure baseline circulating corticosterone were collected within three minutes of moving the rat home cage from the colony room. Peak stress-induced corticosterone was measured from blood samples collected 30 minutes after stress initiation, and recovery level corticosterone was measured in samples collected 90 minutes after stress initiation. After testing, rats were returned to the colony room. Samples were kept on ice until centrifuged. Serum was pipetted into aliquots and stored at -80º C until analysis. Serum samples were analyzed using RadioImmunoAssay (RIA, MP Biomedicals, Solon, OH) to determine corticosterone concentrations.

Innate Immune Response: To measure innate immune reactivity, rats were challenged with lipopolysaccharide (stereotype O111:B4) diluted in sterile endotoxin free 0.9% saline (LPS, 25 µg/kg, Sigma Aldrich, MO, catalog #: L3012) to initiate an innate immune reaction. The protocol was similar to that of Michael et al [201]. Briefly, at 08:00 h a baseline blood sample was collected and then rats were immediately injected

via subcutaneous (s.c.) with 0.5mL LPS. Additional blood samples were collected every two hours over an 8-hour period. Samples were kept on ice until centrifuged. Serum was pipetted into aliquots and stored at -80º C until analysis. IL6, TNFα, and IL10 were measured at each time point using a multiplex cytokine assay (Meso Scale Discovery, NJ).

Adaptive Immune Response: To measure adaptive immune reactivity, rats were challenged with Keyhole Limpet Hemocyanin diluted in sterile endotoxin free 0.9% saline (KLH, 500 µg/kg, MilliporeSigma, MA, catalog #: 374825). On day 0, a blood sample was collected to measure baseline circulating corticosterone concentration, and then rats were immediately injected s.c. with 0.5 mL of KLH. Blood samples were collected on days 7, 14, 21, and 28 to measure circulating corticosterone concentrations. Samples were kept on ice until centrifuged. Serum was pipetted into aliquots and stored at -80º C until analysis. On day 29, rats were re-exposed to KLH (100 µg/kg) via a 0.1 mL hind foot pad injection in one foot, and 0.1 mL of sterile endotoxin free 0.9% saline in the other hind foot pad as a reference. 24 hours later, swelling was measured to determine the delayed-type hypersensitivity reaction. Serum samples were analyzed using RadioImmunoAssay (RIA, Company, Location) to determine corticosterone concentrations.

Heart Rate: To measure basal heart rate and in response to a stressor, rats were placed in a restrainer and heart rate and blood pressure were measured non-invasively using the IITC cardiac machine (IITC Life Sciences, CA). Rats were placed in the restraint tube and allowed to warm in a warming chamber for 10 minutes. After this period, heart rate was measured at three consecutive time periods that lasted five minutes each. A total of

five consecutive measures were collected and averaged within each time period.

Fecal Sample Collection: Fresh fecal samples were collected from rats and stored at -80º C until analysis. Samples were sent to the Lamendella Lab at Juniata College for 16S RNA analysis. Shannon index was calculated at the family level and is a measure of biodiversity that accounts for both richness and evenness. Pielou's evenness and Faith's Phylogeny values were calculated by the Lamendella Lab. Pielou's evenness is an index that measures diversity along with species richness. Faith's Phylogeny measures the diversity covered based on the phylogenic tree and the length of the branches. Combining these three metrics enables more detailed accounting of microbial diversity for each individual.

Data and Dimension Reduction

Behavior: Behavior data was reduced using factor analysis. Dimension reduction was described in detail in [207]. Briefly, we used factor analysis to determine behaviors that co-varied on each test at each time point. Exploratory Factor Analysis was conducted in IBM SPSS 26.0 using a correlation matrix and the principal axis factoring method. We used Varimax rotation with Kaiser Normalization due to the relatively small sample size, and factor scores were calculated for each individual using the regression method. Kaiser–Meyer–Olkin measure of sampling adequacy (KMO) and Bartlett's Test of sphericity (KMO > 0.5, Bartlett's significance < 0.05) were examined to determine relative validity of the factor structure for each behavior test. To interpret factors, we only considered those behaviors that had loadings of greater than 0.30. In addition to the factors identified as temperaments, we also analyzed specific behaviors that have been used to estimate temperaments. This included *latency to interact with novelty* which has

been used to estimate Exploration, *time spent in the center* which is used to estimate Boldness, *total distance travelled* an estimate of Activity, *average time spent with conspecifics* a measure of Sociability, and *latency to attack* a measure of Aggression. Previously we have shown that these behaviors are consistent within an individual across time [207].

Physiology: Physiological data were reduced using the same exploratory factor analysis methods described for behavioral data. This method was used to obtain comprehensive variables of co-varying physiological processes for further analysis. From the factor analysis, we selected factors with variables that logically co-varied (e.g. a pro-inflammatory cytokine reactivity factor included IL-6 and TNFα concentrations, and a gut microbiome alpha diversity factor included Faith's Phylogeny, Pielou's evenness and Shannon index). Some physiological systems only had one metric (e.g. adaptive immune response), while others involved multiple metrics (e.g. innate immunity). For physiological systems with one variable, we used that single metric to compare to the temperaments. For physiological systems with multiple metrics, we used factor analysis to condense the multiple variables into a factor score and then compared this value to the temperaments.

Statistical analyses

Creating physiological profiles of temperament: We used the temperament traits identified from [207] along with specific behaviors that were shown to be consistent across time. We used correlational analysis to determine significant positive or negative relationships between behavior and physiology. Sample sizes for physiological analyses were: N =54 for HPA reactivity and Cell-mediated response, N=52 for HPA Activity,

Microbiome, Innate Immune Pro-inflammatory and Anti-inflammatory response, N=51 for Sympathetic Activity, N=46 for Heart Rate. Since sample sizes varied across physiological systems, we did not run an omnibus correlation with all physiological metrics at once, but each system was independently analyzed against all temperaments to prevent sample size reductions.

Determining if physiological processes are predictors of temperament: We used Least Absolute Shrinkage and Selection Operator (LASSO) for variable selection [208] and then used multiple linear regression to determine which physiological processes are significant predictors of temperament. The tuning parameter used for LASSO was from the glmnet package in RStudio. Significance was identified as $p<0.05$.

Results

Temperament dimension reduction:

We named factors based on the behaviors that loaded heavily on the factor (>0.30). The factors did not completely align with those identified by [12], but provided more complex characterization of covarying behaviors that may reflect temperaments. Some of these factors had the same behaviors that consistently loaded on the factor at all test ages and were more complex identifiers of consistent behaviors. The full results of this analysis can be found in [207].

Novel Social: Across the three time points, we identified the following three factors as: Social Boldness, Safe Activity, and Physical Exploration (*Supplemental Table 2.1*). Within Social Boldness, behaviors included time spent with and number of approaches toward the novel social partner along with time and distance travelled in the center. Safe Activity was comprised of behaviors focused on the periphery including distance

travelled. Physical Exploration was comprised of behaviors centered on the empty cage.

Novel Physical: Across the three test ages, we identified three consistent factors: Physical Exploration, Boldness, and Safe Activity (*Supplemental Table 2.2*). Physical Exploration and Safe Activity were comprised of similar behaviors seen in the Novel Social test. Boldness was comprised of behaviors in the center of the arena including center entries, time and distance travelled.

Partner Preference: Across time, we identified the following three factors: Familiar Sociality, Social Boldness and Boldness (*Supplemental Table 2.3*). Familiar Sociality was comprised of behaviors directed toward the familiar conspecific. Social Boldness was comprised of the same behaviors as seen in the Novel Social test. Boldness was comprised of the same behaviors as seen in the Novel Physical test.

Social Interaction: Across time, we identified the following factors: Aggression, Sociality and Activity (*Supplemental Table 2.4*). The Aggression factor was comprised of antagonistic behaviors including fighting, rearing and pinning. Sociality was comprised of behaviors including following and total time together. Activity was comprised of behaviors including total distance travelled and following.

Resident Intruder: At each test age, we identified the following factors: Aggression and Submission (*Supplemental table 2.5*). Aggression was comprised of similar behaviors as those seen in the Aggression factor of the Social Interaction test. Submission was comprised of behaviors that included freezing, laying on back and being attacked.

Using intraclass correlation coefficients (ICC) and spearman correlation analysis, we found that Social Boldness and Activity were highly consistent factors both over time and across conditions. Physical Exploration was consistent across contexts, but not across

time, and Aggression and Sociality were not consistent across time or across contexts. For single behavior metrics, we found that *latency to interact with novelty, distance travelled* and *time spent with conspecifics* were consistent across time (ICC) and across contexts. *Time spent in the center* was not consistent across time in the Novel Social and Novel Physical tests, but was in the Partner Preference test. This measure of Boldness was also not consistent across contexts. *Latency to attack* was not consistent across time or contexts.

Physiology Dimension Reduction:

With 12 variables and a six-factor model, 78% of the variance was explained, the KMO was 0.528, and Bartlett's approximate χ^2 was 135.85 (df=66). We did not include cardiac measures in this analysis since it would have reduced the sample size to 42 instead of 54, and resulted in reduced KMO values. Variable loadings for each factor are shown in *Supplementary Table 3.1*. The HPA Activity factor was comprised of positive loadings of the mean basal glucocorticoid measures throughout the study along with glucocorticoid levels at sacrifice. The Innate Immune Pro-Inflammatory factor was comprised of positive loadings of both IL-6 and TNFα metrics of AUCi. The Innate Anti-inflammatory factor was comprised of positive loadings of glucocorticoid levels during the innate immune challenge (AUCg) and negative loadings of TNFα. Finally, the Gut Microbiome factor was comprised of positive loadings of Faith's Phylogeny, Pielou's Evenness and Shannon Diversity. HPA reactivity was a single metric of the glucocorticoid AUCg during the acute restraint stress. Cell-Mediated immunity was comprised of a single measure using the delayed-type hypersensitivity reaction. Heart rate activity was a single measure of the average basal heart rate. Heart Rate Reactivity

was a single measure of the average stress-induced heart rate.

Physiological profile of temperament (factors)

We only considered temperament traits (factors) that we were able to be measure on different tests. These included Social Boldness, Activity, Physical Exploration, Boldness, and Aggression. We used the average factor score for each individual across time and context. Social Boldness had the most physiological systems that were significantly correlated with it (**Figure 3.2**). Specifically gut alpha diversity was significantly positively correlated with Social Boldness (R=0.34, $p<0.01$) while heart rate activity and HPA reactivity were negatively correlated with Social Boldness (Rs=-0.28, -0.27, $p<0.05$ respectively). The behavior factor of Activity was significantly negatively correlated with Heart Rate Reactivity (R=-0.33, $p<0.05$). The other temperaments (factors), Physical Exploration, Boldness and Aggression, were not significantly correlated with any of the physiological systems measured here.

Physiological profile of temperament (single behaviors)

Physiological profiles for all individual behavior measures are in **Figure 3.3**. *Latency to interact with novelty* was positively correlated with cell-mediated immune response and HPA reactivity (Rs=0.27, 0.42, $p<0.05$, <0.01 respectively) while basal heart rate activity and basal HPA activity were trending toward a positive correlation (Rs=0.25, 0.25, $p<0.1$). *Average distance travelled* was negatively correlated with heart rate reactivity (R= -0.32 $p<0.05$). *Average time spent in the center* was positively correlated with innate immune anti-inflammatory response and negatively correlated with HPA reactivity (Rs = 0.32, -0.28, $p<0.05$). *Average unfamiliar time* was negatively correlated with heart rate reactivity (R=-0.28, $p<0.05$) and trending toward a negative

correlation with HPA reactivity (R=-0.24, p<0.1). *Average familiar time* was not significantly related to any physiological system. Finally, *latency to attack* was trending toward a negative correlation with heart rate reactivity and innate immune anti-inflammatory response (Rs=-0.28, -0.25, p<0.1).

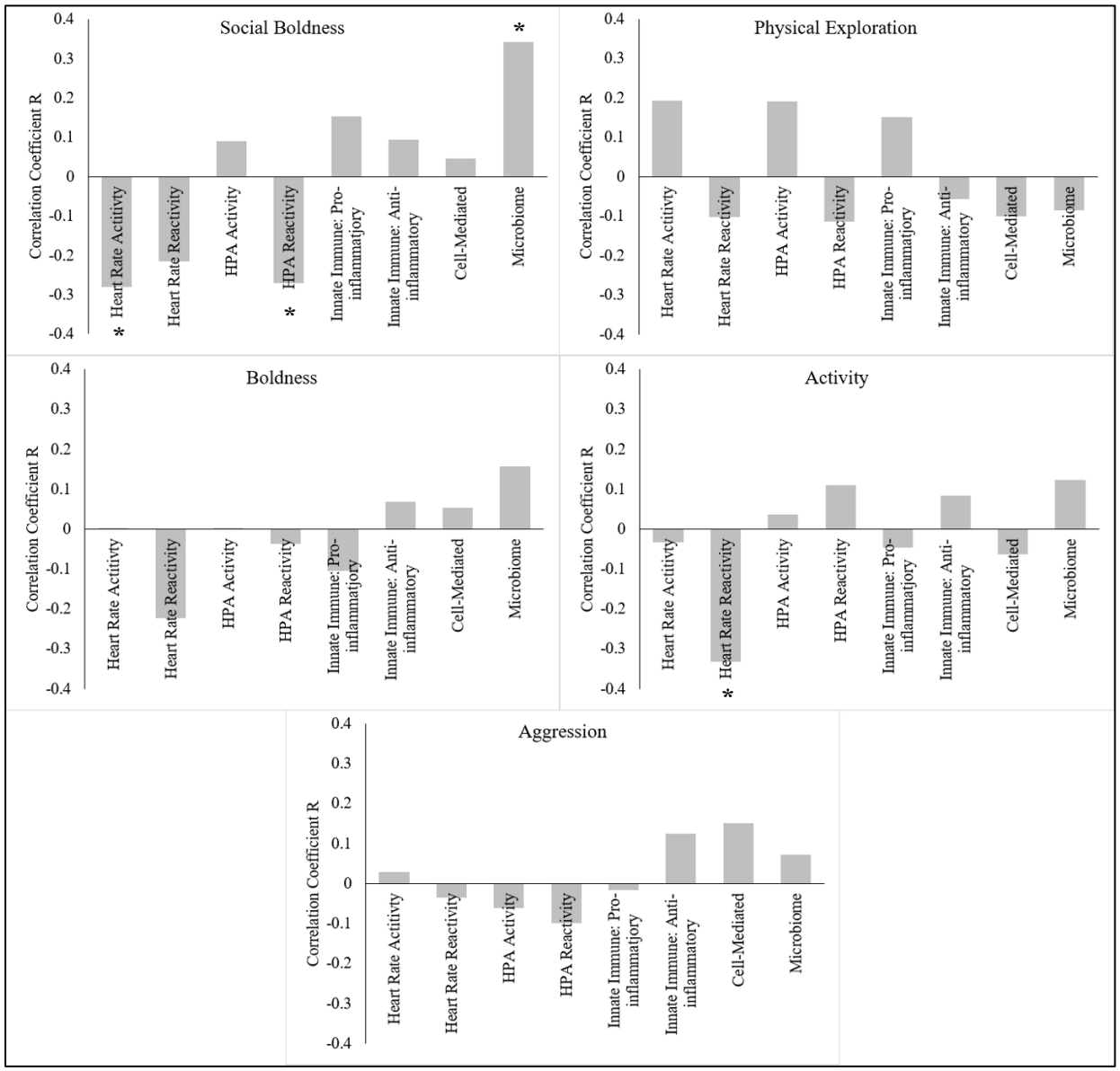


Figure 3.2. Physiological profiles of factor scores used to identify temperament. Correlation coefficients on the y-axis indicate positive and negative correlations of each physiological system to temperaments. Significant correlations are marked by * (p<0.05).

