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## THE ECOLOGICAL AND EVOLUTIONARY EFFECTS OF ENDOCRINE DISRUPTING COMPOUNDS ON SEXUALLY SELECTED TRAITS IN MALE GUPPIES

Kausalya Shenoy

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THE ECOLOGICAL AND EVOLUTIONARY EFFECTS OF ENDOCRINE DISRUPTING  
COMPOUNDS ON SEXUALLY SELECTED TRAITS IN MALE GUPPIES

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DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Arts and Science  
at the University of Kentucky

By

Kausalya Shenoy  
Lexington, Kentucky

Director: Dr. Philip H. Crowley, Professor of Biology  
Lexington, Kentucky

2012

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## ABSTRACT OF DISSERTATION

### THE ECOLOGICAL AND EVOLUTIONARY EFFECTS OF ENDOCRINE DISRUPTING COMPOUNDS ON SEXUALLY SELECTED TRAITS IN MALE GUPPIES

Male mating signals convey important mate-quality information to females and are regulated by androgens. Endocrine disrupting compounds (EDCs) are chemicals that interfere with proper hormonal functioning in exposed animals, causing altered hormone levels and resulting in changed reproductive characteristics, including mating signals. Altered signals can have ecological implications by influencing population and community dynamics and evolutionary implications via trans-generational reduction in signal reliability leading to reduced preference and eventual loss of the signal trait. I examined the effects of exposure to environmentally relevant concentrations of atrazine, a widely used herbicide and EDC, on mating signals and behaviors in male guppies, a sexually dimorphic freshwater fish. Guppies were exposed either during adulthood or embryonic development. Prolonged atrazine exposure during adulthood reduced the size of the carotenoid-based ornament, the number of courtship displays performed, and aggression towards competing males. Embryonic exposure did not affect survival to adulthood and the time to develop male-specific morphologies. But there was a trend for smaller genitalia, and the ornament size was significantly increased. Possible increases in immunocompetence as a result of slight estrogenicity may have allowed for greater carotenoid allocation to the ornament. Embryonic exposure also resulted in reduced courtship behavior, forced copulatory attempts and aggression towards competitors; female guppies found these males less attractive. The low dose had the strongest effects with embryonic exposure, indicating the importance of low-dose exposures. These studies highlight the effects of low and environmentally relevant doses of atrazine on mating signals and behaviors in exposed wildlife. A mathematical model was used to understand the evolutionary effects of EDCs on the optimal allocation of carotenoids between ornament and immunocompetence. Animals obtain carotenoids through their diet, and allocate some of this to enhance immune function and the rest to ornaments for mate attraction. The model replicates the disruption of carotenoid-based ornaments as a result of EDC-exposure, and predicts that signal reliability will be reduced. The model simulates an evolutionary shift in the optimal allocation if exposure spanned multiple generations, but signal reliability is not restored. Including additional selective forces like predation further suppresses signal reliability.

Keywords: atrazine, carotenoid-based ornament, mating behaviors, *Poecilia reticulata*, sexual signaling

Kausalya Shenoy  
May 16, 2012

THE ECOLOGICAL AND EVOLUTIONARY EFFECTS OF ENDOCRINE DISRUPTING  
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It has been an exciting, adventurous, rough yet rewarding journey to get to this point. I had grand ideas and complicated experiments designed. Many ideas crashed and burned, and of those that survived many ambitious ones never took off; the few worthy ones remained to be presented here. Today I have a dissertation I am proud of, albeit a fraction of the original grand plan! Many people helped along the way, and I would like to thank each one of them.

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## TABLE OF CONTENTS

|   |             |
|---|-------------|
| <b>ACKNOWLEDGEMENTS.....</b>  | <b>III</b>  |
| <b>LIST OF TABLES .....</b>   | <b>VIII</b> |
| <b>LIST OF FIGURES .....</b>  | <b>IX</b>   |
| <b>CHAPTER ONE. INTRODUCTION .....</b>  | <b>1</b>    |
| <b>CHAPTER TWO. ENDOCRINE DISRUPTION OF MALE MATING SIGNALS: ECOLOGICAL AND<br/>EVOLUTIONARY IMPLICATIONS .....</b>                               | <b>3</b>    |
| Summary .....   | 3           |
| Introduction .....  | 3           |
| Male Mating Signals and Female Receivers – Definitions .....  | 4           |
| Altered Signals and Responses .....   | 5           |
| Hormonal Regulation of Mating Signals and Female Responses .....  | 7           |
| Endocrine disruption of mating signals .....  | 9           |
| Ecological Implications of Altered Male Mating Signals .....  | 10          |
| Evolutionary Implications of Altered Mating Signals .....   | 12          |
| Future directions .....   | 14          |
| <b>CHAPTER THREE. ENVIRONMENTALLY REALISTIC EXPOSURE TO THE HERBICIDE ATRAZINE ALTERS<br/>SOME SEXUALLY SELECTED TRAITS IN MALE GUPPIES .....</b> | <b>27</b>   |
| Summary .....   | 27          |
| Introduction .....  | 27          |
| Methods .....   | 29          |
| Ethics statement .....  | 29          |
| Treatments.....   | 29          |
| Animals .....   | 29          |
| Color measurements.....   | 29          |
| Behavior trials .....   | 30          |
| <i>Trials without competing males</i> .....   | 30          |
| <i>Trials with competing males</i> .....  | 30          |
| Data Analyses.....  | 31          |
| <i>Mortality</i> .....  | 31          |
| <i>Area of Orange Spots, Intensity of Orange Spots, and Mating Behaviors in the Absence of<br/>Competition</i> .....                              | 31          |
| <i>Mating behaviors in the presence of competition</i> .....  | 31          |
| Results.....  | 32          |
| Mortality .....   | 32          |
| Color .....   | 32          |
| Mating behaviors in the absence of competing males .....  | 33          |
| Mating behaviors in the presence of competing males .....   | 33          |
| Discussion .....  | 34          |
| Differential susceptibility to atrazine .....   | 34          |
| Impaired mating signals and implications for sexual selection.....  | 34          |
| Atrazine.....   | 36          |
| <b>CHAPTER FOUR. THE EFFECTS OF PRENATAL EXPOSURE TO ATRAZINE ON BROOD CHARACTERISTICS<br/>AND MALE SECONDARY SEXUAL TRAITS IN GUPPIES .....</b>  | <b>43</b>   |
| Summary .....   | 43          |
| Introduction .....  | 43          |



|   |           |
|---|-----------|
| Methods .....   | 45        |
| Study System .....  | 45        |
| Animal care .....   | 45        |
| Experimental Treatment .....  | 46        |
| Measurements .....  | 46        |
| Data Analyses .....   | 46        |
| Results .....   | 47        |
| Mortality, Brood Size and Sex Ratios .....  | 47        |
| Development of male-specific traits .....   | 47        |
| Size of gonopodium and orange spots .....   | 48        |
| Discussion .....  | 48        |
| <b>CHAPTER FIVE. PRENATAL EXPOSURE TO LOW DOSES OF ATRAZINE AFFECTS MATING BEHAVIORS IN MALE GUPPIES.....</b>                                   | <b>56</b> |
| Summary .....   | 56        |
| Introduction .....  | 56        |
| Methods .....   | 57        |
| Study System .....  | 57        |
| Treatments .....  | 58        |
| Animal Care .....   | 58        |
| Behavior trials .....   | 58        |
| <i>Trials without competing males</i> .....   | 59        |
| <i>Trials with competing males</i> .....  | 59        |
| <i>Attractiveness to females</i> .....  | 59        |
| Data Analyses .....   | 60        |
| <i>Mating behaviors without competition</i> .....   | 60        |
| <i>Mating behaviors in the presence of competition</i> .....  | 60        |
| <i>Attractiveness to females</i> .....  | 61        |
| Results .....   | 61        |
| Behaviors in the absence of competitors .....   | 61        |
| Behaviors in the presence of competitors .....  | 62        |
| Attractiveness to females .....   | 63        |
| Discussion .....  | 63        |
| Implications of altered mating behaviors .....  | 64        |
| Timing of exposure .....  | 66        |
| <b>CHAPTER SIX. ENDOCRINE DISRUPTION AND THE CAROTENOID MODULATION OF THE IMMUNOCOMPETENCE HANDICAP HYPOTHESIS: AN EVOLUTIONARY MODEL .....</b> | <b>73</b> |
| Summary .....   | 73        |
| Introduction .....  | 73        |
| Methods .....   | 75        |
| The Guppy System .....  | 75        |
| Model Overview .....  | 76        |
| Details of the model .....  | 77        |
| <i>Quality</i> .....  | 77        |
| <i>Finding the optimal allocation strategy</i> .....  | 78        |
| <i>Baseline runs of the model</i> .....   | 78        |
| <i>EDC</i> .....  | 78        |
| <i>Predation</i> .....  | 78        |
| Calculating fitness .....   | 79        |
| Results .....   | 80        |
| Optimal x .....   | 80        |

|  |            |
|--|------------|
| Ornament size.....   | 80         |
| Immune response .....                                      | 80         |
| Signal reliability .....                                   | 80         |
| Fitness .....  | 80         |
| Predation pressure.....                                    | 80         |
| Important parameters.....                                  | 81         |
| Discussion .....   | 81         |
| Patterns in immunocompetence .....                         | 82         |
| Signal reliability .....                                   | 82         |
| Shift in allocation strategy .....                         | 82         |
| Predation .....  | 83         |
| <b>Appendix 6.1. Relationships between variables .....</b> | <b>85</b>  |
| <b>CHAPTER SEVEN. FUTURE DIRECTIONS .....</b>              | <b>104</b> |
| <b>VITA .....</b>  | <b>106</b> |
| <b>REFERENCES .....</b>                                    | <b>108</b> |

## LIST OF TABLES

|  |    |
|--|----|
| <b>Table 2.1.</b> Empirical studies showing altered reproductive traits in male vertebrates after contaminant-exposure.....  | 16 |
| <b>Table 3.1.</b> Pearson's correlation coefficients between variables of mating behavior in the presence of competing males.....  | 37 |
| <b>Table 3.2.</b> Intercept and slope estimates of each treatment group from the ancova of the effects of treatments on mating behaviors in the presence of competing males..... | 38 |
| <b>Table 5.1.</b> Means and slopes for the focal males' behaviors regressed against the paired control group males' behaviors .....  | 67 |
| <b>Table 6.1.</b> Assumptions made in the model .....  | 89 |
| <b>Table 6.2.</b> Parameter names, symbols and definitions with units and default values .....   | 90 |
| <b>Table 6.3.</b> Variables, definitions, and their range of values .....  | 93 |

## LIST OF FIGURES

|  |     |
|--|-----|
| <b>Figure 2.1.</b> Discriminability between signal trait values represented by individuals of two differing quality values .....                               | 19  |
| <b>Figure 2.2.</b> Female responses to the altered male signals.....   | 20  |
| <b>Figure 2.3.</b> Altered female responses to male traits as a result of endocrine disruption.....  | 22  |
| <b>Figure 2.4.</b> Characteristics of altered male signals after exposure to EDCs.....   | 24  |
| <b>Figure 2.5.</b> Mechanism by which a trait might evolve into a signal or be lost .....  | 25  |
| <b>Figure 3.1.</b> Treatment effects on change in proportion of body area covered by orange.....   | 39  |
| <b>Figure 3.2.</b> Treatment effects on mating behaviors.....  | 40  |
| <b>Figure 3.3.</b> Treated males' behaviors in relation to paired control males' behaviors for each response variable measured .....                           | 42  |
| <b>Figure 4.1.</b> Proportion of guppies surviving until the end of the experiment in each treatment group.....  | 52  |
| <b>Figure 4.2.</b> Brood sizes of adult female guppies exposed to the treatment .....  | 53  |
| <b>Figure 4.3.</b> Average age at development of secondary sexual characteristics of male guppies that were prenatally exposed to the treatment .....          | 54  |
| <b>Figure 4.4.</b> Size of secondary sexual characteristics of male guppies that were prenatally exposed to the treatment.....                                 | 55  |
| <b>Figure 5.1.</b> Likelihood of performing mating behaviors .....   | 69  |
| <b>Figure 5.2.</b> The effect of treatments on the number of mating behaviors performed .....  | 71  |
| <b>Figure 5.3.</b> Strength of the female's preference (SFP) for treated male compared to the paired control male.....   | 72  |
| <b>Figure 6.1.</b> The causal relationships between the variables that ultimately affect fitness .....   | 94  |
| <b>Figure 6.2.</b> Optimal proportion of carotenoids (x) allocated to the ornament.....  | 96  |
| <b>Figure 6.3.</b> Ornament size, defined as the proportion of body area covered by orange spots, corresponding to the optimal allocation of carotenoids ..... | 97  |
| <b>Figure 6.4.</b> Immune response corresponding to the optimal allocation of carotenoids .....  | 100 |
| <b>Figure 6.5.</b> Signal reliability .....  | 101 |
| <b>Figure 6.6.</b> Fitness, as measured by lifetime reproductive success, corresponding to the optimal allocation of carotenoids.....                          | 103 |

## CHAPTER ONE. INTRODUCTION

Animal mating systems have been extensively studied, especially since Darwin (1859, 1871) proposed his theory of Sexual Selection. Much is known about inter- and intra-sexual interactions and the behaviors and morphologies involved in mating success of both males and females of numerous species. Biologists have examined the proximate causes of these traits as well as the evolutionary pressures selecting for or against these traits. In recent years, increasingly more attention is being focused on understanding how anthropogenic interferences are shaping mating systems. Of notable importance are endocrine disrupting compounds (EDCs) which are chemicals, natural or synthetic, that interfere with proper hormonal functioning in exposed animals. Most EDCs have anthropogenic sources such as agrochemicals, industrial effluents, sewage, plastics, cosmetics, pharmaceuticals, and so forth. Contamination by EDCs pervades aquatic and terrestrial ecosystems, including all trophic levels. Many EDCs disrupt the functioning of gonadal hormones either by blocking or binding to hormone receptors, or upregulating or downregulating crucial enzymes involved in hormonal pathways. Disrupting hormonal pathways jeopardizes the production of traits regulated by hormones, including mating signals, ornaments and behaviors, with implications for mating system ecology. I was particularly drawn to this idea that sublethal EDC-exposures can have subtle and latent effects such as altered mating systems, which became the central theme of my dissertation. These effects can have long-term effects on population dynamics with crucial implications for wildlife health and conservation.

Mating signals, including ornaments and courtship behaviors, are honest indicators of the bearer's quality as a potential mate. Signal honesty is maintained by the cost of producing the signal, the potential risks of expressing the signal, and social costs of dishonestly expressing a signal. Hamilton and Zuk (1982) proposed that in birds, the health of an individual was reflected in the condition of its plumage, and those that were infected by parasites had shabbier plumage than those in better health. So, females that chose to mate with more colorful males were indirectly choosing mates who could better resist parasitic infections, thus ensuring better genes for their offspring. Folstad and Karter (1992) proposed the "Immunocompetence Handicap Hypothesis" to include the role of testosterone and immunocompetence in sexual selection. They proposed that testosterone is a double-edged sword, enhancing sexual signaling on the one hand, but suppressing immune functioning on the other. Increased testosterone during breeding seasons raises metabolic rate, which leads to the production of free radicals that damage cells of the immune system. They suggested that testosterone, via the costs of suppressed immunocompetence, maintained the honesty of the signals.

Courtship behaviors fall under the category of honest signals, because individuals in poor health are unable to display optimally. Androgens are necessary for the proper expression of displays, and are correlated with the frequency of displays, which in turn correlates with female mate preference. Immunocompetence is biochemically linked to courtship displays in a manner similar to the way it is linked to ornament expression.

Male ornaments include exaggerated morphologies, plumage and color patterns used to attract females. I was particularly fascinated by carotenoid-based ornaments, which are yellow, orange or red colored ornaments. These colors are produced by carotenoids, which animals must obtain through their diet. Lozano (1994) hypothesized that because carotenoids were free-radical scavengers and overall modulators of the immune system, carotenoids must either be allocated to immune functioning or to the ornament for mate attraction. Males that could bear the cost of a somewhat suppressed immune system could allocate more carotenoids to their ornament and thus appear more colorful; in this way, carotenoid-based ornaments are honest signals of the bearer's immunocompetence. Alonso-Alvarez and colleagues (2007) extended this hypothesis to the balance between ornament expression and overall oxidative stress. The "Oxidation Handicap Hypothesis" states that carotenoids can be allocated to ornament

coloration or to quench free radicals and relieve the body of oxidative stress. This central trade-off in the allocation of carotenoids has been especially interesting to me, and I plan to devote much future research to this and related hypotheses.

