MALES, MASCULINITY AND IMMUNITY: A TEST OF THE IMMUNOCOMPETENCE HANDICAP HYPOTHESIS IN FATHEAD MINNOWS

by

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ABSTRACT

Mate selection by females typically favors males with more prominent sex specific traits or ornamentation. Previous studies have shown that the prominence of such male traits is positively correlated with androgen (e.g., testosterone) levels. An increase in secondary sexual characteristics enhances the likelihood of attracting a mate and that such a male will be able to reproduce. However, there is also evidence to suggest that increased androgen levels may impair immune function. The immunocompetence handicap hypothesis addresses the relationship between androgens, sexual ornamentation, and immune function. Some studies have provided evidence to support this hypothesis, while others have provided evidence suggesting that another hypothesis known as the stress-induced immunocompetence handicap hypothesis is more appropriate because the stress hormone, cortisol, rather than testosterone, is a key factor in regulating both the expression of sexual ornamentation and immune function. The present study evaluated the immunocompetence handicap and stress-induced immunocompetence handicap hypotheses by utilizing male fathead minnows (*Pimephales promelas*) divided into two groups of fish, one with high ornamentation and another with low ornamentation. Immune function was measured using a pathogen resistance challenge, spleen mass, and gene expression. Duds were shown to have significantly larger spleen indexes and lower immune gene expression than the studs indicating increased immunocompetence. No differences in plasma androgen levels were detected. However, duds had significantly higher levels of cortisol than studs indicating that differences in male ornamentation and immune function between the studs and duds are likely driven by stress hormones. These results best support the stress-induced immunocompetence handicap hypothesis.

ACKNOWLEDGEMENTS

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INTRODUCTION

Immunocompetence Handicap Hypothesis

In his analysis of mate selection, Zahavi (1975) first summarized the behavior of females selecting males based on secondary sexual characteristics despite those characteristics imposing a disadvantage on the male. This work suggested that males with more prominent or elaborate secondary sexual characteristics (*i.e.,* bright plumage in birds) have an increased likelihood of attracting a mate and an increased risk of predation (Zahavi 1975). In addition to the risk of predation, increased secondary sexual characteristics have also been shown to come at the cost of decreased immune function (Kurtz et al. 2007, Mougeot et al. 2004). The Immunocompetence Handicap Hypothesis (Fig. 1) states that an increase in androgens (*i.e.,* testosterone) in males increases secondary sexual characteristics and in turn, increases the likelihood of attracting a mate. However, the increase in androgens also leads to a decrease in immune function (Folstad and Karter 1992). The Immunocompetence Handicap Hypothesis hinges on three critical assumptions, which are described below:

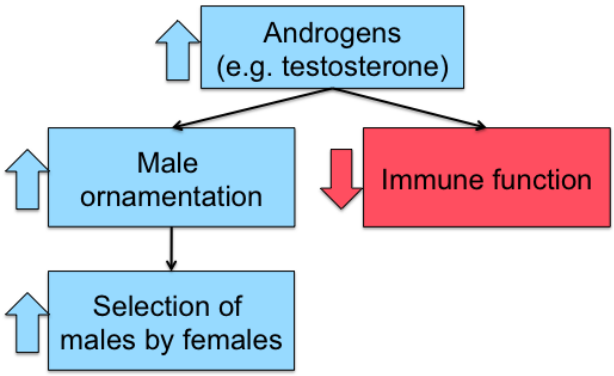


Figure 1. Flow chart of Immunocompetence Handicap Hypothesis assumptions

Assumption 1: Males with more prominent secondary sexual characteristics have increased mating success.

Darwin first described the selection of secondary sexual characteristics of males, such as bright coloration and bird songs, as being different from ordinary natural selection because it was driven by female preference (Darwin 1871). Since Darwin's writings, many scientists have conducted research to understand more about mate selection by females (Dewsbury 1982, Fleming and Gross 1994, Berglund et al. 1996, Hudman and Gotelli 2007, Tiemann 2007, Divino and Tonn 2008, Jacob et al. 2009). Secondary sexual characteristics of preferred males are often exaggerated in size or complexity beyond the optimal development for survival, which leads to the prediction that these traits are selected for by mates as opposed to other factors in the environment (Pomiankowski and Moller 1995). In the common minnow (*Phoxinus phoxinus*), body size and breeding tubercles, projections on the snout used during spawning, have been shown to positively correlate to male reproductive success and territoriality by using genetic markers to determine paternity (Jacob et al. 2009). Similarly, fathead minnows (*Pimephales promelas*) have shown associations between morphologies such as body size, length, tubercle number, and size of dorsal pad, a collection of mucus secreting cells used in parental care, and the ability to compete for a nesting site, a necessary quality for mating success (Sellin Jeffries et al. unpublished, Smith and Murphy 1974). In summary, many studies have shown that an increase in secondary sexual characteristics is associated to an increase in male mating success.

Assumption 2: More prominent secondary sexual characteristics are a result of increased levels of androgens.

Testosterone and its androgenic derivatives have been shown to be strongly associated with ornamentation in many animals including mammals, birds, and fish (Zuk et al. 1995, Evans et al. 2000, Ditchkoff et al. 2001, Gonzalez et al. 200, Martinovic et al. 2007, Burgess at al. 2012). For example, antler size in white-tailed deer (*Odocoileus virginianus*), and tusk size in male dugongs (*Dugong dugon*), marine mammals, have been shown to positively correlate with plasma hormone levels of testosterone (Ditchkoff et al. 2001, Burgess et al. 2012). Implantation of testosterone has been shown to increase comb size, comb color, and spur length in male red jungle fowls (*Gallus gallus)* and bib size in male house sparrows (*Passer domesticus*) (Zuk et al. 1995, Evans et al. 2000). The androgenic chemical, methyltestosterone, has been shown in fathead minnows to increase the size of breeding tubercles in males and lead to the formation of breeding tubercles in females, suggesting the importance of androgens for male traits (Ankley et al. 2001). Overall, many studies have supported the concept that an increase in androgens of an organism correlates to more prominent secondary sexual characteristics.