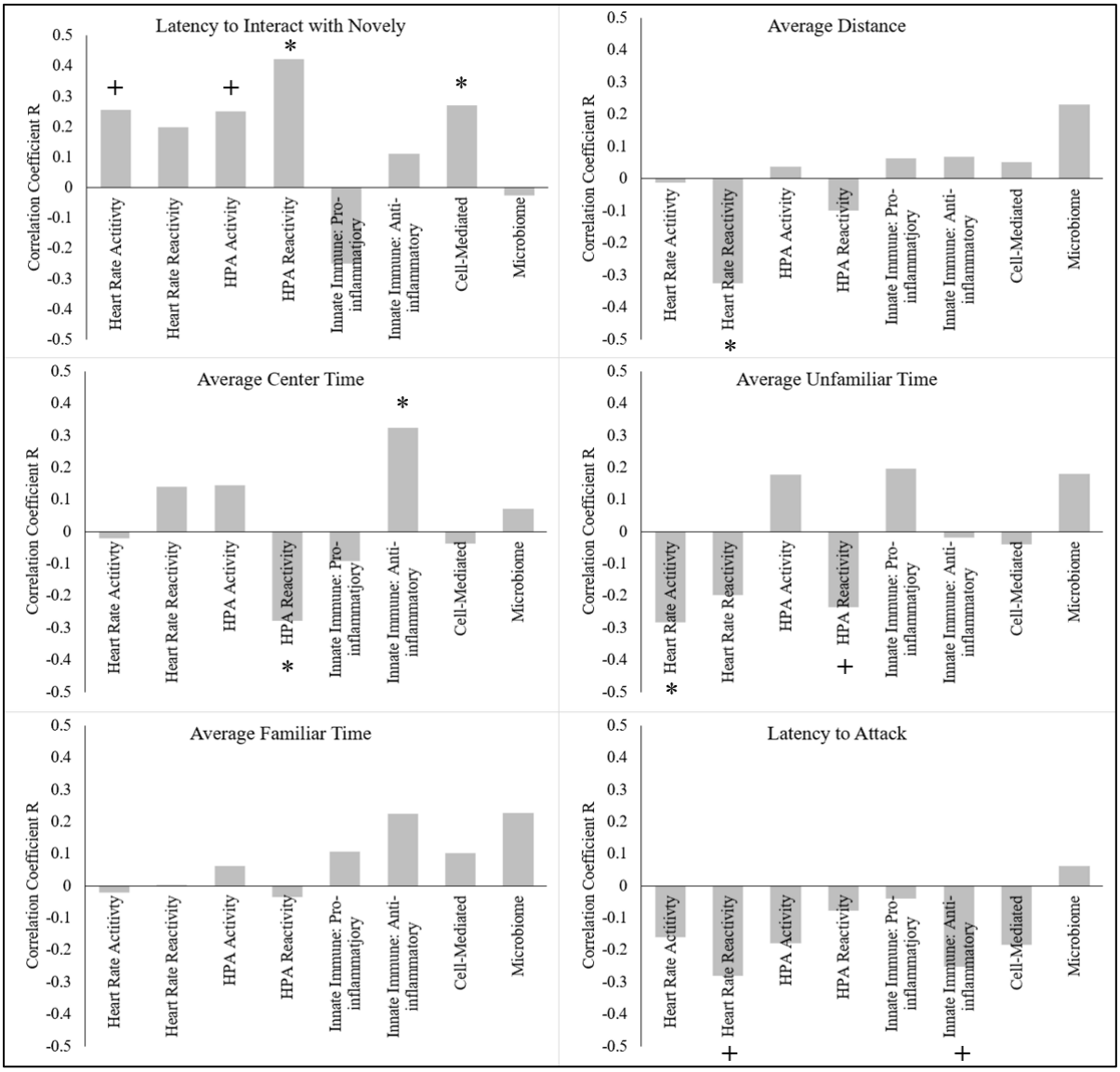


Figure 3.3. Physiological profiles of single behavior metrics used as proxies for temperament. Correlation coefficients on the y-axis indicate positive and negative correlations of each physiological system to temperaments. Significant correlations are marked by * (p<0.05). Trends toward significance are designated by + (p<0.10).

Physiological mechanisms predictive of temperament

Table 3.1 summarizes the descriptive statistics and analysis results. The model composed of all but two physiological variables (Heart Rate Reactivity and Cell-Mediated Immunity) resulted in the best model to predict *latency to interact with novelty* (R^2 = 0.418, p<0.01). However, only HPA Activity, HPA reactivity and Heart Rate

Activity were significant predictors within this model. *Average unfamiliar time* was predicted by Heart Rate Activity (R^2=0.10, p<0.01). HPA activity and innate immune anti-inflammatory contributed to the best fit model in predicting *average familiar time*, but this model was not a significant (R^2=0.11 , p>0.05). *Center time* and *latency to attack* did not have any significant physiological predictors. Heart Rate Reactivity was the only physiological mechanism that significantly predicted *total distance travelled* (R^2=0.11, p<0.05).

The model containing microbiome and HPA Reactivity was the best fit in predicting Social Boldness (R^2 = 0.151, p<0.05). However, only the microbiome was a significant predictor. Physical Exploration, Boldness and Aggression did not have any significant physiological predictors. The best fit model for Activity was composed of physiological mechanisms that included HPA Reactivity and Heart Rate Reactivity (R^2=176, p<0.05). However, only Heart Rate Reactivity was a significant predictor.

Table 3.1. Linear regression analyses for physiological mechanisms that predicts temperament. Significant models and significant predictors are indicated by * (p<0.05). A . indicates predictors that are trending toward significant (p<0.10).

		R^2	Adj R^2	B	SE B	β	P
Latency to interact with novelty		0.418	0.318				0.002*
	Intercept			-1.250	1.160		0.289
	HPA Activity			0.199	0.099	0.270	0.050*
	Gut alpha diversity			0.161	0.118	0.199	0.180
	Pro-inflammatory cytokine response			-0.144	0.105	-0.191	0.176
	Anti-inflammatory cytokine response			0.990	0.103	0.130	0.311
	HPA Reactivity			0.003	0.001	0.369	0.011*
	Heart Rate Activity			0.003	0.001	0.327	0.030*
Average Unfamiliar Time		0.10	0.087				0.030*
	Intercept			66.710	12.06		<0.0001
	Heart Rate Activity			-0.070	0.031	-0.331	0.032
Average Familiar Time		0.11	0.061				0.110
	Intercept			29.210	1.91		<0.0001
	HPA Activity			2.410	2.09	0.176	0.250
	Anti-inflammatory cytokine response			3.660	2.16	0.258	0.098 .
Average Center Time							NA
	Intercept			7.470	0.46		<0.0001
Average Latency to Attack							N/A
	Intercept			94.700	13.04		<0.0001
Average Distance Travelled		0.11	0.083				0.030*
	Intercept			57.210	9.48		<0.0001
	Heart Rate Reactivity			-0.049	0.023	-0.325	0.036
Social Boldness		0.151	0.107				0.040*
	Intercept			1.638	1.015		0.100 .
	Gut alpha diversity			0.207	0.109	0.310	0.041*
	HPA Reactivity			-0.002	0.0012	-0.266	0.081 .
Physical Exploration							NA
	Intercept			-0.270	0.067		0.632
Boldness							NA
	Intercept			0.032	0.089		0.711
Activity		0.176	0.134				0.022*
	Intercept			0.406	1.066		0.723
	HPA Reactivity			0.001	0.0009	0.241	0.101 .
	Heart Rate Reactivity			-0.004	0.002	-0.359	0.014*
Aggression							NA
	Intercept			0.056	0.079		0.441

Discussion

We used five behavior tests and five physiological tests to determine the relationship between multiple temperaments and physiological systems within an individual. This study provides a broader understanding of how temperament and physiological systems interact under basal conditions. We found that temperaments that were highly consistent across time and contexts, including Social Boldness and Activity, had more significant physiological systems associated with them in comparison to temperaments that were not consistent across time or context. Additionally, we found that single-behavior metrics of temperament had many more significant associations with physiological systems than multi-behavior metrics of temperament. Social Boldness was positively correlated with the gut microbiome alpha diversity and negatively correlated with heart rate activity and HPA reactivity. In the current study, the Social Boldness temperament was highly consistent across both time and contexts, suggesting that it is not a flexible trait. Social Boldness, as we define it, has not been identified or heavily studied in the literature. This temperament is a combination of behaviors that focus on interactions with a novel social partner and risky areas such as the center of the arena. Based on our analyses we found that individuals that were Socially Bold had greater gut microbiome alpha diversity, lower glucocorticoid reactivity to stressors, and lower basal heart rates. An important factor in this study is that rats all lived in the same environment and received the same diets, yet we still observed these unique temperament-related physiological biases. Studies on gut microbial diversity suggest that diet may drive microbiome diversity among individuals ([209]cf.[210]). However, all animals in the current study received the same standard food, suggesting a more complex and

potentially adaptive life history strategy causing significant differences between high- and low-socially bold individuals. This suggests that these physiological biases may somehow support or lead to the temperamental behavioral repertoire. Alternatively, at the very least the current results indicate that temperament-specific physiology is not necessarily a result of individuals exposing themselves to different environmental stimuli that then cause physiological differences among temperaments.

From an evolutionary perspective, animals that frequently expose themselves to dangerous conditions and interact with many social partners (i.e. Socially Bold) may preferentially survive if they have physiological underpinnings that support risky and socially diverse environments. For example, a more diverse gut microbiome may help Socially Bold individuals be resilient to infection or changing environments [193]. Similarly, lower hormonal responses to stressors and lower basal heart rates may prevent over-reactivity to novel stimuli and potential perceived stressors. Previous studies are inconsistent in their findings of the link between gut microbiome diversity and behavior (reviewed in [211]), however, many times these studies did not have multiple measures of diversity but only used one metric, usually the Shannon Diversity Index. However, Christian and colleagues [210] found similar results to ours, showing more extraverted male children had higher Phylogenetic diversity and Shannon Diversity. In the current study, our gut microbiome alpha diversity factor was comprised of the Shannon Diversity Index, Peilou's Evenness, and the Faith Phylogeny Index. This suggests that individuals who are Socially Bold have high richness, evenness and a larger number of unique phylogenies compared with low socially bold individuals. Further, from an evolutionary perspective, individuals vary in their responses to stressors and lower reactive

glucocorticoid levels have previously been shown as an adaptive strategy, especially for individuals who are living in changing or risky environments [212,213]. Our study aligns with this in that rats that tend to expose themselves to risky areas and new social partners had lower glucocorticoid stress reactivity than those who are not socially bold. Heart rate isn't commonly studied in natural settings especially at basal levels, but from a physiological perspective, elevated basal heart rate is linked to heightened anxiety and reactivity [36,214,215], further demonstrating that Socially Bold rats may be better physiologically prepared for risky environments. However, it is important to note that while Sprague-Dawley rats are outbred, they have not experienced natural selection for a while, so these relationships between temperament and physiology may have been selected for long ago and have been maintained in the gene pool. Put together, we see that Socially Bold rats maintain a physiological profile that best prepares them for risky environments and interacting with novel social partners.

The other highly consistent temperament was Activity. This is different from the behavior activity, which is a single measure of distance travelled or lines crossed and is a highly studied behavior in drug and addition research due to its high within-individual consistency [216], but few studies have verified this. However, the temperament Activity is not frequently studied and there are even fewer studies that relate it to physiological systems [197]. We found that Activity was negatively correlated with heart rate reactivity, meaning individuals who were more active had lower heart rates compared to those that are less active. Similar results have been shown in human studies, where individuals who are highly active had lower heart rates during acute stressors as compared to less active individuals [215,217,218]. However, studies in animal models

are not congruent, with some showing positive correlations between Activity and heart rates [219,220], other showing negative correlations [221] or no relationship at all [222]. It is possible these differences are due to the methodologies used. [219,220] conducted their studies in field settings on wild rodents by measuring heart beats after an acute stressor while [221] conducted research on rats in a controlled laboratory setting and [222] studied zebrafish without a stressor. While we did not find any other significant relationships between Activity and the other physiological systems, other studies have found varying results between Activity and HPA activity and HPA reactivity. Specifically, we see in mammals, Activity is positively correlated with basal HPA activity [223–225], while in birds we see one negative relationship [124] and in marmots and fish we see no relationship [226,227]. Activity has varying relationships with HPA reactivity as well with most studies showing positive relationships ([228–231] cf: [232,233]). Immune responses are highly understudied in relation to Activity. In the current study, we found no relationship between innate or cell-mediated immune responses and Activity, suggesting that immunity and Activity might not strongly influence each other. These results along with previous studies further demonstrate that it is imperative to include Activity as a temperament metric in order to determine if there are any physiological processes that are regularly associated with this relatively consistent behavioral trait. Based on the current study, the results suggest that Activity is not highly associated with these physiological outcomes and might be related to other physiological systems that we did not measure that can influence life history strategies, health outcomes and ecological consequences.

The other temperament traits, Physical Exploration, Boldness and Aggression,

were not related to any of the physiological systems measured. We found this surprising, especially for Physical Exploration, which is highly studied across a variety of taxa especially in relation to glucocorticoid secretion [197]. Physical Exploration and Boldness estimates were consistent across time, but not across contexts, suggesting that these behavioral responses are flexible traits that are adjusted in response to the specific environment that an animal experiences. The lack of any relationships between Physical Exploration/Boldness and physiology might be one reason that these behaviors are flexible. It is possible that a consistent physiological profile is one mechanism that leads to a consistent behavioral profile. Another possible explanation is that tests that focus on exploration of physical objects are not as highly associated with physiological mechanisms compared to measures of social exploration [128,166,234]. Results from the current study provide further evidence of this. Aggression was not highly consistent across time or conditions in this study, so it is possible that our multi-behavior metrics of this temperament did not accurately characterize this temperament trait. Conversely, the inconsistency of this trait both across time and context might be due to the time limitation of the test or that Sprague-Dawley rats are not a particularly highly aggressive strain, thus resulting in the observed null results.

