ABSTRACT

Title of Thesis: **PERSONALITY TRAITS IN THE BUDGERIGAR *(Melopsittacus undulatus)***

Taylor Callicrate, Master of Science, 2008

Thesis Directed By: Professor Inmaculada Estevez
Department of Animal and Avian Sciences

This study investigated bold-shy personality in the budgerigar, *Melopsittacus undulatus*. Adult budgerigars (14 females, 9 males) fed either a control diet, or one supplemented with docosahexaenoic acid (DHA), were subjected to seven behavioral tests and two immunocompetence assays. Behavioral responses were categorized by context: fear, feeding, or activity. Correlations were obtained within contexts and among immunocompetence variables and all behavioral variables. Kruskal-Wallis analysis was used to investigate effects of gender and DHA on all variables. Budgerigars behaved consistently within activity and feeding contexts. Males had higher feeding rates, and their feeding responses were negatively correlated with a measure of innate immunity. Cluster analysis characterized birds by activity levels; bold birds were highly active and shy birds were less active. The results of this study suggest that budgerigars exhibit consistent behaviors in two contexts, feeding and activity, which are unrelated to each other, and that activity is the predominant personality trait.

PERSONALITY TRAITS IN THE BUDGERIGAR *(Melopsittacus undulatus)*

By

Taylor Eilers Callicrate

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Advisory Committee:
Professor Inmaculada Estevez, Chair
Assistant Professor Frank Siewerdt
Dr. Liz Koutsos

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Chapter 1 LITERATURE REVIEW

1.1 Introduction to animal personality

Behavioral studies have traditionally focused on differences between populations, groups, or treatments (Groothuis & Carere, 2005). A more recent trend is to examine variation in personalities which are defined as consistent individual differences across a diversity of situations (Sih et al., 2004; Bell, 2007a; Bell, 2007c; Carere & Eens, 2005; Carere et al., 2005; Groothuis & Carere, 2005).

The question of animal personalities has not been given much attention until recently, perhaps due to reluctance of scientists to use the terminology and/or methodology of human behavioral research. It may also be attributable to a lack of understanding of the importance of personalities in animals (Groothuis & Carere, 2005; Réale et al., 2007; Smith & Blumstein, 2008) and how it may affect their life history. However, the existence of personalities may help to explain the maintenance of variation in behavioral traits, diversity of behavioral strategies and also the occurrence of non-adaptive behaviors (Sih et al., 2004; Sih & Johnson, 2005; Réale et al., 2007; Smith & Blumstein, 2008). Furthermore, the study of differences in animal personality may be useful in understanding ecological aspects such as species dispersal[1], invasiveness, distribution, or response to environmental change (Dingemanse et al., 2003; Sih et al.,

[1] Dingemanse et al. (2003) found that there was a positive correlation between parental exploration score and offspring dispersal in free-living great tits. This effect was corroborated by their finding that birds who migrate to an area were faster explorers than native-born birds.

2004), and more importantly can provide an insight into the question of why individuals may deviate from the 'optimal' (Bell, 2007b). Individual variation in specific behaviors is often non-random, indicating that individual differences or personalities may be acted upon by natural selection (Dall et al., 2004; Carere & Eens, 2005).

Traditionally, behaviors have been studied based on categories, such as reproduction, predator avoidance, or foraging, since selection should favor independent optimal behavior in each context (Bell, 2007b). Behaviors in each category have been treated as independent, and individual differences have been examined within those categories (Wilson et al., 1994; Bell, 2007b). If individuals actually differ in a consistent manner across contexts (for example, if an individual is always highly active), or if suites of behavior are correlated within a situation (such as activity and aggression during the feeding context), it may be more appropriate to study the behaviors together (Groothuis & Carere, 2005). Similarly, behaviors that evolved "as a package" should be studied together as behaviors occurring in one context might be related to or dependent upon behaviors in another (Carere & Eens, 2005). Negative behavioral associations or correlations require equal consideration, as they might be indicative of adaptive strategies for risky behavior (Sih et al., 2004).

Differences in personalities have been implicated as being related to fitness tradeoffs, although it is unclear as to whether such tradeoffs are the result of the influence of personality, or if life history tradeoffs instigate the development of personality. Either way, personality could be a key component to understanding life history patterns (Boon et al., 2008) and phenomena such as dispersion and invasiveness (Dingemanse et al., 2003).

The effects of personality differences on adaptability are especially relevant in areas subject to a high degree of environmental change, such as places under heavy human influence (Sih et al., 2004). The limited plasticity – consistent behavioral responses across contexts - which can result from personality traits may affect how animals will cope with change (Drent et al., 2002). On the other hand, species which harbor greater variation in their personality types or a greater range of flexibility may thrive in human-influenced areas. Additionally, personality has been shown to be related to fitness[2] (reviewed in (Dingemanse & Reale, 2005) as it may lead, for example, to differential predation risk for the members of a group (Quinn & Cresswell, 2005).

The reactions of individuals of differing behavioral profiles should be carefully considered in animal experiments, as personality type may affect the way animals respond (Groothuis & Carere, 2005) and cope with the environment. This includes experiments in natural and artificial conditions (Carere & Eens, 2005). For example, aggressive fish in commercial aquaculture conditions may be better able to compete for resources, and consequently grow faster than less aggressive fish (Huntingford & Adams, 2005). Differences in personality may lead to varying degrees of susceptibility to stressors in captivity, which has implications for animal welfare (Carere & Eens, 2005; Groothuis & Carere, 2005).

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[2] In great tits, for example, individual birds have different optima under different environmental conditions Dingemanse, N. J., Both, C., Drent, *P*. J. & Tinbergen, J. M. 2004. Fitness consequences of avian personalities in a fluctuating environment. *Proceedings of the Royal Society of London,* **271,** 847-852.)

Personality offers a new paradigm for studying animal behavior in a way that takes into account individual strategies and relationships between behaviors. It could potentially be important for ecological as well as welfare issues.

1.2 Personality terminology

Despite the ubiquitous discussions on defining animal personality (also referred to as behavioral syndromes, temperament, and coping style, [(Gosling, 2001)]), there is not yet a consensus in the literature as to which definition is most adequate.

Groothuis and Carere (2005) have produced both a basic definition, describing personality as consistent individual differences in behavior across a variety of contexts or within one context across a variety of situations, and a more complex definition, which gives details about the nature of personality: (1) individuals' differences in behavior should be consistent over time; (2) differences in the same behavior(s) between individuals should be consistent across different scenarios (contexts); (3) relationships between behaviors should not vary depending on the situation; (4) there should be an objective, ideally quantitative, way to measure all behaviors in question (Groothuis & Carere, 2005).

Carere and Eens (2005) describe personality as suites of correlated behaviors that are expressed across different situations and suggested that these consistent behavioral features endow individuals with discernable predispositions. Similarly, Bell (2007a) has proposed that personality, which she refers to as a 'behavioral syndrome', is defined by a correlation between individuals' rank-order differences in behavior through time or across situations, which is equivalent to Carere and Eens' (2005) definition. Bell (2007a)

also defines personality with respect to an individual (rather than personality in general) as being an individual's specific configuration of behaviors.

According to Smith (2008), the term 'personality' can be defined by three different situations: (1) correlated behaviors within a given context (this has been demonstrated for dumpling squid (*Euprymna tasmanica*), which show correlated behaviors within, but not between, contexts of feeding and predator threat [(Sinn & Moltschaniwskyj, 2005)]); (2) correlated behaviors in different contexts within the same situation (for example, heightened aggression when there is a conspecific present, regardless of the situation); (3) correlated behaviors across contexts and situations (heightened aggressiveness or boldness during contexts of feeding, parenting, and mating in all situations- various levels of predator threat, group size, season, etc.). Generally, situation (2) is uncommon and remains un-discussed; most personality studies fit under categories (1) and (3) (Smith & Blumstein, 2008).

Finally, some authors also make a distinction between *situations* and *contexts*. Contexts are broader categories of behavior, such as mating, feeding, antipredator, parental care, or dispersal, whereas situations are specific instances within a context, such as feeding alone, feeding socially, and feeding in the presence of a predator (Sih et al., 2004). Many authors use 'context' and 'situation' interchangeably, and in those cases they are usually defining personality as correlated behaviors across contexts. However, personality can also manifest as correlated behaviors within a context across situations.

For the purposes of this study, we will be working under the definition that personality is evidenced by individuals showing correlated behaviors within or between contexts.

1.3 Evolution and Behavioral Syndromes

If behavioral syndromes are adaptive, then differences may occur between populations as result of environmental differences or ecological constraints indicating that selection can act to change behavioral syndromes (Carere & Eens, 2005). For example, differences exist in the degree of boldness of juvenile poeciliid fish (*Brachyraphis episcope*), as measured by emergence from a shelter and exploration of a novel environment, depending on whether the fish were from a downstream or upstream site. The two sites differed in predation risk to the fish, indicating that degree of boldness was adjusted as result of the differences in the environment (Brown & Braithwaite, 2004). The concept that individuals with specific personalities perform better under certain conditions may help to explain the maintenance of variation in animal behavior (Dingemanse et al., 2004; Brydges et al., 2008).

A negative side effect of animal personalities is that correlation between behaviors across contexts or situations can limit optimization of behavior (Dall et al., 2004; Groothuis & Carere, 2005; Bell, 2007a). Although it may be ideal for a set of behaviors to be correlated in one context, the same association may be disadvantageous in another, potentially having negative effects on fitness. For example, in a study of anti-predation behavior in chaffinches (*Fringilla coelebs*), hypoactive chaffinches (in good physical condition) froze when there was a high-risk predator threat, as opposed to an

active escape response (Quinn & Cresswell, 2005). This response appears to be due to a lack of plasticity[3]. In some cases behaviors are correlated within, but not between contexts. A good example is the dumpling squid, which shows correlated behaviors within feeding context and predator threat context, but not between the two, alleviating the potential negative effects of limited plasticity related to personality (Sneddon, 2003).

If personality can have such a high cost to animals in terms of fitness due to lack of plasticity, why is it maintained? One hypothesis is that an individual's behavioral syndrome may change slowly, preventing behavioral variation across contexts (Sih et al., 2004). Changing an individual's level of aggressiveness, for example, might require the individual to "entirely rewire neural machinery" (Bell, 2007a). Also, multiple behaviors might be correlated because of genetic mechanisms such as pleiotropy (van Oers et al., 2005), and changing that association might be impossible (reviewed in Sih 2004, van Oers (2005). When there is little information available about the specifics of the environment (for example, rates of predator attack are unknown), it may be advantageous to behave in a consistent manner since it would be difficult, and highly costly, for an animal to predict and make the needed behavioral changes for each scenario (DeWitt et al., 1998; McElreath & Strimling, 2006). Although it may be ideal for an animal to be

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[3] Incidentally, the authors present an intriguing alternative explanation for the hypoactive birds freezing. If the hypoactive birds are reactive to their environment (relying on external signals as opposed to being proactive and relying on internal signals), then they may have assessed that escape from the cage was impossible and elected to freeze even though they were apparently intended victims of the hawk Quinn, J. L. & Cresswell, W. 2005. Personality, anti-predation behaviour and behavioural plasticity in the chaffinch *Fringilla coelebs*. *Behaviour*, **142,** 1377-1402.

able to always adapt to changes in the environment, maintaining a constant strategy is one way to compensate for highly unpredictable situations. Reduced plasticity may also be advantageous as it would limit effort spent on evaluating and changing strategies, especially if it is difficult to predict the best strategy.

1.4 What maintains personality variation?

Theoretically, there is an optimal behavioral response to any given context which should lead to a loss of variation in heritable traits which are acted on by selection. Personality traits defy this idea, as they are heritable, selectable, and may be highly variable within a population (Wolf et al., 2007). Variation in personality may be maintained because optimality of each strategy depends on the frequency of existing phenotypes in the population (Wilson et al., 1994). Personalities could develop from frequency-dependent selection if a proportion of individuals performs each strategy, rather than individuals randomly performing strategies at fixed probabilities. Alternatively, personality variation in a population may be maintained as individuals adapt their strategy according to their own or others' conditions (Punzalan et al., 2005; Sinervo & Calsbeek, 2006).