My first dissertation chapter reviews the literature in the fields of sexual selection, evolution and toxicology to synthesize a hypothesis that prolonged and trans-generational exposure of populations to EDCs can reduce the reliability of male mating signals with important ecological and evolutionary implications. I suggest that reduced signal reliability can cause females to lose the preference for the signal trait and result in the eventual loss of the trait from the population. Altered evolutionary trajectories as a result of anthropogenic disturbances have been documented, but are yet to be found in the context of EDC-contamination. My literature review indicates that such a phenomenon is very possible and merely awaits discovery.

The second chapter examines the effects of prolonged exposure to environmentally relevant doses of a common herbicide atrazine on ornaments and mating behaviors in adult male guppies. The results indicated that realistic exposures to atrazine can reduce ornament size and decrease the frequency of courtship displays and aggression towards competitors. In the third and fourth chapters, I tested for the effects of embryonic exposure to realistic concentrations of atrazine on the development and expression of male reproductive characteristics in guppies. Although the development of male genitalia and ornaments was not delayed, I found that there was a trend for smaller genitalia, and surprisingly, significantly increased ornament size at adulthood. In Chapter Four, I show that embryonic exposure to atrazine reduced courtship displays, mating frequency, and aggression in male guppies at adulthood. In both of these chapters, non-monotonic responses were observed, with the low dose of atrazine having stronger responses than the high dose.

In Chapter Five, I use a mathematical model to predict how EDC-exposure will disrupt the optimal balance of carotenoid allocation between ornament and immunocompetence to reduce signal reliability. I test whether this optimal allocation will shift, either as a plastic response or an evolutionary response, in populations exposed to EDCs for multiple generations. I then predict whether signal reliability can be restored under the shifted optimum, and also how additional selection pressures like predation can compound the effect of EDCs in the short and long-term.

Overall, my dissertation represents an attempt at understanding how EDC-exposure can alter mating systems via disrupted biochemical pathways. More work is required to properly understand these mechanisms. In the ecotoxicology literature, much emphasis has been laid on the effects of contaminants on the health of individuals, but little research has focused on evolutionary trajectories. I believe I have laid the foundation for some novel and informative future research in evolutionary ecotoxicology.

## CHAPTER TWO. ENDOCRINE DISRUPTION OF MALE MATING SIGNALS: ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS

### SUMMARY

Endocrine disrupting chemicals (EDCs) are chemicals that interfere with proper hormonal functioning in exposed animals. They enter the natural environment through multiple sources, and many non-target wildlife species are exposed to them via several modes. Exposure causes altered hormone levels, importantly gonadal hormones, resulting in changed reproductive characteristics. Vertebrate male mating signals convey important mate quality information to females. These signals are dependent on androgens for their production and maintenance. Female responses to signals depend on estrogens. Disrupting these pathways jeopardizes signal production and reception, which has implications for mating system ecology. Besides affecting various aspects of the vertebrate physiology, EDCs can impair hormonal functioning by binding to or blocking hormone receptors, or by altering production and function of hormones or hormone receptors. We consider the ecological implications of multi-generational signal disruption by EDCs. Altered signals can influence population dynamics and sex ratios; local extinctions are possible. Community-level dynamics may be affected via interspecific dependence on signals or population fluctuations. We then address the evolutionary effects of EDC-altered male mating signals in vertebrates and discuss how females may respond to altered signals over evolutionary time. Trans-generational reduction in signal reliability can lead to reduced preference and eventual loss of the signal trait and to the evolution of new traits as signals of mate quality. Genetic divergence between endocrine disrupted and undisrupted populations may result, perhaps giving rise to speciation. Finally, we recommend areas of research to further explore some of the issues addressed in this review. We suggest field surveys to document existing alterations in mating systems and genetic divergence in endocrine disrupted populations. Long term mesocosm studies and mathematical models would be useful to predict the fate of mating signals and female responses as a result of prolonged endocrine disruption. EDCs have been the focus of ecotoxicology for some time now, and we feel that this analysis should now enter the realm of evolutionary biology to determine the subtle, yet far-reaching effects on exposed non-target wildlife.

### INTRODUCTION

Endocrine disruption, the interference with proper hormonal functioning by exogenous compounds, has explicitly been a focus of ecological research for almost two decades now (since Colborn 1991). Some of the most important effects of these endocrine disrupting chemicals (EDCs) are the sub-lethal effects on populations caused by reproductive malfunctions. The most important of these are effects on reproductive traits, and these can have long term ecological as well as evolutionary significance, as we will emphasize. Endocrine disruptors can alter reproductive success by affecting all aspects of the reproductive system, including gonadal formation, production of hormones and gametes, sex determination (Basrur 2006), formation of egg shells (Porter and Wiemeyer 1969), and production (Kelce and Wilson 1997; Basrur 2006) and maintenance of mating signals and behaviors (Palanza and vom Saal 2002; Milnes et al. 2006). Several reviews have addressed endocrine disruption from ecological (Colborn et al. 1993b; Colborn 1995; Jones and Reynolds 1997; Crews et al. 2000; Guillette et al. 2000; Wingfield 2008), physiological (Basrur 2006; Milnes et al. 2006; Patisaul and Adewale 2009) and clinical (Toppari et al. 1996; Kelce and Wilson 1997) perspectives.

Endocrine disruptors can have lasting effects in local populations. Many of these chemicals are persistent in soil and animal tissue at low concentrations and can bioaccumulate (Smolen

and Colborn 1997); their effects are observable even at supposedly safe levels. The effects can be multi-generational either by exposure to soils containing persistent endocrine disruptors or via maternal effects (Clotfelter et al. 2004). Multi-generational changes in mating signals and behaviors in a local population can be of ecological significance if reproductive success is altered, and of evolutionary significance if populations evolve genetic responses to these alterations. Evolutionary effects may include altered mating systems or development of resistance to endocrine disruptors. If these changes are hereditary, intraspecific differences across populations may lead to genetic divergence.

In this review we address the effects of endocrine disruptors on mating signals and behaviors in male vertebrates and show how these changes can have ecological and evolutionary significance in wild populations. We focus primarily on the estrogenic and anti-androgenic effects of endocrine disruptors in vertebrate systems, as these are the most common effects of EDCs (Guillette 2006); androgenic contaminants are found mainly in livestock feedlot wastes (Soto et al. 2004; Kolok et al. 2007; Khan et al. 2008) and a few pesticides (Barbaglio et al. 2006). First, we outline concepts surrounding signaling theory and address altered signals and responses with the goal of understanding the implications of EDC-altered signals. Next, we review the control of these mating signals by androgens, and then elucidate the mechanisms by which EDCs can disrupt the production and maintenance of these signals. We then explore the ecological and evolutionary implications of altered mating signals and female responses as a result of prolonged EDC-exposure. Finally we recommend areas of research that could further explore some issues addressed in this article. We focus mainly on EDCs disrupting male signals, their potential for altering female responses, and the resulting implications for mating systems. Alteration of female signals or other reproductive characteristics, like number of eggs or size of eggs, is a separate and complex topic in itself and will not be considered in this review.

## MALE MATING SIGNALS AND FEMALE RECEIVERS – DEFINITIONS

For present purposes, we define **mating signals** as male morphological and behavioral traits that may be capable of transmitting information about the transmitter's quality and of influencing his reproductive success via female choice. Male **quality** expresses benefits that the male provides to the female, which directly or indirectly increase her fitness. **Displays** are behaviors aimed at attracting the attention of females and consequently showing off specific morphologies. Zahavi (1975) suggested that signals are costly to produce and maintain, "confer[ring] a handicap on survival". These costs may include metabolic costs of production and maintenance, and predation and parasitization risks due to the conspicuous nature of the signal or the added burden of a large ornament. In some systems where there are neither metabolic nor predatory costs on signal production and maintenance, social rules enforce signal honesty (Harris sparrows, *Zonotrichia quereula*: Rohwer and Rohwer 1978; house sparrows, *Passer domesticus*: Moller 1987). Signals thereby allow for assessment of quality, as implied by studies showing positive correlations between signal strength and male quality. For example, acquired resistance, a measure of quality in guppies (*Poecilia reticulata*), correlates with sigmoidal displays and orange color spots (Lopez 1998); foraging ability, another heritable trait that measures quality in guppies, is associated with coloration (Karino et al. 2005); bill color of male blackbirds (*Turdus merula*) correlates with parental abilities (Preault et al. 2005); and redness of northern cardinals (*Cardinalis cardinalis*) is strongly associated with quality of territory the male defended (ability to defend a good territory was the measure of quality; Wolfenbarger 1999). A signal is a reliable indicator of quality if there is a strong correlation between trait values expressed by the males and their quality, as defined according to the species (Maynard Smith and Harper 2003).



A signal has to be detectable, discriminable and assessable. We define detectability as the probability that the signal stands out from noise and is thus recognizable as a signal; discriminability as the probability that the receiver can accurately discern the level of the signal compared to the range of signal values produced by other individuals in the population; and assessability as the probability that the female can accurately associate the signal value with the appropriate quality level. Females receive these signals and respond more strongly to males expressing higher trait values (Grafen 1990). We refer to the measure of the females' response to a given quality as responsiveness. A female's response to a signal (i.e. how much she invests – time, energy, eggs – in that particular mating event) may be hereditary (Pomiankowski 1987), and a response has a cost to the female, either metabolic or through predation risk, or as lost mating opportunities (Pomiankowski 1987). Females should have distinct thresholds in true trait value below which they do not respond and above which they respond maximally. But trait values are perceived with error (Hoelzer 1989) and females differ in their threshold magnitude, resulting in responsiveness that can be expected to increase in a sigmoid fashion with male trait value. For each quality value there is a “best” response that maximizes female fitness (Grafen 1990).

To describe discriminability, I refer to Figure 2.1: here the x-axis represents the value of the trait (for example, wavelength being reflected, reflectance of a particular wavelength, frequency of sound waves, etc.), while the y-axis represents frequency density. Two representative curves are shown, each representing a particular mean trait value corresponding to a quality value. For explanatory purposes in this review, the curves represent a mean high trait value ( $\bar{T}_H$ ) produced by a high quality male (expressing quality  $Q_H$ ), and a mean low trait value ( $\bar{T}_L$ ) produced by a low quality male (expressing quality  $Q_L$ ). The y-axis then represents frequency of trait values expressed by males of quality  $Q_H$  and  $Q_L$ . The closer these curves are to each other, the less discriminable is the high trait value from the low trait value. Discriminability is thus a function of the difference between high and low-quality trait means, divided by their common standard deviation (cf. effect size of Cohen 1969). In reality, quality values will occur along a continuum, and corresponding trait values will also occur continuously. Discriminability would then refer to the probability that a trait value from a  $Q_H$  distribution is greater than a trait value from a  $Q_L$  distribution, for any pair of trait values selected randomly from the two distributions. Discriminability could be reduced either by smaller difference between the means, or by increased variance.

## ALTERED SIGNALS AND RESPONSES

Here I address altered signals and their implications, with a view to discussing implications of EDC-altered signals further in the article. **Altered signals** are traits used for signaling quality whose means have shifted substantially in a short span of time. After the change, these traits may or may not continue to signal the true quality of the signaler. Signals may be altered due to novel selection pressures like habitat changes (e.g. see the case study of great tits, *Parus major*, reviewed by Slabbekoorn and Ripmeester 2008). It is possible that the altered signal may continue to be discriminable as well as reliable, remaining a dependable indicator of quality, even if it is altered. Females may undergo an assessability lag, during which the sigmoid response curve is displaced along the axis of male quality because of decline in trait values (Figure 2.2A). Eventually under selection, by shifts in behavior or by evolutionary change, this curve may shift back to its former position along the quality axis. Consequently, if the trait values remain constant, the female response curve shifts along the axis of male trait values so that the new trait values generate responses similar to those previously associated with high and low male qualities (Figure 2.2B). Further, if endocrine disruption were to induce estrogenicity, females could become more responsive to male displays (as seen in ovariectomized African clawed frogs, *Xenopus laevis* treated with a combination of estradiol and progesterone: Kelley

1982; tungara frogs, *Physalaemus pustulosus* during periods of high circulating estradiol: Lynch *et al.* 2005), thus shifting the female response curve to the left and leading to less discrimination between high and low signal values (Figure 2.3A). In other words, females could become less choosy. An alternate possibility is that females become more choosy (as seen in zebra finches, *Taeniopygia guttata* treated with estradiol: Vyas *et al.* 2008; Vyas *et al.* 2009) by responding even lower to low signals and higher to high signals (Figure 2.3B). In this case, the sigmoid response curve has a steeper slope about the same inflexion point. A similar change in the curve, but with a shallower slope, could also produce less choosy responses. The exact theoretical mechanism by which estradiol produces choosier or less choosy female responses is not clear, but regardless there is evidence for both cases.

Some alterations lower the discriminability of the signal. In Figure 2.4, suppose after exposure to endocrine disruptors the high trait curve moves closer to the low trait curve (Figure 2.4B), or varied responses to the endocrine disruptor within the population lead to increased variances (Figure 2.4C); then the signal will be less discriminable or less reliable, respectively. Lowered discriminability implies a smaller range of trait values within the population, and does not necessarily imply unreliability of the signal. However, increased variance can reduce the correlation between trait value and quality, thereby reducing reliability (Figure 2.4C). Females may then be unable to rely on these traits to assess male quality. Not all altered signals are rendered unreliable, but they may be less reliable.

I assume that the exogenous compound responsible for signal alteration does not generally affect quality itself. However this may not be the case in all situations. For example, if the compound is an anti-androgenic or estrogenic compound, the same vector might enhance immunocompetence by reducing androgen levels (Folstad and Karter 1992), or reduce immunocompetence via other pathways (reviewed in Ahmed 2000; Christin *et al.* 2003; Brodtkin *et al.* 2007; Markman *et al.* 2008). In many animals, immunocompetence is a measure of quality (Hamilton and Zuk 1982; Lopez 1998). Such more complex cases are beyond the scope of this review.

In a system where a trait has evolved to be a reliable signal, and alterations render it either less reliable or unreliable, it becomes costly for females to depend on that signal as an indicator of quality. In such situations, one might expect females to stop responding to the signal, or respond more weakly. As illustrated in Figure 2.5, the loss or weakening of the signal-quality correlation can lead to a cyclic pattern of reduced female responsiveness to the signal and to the consequent further weakening of the signal-quality correlation. Reduced female choosiness (as in Figure 2.3A) relaxes selection pressures on the signal to be discriminable and through a similar cyclical pattern leads to the signal becoming a less reliable indicator of mate quality. A combination of effects of EDCs on both male signals and female responses can hasten the process of rendering the signal unreliable (Figure 2.5). Conversely, increased female choosiness (as in Figure 2.3B) raises selection pressures to make signals more discriminable. If males are able to respond to these pressures, the signal can become a more reliable indicator of mate quality; but if males are unable to make their signals more discriminable, choosier females face high costs of unreliable signals and are likely to stop using the trait as a quality indicator. Other traits may evolve to become primary indicators of quality, while the earlier trait might disappear (Morris *et al.* 2005) or become secondary cues in female mate assessment (Scheffer *et al.* 1996; Seehausen and van Alphen 1998; reviewed in Candolin 2003).

Signals evolve to be specifically received by conspecifics and often serve as mate-recognition factors (also reviewed in Ptacek 2000; Schluter 2001; courtship songs of sympatric flies, *Drosophila ananassae* and *D. pallidosa*: Yamada *et al.* 2002; mating preferences of the swordtail fish, *Xiphophorus pygmaeus*: Hankison and Morris 2003). If received and responded to by heterospecifics, hybridization may occur. This can happen in closely related sympatric species with similar signals (bluegill sunfish, *Lepomis macrochirus*, and pumpkinseed sunfish, *L. gibbosus*: Konkle and Philipp 1992). Changes between populations in sexual traits are quite

often driven by sexual selection (reviewed in Panhuis *et al.* 2001). The coevolution between signalers and receivers (males and females respectively, in the case of this review) based on the trait strengthens the linkage between mate quality advertising and assessment. Altered signals could hence blur the distinction between closely related sympatric species, leading to hybridization and possibly loss of species (Seehausen *et al.* 1997; Seehausen and van Alphen 1998).

## HORMONAL REGULATION OF MATING SIGNALS AND FEMALE RESPONSES

Testosterone, an important androgen, is believed to play a key role in the development and maintenance of ornaments and other signals in male vertebrates (Hillgarth and Wingfield 1997). Synthesis of 5 $\alpha$ -dihydrotestosterone (DHT) from testosterone is necessary for the development of external sexual organs (Neubert 2002). Aromatization of testosterone into estrogen in the brain is essential for the development and execution of appropriate male behaviors (Ball and Balthazart 2004).