Besides using complex, multi-behavior metrics to define temperaments, an alternative and more common method is to use a single behavior metric as a proxy for individual temperament traits. Following this method, we used *latency to interact with novelty* to measure Exploration, *time spent in the center* to measure Boldness, *total distance travelled* to measure Activity, *average time spent with conspecifics* to measure Sociability, and *latency to attack intruder* to measure Aggression. We found that

individuals who showed long latencies to interact with novelty (Low Explorers) had significantly higher HPA reactivity and cell-mediated immunity, and a trend toward higher basal heart rate and basal HPA activity, and lower pro-inflammatory responses compared to High Exploratory individuals. The elevated glucocorticoid reactivity is similar to results of previous studies across taxa [68,124,201,235–238], however the dampened pro-inflammatory and elevated cell-mediated immune response does not align with previous work. This difference in findings may have resulted from the fact that animals in the current study were exposed to restraint stress prior to immune challenges, which was not conducted in these prior studies. This stress exposure prior to immune challenges may have caused differing results compared to what was seen previously. However, this is an important result because in natural settings animals will be exposed to multiple stressors, which may shift the innate and adaptive immune systems. With the more comprehensive physiological sampling in the current study, we may have observed results that are more likely to occur in natural settings. The *distance travelled* metric results were similar to the factor score of Activity; showing highly active individuals had lower heart rates during an acute stressor. Individuals who spent more time in the center of the arena (Bolder) had higher anti-inflammatory cytokine responses and lower HPA reactivity. The lower HPA reactivity in bold individuals is similar to results from the Social Boldness factor score, however the anti-inflammatory cytokine production is distinct. Bolder individuals may have heightened immune responses because they have increased exposure to novel antigens, as a result of their Bold behavior [239]. However, in the current study, these animals were not exposed to variable immune environments that would result in this, again, suggesting possible co-regulation of behavior and

physiology. To measure sociability, we used an average of the time spent with both the unfamiliar and familiar conspecific in the partner preference test. We found that only time with the unfamiliar partner was negatively correlated with basal heart rate and trended toward a negative correlation with HPA reactivity. Time spent with an unfamiliar partner is more closely related to the Social Boldness metric, indicating that sociability, broadly speaking, is not necessarily related to physiology, but that willingness to engage a novel social partner *per se* may be more closely related to underlying physiology. Finally, individuals that had high latencies to attack (Low Aggressive) showed a trend toward having lower heart rates during an acute stressor and lower anti-inflammatory cytokine responses. While these are only trends, it suggests that individuals who are more aggressive have higher heart rates and anti-inflammatory reactions. This may support an aggressive phenotype that is often considered reactive and quick to respond to stimuli, which may result in elevated sympathetic reactions including elevated heart rates [240–242].

We also determined whether physiological system response biases could be used to predict temperaments. We found that HPA reactivity was a modest predictor of two multi-behavior metrics and a relatively strong predictor of one of the single-behavior metrics of temperament (Activity, Social Boldness, *latency to interact with novelty*). On the other hand, cardiac activity and reactivity was also a significant predictor of temperament (Activity, *latency to interact with novelty*, *time spent with unfamiliar partner* and *average distance travelled*).

The data show that the most highly consistent single-metric of temperament, *latency to interact with novelty*, was predicted by multiple physiological systems (HPA

activity and reactivity, gut alpha diversity, pro-inflammatory cytokine response, anti-inflammatory cytokine response, and cardiac activity). *Latency to interact with novelty* has been widely used to measure Exploration across species [128,166,207,224,243,244]. Focusing on those physiological systems that were significant predictors within the model (HPA activity and reactivity, and cardiac activity), we saw that these systems might have the strongest influence on this particular metric of temperament. Previous studies have connected the HPA axis to latency to interact with novelty [68,128,165,236,245]. However, thus far, results on this relationship are mixed with some studies showing increased glucocorticoid concentrations and latency to interact with novelty while others show the opposite or no relationship [197]. These differences are likely due to the type of stressor animals experienced since we see similar results regardless of taxa, age, sex and environmental conditions. Additionally, cardiovascular function has been linked to Exploration in human studies (reviewed in [81,214,246,247]), but few have measured this in animal models and show positive relationships in rodents [220,248] and no relationship in larger mammals [249,250]. These systems, HPA function and cardiovascular function, are relatively fast acting systems that work within seconds to minutes as compared to other systems such as the immune system that can take several hours or the gut microbiome that can take several days to significantly change. These faster acting systems might have a more direct influence on behavior, especially *latency to interact with novelty* and be the reason why this temperament is so highly consistent while other behaviors are less so. Since HPA function and cardiovascular function positively predict *latency to interact with novelty*, it suggests that individuals who have high basal and stress-induced glucocorticoids and elevated basal

heart rates are likely to have longer latencies to interact with novelty. This might be an adaptive coping mechanism for individuals who tend to avoid novel situations that they perceive as potential threats. Additionally, those that frequent novel situations and have low latencies to interact with novelty, have lower basal and stress-induced glucocorticoids along with lower heart rates, which might enable them to minimize energy mobilization during frequent interactions with novelty.

However, with other equally-consistent single-metric temperaments (e.g. *distance travelled* and *time spent with conspecifics*), we do not necessarily see as many relationships with physiological systems, suggesting that physiology might not have as strong an influence on these other temperaments. This might suggest that these temperaments might not have been co-selected with these physiological systems over time as compared to other temperaments such as Exploration and Social Boldness.

It is important to note the limitations of this study. One in particular is the sample size. Some of these results may have been influenced by low statistical power. While a sample size of 54 is large for both laboratory and field studies, in the context of measuring multiple variables, it is the case that more individuals would have resulted in better predictive power within the correlation and multiple regression analyses. Additionally, due to equipment malfunction, not all individuals were measured during the heart rate monitoring procedure. Future studies should use larger sample sizes to ensure enough statistical power to analyze multiple physiological systems and temperament traits.

While taking these limitations into account, this is still one of the first studies to measure multiple temperaments and multiple physiological systems within the same

individuals. We recognize that continuously challenging animals with different physiological tests may have amplified results. For example, exposing animals to an acute restraint stress may have heightened glucocorticoid basal levels which might have influenced cytokine production in the innate immune challenge, which may have further influenced T-helper cell production in the adaptive immune challenge. However, animals were given a minimum of a week between each test, with 10 days between immune challenges, to allow animals to recover. Understanding how these physiological mechanisms interact is another important area that needs to be further studied. By collecting multiple measures within the same individual, we determined that all physiological mechanisms are highly interconnected, providing further proof of a physiological profile for each temperament (*See Supplementary Data 3.1*). This trends with human studies that measure multiple systems to identify problems or reactions [251]. This research is further proof that it is important to measure across disciplines to better understand and obtain a more holistic perspective of the individual to understand the population. Understanding physiological profiles for each temperament provides a more comprehensive method to determine both causes and consequences of persistent temperament traits. Once we understand the physiological profiles of temperament, we can begin to determine mechanisms that make some temperaments more flexible than others, mechanisms that influence survival and fitness of different temperaments, and even environmental conditions that may favor one temperament over others. Understanding the connections between physiological mechanisms and temperament will enable future studies to understand evolutionary adaptations of these traits and why we see variability in ecological outcomes from the same populations.

Chapter 4:

Chronic Basal Glucocorticoid Influence on the Physiological Network and Temperament

Abstract

Behavioral variation between individuals is commonly observed within populations. To understand this variation, studying the underlying physiology in relation to temperament is key. Both the physiological network and temperament traits within an individual can either be flexible or rigid, providing advantages and disadvantages in different environments. In this study, we determined the relative flexibility of the physiological network and associated temperament. We non-invasively manipulated basal glucocorticoid (GC) levels and determined changes in other physiological systems and temperament. Our objectives were to 1) determine the flexibility of the temperament traits of Exploration and Social Boldness, and 2) determine the flexibility of associated physiological mechanisms. We had two study groups comprised of 34 Sprague-Dawley rats: controls (N=17) and GC-treated (N=17). Control animals received 1% ethanol in their drinking water and GC-treated animals received 30μg/mL of corticosterone. We measured temperament traits and other physiological mechanisms including the hypothalamic pituitary adrenal (HPA axis), the cell-mediated immune response and heart rate before and during the manipulation. We found that the GC treatment group had significantly increased basal GCs and significantly decreased stress-induced GCs, cell-mediated immune activity, stress-induced heart rate and Exploration compared to control rats. The Social Boldness temperament trait did not significantly change with GC treatment. Based on the changes in the physiological network and the correlation to changes in Exploration, it is likely that the flexibility of Exploration is driven by the underlying physiological network while Social Boldness is a rigid temperament that does not alter with the changing physiological network.

Introduction

Individual variability is driven by unique physiological and behavioral interactions that result in the organism-specific responses to their environment. To understand how the individual responds to their environment, we need to study both the internal physiological function and the external consistent behaviors that they present. Physiological traits are key in understanding the relationship between temperament and ecological outcomes. Additionally, different physiological systems have been positively and negatively associated with different temperament traits [197]. However, there are still few studies that have linked multiple physiological mechanisms to temperament [101,197]. This has resulted in a limited and disjointed understanding of the interaction between the physiological network and temperament [252].

Physiological network

The physiological network is composed of integrated physiological systems that are co-regulated [205,252,253]. Specifically, different physiological systems (such as the endocrine vs. immune system) within the network signal to one another and so, at a functional level, a change in one system will result in a change in another system (e.g. chronically-elevated glucocorticoid production may alter immune function). This suggests that the whole network can be relatively plastic in response to changing environments. Furthermore, the relative flexibility or rigidity of the physiological network may present advantages in different ways [254–258]. For example, in a constant environment with little change, a rigid physiological network may be more advantageous than a more flexible network. However, in changing environments, which are becoming more common, adaptation is key to survival and thus a more flexible physiological

network may be more advantageous.

In the current study, we sought to determine the relative flexibility of the physiological network and associated temperament. We tested this by manipulating one portion of the physiological network—basal glucocorticoid levels. We focused on glucocorticoids (GCs) because they are key regulators in the physiological network as a result of their broad influence on multiple physiological systems, including immune function, gut microbiome, and cardiac function (See *Supplemental Data and Figure 3.1*). GCs are a steroid hormone that produce a variety of effects that center on glucose metabolism in response to both daily metabolic cyclic activities and stressful events. GC production is stimulated by the hypothalamic pituitary adrenal (HPA) axis, and circulating GCs can produce a wide variety of physiological and behavioral traits under baseline conditions and in the response to stressful stimuli [72,259]. While basal GC levels maintain energy balance and expenditure, moderate elevation of baseline GCs can affect innate immune system activity by assisting in anti-inflammatory cytokine production [260]. Acute stress-induced GCs result in suppression of unnecessary activities for immediate survival, such as reproduction and immune regulation, and activation of glucose mobilization which can support the response to stressors [72,259], which works congruently with the sympathetic response [261,262].

While acute influences of GCs are advantageous to the physiological profile, chronic elevations can result in dysregulation across multiple systems (**Figure 4.1**). Immune function is impacted by chronically-elevated GCs in multiple ways. This includes suppression of leukocyte numbers, trafficking and function, and changes in cytokine balance. For example, chronically-elevated GCs can lead to dampened adaptive

cell-mediated immune responses which can lead to poorly regulated inflammation [260,263–266]. Chronically-elevated GCs can also cause dampened T-helper 1 (T_H1) cell-mediated responses by targeting dendritic cell communication and downregulating pro-inflammatory cytokines [96,267]. However, this downregulation of T_H1 leads to enhanced T-helper 2 cell (T_H2) production resulting in greater humoral immune responses including elevated immunoglobin in the blood (IgG and IgM) [268–270]. The impact of elevated GCs on autonomic function is not well understood. Some long-term studies demonstrate chronically-elevated GCs can cause increased baseline heart rate [261,271] or have no effect [272–274]. Previous work demonstrates that glucocorticoid and mineralocorticoid receptors (GR and MR) mediate GC interactions with autonomic functions (reviewed in [275]), further showing the interconnectedness of multiple systems. In turn, other systems can have large impacts on the rest of the profile. For example, immune function has been shown to have influences on other systems as well (Box 1).

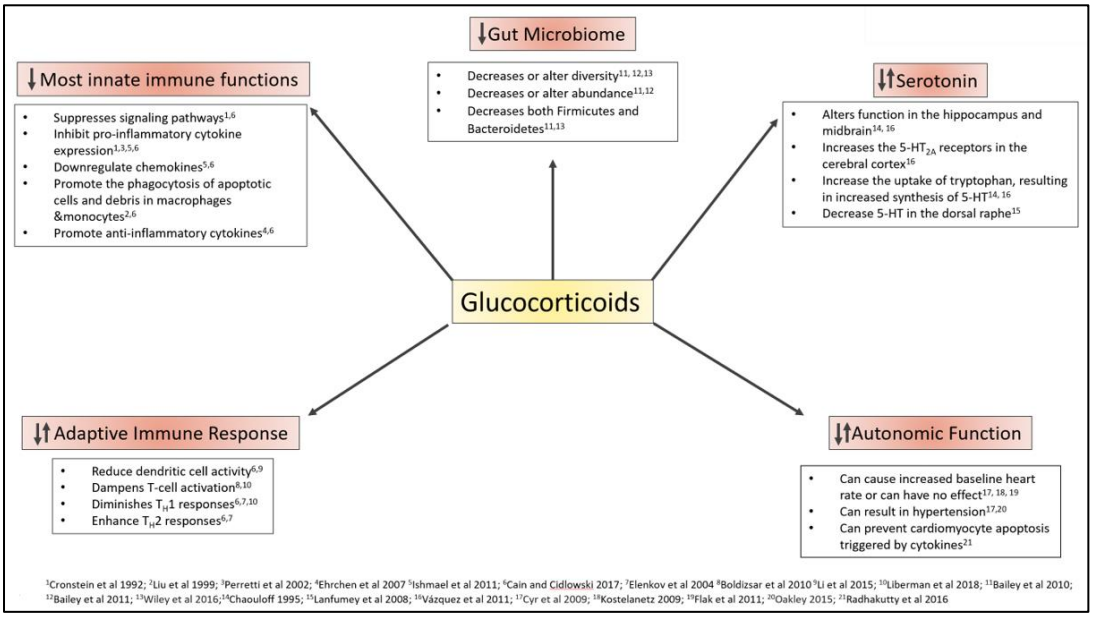
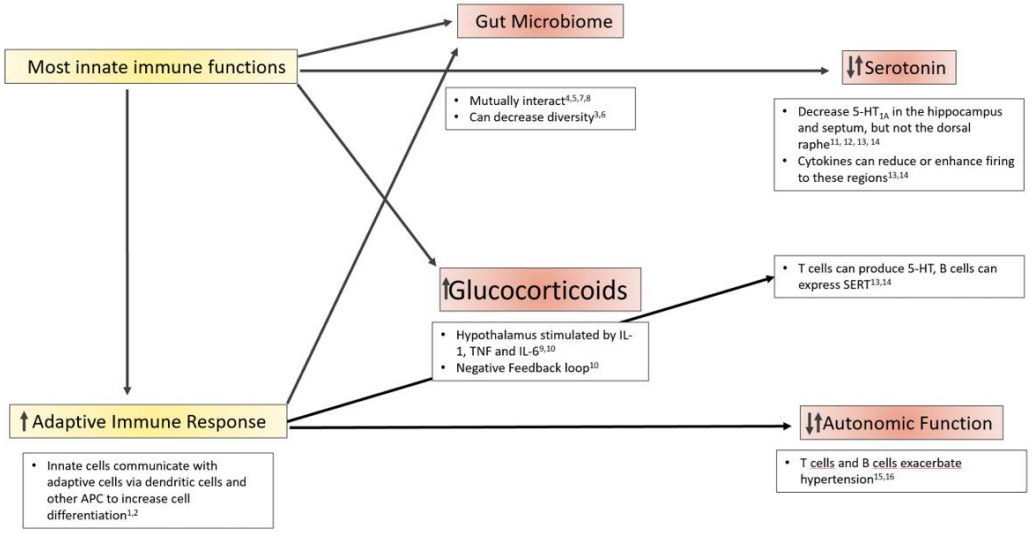


Figure 4.1. Depiction of chronic GC production influence on other physiological systems.