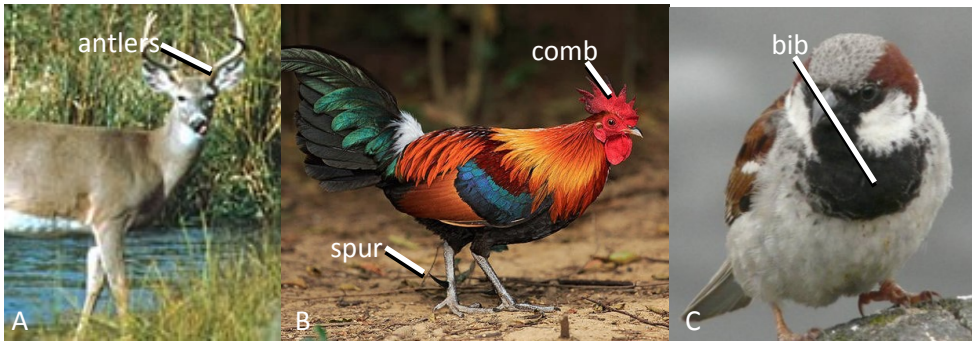


Figure 2. Secondary sexual characteristic examples A. white-tailed deer (New Hampshire Public Television) B. red jungle fowl (Bennett) C. house sparrow (Dawson)

Assumption 3: Increased levels of androgens create a handicap by decreasing the males' ability to fight infection.

Studies have used various methods to show that testosterone and other androgens may be immunosuppressive (Ashmed et al. 1987, Evans et al. 2000, Mougeot et al. 2004, Kurtz et al. 2007, Mills et al. 2009, van Oers et al. 2011). The interaction between androgens and the immune system may not be surprising because organs associated with immune function, such as the thymus and spleen, often have receptors for gonadal steroids (e.g. testosterone) (Grossman 1984). A correlation between androgens and immune function has been observed in many organisms including male bank voles (*Clethrionomys glareolus*) reduced antibody production after testosterone implantation (Mills et al. 2009). Birds with testosterone implants were shown to be infected with significantly more coccidia (*Isospora ssp.*) eggs than control males one month after androgen treatment (Mougeot et al. 2004). In addition, after implantation of 11-ketotestosterone, the dominant male androgen in fish, three-spined sticklebacks (*Gasterosteus aculeatus*) displayed signs of immunosuppression indicated by reduced phagocytic activity, which is necessary for destroying pathogens (Kurtz et al. 2007). These studies demonstrate evidence that androgen production may lead to a decrease in male immune function.

Stress-Linked Immunocompetence Handicap Hypothesis

Although many studies suggest that androgens have a profound effect on immune function, other results have shown that the relationship is more complex because the two factors have shown little or no correlation (Duffy et al. 2000, Peters 2000). An alternative hypothesis known as the Stress-Linked Immunocompetence Handicap Hypothesis (Fig.

3) suggests that glucocorticoids serve an important role in regulating secondary sexual characteristics and immune function. The foundation of this hypothesis challenges the second assumption (*More prominent secondary sexual characteristics are a result of increased levels of androgens.)* and third assumption (*Increased levels of androgens create a handicap by decreasing the males' ability to fight infection)* of the Immunocompetence Handicap Hypothesis and serves to replace it with the following assumptions:

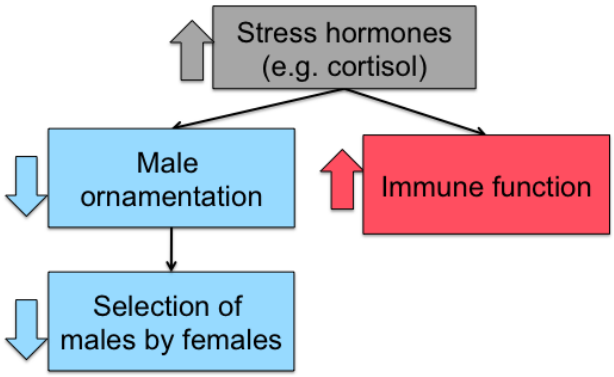


Figure 3. Flow chart of Stress-Induced Immunocompetence Handicap Hypothesis assumptions

Replacement Assumption for Immunocompetence Handicap Assumption 2: Males with more prominent secondary sexual characteristics have decreased levels of glucocorticoids.

The stress response of an organism is thought to divert energy away from the maintenance of secondary sexual characteristics (Vitousek et al. 2014). Studies have shown that organisms may respond to increases in glucocorticoids by reducing expression of ornamentation including melanin-based coloration (Roulin et al. 2008, Saino et al. 2002). Feather coloration in male barn owls (*Tyto alba)* has been shown to significantly decrease in saturation upon corticosterone implantation (Roulin, 2008).

Even without implantation, common snipes (*Gallinago gallinago*) with dark underwings have been shown to have a lower heterophil/lymphocyte (H/L) ratio than those with light underwings, indicating that darker colored snipes have a stronger ability to resist stress (Minias et al. 2014). Barn swallows *(Hirundo rustica)* have also been shown to have significantly lower concentrations of plasma corticosterone than their shorter-tailed counterparts (Saino et al. 2002). These studies suggest that not only can glucocorticoids lead to alterations in secondary sexual characteristics when injected, but organisms may be predisposed with a lesser or greater ability to respond to stress depending on their present secondary sexual characteristics.

Replacement Assumption for Immunocompetence Handicap Assumption 3: Increased levels of glucocorticoids cause alterations in the ability of males to fight an infection.