The physical state and condition may limit the behavioral options of an individual and can be affected by the individual's previous behavioral choices. In a recent study great tits (*Parus major*) were used to test the stability of individual differences and behavioral profiles in a population over time. Birds from the third and fourth generations of two selected lines were tested both as juveniles and as adults over a two to three year

time span. The authors found that scores for two types of exploratory test and a composite exploratory differed between fast and slow birds, both as juveniles and as adults. Juvenile and adult scores were correlated for fast birds, but slow birds showed no correlation at all between their juvenile and adult scores. Slow birds became significantly faster as adults, whereas fast birds had greater personality stability than slow birds. Personality at the individual level was much less consistent, with the exception that the level of aggressive behavior was maintained over time (Groothuis & Carere, 2005).

Life history strategies based on fitness tradeoffs have also been proposed as a mechanism for maintaining individual variation (Clark, 1994; Stamps, 2007; Wolf et al., 2007). For example, two opposing strategies may be undergoing early reproduction with the risk of limited resources vs. reproducing later, waiting for periods of better resource availability to insure survival (Wolf et al., 2007). Late reproducers would likely be more risk-averse (shy), whereas the earlier ones would take greater risks (bold), thereby maintaining individual differences due to the tradeoff in reproductive age. A good example of life history tradeoffs can be seen in the dumpling squid. For this species, it was found that individuals that were closer to the end of life and had a more urgent need to find mates and forage to enable reproduction were bolder, more likely to flee from predators, and less likely to abandon a feeding area if threatened by predators (Sneddon, 2003). However, it is important to note that the relationship between personality and life-history patterns is bidirectional. If an individual with an early-reproduction life history strategy develops a bold personality that allows it to acquire enough resources, it could potentially switch strategies and reproduce later, thus altering its behavioral strategy to shy/risk-averse (Bell, 2007a). By reproducing later, an animal would have the advantage

of having more resources both for itself and its offspring, possibly allowing a higher quality of offspring and higher probability of survival for itself.

Individual differences in behavioral strategies can also emerge from differences in growth patterns (fast or slow growth) (Stamps, 2007) or as result of changes in environmental conditions and subsequent changes in selection pressures (Dingemanse et al., 2004). Smaller poeciliid fish are more likely to emerge from a shelter and explore a novel environment (indicating a greater degree of boldness) as compared to larger individuals (Brown & Braithwaite, 2004); for wild great tits, the abundance of food in a particular year affects whether slow- or fast- exploring birds will have a survival advantage (Dingemanse et al., 2004).

1.5 Discovering the axes of personality

Factor analysis, principle components analysis (PCA), or discriminant function analysis can be used to identify axes of personality[4] (eg, bold-shy, aggressive-passive) based on the responses of individuals to various situations and contexts (Bell, 2007b; Réale et al., 2007). Each individual's personality is described by their placement on each axis. The procedure of testing and measuring responses is known as coding of behaviors (Gosling, 2001). The PCA approach was used in dumpling squid to reveal four components which explain over 75% of the individual variation: shy avoidance-bold aggression, activity, reactivity, and bury persistence (Sinn & Moltschaniwskyj, 2005).

[4] Axes of personality are ranges of personality types, such as bold to shy or aggressive to passive.

Various measures of behavior, such as tonic immobility duration, latency to feed after a simulated predator threat, and willingness to cross a barrier may be used for factor analysis, principle components analysis (PCA), or discriminant function analysis. Measures of behavior must be chosen on a species-specific basis to make sure they are appropriate for the species and testing facilities in question (Plusquellec et al., 2001; Van Reenen et al., 2004; De Palma et al., 2005; Muller & Schrader, 2005; Kilgour et al., 2006).

Although it is possible to identify a large number axes of personality using methods such as PCA, important personality axes are generally those that represent tradeoffs, span a variety of contexts, and tend to be stable over time. Dichotomous axes, those labeled with two extremes of behavior, often represent tradeoffs with important fitness consequences to each strategy and therefore have a high evolutive value. The **active-inactive axis** is especially important for prey animals. Individuals that are always highly active may obtain more food, but will also incur a higher risk of predation in the presence of predators (Sih, 1987). This axis may be a key trait that links behavior to feeding rate, metabolic expenditures, and predation risk (Sih et al., 2004).

The **aggressive-passive axis** refers to an individual's propensity to attack conspecifics or prey items (Sih et al., 2004). Individuals may change their level of aggressiveness depending on the situation (such as changes in group dynamics or resource availability; (Estevez et al., 2007), but some are consistently more aggressive than others (Sih et al., 2004). A classic example of an animal exhibiting an aggression axis is the funnel web spider (*Agelenopsis aperta*). Spiders classified as aggressive showed increased attack tendencies against other spiders and prey and exhibited reduced

latencies to recover from a simulated predator attack (Riechert & Hedrick, 1993). Frequencies of aggressive behavior in greyleg geese (*Anser anser*) in two feeding situations (high and low density of food in an experimental food patch) and during a low-density social situation have been found to be consistent Kralj-Fiser et al. (2007).

The **shy-bold axis** affects properties such as dispersion, mate attractiveness, and survival (Reale et al., 2000; Fraser et al., 2001; Dingemanse et al., 2003). Bold animals tend to be more active, are more likely to feed under high predation risk, and are more likely to inspect predators or novel objects; they also learn more quickly (Sneddon, 2003; Sih et al., 2004) (Frost et al., 2007). Although the shy-bold axis incorporates activity, it is broader in concept than the activity axis, since it take into account other behaviors. Bold rainbow trout, for example, were observed to master a feeding task more quickly than shy ones (Sneddon, 2003).

A more holistic personality type is the **proactive-reactive axis**, which takes into account a broader set of behavioral contexts: exploratory behaviors, aggression, fear, and response to the environment (Carere et al., 2005). Proactive individuals tend to dominate and outcompete reactive ones in a stable environment, whereas reactive ones appear to respond better to changing environments. To be classified as proactive, an individual must be bold and aggressive, highly active and exploratory. Contrarily, reactive individuals are sensitive to external stimuli and cautiously adjust to environmental change (Sih et al., 2004). Groothuis et al. (2005) indicated that that the proactive-reactive axis could be a fundamental descriptor in the organization of animal personalities.

1.6 Mechanisms of personality

Potential proximate mechanisms for personality variation are not well-understood outside of a few model species (Sih et al., 2004) such as mice and primates. However, it is apparent that genetics, individual experiences, and neuroendocrine factors can interact to govern personality development. For example, in *Drosophila melanogaster*, the *´for´* gene is known to affect flies' activity level during foraging by affecting the activity of a cGMP-dependent protein kinase (reviewed in Sih et al. 2004). Heritability of personality traits can be measured using standard quantitative genetic mechanisms (Sih et al., 2004). In great tits the realized heritability of early exploratory behavior has been quantified at 0.54 ± 0.05 (Drent et al., 2002), whereas broad-sense heritability (including both additive and non-additive effects) for exploration was estimated to be between 0.33 and 0.25 for free-living great tits (Drent et al., 2002). van Oers et al. (2005) suggested that heritability measured in captive populations should be interpreted with caution because heritability estimates are affected byenvironmental variation (van Oers et al., 2005).

Besides heritability, genetic correlations for behaviors have been quantified in mice (Sluyter et al., 1995; Bult & Lynch, 2000; Simoncic et al., 2008; Hill, 2000) and great tits (Groothuis & Carere, 2005). In great tits high risk-taking and exploratory behavior were found to be highly correlated at the genetic level. Evidence was found for pleiotropic effects, as well as additive and dominance effects, but not for sex-dependent expression. But environmental conditions also interacted with genetic factors. In an experimental setup with great tits, poor food availability resulted in chicks of the slow exploration line becoming faster explorers than their parents, whereas fast exploration

chicks became more aggressive than their parents. The authors concluded that gene-environment interaction could indeed uncouple correlated behaviors (Groothuis & Carere, 2005). This suggests that the early environment can serve as a mechanism to bring an individual's personality closer to optimality for given conditions (Groothuis & Carere, 2005).Unfortunately, further genetic information about personality is limited, especially in 'non-model' and free-living populations (Sih et al., 2004; van Oers et al., 2005). An especially important question for investigation is why some personality types are more abundant in certain environmental conditions than in others, highlighting the relevance of gene-environment interaction (Sih & Bell, 2007).

Similarly to the role of the environment, the individual's previous experiences may interact with genetics to influence an individual's personality (Frost et al., 2007). Experience could affect personality through two potential routes: by altering a fixed trait (such as physical traits) or by affecting learning and life history choices (Sih et al., 2004). Early experience, such as early juvenile social interactions or mother-infant interactions, have been shown to influence personality in rats (Meaney, 2001), although events that occur later in life could also have an impact. The latter would be especially relevant in species where the environment experienced by young animals is drastically different from that experienced by adults, or in species with complex lifecycles (Sih et al., 2004).

A clear interaction between genetic and environmental factors was demonstrated in a study with poeciliid fish. In this study the authors observed that boldness appeared to derive from both heritable and experiential components (Brown et al., 2007). Individuals from two different populations, with either high or low predation risk, were caught and bred, with other members of their original population, in the laboratory to produce first-

generation captive fish from both populations. An open-field assay was used to assess boldness, and fish were tested either with or without the experience of previously being 'chased' with a net. The first-generation offspring behaved similarly to their parents (bold for fish whose parents were from the low predation risk population, and shy for fish whose parents had a high predation risk) on the open-field, indicating a heritable component to boldness; in contrast, all fish showed increased boldness after being chased with the net, indicating that experience has an effect as well (Brown et al., 2007).

Finally, neuroendocrine mechanisms are capable of altering personality traits. Many factors mediate the impact of hormones on behavior, such as hormone synthesis and breakdown, variation in receptors, or hormone-hormone interactions (Sih et al., 2004), making it complicated to fully examine the effects of hormones on personality. However, some studies have demonstrated a direct link. In zebra finches (*Poephila guttata*), increased corticosterone levels after a stressful experience produced greater exploration and risk-taking behavior (Martins et al., 2007). In male greylag geese, individuals' baseline levels of corticosterone metabolite and testosterone were found to be consistent across contexts of feeding and handling (antipredation), which may be partly responsible for maintaining personality traits in the geese (Kralj-Fiser et al., 2007).

1.7 Docosahexaenoic acid, behavior, and immunity

Docosahexaenoic acid (DHA), a very long chain polyunsaturated fatty acid of marine origin, is a major component of brain tissue (Kidd, 2007) and has been shown to affect the development and functioning of neurons and photoreceptors (Lauritzen et al., 2001), improving cognitive ability (Cohen et al., 2005). In chickens, supplementing the

maternal diet with DHA has been found to increase brain DHA content of offspring (Ajuyah et al., 2003; Pappas et al., 2006).

In addition, DHA has been shown to have an effect on immune function. When introduced into the diet, it is integrated into the membrane phospholipids of immune system cells, modulating immune function (Wu & Meydani, 1998). *In vitro* culture of immune cells with DHA has been shown to produce anti-inflammatory effects (Wu & Meydani, 1998; Calder, 2001). In animal studies, feeding DHA in the form of fish oil[5] has been found to have a positive anti-inflammatory effect (reviewed in Calder 2001). However, in many mammal studies conducted, fish oil has been fed at very high levels, negatively impacting acquired immune response to foreign antigens (see review in (Calder, 2001), and in some cases, increasing susceptibility to viral or bacterial pathogens (Fritsche et al., 1997; Byleveld et al., 1999). Several studies have demonstrated that the effect of fish oil on pathogen susceptibility may depend on many variables, including the nature of the specific pathogen, immune aspects required to interact with the pathogen, or properties of the study such as animal model used or pathogen delivery method (Rubin et al., 1989; Clouva-Molyvdas et al., 1992; Calder, 2001). In addition, fish oil has been found to have a positive impact on the host's ability to resist bacterial endotoxin (Mascioli et al., 1988; Mascioli et al., 1989). Immunosuppressive effects of fish oil may also be alleviated by insuring that there are adequate levels of vitamin E in the diet (Wu & Meydani, 1998; Turini et al., 2001). Furthermore, in human studies examining the

[5] It is important to note that DHA may also be derived from algae.

effect of DHA, no negative effect was found on acquired immunity (Kelley et al., 1999), natural killer cell activity, or lymphocyte proliferation (Thies et al., 2001a; Thies et al., 2001c; Thies et al., 2001b). Turini et al. (2001) found that supplementation with fish oil (containing DHA) resulted in increased phagocytic activity by immune cells.

