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Research paper

## Behavioral phenotype relates to physiological differences in immunological and stress responsiveness in reactive and proactive birds

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## ABSTRACT

It has now been demonstrated in many species that individuals display substantial variation in coping styles, generally separating into two major behavioral phenotypes that appear to be linked to the degree of physiological stress responsiveness. Laying hens are perfect examples of these dichotomous phenotypes; white laying hens are reactive, flighty, and exhibit large hormonal and behavioral responses to both acute and chronic stress, while brown laying hens are proactive, exploratory, and exhibit low hormonal and behavioral responses to stress. Given the linkages between stress physiology and many other body systems, we hypothesized that behavioral phenotype would correspond to additional physiological responses beyond the stress response, in this case, immunological responses. Because corticosterone is widely known to be immunosuppressive, we predicted that the reactive white hens would show more dampened immune responses than the proactive brown hens due to their exposure to higher levels of corticosterone throughout life. To assess immune function in white and brown hens, we compared febrile responses, corticosterone elevations, feed consumption, and egg production that occurred in response to an injection of lipopolysaccharide (LPS) or saline, inflammatory responses to phytohemagglutinin (PHA) injection in the toe web, innate phagocytic activity in whole blood, and antibody responses to an injection of Sheep Red Blood Cells (SRBCs). Contrary to our predictions, white hens had significantly greater swelling of the toe web in response to PHA and showed a greater inhibition of feeding and reproductive output in response to LPS. These results indicated that reactive individuals are more reactive in both stress and immunological responsiveness.

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## 1. Introduction

Animals of the same species can often be divided into behavioral phenotypes that appear to suit them for life in different environments. These distinct phenotypes are characterized by consistent variation in aggressive, fear, and exploratory behaviors, and also by differences in physiological responses to stress (Baugh et al., 2013, 2012; Carere et al., 2003, 2010; Cockrem, 2012; Fraisse and Cockrem, 2006; Groothuis and Carere, 2005). Scientists now define these behavioral phenotypes using the terms “reactive” and “proactive” (Koolhaas et al., 1999; Groothuis and Carere, 2005). Proactive animals tend to have a bold and fast response to novel environments or stimuli (i.e., they are quick explorers, less fearful, and more aggressive) and produce smaller physiological responses (e.g. corticosterone elevations) to acute (Carere et al., 2010; Cockrem, 2007) and chronic stressors (Pusch et al., 2017).

Reactive animals tend to have a slow and shy response (i.e., they are cautious explorers, more fearful, and more passive) and produce stronger physiological responses to acute (Carere et al., 2010; Fraisse and Cockrem, 2006) and chronic stressors (Pusch et al., 2017).

While studies characterizing behavioral phenotypes have focused on the responses of animals to stressful stimuli, the differences in the activity of the hypothalamic-pituitary-adrenal axis between these behavioral groups may impact many downstream physiological and behavioral processes including foraging, metabolism, and immunity (Baugh et al., 2012; Carere et al., 2010, 2003; Cockrem, 2007; Groothuis and Carere, 2005, Koolhaas, 2008; Koolhaas et al., 2010). These reactions are integral to an organism's ability to respond to a stressor, and to return to, and maintain, homeostasis. Indeed, other physiological responses have been shown to co-vary with behavioral phenotype (Huff et al., 2013; Koolhaas, 2008; Koolhaas et al., 1999; Korte et al., 1996; van Oers et al., 2011). For example, mice with short attack latencies (SAL) had low plasma corticosterone levels and greater levels of serotonin receptor mRNA and serotonin receptor binding sites in

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the hippocampus than mice with long attack latencies (LAL) (Korte et al., 1996), and differences in body temperature and breathing rate have been shown in great tits with different behavioral phenotypes (Carere and van Oers, 2004). Work in great tits suggests that regulation of testosterone production may also either contribute to or result from the divergent characteristics of behavioral phenotypes (van Oers et al., 2011).

Of particular relevance to the current paper are the documented relationships between behavioral phenotype and immunological responsiveness. It is now relatively well-known that stress and the resulting production of glucocorticoids can depress immunological function (e.g. Saino et al., 2003, Yang et al., 2015, reviewed in Capitanio, 2011). It has thus been predicted that reactive individuals, which react more highly to stress, would have depressed immunological responses compared to proactive individuals (Capitanio, 2011). This idea has been tested in some mammalian and avian species. Hessing et al. (1995) compared the responses of proactive and reactive pigs to innate and adaptive immune challenges and found that proactive pigs had higher innate immune responses but lower adaptive immune responses when compared with reactive pigs (Hessing et al., 1995), though a second study on pigs showed no relationship (Geverink et al., 2004). In another study, reactive and proactive mice (termed passive and active) given a stress challenge showed differential production of pro-inflammatory cytokines in the hypothalamus and spleen; proactive/active mice had higher levels of interleukin 1- $\beta$  in both hypothalamus and spleen, and reactive/passive mice had higher levels of IL-2 in the spleen compared with proactive/active mice (De Miguel et al., 2011). These cytokines were not measured in response to a challenge, however, so it is difficult to predict how this relates to differences in susceptibility to disease. There is now a substantial body of work in primates examining the idea that behavioral phenotypes predict immunological responsiveness (reviewed by Capitanio, 2011). For example, rhesus monkeys that were highly social had the higher antibody responses compared to conspecifics that were less social, and similarly, in cynomolgus monkeys, highly affiliative behavior was predictive of a better immune response. In pigtailed and bonnet macaques, individuals that produced more distress signals (and thus were more reactive), had lower proliferative responses to conovalin A (Con-A) and phytohemagglutinin (PHA).