The assumption that stress impacts immune function is based on the idea that corticosteroids are able to alter the immune system either through suppression or redistribution of lymphocytes (Arzt et al. 1994, Dhabhar 1998). Cortisol treatment in the common carp (*Cyprinus carpio*) significantly decreases the production of superoxide anion (a compound produced during inflammation) in kidney leucocytes and down-regulates the expression of immune-related genes in the kidney (Kawano et al. 2003). Although these factors may seem like a disadvantage to the organism, studies have actually shown cortisol to be protective against pathogens. Acute and chronically stressed mice (*Mus musculus*) have shown to have significant resistance to the bacteria *S. aureus* compared to the control by measuring bacteria levlesin blood. In this study, inflammatory cytokines, interleukin-6 (IL-6) and interleukin-10 (IL-10) were found to be significantly

increased when stressed groups were infected with the pathogen. Additionally, these interleukins were significantly higher in the acutely stressed group than the chronically stressed group. However, the opposite effect was seen with inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interferon gamma (INF-ϒ) (Mahanti et al. 2015). Combined, these studies suggest that corticosteroids may play an important role in protecting an organism from invading pathogens.

Previous Studies and Objectives

Male fathead minnows (*Pimephales promelas*) serve as a valuable model to evaluate mate selection because of distinct secondary sexual characteristics including the dorsal fat pad, breeding tubercles, and coloration (Powlowski et al 2004). Previous studies have confirmed that large body size, dark banding pattern, large number of tubercles, and large dorsal fat pad may serve as indicators of selection of males by females (Hudman and Gotelli 2007, Sellin Jeffries et al. unpublished). These characteristics are also easily measured by scoring systems, such as the metrics for dorsal fat pad score (Ankley et al. 2001).

Figure 4. Coloration and dorsal fat pad (1)

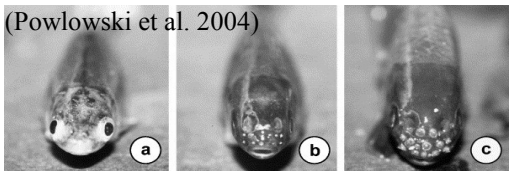


Figure 5. Breeding tubercles extending across snout in males (b, c) compared to female (a)

In a previous experiment from Sellin Jeffries et al., males were separated by their ability to guard a nest, a necessary quality for mating success. The most successful 20% and least successful 20% were shown to significantly differ with regard to the

prominence of their secondary sexual characteristics. The most successful 20% were significantly larger in mass and length, had significantly more tubercles, and had higher dorsal pad scores than the least successful 20% of males suggesting that males with increased levels of cortisol had less prominent secondary sexual characteristics. This study revealed neither group as having different testosterone or 11-ketotestosterone plasma concentrations. However, plasma cortisol concentrations were significantly higher in the least successful 20% of males compared to the most successful 20% of males. This previous study forms a basis for the present study by establishing a connection between features associated with male reproductive success and plasma cortisol levels (Sellin Jeffries et al. unpublished).

In the previous study, male-to-male competition may have induced stress and therefore been a confounding factor for studying cortisol. The overall goal of the current study was to test the Immunocompetence Handicap Hypotheses in fathead minnows under conditions that minimize stress.

The specific objectives of this study were:

Objective 1: To determine whether androgens play a role in the Immunocompetence Handicap Hypothesis for fathead minnows.

To complete objective 1 the following hypotheses were tested:

Hypothesis 1: Males with more prominent secondary sexual characteristics have increased levels of androgens.

Hypothesis 2: Increased levels of androgens cause alterations in the ability of males to fight an infection.

Objective 2: To determine whether glucocorticoids plays a role in the Immunocompetence Handicap Hypothesis for fathead minnows.

To complete objective 2 the following hypotheses were tested:

Hypothesis 3: Increased levels of glucocorticoids cause alterations in the ability of males to fight an infection.

Hypothesis 4: Males with more prominent secondary sexual characteristics have decreased levels of glucocorticoids.

MATERIALS AND METHODS

Animals

Adult male and female fathead minnows from Aquatic Biosystems were utilized in this study. The minnows were housed in 20L aquaria with constantly aerated dechlorinated water at 25-27 °C under a photoperiod of 16 hours light: 8 hours dark. Each aquarium was cleaned daily via a static renewal system in which feces were removed and 1/3 of the water was replaced. Fish were fed twice daily with commercially available flake food (Tetramin, Blacksburg, VA). Temperature and deaths were recorded daily.

Experimental Design

A total of 150 adult male fathead minnows were randomly selected from 200 males for study inclusion. The 150 males were divided equally into a total of 15 20L aquaria (10 males/tank). 10 female minnows were also added to each tank in order to decrease competition between males. Aquaria were checked for neutral pH (pH=7-8) and temperature within 1 degree of their holding tank before transfer. Each tank was equipped

with three nests consisting of longitudinally cut 7.6 cm diameter PVC pipe. Fish were monitored for 15 days at constant aquaria conditions (25-27 °C, constant aeration, 1/3 water daily renewal). On day 15, the top 20-30% and bottom 20-30% of males were selected from each tank based on secondary sexual characteristics (number and prominence of breeding tubercles, body size, coloration, and size of dorsal pad) resulting in the selection of 40 studs and 40 duds, of which 20 from each group were selected for tissue collection.

Tissue Collection

Tissues from 20 studs and 20 duds were collected on day 15 of constant 20L aquaria conditions. Minnows were euthanized with a lethal dose (0.3 g/L) of tricaine mesylate (MS-222), weighed and, measured for length. Blood, liver, kidney, spleen, brain, gonad tissues were collected, frozen in liquid nitrogen, and stored at -80°C for gene and hormone analysis. Liver, gonad, and spleen samples were weighed to determine the mass indexes. Fish heads were stored in 10% formalin solution to be used for secondary sexual characteristic quantification.

Secondary Sexual Characteristic Quantification

The dorsal pad size, tubercle number and prominence, and coloration were recorded. These factors were measured immediately after euthanization, formalin tubes were numbered, and these characteristics were measured again using the fish heads preserved in formalin. Scoring for dorsal pad, tubercles, and coloration were done as listed in tables 1-3. Dorsal pad scoring was done according to the system originated from (Danylchuck and Tonn 2001).