Although DHA is an important component in the tissues of avian species (Ajuyah et al., 2003; Speake et al., 2003) and has been shown to have an impact on behavior and cognition in mammals, no investigation has examined the effects of DHA on avian behavior or immunity.

Chapter 2 PERSONALITY TRAITS IN THE BUDGERIGAR *(Melopsittacus undulatus)*

2.1 Abstract

This study investigated the existence of a bold-shy personality axis in the budgerigar, *Melopsittacus undulatus*. Adult budgerigars (23 total; 14 females, 9 males) from one of two home pens- control diet, or supplemented with docosahexaenoic acid (DHA) - were subjected to tests in various behavioral contexts. Within each context the behavioral responses of the birds were categorized by fear, feeding, and activity. In addition, budgerigars were subjected to two mmunocompetence tests. Correlations were obtained within fear, feeding, activity and among all behavioral and immunocompetence variables. Kruskal-Wallis analysis was used to investigate possible effects of gender and dietary supplementation with DHA on behavioral and immune variables. Behavioral response variables in the feeding and activity categories were positively correlated, suggesting that budgerigars behaved consistently as bold or shy. There were significant gender differences for feeding and immune variables. Males generally had higher feeding rates, and their feeding responses were negatively correlated with percent of bacteria killed in the whole blood killing assay, a measure of innate immunity. Females had better scores for percent of bacteria killed in the whole blood killing assay. Regarding the effect of DHA, the only variable where a significant effect was found was that supplemented birds had a higher activity level in the presence of a group of familiar conspecifics. Taken together, the results of this study suggest that budgerigars exhibit bold-shy personality in two traits, feeding and activity, which are unrelated to each other as suggested by lack of

correlations between behavioral contexts. Additionally, male budgerigars who are bold in feeding have lowered innate immunocompetence, possibly because of the effects of testosterone.

2.2 Introduction

Budgerigars (*Melopsittacus undulatus*) in the wild are nomadic birds who inhabit most of the interior portion of Australia, migrating to follow patterns of vegetation growth (Wyndham, 1983). They live in large dynamic groups and are colony breeders, nesting opportunistically when conditions are favorable, usually in the hot months (Wyndham, 1980b). Flocks, composed of mixed genders and ages, feed on the seeds of grasses and plants on the ground during the early morning and late afternoon. Depending on the plants growing in a given area, budgerigars may eat close to thirty distinct types of seeds in a single day. Common types include *Panicum decompositum, Iseilema membranaceum*, *Atriplex spongiosa*, *Bassiea quinquecuspis,* and *Astrebla* species (Wyndham, 1980b). During the hot middle portion of the day, flocks of budgerigars roost in leafy trees, commonly *Eucalyptus microtheca*, *Acacia longifolia*, *Bauhinia cunninghamii*, or *Santalum acuminatum*. Holes in such trees are also utilized for nesting. Group size changes depending on the activity, with smaller groups (less than 100 birds) occurring during migration, and larger groups (sometimes with over 1,000 birds) foraging or roosting together (Wyndham, 1980a).

The budgerigar has become the most readily available and most popular pet parrot (Engebretson, 2006) and is frequently used as an animal model in learning and auditory/vocal development research (Brittan-Powell, 1997; Farabaugh et al., 1998; Hile

& Striedter, 2000; Plummer & Striedter, 2000; Mello, 2002; Striedter et al., 2003; Brittan-Powell & Dooling, 2004). Budgerigars have been an important model species for understanding Australian arid-land nomadic birds (see (Wyndham, 1980b). Although behavior of captive budgerigars has been described for both breeding (Brockway, 1964b) and non-breeding birds (Brockway, 1964a), personality traits have not been studied. Personality has important ecological (Dingemanse & Reale, 2005) and welfare implications (Huntingford & Adams, 2005). Understanding of how physical and social conditions affect personality may help to improve the conditions in which captive animals are maintained (Cavigelli, 2005).

DHA, a long-chain polyunsaturated fatty acid, is an essential component of the brain. Because of its role in brain development and cognitive function (Wainwright et al., 1994; Lauritzen et al., 2001; Wainwright, 2002; Cohen et al., 2005) DHA is likely to have an effect on behavior and personality development. In humans, DHA may help alleviate attention deficit hyperactivity disorder and depression, although eicosapentaenoic acid (a related n-3 polyunsaturated fatty acid) may have a greater effect than DHA (Kidd, 2007; Ross et al., 2007). Additionally, DHA may result in elevated immunocompetence (Wu & Meydani, 1998).The effect of DHA on avian behavior and personality, however, is unknown.

The objectives of this study was to examine the nature of personality traits in the budgerigar, comparing budgerigars fed a standard diet to those supplemented with docosahexaenoic acid (DHA). Budgerigar personality was investigated with respect to the shy-bold personality axis. Dichotomous axes, those labeled with two behavioral extremes, often represent tradeoffs where there are consequences to each strategy, and are

therefore evolutionarily and ecologically valuable to study (Wilson et al., 1994; Sih et al., 2004). Tests can be designed to assess, for example, boldness across a range of contexts or situations. It is then relatively easy to calculate correlations for the response variables or for boldness rankings for each test (Stamps, 2007). The presence of significant correlations indicates consistent behavioral responses, which is in turn indicative of personality traits.

We chose to focus on the shy-bold axis because the tradeoffs it represents are relevant to the demands faced by a prey species such as the budgerigar. While acquiring resources, prey species must choose between drawing the attention of predators through high activity, or discretion in exploration. Bold animals are more active and likely to inspect novel objects or predators; they also learn more quickly than shyer individuals (Sneddon, 2003; Sih et al., 2004); (Frost et al., 2007).

We subjected budgerigars to tests in feeding, exploration, anti-predation, and socialization contexts and measured behavioral responses which could be used to asses boldness. Most tests were conducted with one bird at a time, to remove the influence of social factors. Budgerigars exhibit high degree of group cohesion, with birds maintaining tighter group formation while foraging, flying, and roosting, and conducting maintenance behaviors as a group (Brockway, 1964a; Wyndham, 1980a), so it is important to remove the group structure to determine the boldness of individuals.

Locomotory behaviors were measured for tests in feeding, socialization, and exploration contexts, since bold budgerigars should be more active and willing to explore. Latencies to investigate a novel object or cross a visual barrier were also measured to indicate boldness in exploration. Feeding tests were conducted with both

solitary and paired birds to investigate both willingness to eat without the protection of a social group and boldness in competing for food. Additionally, one test measured latency to eat after a simulated predator threat. Length of tonic immobility (TI) reaction induced by simulated capture by a predator was measured as an indicator of fearfulness (Jones, 1986). It is predicted that bolder animals should be less fearful in the presence of the predator. To determine boldness in the social context, birds were tested with both familiar and unfamiliar conspecifics, as bolder budgerigars should be more willing to investigate unfamiliar birds.

Finally, we submitted the budgerigars to two tests of immunocompetence designed to assess their innate and adaptive immunity. Personality may be related to immunocompetence if personality traits are moderated by the endocrine system (for example, testosterone may affect both boldness and immunocompetence) (Hamilton & Zuk, 1982; Folstad & Karter, 1992; Zuk & Stoehr, 2002; Roberts et al., 2004).

We hypothesized that budgerigars would exhibit consistencies in their behavior within and across contexts, as indicated by significant correlations between behavioral responses in each context and between contexts. Furthermore, we predicted that individuals could be characterized as bold or shy based on their behavioral responses, using cluster analysis. Finally, we hypothesized that budgerigars fed with supplemental DHA would differ in behavioral and immune response. We predicted that birds who had received supplemental DHA in their diet would show elevated innate and constitutive immunity.

2.3 Materials and Methods

2.3.1 Experimental animals and facilities

For this study we used 23 budgerigars- 14 females and 9 males - obtained in January 2007 at approximately four months of age from a commercial pet store (PetCo, Beltsville, MD). Birds were randomly divided into two groups and housed in two home pens (228 cm wide x 290 cm tall x 427 cm long). The pens were constructed of 2.54 cm diameter PVC frame with black plastic mesh (2.54 cm square openings) walls in the Animal Wing of the Animal and Avian Sciences Building at the University of Maryland. Birds were maintained on a light schedule of 16 hours of light followed by 8 hours of dark, a constant temperature of 24 C and a relative humidity of 50%. Each home pen was fitted with seven wooden budgerigar nest boxes.

Water was available from tube drinkers located at three places along the wall of each home pen. Feed was placed in two dishes on the floor of each home pen each morning. Birds were gradually converted from a seed to a pellet diet over approximately one month. Subsequently, food was available *ad libitum* in two dishes on the floor of each home pen. Food was replaced daily. Birds in each home pens were randomly assigned to one of two diets. In one pen the 12 birds composing the group were fed an extruded diet designed for small breeding psittacines (Table 2-1; Mazuri Exotic Animal Nutrition, St. Louis, MO). Birds in the second pen, with 11 individuals, were fed the same diet but enriched with 2.5 g/kg of docosahexaenoic acid sourced from dried *Schizochytrium* algae (Martek Biosciences Corporation, Winchester, KY). The level of DHA was chosen to allow birds to consume a quantity approximately equal to 0.04% of

bodyweight daily. This ratio is slightly lower than that used by the same authors in order to reduce the risk of negative effects such as increased embryonic mortality and reduced hatchability as seen in domestic chickens (*Gallus gallus*) (Pappas et al., 2006). Birds received the pelleted diets for approximately five months prior to the start of the experiment, and continued eating the pelleted diets for the duration of testing. Diets were formed using a cold pelleting method which did not require heat extruding after the addition of DHA. This method prevented the DHA from being deactivated. Supplemental broccoli (approximately 20 grams per pen per day) and hardboiled chicken eggs (1 per pen per day) were occasionally available to all birds.

All birds arrived from the supplier with one numbered metal leg ring each, but because some of them had duplicate numbers we added a second ring to several birds. Applying the rings was a non-invasive, non-painful procedure: to apply, a tool was used to stretch the band and place it over the leg. In addition to keeping a record of individual identification numbers, gender for each bird was determined and recorded. Male birds were those whose ceres were dark blue in color; females were those whose ceres were mauve, pink, or very pale blue.

Table 2-1 Mazuri small bird breeder diet

Guaranteed Analysis		*MINERALS*	
Metabolizable energy, kcal/g	3.45	**Ash, %**	**6.1**
Crude Protein not less than	0.18	Calcium, %	1.20
Crude Fat not less than	0.065	Phosphorous, %	0.87
Cruder Fiber not more than	0.045	Phosphorous (non-phylate), %	0.60
Ash not more than	0.09	Potassium, %	0.66
		Magnesium, %	0.16
		Sodium, %	0.13
		Chlorine, %	0.24
Approximate Nutrient Composition		Iron, ppm	380
NUTRIENTS		Zinc, ppm	100
Protein, %	**19.5**	Manganese, ppm	100
Arginine, %	1.02	Copper, ppm	14
Cystine, %	0.31	Cobalt, ppm	0.39
Glycine, %	0.78	Iodine, ppm	1.3
Histidine, %	0.48	Selenium, ppm	0.20
Isoleucine, %	0.93		
Leucine, %	2.06	*VITAMINS*	
Lysine, %	0.90	Vitamin K (as menadione), ppm	3.0
Methionine, %	0.58	Thiamin Hydrochloride, ppm	11
Phenylalanine, %	1.00	Riboflavin, ppm	15
Tyrosine, %	0.67	Niacin, ppm	110
Threonine, %	0.70	Pantothenic Acid, ppm	20
Tryptophan, %	0.21	Choline Chloride, ppm	1500
Valine, %	0.96	Folic Acid, ppm	4.8
		Pyridoxine, ppm	12
Fat, %	**8.4**	Biotin, ppm	0.73
		Vitamin B_{12}, mcg/kg	44
Fiber (Crude), %	**2.7**	Vitamin A, IU/kg	10
Neutral Detergent Fiber, %	12.6	Vitamin D_3 (added), IU/gm	1.9
Acid Detergent Fiber, %	3.7	Vitamin E, IU/gm	180

2.3.2 Experimental design

All testing was carried out in a separate room adjacent to the room housing the home pens. All tests except for tonic immobility (TI) took place in a test pen. Dimensions of the test pen were 137 cm high, 137 cm long and 141cm wide. The pen frame was constructed of blue metal pipe and the sides and ceiling were covered with 2.54 cm square black plastic mesh. Black plastic sheeting was used to cover the sides and back of the pen to limit visual stimuli to the birds. For some tests, black plastic sheeting also covered the front of the pen, in which case the bird was observed by video camera. The experimenter sat in a chair approximately 61 cm in front of the center of the test pen, and the areas to the side and behind the experimenter's chair were also covered with black plastic sheeting. Schematic diagrams for test pen configuration for each test can be found in Figure 2-1.

