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Parasite Infection Mediates Trait Tradeoffs in *Fundulus heteroclitus*

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in the Department of Biology

By

Sarah Dunn

Under the mentorship of Dr. Tavis Anderson

ABSTRACT

To be successful, an animal must eat, grow, and reproduce. With limited resources, there are tradeoffs between these critical life history parameters but the direction of the tradeoffs is largely unknown in a changing environment. To determine whether environmental context affects life-history tradeoffs, I surveyed and quantified investment into reproduction, growth, and a proxy for immunity (parasitism), in the mummichog, *Fundulus heteroclitus*, a common inhabitant of salt marshes in Georgia. Three salt marsh sites along coastal Georgia (Shellman Bluff, Skidaway Island, and Tybee Island) were selected using a proxy for anthropogenic disturbance (impervious surface), which also fell along a gradient in chronic stress. I measured reproductive investment, parasitism as a proxy for immunity, and fish condition. I found that parasitic infection, my proxy for immune investment, affected a fish’s investment into reproduction but that there were only differences in fish that were chronically stressed. Specifically, in stressed environments fish appeared to invest in reproduction to the detriment of immunity and body condition. However, in environments with fish that were less stressed, investment into growth, immunity, and reproduction was maintained almost equally. These data reveal how environmental context can affect important life-history tradeoffs, and suggest that though individuals may be able to reproduce in stressful conditions, they may suffer more from infectious disease.

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Sarah M. Dunn
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Introduction

To survive and persist, an animal must acquire enough food, be able to reproduce, avoid being eaten, grow, and use its immune system to prevent infection. However, each of these important traits is costly to maintain, which can lead to tradeoffs. That is, investment or allocation into one trait comes at a cost to another trait (Ricklefs & Wikelski 2002). Traditionally, studies examining tradeoffs quantify one trait (e.g., reproduction) after altering investment into another trait (e.g., immunity) (Stahlschmidt et al. 2013a). These two-trait studies have greatly advanced our understanding of tradeoffs, but organisms are typically under selective pressure to optimize more than two traits simultaneously. For example, in addition to immune function and reproduction, size and growth is critical to animal fitness (mating, food acquisition, and predator avoidance).

In addition to physiological state, an organism's investment into life history parameters may also be affected by stressful environments. Previous research has defined stress as chemical and physical implications that cause reactions in the organism that possibly contribute to an increase in disease and death (Rottmann et al. 1992). Not all stress is considered harmful to the organism and can also be simply defined as an increase in the demand for energy when an organism is found in an altered state. The term stress was separated into two phases (Sale 1985). The first, “distress” which promotes alteration to the physiology of an organism and possible decrease of legitimacy of the organisms life (Selye 1985). The second, “eustress” which occurs under stimulations that increase biological performance (Selye 1985). Prior research has shown that under stressful conditions, allocation to storage or maintenance can take precedence over allocation to reproduction (Perrin et al. 1990; Rogowitz 1996). However, both reproduction and immune function may be maintained if conditions are favorable (e.g., French et al. 2007a,b;
Shoemaker et al. 2006; Xu et al. 2012) which suggests that tradeoffs may be facultative or resource-dependent. Thus, in stressed conditions, an individual should invest energy in one trait, leading to a trait tradeoff as less energy can be allocated to other life history traits. Alternatively, in a low stress environment an organism will be able to maintain all bodily functions.

Parasitism, which is defined as an interaction in which the parasite is physiologically dependent upon the host, can be influenced by an individual’s immune function. An individual with a robust immune response is more likely to have a lower parasite load; conversely, an individual with a poor immune response is more likely to have a high parasite load (Jones 2001). This is particularly true for fish infected with parasites that exhibit complex multiple host life cycles. These parasite species have a life cycle that may include fish (or bird) definitive hosts and several intermediate invertebrate hosts and their life stages frequently are found in the visceral organs. Prior research using model fish systems have demonstrated elegant immune responses to parasitic infections (Jones 2001). Thus, parasite infection can dramatically affect an organism’s energy allocation budget and the dynamics of trait tradeoffs.