Table 1. Dorsal pad scoring criteria.

Score	Dorsal Pad
0	No visible dorsal pad
1	Epidermis between head and dorsal fin becoming 'spongy' along medial ridge
2	Dorsal pad increasing in width; thickening but only a slight nape behind head
3	Dorsal pad wide and thick, forming a sharp nape posterior to head when viewed laterally

Table 2. Tubercle scoring criteria.

Score	Tubercles
0	No visible tubercles
1	Tubercles visible but with height less than radius and have single point
2	Tubercles visible having a single point whose height is nearly equivalent to its radius
3	Tubercles enlarged having a radial base with grooves emerging from the center
4	Tubercles pronounced; quite rounded with less definition and structure

Table 3. Coloration scoring criteria.

Score	Head and Body Coloration
1	Light in color, not distinguishable from female color, head and body same color
2	Medium in color, distinguishable from female color, head slightly darker from body
3	Dark, grey or black in color across dorsal side and head, head dark grey or black

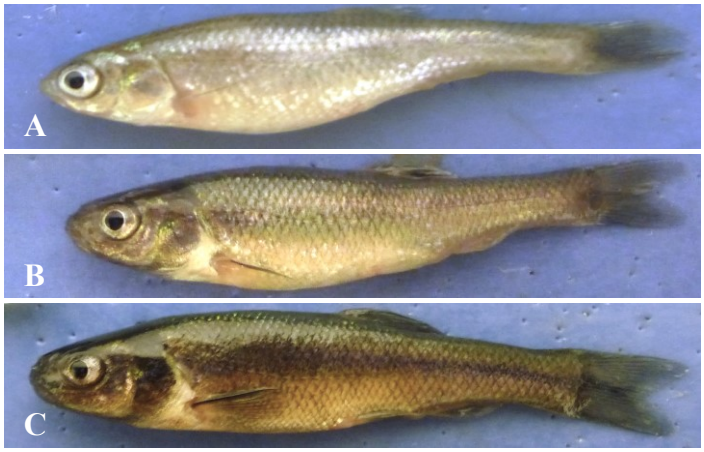


Figure 6. Examples of coloration score criteria A. score of 1 B. score of 2 C. score of 3

Yersinia ruckeri Preparation

Gram-negative bacterial pathogen *Y. ruckeri* was incubated for 16 hours overnight at 27°C on an orbital shaker in 4 tubes each with 4 mL nutrient broth inoculated with a single isolated colony. The overnight cultures were combined into a single 15 mL conical

tube, centrifuged at 3900 rpm for 6 minutes, washed with Hank's Balanced Salt Solution (HBSS), and centrifuged for an additional 6 minutes. The cultures were then suspended in 10 mL HBSS. The OD_{600} of 0.658 was used for injections. This optical density was utilized for plating on nutrient agar for colony forming units (CFU).

Yersinia ruckeri Injections

A second set of 20 studs and 20 duds were anesthetized via a sub-lethal dose (0.15 g/L) MS-222 and utilized for immune response trials. Prior to injection, fish were weighed, measured by length, and assessed for secondary sexual characteristics including color, tubercle number, and dorsal pad score. 10 µL/g bacterial suspension was administered using a ½ cc syringe with 27 ½ g needle and New Era syringe pump. A total of 20 "studs" and 20 "duds" were injected. "Studs" were placed in one tank and "duds" were placed in another tank. Mortality was recorded for 14 days.

Plasma Analysis

Plasma samples from the first set on 20 studs and duds were analyzed for cortisol, and 11-ketotestosterone (11-KT) using Cayman Chemical Company Enzyme Immunoassay (EIA) kits. Plasma was diluted 1:50 and 1:100 for cortisol and 1:1000 for 11-KT in EIA buffer. 11-KT and cortisol plates were loaded and placed in a cold room (3 °C) to incubate for 18 hours. The 11-KT and cortisol plates were then incubated for 90 minutes at room temperature on an orbital shaker. All plates were read at 405 nm wavelength using the BMG Labtech FLOUStar Omega operated with Omega software version 1.20.

Gene Expression Analysis

A total of 16 samples were utilized for gene expression analysis. From the 40 total fish selected for gene expression, the top 8 and bottom 8 based on mass, tubercle number, tubercle score, and dorsal pad score were used for analysis. Tissues collected on day 15 for gene expression analysis were homogenized with the QSonica tissue sonicator (QSonica, Farmingdale, NY) and combined with lysis buffer. RNA was extracted from homogenates via the Maxwell 16 LEV simplyRNA Purification Kit (Promega, Madison, WI) per manufacturer protocol (Sellin Jeffries et al. 2014). Total RNA was quantified via the NanoDrop 1000 (ThermoScientific, Wilmington, DE). A RNA quality check was completed on a 46-sample set using Experion RNA StndSens chips (Bio-Rad, Hercules, CA). Loading buffer and denatured RNA were mixed and loaded on chip and the assessment was used to further verify the quality of RNA samples. Extracted RNA was combined with reverse transcriptase and reaction mix (consisting of oligo(dT) and random hexamer primers) from the iScript cDNA Synthesis Kit and then RNA was converted to cDNA using a TC-100 thermal cycler (Bio-Rad, Hercules, CA).

Sequences for mRNA were acquired from the National Center for Biotechnology and then converted into primer sequences using Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primer parameters were adjusted to 18-22 base pairs in length, an amplicon size of 75-150, temperature of 55-63°C and a GC% of 40-60. Optimal annealing temperature for each primer was determined using qPCR reactions on a thermal gradient of 48-62°C. Sequences were acquired from fathead minnows *(Pimephales promelas)*, zebrafish *(Danio rerio)*, common carp (*Cyprinus carpio)*, and grass carp (*Ctenopharyngodon idella*) as listed in table 3. Primers were constructed as a consensus sequences if a gene sequence was not

found for *Pimephales promelas,* but the gene had multiple results from other species listed previously. Consensus sequences were designed using the programs MEGA5 version 2.2 and Sequencer 5.1 software. The consensus sequences for C3 was constructed and confirmed in a previous experiment (Thornton et al. unpublished).