Starting 30 cm from the front of the test pen, nine horizontal lines were marked across the floor with chalk every 15 cm to facilitate tracking of bird movements. Each line was numbered at each end, next to the walls. In most tests, bird position was recorded each time a bird crossed a line using the Observer software (Noldus, Inc, Wageningen, The Netherlands).

A small cardboard box (19 cm tall x 27 across x 13 cm deep) was attached on the floor against the center of the front wall of the test pen and was used as a starting box for all experiments. This box had a lid which could be opened from outside the test pen by the experimenter. At the beginning of each experiment, the focal bird was placed in the starting box for three minutes, at which point the experimenter opened the lid from outside the test pen, leaving it open for the duration of the experiment.

Most tests required birds to walk across the floor and birds were not permitted to perch on the sides or ceiling of the test pen. If a bird landed on the plastic mesh on the sides of the test pen during the test, the mesh near where they were perching was gently tapped with a dowel. Taps were not recorded and were included in flying time. Perching on the sides or ceiling was a rare occurrence, especially as the experiment progressed past the first two tests.

Unless otherwise noted, each bird was subjected to each test once. Any hens that were nesting or feeding chicks during the testing period were excluded from that test. No males were excluded from testing, as it was unlikely that the short testing periods would hinder their ability to provide for their mates or offspring.

Birds were always tested in random order. Random ordering of birds for each test was generated using the Research Randomizer (www.randomizer.org). Order of tests was as follows: tonic immobility, barrier threat, open field, tendency to flock, novel object, feeding rate, intense food competition, predator threat, color learning, and immunity assays. This order was selected so that tests occurred in order of increasing complexity. The intense food competition and color learning tests were dropped from the experiment (please see Appendix for more information). Behavioral data was collected using The Observer® (Noldus, Inc.) and the Chickitizer© (Sanchez & Estevez, 1998). Bird identification number and gender were recorded in connection with each response variable. Variables measured for all tests are listed in Table 2-2.

All birds were subjected to two immunological assays conducted during January 2008, after all behavioral testing had ended. Blood was collected from the brachial vein for use in the whole-blood killing assay. At that time, birds were inoculated with whole

rabbit blood in Alsever's solution (HemoStat Laboratories, Dixon, CA) for the hemolysis-hemagglutination assay. Four days later blood was collected from the jugular vein for the hemolysis-hemagglutination assay using the jugular vein collection method.

Figure 2-1 Schematic diagrams of test pen setup for each test

Diagrams are not to scale.

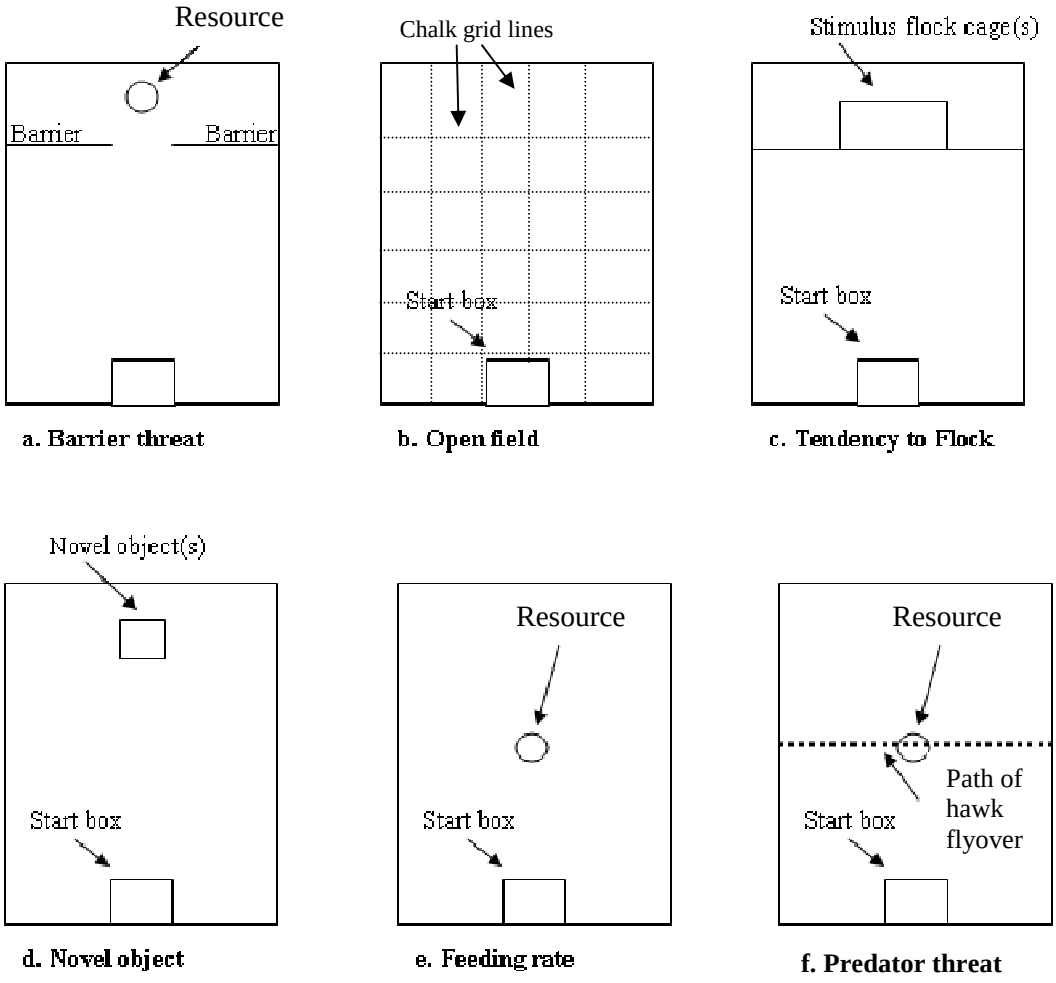

Table 2-2 Variables Measured Throughout the Experiment

Test	Variable
Tonic Immobility	Duration of TI response (s)
Open Field	Total distance traveled (cm)
	Net distance traveled (cm)
	Percentage of points in the core
	Minimum convex polygon (cm^2)
Barrier Threat	Latency to cross barrier (s)
	Activity level (# lines crossed)
Tendency to Flock	Activity level in presence of familiar flock (# lines crossed)
	Activity level in presence of unfamiliar flock (# lines crossed)
Novel Object	Activity level in presence of unattractive object (# lines crossed)
	Activity level in presence of attractive object (# lines crossed)
Feeding Rate	Eating score alone (# seeds eaten)
	Eating score with one competitor (# seeds eaten)
	Competitive ability (% seeds eaten by focal in competition test)
Predator Threat	Latency to resume eating after predator threat (s)
Whole Blood Killing	Percent of bacteria killed by leukocytes
Hemolysis-Hemagglutination	Lysis score after 20 minutes with antigen
	Agglutination score after 20 minutes with antigen
	Lysis score after 90 minutes with antigen
	Agglutination score after 90 minutes with antigen

2.3.3 Tonic immobility

Tonic immobility (TI) is a recognized measure of fear response to being restrained or captured (Jones, 1986; Marin et al., 2001), and is thought to be an anti-predator reaction. We measured TI according to the method of Marin et al. (2001). Every bird was tested three times in random order over the course of a week to determine the repeatability of TI. Before the TI procedure each bird was individually removed from its home pen using a net designed for caged birds. The tested bird was carried by hand to a room out of sight of other birds. The bird was placed on its left side or back over a small towel on a table, facing away from the handler, who gently restrained the bird for 15 seconds by keeping the right hand against the bird's right wing or against the point of the keel bone and the left hand (gloved) cupped over the head. Birds were given up to 6 immobilizations to sustain TI for a minimum of 10 seconds. Duration of TI was recorded through a maximum period of 10 minutes. If a bird escaped in between TI immobilizations, it was placed in a holding box for three minutes before resuming the experiment.

2.3.4 Open Field

This is a common test in the context of exploration (Brown et al., 2007; Boon et al., 2008). Each bird was allowed to explore the empty test pen, which had ten horizontal and ten vertical lines chalked on the floor, forming a 10 cm x 10 cm grid (Fig 2-1b). Black plastic sheeting was used to cover the front of the test pen, preventing birds from clinging to the plastic mesh. It was important to minimize birds perching on the plastic mesh since this test required the experimenter to constantly record bird position on the

floor. If a bird landed on the ceiling, the area where it perched was tapped gently with a dowel. A tablet PC with the Chickitizer software (Sanchez & Estevez 1998) was used to record for ten minutes each bird's movement across the open field test. If birds flew, their position was recorded as being outside of the grid. From the bird's movements, total and net distances travelled were calculated. Total distance was defined as the sum of all the Euclidean distances[6] between successive locations of the trajectory during the observation period, and net distanced was defined as the Euclidian distance between the initial and final position on the grid (Leone & Estevez, 2008). Use of central vs. peripheral areas was also calculated. The periphery was defined as the area within 10 cm of the walls of the enclosure, whereas the remaining area was considered as center. The percentage of locations in the center was calculated, standardizing for the relative areas of the core and periphery by dividing the number of points in either zone (center or periphery) by the area of that zone before proceeding with analysis. Total and net distance and core vs. periphery calculations were all done using SAS (v 9.1, SAS Institute, Cary, NC). Additionally, a minimum convex polygon (Mohr, 1947) was constructed with all the observed locations for each particular bird.

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[6] Euclidian distance formula.

$$\sum_{i=1}^{m-1} \sqrt{(x_{i+1} - x_i)^2} + (y_{i+1} - y_i)^2$$

2.3.5 Barrier Threat

The barrier threat measures risk-taking behavior in the context of exploration. In the test pen each experimental bird had an opportunity to cross an opaque barrier to gain access to visible resources (Fig 2-1a). For this test a barrier made from black plastic sheeting was hung 30 cm from the back of the test pen with a 10-cm-wide gap in the middle of it. The barrier spanned the entire height of the testing pen. Two target objects were placed behind the barrier, centered with the gap to allow for visibility. One object was a Petri dish containing approximately 10 grams of seeds which was placed on the floor 10 cm from the back wall of the pen. Hung directly above it, 40 cm from the ceiling, was the second object, a small perch platform 10 cm^2 wide made from a dowel frame and plastic mesh. The test was stopped when a bird had physically interacted with one of the objects, or after 30 min if no interaction occurred. Latency to cross the barrier and number of lines crossed during the test were recorded using the Observer. All lines crossed during the test were counted, regardless of the direction in which the bird was moving. Birds that did not cross the barrier were assigned a maximum score (1,800 seconds). The time and number of lines crossed were considered measures of activity level (Moretz et al., 2007)

2.3.6 Tendency to flock

This is a test of boldness in the social context. The test pen contained a cage (33 cm high x 49 cm long x 67 cm wide) centered approximately 15 cm from the back wall (Fig 2-1c). The cage contained the 'stimulus flock', which was comprised of three experimental birds either from the focal bird's home pen (familiar flock) or the other home pen (unfamiliar flock). Tests were conducted to assess the response of the focal bird, which had the choice of remaining by itself, exploring the pen, or approaching the

caged stimulus flock. Each focal bird was tested three times: the first two times, once each with a familiar or unfamiliar stimulus flock, and the third time with both stimulus flocks concurrently. For the test with both stimulus flocks, two identical cages were placed adjacently 30 cm from the back wall. To prevent the focal bird from climbing on or going behind the stimulus flock cage, a barrier made of black plastic sheeting was hung from the ceiling of the test pen directly above the front of the stimulus flock cage. The barrier had an opening through which the front of the stimulus flock cage was visible. To the focal bird, the stimulus flock cage front appeared flush with the back wall of the test pen. All stimulus flock birds were allowed ten minutes to habituate to their cages before any testing began. Each stimulus flock was provided with a water bowl in the cage during the time of testing.