To date, there are few studies that consider the tradeoffs between three important life history traits simultaneously. The goal of my project is to advance the understanding of tradeoffs to a complex-trait system within environmentally realistic situations. My thesis used a trait tradeoff system in the common killifish, *Fundulus heteroclitus*, and quantified: 1) reproductive investment via a gonadosomatic index; 2) the intensity of parasite infection; and 3) investment into body maintenance and condition (Fulton’s Condition Factor). These data were collected from fish in salt marsh areas that represent a gradient of human disturbance: this disturbance affects chronic stress levels of the fish, which should affect whether tradeoffs occur. I have two predictions: first, given prior studies, I expected to see reduced reproductive output in heavily
parasitised fish that are chronically stressed; alternatively, reproductive output will remain constant as infected fish will decrease investment in immunity. In addition, I only expected to see these trade-offs in suboptimal environmental conditions: specifically, in fish that are chronically stressed, I expected to see differential investment into traits, and in those fish that are in good condition, I expect to see no change in investment into traits (Figure 1).

**Materials and Methods**

*Site Selection*

I surveyed three salt marsh sites on the coast of Georgia that reflected a gradient in urbanization. The sites included a salt marsh on the western side of Tybee Island (32°01′29″N, 80°90′18″W), the Saltmarsh Ecosystem Research Facility at Skidaway Island (31°97′57″N, 81°03′26″W), and a salt marsh on the western side of the city of Shellman Bluff (31°58′07″N, 81°36′49″W). The three salt marshes formed a gradient defined by the urbanization of the surrounding area. The gradient placed Shellman Bluff as the lowest urbanized area due to least amount of anthropogenic disturbance, Skidaway Island followed as an intermediate urbanized area. Tybee Island was the highest level on the gradient due to the highly urbanized area that surrounds the salt marshes of Tybee.

*Sample Collection*

My study focused on the common killifish, *Fundulus heteroclitus*. The species was selected because it is a highly abundant resident marsh species along the east coast of North America, plays an important role in marsh food webs (Anderson and Sukhdeo 2011), and has a wide range of possible helminth parasites (Harris and Vogelbein 2006). The fish were caught by randomly distributing minnow traps through each site baited with dry dog food. Each site was sampled using a minimum of 5 minnow traps placed more than 20 meters apart through rivulets
in each marsh. Following one tidal period, traps were collected and killifish were identified and a random subsample of killifish were collected for further analysis.

**Quantifying chronic stress in fish**

A potential marker for chronic stress is glucose levels in viscera. Glucose is a carbohydrate broken down to fuel cells and organism functions. In high stress environments, individuals are likely to have higher glucose levels due to the higher demand for energy (Ceriello et al. 1996; Vijayan and Moon 1994).

Fish condition and stress level were assessed using a glucose assay. The glucose level was assessed for 45 of the fish from the three collection sites using a Glucose (HK) Assay Kit (Sigma-Aldrich #GAHK-20). For the reaction to occur, adenosine triphosphate (ATP) phosphorylates the glucose (Bellaloui et al. 2013). The reaction is catalyzed by the enzyme hexokinase which forms glucose-6-phosphate (G6P) (Bellaloui et al. 2013). G6P is oxidized when the compound was manipulated by the oxidized nicotinamide adenine dinucleotide (NAD).

The oxidation reaction is catalyzed by the enzyme glucose-6-phosphate dehydrogenase (G6PDH) and forms the compound 6-phosphogluconate. The process of oxidation for the reaction is understood as the equimolar total of NAD being reduced to the compound NADH and consequently the sequential rise in absorbance at 360 nm is proportional to the glucose concentration found in the liver sample (Bellaloui et al. 2013).

Each fish liver was thawed at room temperature, homogenized with 1 ml of deionized water to obtain uniform particles. The extract was diluted to obtain a range of 0.05-5 mg glucose ml-1. Then 200 microliters of the homogenized liver was placed into a cuvette with 1.8 ml of Glucose Assay Reagent and incubated at room temperature for 15 min. A sample blank consisting of 200 microliters of sample and 1.8 ml of deionized water, and a reagent blank
consisting of 1.8 ml of Glucose Assay Reagent and 200 microliters of deionized water were also prepared. To create a standard curve, I mixed four standards of known glucose concentration at 0 mg/ml, 0.1 mg/ml, 0.25 mg/ml, 0.50 mg/ml. After 15 minutes of incubation, the absorbance was read at 360 nm using a Thermo Fisher Scientific Spectronic 200 spectrophotometer. The concentration of glucose was expressed as mg/ml.