Box 1. Immune function affects glucocorticoids, gut microbiome and cardiac function: The immune system is comprised of the innate and adaptive branches. These two branches protect the body from infectious disease. The innate immune system provides rapid responses to foreign substances while the adaptive immune system is delayed and targets more specific antigens. Within this system, the innate immune branch signals the specialized T-helper cells that stimulate complex cellular and humoral responses of the adaptive immune system (reviewed in [420]). These systems also influence other physiological systems including glucocorticoids. For example, inflammation from the activation of the innate immune system can increase GC production [97,284,421]. The interaction of the immune system and GCs play a key role in gut microbiome diversity. Elevated GCs can lead to heightened antimicrobial peptides that decrease gut microbiome diversity [326,422–425]. However, the immune system has co-evolved with the gut microbiome in that they regulate each other and crosstalk to sustain the health of the body [326,426–428]. Specifically, innate and adaptive immune mechanisms complement each other to maintain mutualism between the microbes in the gut and the immune system [206].



[1]Parkin and Cohen 2001; [2]Parham, 2014; [3]Bailey et al 2009; [4]Slack et al 2009; [5]Clarke et al 2010; [6]Bailey et al 2011; [7]Min et al 2015; [8]Fung et al 2017; [9]Dunn 2000; [10]Cain and Cidlowski 2017; [11]Finocchiaro et al 1988; [12]Kushnir-Sukhov et al 2007; [13]Leon et al 2007; [14]Baganz and Blakely 2013; [15]Abais-Battad et al 2015; [16]Mikolajczyk and Guzik 2019

Box Figure 4.1. Influence of immune function on other physiological systems.

While much research has been conducted on the influence of chronic GC production on other physiological systems, little research has been conducted on how GCs simultaneously influence multiple systems within the same individual, and how these changes may influence consistent behaviors (i.e. temperament). Within this

manuscript we explore how chronic elevations of GCs influence other systems and how individual variation plays into this [73,276].

Temperament and the Physiological Profile

All of these physiological systems and their interactions are associated with a variety of temperaments, especially Exploration. Exploration is one of the most common temperaments studied and is defined by an individual's interactions with novel situations. Individuals that are high explorers disperse frequently and extensively and are exposed to more novel social partners, environmental conditions and antigens compared to low explorers that infrequently emigrate (reviewed in [277]). High exploratory individuals are characterized as having increased sympathetic regulation [278], low to moderate HPA baseline values and reactions to stressors [68,128,245,279–282], and immune function that is suited toward cell-mediated responses, with minimum energy toward slower, longer-lasting responses to novel antigens (i.e. humoral immunity) or fast-acting non-specific defense to antigens (i.e. innate immunity) [79,81,225,283,284]. For example, invasive cane toads that are high dispersers (i.e. high explorers) have decreased innate and humoral immunity, but enhanced cell-mediated immunity when exposed to novel antigens in wild settings [285]. However, it is important to note that the above results can vary across studies (e.g.[286–288]). These underlying physiological mechanisms that are associated with Exploration can be categorized as a physiological profile.

In comparison to Exploration, Social Boldness is not commonly identified as a temperament trait, but has been identified in green anoles [289]. Previous work that we have conducted categorizes Social Boldness as a temperament trait that is expressed in behaviors that relate to interactions with novel social partners and risky situations [207].

We have previously shown that Social Boldness is a highly consistent trait across time and conditions in Sprague-Dawley rats [207]. Additionally, we have shown that this trait is positively correlated with gut microbiome alpha diversity and negatively correlated with sympathetic activity and HPA reactivity (Chapter 3). Last, we have seen that gut microbiome alpha diversity positively predicts Social Boldness and that HPA reactivity showed a trend to being a negative predictor. From our previous study, only Social Boldness and Activity were highly consistent traits across time and conditions and were also significantly correlated with physiological mechanisms. This suggests that only behavioral traits that are highly consistent may show strong associations with physiological systems.

Previous studies that we conducted showed several correlations between temperament and regulation of several physiological systems. To better understand the causal relationship between physiology and temperament, we need to manipulate one aspect of physiology to determine if and how temperament and other physiological systems change. In this study, we manipulated glucocorticoids by increasing basal circulating levels to determine effects on individual temperament and physiological systems. Our objectives were to 1) determine the flexibility of the temperament traits of Exploration and Social Boldness, and 2) determine the flexibility of associated physiological mechanisms.

Methods

Animal Model:

Adult male Sprague-Dawley rats (N=34, 60 days of age, from Charles River Laboratories, Raleigh, NC) were maintained on a 12L:12D light schedule (lights off at

10:00 h and on at 22:00 h EST) and housed individually in plastic cages (43.5 × 23.5 × 20.5 cm). Cages contained a large red tube and a wooden chew stick for enrichment. Food and water were available *ad libitum*. The colony room was sustained at 22°C with an average 50% humidity. Rats were allowed to acclimate to laboratory housing and handled daily for 10 days prior to testing. All methods in this study were approved by the Pennsylvania State University Institute for Animal Care and Use Committee (PROTO202001673).

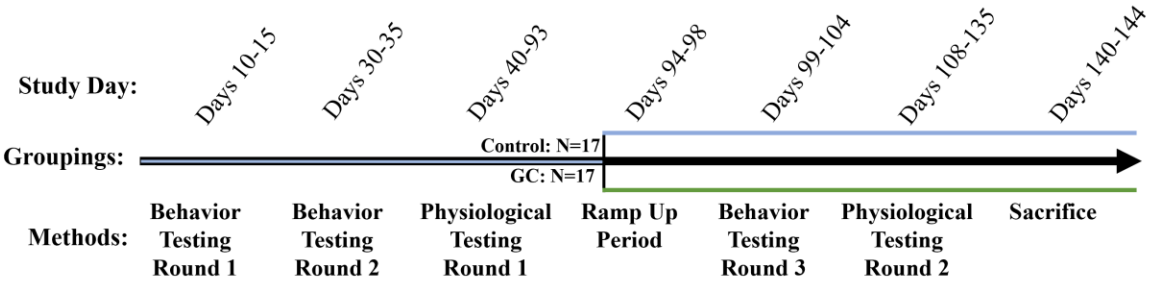


Figure 4.2. Timeline depicting duration of study and procedures. At the beginning of the study, all animals received normal tap water (depicted by light blue line outlined in black). During this time, we conducted two rounds of behavior testing and one round of physiological testing. After this, animals were equally divided into two groups (Control and GC). For the remainder of the study, the Control group received 1% ethanol in their drinking water (depicted by diverging darker blue line) and after the ramp up period, the GC group received 30µg/mL (depicted by diverging green line) in their drinking water.

The study timeline can be seen above (**Figure 4.2**). We conducted two rounds of behavior testing at the start of the study to determine individual consistency of exploration and social boldness behaviors. We then conducted a battery of physiological challenges on animals to determine baseline physiological activity and physiological reactivity. Next, we divided animals into two groups: Control and GC that contained equal numbers of high-, low-, and mixed-explorers and high-, low-, and mixed-socially bold individuals in each group (based on results of rounds 1 and 2 behavior tests). Animals in the GC group were exposed to increasing concentrations of GC in their

drinking water on consecutive days of a five-day ramp up period (Day 1: 7.5 µg/mL, Day 2 and 3: 15 µg/mL, Day 4 and 5: 30µg/mL). After this, a third round of behavior testing was conducted followed by a second round of the same physiological testing. At the end of the study, rats were sacrificed and a final blood sample was collected.

Behavior Tests:

Behavior testing is described in [207]. Briefly, we used three behavioral tests, each conducted at three different times. The first and second round of testing were conducted prior to GC manipulation, while the third round was conducted during the GC manipulation. Animal test order was randomized within each test and the order of the behavior tests within each round was changed. All tests were performed during the active/dark phase (13:00-15:00 h) in a room separate from the colony room. Each test lasted 5 minutes, after which time rats were immediately returned to their home cage. AnyMaze software (Stoelting Company, Wood Dale, IL) was used to code behavior in the three behavior tests.

Novel Social Test: This test was used to measure Exploration, and Social Boldness [207]. The dimensions of the arena were 120cm x 120cm with 46cm walls. The arena floor was coated with fresh sawdust bedding. The arena contained two cages in diagonal corners from each other - one empty and the other with a novel same-sex rat of similar size and age as the test rat. Each study rat, within its enrichment tube, was placed into one of the free corners of the arena.

Novel Physical Test: This test was used to measure Exploration [68,207]. The test arena was the same size and height as the Novel Social Arena, but instead of two cages, there were three rat-sized objects placed in three of the four corners of the arena. Study rats,

within the enrichment tube from their home cage, were placed in the free corner of the arena.

Partner Preference Test: This test was used to assess Social Boldness [207]. The Partner Preference Test arena was the same size as for the Novel Social Test arena with two additional walls that divided the arena into three equal sections of 40cm x 120cm. The internal walls each had a 5-inch square cutout at the bottom to allow for passage between arena sections. In one section a familiar rat was placed in a cage and in the other side section an unfamiliar rat was placed in a cage. (The familiar stimulus rat was the one used in the previous Novel Social Test whereas the study rat had never interacted with the unfamiliar stimulus rat.) The center section was left empty and the study rat, along with its red enrichment tube, was placed into this section of the arena.

Physiological Tests:

Physiological testing was conducted twice; once before and once during the GC manipulation period. This enabled us to determine individual change in physiology based on the manipulation. Rats were weighed once a week to monitor changes due to age and due to GC treatment.

Overall physiology procedures: All physiological tests were conducted during the active/dark phase (13:00-15:00 h) except for the Innate Immune challenge, which extended from 08:00 to 16:00 h. All blood was collected initially by tail tip amputation and then removal of the scab at the wound site for further blood collection throughout the study.

Glucocorticoid Manipulation: Rats in the GC treatment group received 30µg/mL of corticosterone with 1% ethanol as a vehicle in their drinking water. Control rats were

exposed to 1% ethanol in the drinking water. Rats had a five-day ramp up period where the GC concentration in the water bottle went from 7.5 on Day 1, to 15 µg/mL on Days 2 and 3, to 30µg/mL on Days 4 and 5. After this ramp up period, GC treated rats consistently had 30µg/mL corticosterone and control rats had vehicle-laced water in light sensitive bottles. This was the only water that rats had to drink. Bottles were changed every other day and weighed daily.

Glucocorticoid Response to Acute Restraint: To measure circulating GC response to a physical acute stress, rats were placed in a restraint tube for 30 minutes. To measure baseline GC concentrations, blood samples were collected within three minutes of moving the rat's cage from the colony room. To measure stress-induced GC concentrations, blood samples were collected 30 minutes after rats were first placed in the restraint tube. And to measure recovery GC levels, blood samples were collected 90 minutes after restraint stress initiation. After testing, rats were returned to the colony room. After collection, blood samples were immediately put in ice until centrifuged. After centrifuged, serum was pipetted into aliquots and stored at -80º C until analysis. Serum samples were analyzed using an ELISA (ELISA, Arbor Assay, MI) to determine corticosterone concentrations.

Adaptive Immune Response: In the Physiological Testing Round 1, to measure adaptive immune reactivity, rats were challenged with Keyhole Limpet Hemocyanin diluted in endotoxin free 0.9% saline (KLH, MilliporeSigma, MA, catalogue # 374825). On day 0, a blood sample was collected and then rats were immediately injected s.c. with KLH (0.5mL, 500µg/kg). Additional blood samples were collected on days 7, 14, 21, and 28 for future measures of antibody production and circulating corticosterone concentrations.

On day 29, rats were re-exposed to KLH (100µg/kg) via a 0.1 mL hindfoot pad injection and 0.1 mL of endotoxin free 0.9% saline in the other hindfoot pad. Swelling was measured 24 hours later and a relative hindfoot swelling response was calculated as the difference in swelling between the KLH- and saline-exposed foot pad to estimate the delayed-type hypersensitivity response.

In Physiological Testing Round 2, we measured cell-mediated immune responses by challenging rats with methylated Bovine Serum Albumin (mBSA, Millipore-Sigma, MA, catalogue #A1009). On day 0, we collected a blood sample to measure basal GC levels and then immediately injected rats s.c. with 0.1mL of mBSA (200µg) into their caudal tail fold. An additional blood sample was collected on day 7 for future measures of antibody production and circulating corticosterone concentrations. After this blood collection, rats were immediately re-exposed to mBSA (200µg) via a 0.1mL hindfoot pad injection and 0.1mL of endotoxin free 0.9% saline in the other hindfoot pad. Similar to the KLH-exposure protocol, swelling was measured 24 hours later and a difference in swelling between saline and mBSA exposed foot pad was calculated to estimate delayed type hypersensitivity response.

Heart Rate: To measure heart rate in response to a stressor, rats were placed in a restrainer and heart rate and blood pressure were measured non-invasively using the IITC cardiac machine (IITC Life Sciences, CA). Rats were placed in the restraint tube and allowed to warm in a warming chamber for 10 minutes. After this period, heart rate was measured at three time periods. Within each time period, five consecutive measures were taken and averaged.

Temperament Analysis:

To categorize Exploration, we used a single measure of latency to interact with novelty in the Novel Social and the Novel Physical tests. We used a median split to categorize individuals as High or Low Exploratory. We then compared individuals between the two tests to determine if they were consistently High or Low Explorers. If they had high latencies in one test (Low Explorer) and low latencies in the other test (High Explorer), we categorized them as Mixed Explorers.