Table 4. Primer sequences used in this study.

Gene*	Derived Species and Literature	Primer Sequence (5'-3')	Annealing Temp. (°C)
11b-HSD	*Pimephales promelas*	Forward: GAGCTCCAGAAGACCTGCTC Reverse: CCAGTTTGGCCTTGGTGT	57
AR	*Pimephales promelas* (Kolok et al. 2007)	Forward: GTTTCCGTAACCTGCATGTGG Reverse: CGCGCATTAGCGTTCTTGTA	60
Aromatase	*Pimephales promelas* (Ankley et al. 2007)	Forward: TGCTGACACATGCAGAAAAACTC Reverse: CAGCTCTCCGTGGCTCTGA	60
AVP	*Danio rerio*	Forward: AGAGAGCTGCGCTGTAGACC Reverse: AGACGCAGCAGAGTTTCTCC	51
B-actin	*Pimephales promelas*	Forward: GGCTGTTTTGTCCCTGTACG Reverse: AGGGCGTAACCCTCGTAGAT	58
C3	Consensus-*Danio rerio*/Cyprinus carpio/*Ctenopharyngodon idella* *(Thornton et al. unpublished)*	Forward: GTGCCAGTGTGCAGAAGAAA Reverse: TTCCCCTCAACATCCTCATC	60
CRF	*Pimephales promelas*	Forward:TCCCTGGATCTGACCTTTCA Reverse: TCATGATGGAAAAGCAGCAC	60
GCR	*Pimephales promelas* (Filby and Tyler, 2007)	Forward: CGTCAATGGTTCCTCAACCT Reverse: TGGTGTGCTCCTCAAGAGTG	60
HSP70	*Pimephales promelas*	Forward TGGGCTCAATGTCCTCAGAAT Reverse: CTGCTCCTTTGCCTTTGTCAA	61
L8	*Pimephales promelas* (Kolok, 2007)	Forward: GCCCATGTCAAGCACAGAAAA Reverse: ACGGAAAACCACCTTAGCCAG	63.8
MR	*Danio rerio*	Forward: AAGCCCAACAACAAATGGAG Reverse: GATGAGTTCCTGGGACTGGA	60
StAR	*Pimephales promelas* (Kolok, 2007)	Forward: CTTGAACAGCAAACAGATGACCTT Reverse: CTCCCCCATTTGTTCCATGT	60
TNF-a	*Cyprinus carpio*	Forward: TGCTTCACGCTCAACAAGTC Reverse: GCTGCCTTGGAAGTGACATT	54
Uro	Consensus *Danio rerio/Cyprinus carpio*	Forward: GAGTTTTCCAAACGGAACGA Reverse: TGGAAAAGAGTGCATTGAGC	60

* Abbreviations: 11b-HSD (11β-hydroxysteroid dehydrogenase), AR (Androgen receptor), AVP (arginine vasopressin), C3 (complement), CRF (Corticotropin-releasing factor), GCR (Glucocorticoid receptor), HSP70 (Heat-shock protein 70), L8 (Ribosomal protein L8), MR

(Mineralocorticoid receptor), StAR (Steroidogenic acute regulatory protein), TNF-a (Tumor necrosis factor alpha), Uro (Urotensin)

Primers listed in table 4 were used for qPCR by CFX Connect real-time PCR detection system and CFX Manage Software version 3.0 (Bio-Rad, Hercules, CA). Each utilized reaction well contained 0.40 μL cDNA, 4.30 μL nuclease free H_2O, 0.30 μL primer mix, and 5.00 μL SsoAdvanced Universal SYBR Green Supermix. Relative gene expression was detected based on the standard curve of serial diluted cDNA samples. The expression of each gene was normalized using ribosomal protein L8 as a reference gene for gonad, kidney, spleen, and liver samples and beta actin as a reference gene for brain samples.

Next-Generation Sequencing

Sequencing was later conducted in liver cDNA samples by utilization of Next Generation Sequencing, a technique in which the entire genome or transcriptome of samples can be detected (Morozova, 2008). Sequencing was sponsored by a workshop conducted by the Genome Consortium for Active Teaching NextGen Sequencing Group and consisted of Illumina Miseq 100 base pair sequencing with paired end reads.