*Quantifying parasite infection and investment in reproduction*

To determine whether the fish was infected with parasites, I humanely euthanized and necropsied ten fish from each site. The fish was placed into a buffered solution of 300 mg/L tricaine methanesulfonate (MS-222) until cessation of opercula movement and muscle contractions terminated. The fish were then weighed and measured, and a comprehensive necropsy to quantify helminth parasites was conducted. Specifically, the exterior of the fish was examined for ectoparasites whereby each fin and each gill arch was removed for a close examination under a stereomicroscope. The viscera were removed and internal body organs (heart, liver, spleen, swim bladder, gall bladder, digestive tract, gonads) were individually examined under a stereomicroscope. All of the major organs were removed and weighed individually following examination. Each liver was collected and immediately placed in a freezer (-81 C) for subsequent use in a quantitative glucose assay. All helminths were heat fixed and stored in a solution of 70% ethanol until staining and identification. Nematodes were placed in a solution of 5% glycerol and 70% ethanol and were identified after approximately 2 weeks. All parasites were identified using keys (Yamaguti 1958; Schell 1970; Anderson et al. 1974) and primary literature (see Harris and Vogelbein 2006 and references therein).
To determine investment in reproduction I calculated the gonadosomatic index (GSI) for each fish, which is determined as GSI = [Gonad Weight / Total Tissue Weight] x 100 (Barber and Blake 2006).

**Quantifying fish condition**

To quantify the fish’s condition I calculated Fulton’s Condition Factor, K. The formula is $K=100 \times (W / L^3)$ where the K is the coefficient of condition, W is the fish’s weight in grams (g), and L is the fish’s length in millimeters (mm) (Barnham and Baxter 1998). The length is cubed in the equation because the growth of the fish in weight is proportional to volume growth (Barnham and Baxter 1998). The value produced for K is influenced by many factors of the fish, such as age. More importantly, K can be influenced by reproductive organ weight, which varies depending on developmental stage of the reproductive system. For an appropriate comparison, fish must be collected at the same time of the year (Barnham and Baxter 1998): all my fish were collected at the same time point.

**Statistical analysis**

My data includes fish level metrics for: 1) relative body condition (K); 2) a proxy for reproductive fitness (GSI); and 3) a proxy for immunity (intensity of parasite infection). These data points are collected from the fish at three sites and each site reflects a level of chronic stress (measured as glucose concentration in fish liver).

To understand whether there were significant tradeoffs between immunity, reproduction, and locomotion, I used linear mixed-effects models (LMEM). These models attempt to explain sources of variability across a set of individual units of observation as a function of a series of independent variables, random sources of variability, and error. This method allowed me to determine whether there are significant effects of parasitism on: 1) allocation of energy to
reproduction; and 2) allocation of energy to body maintenance. In these models, the dependent variable ($y$) is the gonadosomatic index, and the fixed factors are as follows: (1) $x_1$, fish condition (K); (2) $x_2$, intensity of parasite infection; and (3) $x_3$, index of chronic stress (glucose concentration). The random factors consider spatial variability, and assume that our marshes are random samples from a larger population, and, consequently, they model site variability, with 3 levels (Tybee, Skidaway, Shellman Bluff). To assess the significance of fixed factors, we used parametric bootstrapping, with the estimation of parameters using restricted maximum likelihood.

**Results**

For this analysis, 46 fish were examined, 17 from Shellman Bluff, 16 from Skidaway Island, and 13 from Tybee Island. For the glucose assay, 45 livers were homogenized and tested for glucose concentrations (mg/ml) as a proxy for chronic fish stress. The mean glucose concentration at Shellman Bluff was $0.51 \pm 0.07$ standard error, the mean concentration at Skidaway was $0.30 \pm 0.08$ standard error, and the mean concentration at Tybee was $0.60 \pm 0.12$ standard error (Figure 2).

The 46 fish which were weighed and measured were analyzed for the average condition of the fish at each site. The mean Fulton’s Condition Factor was for Shellman Bluff was $1.05 \pm 0.04$ standard error, for Skidaway Island was $1.25 \pm 0.05$ standard error, and for Tybee Island $1.32 \pm 0.09$ standard error (Figure 3).