To categorize Social Boldness, we measured multiple behaviors in the Novel Social and Partner Preference Tests. We used a factor analysis to condense behaviors into latent variables and identified the Social Boldness factor based on those that loaded behaviors that focused on interactions with novel social partners and with risky situations. We used a median split of the scores to categorize individuals as Socially Bold or Non-Socially Bold. We then compared individuals between the two tests to determine if they were consistently Socially Bold or Non-Socially bold. If they had high scores from the factor analysis in one test (Socially Bold) and low scores in the other test (Non-Socially Bold), we categorized them as Mixed Socially Bold.

Statistical Analysis:

Effects on body weight were calculated using a repeated measures analysis using an ANOVA. We used a T-test to measure the change in body weight from the start of the GC manipulation to the end of the study between the Control and Treatment groups. Changes in both temperament and physiology were analyzed by calculating the difference in a specific measure from time point one to time point two. We used a two-way ANOVA to analyze physiological and behavioral data with GC treatment, original temperament category, and GC treatment x temperament category effects. We used

Tukey's HSD for post-hoc analysis. We used Levene's test to determine homogeneity of the data and Shapiro-Wilk test to determine normality.

Results

Treatment effect on Body Weight:

There was a main effect of time showing rats had significant increases of body weight across the study ($F(17,544)=820.305$, $p<0.05$) (**Figure 4.3**). However, the GC treatment did not influence body weight from the control group ($F(17,544)=0.766$, $p>0.05$) or across time ($F(1,32)=0.574$, $p>0.05$). Finally, there was no significant change in body weight from when the GC manipulation started to the end of the study between the control ($M= 35$, $SD= 12.58$) and GC treated (22.53, $SD=25.54$) groups ($t(23.31) = 1.8059$, $p>0.05$).

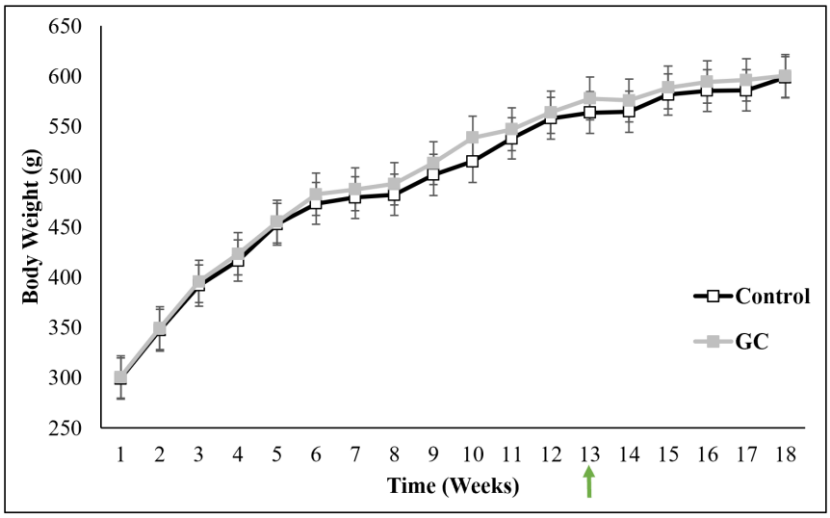


Figure 4.3. Rat body weight over time. There was no difference in rat weight between treatment groups ($p>0.05$). All rats regardless of treatment had significant increases in weight across the study ($p<0.05$). The GC treatment started on week 13 indicated by the green arrow. There was no significant difference in the change in body weight between the two treatments from the start of the manipulation until the end of the study.

Treatment effect on Glucocorticoids:

There was a main effect of treatment in changes in GC levels during the acute

restraint stress. Rats in the GC treatment had significantly higher basal GCs, and lower stress-induced GCs compared to control individuals (basal: $F(1,28) = 44.145$, $p < 0.00001$; stress-induced: $F(1, 28) = 55.367$, $p < 0.0001$), but we found no change in recovery GC levels ($F(1,28) = 2.233$, $p > 0.05$) (**Figure 4.4**). Temperament did not have an effect on changes in GC levels during the restraint stress tests (Basal: $F(2,28) = 0.058$, $p > 0.05$; Stress-induced: $F(2,28) = 0.605$, $p > 0.05$; Recovery: $F(2,28) = 0.440$, $p > 0.05$) and there was no interaction effect between treatment and exploration temperament (Basal: $F(2,28) = 1.466$ $p > 0.05$; Stress-induced: $F(2,28) = 1.115$, $p > 0.05$; Recovery: $F(2,28) = 2.679$, $p > 0.05$). Within the GC group, during GC exposure, Low- and Mixed-explorers had significantly higher stress-induced GC concentrations compared to High-Explorers (**Figure 4.5**, $F(2,16) = 5.956$, $p < 0.05$).

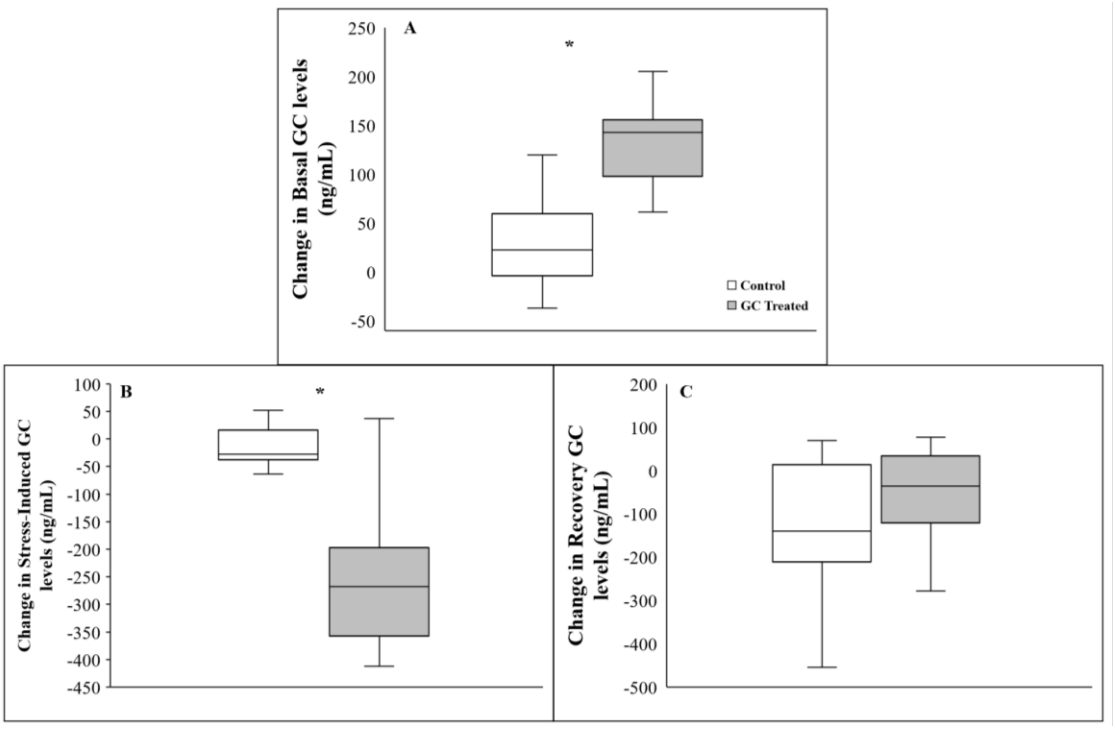


Figure 4.4. Change in circulating GC concentrations from Physiological Test Round 1 to 2 during the acute restraint stress tests. GC treated rats (grey) had (A) a significant increase in basal circulating GC concentrations at baseline and (B) a significant decrease in stress-induced concentrations compared to controls (white) (* $p<0.05$). (C) Change in GC levels at the recovery time point were not significantly different between the control and GC groups.

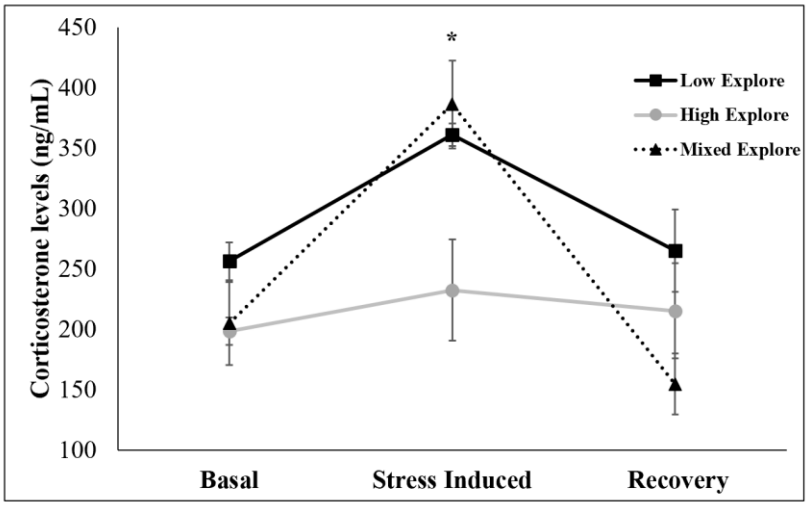


Figure 4.5. GC levels during the second acute restraint stress in the GC-treated animals. Low (—■—) and Mixed (···▲···) Explorers had significantly higher stress-induced GC concentrations compared to High-Explorers (—●—) (* indicates $p<0.05$). There were no temperament-specific differences in GC concentrations at the basal or recovery time points.

Treatment effect on Exploration:

GC-treated animals became significantly slower to interact with novel social partners during the GC treatment phase compared to controls ($F(1,28)= 75.477$, $p<0.0001$). We also found a main effect from the exploration category and an interaction effect of treatment x exploration category effect (**Figure 4.6**). Based on the exploration categories, we saw a significant difference in the change to interact with the novel social partner between the High and Low Explorers ($F(2,28)= 3.529$, $p<0.05$). In GC treated animals, Low Explorers had a significantly greater increase in latency to interact during GC exposure compared to Mixed and High Explorers, who were not different from each other ($F(2,28)=7.243$, $p<0.01$). There was no significant main effect of treatment differences in latency to interact with novel objects in the Novel Physical test ($F(1,28)=4.016$, $p>0.05$).

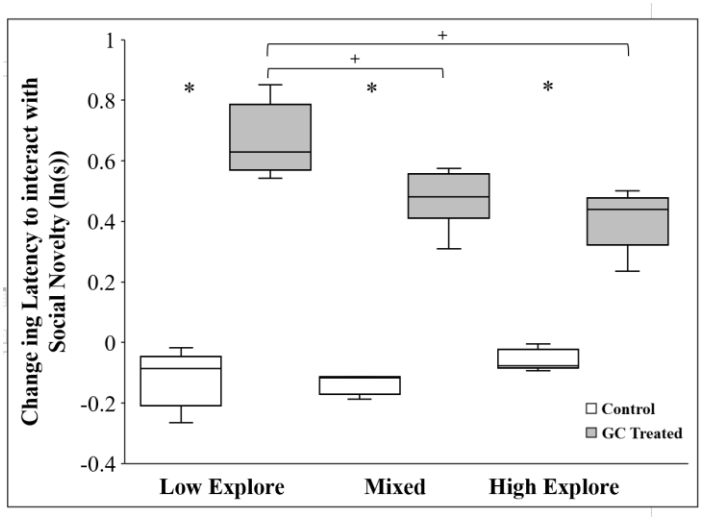


Figure 4.6. Change in latency to interact with novelty in the Novel Social Test from Behavior Testing Rounds 1 and 2 to Round 3. Rats in the GC treatment group (grey) had a significantly greater increase in latency to interact with novelty compared to controls (white) (* $p<0.0001$). In addition, a treatment x exploration category effect indicates that GC-treated Low Explorer showed the greatest increase in latency to interact compared to Mixed and High Explorers (+ $p<0.05$).

Treatment effect on Social Boldness:

Social Boldness behavior was not affected by treatment regardless of behavior test (**Figure 4.7**). Rats that were treated with GCs had no significant change in Average Social Boldness scores compared to controls ($F(1,28)=0.601$, $p>0.05$). Additionally, there was no main effect of Social Boldness Category ($F(2,28)=2.335$, $p>0.05$) and no interaction effect ($F(2,28)=0.159$, $p>0.05$).

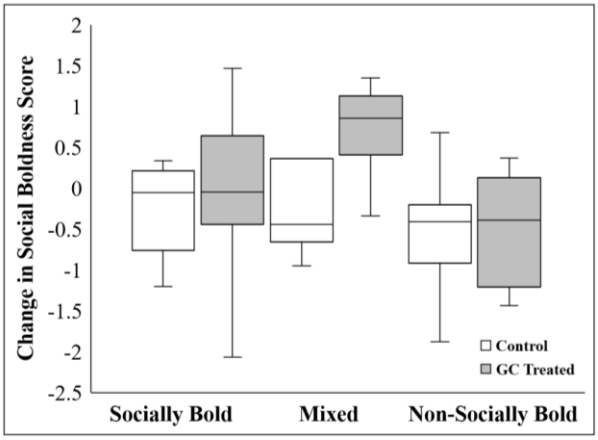


Figure 4.7. Change in Social Boldness from Behavior Testing Rounds 1 and 2 to Round 3. GC treatment did not lead to significant changes in Social Boldness scores.

Treatment effect on Cell-Mediated Immune Response:

We found treatment, exploration category, and a treatment x exploration category effects on the cell-mediated immune response (**Figure 4.8**). Compared to the first cell-mediated test round, rats treated with GC had a significant decrease in hindfoot swelling during the second test round when exposed to the GC manipulation compared to unexposed control rats ($F(1,28)= 40.242$, $p<0.00001$). Additionally, in the GC treatment group, rats that were Low and Mixed Explorers had significant suppressed swelling ($F(2,28)= 5.372$, $p<0.05$), but High Explorers were not different from controls. In the GC group, High Explorers were significantly different from Mixed and Low Explorers ($F(2,28)= 4.925$, $p<0.05$).