For the purpose of this review male traits will be subdivided into male-specific anatomies, male-specific morphologies and male-specific behaviors, all relating to reproductive function. "Anatomy" will refer to internal organs, such as the larynx, vomeronasal organ and brain, responsible for the production or perception of signals, while "morphology" will refer to external features such as color patterns, plumage and other ornaments. "Behavior" will refer only to sex-specific reproductive behaviors. I will explore the role of androgens in the development and expression of these phenotypes.

Androgens are crucial for the proper development of **sexually dimorphic anatomies**. The example of the development of the larynx in anurans, illustrated below, provides a well-studied example. Males of most anuran species vocalize as a mating signal. Females of most species do not vocalize, while those that do have a different vocalization pattern from males. It is the difference in larynx structures that permits this difference between males and females.

Male vocalization requires certain specific muscle types that develop only in the presence of androgens. Male and female *X. laevis* produce distinctly different calls (Tobias *et al.* 1991). Production of male calls requires certain muscle types (fast twitch) that do not develop in females (Sassoon *et al.* 1987; Marin *et al.* 1990; Tobias *et al.* 1991). A larynx specific myosin heavy chain gene (LM) isolated from laryngeal muscles of *X. laevis* is responsible for conversion of laryngeal muscle type from slow twitch to fast twitch after metamorphosis (Catz *et al.* 1992). Expression of LM is regulated by endogenous androgens, especially DHT (Catz *et al.* 1995); the maintenance of fast twitch muscle fibers also requires androgens (Potter *et al.* 2005). Similarly, the syrinx in song-birds is an androgen-sensitive organ (Gong *et al.* 1999; reviewed in Wade 2001): female zebra finches with increased testosterone increase the size of their syrinxes (Gong *et al.* 1999; Wade and Buhlman 2000), while castrated males have reduced syrinxes, which can be reversed with testosterone or androsterone treatment (Harding *et al.* 1983). This has mainly been explored in zebra finches.

Males of many vertebrate species have conspicuous **morphologies** like bright plumage, large tails, and characteristic color patterns that signal the individual's quality to conspecific females. Development and maintenance of these sexually dimorphic morphologies is believed to be regulated by androgens and has been well studied in various taxa (red jungle fowl, *Gallus gallus* combs: Zuk *et al.* 1995; house sparrow badges: Gonzalez *et al.* 2001; superb fairy-wren, *Malurus cyaneus* plumage: Peters *et al.* 2001; guppy coloration: Jayasooriya *et al.* 2002; Strasser and Schwabl 2004; zebra finch beaks: McGraw *et al.* 2006; cirri lengths of Shermani salamanders, *Plethodon shermani*, used in uptake of pheromones: Schubert *et al.* 2006; cichlid *Pundalimia nyererei* nuptial coloration: Dijkstra *et al.* 2007).

The role of androgens in mating behaviors of vertebrates has been studied quite extensively in different taxa. **Male reproductive behaviors** may be defined as actions

performed by males that increase mating success, reproductive success and offspring survival. These include territoriality, courting females, vocalization, mounting females, building nests, and parental care. Studies from different taxa have shown the dependence of these behaviors on androgens. 11-Ketotestosterone (KT), a potent teleost androgen was found to be required for parental care in bluebanded goby (*Lythrypnus dalli*) (Rodgers et al. 2006), courtship behavior in mosquitofish (*Gambusia holbrooki*) (Toft and Guillette 2005) and guppies (Baatrup and Junge 2001; Bayley et al. 2002), and female recognition and courtship in goldfish (*Carassius auratus*) (Thompson et al. 2004b).

Studies on brain regions responsible for sexual behavior in birds and mammals have shown that the preoptic area (POA) and medial preoptic nucleus (POM) in the brain control these behaviors (reviewed in Ball and Balthazart 2004). These regions are sexually dimorphic and larger in males than in females. They are rich in androgen receptors. Castration causes these regions of the brain to shrink and corresponds to a reduction in sexual behavior, while implanting testosterone in the POA or POM can restore sexual behavior to pre-castration levels. Electrolytic lesions of the POM also disrupt sexual behaviors. It is believed that aromatization of testosterone into estrogen and DHT in these regions of the brain are responsible for male sexual behaviors (Ball and Balthazart 2004).

Empirical studies have correlated testosterone levels with mating behaviors in several bird species (singing, pursuing females, aggression towards males, territoriality in red grouse, *Lagopus lagopus scoticus*: Watson and Parr 1981; altered mating systems from monogamy to polygyny in white-crowned sparrows, *Zonotrichia leucophrys pugetensis*, and song sparrows, *Melospiza melodia*: Wingfield 1984; crowing and mounting attempts in Japanese quail, *Coturnix coturnix japonica*: Balthazart et al. 1990; bower features in satin bowerbirds, *Ptilonorhynchus violaceus*: Borgia and Wingfield 1991; extra pair copulations and time spent with paired female in mallard ducks, *Anas platyrhynchos*: Davis 2002). Similar patterns are also seen in amphibians (vocalization pattern in *X. laevis*: Kelley 1978; calling rates in tungara frogs: Marler and Ryan 1996; also reviewed in Moore et al. 2005; clasping behavior in *X. laevis*: Potter et al. 2005; activity levels associated with recognizing female pheromones in Shermani salamanders: Schubert et al. 2006).

Estradiol has been implicated in several taxa as the primary driver for female responses to male mating signals. In female tungara frogs, estradiol treatment increases receptivity, a positive response to a conspecific male call (Lynch et al. 2006; Chakraborty and Burmeister 2009), and permissiveness, a positive response to any male call, including unattractive calls (Lynch et al. 2006). Similarly, female tungara frogs were more receptive to male calls, and less choosy between attractive and unattractive calls during amplexus, the stage associated with increased levels of circulating estradiol when females are ready to oviposit (Lynch et al. 2005). In another study estradiol treatment made the frogs more choosy (Lynch et al. 2006); but here the females chose between an attractive conspecific call and a hybrid artificial call, which may simply resemble a heterospecific call rather than an unattractive conspecific call.

Female *X. laevis* treated with a combination of estradiol and progesterone increase receptivity to males (Kelley 1982). Similar estrogenic control of female responses to male mating behaviors has been seen in zebra finches (Vyas et al. 2008; Vyas et al. 2009), where estrogen treated females were more receptive to male songs, but also more discriminating between complex and simple male songs. Female white throated sparrows (*Zonotrichia albicollis*) implanted with estradiol responded to male songs with “copulation solicitation displays”, which corresponded to an increased expression of *zenk* (also known as *egr-1*) expression in the auditory forebrain (Maney et al. 2006). *Zenk* expression is associated with positive responses to male songs. Another study on the same species showed that *zenk* expression increased in nine brain regions thought to be part of the “social behavior network” in response to male songs only in estradiol treated females (Maney et al. 2008). Similarly, female tungara frogs treated with human chorionic gonadotropin (which stimulates the production of

gonadal hormones) showed higher expression of *egr-1* in the auditory midbrain in response to male choruses (Lynch and Wilczynski 2008). This mechanism is dependent on catecholamines, which allow the brain to respond selectively to important stimuli (LeBlanc *et al.* 2007). Estrogen treatment of female white throated sparrows increased density of catecholaminergic innervation in the auditory forebrain and the number of catecholaminergic cells in brain regions involved in song learning and production (LeBlanc *et al.* 2007).

## ENDOCRINE DISRUPTION OF MATING SIGNALS

The term endocrine disruption refers to the interference with proper functioning of the endocrine system due to exposure to exogenous compounds. Common types of EDCs include organochlorides, organophosphates, polychlorinated biphenyls (PCBs), phthalates, synthetic hormones and hormone-blockers, and phytoestrogens. These enter the natural environment via such sources as pesticides, industrial effluents, pulp mill effluents, plastics and sewage. Significant routes of exposure include direct exposures from living in contaminated soil or water, as well as indirect exposures through eating contaminated prey (Markman *et al.* 2007; Markman *et al.* 2008; Park *et al.* 2009; Walters *et al.* 2010). It is important to note that EDCs are often irregularly distributed along a landscape, exposing individuals differentially within a population.

Many ecological stressors that are not endocrine disruptors by definition also alter mating signals (acidification: Lorenz and Taylor 1992; hypoxia: Abrahams *et al.* 2005; heavy metals: Gorissen *et al.* 2005; Kuperberg *et al.* 2009; Ortiz-Santaliestra *et al.* 2009; Hallinger *et al.* 2010). Exploring these mechanisms is beyond the scope of this review. Here I restrict my discussion to EDCs, but note some effects of non-EDC contaminants and stressors (along with EDCs) on mating signals in Table 2.1.

Goksoyr *et al.* (2003) list the mechanisms by which EDCs can alter the natural functioning of hormones: (i) agonistically, by binding to hormone receptors and mimicking natural hormones; (ii) antagonistically, by blocking or altering the binding of natural hormones to hormone receptors; (iii) altering production and breakdown of natural hormones; and (iv) altering production and function of hormone receptors.

Estrogenic EDCs are agonistic and compete with estradiol to bind to estrogen receptors (ER- $\alpha$  or - $\beta$ ) (Kuiper *et al.* 1998), or increase endogenous estrogens by inducing aromatization (Fan *et al.* 2007). Exposure to estrogenic EDCs is associated with decreased circulating testosterone levels in male vertebrates (e.g. 4-tert-octylphenol (OP): Blake and Boockfor 1997; bisphenol-A: Takao *et al.* 1999; o,p'-DDT: Mills *et al.* 2001; endosulfan: Saiyed *et al.* 2003; Kim *et al.* 2007; atrazine: Hayes *et al.* 2010; Nakamura *et al.* 2010; Victor-Costa *et al.* 2010), thereby altering androgen-regulated mating signals. The mechanism by which estrogenic EDCs decrease testosterone production in testes appears to be via the reduced expression of steroidogenic enzymes (estradiol and diethylstilbestrol: Bartke *et al.* 1977; di(n-butyl) phthalate: Thompson *et al.* 2004a; OP: Kim *et al.* 2007; PCB: Murugesan *et al.* 2008) and altered cholesterol metabolism and transport (di(n-butyl) phthalate: Thompson *et al.* 2004a; OP: Kim *et al.* 2007) in Leydig cells.

EDCs that have anti-androgenic effects in vertebrates prevent the binding of androgen with the androgen receptor (AR), thereby inhibiting transcription of AR-dependent genes. Binding of androgen to AR causes a conformational change in AR that is required for stabilization against proteolytic degradation, and for the AR dimerization necessary for transcriptional activation (Quigley *et al.* 1995; Zhou *et al.* 1995). Mechanisms by which anti-androgens inhibit the functioning of AR-dependent gene transcription are by preventing AR binding to DNA or by preventing initiation of transcription (Truss *et al.* 1994); most of the studied environmental anti-androgens function by the former mechanism (Kelce and Wilson 1997). This disruption of AR-dependent gene transcription can interfere with sex differentiation, development of sexually dimorphic anatomies, morphologies and behaviors, production of sperm, and maintenance of

sexually dimorphic morphologies and behaviors (as illustrated in Table 2.1), ultimately affecting the individual's reproductive success.

Evidence of contaminant induced alteration of male mating signals is seen in various taxa exposed to a range of chemicals. Table 2.1 lists examples of altered expression of male mating signals by exposure to various EDCs as well as non-endocrine contaminants. In most cases, exposure to the contaminant reduced the expression of the signal compared to the controls. Edwards and Guillette (2007) suggest that the increased gonopodial length of nitrate-exposed mosquitofish could be due to the inhibition of steroid synthesis because of the deactivation of P450 enzymes by nitrates. Of special interest is the paradoxical positive effect of EDCs on song characteristics accompanied by increased HVC volume, the brain region involved in singing, in the case of European starlings (*Sturnus vulgaris*), which increased their attractiveness to female starlings (Markman *et al.* 2008). These results are supported by evidence for the role of estrogens in the development of male-specific song nuclei in the brain of songbirds (Schlinger 1997; Holloway and Clayton 2001). However the increased song complexity was accompanied by reduced immunocompetence, which has implications for fitness (Markman *et al.* 2008). It is not clear whether the increased song complexity was proportional to mate quality in this study. But if immunocompetence can be considered a measure of mate quality, then the increased song complexity and decreased immune responses as a result of EDC-exposure only blurs the correlation between signal and quality, leading to females being attracted to individuals of perhaps lower quality than expected. However, these results are not consistent with other studies; Iwaniuk *et al.* (2006) showed a decreased volume of brain regions involved in singing and sexual activities in American robins (*Turdus migratorius*) that came from nests with eggs containing high DDT levels. Heavy metals also appear to negatively affect song characteristics (see Table 2.1 for details). Hence the effects of EDCs on birdsong appear to depend on the particular pollutant, and the exact mechanisms of action are not clear. The effects of various pollutants on reproductive behaviors of fish including courtship behaviors, parental care and aggression have been reviewed in Jones and Reynolds (1997).

## ECOLOGICAL IMPLICATIONS OF ALTERED MALE MATING SIGNALS

Signal disruption due to feminization of males exposed to EDCs can have significant ecological effects, if the contaminant affects a large section of the population. Altered mating signals can include size of ornaments, intensity of colored signals, pheromonal production, and courtship behaviors such as displays, nest building, and competitiveness (reviewed in Clotfelter *et al.* 2004). Females discriminate against males with EDC-altered mating signals (e.g. Arellano-Aguilar and Garcia 2008; Secondi *et al.* 2009), thus directly affecting the males' fitness. Altered demographics, and local extinctions in extreme cases, are possible if females do not adapt or otherwise adjust to the altered signals soon enough. If the altered signal is not reliable, both males and females incur higher costs that reduce reproductive success. Costs to males include reduced mating opportunities; costs to females include possible reductions in offspring number and quality. Similar costs also arise for females if endocrine disruption renders them less choosy; in this case it is also unduly costly for males to have discriminable signals. If however, females are rendered more choosy, they incur heavier costs by depending on unreliable signals. Increased choosiness can increase the frequency of extra-pair copulations (EPCs) in species where this occurs (Kempnaers *et al.* 1997). Increased mating frequency increases fitness costs such as predation risks, energy costs, the risk of pathogen transfer, and so forth (Keller and Reeve 1995). Increased EPCs can further alter demographics by decreasing effective population sizes. Fitness costs to females and propagation of lower quality individuals as a result of signal mis-communication have consequences for population persistence (Kokko and Brooks 2003). The effects on individuals have obvious links to population level effects via reduced reproductive success resulting from signal mis-communication and supernormal clutch

sizes (with reduced success) from female-female pairs as a result of reduced availability of male partners (Hunt and Hunt 1977; Ryder and Somppi 1979; Fox 1992).

The effects of mate choice on population dynamics have been reviewed by Quader (2005), and the implications are relevant to altered mating signals. It has been suggested by several authors that environmental changes are more threatening to species with higher degrees of sexual selection (Kokko and Brooks 2003; Morrow and Pitcher 2003); altered mating signals in such species could affect population viability. The resulting low fitness of individuals in these contaminated habitats can produce ecological sinks (Matson *et al.* 2006). Sex ratios can be skewed due to decreased predatory risks for males as a result of less obvious ornaments. This can also have community and ecosystem level effects if population sizes fluctuate uncharacteristically (e.g. Whiles *et al.* 2006; Brown and Lawson 2010). Altered mating signals can affect parasite-host relationships where parasites detect hosts by their mating signals (Zuk and Kolluru 1998).

Gomulkiewicz and Holt (1995) developed a population growth model to predict when populations would avoid extinctions in novel environments that reduce fitness. They defined a species-specific critical threshold level of population size,  $N_c$ , below which the population is at risk of extinction due to demographic stochasticity. Reduced reliability of signals under severely altered environmental conditions could trigger a decline below  $N_c$ . In this case natural selection will drive altered female preferences to the extent that these are genetic; sexual selection on males may produce greater investment in signaling to increase signal expression despite endocrine disruption, potentially causing population size to increase above  $N_c$ . In the model, the degree of initial maladaptation to the novel environment  $\beta_0$  depends directly on the difference between the initial mean trait value and the optimal trait value for the altered environment. Initial maladaptation also depends inversely on the variance in the trait value about the mean. Assume the trait is female responsiveness, the ability to correctly assess male quality and respond accordingly. Altered signals might require females to alter their responses (as in Figure 2.2). Hence their original responsiveness will be a maladaptation. The farther this is from the responsiveness required to maximize fitness in the face of altered male signals, and the lower the original variation in this trait was, the higher the degree of maladaptation. If  $\beta_0$  is very high as a result of strong coevolution between the trait and female responsiveness over the years, then there is a higher chance of local extinctions. The assessability lag (during which females shift their response curve to adapt to the new trait values) will be a crucial factor in determining whether the population will be able to rise above  $N_c$  before stochasticity eliminates it.