Stimulus flocks were formed by splitting all experimental birds randomly into groups of three from the same home pen. Each bird served as a stimulus flock member at least twice. For a given session of testing, one randomly chosen stimulus flock was placed in the cage in the test pen. After the stimulus flock habituation period, up to seven focal birds were subsequently tested using that stimulus flock. Focal birds were chosen randomly out of the pool of all experimental birds. Each test lasted 10 minutes. After all experimental birds had been tested with both familiar and unfamiliar stimulus flocks, testing procceded with both the familiar and unfamiliar flocks simultaneously.

Latency to cross each line and total number of lines crossed during the test 10 minute period were recorded. Time spent in close proximity (within 10 cm) to the stimulus flock, and behaviors performed during that time were recorded. These behaviors

included sitting, pacing, while within 10 cm of the flock , and chewing or climbing the front of the flock's cage.

2.3.7 Novel object

This test focused on fear and risk-taking behavior in an exploratory context. The test was administered to each bird three times. The test was given once with a positive novel object, which consisted of a Petri dish with approximately 10-12 mealworms (*Tenebrio mollito)* in about a teaspoon of sawdust, and once with a negative novel object, a wadded 10-cm diameter bundle of translucent white plastic sheeting. The third time, the birds were tested with the positive object and a familiar object consisting of a plastic mesh basket containing Spanish moss to provide foraging opportunities. These objects were placed side-by-side to determine if novelty or familiarity was preferred for positive objects.

The test pen floor was set up with marked lines traced with chalk as in previous tests. The novel object(s) was/were placed 15 cm from the back of the center of the pen on the floor (Fig 2-1d). Birds were given both objects independently in random order before being tested with two objects. In the third test, the two positive objects (Petri dish and Spanish moss) were placed side-by-side, approximately 10 cm apart. Birds were given ten minutes for each test. The number of lines crossed (measured in the same way as for the barrier threat) and time spent in close proximity (within 15 cm) to the objects was recorded.

2.3.8 Feeding rate

This is a test of competitive ability in the feeding context. Birds were allowed *ad libitum* access to a Petri dish of millet seeds for ten minutes, either by themselves or in groups of two. The Petri dish was located approximately 30 cm from the door of the start box on the floor of the test pen (Fig 2-1e). This test allowed us to determine changes in feeding ability in an individual vs. a competitive situation. Birds were not fasted before receiving this test; they were fed as usual.

Prior to the test all birds first received two training sessions in groups of five birds from the same home pen. During a training session, the five birds were placed directly into the test arena and were allowed to explore the resources freely for 20 minutes. After all birds were trained, they were tested in groups of two, and then again alone. Each pair of birds was randomly chosen from the same home pen. Both individual and pair trials were video recorded. Two video cameras were used: one with an overhead view of the Petri dish, and one at floor level with a side view of the dish. The front of the test pen was covered with black plastic sheeting to minimize distractions to the birds during the test. Data were collected with the use of a closed-circuit TV monitor.

Eating a seed was defined as follows. After grasping a seed from the bowl with its beak, the bird raised its head to hull the seed before consuming it. As birds often manipulate several items such as empty seed hulls while searching for edible seeds, it is important to note that the bird *must* have brought its head up and hulled a seed in order for a seed to be counted as being eaten.

The number of seeds eaten by each bird in a 15 minute period was recorded for both the pair and individual trials. For the pair test, number of seeds eaten by each bird

was summed to get the total number of seeds eaten in that trial. The percentage of seeds eaten by a specific bird in its pair test was calculated as a measure of competitive ability.

2.3.9 Predator threat

This test measures risk-taking behavior after an imminent threat consisting of the flyover of a model hawk. The setup was identical to the feeding rate trial, except that a string was stretched over the pen to allow the experimenter to pull a model hawk silhouette across the pen (Fig 2-1f). The hawk was made of a cutout of black plastic sheeting with dowel supports, and measured approximately 30 cm (wingspan) by 15 cm (from beak to end of tail). It was pulled across the pen at a slow glide (approximately 28 cm/second[7]) over the middle of the pen. Hawk flyover began after the focal bird had been consuming seeds for approximately 10 seconds. Birds were given 15 minutes to begin eating seeds after the starting box was opened, and 20 minutes to resume seed-eating after the hawk flyover finished. If a bird did not begin to consume seeds within 15 min after leaving the starting box, it was returned to the home pen and re-tested at a later date, up to two additional times (for a total of three opportunities to complete the test). Once hawk flyover occurred, the trial ended when the bird consumed a seed or when 20 minutes had passed. Latency to resume eating was recorded. Birds who did not resume were given a maximum score of 1200 seconds.

[7] Calculated from the average time it took to 'fly' the hawk a known distance.

2.3.10 Microbicidal assay

This test is a measure of innate immune response 30 minutes after a challenge. It is designed to assess the bactericidal activity of leucocytes in whole blood (Millet et al., 2007). Protocol for this assay followed the methods of Millet et al. (2007). Briefly, sterile blood samples were taken from all birds in January, 2008, after approximately 11 months of feeding the experimental and control pelleted diets. Blood collection methods used for this assay are further described in section 2.3.12. The microbicidal assay procedure was carried out immediately post-collection. Blood samples were diluted with pre-warmed media (1:10 ratio with CO_2-independent media, Gibco-Invitrogen, CA, California), then inoculated with *Escherichia coli* (100 CFU per 50 µL diluted blood), and their ability to kill the bacteria was measured. The diluted blood and bacterial solution was vortexed, then incubated at 34-38˚C for 30 minutes, then 50 µL aliquots were then pipetted in duplicate and spread onto nutrient agar plates. Plates were inverted and incubated at room temperature in the laminar flow hood for 24 hours, at which point the number of colonies per plate was counted. Large uncountable plaques were assigned a colony value of ten. The baseline number of colonies was determined by plating and incubating the bacterial culture alone. Additionally, a test for sterility was conducted by incubating plates with just the CO_2-independent media. Antimicrobial activity of the blood was defined as the proportion of bacterial inoculum killed: (1 – (live colonies after 24 hr incubation / number CFU inoculated)). *E. coli* was acquired in the form of a lyophilized pellet (approximately 2.8 x 10^3 bacteria per pellet; between 1,000 and 10,000 colony forming units, American Type Culture Collection #8739). This microbe was selected because Millet et al. (2007) found that this organism was more susceptible to the bactericidal

action of whole cockatiel (*Nymphicus hollandicus*) blood than *Candida albicans*, *Staphylococcus aureus*, or other strains of *E. coli*. All work involving *E. coli* was carried out in a laminar flow hood. An *E. coli* pellet was reconstituted according the manufacturer directions immediately prior to conducting the assay.

2.3.11 Hemolysis-hemagglutination assay

This assay was designed to test the birds' innate humoral immunity by measuring the ability of their natural antibodies to recognize a recently introduced protein (Koutsos et al., 2003; Matson et al., 2005). A total of 15 birds were inoculated with 100 μL whole rabbit blood in 50% Alsever's solution (Hemostat Laboratories, Dixon, CA) injected subcutaneously in two areas of the pectoral region. Inoculation was performed immediately following brachial blood collection for the microbicidal assay.

Four days after inoculation, blood was collected from birds using the jugular vein collection method (see section 2.3.13); the plasma fraction was then isolated by centrifugation at 600 rpm in sterile Eppendorf tubes. Complement was denatured by heat inactivation. 25 μL of plasma sample was added to a 96-well round-bottom assay plate and serially diluted by a factor of twowith phosphate buffered saline; dilutions ranged from 1 in column 1 to 1/1024 in column 11, with a negative control of phosphate-buffered saline only in column 12. Next, 25 μL of 1% rabbit red blood cell suspension was added to all wells. The plate was then sealed with Parafilm M, gently vortexed, incubated in a 37˚ C water bath for 90 minutes and allowed to settle at room temperature. Hemolysis and hemagglutination scores for each plasma sample were determined

according to the standard described in Matson et al (2005). Each well in a row was labeled with a titer number from 1 – 11, with well 12 as a negative control.

2.3.12 Brachial vein blood collection method

Birds were individually captured using a net designed for caged birds and moved to another room out of sight of the rest of the colony during blood collection. Feathers were pushed away from the brachial vein and dander was removed by swabbing with an ethanol wipe. The area was allowed to air-dry for approximately 20 seconds, as drying is important to the sterilization process (Millet et al., 2007). The vein was punctured using a 28 gauge insulin syringe needle. Blood was drawn from the puncture wound into two 25 μL capillary tubes and was placed on ice immediately. Approximately 50 μL of blood was collected per bird. To halt bleeding, light pressure was subsequently applied to the puncture wound. Blood collection occurred no later than five minutes after capture to prevent elevated levels of corticosterone, a stress hormone which has a immunosuppressive effects (Saino et al., 2003; Millet et al., 2007). The two capillary tubes from each bird were allowed to drain into one Eppendorf tube in which the blood was transported to the lab. Blood was collected from the birds in a random order and each bird's sample was assigned a random number for processing.

2.3.13 Jugular vein blood collection method

Birds were taken out of their home pens and sorted into two holding containers depending on their home pen. The holding containers were moved to a separate surgery room for blood collection. For collection, each bird was gently removed by hand from its

holding pen. One person held the bird and located the right jugular vein. A second person then removed feathers and dander by swabbing with an ethanol wipe. The second person also collected the blood. The vein was punctured using a 25 gauge needle on a 1-ml syringe and 0.1 ml of blood was collected. Each bird's blood was immediately transferred to an Eppendorf tube in an ice bucket for transport to the lab. To halt bleeding, light pressure was applied to the puncture wound. The order of birds was random and each bird's sample was assigned a random number for processing.

2.3.14 Statistical analysis

SAS statistical analysis software (v 9.1, SAS Institute, Cary, NC) was used for the analysis, and all tests of significance were done at the $\alpha = 0.05$ level. Each variable analyzed represented the response of individual birds during one of the tests. Four response variables (time spent in the presence of the familiar flock, unfamiliar flock, attractive object, and unattractive object) were dropped from the analysis due to low numbers of birds having scores for these variables.

Spearman rank correlation analysis was used to determine if budgerigar behavior was consistent between different situations within three categories: fear, activity, and feeding. Correlation analysis was performed within each category. Table 2-3 presents variable classifications within each category. Additionally, Spearman's procedure was used to evaluate correlations between the immunological variables and all the behavior variables to investigate any relationship between personality and immunity.

Residuals of all behavioral and immunological variables were examined for normality of distribution and homogeneity of variance within each gender group.

Variables from the open field test which included total and net distance traveled, minimum convex polygon area, and percentage of locations in the central area[8] met assumptions for normality and homogeneity of variance. These data were subjected to analysis of variance (ANOVA) using a general linear model to determine the effects of diet and gender. All other variables did not meet assumptions for the ANOVA; therefore, diet and gender differences involving those variables were analyzed using the Kruskal-Wallis test.

The categories of fear, feeding, activity, and immunity were also used to create composite variables for cluster analysis to determine if birds could be clustered into bold and shy groups based on their relative boldness in those areas. Bold animals are those that show high activity, competitive ability, and feeding rates, and low fear, and shy animals show low activity, competitive ability, and feeding rates, and high fear. Therefore, we expected that birds could be placed along a bold-shy axis and clustered based on their behavioral responses.

[8] Percentage of locations in the central area was calculated based on standardized counts for number of locations in the core and in the periphery. The core was calculated to represent 66.9% of the total open field, so all counts for number of points in the core were divided by .669 to give standardized counts for each bird; the periphery was calculated to be 33.1% of the area of the open field, so each bird's count for number of locations in the periphery was divided by .331. From there, percent of locations in the core was calculated as (standardized count of locations in the core/standardized total locations, core and periphery)*100.