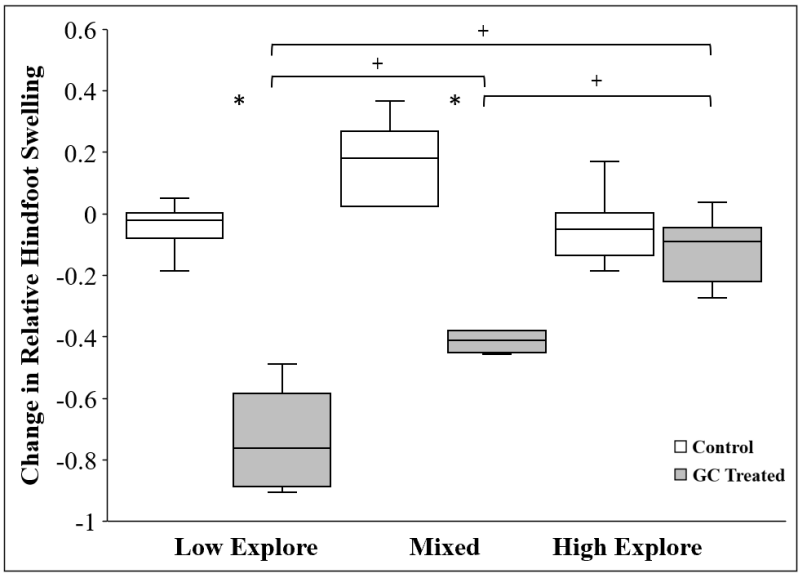


Figure 4.8. Change in hindfoot swelling response to antigen re-exposure from Physiological Test Round 1 to 2. Rats in the GC treatment (grey) had significant a decrease in swelling responses compared to controls (*p<0.05). However, High Explorers in the GC treatment were not significantly different from High Explorers in the Control group. Within the GC treatment, Low Explorers had significantly higher changes (more suppressed) in swelling response compared to Mixed and High Explorers +p<0.05).

Treatment effect on Cardiac Function:

GC treatment led to a significant decrease in heart during the first heart rate measure (collected 10 minutes after animals were placed in the restraint tube) compared to controls ($F(1,28)=13.691$, $p<0.001$) (**Figure 4.9**). All other heart rates measurements did not show any significant changes based on treatment or temperaments.

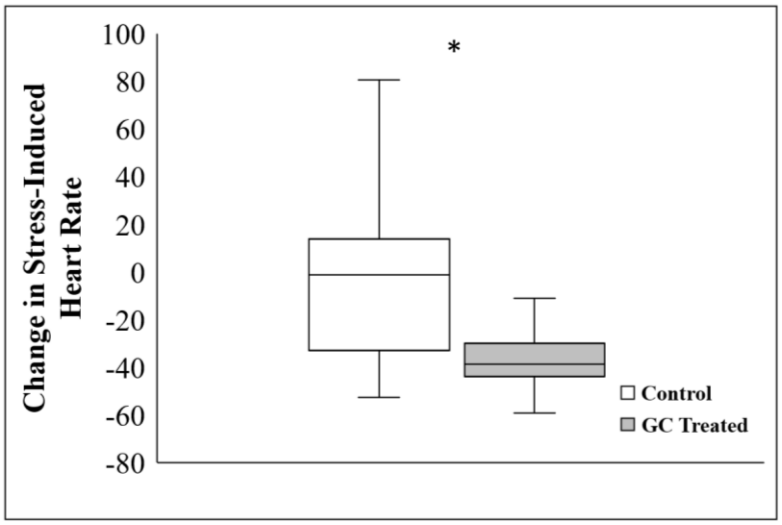


Figure 4.9. Change in stress-induced heart rate from Physiological Test Round 1 to 2. GC treated animals (grey) had a significant decrease in stress-induced heart rate during GC exposure compared to untreated controls (white) ($p<0.001$).

Discussion

To determine the influence of slight, but chronic elevations in circulating basal GCs on temperament and associated physiological mechanisms, we added GC to rats' drinking water and measured changes in behavioral and physiological processes. We found that animals in the GC treatment group had significantly increased basal GCs and latency to interact with novelty from before to during the GC manipulation, and significantly decreased stress-induced GCs, cell-mediated immune activity, and stress-induced heart rate compared to control rats. The Social Boldness temperament trait did not significantly change with GC treatment.

Treatment effect on GCs

We determined that our GC treatment increased basal circulating GCs, which other studies have also shown using the drinking water methodology [290–293] or conducting chronic stress experiments [294,295]. Other methods include using a steroid-filled implant [296–298] or direct steroid injections [299,300]. While these methods are

useful in other studies, we wanted to use a minimally-invasive method that mimics the normal glucocorticoid circadian rhythm [301,302]. Interestingly, the chronic low-grade GC treatment also led to a suppressed GC response during an acute restraint stress where rats' GC levels were significantly reduced at the peak stress-induced time period. Other studies that have used chronic stress paradigms have found similar results with respect to attenuated stress-induced GCs [271,303–305]. As Rich and Romero [271] stated there are three possibilities for these results: 1) habituation, 2) exhaustion, or 3) downregulation. We can confidently rule out the first (habituation) because rats in the control group did not have a significant change in their stress-induced GCs from the first to second time point. It is possible that rats experienced exhaustion as defined by Rich and Romero: the state in which an animal can no longer compensate for sustained stress and effects become life-threatening [271]. However, animals would have likely had other external markers and signs such as reduced weight, which we did not find. Therefore, we theorize that the decreased stress-induced GC response resulted from a downregulation effect. Downregulation refers to suppression that is biological in nature such as decreased receptors that are not necessarily related to cognitive induction (like habituation).This is likely an adaptive response to minimize negative effects of chronic GC production [259,306]. Previous studies show that chronic GCs lead to downregulation of GR in the hippocampus and weaker negative feedback to the HPA axis resulting in dysregulation [259,307–312].

GC influence on Temperament

Exploration and Social Boldness have both been shown to be highly consistent across time and conditions [207]. Previous work from our lab shows that basal GC

production along with GC reactivity and basal heart rate were significant predictors of Exploration while gut microbiome alpha diversity was a significant predictor of Social Boldness, although GC reactivity showed a trend toward being a significant predictor (Chapter 3). This suggests that Social Boldness is a rigid temperament trait not driven by GC production, and that it might not be driven by the physiological mechanisms that we measured in the current study. On the other hand, the significant decrease in Exploration (i.e. increased latency to interact novelty) in GC-treated animals suggests that a Low-Exploration temperament may be a result of elevated basal circulating GC concentrations and that Exploration is a flexible temperament. In addition, because we saw an overall attenuated GC response in GC-treated rats, it is likely that chronically-elevated basal GC concentrations might drive both responses – decreased Exploration and an attenuated GC response to a physical challenge. Other studies that used acute GC administration protocols have found similar results with increased GC concentrations in circulation resulting in decreased Exploration [313–315], but see [81] that showed no change in Exploration. However, few studies have chronically manipulated basal GC levels and determined the influence on temperament, especially in animal models. Additionally, we found that experimentally chronically elevated basal GC concentrations attenuated stress-induced GC concentrations, but that rats retained their individual differences in GC reactivity, with High-Explorers still having the lowest GC response to restraint stress compared to Low-Explorers. Another interesting result is that we only saw changes in Exploration in the Novel Social test, but not the Novel Physical test. Previous studies from our lab have found that there are stronger associations between GC production and the Novel Social test compared to the Novel Physical test [128] suggesting that Novel

Social tests might be more accurate at identifying Exploration tendencies while the Novel Physical test may be testing other lower-risk types of Exploration and is not a flexible temperament.

Social Boldness did not change as a result of GC treatment. This lack of change was expected since our prior work showed that basal circulating GC concentrations are not predictive or correlated with Social Boldness (Chapter 3). However, in our previous study, HPA reactivity was negatively correlated with Social Boldness and trending toward a negative predictive variable (Chapter 3). This suggests that this downstream consequence of GC treatment did not influence Social Boldness, further supporting the theory that this is an inflexible temperament. Given that our prior work showed a positive correlation between Social Boldness and gut microbiome alpha diversity, future studies should continue to analyze how chronically-elevated basal GC production influences the gut microbiome diversity in relation to temperament.

GC influence on Cell-Mediated Immune Response

Within this study we saw an attenuated cell-mediated immune response in rats exposed to the GC treatment. This was expected as previous studies have shown that chronically elevated GCs dampen this response [260,264,266,267,316]. When we compared these results among Exploration categories, we found that Low Explorers had the most attenuated response followed by Mixed Explorers, and that High Explorers were no different from controls. The most suppressed response in Low Explorers is to be expected as their reactive GC levels were significantly greater than the High Exploratory individuals, and it is likely that the addition of more GCs resulted in a more significant effect on this adaptive branch of the immune system [81]. Further, High Explorers did not

have a significant change in swelling response based on the treatment, and this is to be expected given that the High Explorers had an attenuated restraint stress-induced GC response. In our previous study (Chapter 3), we reported Exploration was positively correlated with a cell-mediated immunity and HPA reactivity. With these physiological systems shifting based on elevated GCs, it provides further evidence of the flexibility of both Exploration and Exploration's physiological profile.

GC influence on Cardiac Function

Stress-induced heart rates were attenuated in the GC treated animals. Other studies have found similar results [261,317] and few have found the opposite [318]. However, we do not see changes in other heart rate measures at other time periods. A normal acute stress response is characterized by activation of the sympathetic response through elevated heart rate and blood pressure (first wave) followed by elevated GCs (second wave), resulting in these systems working in tandem to cope with the stressor until it is removed, and these systems return to basal levels. However, in chronic stress situations, the HPA axis is in dysregulation resulting in changes to the sympathetic response [261,319–321]. For example chronic GC exposure can lead to persistent activation of cardiomyocyte GR and MR resulting in tissue damage [320–323]. Based on our results, we saw that chronic basal elevation in GCs resulted in dampening of both the first wave and second wave of the stress response. This change in the physiological system was not linked to Exploration or Social Boldness, suggesting that it might not be a substantial contributor to the flexibility of either.

Future studies should include analyses of other physiological systems and test how GCs influence other physiological systems. For example, the gut microbiome is

highly influenced by the HPA axis through GC production [324,325]. Specifically, microbial diversity decreases when GC concentrations are chronically increased [326–328]. In addition, exploratory analyses with larger studies could model which aspect of the altered physiological profile appear to be most closely associated with changes in behavior.

Conclusion: We found that chronic elevations in basal GCs resulted in alterations in parts of the physiological network, especially in HPA axis regulation, the cell-mediated immune response and cardiac function. With HPA axis regulation and cell-mediated immune responses being significant systems within Exploration's physiological profile (Chapter 3), it likely that the flexibility of Exploration is driven by the flexibility of the underlying physiology we tested. However, Social Boldness was inflexible based on the GC treatment and was not related to the flexible physiological systems we measured. This study shows how manipulation of one physiological system can influence other systems and result in changes to some temperaments, further providing evidence of the importance of identifying the physiological profiles of temperaments.

Chapter 5:

Conclusion

Summary of Findings

Within this dissertation, I conducted the comprehensive studies to identify a range of physiological processes associated with temperament. Within the initial literature review (Chapter 1), I showed that this area of study is lacking comprehensive studies that consider more than one physiological system, and suggested potential avenues to advance this field. Before we could begin to look at the associations between temperament and physiology, I needed to characterize a range of the temperament traits in my model organism. I compared two methodologies that are commonly used to quantify temperament and determined the relative consistency associated with each method across time and conditions (Chapter 2). In Chapters 3 and 4, I measured multiple physiological components to identify physiological profiles for several temperaments, and determined how a flexible these profiles and temperaments are by altering one physiological system. Taken together, we see that temperament and physiology are complexly associated and that a shift in one aspect of the physiological profile can lead to changes in other aspects of physiology and temperament.

In Chapter 1, my co-authors and I identified the gaps in studying relative animal temperament. We used the five categories of temperament identified by Réale and colleagues [12], which many in the literature utilize. Based on the review of the current literature, I found that most studies analyze the relationship between temperament and physiology in one-to-one comparisons, with one temperament trait and one physiological system. Additionally, across studies we saw considerable variation in results and that the majority of studies focused on HPA re/activity and its association with different

temperaments. We proposed other physiological systems to sample and compare to temperament across a wider range of taxa. Finally, based on the literature, we hypothesized about potential physiological profiles of different temperaments. We concluded that to understand the rigidity or flexibility of temperaments, and to determine their long-term consequences, we need information on inter-related physiological systems that underlie temperament.

This drove the remainder of my dissertation work to understand the relationship between temperament and physiology within an individual. The first question we asked was: How do we measure temperament? Based on the literature review from Chapter 1, it was evident that scientists use two methodologies: single-behavior and multi-behavior metrics. The single-behavior metric method is especially practical in field studies when time is of the essence. To use this single-behavior metric, researchers need to determine which behavior to use as a proxy for temperament. For example, Boldness is defined as the risk an individual is willing to take. In many wild species, being in an open area with limited cover or places to hide is considered risky and thus, an individual spending an extended time in this open area is considered bold. This reflects a single-behavior metric of Boldness. On the other end of the spectrum, multi-behavior metrics are becoming more frequently used in studies. In these instances, scientists collect multiple behaviors during a behavior test and use dimension reduction analysis to determine what behaviors covary to identify temperaments. This method results in composite factor scores that can then be identified based on covarying behaviors. Both methods have frequently been used in laboratory and field conditions. In Chapter 2, we wanted to determine if one method led to more consistent estimates of temperament across time and conditions. We found

that multi-behavior metrics did not necessarily provide better estimates of consistent behavioral traits over time compared to a single behavior. Many of the behaviors that contributed to consistent factors were themselves individually consistent over time, and in some cases more consistent than the composite factor scores. The multi-behavior metrics that were consistent across time and conditions were Social Boldness and Activity. Social Boldness is not a temperament trait that has been previously identified while Activity is one of the five temperament traits identified by Réale and colleagues [12]. Based on this, we suggested that future studies should conduct a battery of tests and use a combination of single- and multi-behavior metrics to determine which produce the greatest consistency.

Once we identified and characterized different temperaments, we determined how they are associated with multiple physiological mechanisms. The goal of Chapter 3 was to create a physiological profile for each temperament trait that we observed in Chapter 2. We collected multiple measures of physiological processes including HPA axis, innate immune system, adaptive immune system, gut microbiome and cardiac function. We found that temperaments that were highly consistent were also correlated with more physiological systems than temperaments that were not consistent. We analyzed the relationship between the single-metric behaviors and the physiological systems and found that more single-behavior metrics of temperament were more often correlated with physiological systems as compared to the multi-behavior metrics of temperament. Additionally, we found that some physiological systems were highly predictive of temperament. Specifically, HPA axis reactivity was predictive of multiple temperaments (both multi-behavior and single-behavior metrics) while cardiac activity and reactivity

and HPA activity were also significant predictors of some temperament traits. Last, we found that latency to interact with novelty, which is used as a measure of Exploration, was closely associated with multiple physiological systems. This builds on prior research showing that latency to interact with novelty is associated with cell-mediated immune function and HPA re/activity across taxa [68,124,201,235–238]. Social Boldness was correlated with the gut microbiome alpha diversity, cardiac activity and HPA reactivity, but was only significantly predicted by gut microbiome alpha diversity. This was the first step in identifying the physiological profiles of temperament and understanding how physiology might influence temperament.