Exposure to EDCs can have physiological implications that might be important for carotenoid-based signal expression. The carotenoid allocation trade-off between mating signal and immunocompetence plays an important role in the evolution of many mating systems (Hill 1991; Folstad and Karter 1992; Zuk 1992; Lozano 1994); disruption of this relationship can have ecological and evolutionary implications. Elevated androgen levels retard an individual's immune response (Folstad and Karter 1992), leading to the honesty of androgen-regulated signals (Immunocompetence Handicap Hypothesis: Folstad and Karter 1992). Carotenoids can function as immunoenhancers (McGraw and Ardia 2003; Grether *et al.* 2004; but see also Kolluru *et al.* 2006). Also androgens are thought to have a positive effect on bioavailability of plasma carotenoids because of increased dietary intake due to elevated androgens, increased mobilization of carotenoids from body stores, or increased absorption efficiency of ingested carotenoids as a result of elevated androgen levels (Blas *et al.* 2006). Most carotenoid-based signals are believed to be truthful signals because a high quality individual (defined by his immunocompetence) can allocate more carotenoids to the signal and less to his immune system (Faivre *et al.* 2003; Alonso-Alvarez *et al.* 2004). Besides affecting androgen levels, EDCs can directly or indirectly impact immunocompetence (reviewed in Ahmed 2000; Ndebele *et al.* 2003; Hayes *et al.* 2006b; Inadera 2006; Brodtkin *et al.* 2007; Filby *et al.* 2007), circulating plasma carotenoid levels, and consequently the allocation of carotenoids to the different

functions. Altered immune responses are crucial to population dynamics especially in environments with high pathogen and parasite densities. Thus understanding how EDCs might affect immune responses and allocation of carotenoids can be of ecological importance.

## EVOLUTIONARY IMPLICATIONS OF ALTERED MATING SIGNALS

Evolved resistance to pollutants in target and non-target species has been documented; a large body of literature exists on this topic (reviews: Dover and Croft 1986; Roush and McKenzie 1987; Croft and van de Baan 1988; Leibeck and Capinera 1995; Bard 2000; here I cite a few examples: Hemingway and Ranson 2000; Twigg et al. 2002; Brammell et al. 2004; Lopes et al. 2008; Brausch and Smith 2009; Arzuaga and Elskus 2010). If individuals develop resistance to EDCs, their mating signals may not alter. However, there are bound to be differential levels of resistance within a population with some individuals being better able to resist the pollutant than others; hence the degree of signal alteration will differ accordingly. Whether this will correlate with quality cannot be predicted. If resistance correlates with quality, then the altered signal may also correlate with quality, maintaining signal reliability; otherwise, signals may become less reliable. Further, some populations may evolve resistance, while others may not. This is an important topic that cannot be addressed within the scope of his article. I restrict my discussion to situations where signals are subject to being altered by EDCs.

Altered signals in local populations due to persistent multi-generational exposure to EDCs can have significant evolutionary implications. Genetic divergence in the form of resistance to EDCs has been detected (Elskus *et al.* 1999; Crews *et al.* 2000). Divergence as a result of sexual selection in altered habitats has been documented (Seehausen et al. 1997; Craig and Foote 2001). However, divergence due to selection on altered signals resulting from EDCs has not been examined. Vertebrate and invertebrate traits can evolve rapidly across a few generations (Thompson 1998; Lande et al. 2001), notably including reproductive proteins (Clark et al. 2006). Labonne and Henry (2010) showed with a simulation model that phenotypic divergence in response to natural and sexual selection can occur within twenty generations; the simultaneous evolution of female preference is somewhat slower, taking up to two hundred generations or longer. It is worth examining whether these anthropogenic factors can lead to varied responses of females to male signals, disappearance of signals in some populations, or evolution of novel signals in others.

Many ecological and behavioral factors can cause or facilitate disruptive selection and ultimately speciation. The most common of these factors are female preference for male phenotypes (Seehausen et al. 1999; Ptacek 2000) and environmental changes (passerine birds: Barraclough et al. 1995; mouse subspecies, *Mus musculus domesticus* and *M. m. musculus*: Laukaitis et al. 1997; cichlid fishes: Seehausen et al. 1997; anurans: Ptacek 2000; Little Greenbul, *Andropadus virens*: Slabbekoorn and Smith 2002). Craig *et al.* (2001) discovered a polymorphism in sockeye salmon (*Oncorhynchus nerka*) as a response to altered environment. Anadromous sockeye landlocked in freshwater lakes are nonanadromous and are called kokanee. Both sexes of sockeye and kokanee turn bright red at maturation. However, fresh water systems are poor in nutritional resources and should provide relatively low levels of carotenoids. Sockeye bred in fresh water remain green due to a lack of carotenoids. Apparently because of a sexual preference for the color red, kokanee evolved more efficient mechanisms of carotenoid utilization, thus diverging genetically from the ancestral sockeye (Craig and Foote 2001). This is a clear example of genetic divergence driven by selection on a signal altered due to habitat modification.

Male signal production and female response to the received signal have coevolved (Arnqvist and Rowe. 2005) such that females can adjust their response to varying levels of signal quality. In recent decades, rapid environmental changes have led to drastic alterations of habitats of many animals, with important implications for mating interactions. For example,



turbidity reduces perceptivity of visual signals in aquatic species (Seehausen *et al.* 1997), structural modifications of habitats alter perception of auditory signals (Telford *et al.* 1989; Gerhardt 1992), and noisy environments cause animals to vocalize less (Lengagne 2008). If novel environmental changes prevent the signal perceived by the female from reliably reflecting quality, will female responses shift accordingly? A different disruption of these signaling systems can occur when environmental changes affect the male trait, causing him to alter the transmitted signal. In this case, females perceive signals as they are produced, but it is the production that is disrupted. But female perception of signals can also be altered by EDC-exposure, as discussed above. In any case, environmental changes are causing a discrepancy between male traits and female assessment of male quality.

Female responses to male signals can be a strong driving force for genetic divergence. In a population where sufficient numbers of individuals are exposed to endocrine disruptors, the exposed males may no longer be able to produce signals that correspond to their quality. There may be reduced discriminability as well as reliability (Figure 2.2). Such ideas have been suggested by other authors (Secondi *et al.* 2009) but remain to be tested. Females should rapidly lose the preference across generations if the trait no longer signals true quality. This process should be hastened by altered female choosiness (Figs 3 and 5).

Males of several swordtail fish species of the genus *Xiphophorus* have tails with long swords apparently to indicate larger body size to females (Basolo 1998a, b; Rosenthal and Evans 1998). Females of the descendent species *X. nigrensis* have lost their preference for the sword, yet the males have not lost their swordtails. Basolo (1998a) and Rosenthal, Wagner and Ryan (2002) postulate that increased predation risks of associating with sword-bearing males, or scarcity of heterospecifics reducing the need for strong species recognition, may have undermined preference for the ornament. If production and maintenance of the sword were expensive, there would be natural selection pressures on males to lose the sword. Though the sword is a relatively inexpensive ornament with regard to metabolism (Rosenthal *et al.* 2002), predation risks associated with a conspicuous ornament are high, and the persistence of the trait is perplexing (Rosenthal *et al.* 2002). It is possible that the sword has other purposes, such as determining dominance in intraspecific competition (Rosenthal *et al.* 2002).

But sexually selected male traits can be lost (reviewed in Wiens 2001). Swordtail fish species show a range of morphological and behavioral signals. Males of the species *X. continens* do not have any of the reproductive characteristics that other swordtail males exhibit as mating signals (Morris *et al.* 2005). These males are small and lack swords and color patterns (vertical bars). A lack of female preference for large males and low male-male-competition may account for the loss of large body size in these males, but the loss of vertical bars and swords remain unexplained (Morris *et al.* 2005).

How do females then assess mate quality? A mutant female may use a different indicator to assess male quality that may not require androgens for maintenance. The cichlid fishes of the eutrophic Lake Victoria use body size to assess mates, whereas they were using color before the disruption of visual signals by increasing turbidity (Seehausen *et al.* 1997). There is a higher rate of hybridization in these lakes, leading to the loss of species and possibly emergence of new ones if hybridized fishes persist. Such situations may lead to the evolution of a new signal of quality that females use for mate assessment by starting a whole new sequence of coevolutionary signal production and response between males and females (as illustrated in Figure 2.5). Conversely, decreased female choosiness might lead to a scramble-competition mating system where females bias male mating success through resistance and cryptic choice (Dunn *et al.* 1999; Blanckenhorn *et al.* 2001; Nahrung and Allen 2004), with sexual selection acting more on male traits enabling better access to females (Dunn *et al.* 1999; Bertin and Cézilly 2003; Alcock and Kemp 2005; Bertin and Cézilly 2005; Greene and Funk 2009; Lu *et al.* 2010).

If the effects of EDCs span many generations, there is the possibility of a mutation in female preference that can be heritable. Female preferences for certain male traits are believed to be heritable (Lande 1981; Kirkpatrick 1982; Pomiankowski 1988; Haesler and Seehausen 2005). Labonne and Hendry's (2010) simulation of guppy evolution suggested two hundred generations or more for detectable changes in female preference; for guppies, this can translate to about seventy years. Certain chemicals are extremely persistent in the environment. DDT, one of the most persistent organic pollutants, has a half-life in soil of upto 15 years, depending on the type of soil (HSDB <http://toxnet.nlm.nih.gov/>). This means that a hundred years later approximately 1% of the current amount of DDT will still be present in the soil. This may be significant in areas of intense use of the chemical. Further, some chemicals may continue to be used by humans for many years. Can this time frame be enough to produce genetic divergence? Also, would genetic changes persist in an environment free of endocrine disruptors?

With this in mind, I expect a population of animals exposed to endocrine disruptors to alter their mating systems relative to unexposed populations. Evolution of resistance should not affect this prediction due to differential degrees of resistance within a population. Females may be less choosy with less discriminable mating signals, or females may rely on different indicators of male quality. It is also possible that females could evolve more astute sensory systems. In extreme cases where signals become totally unreliable, a new system of mate quality assessment may evolve – a system different from that of conspecifics not exposed to endocrine disruptors. If the original male trait was based on pre-existing sensory biases, the female preference may re-establish in the population, leading to the reemergence of that trait as a signal. Otherwise, in rare cases, this genetic divergence might lead to speciation if the new male trait used for quality assessment becomes elaborated under selection associated with refocused female preference. To my knowledge, speciation as a result of EDC-exposure has not been documented, but genetic divergence in the form of resistance has been observed (Elskus *et al.* 1999; Crews *et al.* 2000). EDCs often do not affect entire populations due to their irregular distribution in the environment; further there is usually gene flow between exposed and unexposed groups. However, when large amounts of persistent EDCs cover an extensive area (e.g. Lake Apopka: Woodward *et al.* 1993), animal populations inhabiting these places might diverge from their unexposed relatives after multiple generations of exposure and relative isolation.

## FUTURE DIRECTIONS

Field studies on altered male reproductive characteristics have been conducted in EDC contaminated habitats (reviewed in Rattner 2009). Of further interest would be to study whether endocrine disruption alters female responses to EDC-exposed and unexposed males, and to quantify fitness costs of these altered responses. To understand the evolutionary implications of endocrine disruption, we need data on whether the relationship between a male mating signal and the measure of quality for that species is comparable in contaminated versus uncontaminated habitats. Quantifying the allocation of dietary pigments like carotenoids, as well as those synthesized *de novo* like melanin and pterins, to various physiological and reproductive functions, would provide insights into the effects of endocrine disruption on the value of these pigments as mediators of honest signals. Mathematical models could be developed to project the fate of a signal in disrupted environments. Long term mesocosm experiments using species with short generation times might provide better insights into these questions. Larger variances in measured traits as a response to EDCs might imply variance within the population in resistance to the contaminant, raising questions about the mechanics of differential development of resistance to EDCs among individuals.

Genetic divergence, and perhaps speciation could be extreme results of persistent exposure of local populations to endocrine disruptors; however, there is no direct empirical evidence of such phenomena. When responses occur on evolutionary time scales, experiments to test these hypotheses might be unrealistic in many systems. Comparative genomic studies of species in contaminated and un-contaminated areas could provide us with information on genetic divergence between endocrine disrupted and non-disrupted populations. Mathematical models could project the fate of populations with disrupted signaling systems. While ecological and physiological implications of EDCs have received scientific focus, it is now necessary to divert these efforts to the understanding of rapid evolutionary changes in local populations exposed to EDCs over several generations.

**Table 2.1.** Empirical studies showing altered reproductive traits in male vertebrates after contaminant-exposure. The arrows indicate the direction of change compared to controls. See Jones *et al.* (1997) for a review of the effects of pollutants on reproductive behaviors in various fish species.

| Species  | Trait disrupted                               |   | EDC   | Reference  |
|--|---|---|---|--|
| Guppy<br>( <i>Poecilia reticulata</i> )                              | Sigmoid display rate                          | ↓ | Flutamide, DDE, Vinclozolin                   | (Baatrup and Junge 2001)                                       |
|  | Time spent in mating behaviors                | ↓ | Flutamide, DDE, Vinclozolin                   | (Baatrup and Junge 2001)                                       |
|  | Area of orange color spots                    | ↓ | Vinclozolin, Flutamide<br>4-tert-Octylphenol  | (Baatrup and Junge 2001)<br>(Toft and Baatrup 2001)            |
|  | Gonopodium length                             | ↓ | Vinclozolin                                   | (Bayley <i>et al.</i> 2002)                                    |
| Three-spined<br>stickleback<br>( <i>Gasterosteus<br/>aculeatus</i> ) | Nest building behavior, courtship<br>behavior | ↓ | Fenitrothion                                  | (Sebire <i>et al.</i> 2009)                                    |
|  | Courtship behavior                            | ↓ | Perchlorate                                   | (Bernhardt and von Hippel 2008)                                |
| Atlantic salmon<br>( <i>Salmo salar</i> )                            | Olfactory response to female<br>pheromones    | ↓ | Atrazine<br>Atrazine, simazine                | (Moore and Waring 1998)<br>(Moore and Lower 2001)              |
| Medaka<br>( <i>Oryzias latipes</i> )                                 | Courtship behavior                            | ↓ | Tributyltin, mixture of<br>tributyltin + PCBs | (Nakayama <i>et al.</i> 2004)                                  |
| Amarillo fish<br>( <i>Girardinichthys<br/>multiradiatus</i> )        | Median fins                                   | ↓ | Methyl parathion                              | (Arellano-Aguilar and Garcia<br>2008)                          |
|  | Courtship behaviors                           | ↓ | Methyl parathion                              | (Arellano-Aguilar and Garcia<br>2008)                          |
| Sand goby<br>( <i>Pomatoschistus<br/>minutus</i> )                   | Courtship                                     | ↓ | 17 $\alpha$ -ethynyl estradiol                | (Saaristo <i>et al.</i> 2009)                                  |
|  | Leading female to nest                        | ↓ | 17 $\alpha$ -ethynyl estradiol                | (Saaristo <i>et al.</i> 2009)                                  |
|  | Completion for nesting sites                  | ↓ | 17 $\alpha$ -ethynyl estradiol                | (Saaristo <i>et al.</i> 2009)                                  |
| Mosquitofish<br>( <i>Gambusia holbrooki</i> )                        | Gonopodium length                             | ↓ | Nitrate                                       | (Edwards and Guillette 2007)                                   |
| Fighting fish<br>( <i>Betta splendens</i> )                          | Opercular display                             | ↓ | Hypoxia                                       | (Abrahams <i>et al.</i> 2005; Kuperberg<br><i>et al.</i> 2009) |