Table 2-3 Classification of variables according to category and mean ± SE of all original variables measured for all birds

Reduced Variable	*Original Variables*	*Mean ± SE*	*N*	*Test of Original Variable*
Fear	Duration of TI response (s)	72.79 ± 24.01	19	Tonic Immobility
	Percentage of locations in the central areas (%)	18.726 ± 9.413	14	Open Field
	Latency to cross barrier (s)	870.17 ± 198.51	18	Barrier Threat
	Latency to resume eating after predator threat (s)	821.28 ± 176.61	18	Predator Threat
Activity	Total distance traveled (cm)	169.07 ± 39.41	14	Open Field
	Net distance traveled (cm)	70.74±13.23	14	Open Field
	Minimum convex polygon (cm^2)	2391.96±753.96	14	Open Field
	Activity level (# lines crossed)	5.39 ± 1.27	18	Barrier Threat
	Activity level in presence of familiar flock (# lines crossed)	5.79 ± 1.18	19	Tendency to Flock
	Activity level in presence of unfamiliar flock (# lines crossed)	4.17 ± 1.57	18	Tendency to Flock
	Activity level in presence of unattractive object (# lines crossed)	2.67 ± 1.00	15	Novel Object
	Activity level in presence of attractive object (# lines crossed)	2.71 ± 0.97	17	Novel Object
Feeding	Eating score alone (# seeds eaten)	83.53 ± 31.19	19	Feeding Rate
	Eating score with one competitor (# seeds eaten)	92.50 ± 34.97	20	Feeding Rate
	Competitive ability (% seeds eaten by focal in competition test)	35.00 ± 7.97	20	Feeding Rate
Immunity	Percent of bacteria killed by leukocytes	69.67 ± 9.56	15	Whole Blood Killing
	Lysis score after 20 minutes with antigen	1.28 ± 0.38	14	Hemolysis-Hemagglutination
	Agglutination score after 20 minutes with antigen	4.29 ± 0.68	14	Hemolysis-Hemagglutination
	Lysis score after 90 minutes with antigen	0.79 ± 0.35	14	Hemolysis-Hemagglutination
	Agglutination score after 90 minutes with antigen	3.14 ± 0.71	14	Hemolysis-Hemagglutination

2.4 Results

2.4.1 Correlates of personality

Means and standard errors for all variables are presented in Table 2-3.

Significant correlations were found for the variables measured within the feeding and activity behavioral types, but not within the fear behavioral type. In the activity behavioral type, seven positive correlations were found, involving seven out of eight activity variables: total distance traveled and net distance traveled (r_S = 0.552, N = 14, *P* = 0.041); total distance traveled and minimum convex polygon area (r_S = 0.886, N = 14, *P* = <0.0001); total distance traveled and activity level in the barrier threat test (r_S = 0.701, N = 12, *P* = 0.011); total distance traveled and activity in the presence of the attractive object (r_S = 0.658, N = 11, *P* = 0.028); minimum convex polygon area and activity level in the barrier threat test (r_S = 0.729, N = 12, *P* = 0.007); minimum convex polygon area and activity level in the presence of the attractive object (r_S = 0.630, N = 11, *P* = 0.038); and activity levels in the presence of the familiar and unfamiliar flocks (r_S = 0.515, N = 18, *P* = 0.029).

All three variables within the feeding behavioral type were positively correlated with each other: seeds eaten alone and seeds eaten in competition (r_S = 0.773, N = 19, *P* = 0.0004); seeds eaten alone and competitive ability (r_S = 0.624, N = 17, *P* = 0.004); seeds eaten in competition[9] and competitive ability[10] (r_S = 0.964, N = 20, *P* = <0.0001).

[9] Number of seeds eaten by the focal in the competitive section of the feeding rate test.
[10] Percent of the total seeds eaten by both birds in the competitive feeding rate test that were eaten by the focal.

Regarding the immune variables, lysis at 20 minutes was found to be negatively correlated with latency to resume eating after the predator threat (r_S = -0.610, N = 13, *P* = 0.027) and with net distance traveled (r_S = -0.874, N = 8, *P* = 0.005). Contrarily, lysis at 20 minutes was positively correlated with latency to cross the barrier (r_S = 0.585, N = 12, *P* = 0.046). Percent bacteria killed was negatively correlated with the number of seeds eaten alone (r_S = -0.598, N = 15, *P* = 0.024) and in competition (r_S = -0.663, N = 15, *P* = 0.010), and competitive ability (r_S = -0.555, N = 14, *P* = 0.0394).

Regarding gender effects, the Kruskal-Wallis test indicated the existence of significant differences between males and females in the number of seeds eaten alone or in competition, and percent of bacteria killed (see section 2.4.2 below for results of the Kruskal-Wallis test). Because of the gender differences, correlations involving the above mentioned variables were obtained for each gender separately. Only competitive ability and number of seed eaten alone were correlated for females, whereas for males all three feeding variables, including number of seed eaten alone and in competition and competitive ability were correlated pairwise.

Percent bacteria killed had no significant correlations with any of the feeding variables for females at the 5% significance level. Contrarily, in males percent bacteria killed was negatively correlated with the number of seeds eaten in competition (r_S = -0.943, N = 6, *P* = 0.005) and competitive ability (r_S = -0.943, N = 6, *P* = 0.005) and also was found to be correlated with minimum convex polygon area (r_S = 1.00, N = 3, *P* = <0.0001).

A second Spearman correlation analysis was performed on rank scores for boldness between behavioral categories. Variables were classified within the behavioral categories of feeding, fear, or activity (see Table 2-3). Values for each variable were ranked from the highest to the lowest. Feeding and activity ranks were therefore given from boldest to shyest (most eating to least; highest activity level to lowest activity level) and fear ranks were given from shyest to boldest (longest latency to shortest). The bird with the highest value for each variable was assigned a rank of one, the second a two, and so on. Ties were given the average of the sum of their un-tied ranks. Once all the variables within each behavioral category were ranked, each bird received an average rank for boldness within each category. Correlations were calculated between average boldness ranks for all behavioral categories, and we found no significant correlations between rank scores.

2.4.2 Gender and dietary treatment differences

Males ate more seeds alone ($H_1 = 5.97$, $P = 0.015$) and in competition ($H_1 = 4.20$, $P = 0.040$), while females had better scores than males for percent of bacteria killed ($H_1 = 5.13$, $P = 0.023$). Means and standard errors based on gender are presented in Table 2-4. No other significant effects of gender were found. However, trends towards significant gender effects were detected, with males potentially having a shorter latency to resume eating after the predator threat ($H_1 = 3.20$, $P = 0.074$) and better competitive ability ($H_1 = 3.58$, $P = 0.059$).

Additionally, a significant difference was found in the activity level in the presence of the familiar flock between the two dietary treatments ($H_1 = 3.99$, $P = 0.046$). Birds receiving DHA in their diet had a higher average activity level than those on a control

diet. Means and standard errors for all variables based on dietary treatment are listed in Table 2-5.

No significant effect of gender or diet or the interaction between the two was found for the open field test variables total distance ($F_{1,11} = 1.16$, $P = 0.348$) and net distance ($F_{1,11} = 1.53$, $P = 0.259$) traveled, minimum convex polygon area ($F_{1,11} = 2.01$, $P = 0.181$), or percent of locations in the center of the testing arena ($F_{1,11} = 0.05$, $P = 0.948$).

Table 2-4 Mean ± SE of all variables measured by gender

Variable	*Females*	*N*	*Males*	*N*	*P value*
Latency to cross the barrier	902.10 ± 272.08	10	830.25 ± 307.77	8	N.S.
Activity level in the barrier threat	5.90 ± 2.03	10	4.75 ± 1.45	8	N.S.
Latency to resume eating after the predator threat	997.91 ± 208.90	11	543.71 ± 303.22	7	N.S.
Seeds eaten alone	12.91 ± 11.34	11	180.63 ± 57.99	8	0.015
Duration of the tonic immobility reaction	84.10 ± 40.97	10	60.22 ± 24.55	9	N.S.
Seeds eaten in competition	31.08 ± 9.92	12	184.63 ± 77.77	8	0.040
Competitive ability	20.31 ± 6.91	12	57.04 ± 14.24	8	N.S.
Activity in the presence of the unattractive novel object	4.14 ± 1.77	7	1.38 ± 0.96	8	N.S.
Activity in the presence of the attractive novel object	2.09 ± 0.73	11	3.83 ± 2.50	6	N.S.
Activity in the presence of the familiar flock	5.73 ± 1.73	11	5.88 ± 1.64	8	N.S.
Activity in the presence of the unfamiliar flock	3.55 ± 1.23	11	5.14 ± 3.70	7	N.S.
Lysis at 90 minutes	0.44 ± 0.34	9	1.40 ± 0.75	5	N.S.
Agglutination at 90 minutes	3.00 ± 0.97	9	3.40 ± 1.08	5	N.S.
Lysis at 20 minutes	1.11 ± 0.48	9	1.60 ± 0.68	5	N.S.
Agglutination at 20 minutes	4.11 ± 0.75	9	4.60 ± 1.47	5	N.S.
Percent of bacteria killed	84.89 ± 8.74	9	46.83 ± 16.80	6	0.023
Percent of locations in the central area	20.27 ± 11.01	9	25.00 ± 19.36	5	N.S.
Total distance traveled	213.31 ± 53.57	9	89.45 ± 36.62	5	N.S.
Net distance traveled	86.77 ± 17.79	9	41.90 ± 11.41	5	N.S.
Area of the minimum convex polygon	3379.00 ± 1006.33	9	615.30 ± 547.46	5	N.S.

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Table 2-5 Mean ± SE of all variables measured by dietary treatment

Variable	*DHA*	*N*	*Control*	*N*	*P*
Latency to cross the barrier	1011.11 ± 311.84	9	729.22 ± 255.48	9	N.S.
Activity level in the barrier threat	5.22 ± 1.93	9	5.56 ± 1.76	9	N.S.
Latency to resume eating after the predator threat	779.88 ± 251.05	8	854.40 ± 257.98	10	N.S.
Seeds eaten alone	117.33 ± 54.49	9	53.10 ± 33.24	10	N.S.
Duration of the tonic immobility reaction	59.89 ± 30.28	9	84.40 ± 37.76	10	N.S.
Seeds eaten in competition	115.80 ± 68.60	10	69.20 ± 18.39	10	N.S.
Competitive ability	30.00 ± 13.26	10	40.00 ± 9.31	10	N.S.
Activity in the presence of the unattractive novel object	1.00 ± 0.53	7	4.13 ± 1.71	8	N.S.
Activity in the presence of the attractive novel object	3.44 ± 1.66	9	1.88 ± 0.93	8	N.S.
Activity in the presence of the familiar flock	8.63 ± 1.63	8	3.73 ± 1.63	11	0.046
Activity in the presence of the unfamiliar flock	1.71 ± 0.75	7	5.73 ± 2.45	11	N.S.
Lysis at 90 minutes	0.75 ± 0.75	4	0.80 ± 0.42	10	N.S.
Agglutination at 90 minutes	4.50 ± 1.89	4	2.60 ± 0.65	10	N.S.
Lysis at 20 minutes	1.50 ± 0.96	4	1.20 ± 0.42	10	N.S.
Agglutination at 20 minutes	4.75 ± 0.85	4	4.10 ± 0.91	10	N.S.
Percent of bacteria killed	77.60 ± 19.51	5	65.70 ± 11.11	10	N.S.
Percent of locations in the central area	25.06 ± 13.48	7	18.86 ± 14.26	7	N.S.
Total distance traveled	161.70 ± 50.86	7	176.45 ± 64.24	7	N.S.
Net distance traveled	73.22 ± 24.36	7	68.27 ± 12.76	7	N.S.
Area of the minimum convex polygon	1728.86 ± 1167.14	7	3055.07 ± 980.58	7	N.S.