In the next study I determined the relative flexibility of the underlying physiological profile by shifting one physiological system to determine if/how this shifts temperament and other physiological systems. I challenged a new cohort of rats with a low-dose corticosterone (GCs) treatment by adding it to their drinking water and repeated experiments from Chapter 2 and 3. GCs are associated with multiple temperaments (as seen in Chapter 1) and also significantly influence other physiological systems ([72,96,259,329,330] *See Supplemental Data 3.1*). Rats exposed to GCs in their drinking water showed a significant change in Exploration (i.e. lower latency to interact with novelty), but not in Social Boldness. The fact that Exploration changed but not Social Boldness can possibly be explained by results from Chapter 3, where I determined that Exploration was significantly predicted by basal HPA axis activity. This could be further support that the HPA axis is a significant driver of changes in temperament. However, Social Boldness might be driven by other physiological systems that I did not measure in this dissertation. Additionally, GC treated rats also significantly lower cell-mediated

immune responses and lower stress-induced heart rates. The GC induced decrease in cell-mediated immunity was driven by low and mixed exploration rats. It is possible that the attenuated GC response in the GC treated high-explorers drove the non-significant change in swelling response, but future studies need to identify the influence of dampened GC production on cell-mediated immunity [331]. It is also possible that other physiological testing may have influenced this result.

This research is the first step in understanding how physiology and temperament are connected. Current research is focusing more toward understanding individual variation, especially in temperament and its relation to physiology (reviewed in [332]). While the field still focuses on only a few physiological mechanisms [197], more recent research is embracing the idea of physiological profiles associated with temperament and determining the flexibility of these profiles [258,333–335]. This dissertation sheds light and provides valuable background data and information for future studies. There are still many ways to expand and broaden this research to truly understand the connections we observe. To uncover and understand these relationships, research needs to delve further into the physiology of the individual and focus on genetics. By identifying genes that regulate temperament, physiology and interactions among them, we can better understand the heritability of these traits. I expand on this area in the future directions.

Ecological Significance and Critiques

Within this dissertation I elude to the significance of this research to the field of ecology. The connection between temperament and underlying physiological systems can help us better understand individual variability and better predict different ecological outcomes in varying environments. From this, we can then better understand the

ecosystem dynamics we observe. This field is still expanding and there is little congruence [101] resulting in many critiques to both animal behavior and the connection to physiological mechanisms.

There are caveats that should be considered when studying temperament. For example, terminology used is a glaring problem in this field. Ecologists use terms such as "personality" and "temperament" interchangeably to discuss individual differences in behavior that are consistent across time and/or conditions. This has resulted in confusion for many. These terms are not interchangeable in the psychology literature, where these terms originate. While animal behavior and psychology are two distinct fields, not using the same terminology between them prevents cross-disciplinary research. However, even within the definition, novices to the field think that these behaviors must be consistent both across time and contexts. This is not the case within this definition as behaviors have been measured across time and found to be repeatable, but not across contexts or vice versa. This brings up the need to study what is driving these differences in consistency that we are observing. Within my third and fourth chapter of my dissertation I dove into this area to better understand how underlying physiology might be driving the differences we see and if these physiological systems, along with the behavioral traits observed, are plastic and can change in different environments.

Using a battery of physiological tests needs to be critiqued as well. Many studies only focus on one or, at most two, physiological systems to determine how they interact and/or how they relate to behavioral traits. While this prevents a full conceptualization of how these physiological systems are interacting and influencing behavior, it does enable researchers to note that one physiological test did not influence others. However, within

my research, I conducted the battery of physiological tests to determine how they interact and influence behavior. My results show that this physiological testing may have influenced behavior tests, but more data would be needed to support this claim. Regardless, it is still an important caveat within my research to note that within Chapter 3 and 4, the last physiological results collecting (the cell-mediated immune response) could have been influenced by the acute restraint stressors along with the innate immune challenge. The reason we conducted these tests was to simulate what any human or animal might go through in a normal period of time to better understand how these systems interact.

Another important critique within animal behavior research field is the five common traits that are proposed by Réale and colleagues [12]. Réale and colleagues specifically said that the five traits they identified were only to be used as starting points for ecologists studying temperament. However, it seems many have narrowed their viewpoint to using only these, especially when using single-behavior metrics of temperament and their definitions of these categories vary greatly, resulting in incongruent results. Within my research, I used these terms based on the definitions Réale and colleagues used to identify the single-behavior metrics and compare our results to other studies. However, when using multiple-behavior metrics, we found multifaceted behavioral traits that combined some of the traits Réale and colleagues identified. Future studies should use this methodology to better understand how specific behaviors are interconnected and might result in a single consistent temperament trait.

Finally, another critique within this field is the methodology used to study temperaments. Within Chapter 1, I noted that while many studies only analyzed one

temperament trait, they also did not determine the reliability or repeatability of this trait across time or context and relied on previous studies that have measured this technique. While it may be difficult to measure consistency across time in wild populations, it is imperative that this is conducted within each study to determine the validity of the behaviors being measured. That is why within Chapter 2 of my dissertation I conducted the behavior testing at three time points to determine which of these behavioral traits are consistent across time or across test.

Human Translational Significance

While much of this dissertation focused on ecological implications, it is important to note the important translation aspect of this research to humans. The use of animals as model species for human clinical research has been a common methodology for many years [336,337]. Using animal models provides insight into mechanisms of disease and developing new treatments that would be difficult to test in humans. Historically, in the USA animal behavior was described in relation to human behavior, and used as a model to understand human behavior. While both human psychology and animal behavior methodologies were conducted and developed at the same time, animal behavior research is just catching up to studying consistent individual differences in behaviors (temperament). This expansion was driven by the realization that temperaments were not exclusive to humans and can be documented in a variety of taxa. While both research disciplines have studied an array of temperament traits, I will focus on Behavioral Inhibition (BI). BI is defined as a behavioral tendency to show fearful reactions toward unfamiliar people or situations [214]. BI has been associated to many negative health outcomes including depression and anxiety [338–343], asthma and allergies [344–346]

and overall shorter lifespans [347,348]. These negative health outcomes are associated with physiological mechanisms that co-occur with BI individuals such as over-stimulation of the sympathetic nervous system [246,247,346] and elevated glucocorticoid re/activity [349–353]. Based on the definition and the physiological mechanisms associated with human BI, it is clear that BI is similar to Exploration studied in animal models. For example, Low Explorer rats have elevated stress-induced GC production, dampened cell-mediated immune responses, and heightened sympathetic reactivity [68,81,201]. Much of my current research aligns with these previous studies. Much work has been done in the past two decades using animal models to study the underlying mechanisms of BI and applying this for clinical interventions to ameliorate the maladaptive consequences [81,344,354,355]. However, future studies should be more interdisciplinary and conduct complementary studies in humans and animals to better understand the similarities and differences observed. By understanding individual differences within biological systems in both human and animal species, we can identify potential universal systems that orchestrate temperament and result in differing health and developmental trajectories.

Future Directions

This research should continue in multiple complementary directions. I will discuss two avenues. The first is to take a mechanistic approach and to understand how genetics along with other physiological systems potentially drive or are affected by temperament. The second is broader and involves characterizing sex differences and species differences in physiological and temperament interactions.

Genomics Research

One key area that is currently understudied is how behavioral and physiological traits co-occur. Future research should take advantage of the genomic data to start to answer these questions. Large-scale genomic data and analyses allow for global estimates of gene expression patterns related to temperament, physiological processes, and health-related outcomes [97,259]. The genetic precursors that regulate physiology provide building blocks to infer complex physiological profiles associated with behavioral traits. Since temperaments are likely influenced by a multitude of genes, this work should involve whole-genome profiling [97,356]. Genome-Wide Association Studies (GWAS) are a powerful tool to evaluate the association between each genotype marker and the phenotype of interest [357]. GWAS can be used to identify pleiotropic effects on multiple phenotypes, but this is still a growing field and statistical analyses are not consistent across situations [358,359]. GWAS has been used to identify underlying genes associated with specific behaviors [360,361] and is being used to identify genes that coregulate temperament and underlying physiology within humans [362–364]. Based on these studies, future work can identify potential genes that express both animal temperament and physiological mechanisms. However, GWAS should not be used as the only method to identify direct pathways; additional factors such as environment and other downstream physiological consequences should be taken into account when interpreting results [365,366]. While GWAS and other whole genome methods have been used, many researchers still use the Candidate Gene (CG) approach, which is used to investigate how alleles within a gene relate to phenotypic traits [367]. With this method, researchers select genes based on a-priori hypotheses about specific mechanisms that may influence phenotypes of interest. While using CG can provide much more targeted information and

test hypotheses, it is still limited by prior knowledge about the physiological and biochemical relationships to the phenotype of interest. However, CG has been frequently used to understand underlying mechanisms of behavior and overtime has built a solid foundation of understanding how specific genes contribute to behavioral variation [368–370]. Future studies will need to consider both methodologies and determine which method is best to identify underlying mechanisms of temperament, physiology and their interactions.

Other Physiological Mechanisms

While neuroendocrine, immune and sympathetic function are frequently studied, research on other physiological systems will further our understanding of the functional significance of behavioral traits. For example, metabolic rate is an important physiological mechanism that is highly studied in laboratory conditions and less studied in ecological settings. Metabolic rates provide an indicator of health as an estimate of energy stored, required, and expended. Studies have shown relationships between metabolic rate and behavior in a variety of organisms [106,371] showing that this physiological process and behavior may be interrelated. In particular, basal metabolic rate (BMR) is a measure of the minimal energy expenditure in individuals at rest and has been shown to be highly repeatable and consistent [372]. BMR has also been shown to be positively correlated to activity, risk taking, exploration, and aggression [69,135,373–378]. The relationship between BMR and these temperament traits is functionally significant and adaptive. For example, individual differences in metabolism may affect an individual's capacity to engage in these behaviors so it is adaptive for high explorers or bold individuals to have higher metabolic rates and higher energy budgets to carry out

these behaviors. Additionally, Careau and Garland [375] made a valid point stating that temperament is related to other life history traits such as growth and fecundity. To be able to carry out these life history traits, individuals have adapted to have adequate BMR.

In Chapter 4 I focused on the influence of GCs on other physiological systems and temperament. However, other highly-studied hormones could be incorporated into future studies, such as testosterone. Previous studies have found that circulating testosterone is not associated with some temperaments such as exploration, boldness and aggression [379–381] but others have found exogenous testosterone is elevated in aggressive individuals [382–384]. Additionally, testosterone is associated with GCs through inhibitory receptor interactions [385,386] and is shown to have inhibitory effects on GC production [387]. Also, exogenous GCs result in decreased testosterone production [388,389] showing that this is a bidirectional relationship. Testosterone has been shown to have direct effects on immune function [390] by binding to receptors on lymphocytes [391,392] or indirect effects such as interactions with GCs that then regulate immune function [393–395]. Finally, the gut microbiome and testosterone interact and can also have bidirectional effects [396–398], but the mechanisms that drive these interactions are poorly understood. Future studies could incorporate testosterone to better understand how physiological mechanisms interact and how covariation of these systems influence temperament.

While hormones such as GCs and testosterone are well-studied in connection to animal behavior and temperament, there is little research on other endocrine systems such as fast-acting hormones including epinephrine, norepinephrine, oxytocin and vasopressin. These hormones have been shown to influence specific behaviors such as sociability and

aggression (reviewed in [103]), but have not been widely studied in relation to other temperaments. While it is difficult to measure these in field settings, collecting blood samples or conducting laboratory studies may be the first step in understanding their relationship to other physiological systems and temperament. For example, epinephrine and nor-epinephrine have been highly studied as they are both part of the acute stress response [259], but there is limited understanding of the relationship of these hormones to temperament [399]. Recent studies have analyzed the relationship between oxytocin and the microbiome [400] finding positive correlations between oxytocin concentrations and gut microbiome diversity. Additionally, previous studies show that oxytocin downregulates stress-induced GC [401] and potentially works congruently with the gut microbiome to produce this effect [402,403].

Characterizing sex differences

Across all disciplines, studies in females are lacking [404–406]. However, we do know that previous studies show that female Sprague-Dawley rats produce different immune and stress responses compared to males [347,407,408]. Additionally across a variety of species, we see that females have a marked difference from males in behavioral responses to the same environment cues (rats: [347,407,409,410]; mice: [411]; fish: [412]; voles:[413]; humans: [414]). These differences are, in part, likely due to differences in brain function that were organized during early life by sex steroids [415–417]. Future studies should start by looking at the relationships between temperament and physiology in female laboratory animals. If scientists are worried about the estrus cycle, and changing sex steroids during this cycle, other females that have facultative reproductive physiology can be used. For example, in prairie voles, females are induced

ovulators and do not display ovarian activation but are induced into estrus by pheromonal exposure from a novel, conspecific male [418]. These animals have been used in previous studies to understand the relationship between oxytocin, social isolation and aging, which has been used to model human processes [401]. However, it is still important to know how other physiological systems may change during the estrus cycle due to changes in sex steroids and will be invaluable in determining the consistency in temperaments across a normal reproductive cycle. Work in females provides further evidence to determine the physiological mechanisms that influence or are associated with temperament.

Characterizing species differences

While rat models are frequently used in biomedical and psychology research, future studies should consider other species to characterize potential differences in the relationships between temperament and physiological systems. This would provide insight into temperament-specific individual variability in survival, reproductive success and overall fitness. In a recent paper that I wrote with Dr. Sonia Cavigelli, we identified the gaps in studying temperament and physiology in ecology [101]. We discussed model species and understudied taxa in relation to temperament and physiology.

Model species that are frequently used include great tits, three-spined sticklebacks, rhesus macaques and laboratory rodents. However, even within these model species there is still much that is unknown. For example, within great tits, there are many studies on the relationship between boldness, exploration and GC production, but few have considered other physiological mechanisms such as immune function, metabolic rate, or other hormones. Within rodent models, rats have predominantly been used to study temperament and physiology and mouse models have been used to study specific

behaviors and physiology. It is more cost-effective to create unique genetic lines within mouse models as compared to rats, so future studies should consider incorporating more mouse models to be able to study the genetic underpinnings of temperaments and physiological systems. However, three-spined sticklebacks and rhesus macaques have been used to model human temperament and physiological underpinnings for a much longer time and now more manipulation studies are being conducted to determine causal relationships between temperament and physiology.

We also identified specific taxa that future studies should incorporate into their research. Reptiles and amphibians are the least studied taxa in relation to temperament and physiology. This would be an important taxa to build on to better understand differing physiology from mammals in particular. Birds are another understudied taxa when considering the relationship between temperament and physiology. There are many immunology studies in different bird species and some species have been highly studied in relation to behavior, but it appears that there is not a lot of cross over between these two areas. With bird physiology changing each season and incorporating in migration patterns, future studies would benefit from understanding why individual differences occur within populations or flocks and how seasonality can affect temperament and physiology. Insects and arachnids are another understudied taxa. We see studies on sociality and boldness within this taxa, but very few studies on the relationships of temperaments to physiology.