The 46 fish which were weighed and measured were analyzed for the average condition of the fish at each site. The mean gonadosomatic index was for Shellman Bluff was $1.9 \pm 0.30$ standard error, for Skidaway Island was $2.5 \pm 0.35$ standard error, and for Tybee Island $1.1 \pm 0.18$ standard error (Figure 4).
A total of 46 fish were studied from the three marshes. Seven taxa of metazoan parasites were identified: these included *Contracaecum* spp. nematodes found encysted as larval stages in the liver and and viscera; the adult digenean *Lasiotocus minutus* and one unidentified larval digenean metacercaria; the ectoparasitic monogenean species *Swingleus anci*strus; the copepod *Ergasilus funduli*; the branchiuran species *Argulus funduli*; and the cestode *Glossocercus* spp. as a metacestode stage encysted in the visceral organs. These taxa infected more than 65% of the killifish examined, the prevalence by site was 68%, the mean intensity of infection by site was 11.13.

There was statistical evidence to suggest an association between parasite infection status of in *F. heteroclitus* and investment in reproduction (Table I: log likelihood = -46.69, d.f. = 4, \( p = 0.048 \)). In addition, there was statistical evidence to suggest an association between chronic stress in *F. heteroclitus* and investment in reproduction (Table I: log likelihood = -47.40, d.f. = 4, \( p = 0.026 \)). There was no evidence to suggest that body condition had an effect on investment in reproduction (Table I: log likelihood = -50.03, d.f. = 4, \( p = 0.277 \)). Similarly, there was no statistical support for an stress \( \times \) infection \( \times \) body condition interaction, on the investment into reproduction in *F. heteroclitus*.

**Discussion**

A key finding in my data is that parasitic infection, my proxy for immune investment, affected a fish’s investment into reproduction. This supports previous studies that have demonstrated important tradeoffs between life-history traits such as reproduction and immunity (Stahlschmidt et al. 2013). However, in my study this dynamic tradeoff between immunity and reproduction only occurred in situations where fish were under chronic stress. In sites where fish were under high stress, an individual fish would tend to invest heavily in reproduction but not
into immunity. These data join a growing body of literature that suggests studying tradeoffs in realistic environments is likely to provide different results than a study in ideal “lab” environments. Specifically, under stressful conditions, data has been collected to show that allocation to storage or maintenance can take precedence over allocation to reproduction (Perrin et al. 1990; Rogowitz 1996). However, the opposite has also been shown to occur; that both reproduction and immune function may be maintained if energy is in abundance or if conditions are favorable (e.g., French et al. 2007a,b; Shoemaker et al. 2006; Xu et al. 2012). In my system, fish in stressed conditions (i.e., those with elevated liver glucose concentrations), invested their energy in reproduction and body maintenance. Alternatively, those fish in a relatively low stress environment appeared to be able to that the standard paradigm of trait tradeoffs may not always be correct, and that trait tradeoffs could be facultative.

The concept that an organism under chronic stress does not perform well is widely accepted (Sanders 1983). Two sites, Shellman Bluff and Tybee Island, were classed as “disturbed” because the fish were found to have high glucose levels in their levels; this is indicative of chronic stress. Alternatively, Skidaway Island was classified as “pristine” because fish tended to have lower liver glucose levels which suggest that they weren’t living in a stressful environment. Our data demonstrate that fish under chronic stress (e.g., higher glucose levels) at Shellman Bluff invested a significantly smaller amount of energy into immunity, using the majority of their energy to maintain reproductive output (Fig. 4). This impacted the fish’s ability to maintain body condition (Fig. 3), and fight infection, evidenced by higher parasite levels in those fish found in Shellman Bluff (Fig. 5). The second site that had fish that were chronically stressed was Tybee Island: at this site, the fish maintained body condition and immunity, but did not invest into reproduction. However, in our site where fish were not under stressful conditions
(Skidaway Island), all life-history traits were maintained. These data support prior research, such as work by French et al. (2007) and Shoemaker et al. (2006) who have demonstrated that both reproduction and immune function are maintained in favorable conditions.