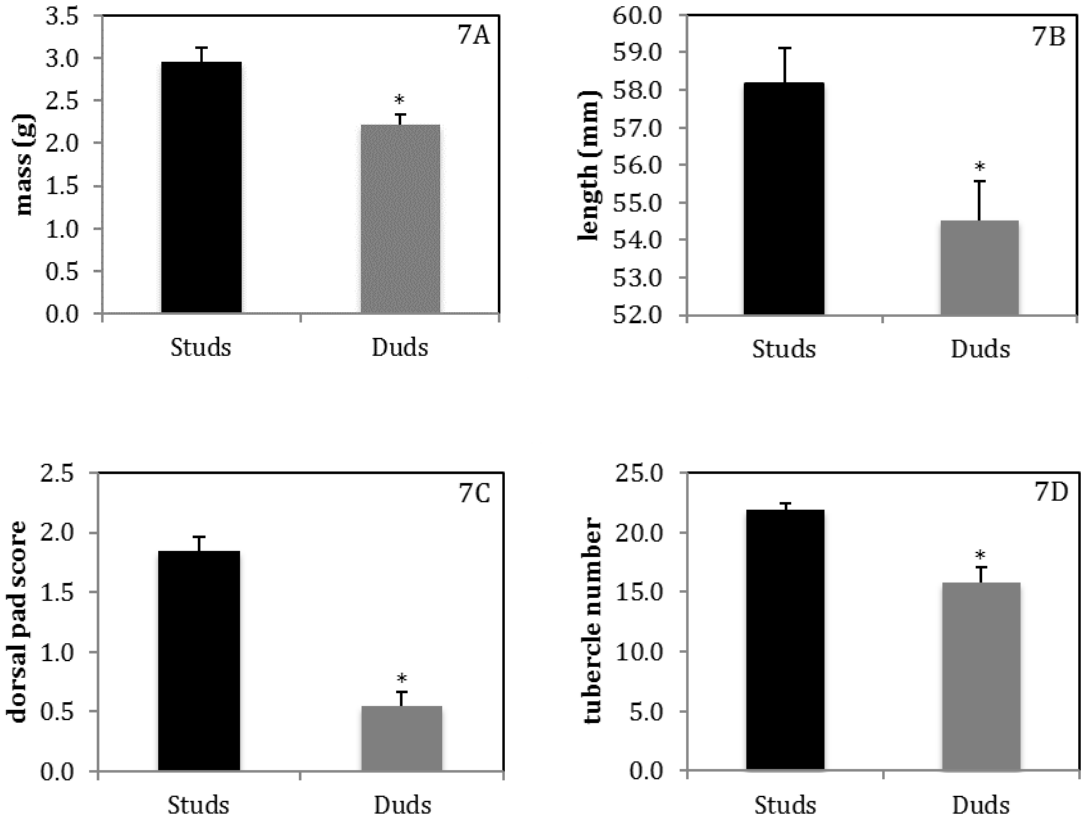
Statistical Analysis

Spleen, gonad, liver standard indexes were determined by dividing the tissue mass by the whole organism mass. Significant differences for tissue indexes, coloration, mass, tubercle number, and gene expression were determined using a one-way analysis of variance (ANOVA) using the statistical software JMP 10.0. Significant unequal variance in coloration was accounted for by using a Wilcoxon 2-Sample Test.

RESULTS

Mass and Secondary Sexual Characteristic Analysis

Results for mass and secondary sexual characteristics are displayed in figures 7A-7F after separation into respective groups based on those secondary sexual characteristics. The selected 20 studs and 20 duds were confirmed to significantly differ across whole body mass (ANOVA, p-value = 0.0007), length (ANOVA, p-value = 0.0140), dorsal pad score (ANOVA, p-value < 0.0001), tubercle number (Wilcoxon test, p-value <0.0001), tubercle score (ANOVA, p-value < 0.0001), and coloration (Wilcoxon 2-Sample Test, p-value < 0.0001).



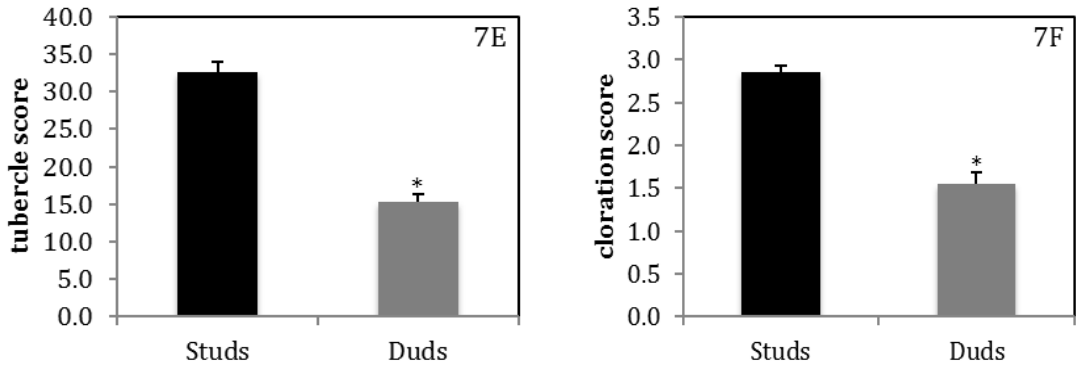


Figure 7. Mean measures of physical characteristics of studs and duds: (A) body mass, (B) length from snout to tail, (C) dorsal pad score, (D) tubercle number, (E) tubercle score, (F) coloration score. * Represents significant difference with p value < 0.05. Error bars represent mean standard error.

Secondary Sexual Characteristics and Spleen Index

Organ indices for liver, gonad, and spleen tissues are displayed in figures 8A-8C. No differences were found between the studs and duds with regard to liver standard index (ANOVA p-value > 0.12) or gonad standard index (ANOVA p-value > 0.12) Spleen tissue had the only index with a significant difference between the studs and duds. Duds were found to have a significantly larger spleen index compared to the studs (Wilcoxon 2-Sample Test, p-value = 0.0325).

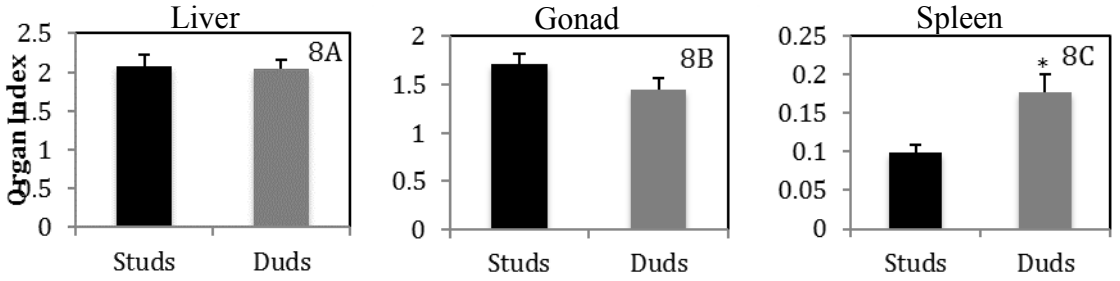


Figure 8. Mean standard indexes of studs and duds: (A) liver standard index (LSI), (B) gonad standard index (GSI), (C) spleen standard index (SSI) * Represents significant difference with p value < 0.05. Error bars represent mean standard error.

Secondary Sexual Characteristics and Pathogen Resistance

The survival plot of the studs and duds is shown in figure 9. There were no significant differences in overall survival or time of death between the studs and duds (survival time analysis p-value = 0.83).