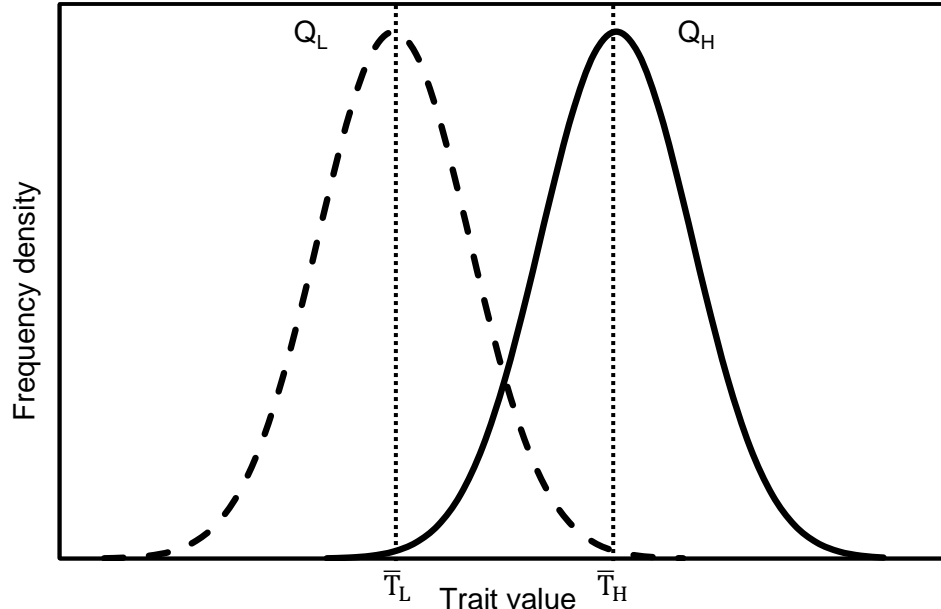
Table 2.1, continued

|  |  |   |   |  |
|--|--|---|---|--|
| Palmate newt ( <i>Triturus helveticus</i> )          | Secondary sexual traits (combination of filament length, tail height, hind foot web area)            | ↓ | Nitrate   | (Secondi <i>et al.</i> 2009)                                 |
|  | Olfactory cues   | ↓ | Nitrate   | (Secondi <i>et al.</i> 2009)                                 |
| Iberian newt ( <i>Lissotriton boscai</i> )           | Total courtship time   | ↓ | Acidification   | (Ortiz-Santaliestra <i>et al.</i> 2009)                      |
|  | Persuasion index   | ↓ | Acidification   | (Ortiz-Santaliestra <i>et al.</i> 2009)                      |
|  | Time to begin courtship  | ↓ | Acidification   | (Ortiz-Santaliestra <i>et al.</i> 2009)                      |
| Table 2.1, continued                                 |  |   |   |  |
| African clawed frog ( <i>Xenopus laevis</i> )        | Larynx size  | ↓ | Atrazine  | (Hayes <i>et al.</i> 2002a)                                  |
|  | Size of nuptial pads   | ↓ | Atrazine  | (Hayes <i>et al.</i> 2010)                                   |
|  | Size of breeding glands  | ↓ | Atrazine  | (Hayes <i>et al.</i> 2010)                                   |
|  | Larynx structure resembled female larynges   | ↓ | Atrazine  | (Hayes <i>et al.</i> 2010)                                   |
| Alligator ( <i>Alligator mississippiensis</i> )      | Penis size   | ↓ | pp-DDE <sup>1</sup>   | (Guillette <i>et al.</i> 1996; Guillette <i>et al.</i> 1999) |
| Japanese quail ( <i>Coturnix coturnix japonica</i> ) | Strutting and copulatory behavior  | ↓ | Flutamide   | (Adkins-Regan and Garcia 1986)                               |
|  | Reproductive behaviors   | ↓ | Ethinylestradiol, diethylstilbestrol                                    | (Halldin 2005)   |
| European starling ( <i>Sturnus vulgaris</i> )        | Song characteristics (time spent singing, number of song bouts, song bout duration, repertoire size) | ↑ | mixture of estradiol + dioctylphthalate + bisphenolA + dibutylphthalate | (Markman <i>et al.</i> 2008)                                 |
|  | HVC (brain area controlling song)  | ↑ | mixture of estradiol + dioctylphthalate + bisphenolA + dibutylphthalate | (Markman <i>et al.</i> 2008)                                 |
| American robin ( <i>Turdus migratorius</i> )         | Brain regions involved in singing and sexual behavior  | ↓ | DDT   | (Iwaniuk <i>et al.</i> 2006)                                 |
| Carolina Wren ( <i>Troglodytes aedon</i> )           | Song characteristics (tonal frequency, note types per song,  | ↓ | Mercury   | (Hallinger <i>et al.</i> 2010)                               |

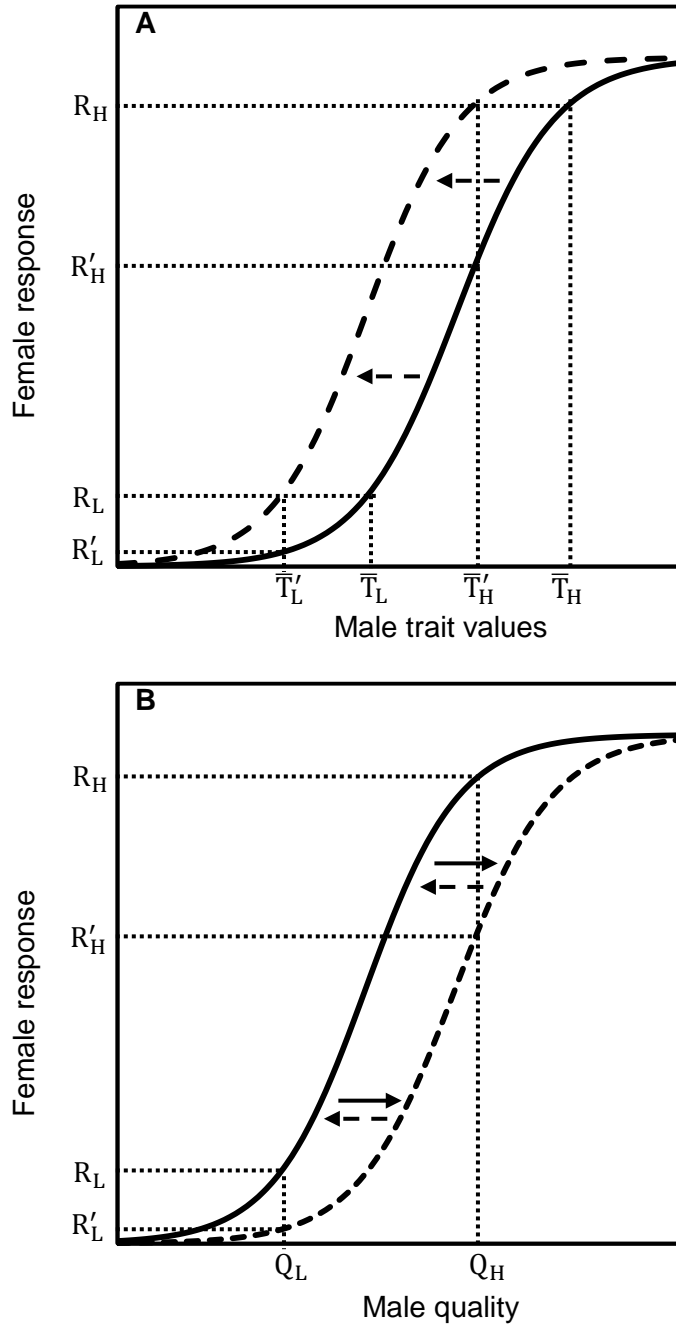
<sup>1</sup> Note: These studies were conducted on alligators inhabiting contaminated lakes. The contaminant is suspected to be pp-DDE, but it is possible that other contaminants might have induced the change in the measured trait.

Table 2.1, continued

|  |  |   |         |                                |
|--|--|---|---------|--------------------------------|
| <i>ludovicianus</i> ),<br>House Wren<br>( <i>Troglodytes aedon</i> ) | average strophe length, peak<br>frequency of most repeated<br>element)   |   |         |                                |
| Song sparrow<br>( <i>Melospiza melodia</i> )                         | Song characteristics (tonal<br>frequency, peak frequency and<br>bandwidth of buzz notes, number<br>of note types per song) | ↓ | Mercury | (Hallinger <i>et al.</i> 2010) |
| Great tit<br>( <i>Parsus major</i> )                                 | Amount of song, repertoire size  | ↓ | Lead    | (Gorissen <i>et al.</i> 2005)  |



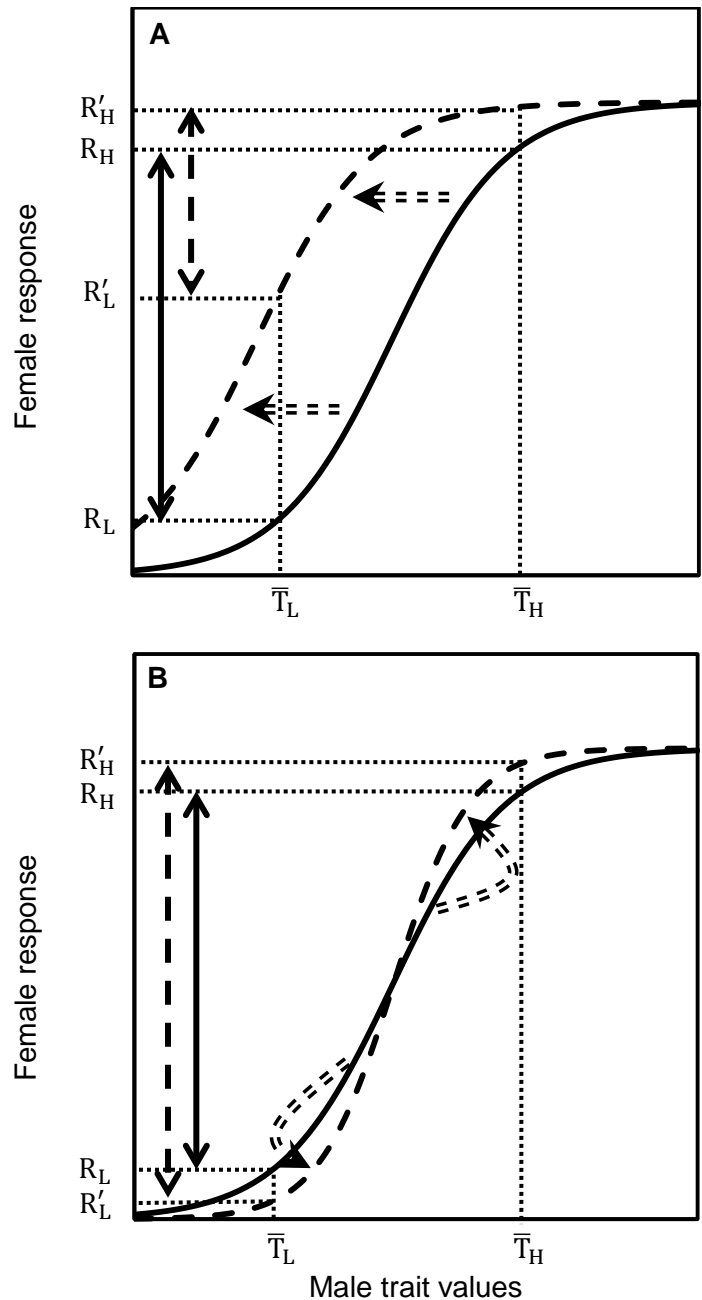
**Figure 2.1.** Discriminability between signal trait values represented by individuals of two differing quality values. The solid curve represents the trait value ( $T_H$ ) produced by the high quality male ( $Q_H$ ) while the broken curve represents the trait value ( $T_L$ ) produced by the low quality male ( $Q_L$ ). The means are represented by  $\bar{T}_H$  and  $\bar{T}_L$  respectively. Discriminability is a function of the difference between high and low-quality trait means, divided by their common standard deviation (effect size: Cohen 1969). The higher the discriminability, the greater is the probability that the high trait stands out distinctly from the low trait. The overlapping areas allow for incorrectly identifying the low trait as being produced by the high quality male or *vice versa*.



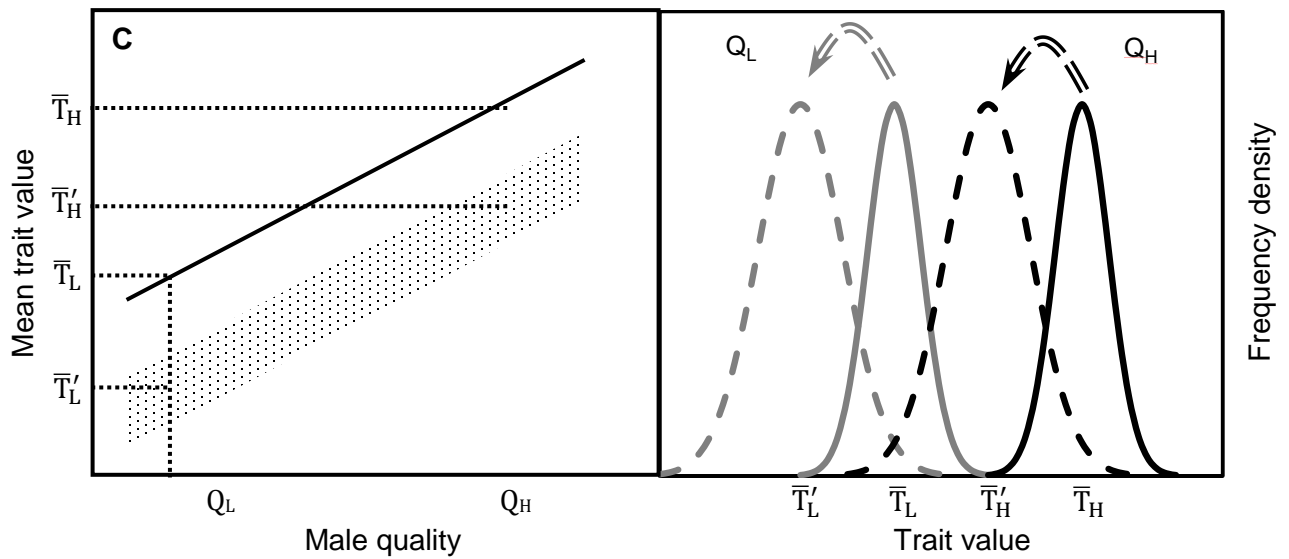
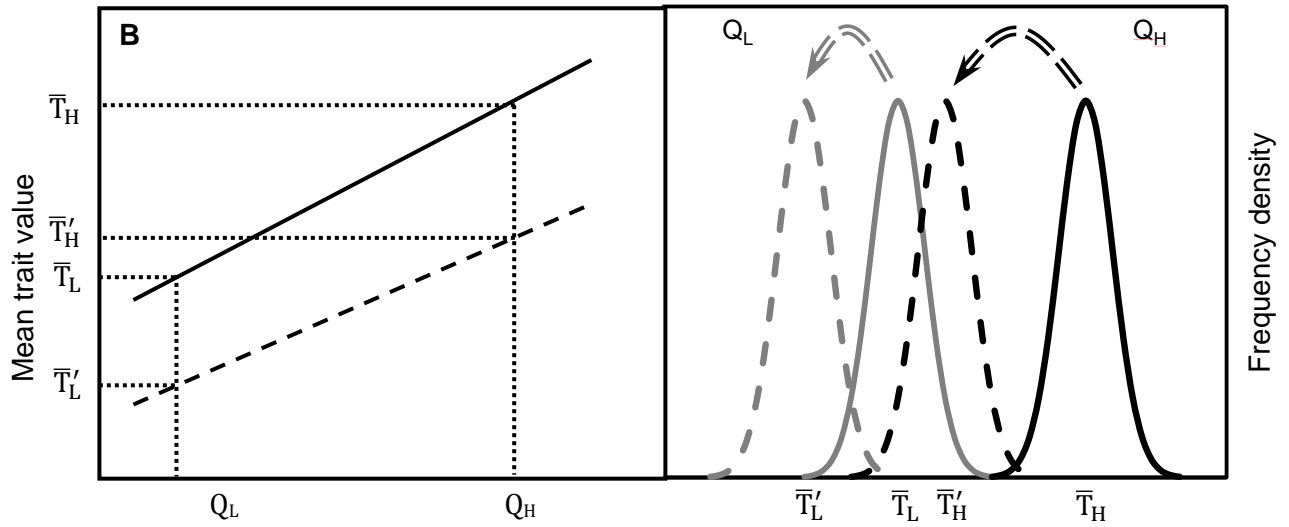
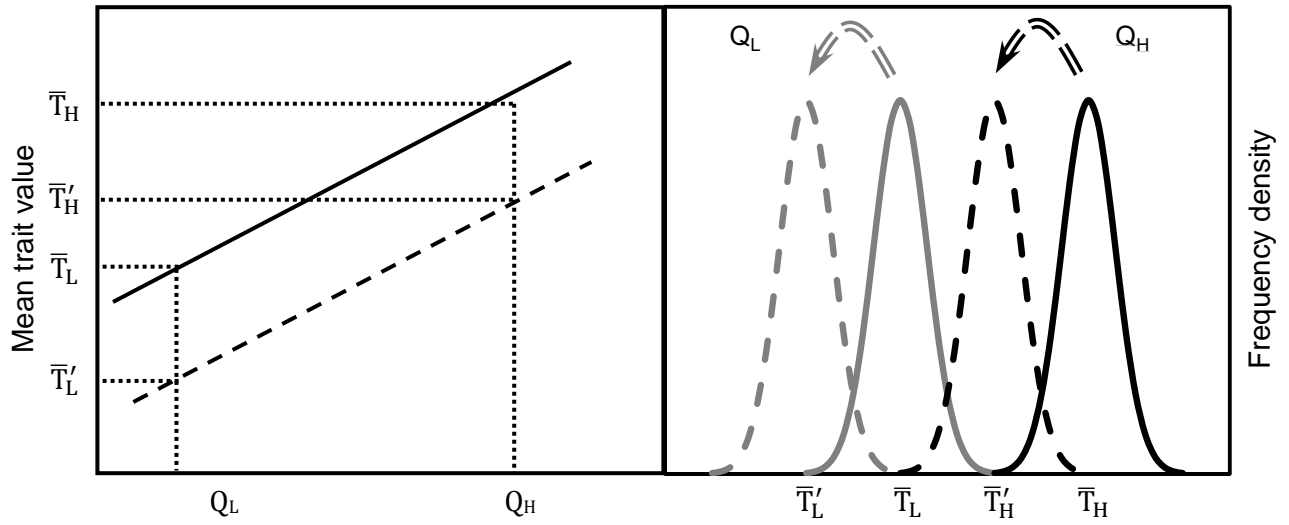
**Figure 2.2.** Female responses to the altered male signals. The solid line represents original female responsiveness to unaltered signals. (A) After endocrine disruption, females respond  $R'_H$  and  $R'_L$  respectively to the altered mean trait values  $\bar{T}'_H$  and  $\bar{T}'_L$ , which is a lower than  $R_H$  (response to  $\bar{T}_H$ ) and  $R_L$  (response to  $\bar{T}_L$ ). This reduced response increases fitness costs to both males and females, imposing selection pressures on females to alter their responses. Hence, across generations, female responses should adjust to the altered signals such that a strong response ( $R_H$ ) should now match the altered mean high trait value  $\bar{T}'_H$  and the weak response ( $R_L$ ) should match  $\bar{T}'_L$ . The time taken for this shift is the assessability lag. (B) After endocrine disruption, both high quality and low quality males signal lower than their original means. Females will associate these lowered trait values with lower quality and respond more weakly