Conclusion: One of the main questions ecologists ask is why we see variations within populations. Why do individuals have different fitness outcomes, yet within a population they usually have similar experiences? To understand this variation, we can study the

consistent behavioral differences observed in a population (i.e. temperament). While studying temperament is the first step in identifying and categorizing the variation observed, this doesn't completely explain how these differences originate. Incorporating physiology can help scientists better understand this variation. I created physiological profiles of temperaments observed in Sprague-Dawley rats to better understand how physiology and temperament interact. From this, I manipulated one system to better understand how these systems interact and if they can change the behavioral traits observed. This dissertation provides a broader perspective on different physiological systems associated with temperament and provides essential information necessary to understand the functional significance of temperament. The research conducted provides key methodologies and a foundation for future studies to conduct cross-disciplinary research on the physiology and behavior that drive differences in survival, fitness, and health.

Appendix

Supplemental Table 1.1 and Supplemental References for Chapter 1 can be found at this link: https://royalsocietypublishing.org/doi/suppl/10.1098/rspb.2021.2379

Supplementary Table 2.1. Exploratory factor analysis matrix for the three rounds of the Novel Social Test. The factor structure was subject to Varimax rotation with Kaiser Normalization due to the relatively small sample size. Each factor shows the percent of variance explained by that factor. To interpret factors, we only considered those behaviors that had loadings of greater than 0.30.

	Round 1				Round 2				Round 3			
Factor:	1(30%)	2(22%)	3(15%)		Factor:	1(34%)	2(25%)	3(15%)	Factor:	1(40%)	2(18%)	3(16%)
NS1sApp	0.888				NS2sApp	0.934			NS2sApp	0.65		
NS1sTime	0.822				NS2sTime	0.603	-0.534		NS2sTime	0.703		
NS1eApp			0.584		NS2eApp			0.904	NS2eApp		0.776	
NS1eTime			0.83		NS2eTime			0.634	NS2eTime		0.948	
NS1pEnt		0.747			NS2pEnt	0.377	0.759		NS2pEnt			0.677
NS1pTime	-0.625	0.344	-0.51		NS2pTime	-0.455	0.613		NS2pTime	-0.579		0.302
NS1pDist		0.883			NS2pDist		0.741		NS2pDist			0.729
NS1cEnt					NS2cEnt	0.901			NS2cEnt	0.805		
NS1cTime					NS2cDist	0.724		0.326	NS2cDist	0.805		
NS1cDist	0.664	0.576			NS2cTime	0.311			NS2cTime	0.712		

Supplementary Table 2.2. Exploratory factor analysis matrix for the three rounds of the Novel Physical Test. The factor structure was subject to Varimax rotation with Kaiser Normalization due to the relatively small sample size. Each factor shows the percent of variance explained by that factor. To interpret factors, we only considered those behaviors that had loadings of greater than 0.30. At Round 3, the factor analysis only converged with two factors.

	Round 1				Round 2				Round 3		
Factor	1(31%)	2(18%)	3(15%)		Factor	1(37%)	2(21%)	3(11%)	Factor	1(46%)	2(19%)
NP1oApp		0.302	0.457		NP2oApp			0.61	NP3oApp	0.773	0.384
NP1oTime			0.742		NP2oTime				NP3oTime	0.529	0.313
NP1pEnt		0.387			NP2pEnt		0.53	0.52	NP13pEnt		0.678
NP1pTime		0.549	-0.549		NP2pTime		0.919		NP3pTime	-0.754	
NP1pLat					NP2pLat		-0.644		NP3pLat		-0.523
NP1pDist		0.972			NP2pDist		0.589	0.622	NP3pDist		0.726
NP2cEnt	0.892				NP2cEnt	0.749		0.406	NP3cEnt	0.838	
NP1cTime	0.594				NP2cTime	0.775			NP3cTime	0.762	
NP1cLat	-0.59				NP2cLat	-0.392		-0.548	NP3cLat	-0.691	
NP1cDist	0.877				NP2cDist	0.887		0.383	NP3cDist	0.835	

Supplementary Table 2.3. Exploratory factor analysis matrix for the three rounds of the Partner Preference Test. The factor structure was subject to Varimax rotation with Kaiser Normalization due to the relatively small sample size. Each factor shows the percent of variance explained by that factor. To interpret factors, we only considered those behaviors that had loadings of greater than 0.30.

Round 1				Round 2				Round 3			
Factor:	1(36%)	2(17%)	3(15%)	Factor:	1(43%)	2(23%)	3(12%)	Factor:	1(51%)	2(16%)	3(13%)
PP1Dist	0.457			PP2Dist		0.348	0.843	PP3Dist	0.546	-0.488	0.427
PP1fApp	0.767		0.467	PP2fApp	0.928			PP3fApp	0.88		
PP1fTime	0.805			PP2fTime	0.884			PP3fTime	0.875		
PP1fLat	-0.601			PP2fLat	-0.616		-0.436	PP3fLat	-0.608	0.31	
PP1cEnt			0.801	PP2cEnt			0.896	PP3cEnt	0.416	-0.528	
PP1cTime	-0.700	-0.335		PP2cTime	-0.694	-0.42		PP3cTime	-0.536	0.469	-0.566
PP1cLat			-0.764	PP2cLat	-0.331			PP3cLat		0.846	
PP1uApp		0.858		PP2uApp		0.868		PP3uApp			0.879
PP1uTime		0.866		PP2uTime		0.984		PP3uTime			0.785
PP1uLat		-0.325		PP2uLat		-0.543	-0.459	PP3uLat		0.872	

Supplementary Table 2.4. Exploratory factor analysis matrix for the three rounds of the Social Interaction Test. The factor structure was subject to Varimax rotation with Kaiser Normalization due to the relatively small sample size. Each factor shows the percent of variance explained by that factor. To interpret factors, we only considered those behaviors that had loadings of greater than 0.30.

Round 1					Round 2					Round 3				
	1	2	3	4		1	2	3	4		1	2	3	4
Factor:	(31%)	(21%)	(13%)	(9%)	Factor:	(39%)	(19%)	(14%)	(9%)	Factor:	(29%)	(21%)	(16%)	(9%)
SI1Dist				0.893	SI2sDist		0.58			SI3sDist			0.736	
SI1TT	0.606	0.437		0.302	SI2sTT	0.743				SI3sTT	0.366	0.833		
SI1AS	-0.32				SI2sAS				0.855	SI3sAS	-0.406	0.66	-0.493	
SI1Pinn	0.592				SI2sPinn	0.835				SI3sPinn	0.697	0.707		
SI1Fight	0.711		0.571		SI2sFight	0.896				SI3sFight	0.54			
SI1F		0.467	-0.45	0.327	SI2sF			0.96		SI3sF				
SI1Rear	0.693				SI2sRear	0.775				SI3sRear	0.732			
SI1Mount		0.966			SI2sMount	0.541				SI3sMount	0.585			0.689
SI1RA			0.781		SI2sRA		0.873			SI3sRA			0.35	-0.39

Supplementary Table 2.5. Exploratory factor analysis matrix for the three rounds of the Resident Intruder Test. The factor structure was subject to Varimax rotation with Kaiser Normalization due to the relatively small sample size. Each factor shows the percent of variance explained by that factor. To interpret factors, we only considered those behaviors that had loadings of greater than 0.30.

Round 1			Round 2			Round 3		
Factor:	1(30%)	2(21%)	Factor:	1(33%)	2(24%)	Factor:	1(40%)	2(18%)
RI1Lat		0.303	RI2Lat	-0.654		RI3Lat		-0.604
RI1Mov	-0.477		RI2Mov		-0.568	RI3Mov	0.937	
RI1Attack		-0.981	RI2Attack	0.673		RI3Attack	0.492	0.831
RI1BeAttack	0.668		RI2BeAttack	0.892		RI3BeAttack	0.655	
RI1Lay	0.72		RI2Lay	0.527	0.428	RI3Lay	0.494	
RI1Sniff			RI2Sniff		-0.5	RI3Sniff		
RI1AnoGen		0.424	RI2AnoGen			RI3AnoGen	0.431	-0.335
RI1Freeze	0.602	0.359	RI2Freeze		0.94	RI3Freeze	0.591	

Supplementary Table 3.1. Exploratory factor analysis matrix for the Physiological Systems measured. The factor structure was subject to Varimax rotation with Kaiser Normalization due to the relatively small sample size. Each factor shows the percent of variance explained by that factor. To interpret factors, we only considered those behaviors that had loadings of greater than 0.30. The factor structure would only converge with six factors.

Factor:	1 (22%)	2 (17.5%)	3 (13.9%)	4 (11.5%)	5 (10.1%)	6 (8.8%)
Relative Hindfoot Swelling					0.619	
KLH GC levels (AUCg)					0.706	
LPS GC levels (AUCg)						0.577
Sacrifice GC levels	0.810					
Mean Basal GC levels	0.803					
IL6 (AUCi)				0.708		
TNFα (AUCi)				0.666		-0.565
IL10 (AUCi)			0.696			
Faith Phylogeny Diversity		0.303	0.698			
Pielou's Evenness		0.740				
Shannon Diversity Index		0.809	0.397			

Supplemental Data 3.1. Physiological network analysis:

Previous studies have conducted some analyses on the connection between multiple physiological systems. For example, reviews have been written on the connection between the HPA axis, gut microbiome and immune function or the immune function and HPA axis or any variation thereof (i.e. [96,261,271,324,325,419]). However, to date there are few if any studies that have tested/analyzed multiple physiological systems without any manipulation. I conducted further analyses from the first project (which uses data from Chapter 3) to determine the correlations between different physiological systems. From this, I have found that all systems are equally positively or negatively associated with each other (*Supplemental Figure 3.1*).

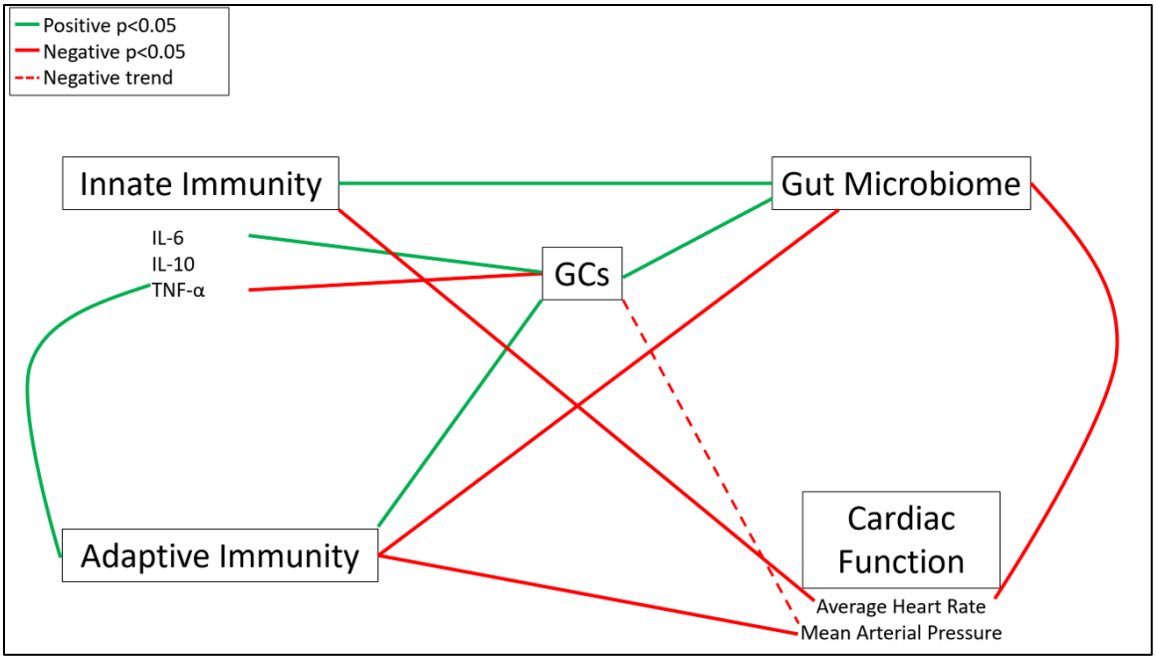
Glucocorticoids: Glucocorticoids (GCs) were positively correlated with gut microbiome alpha diversity (R=0.34 , $p<0.05$), cell-mediated immunity (R=0.54, $p<0.05$) and IL6 cytokine production (R=0.29, $p<0.05$). However, GCs were negatively correlated with TNFα (R=-0.32, $p<0.05$) and trending toward a negative correlation with stress-induced mean arterial pressure (R=-0.26 , $p<0.1$).

Innate Immunity: All cytokines were positively correlated with gut microbiome alpha diversity (Rs=0.29(IL6), 0.37 (TNFα), 0.35(IL10), $p<0.05$) and negatively correlated with average heart rate (Rs=-0.43(IL6), -0.33 (TNFα), -0.36 (IL10), $p<0.05$). During the innate immune challenge, IL6 was positively correlated with GC production (R=0.29, $p<0.05$) while TNFα was negatively correlated with GC production (R=-0.32, $p<0.05$). TNFα production was also positively correlated with the Cell-Mediated Immune Response (R=0.29, $p<0.05$). IL10 was not independently correlated with any of the other physiological functions.

Adaptive Immunity: Cell-mediated immune function was positively correlated with GC production during the adaptive immune challenge (R= 0.54, $p<0.05$) and positively correlated with TNFα (R=0.29, $p<0.05$). This adaptive immune measure was negatively correlated with gut microbiome alpha diversity (R= -0.32, $p<0.05$) and basal mean arterial pressure (R=-0.34 , $p<0.05$).

Gut Microbiome: The gut microbiome alpha diversity was positively correlated with the innate immune cytokine production (Rs=0.29(IL6), 0.37 (TNFα), 0.35(IL10), $p<0.05$) and GC concentrations during the acute restraint stress (R=0.34 , $p<0.05$). However, gut microbiome alpha diversity was negatively correlated with the cell-mediated immune response (R= -0.32, $p<0.05$) and basal average heart rate (R=-0.40, $p<0.05$).

Cardiac Function: Two metrics were used to measure cardiac function: average heart rate and average mean arterial pressure. Average heart rate was negatively correlated with all innate immune cytokines (Rs=-0.43(IL6), -0.33 (TNFα), -0.36 (IL10), $p<0.05$) and fecal gut microbiome alpha diversity (R=-0.40, $p<0.05$). Mean arterial pressure was negatively correlated with cell-mediated immune function (R=-0.34 , $p<0.05$) and trending toward a negative correlation with acute stress induced GCs (R=-0.26 , $p<0.1$).



Supplemental Figure 3.1. Correlation Analysis of all physiological systems that were measured in Chapter 3. Solid red lines indicate significant negative correlations ($p<0.05$). Solid green lines indicate significant positive correlations ($p<0.05$). Dashed red lines indicate a trend toward a negative correlation ($p<0.10$).

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