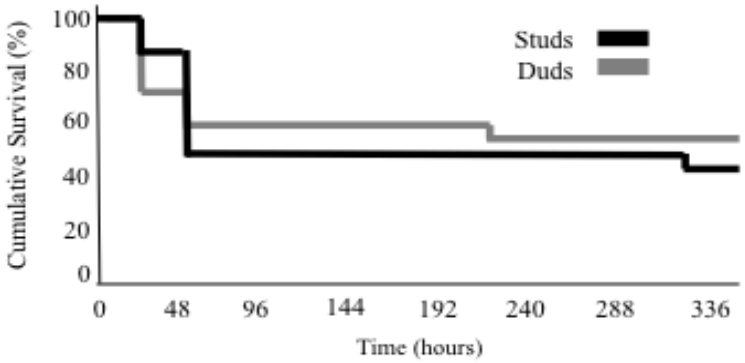


Figure 9. Cumulative Survival of studs and duds injected with *Y. ruckeri* over 336 hours (14 days)

Secondary Sexual Characteristics and Plasma Hormone Levels

Plasma hormone levels for 11-ketotestosterone and cortisol are shown in figure 10. No significant differences were seen between studs and duds for 11-ketotestosterone (ANOVA p-value = 0.40). Duds were found to have a significantly higher average level of cortisol in blood plasma (Wilcoxon 2-Sample Test p value = 0.045).

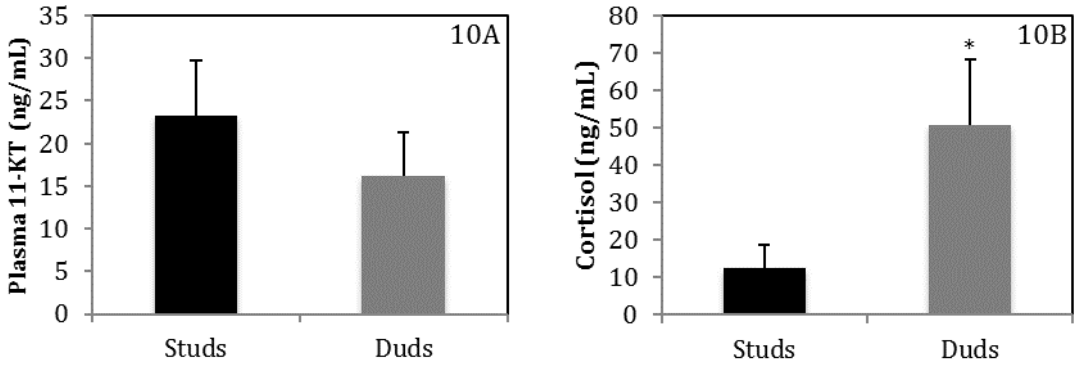


Figure 10. Average plasma hormone concentration: (A) 11-ketotestosterone, (B) cortisol. * Represents significant difference with p value < 0.05. Error bars represent mean standard error.

Targeted Gene Expression

Relative gene expression of fathead minnow studs and duds in kidney, gonad, spleen, liver, and brain tissues are shown in table 5. No significant differences were found between the two groups for the genes listed in table 5.

Table 5. Relative expression of genes related to immune function, stress, and reproduction in stud and dud fathead minnows. No significant differences were found. All data represented as mean ± standard error mean, n=6-8

Tissue	Gene	Studs	Duds
Kidney	GCR	1.51±0.09	1.25±0.09
	MR	1.34±0.11	1.22±0.08
	StAR	1.18±0.18	0.85±0.18
	TNF-a	0.52±0.14	0.30±0.02
Gonad	AR	0.62±0.09	0.57±0.33
	Aromatase	1.46±0.30	0.89±0.25
	GCR	0.95±0.10	1.14±0.06
Spleen	GCR	2.55±0.31	2.11±0.43
	TNF-a	1.64±0.48	4.13±2.66
Liver	AR	0.75±0.07	0.89±0.05
	C3	0.85±0.09	1.13±0.12
	HSP70	0.53±0.28	0.41±0.12
Brain	AR	0.22±0.01	0.69±0.55
	Aromatase	0.43±0.13	2.63±2.37
	AVP	1.03±0.10	0.79±0.49
	CRF	1.00±0.11	6.26±5.77
	GCR	1.41±0.31	2.66±1.85
	StAR	0.46±0.14	3.09±2.82
	Uro	1.10±0.11	4.43±0.11

Next Generation Sequencing

Next-Generation sequencing revealed differences between studs and duds for immune function related genes (Table 6) and metabolic related genes (Table 7). All genes listed in tables 6 and 7 were found to be significantly higher expressed in studs than duds as demonstrated by the fold change and p-values (p-value < .05).

Table 6. Next-Generation sequencing data for immune related genes

Gene	P-value	Fold Change (log) Studs/Duds
complement component c3a precursor	0.00	8.09
interleukin-1 receptor type 1-like	0.00	7.49
coronin-1A	0.01	7.46

Table 7. Next-Generation sequencing data for metabolism related genes

Gene	P-value	Fold Change (log) Studs/Duds
ATP synthase subunit mitochondrial	0.00	9.42
insulin receptor substrate 1-b	0.00	6.90
ribonuclease like 2 precursor	0.00	8.74

DISCUSSION

Secondary sexual characteristics between the studs and duds were found to be significantly different across all measured characteristics. This confirms that the groups were correctly separated into high secondary sexual characteristics (studs) and low secondary sexual characteristics (duds). This separation allows for the basis of the immunocompetence handicap and stress-induced immunocompetence handicap hypotheses (Zahavi 1975)(Vitousek et al. 2014).

Hypothesis 1: Males with more prominent secondary sexual characteristics have increased levels of androgens.