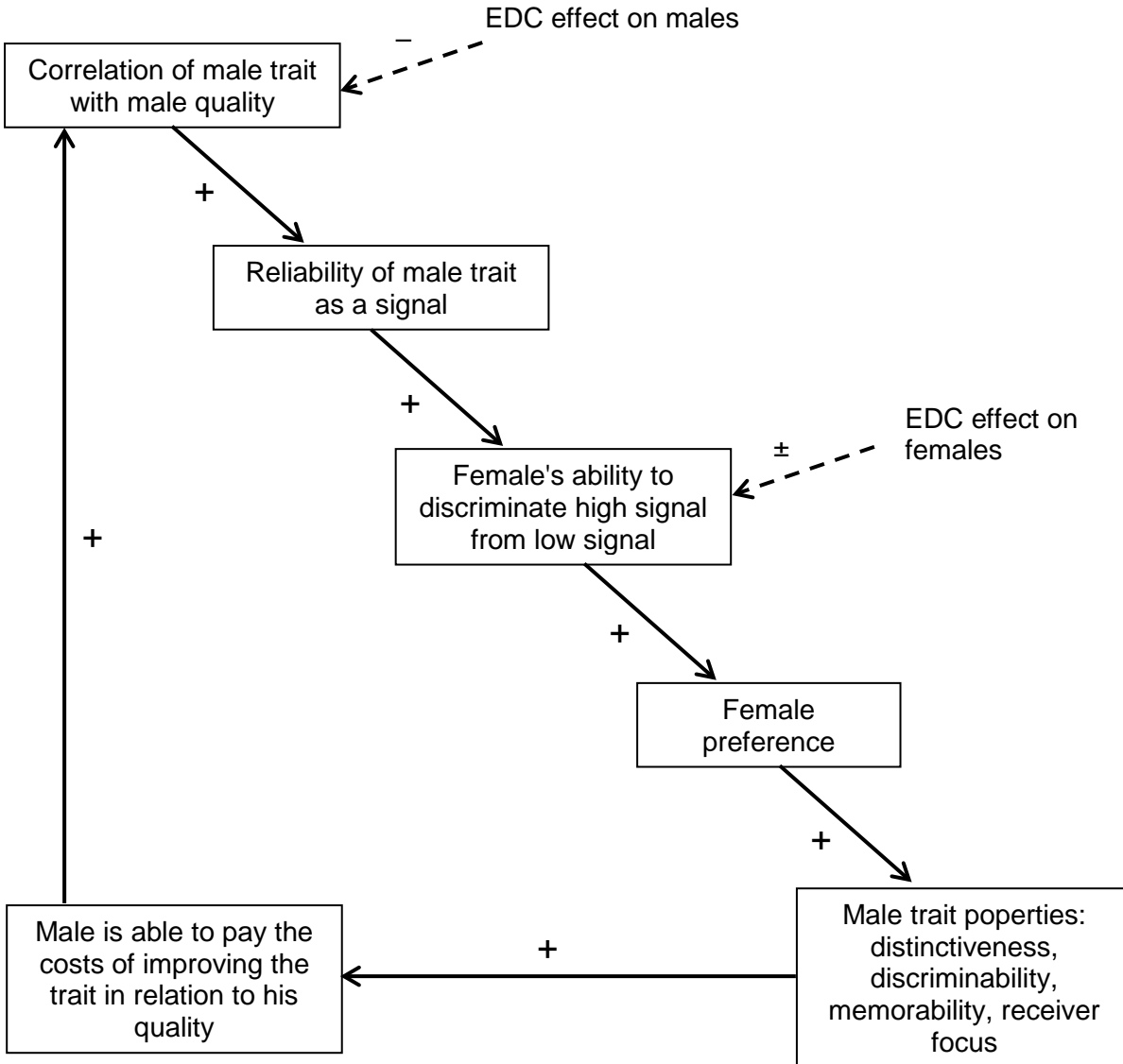
leading to a shallower response curve (broken line). Hence there is a shift in female responses to male quality, which follows the shift (solid arrow) in female responses to trait values. Eventually, females adapt to the altered trait values and their responses to quality shift back to the original (broken arrows).



**Figure 2.3.** Altered female responses to male traits as a result of endocrine disruption. (A) Exogenous estrogens increase female responses to displaying males, which moves the female response curve to the left (dashed line). Hence for the same male trait value, females respond more strongly. This also reduces the difference in response to a high trait value versus a low trait value, thereby reducing female discrimination between two trait values. Female choosiness (i.e. discrimination between high and low trait values) is depicted by the solid and dashed double ended arrows between the high and low responses, before and after EDC exposure respectively. Note that this reduced discrimination is particularly clear when the two male traits are somewhat symmetrical on either side of the inflexion point. (B) Exogenous estrogens could alter the response curve by shifting it about the inflexion point with a steeper slope. This would produce choosier responses to the same male traits.



**Figure 2.4.** Characteristics of altered male signals after exposure to EDCs. High quality males ( $Q_H$ ) produce a mean trait value  $\bar{T}_H$  before disruption and  $\bar{T}'_H$  after disruption. Low quality males ( $Q_L$ ) produce a mean trait value  $\bar{T}_L$  before disruption and  $\bar{T}'_L$  after disruption. Panels on the left associate male trait value with male quality. Regressions will generally have variation around the lines, but this has not been shown (except in panel C) for clarity's sake. Panels on the right are frequency density plots of male trait values in the population expressed by high quality and low quality males. Solid lines represent trait values before endocrine disruption and broken lines after endocrine disruption. In the frequency density plots, black curves represent high trait values, and gray curves represent low trait values. (A) The slope of the regression line after disruption may be the same as that of the regression line before disruption; in this case the signal will continue to be reliable (same slope) and discriminable (same  $d$ ). (B) If exposure to EDC causes a proportional reduction in trait values, then the slope will become lower, reducing reliability as well as discriminability (by reducing the mean between  $T'_H$  and  $T'_L$  curves). (C) Response to the endocrine disruptor will not be similar by all individuals of a given quality in the population. This will increase the variance of the frequency density curves. The slope may or may not remain the same after disruption (for simplicity's sake, it has been depicted as unchanged in this figure). Increased variance will reduce discriminability, as well as the reliability (by a reduction in correlation between trait value and quality). If this is combined with a reduced slope, then the outcome will be an even less discriminable and reliable signal.



**Figure 2.5.** Mechanism by which a trait might evolve into a signal or be lost. If there is a positive correlation between the male trait and male quality within the population, the trait has a stronger probability of becoming reliable as a signal of true quality. The more reliable a trait is as a signal, the stronger the selection on females to discriminate between high and low signalers, thereby strengthening female preference. As female preference strengthens, there is selection on the male trait to become more distinctive, discriminable, and increase specificity to be received by desired receivers only. There are generally costs associated with improving these traits, and high quality males are able to afford these costs better than low quality males (Grafen 1990; Moller & Lope 1994). This feeds back into further strengthening the correlation between variation in the trait and variation in male quality in the population. Conversely, if the correlation between trait and male quality is reduced (due to exposure to EDCs) as in Figure 4, there will be subsequent negative impacts at all stages of the chain causing a weakening of the male trait properties and a further weakening of the correlation between trait and quality. Lowered female

choosiness (due to EDC exposure) as in Figure 2.3A can also lead to the same result. This process is expedited when both males and females are affected by EDCs. Increased choosiness increases costs to females if males, due to endocrine disruption, are unable to make their signals more discriminable; hence this too leads to a similar outcome.

## CHAPTER THREE. ENVIRONMENTALLY REALISTIC EXPOSURE TO THE HERBICIDE ATRAZINE ALTERS SOME SEXUALLY SELECTED TRAITS IN MALE GUPPIES

### SUMMARY

Male mating signals, including ornaments and courtship displays, and other sexually selected traits, like male-male aggression, are largely controlled by sex hormones. Environmental pollutants, notably endocrine disrupting compounds, can interfere with the proper functioning of hormones, thereby impacting the expression of hormonally regulated traits. Atrazine, one of the most widely used herbicides, can alter sex hormone levels in exposed animals. I tested the effects of environmentally relevant atrazine exposures on mating signals and behaviors in guppies, a sexually dimorphic freshwater fish. Prolonged atrazine exposure reduced the expression of two honest signals: the area of orange spots (ornaments) and the number of courtship displays performed. Atrazine exposure also reduced aggression towards competing males in the context of mate competition. In the wild, exposure levels vary among individuals because of differential distribution of the pollutants across habitats; hence, differently impacted males often compete for the same mates. Disrupted mating signals can reduce reproductive success as females avoid mating with perceptibly suboptimal males. Less aggressive males are at a competitive disadvantage and lose access to females. This study highlights the effects of atrazine on ecologically relevant mating signals and behaviors in exposed wildlife. Altered reproductive traits have important implications for population dynamics, evolutionary patterns, and conservation of wildlife species.

### INTRODUCTION

The role of sex hormones in the expression of sexually selected traits has been established in many vertebrate species, especially in males (examples: Zuk et al. 1995; Hillgarth and Wingfield 1997; Peters et al. 2001; Jayasooriya et al. 2002; McGraw et al. 2006). Disruption of the expression or perception of such traits can influence mate choice and evolutionary patterns (Seehausen et al. 1997; Seehausen and van Alphen 1998; Slabbekoorn and Ripmeester 2008; reviewed in Shenoy and Crowley 2011). The increase in various forms of pollution is becoming an important factor in such disruptions (Seehausen et al. 1997; Mockford and Marshall 2009) and is hence instrumental in shaping evolutionary trajectories. A common form of pollution is caused by endocrine disrupting compounds (EDCs), which interfere with proper hormonal functioning. These compounds can be natural or synthetic in origin, including organochlorines, organophosphates, polychlorinated biphenyls (PCBs), phthalates, synthetic hormones and hormone-blockers, and phytoestrogens. Many of them have anthropogenic sources such as pesticides, industrial effluents, pulp mill effluents, plastics and sewage. Significant routes of exposure include direct exposures from living in contaminated soil or water, as well as indirect exposures through eating contaminated prey (Markman et al. 2007; Markman et al. 2008; Park et al. 2009; Walters et al. 2010). EDCs can alter reproductive success by affecting all aspects of the reproductive system, including gonadal formation, production of hormones and gametes, sex determination (Basur 2006), formation of egg shells (Porter and Wiemeyer 1969), and production (Kelce and Wilson 1997; Basur 2006) and maintenance of mating signals and behaviors (Palanza and vom Saal 2002; Milnes et al. 2006).

The effects of EDCs on wildlife have been receiving increasing attention in the literature in recent years. While earlier toxicological studies focused on mortality effects from acute exposures, ecotoxicologists are now focusing on sub-lethal effects of more realistic exposures. Sub-lethal effects can be subtle yet far-reaching by influencing population and community dynamics through cascading effects. Population level effects may include altered demographics

(Willingham 2005; Kidd et al. 2007; Kristensen et al. 2007; Cotton and Wedekind 2009) and mating systems (Hunt and Hunt 1977; Saaristo et al. 2009; Secondi et al. 2009; Partridge et al. 2010). This can affect community dynamics by impacting species closely associated with the focal species. Multi-generational effects due to persistence of pollutants in the environment across generations, or via maternal transfer, can affect evolutionary trajectories of these species as a result of altered sex ratios and mating systems.

The current study focused on the effects of atrazine, a widely used triazine herbicide. Atrazine is the second most commonly used pesticide in the US (Grube et al. 2011). It is resistant to degradation, and its half-life in surface waters can be over 700 days (Solomon et al. 1996; Comber 1999). Many animal species that spend all or part of their life cycle in water can be exposed to significant levels of the chemical for a considerable part of their life. Concentrations of atrazine in water bodies around agricultural fields are expected to be in the range of 19-194 ppb (90 day average) depending on the type of crop and application rate (USEPA 2006a). Non-target species inhabiting water bodies around agricultural fields are particularly at risk for exposure to atrazine. Atrazine induces aromatization of testosterone to estradiol (Hayes 2005; but see Hecker et al. 2005; Fan et al. 2007), thereby causing an estrogenic effect in exposed individuals. Several studies have demonstrated the feminizing effects of atrazine in amphibians (Hayes et al. 2002b; Hayes et al. 2002c, 2003; Hayes et al. 2010), yet the number of studies with ambiguous and conflicting results (Solomon et al. 2008; Rohr and McCoy 2010) contributes to preventing policy changes regarding the use of this pesticide.

Here, I tested whether prolonged exposure to atrazine can alter male mating signal expression, including ornamentation and mating behaviors. I used guppies (*Poecilia reticulata*) as a model organism to test these questions, as guppies have distinct sexual dimorphism, their mating signals and behaviors have been well characterized (Houde 1997), and the role of sex hormones in the expression of these traits has been explored (Jayasooriya et al. 2002; Hallgren et al. 2006). Further, guppies have been used for testing similar questions in other ecotoxicological studies (Baatrup and Junge 2001; Toft and Baatrup 2001; Kristensen et al. 2005). Guppies are small tropical fish native to Trinidad and parts of South America. They are especially useful for testing hypotheses related to sexual selection. Males have different colored spots on their body and fins (Houde 1997); they perform characteristic courtship displays (called "sigmoid" displays) and attempt forced copulations. Mating is predominantly through female mate choice; females respond to courtship displays and to males with larger and brighter orange spots (Houde 1997; Kodric-Brown and Nicoletto 2001), but avoid forced copulatory attempts (Houde 1997; Evans et al. 2003).

Although the pattern and intensity of orange spots are mostly governed by genetics (Haskins et al. 1961; Houde 1992), there is indication that androgens are required for their expression (Haskins et al. 1961; Baatrup and Junge 2001; Toft and Baatrup 2001; Bayley et al. 2002; Devasurendra et al. 2007; Gordon et al. 2011), as well as for performing courtship displays (Baatrup and Junge 2001; Bayley et al. 2002; Bayley et al. 2003). Shenoy and Crowley (Shenoy and Crowley 2011) discuss in detail how hormones may be involved in the expression of sexual signals. An aromatase inducer like atrazine can alter hormonal balances by (1) increasing the estradiol concentrations, which would increase the estradiol: testosterone ratio, and directly reduce the production of testosterone (Bartke et al. 1977; Kim et al. 2007), and by (2) reducing the concentration of testosterone available for conversion to 11-keto testosterone (Ankley et al. 2002; Ankley et al. 2005), an important teleost androgen required for the expression of secondary sexual characteristics.