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2.4.3 Cluster analysis

The average rank scores for feeding, fear, activity, and immunity were used for factor and cluster analyses. First, scatter plots were generated for each pair of variables to visualize the shape of possible clusters, and it was determined that clusters would be elliptical and irregular. Therefore, the ACECLUS procedure of SAS was used to further reduce the variables through factor analysis by creating new canonical variables. Correlation analysis was used to check the significance of the loading of each original variable onto each of the four canonical variables. See Table 2-6 for loadings and results of the significance tests. Only activity loaded significantly on canonical variable 1; since this variable explained 98.5% of the variance in the data, and had an eigenvalue of 151.3, it was determined that activity was a very important factor in budgerigar personality. Since variable 1 explained such a high proportion of the variance and had by far the highest eigenvalue, it was the sole variable used in the cluster analysis. Table 2-7 shows each bird's loadings on the four canonical variables. A plot of semipartial R^2 suggested that the data would best be represented by three clusters, with these three groups explaining approximately 95% of the original variance. Tree dendogram and cluster memberships are shown in Figure 2-2. Nine birds had missing values for one or more variables and were therefore excluded from the cluster analysis, so the analysis was conducted with 14 birds.

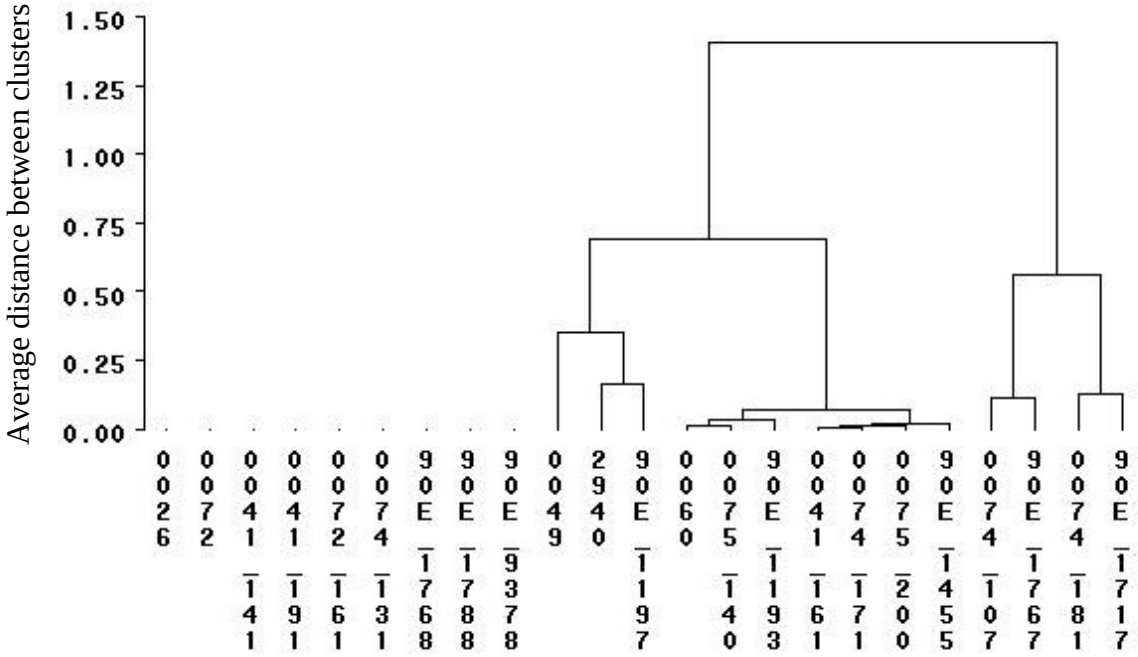
Table 2-6 Standardized factor loadings and the results of the significance tests

Variable	*Can1*	*Can2*	*Can3*	*Can4*
Fear	-3.07	1.83	0.23	-0.26
	0.6997	<.0001	0.2006	0.2698
Feeding	-1.10	0.25	0.52	0.80
	0.1864	0.6626	0.3989	<.0001
Activity	12.67	-0.17	0.24	0.33
	<.0001	0.5123	0.5305	0.8994
Immunity	-1.12	-0.83	0.90	-0.19
	0.8231	0.2319	0.0002	0.1339

Table 2-7 Individual bird loadings onto canonical variables

	Activity	Fear	Immunity	Feeding
Bird	*Can1*	*Can2*	*Can3*	*Can4*
0049	18.767	1.532	-1.201	-0.625
0060	3.832	0.063	0.206	1.870
2940	14.277	3.783	-0.002	0.396
0041-161	2.693	-0.810	1.623	0.039
0074-107	-20.591	0.952	-1.376	1.117
0074-171	2.645	-0.035	-1.365	-0.957
0074-181	-12.976	0.383	1.338	0.390
0075-140	3.583	1.704	0.845	-0.597
0075-200	2.531	-1.060	0.166	0.393
90E-1193	4.235	-0.873	1.346	-0.937
90E-1197	11.446	-4.620	-0.823	0.238
90E-1455	2.915	-1.070	-0.043	0.187
90E-1717	-10.770	0.105	-0.481	-0.251
90E-1767	-22.588	-0.055	-0.233	-1.263

Figure 2-2 Tree dendogram and cluster membership.
Birds with missing value for any variable were not clustered and are shown at left.



2.5 Discussion

Results of this study suggest that budgerigars, small Australian psittacines, have differences in personality, as birds appeared to behave consistently boldly or shyly. This was demonstrated by the fact that budgerigars' behavioral responses within the contexts of activity and feeding were consistently bold or shy, with bold birds having higher activity and higher feeding scores, respectively. According to (Ioannou et al., 2008) high activity and feeding behavior are characteristics of bold individuals, whereas low activity and reduced feeding are characteristics of shyness. In this study birds who exhibited consistently higher activity levels in the barrier threat, open field, tendency to flock, and novel object tests and feeding levels in the feeding competition test were considered to be bold, while in the contrary, those with low activity and feeding levels were classified as shy. We were able to characterize individuals as bold, medium, or shy based on their activity responses, with bold birds being those with high activity, shy birds being those with low activity, and the birds with medium activity falling along the continuum between bold and shy. Our results also suggest that budgerigars do not behave consistently with regard to their fear responses between situations, as indicated by lack of significant correlations between fear variables measured. Two measures of immunity, percent of bacteria killed in the whole blood killing assay and lysis at 20 minutes in the hemolysis-hemagglutination assay, appeared to be related to personality, with percent bacteria killed being gender-specific. The relationship between percent bacteria killed and personality could be affected by testosterone in the male budgerigar. However, supplementation of DHA in the diet had no apparent effects either in terms of bird personality or immunity status. Budgerigar blood was not assayed for the presence of

DHA during the experiment, so it is possible that the lack of effect may have been due to low levels or no DHA being absorbed by the birds. Alternatively, it may be necessary for birds to consume DHA while still developing in order for DHA to be properly absorbed and incorporated into tissues. Finally, inclusion of DHA in the diet may have made the pellets less palatable, lowering feed intake and therefore DHA consumption. Individual bird feed intake was not recorded, so lowered intake levels may have occurred.

Birds' lack of consistent behavioral responses between fear variables could be due to differing levels of threat in the situations in which we measured fear, the open field, barrier threat, and predator threat tests. In the open field, we expected less fearful birds to visit more locations in the center than the periphery of the open field, which was possibly the lowest level of threat in the experiment, since there was not an immediately apparent danger and the entire arena was visible. In the barrier threat, the threat level was elevated, as the bird would have to cross into an unknown area to acquire the resources visible through the barrier. Finally, the 'danger' level was highest in the predator threat test, as an actual 'predator' appeared during testing.

Correlations between variables measuring activity levels showed that individual behavioral responses were consistent across the different tests performed in this study. Activity levels measured in the open field test as total distance traveled and minimum convex polygon area were both positively correlated with activity levels measured as the number of lines crossed in the barrier threat test and in the attractive novel object test. In addition, birds that showed high activity, measured as the number of lines crossed, in the test of exposure to familiar birds also had consistnet activity levels in the test of exposure to unfamiliar birds. These results suggest that budgerigars' responses were consistently

bold or shy as indicated by activity levels observed across the experimental situations in this study. Similar to activity, feeding response was consistently bold or shy as illustrated by the positive correlations between all three variables measured in the food competition test: number of seeds eaten alone, number of seeds eaten in competition, and competitive ability, which was measured as percent of seeds eaten in competition.

Interestingly, results indicate that activity level had the highest loading score for the canonical variable used in the cluster analysis. Therefore, activity likely explained the largest portion of variance in the data. This can suggest that activity level in any context may be a better and more efficient parameter to evaluate personality profiles (either bold or shy) of an individual as compared with a feeding context.

It is relevant to note that although budgerigars showed consistent patterns of personality within either activity or feeding, clearly there was no association between the two. This was evidenced by the absence of correlations between activity and feeding responses across all different experimental situations in this study. The results suggest that bold or shy personalities may not be consistent across contexts, which has been shown for zebra fish (*Danio rerio*) (Moretz et al., 2007) and threespined sticklebacks (*Gasterosteus aculeatus*) (Bell, 2005; Dingemanse et al., 2007), in which individuals from one habitat type exhibited different behavioral consistencies than individuals in other environments. The adaptive hypothesis (Bell, 2005) states that relationships between behaviors should not exist unless the relationship itself is adaptive and that different groupings of behaviors may be adaptive in different selective environments. Differences in personality types between two stickleback populations were thought to be due to differing selection pressures of predation between the two habitats (Bell, 2005). It

is possible that boldness in activity and feeding are coupled in other populations of budgerigars, and that there is not an advantage to their mutual dependence in the population used in this study. Other populations of budgerigars, whose personalities have been shaped by different environments, life history experiences, or genetics, may show different relationships between behavioral contexts. Additionally, independence of personality in feeding vs. activity may confer an evolutionary advantage to budgerigars by allowing individuals to develop optimal responses for each context. If personality in different contexts is not independent, an evolutionary constraint may exist in which the correlation between the two contexts prevents the development of an optimal response for either (Schluter, 1996).

This study also provides some evidence of gender differences in behavioral responses of budgerigars. Generally, males had higher feeding ability, measured as number of seeds eaten, both when feeding in isolation and in a competitive situation in the feeding tests. These results suggest that males were generally bolder feeding strategists as compared to females. Interestingly, there was a negative correlation between number of seeds eaten alone and competitive ability in females, such that females who ate a higher number of seeds alone consumed a smaller percentage of the total number of seeds when in a competitive context. This result indicates that the behavioral response of female budgerigars in the feeding context is affected by the social situation, so that boldness depends on the presence of conspecifics. According to Smith (2008), one definition of personality is correlated behaviors in different contexts within the same situation (in this case, boldness when conspecifics are present). As the feeding

rate test was the only test in this experiment in which budgerigars were presented with a competitive social situation, it is impossible to determine if female budgerigar personality is in fact dependent on the presence of social competitors.

Regarding the parameters of immunity considered in this study, the results of the Kruskal-Wallis test showed a gender effect in percent of bacteria killed, with blood from females being able to kill significantly more bacteria when compared to males (see Table 2-4). Because these gender differences were detected, correlation analysis regarding percent bacteria killed was repeated for each gender. Results of this second analysis failed to detect any significant correlation between percent bacteria killed and any of the feeding variables for females. Contrarily, percent of bacteria killed in males was negatively correlated with both seeds eaten in competition and competitive ability. This suggests that bolder males, those who compete more effectively for food, had lower innate immunity than shyer, less competitive males. All these results taken together may be interpreted as evidence of challenged immune status for males, which may be testosterone dependent. The immunocompetence handicap hypothesis (Hamilton & Zuk, 1982; Folstad & Karter, 1992; Zuk & Stoehr, 2002; Roberts et al., 2004) suggests that secondary sexual characteristics are honest signals of male quality. The size and color intensity of these characteristics are related to the testosterone required to produce them; however, high testosterone levels it are also known to cause immunosuppressive effect which only high quality, possibly dominant males can overcome (Hamilton & Zuk, 1982; Folstad & Karter, 1992; Zuk & Stoehr, 2002; Roberts et al., 2004). Since the immunocompetence handicap hypothesis was proposed, many studies have gathered

evidence that testosterone causes immunosuppressive effects indirectly by increasing corticosterone levels (Evans et al., 2000; Casto et al., 2001; Owen-Ashley et al., 2004).