The relationship between behavioral phenotype and immune function has also been studied in birds, however the number of studies are few and the results do not appear to be consistent. In a study of greenfinches, researchers used calmness in captivity, as indicated by the extent of tail damage that theoretically resulted from attempts to escape the cage, as an indicator of behavioral phenotype. Individuals with less tail damage were considered calmer, and more proactive, while more tail damage was indicative of a reactive individual (Sild et al., 2011). Based on the prediction outlined above as well as the results described in mammals, we would expect that the calmest individuals would have more robust immune responses, and this is, in fact, what was found. Calmer individuals had higher antibody responses, and this study also showed that the same birds had higher oxidative burst responses as well (Sild et al., 2011). The same metric (tail damage), along with the frequency of distress vocalizations, was used in a study of great tits as well, and similar results emerged; calmer (proactive) individuals had lower heterophil:lymphocyte ratios (indicative of lower stress levels) and higher antibody responses than less calm (reactive) individuals (Krams et al., 2013). However a separate study using a different metric of behavioral phenotype showed the opposite results. In great tits, exploratory individuals are considered proactive while less exploratory individuals are considered reactive (Carere et al., 2005). Based on the work cited above in mammals, we would predict, then, that more exploratory

tits would have a more robust immune response, but instead, those more exploratory individuals produced *smaller* swelling responses to PHA compared to less exploratory individuals (van Oers et al., 2011), and a recent study showed in superb fairy wrens that more proactive individuals had *lower* antibody titers than more reactive individuals (Jacques-Hamilton et al., 2017). Putting these together, the studies thus far indicate that behavioral phenotype may correlate with immune responsiveness, but there does not appear to be consistency in direction in which the strength of the innate and adaptive arms of the immune system differ between reactive and proactive individuals.

Overall, work in mammals, and two studies (one in great tits and one in greenfinches) support the prediction that reactive individuals would exhibit depressed immune responses compared to proactive individuals, but the work in wild Great tits and superb fairy wrens does not. The reason for this variation may relate to the metric used to assess behavioral phenotype, though the categorization of great tits and slow versus fast explorers, and the suite of behavioral and physiological characteristics of these groups (including stress reactivity), have been well characterized, and seem to fit very well into the reactive:proactive model of behavioral phenotyping. Thus more studies are needed to examine the relationships between behavioral phenotypes and immunological responsiveness in birds. It is also important to measure multiple arms of the immune system, as we might not expect each component of the immune system to relate similarly to behavioral phenotype. For example, while stress has been linked to inhibited functioning of T- and B-lymphocytes (Saino et al., 2003; Yang et al., 2015), the story is likely much more complex, as chronic stress has been shown to alter cytokine balance from type 1 to type 2 driven responses (Dhabhar, 2009). This means that while one arm of the immune system may be depressed under stressful conditions, another arm may thrive. Indeed, in pigs, active and resistant (i.e. proactive) individuals had higher cell mediated responses while reactive individuals had higher humoral responses (Hessing et al., 1995).

For this study, we examined the relationship between behavioral phenotype and immunity in two strains of captive laying hens known to exhibit marked personality differences (*Gallus gallus*). Despite being the same species, brown laying hens are proactive and have a reduced physiological reaction to acute and chronic stress, while white hens are reactive and have a heightened physiological reaction to acute and chronic stress (Fraisse and Cockrem, 2006; Pusch et al., 2017). To examine differences in immune function between strains, we conducted immunological assessments: (1) a phytohemagglutinin (PHA) skin swelling challenge, a common procedure to elicit and test T-cell proliferation (Feder and Hofmann, 1999), (2) a challenge with lipopolysaccharide (LPS), a bacterial endotoxin that stimulates a febrile response, anorexia, and sickness behavior for a short time period (Johnson et al., 1993), (3) a carbon clearance assay to measure phagocytosis *in vitro* (Spinu et al., 1999), and (4) a sheep red blood cell challenge (SRBC) to assess antibody responsiveness (Onbaşilar and Aksoy, 2005). We predicted the reactive white hens would show more dampened immune responses compared to the proactive brown hens due to their exposure to higher levels of corticosterone during the daily stresses experienced throughout their lives.

## 2. Materials and methods

### 2.1. Animals

This work was conducted using two flocks of female Hy-line brown and Hy-line white W-36 hens ( $n = 30$  per strain for the first two flocks and 20 per strain for the second). Birds were reared in

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