I hypothesized that prolonged exposure to environmentally relevant doses of atrazine would (1) reduce the area and intensity of orange color spots, which are the primary male mating signals in guppies; (2) reduce the frequency of mating behaviors such as courtship displays and forced copulatory attempts (these were considered behaviors related to mating



effort); and (3) in the presence of competing males, reduce the frequency of behaviors related to mating effort and those related to male-male aggression. The third hypothesis was tested because male-male competition is high in many animal species, including guppies, and examining behaviors in the context of mate competition is ecologically relevant. Further, contaminants are often differentially distributed in the landscape, and different individuals in a population may be exposed unequally. Since individuals impacted to varying degrees would be competing together within a population, I tested the third hypothesis by pairing treated males with those that were not exposed to the contaminants. This also standardized the condition of each experimental male's opponent.

## METHODS

**Ethics statement:** The experimental protocol for this study was approved by the University of Kentucky Institutional Animal Care and Use Committee (protocol number 2007-0137).

**Treatments:** 85 guppies were randomly assigned to one of five treatments at 17 fish per treatment. The treatments included a control (no treatment), dimethylsulfoxide (DMSO, 62.5  $\mu\text{L/L}$ ) as the solvent control, atrazine low-dose (1  $\mu\text{g/L}$ ), atrazine high-dose (15  $\mu\text{g/L}$ ), and ethynyl estradiol (2  $\mu\text{g/L}$ ) as the estrogenic positive control. A solvent control was used because atrazine and ethynyl estradiol were dissolved in DMSO; all treatments received the same concentration of DMSO. Atrazine concentrations used were based on USEPA estimated environmental concentrations (USEPA 2006a). Pilot experiments helped determine sublethal ethynyl estradiol concentrations. Concentration of atrazine in the water column in three randomly selected jars per treatment was ascertained by liquid phase extraction with methylene chloride following an adaptation of USEPA Method 619 (USEPA 1993)--which produced 95% recovery of the target compound--and analyzed by gas chromatography/mass spectrometry. The average concentration at the end of one week was determined to be 0.26 ppb and 12.98 ppb for the low- and high-dose respectively, with negligible loss over the 7 days. No atrazine was detected in the control samples. Atrazine (98% purity) was purchased from Chem Service, Inc., through Fisher Scientific, and 17  $\alpha$ -ethynylestradiol (98% purity) was purchased from Sigma-Aldrich. Treatments continued for 16 weeks to simulate a long-term exposure.

**Animals:** Adult male guppies used for this study were descendants of wild-caught guppies from Trinidad. Three populations—Aripo Upper River, Aripo Lower River, Guanapo Upper River—were equally represented in all treatments to account for geographic and genetic variation. All males included showed clear color patterns and gonopodium development (Houde 1997), indicating sexual maturity. During the period of the study, all fish were housed separately in individual glass jars with 1.6 L of aged, pre-aerated, carbon filtered, conditioned water. Tropical fish flake food was fed once each day in *ad libitum* quantities. Room temperature was maintained at an average of 25 °C; the light:dark cycle was set to 12:12 hours. Water was changed once weekly with static renewal of chemical treatments. Mortality was recorded every day.

**Color measurements:** All fish were photographed once before the start of treatments and once after treatments stopped with a Nikon D50 digital SLR camera with a 55 mm telephoto lens and Nikon SB-400 AF Speedlight flash. The shutter speed was set to 1/60 s, aperture to 22 F and film speed to 200 ISO. The flash speed was set to 1/16 s and power to -0.7, and was covered with a single sheet of tissue paper to diffuse the light. All fish were photographed on the left side in the same position relative to the lens and flash. ImageJ 1.43u (Rasband 1997-2011) was used to measure the area of orange spots and body area of each fish. An average value of the red (R), green (G) and blue (B) channels of each orange spot was also measured. Each fish was photographed along with an orange color standard, which was placed in the same position in every picture. Colors were standardized across all pictures by applying a correction factor to each of the average R, G, B values, such that the corrected R value of the fish in the picture to

be measured,  $R_i' = R_i * R_{Sr}/R_{Si}$ , where  $R_i$  is the average R value of the fish in the picture to be measured,  $R_{Sr}$  is the average R value of the color standard on one picture chosen to be the reference picture;  $R_{Si}$  is the average R value of the color standard on the picture to be measured. Similarly,  $G_i'$  and  $B_i'$  were calculated for each picture. A dark orange spot would have a high  $R'$  measure, and lower  $G'$  and  $B'$  measures; on the other hand, a pale orange spot would have a high  $R'$ ,  $G'$  and  $B'$  measure. The repeatabilities of the corrected  $R'$ ,  $G'$  and  $B'$  values were  $r = 0.98$ ,  $r = 0.95$  and  $r = 0.96$ , respectively. Further, a single composite variable comprising of all three color channels was created by inputting the corrected  $R'$ ,  $G'$ ,  $B'$  values in a Principal Components Analysis and extracting one variable. The repeatability of this composite variable was found to be  $r = 0.98$ .

**Behavior trials:** At the end of the 16 week treatment period, the fish were subjected to two sets of behavior trials: the first set assessed behavior of the males towards a female in the absence of competition from another male, and the second set of trials assessed mating behaviors in the presence of a competing male. All trials were conducted within the first four hours after lights turned on and during the last four hours before lights turned off. All trials were conducted blind: the observer did not know the treatment that any of the fish had received and identified males by their color patterns only. Data were recorded in real time. Trial tanks were illuminated with full spectrum light to ensure that all colors were perceived naturally by the other fish in the trial (Endler 1991, 1993). The observer sat in darkness, 1 m away from the tank, to avoid startling the fish; the fish did not appear to notice or be disturbed by the presence of the observer.

*Trials without competing males:* each male was placed in a trial tank of dimensions 30 x 20 x 15 cm (height x length x width) and 7.5 L of water, with one virgin female from the same population. Water used was aged, pre-aerated, carbon filtered, and conditioned, and water temperature was maintained between 23-25 °C. After a 5 minute acclimation period, the fish were observed for 10 minutes. The total number of sigmoid courtship displays, gonopodium swings and copulatory attempts were recorded throughout the trial period. Males frequently swing their gonopodium forward, and this appears to increase in frequency during mating or aggressive interactions; any gonopodium swing greater than 90° was counted.

*Trials with competing males:* These trials were conducted to test whether treatments altered male behaviors compared to an untreated male in the context of competition. Males were paired in the following fashion—each pair consisted of one male from the control group (opponent) and one male (focal male) from one of the other four treatment groups: DMSO, atrazine low-dose, atrazine high-dose, or ethynyl estradiol. Control group males were used in multiple pairs as there were not enough males to be used only once. Control group males were paired with each of the different treatment group males in random order. Males of a pair belonged to the same population. Pairs could not be size matched after matching for population; treatment group males were on average 14% of body area larger or smaller than paired control group males. Each pair was placed in a trial tank of dimensions 30 x 20 x 15 cm (height x length x width) and 7.5 L of water, with a virgin female from the same population. After a 5 minute acclimation period, behaviors were recorded for 10 minutes. At each 10s point, I recorded which male was closer to the female. A male had to be more than one body length ahead of the other male to be “closer”, and received 1 point in such cases. If both males were within one body length of each other, and within at least two body lengths of the female’s vent, they were both recorded as being equally close; in such cases both males received 0.5 points. If both males were further than two body lengths from the female’s vent, they were both recorded as being far from the female and received 0 points for that event. At the end of the 10 minute trial period, each male’s “closeness” points were summed and its ratio to the total number of events gave a measure of proximity. Throughout the whole trial period, I counted for each male the total number of sigmoid courtship displays, copulatory attempts, aggressive displays to the rival male, and attacks on the other male. The number of gonopodium swings was not recorded, as

these happened in quick succession, and the observer could not keep a reliable count for both males.

**Data Analyses:** All data were analyzed in SAS 9.2 (SAS-Institute-Inc 2002-2009). All statistical procedures refer to SAS procedures.

*Mortality:* Univariate survival analyses (LIFETEST procedure) were first used to test which variables (among treatment and population of origin) were to be included in the final model to test for effects on mortality. Based on the log-rank test of equality over strata, population of origin was not included in the model ( $\chi^2 = 3.096$ ,  $p = 0.38$ ). Difference in mortality between treatments was then analyzed using regression analysis of survival data based on the Cox proportional hazards model (PHREG procedure).

*Area of Orange Spots, Intensity of Orange Spots, and Mating Behaviors in the Absence of Competition:* The dependent variables were appropriately transformed to meet the assumptions of parametric tests wherever required. Pearson's product-moment correlations between the measures of color and mating behaviors were analyzed using the CORR procedure. A mixed model ANOVA (MIXED procedure) was used to analyze the treatment effects on (1) *Area of orange spots:* the change in proportion of orange between initial and final readings, (2) *Intensity of orange spots:* the change between initial and final readings of corrected R', G', and B' values, and the composite variable, and (3) *Mating behaviors in the absence of competition:* the number of courtship displays and number of copulatory attempts. The correlation coefficients revealed that the number of gonopodium swings was correlated strongly with the number of courtship displays ( $r = 0.62$ ,  $P < 0.0001$ ) and weakly with the number of mating attempts ( $r = 0.26$ ,  $P = 0.04$ ), and so this variable was eliminated from further analyses. A mixed model ANOVA using the MIXED procedure allows the use of fixed and random factors in the model; the effect of random factors, wherever included in the model, is removed and results are based on least square means that are adjusted for this effect.

Preliminary analyses determined that the control group and solvent control group did not significantly differ from each other for all variables and so the two groups were pooled as a common control group (area of orange spots,  $P = 0.9$ ; R',  $P = 0.22$ ; G',  $P = 0.22$ ; B',  $P = 0.81$ ; composite variable,  $P = 0.27$ ; number of courtship displays,  $P = 0.9$ ; number of mating attempts,  $P = 0.08$ ). Population of origin was input as the random effect wherever it improved the fit of the model as determined by significantly lower Akaike Information Criteria values (henceforth AIC statistics). For behavioral responses in the absence of competition, the identity of the female used for the trial (because females were used in multiple trials) was also included as a random factor, and time of day that the trial was conducted was included as a covariate, wherever these improved the fit of the model as determined by AIC statistics. Planned orthogonal contrasts were used to test whether (1) the atrazine low-dose and high-dose had similar effects on the response variables, (2) the two atrazine groups had significantly different effects on the response variables compared to the pooled control group, and (3) ethynyl estradiol had the strongest effect on the response variables compared to the other groups. One-tailed p-values were reported for these tests because of the clear directionality of the hypotheses. Further, Tukey's post-hoc tests were used to see which groups differed significantly from each other. Effect sizes with 95% confidence intervals of the differences between each of the treatment groups and the pooled control group were calculated as per Nakagawa and Cuthill (2007).

*Mating behaviors in the presence of competition:* The dependent variables were appropriately transformed to meet the assumptions of parametric tests wherever required. Pearson's product-moment correlations between all variables were analyzed using the CORR procedure. Due to the moderate correlations between some of the response variables (see Table 3.1 for correlations), and because all behaviors recorded on a pair of fish occurred during the same trial period, a MANOVA was conducted with the GLM procedure using the focal male's responses from each pair. Covariates and random effects were not included as the GLM procedure is not equipped to handle these additional effects. Each response variable was then

analyzed separately. Since males were paired, and their behaviors were dependent on each other, an ANCOVA was performed with the MIXED procedure to analyze the effect of the treatments on the focal male's behavior in response to his paired opponent's behavior, which was included as the covariate. Covariates were mean-centered within treatments so that mean estimates for each treatment corresponded with the mean value of the covariate. I specifically tested for differences between treatment intercepts (seen by a significant effect of the treatment) and slopes (seen by a significant interaction of treatment by covariate). A negative effect of the treatments on competitiveness would be indicated by a reduced slope and intercept of the relationship described above, compared to the DMSO (solvent control) group. The difference between the competing males in body size (measured by area of body) and proportion of body area covered by orange were input as additional covariates if they improved the fit of the model as determined by AIC statistics. Similarly, population of origin and control male's identity (because males from the control group were used in multiple pairs) were input as random effects wherever they improved the fit of the model. Further, each treatment-control paired data set was analyzed separately for each treatment (DMSO, atrazine low-dose, atrazine high-dose or ethynyl estradiol) with a paired design to test whether the treatment male consistently behaved differently from his paired control opponent, depending on what the treatment was. A mixed model ANOVA (MIXED procedure) was used to test this, with the pair identity input as a random effect with compound symmetry as the covariance structure. Population of origin was also input as a random effect wherever it improved the fit of the model as determined by AIC statistics. Time of day that the trial was conducted, the control male's trial number, the difference in body size and proportion of body area covered by orange between the competing males were input as covariates if they significantly improved the fit of the model.

## RESULTS

**Mortality:** There were no significant effects of the treatments on mortality rate (likelihood ratio test:  $\chi^2 = 6.87$ ,  $df = 4$ ,  $P = 0.14$ ). The ethynyl estradiol group had the highest mortality over the 16 week period (47.06%) but the hazard ratio was not significantly higher than the control group (Hazard ratio = 3.38,  $P = 0.07$ ). The mortality in the other groups was as follows: control 17.65%, DMSO 29.41%, atrazine low-dose 23.53%, and atrazine high-dose 11.76%. At the end of the exposure period, the number of surviving fish in each of the groups was: control = 14, DMSO = 12, atrazine low dose = 13, atrazine high dose = 15, ethynyl estradiol = 9.

**Color:** The treatments had a significant effect on the change in body area covered by orange ( $F_{3,58} = 14.19$ ,  $P < 0.0001$ ; Figure 3.1). This effect was mainly driven by the ethynyl estradiol group, which had a significantly lower proportional area of orange than the pooled controls ( $P < 0.0001$ , effect size  $\pm$  95% confidence interval [ $d \pm 95\% CI$ ] =  $-2.27 \pm 0.89$ ), and all the other groups combined (planned orthogonal contrasts,  $p < 0.0001$ ). The atrazine high-dose appeared to reduce the area of orange ( $d \pm 95\% CI = -0.76 \pm 0.68$ , Figure 3.1), but this was not statistically significant ( $P = 0.098$ ). The atrazine low-dose did not reduce the area of orange ( $d \pm 95\% CI = -0.12 \pm 0.65$ ), and the two atrazine groups differed from each other (planned orthogonal contrasts,  $P = 0.055$ , Figure 3.1). Because of the difference between the two atrazine groups, they did not collectively reduce the area of orange compared to the pooled control group (planned orthogonal contrasts,  $P = 0.15$ ). The Tukey's post-hoc tests brought out significant differences only between the ethynyl estradiol group and each of the other groups. The loss of power resulting from all pair-wise comparisons lead to a lack of statistical evidence for a difference between the atrazine high-dose and pooled control groups (unadjusted  $P = 0.04$ , Tukey's adjusted  $P = 0.14$ ). The treatments did not affect the change in corrected  $R'$  ( $F_{3,57} = 0.29$ ,  $P = 0.83$ ),  $G'$  ( $F_{3,57} = 0.53$ ,  $P = 0.67$ ), and  $B'$  ( $F_{3,57} = 0.19$ ,  $P = 0.90$ ) values, or the composite variable ( $F_{3,57} = 0.31$ ,  $P = 0.82$ ). The planned orthogonal contrasts did not reveal any significant patterns. Population of origin failed to improve the fit of the model for explaining the

variation in body area covered by orange or corrected R', G', B' and the composite variable, suggesting that this factor was not important in explaining the change in color over the study period.

**Mating behaviors in the absence of competing males:** The number of copulatory attempts was weakly but negatively related to the proportion of body area covered by orange ( $r = -0.25$ ,  $P = 0.046$ ); there were no other significant correlations between any of the other measures of color and behavioral variables. The number of courtship displays differed significantly between treatments ( $F_{3, 61} = 9.79$ ,  $P < 0.0001$ ; Figure 3.2). The planned orthogonal contrasts determined that the ethynyl estradiol group displayed significantly less than the other groups ( $P < 0.0001$ ). The two atrazine groups displayed similarly to each other ( $P = 0.40$ ), and together they displayed significantly less than the pooled controls ( $P = 0.01$ ). The effect sizes showed that the ethynyl estradiol group displayed less than the pooled control group ( $d \pm 95\% \text{ CI} = -2.15 \pm 0.65$ ), as did the atrazine high-dose group ( $d \pm 95\% \text{ CI} = -0.64 \pm 0.64$ ), but the atrazine low-dose group did not display less than the pooled control group ( $d \pm 95\% \text{ CI} = -0.56 \pm 0.65$ ). The Tukey's post-hoc tests revealed similar trends, though the lack of power weakened some of these results. The number of mating attempts did not differ between groups ( $F_{3, 58.1} = 2.01$ ,  $P = 0.12$ ), and none of the planned orthogonal contrasts showed significant differences. Population of origin improved the fit of the model to explain variation in courtship display rates, but not the number of mating attempts.