Tybee Island and Shellman Bluff are a microcosm for the coastline of Georgia, and more broadly the United States: in these areas, the population is expected to increase by 46% over the next 15 years, with a concentration in the sparsely populated coastal counties. Further, between 1992 and 1997, developed land increased by 27.4% This development has created a relatively hostile environment for fish in these areas, restricting the amount of energy available for fish and exposing them to an abiotic environment that may or may not facilitate growth and reproduction. My data demonstrated that fish at Skidaway Island invested the highest amount of energy into reproduction: this site is characterized by a relatively low amount of abiotic and anthropogenic disturbance. However, fish collected from Tybee Island or Shellman Bluff had differential investment into reproduction, and had slightly different strategies. Fish at Tybee Island had low reproductive output, but were very large and had the lowest parasite burden. Fish at Shellman Bluff had high reproductive output, but had very high parasite loads, and had relatively poor body condition. The Shellman Bluff situation could be likened to “putting all the eggs in one basket” as individuals were able to produce offspring, i.e., evolutionary fitness, but suffered considerable pathology from parasite infection and are unlikely to live for an extended period of time because they have such poor body condition.

My data demonstrated that Tybee Island produced the fish with the highest body condition; Tybee Island was followed by Skidaway Island and then lastly the fish at Shellman Bluff had the lowest body condition. The extremely low investment into the body size from the fish at Shellman Bluff is offset by the high investment into reproduction. Furthermore, the relatively
high investment into body maintenance for the fish at Skidaway Island can simply be explained by the lack of trait tradeoffs found in the “pristine” environment. The fish at this site are able to equally invest into all three traits as there is likely an abundance of resource and energy. The high investment into body maintenance for Tybee Island in a site that lacks reproductive investment creates an interesting question, as it is a different strategy to fish observed at Shellman Bluff. What about the environment at Tybee Island would prove size and body maintenance advantageous over reproductive investment? One possibility is that Tybee Island is disturbed, but at relatively predictable time points, i.e., a seasonal stressor. If this is the case, it would be beneficial for the fish to invest as much energy as possible into growing/maturing so sexual reproduction could be reached before a disturbance occurs.

One potential limitation of my analysis is that parasite species were not weighted or ranked based on the level of impact or harm that is inflicted on the organism via the parasite (i.e., all parasites were equal). It is clear that “lumping” all parasites together is not realistic but throughout the experiment and analysis I was consistent, i.e., the concept that any parasite has the same affect on the immune response was applied consistently. Thus, this is not a hindrance of our results only a limitation to understanding the true effects of parasites on a host. Further exploration of forming a system of parasitic effect is possible extension to this study.

Therefore, the present study which used field sampling and laboratory analyses, suggests that fish in chronically stressed environments will invest differentially in life history traits whereas those in pristine areas do not. Specifically, in chronically stressed fish, individuals will invest in reproduction to the detriment of immunity and growth, or invest in growth but not in reproduction. Ideally, future approaches to studying trait tradeoffs and life history parameters will focus on using realistic environmental conditions.
References


Figure 1: A representation of potential trait trade-offs between an infected and uninfected host. 1) The investment in immunity will reduce investment in reproduction and maintenance of body condition. 2) The infection or the investment for immunity has no effect on reproduction or maintenance of body condition investment. 3) An uninfected host invests the majority of its energy to reproduction, and secondarily body maintenance.
Figure 2: Mean glucose concentration (mg/ml ± standard error), a proxy for chronic stress, in fish collected from salt marshes along the coast of Georgia, USA: Shellman Bluff, Skidaway Island, and Tybee Island.
Figure 3: Mean Fulton’s Condition Factor (± standard error), a proxy for investment into body maintenance and growth, in fish collected from salt marshes along the coast of Georgia, USA: Shellman Bluff, Skidaway Island, and Tybee Island.
Figure 4: Mean gonadosomatic index (± standard error), a proxy for investment into reproduction, in fish collected from salt marshes along the coast of Georgia, USA: Shellman Bluff, Skidaway Island, and Tybee Island.
Figure 5: Mean parasitic infection (± standard error), a proxy for investment into immunity, in fish collected from salt marshes along the coast of Georgia, USA: Shellman Bluff, Skidaway Island, and Tybee Island.
Table 1: Analysis of deviance for the effects of parasite infection, fish condition, and chronic stress on investment in reproduction within Fundulus heteroclitus.

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<td>Error ($\varepsilon$)</td>
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LRT: likelihood ratio test; *P*-values were obtained with a parametric bootstrap.