The finding that bolder, more effectively competitive males had poorer immune status than shy males may possibly be related to dominance. Dominance status determines the priority of access to resources such as food (Evans et al., 2000), and high dominance status can be accompanied by high levels of testosterone (demonstrated in white-throated sparrows *Zonotrichia albicollis* (Archawaranon & Wiley, 1988). Therefore it would be expected that highly dominant individuals may suffer a challenge to their immune systems through this hormone dependent mechanism. However, bolder, possibly dominant males were also able to consume more food in the feeding test than shy males, and increased access to food has been show to increase immunocompetence in experiments with broiler chickens (Glick et al., 1981); bobwhite quail (*Colinus virginianus*) (Lochmiller et al., 1993); and nestling barn swallows (*Hirundo rustica*) (Saino et al., 1997). The effect of increased access to food by bolder, possibly dominant birds was unlikely to be significant in this experiment, since birds were provided with *ad libitum* access to a pelleted diet in their home pens and likely had sufficient access to food resources over time.

In this context, it seems quite possible that bolder, competitive male budgerigars, when compared to shy individuals, may have a challenged immune status as result of the testosterone hormonal mechanism. However, results must be taken with caution as the hormonal status of the budgerigars in this study was not determined.

Lysis after 20 minutes, a measure taken from the hemolysis-hemagglutination assay, had an unexpected negative correlation with latency to resume eating after the predator threat and with net distance traveled in the open field; it was positively correlated with latency to cross the barrier. Lysis scores were not expected to be correlated with any behavioral variables, as the focus of the hemolysis-hemagglutination assay was to measure constitutive innate immunity using agglutination scores to gauge the effectiveness of birds' natural antibody response to a foreign protein. Because the focus of this assay was on agglutination rather than lysis, part of the procedure involved heat-treating the blood to deactivate complement, which is normally responsible for lysis (Matson et al., 2005). A possible explanation for the presence of lysis might be that budgerigar complement could have a novel amino acid sequence that provides better heat stability than for human or chicken complement (Klasing, personal communication). If this is the case, birds with high lysis scores are exhibiting higher innate immunity, since complement engages natural antibodies in a mechanism that does not require previous exposure to an antigen (Matson et al., 2005). It is also possible that the lysis observed in this case was due to the action of natural antibodies without the aid of lysis, which may cause lysis in a slower, less effective manner without the aid of complement.

If it is true that birds with high lysis scores were showing high innate immunity, the same birds were also bolder as indicated by their shorter latencies to resume eating in the predator threat test. This response is in agreement with a study with crickets (*Gryllus integer*) that reported a negative correlation between duration of freezing response after a predator threat and lysis (Kortet et al., 2007). It was suggested that bolder crickets were able to consume more resources and better maintain their constitutive immunity, a

component of their immune system that does not respond to specific antigens (Kortet et al., 2007), and this may be the case for budgerigars as well. However, budgerigars that were shyer in regards to activity, as indicated by traveling shorter net distances in the open field and having longer latency times to cross the barrier, also had also high innate immunity as indicated by their high lysis scores. Birds with higher innate immunity were bold in the predator threat test but not in the open field or barrier threat. These results regarding lysis could be considered contradictory, since birds that were less fearful in the predator threat should be expected to be less fearful in crossing the barrier, although the level of 'danger' was much higher in the predator threat than in the barrier threat. However, results of the correlation analysis showed no significant correlations between fear variables, so birds' fear responses (latency to resume eating and latency to cross the barrier) are likely to be inconsistent. Since the correlation analysis also suggested that budgerigars should behave consistently with regard to activity level in all contexts, it is surprising that activity level measures from other tests were not correlated with lysis scores. In general, all results regarding correlations between immune and behavioral variables should be taken with caution, as we cannot ensure that an individual bird's immune status was the same at the time of both behavioral and immunological testing.

In general, the results of this study provide some evidence to support the hypothesis that budgerigar personality may be defined by a bold-shy axis. In particular, activity level emerged as an important component to budgerigar personality. Feeding behavior appeared of secondary relevance but was a more noteworthy component of personality in males. Male feeding behavior appeared to be related to innate immunity, possibly through hormonal mechanisms such as the effect of testosterone in highly bold,

possibly dominant birds. Finally, although the results show that budgerigars behave consistently with regards to activity and feeding, no correlations were found between their fear responses in different situations. Overall, the results of this study indicate that activity is an important component of budgerigar personality, and that activity and feeding personality traits are probably governed by independent mechanisms, possibly increasing the ability of budgerigars to behave optimally in different situations. The relationship between behavior and testosterone may help to explain links between male budgerigar personality and immune status.

Chapter 3 Summary and Conclusions

Personality is evidenced by individuals showing correlated behavioral responses within or between contexts. In this study, we subjected 23 adult budgerigars to a sequence of behavioral tests in a variety of contexts and measured their responses in the contexts of fear, feeding, and activity. At the end of the study, two immunocompetence tests were also conducted. Behavioral and immune responses were subjected to correlation analysis to determine the consistency of boldness in responses within the categories of fear, feeding and activity, and to investigate if immune responses were somehow associated with behavioral responses. Kruskal-Wallis analysis was performed to detect potential effects of gender or dietary supplementation with DHA on the birds' responses. Additionally, cluster analysis was performed to group the birds based on their boldness ranking, but only for activity variables, which emerged as the most important components of personality.

Several behavioral responses within the activity category (net and total distances traveled, area of the minimum convex polygon, lines crossed in the barrier threat test, lines crossed in the presence of unfamiliar conspecifics, and lines crossed in the presence of the attractive novel object) were correlated, indicating that budgerigars behave consistently boldly or shyly across different contexts with regards to activity. Variables within the feeding category (number of seeds eaten alone or in a competitive situation and percent of seeds eaten in competition) were also correlated, suggesting that birds were also consistently bold or shy within the feeding context. No variables were significantly correlated within the fear category. Additionally, activity, feeding, and fear responses appear not to be interrelated as indicated by the total lack of correlations

between the categories. In this study we also found that innate immunity, measured by percent bacteria killed, was negatively correlated with two feeding variables (number and percent of seeds eaten in competition). However, this effect was exclusively seen in males, suggesting that males who ingested a larger number of seeds in the test may have a lower innate ability to ward off bacterial infection.

Lysis at 20 minutes was negatively correlated with latency to resume eating after the predator threat and net distance traveled and was positively correlated with latency to cross the barrier in the barrier threat. These results may be explained by results of the general correlation analysis, which suggested that birds respond inconsistently with regard to fear responses, in this case latency to resume eating after predator threat and latency to cross the barrier. The negative correlation between lysis and net distance traveled in the open field is confusing, as general correlation results indicate that budgerigars behave consistently with regard to activity levels; therefore, correlations between lysis and other activity variables would have been expected. This result could be due to changing immune status of individual birds during the period of the experiment.

The Kruskal-Wallis results showed that males ate more than females as measured by all of the feeding variables, and that females had better scores in a whole blood killing test. Finally, the cluster analysis grouped birds into three clusters: high (bold), medium (average), and low (shy) activity levels.

The results of this suggest that budgerigars exhibit consistent responses along a bold-shy continuum within the behavioral categories of activity and feeding, although not within fear responses. Boldness was not consistent across the three behavioral contexts for the budgerigars used in this experiment, a result which is consistent with other

examples in the literature for zebrafish (Moretz et al., 2007) and threespined sticklebacks (Bell, 2005; Dingemanse et al., 2007). Additionally, males which were bolder in the feeding context tended to have lower innate immunity, a result which may be explained by the influence of testosterone. In general, activity was found to be the best indicator of consistent boldness or shyness in budgerigars, although boldness in feeding may be an important personality component in males. The results support our hypothesis that budgerigars would exhibit consistencies in their behavior within contexts, but not the hypothesis that they would also exhibit consistent behaviors across contexts. Finally, our results did not support the hypothesis that budgerigars fed with supplemental DHA would differ in behavioral and immune response.

APPENDICES

4.1 Intense food competition

The food competition trial was intended to measure competitive ability in a more intensive situation than the feeding rate test. Birds were to be tested singly or in groups of two as with the feeding rate trial. During the trial, one seed would be introduced to the pen every thirty seconds in order to create an intensely competitive situation. A beep would be sounded before each seed introduction in order to prepare the birds for the competition. This test was to be conducted under two scenarios: consistent placement of the seed, or random placement of the seed in one of five entry positions.

The test pen was set up with five 1”-diameter PVC pipes (set at 45° angles to the floor) protruding about twenty cm into the pen from the left wall. Pipes were evenly spaced along the wall, and the bottom ends of the pipes were about eight cm from the floor. It was possible to drop seeds into the pen through these pipes while remaining out of sight of the birds. For the consistent placement scenario, seeds would only be dropped into the center pipe. In the random placement scenario, seeds would randomly be dropped into one of the five pipes. All birds would be tested in groups of two and then singly in the consistent placement scenario before being tested in the random placement scenario.

Birds received five rounds of training in groups of three in the test pen set up as described above. Groups were composed of randomly-selected birds from the same home pen. After three minutes in the starting box as a group, birds were released and given one minute to acclimate to the pen before seeds were introduced. At that point, seeds were dropped through the center PVC pipe at 30-second intervals for five minutes. After two rounds of training, only one bird had eaten any of the seeds. For the fourth round of

training, the pen setup was modified so that a small amount (four or five) seeds were initially placed at the bottom of the central PVC pipe. No birds approached the seeds. During the third round of training, a Petri dish containing the non-DHA pellet diet was placed at the bottom of the central PVC pipe in an attempt to induce the birds to approach that location. No birds approached the dish at any point during that training. In the fifth round of training, the pellet dish remained in place, but seeds were dropped in at one-minute intervals to reduce the frequency of disturbance in the pen as the seeds came out of the PVC tube.

Next, we modified the pen setup further because the budgerigars' neophobic reaction to the PVC tubes was not decreasing. PVC tubes were all removed, and a Petri dish containing only ten seeds was added to the center of the pen. Although this scenario resulted in less intense competition that the single-seed delivery, the number of seeds was very limited. Birds received four rounds of training with this setup, in groups of four randomly selected from the same home. Additionally, all birds were feed-restricted for three hours prior to these rounds of training. Birds were given ten minutes after release from the start box to eat the seeds. After the fourth round, no birds had eaten any of the seeds. Possible reasons for the failure of this test might include extreme neophobic reactions to the test apparatus, or possibly the effects of the continued stress of being tested throughout the experimental period.

4.2 Color learning

This was intended to be a test of learning, measuring the birds' ability to associate a specific color, orange, with a food reward. Orange was chosen as it was the complement to the blue floor of the test arena and therefore would be highly visible. In There were six phases of training followed by one testing session per bird. The test pen was initially set up with three Petri dishes placed in a horizontal line across the pen, parallel to the starting box. The sides of the Petri dishes were covered in colored tape, either white or orange, to indicate that they contained food or were empty, respectively. During the training sessions, placement of the dishes in the line was random. During testing, only two dishes were used: one orange and one white. All training and testing sessions were performed using individual birds, and each session lasted ten minutes.

Training was split into two phases, differing in how the birds were introduced into the pen. For the first phase, consisting of four training sessions, birds were released directly into the pen from their transportation container, which was placed on the ground and opened at the door of the test pen. During these four sessions, the line of Petri dishes was at the middle point of the test pen, and there were two orange dishes and one white dish. By the end of the first phase, about half of the birds were eating seeds. The second phase of training utilized the starting box. There were three training sessions in the second phase: in the first session, the Petri dishes remained at the middle point. For the second session and third sessions, the dishes were moved so that they were approximately 15 centimeters from the back of the pen. Additionally, one of the two orange dishes was removed, so that there was one white dish and one orange one. During the second training phase, about half of the birds continued to eat seeds.

During the test, the setup was identical to the final training session: two dishes, white and orange, were placed 15 cm from the back of the pen, evenly spaced along a horizontal axis. Birds were introduced from the starting box and were given ten minutes for the test. Number of visits to each dish were recorded. A visit was defined as the bird coming within five centimeters of a dish. Only five birds (38%) visited any dishes. All of the birds who made visits only visited orange dishes, suggesting that the birds were likely able to learn to associate orange with a food reward. However, due to the low number of birds actually participating in this test, it was dropped from the analysis. Birds may have been unwilling to investigate the dishes because of neophobic reaction combined with stress of being alone in the test situation.

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