This study did not find any correlation between secondary sexual characteristics and androgen levels. 11-ketotestosterone was not found to be significantly different between studs and duds. This result may be specific to fathead minnows because other teleost fish species such as *Lythrurus fasciolaris* have been shown to have increased 11-

ketotestosterone levels corresponding to increased coloration and size (Schade et al. 2012). Although androgens such as testosterone and 11-ketotestosterone are well known to control the differentiation of the male gonads, the link between androgens and the current appearance of a male organism remains unclear (Nakamura 1981)(Sellin Jeffries et al. unpublished). The present study did not see differences in gonadal standard indexes, suggesting that the gonads were comparable in size between the two groups. This study also did not find differences in androgen-related gene expression from qPCR and Next-Generation sequencing although the appearance of other teleost fish has been shown to be affected by activation of androgen receptors (Golan and Levavi-Sivan 2014). Together, these results suggest that androgens may not be the reason for current differences in secondary sexual characteristics of fathead minnows in this experiment.

Hypothesis 2: *Increased levels of androgens cause alterations in the ability of males to fight an infection.*

Although this study did not see differences in androgens and androgen-related genes between studs and duds, differences related to immune function were found. These results suggest that androgens in this study were not associated with immune function. Previous studies have also tested the immunocompetence handicap hypothesis and have not found supportive evidence that testosterone and its derivatives are immunosuppressive (Casagrande and Ton 2011)(Sellin Jeffries et al. unpublished). However, Casagrande and Ton did find that male diamond doves (*Geopelia cuneata)* subjected to an immunological challenge reduced redness and size, traits often associated with androgens (Casagrande and Ton 2011). The degree of secondary sexual characteristics before and after infection were not measured in the current experiment,

but could provide future studies with another way to evaluate testosterone and immune function in fathead minnows. However, other studies have seen a correlation between androgens and immune function. After implantation of 11-ketotestosterone, three-spined sticklebacks (*Gasterosteus aculeatus*) have demonstrated immunosuppression by significantly weaker phagocytic activity, which is necessary for destroying pathogens (Kurtz et al. 2007). The present study and previous research suggest that the relationship between androgens and immune function is not consistent between experimental studies and may be indirect.

Hypothesis 3: Glucocorticoids play a role in the maintenance of secondary sexual characteristics.

The current and previous studies have shown that duds had significantly higher levels of cortisol than studs indicating that differences in male ornamentation could be driven by stress hormones (Sellin Jeffries et al. unpublished, Saino et al. 2002, Roulin et al. 2008, Minias et al. 2014). Next-Generation sequencing results of the present study indicated a set of metabolic related genes that were significantly higher expressed in studs than duds. These genes included ATP synthase subunit of mitochondria, which provides addition insight to previous research regarding metabolic function and stress. Adult male *Fundulus heteroclitus* have displayed significant elevations in blood serum glucose levels when injected with cortisol and mitochondrial mass and DNA content have been observed as an early cellular response to oxidative stress in human cells (Leach and Taylor 1980, Lee et al. 2000). The relationship between cortisol and metabolic function has been previously investigated. However, sufficient research has been lacking regarding larger scale interplay of how secondary sexual characteristics can serve as

predictors of metabolic function. The present study demonstrates that male fathead minnows with greater secondary sexual characteristics may have naturally lower levels of plasma cortisol and increased expression of metabolic-related genes. Because previous work has shown that induction of stress or injection of cortisol is often associated with an increase in metabolic function, it is possible that a heightened expression of metabolic genes is necessary for the studs to compensate for low levels of cortisol.

Hypothesis 4: Glucocorticoids plays a role in regulating the ability of males to fight an infection

Although there were no significant differences between the survival of studs and duds, further differences in spleen index and gene expression suggest that the immune function between the studs and duds differ. This suggests that the immune-related differences between studs and duds in this experiment were not at the whole organismal level, but were still present. There is the potential for an immunocompetence threshold for whole organism survival because differences in survival of studs and duds have been seen when competition is induced (Sellin Jeffries et al. unpublished). There is also a potential for varied survivability depending on what pathogen is used in the immune trials. This experiment utilized one bacterial pathogen (*Y. ruckeri*), and the response to this pathogen may not be representative of other bacterial, viral, and parasitic pathogens.

Standard spleen index was found to be significantly higher in duds than studs, which is of particular interest in this study due to the importance of the spleen in the immune system. Other research involving pathogen injections has found similar results in fish such as rainbow trout (*Oncorhynchus mykiss*) when injected with bacteria (*F. psychorophilum*) (Hadidi, 2008). Since the spleen is responsible for filtering blood and

removing damaged cells, the duds could potentially have a greater filtering capacity (Mebius, 2005). The duds could also have a greater cell population in the spleen, which would demonstrate a heightened activation of the immune system compared to the studs (Montoya, 2005).

Several genes related to immune function were found to be significantly higher expressed in the studs than the duds. These genes included complement component c3a precursor, interleukin-1 receptor type 1-like, and coronin-1A. The complement system in teleost fish such as the fathead minnow have been shown to be involved in many functions including recognition and phagocytosis of microbes and induction of leukocyte migration (Jenkins and Ourth 1993). The interleukin-1 system of ligands and receptors have been shown to be key factors in eliciting signal transduction pathways for an inflammatory response to occur (Wang et al. 2016). Coronin is associated with cytoskeletal movement during phagocytosis (Bricheux et al. 2000). Since these three genes are expressed higher in studs than duds, the studs could potentially have to up-regulate immune genes in order to combat the same pathogen as duds at the same survival rate. Furthermore, increased levels of cortisol could improve the immune system efficiency of the duds compared to the studs and help to explain why studs may use gene expression as a compensatory measure for low immunocompetence.

Overall, this study demonstrates the dynamic correlation between secondary sexual characteristics, androgens, stress hormones, and immune function. Although the studs and duds had similar survival rates, there are profound differences in appearance, gene expression, spleen mass, and cortisol levels. Further research is needed to better understand why some studies have found differences in androgens in relation to

secondary sexual characteristics and if androgens do have a role in the immunocompetence handicap hypothesis.

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