**Mating behaviors in the presence of competing males:** The measures of mating effort were all moderately correlated with each other (proximity to the female and number of courtship displays:  $r = 0.51$ ,  $P = 0.0002$ , number of courtship displays and number of forced copulatory attempts:  $r = 0.40$ ,  $P = 0.0056$ , proximity to the female and number of forced copulatory attempts:  $r = 0.40$ ,  $P = 0.0059$ ; Table 3.1), and the measures of aggression were also moderately associated with each other (the number of aggressive displays and the number of attacks on paired male:  $r = 0.41$ ,  $P = 0.004$ ; Table 3.1). Further, the proximity to a female was negatively associated with the number of aggressive displays ( $r = -0.37$ ,  $P = 0.01$ ; Table 3.1), and this is because males do not focus on the female during aggressive interactions and can often be far from her. The treatments had a significant effect on the focal males' responses as a whole (Wilks'  $\lambda = 0.42$ ,  $F_{15, 119.11} = 2.95$ ,  $P = 0.0005$ ). The treatments did not have significant effects on the proximity or number of mating attempts, or their interaction with their opponent's behaviors; the treatments significantly influenced the number of displays, but this was driven by the effect of ethynyl estradiol rather than either of the atrazine groups (Figure 3.3a-c; Table 3.2). Population of origin improved the fit of the model explaining variation in proximity and number of courtship displays, but not the number of mating attempts. The number of mating attempts was influenced by the difference in body size between the competing males ( $P = 0.04$ ); the larger the focal male was compared to his paired control opponent, the more forced copulatory attempts he made. There was a significant effect of the treatment ( $F_{3, 28.9} = 8.25$ ,  $P = 0.0004$ ) and the interaction of treatment and covariate ( $F_{3, 28.4} = 10.37$ ,  $P < 0.0001$ ) on the number of attacks on the competing male (Figure 3.3d). The atrazine high-dose and ethynyl estradiol treatments significantly reduced the slopes and intercepts of the regression lines between the focal male's behavior and the paired control male's behavior (Table 3.2) compared to the DMSO group. Treatments also affected the number of aggressive displays made to the rival male ( $F_{3, 30} = 4.1$ ,  $P = 0.015$ ; Figure 3.3e) but had no effect on the interaction of treatment and covariate as there was no significant effect of the covariate itself. Both these variables were also influenced by the identity of the paired control male. The analyses of the effects of treatments within pairs showed that the solvent control males did not differ from control males with regard to any of the variables tested (proximity,  $F_{1, 24} = 0.33$ ,  $P = 0.57$ ; courtship displays,  $F_{1, 24} = 0.61$ ,  $P = 0.44$ ; mating attempts,  $F_{1, 12} = 0.31$ ,  $P = 0.59$ ; attacks,  $F_{1, 12} = 0.02$ ,  $P = 0.89$ ; aggressive displays,  $F_{1, 21} = 0.00$ ,  $P = 0.94$ ), and neither did the atrazine low-dose males (proximity,  $F_{1, 22} = 2.97$ ,  $P = 0.10$ ; courtship displays,  $F_{1, 21} = 0.51$ ,  $P = 0.48$ ; mating attempts,  $F_{1, 11} = 0.04$ ,  $P = 0.85$ ; attacks,

$F_{1,11} = 0.40$ ,  $P = 0.54$ ; aggressive displays,  $F_{1,22} = 0.01$ ,  $P = 0.93$ ). The atrazine high-dose males showed lower responses than their paired control males with respect to variables of aggression (attacks,  $F_{1,23.4} = 5.41$ ,  $P = 0.03$ ; aggressive displays,  $F_{1,26} = 11.15$ ,  $P = 0.0025$ ) but not the variables of mating effort (proximity,  $F_{1,26} = 0.34$ ,  $P = 0.56$ ; courtship displays,  $F_{1,26} = 0.83$ ,  $P = 0.37$ ; mating attempts,  $F_{1,13} = 0.04$ ,  $P = 0.84$ ). The ethynyl estradiol males showed lower responses than their paired control males for almost all variables measured (proximity,  $F_{1,13} = 3.50$ ,  $P = 0.08$ ; courtship displays,  $F_{1,12} = 47.56$ ,  $P < 0.0001$ ; mating attempts,  $F_{1,10.5} = 4.96$ ,  $P = 0.05$ ; attacks,  $F_{1,13} = 13.22$ ,  $P = 0.003$ ; aggressive displays,  $F_{1,12} = 31.74$ ,  $P = 0.0001$ ).

## DISCUSSION

**Differential susceptibility to atrazine:** Population of origin did not affect mortality rates, suggesting that guppies from the different populations were not differentially impacted. Atrazine treatments did not influence mortality rates. However, estradiol can be toxic (Herman and Kincaid 1988; Krisfalusi et al. 1998; Robinson et al. 2007), and ethynyl estradiol may have moderately increased mortality in this study, though the trend was not statistically significant.

Although the different populations would vary naturally in the intensity and area of orange (Houde 1997), it is not surprising that they did not respond differently to the treatments, because the response variable analyzed was the change in these variables over the exposure period. On the other hand, the number of courtship displays was influenced by population of origin; it is well known that guppies from different populations display at different rates (Luyten and Liley 1985; Godin 1995; Houde 1997). Similarly, display rates and proximity of the focal male in relation to that of the paired control male were influenced by population of origin. This appears to be an artifact of the inherent difference in courtship intensity between high predation and low predation sites (Luyten and Liley 1985; Godin 1995; Houde 1997). Possibly, in the low predation sites, individuals are more conspicuous in their competitiveness and respond to high displaying competitors by also displaying more. But in high predation sites, individuals may be more cautious in responding similarly. Interestingly, the number of mating attempts in the presence or absence of competitors was not influenced by population of origin; perhaps because sneak copulations are less conspicuous than courtship displays (Luyten and Liley 1985), males in any predation regime would perform these at comparable rates (but see Godin 1995). But it must be noted that the fish in this study had been raised in the absence of predators for a few generations, and some plasticity may account for the lack of anti-predatory behaviors.

**Impaired mating signals and implications for sexual selection:** As seen in other studies examining the effects of EDCs on sexual traits (Baatrup and Junge 2001; Toft and Baatrup 2001; Bayley et al. 2002; Bayley et al. 2003; Bortolotti et al. 2003; Nakayama et al. 2004; Arellano-Aguilar and Garcia 2008; Bernhardt and von Hippel 2008; Larsen et al. 2008; Saaristo et al. 2009), prolonged atrazine exposure reduced courtship display rates, and there was a trend for reduced expression of ornament size. The high dose of atrazine reduced the area of orange by 1%; this can alter female responses to male displays (Long and Houde 1989) such that his reproductive success is significantly reduced by two matings (Houde 1988). Area of orange is a highly heritable trait in guppies (Houde 1997), and any reduction in the area must be due to reduced allocation of carotenoids to the orange spots. Though the preference for orange color varies across populations (Endler and Houde 1995), female guppies generally show a preference for brighter males performing more courtship displays (Houde 1997; Kodric-Brown and Nicoletto 2001; Rodd et al. 2002), and these appear to be honest signals of mate quality (Houde and Torio 1992; Lopez 1998; Grether 2000; Karino et al. 2005). In this study, the number of courtship displays was not related to the proportion of body area covered by orange; but color was associated with mating behaviors in other ways (results not shown): a composite variable including the number of courtship displays and gonopodium swings was moderately correlated with the corrected blue channel,  $B'$ , a measure of intensity of the orange spots,

indicating that color intensity was associated with displays. The number of mating attempts was negatively, albeit weakly, related to the proportion of body area covered by orange, suggesting that less colorful males tended to use sneaker strategies more frequently than more colorful males.

It is particularly interesting that the behavior most affected by atrazine exposure was one believed to be an honest mating signal. Several studies indicate that hormones play an important role in maintaining the honesty of such signals via immuno-suppressing mechanisms: increased testosterone required for the maintenance of sexual signals can damage the immune system, and individuals with an already compromised immunocompetence would be unable to signal effectively (Folstad and Karter 1992; some recent examples: Bókonyi et al. 2008; Setchell et al. 2008; Mougeot et al. 2009). Other mating strategies like forced and sneaky copulations may be governed more by factors such as population sex ratios (Evans and Magurran 1999; Jirotkul 1999a), predation risk (Magurran and Seghers 1990) and dominance hierarchies (Soltis et al. 2001) and are not under selection via hormonal pathways. In this study, the number of forced copulatory attempts was not affected by atrazine exposure. Forced copulatory attempts in guppies are not always successful (Evans et al. 2003) and are under selection pressure via male-male competition (Evans and Magurran 1999) and predation (Magurran and Seghers 1990). It is unclear whether hormones play a role in the expression of this behavior in any species (but see Davis 2002). These patterns then raise the question whether environmentally altered hormone levels could affect the honesty of mating signals, and whether alternate mating strategies might become more dominant in populations impacted by EDCs (Shenoy and Crowley 2011). Experiments testing such ideas would be valuable contributions to the fields of ecotoxicology and evolutionary biology.

A few studies have analyzed the effects of EDCs on male competitive behaviors (Jaeger et al. 1999; Bell 2001; Majewski et al. 2002; Palanza et al. 2002). Male-male competition is high in many species, and an individual's aggression levels can influence his access to mates (Andersson 1994; Earley and Dugatkin 2005). Pollutants are often unequally distributed across landscapes and within habitats. It is thus reasonable to expect individuals who have been impacted differently to compete against each other, especially in species that are migratory or that converge at breeding sites. The results of this study show that atrazine-impaired males in such cases may be at a mating disadvantage compared to those exposed less or not at all. Interestingly, in the presence of a rival male, the measures of mating effort (proximity to the female, number of courtship displays and number of forced copulatory attempts) were altered relatively little by atrazine exposure, but aggression was strongly reduced. I observed that when competing, the two males focused more on aggression and less on mating effort; as a result, treatment effects were stronger for the variables of aggression than for the variables of mating effort. It is pertinent to note that the difference between competing males in body area covered by orange did not influence any competitive behaviors, while differences in body size influenced only the number of mating attempts.

Aggressive displays are employed by animals to discourage the rival from attacking or competing for the resource, thereby circumventing active combat (Maynard Smith and Harper 2003). During behavioral trials, I observed that aggressive displays by one individual did not necessarily provoke aggressive displays by the other; however, attacks by one individual provoked a responding attack from the other, resulting in active fighting. Thus, I did not find a relationship between the number of aggressive displays by the focal and opponent males, but I did detect this relationship in the case of attacks, and atrazine exposure reduced the strength of the relationship. The paired control male's identity influenced the focal male's responses, suggesting that some individuals elicited stronger aggression than others. Despite this effect, the treatments had a significant effect on aggression levels. Further experiments testing whether the reduced aggression translates to reduced reproductive success would be informative. Also, it is important to know whether the EDC-altered aggression levels affect

stress of exposed individuals (Earley and Hsu 2008), thereby influencing survival and self maintenance.

Altered mating signals and behaviors can influence population dynamics in many ways. An increased number of unattractive males in the population would alter the effective sex ratio, as females of many species, including guppies, exercise strong mate preference for sexual traits. A reduction in attractive males can also influence extra-pair mating rates (Kempnaers et al. 1997), which can in turn alter offspring quality, disease transmission rates and predation risk (Quader 2005). EDC-altered sexual traits may not correlate with mate quality thus blurring the relationship between mate quality and signal; this can lead to females making “incorrect” mate choices that reduce their offspring quality and number (Shenoy and Crowley 2011). These and other impacts of altered mate choice on population dynamics have been reviewed by Quader (2005).

Understanding the population level effects of EDC-altered mating signals is important to conservation biology. Many contaminants are persistent and remain in the environment at substantial concentrations for several years (USEPA 2009), spanning multiple generations of short-lived species. Multi-generational disruption of sexual traits can alter evolutionary trajectories (reviewed in Shenoy and Crowley 2011). Future studies that aim to assess the evolutionary effects of altered sexual traits as a result of pollution must evaluate the longterm ecological consequences of chronic and persistent contamination.

**Atrazine:** Several studies of sub-lethal effects of atrazine have demonstrated estrogenic effects (Hayes et al. 2006b; Fan et al. 2007) and negative impacts on measures of reproduction, including fecundity, gonadal morphology, sperm counts, and hormone production (Hayes et al. 2002b; Hayes et al. 2003; Hayes et al. 2006b; Abarikwu et al. 2010; Hayes et al. 2010; Tillitt et al. 2010; also see Rohr and McCoy 2010). A few studies have also examined the effects of atrazine exposure on secondary sexual traits: Hayes and colleagues found that larval exposure to low doses of atrazine reduced larynx size (Hayes et al. 2002b) and structure (Hayes et al. 2010) in African clawed frogs. The larynx is important for vocalization, the primary mating signal in many anuran species; males with smaller larynxes produce suboptimal calls. However, there is still a dearth of literature on the effects of atrazine on sexual traits. The current study advances this issue and should encourage further focus on these key effects.

The low dose of atrazine affected only courtship display rates, and not any of the other variables measured, indicating that at this concentration (a minimum of 0.26 ppb), not all mating signals are impaired in guppies. Whether this concentration may affect mating signals in other species remains to be tested; for example, African clawed frog larvae exposed to atrazine concentrations ranging from 1-200 ppb showed reduced larynges at metamorphosis (Hayes et al. 2002). Where there was an effect of atrazine, especially the high dose, the direction of the effect was similar to that of ethynyl estradiol suggesting that at higher doses clear estrogenic patterns may have arisen. It must be kept in mind that non-sexual behaviors were not measured in this study and so it is possible that the effects of atrazine on sexual behaviors may be due to poor health in general. Regardless, the impacts on sexual traits seen here are significant enough to be of concern. Dose-response studies with a larger range of atrazine concentrations would help determine the concentrations and exposures influencing different end-points in wildlife species. Understanding the effects on sexual traits is especially important because of their subtle yet crucial implications for reproduction and populations dynamics. More studies along these lines will highlight the negative impacts of atrazine on wildlife reproduction. There may be similar effects on human health as well, because the mechanism of action of atrazine is similar across most vertebrate taxa, including humans (Hayes et al. 2006b).

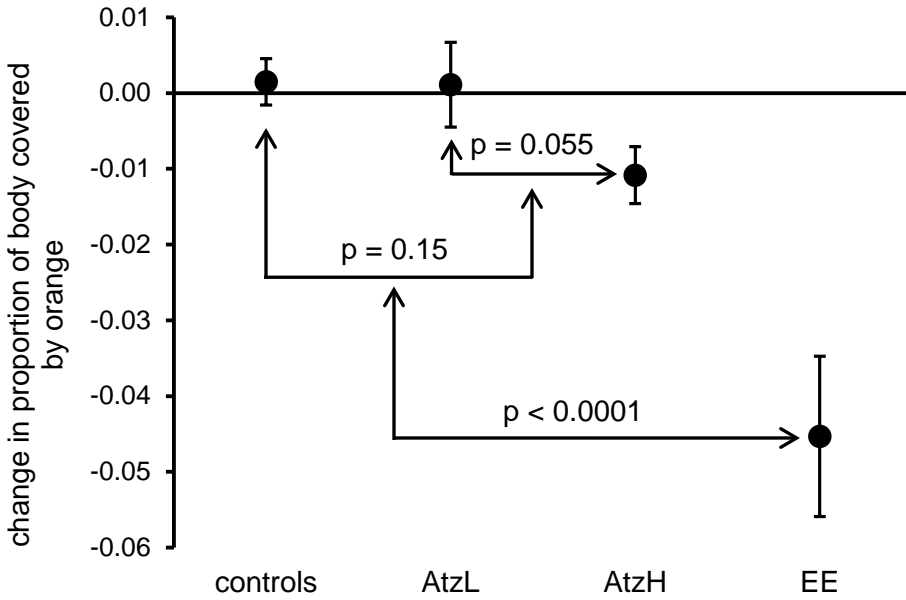


**Table 3.1.** Pearson's correlation coefficients between variables of mating behavior in the presence of competing males.

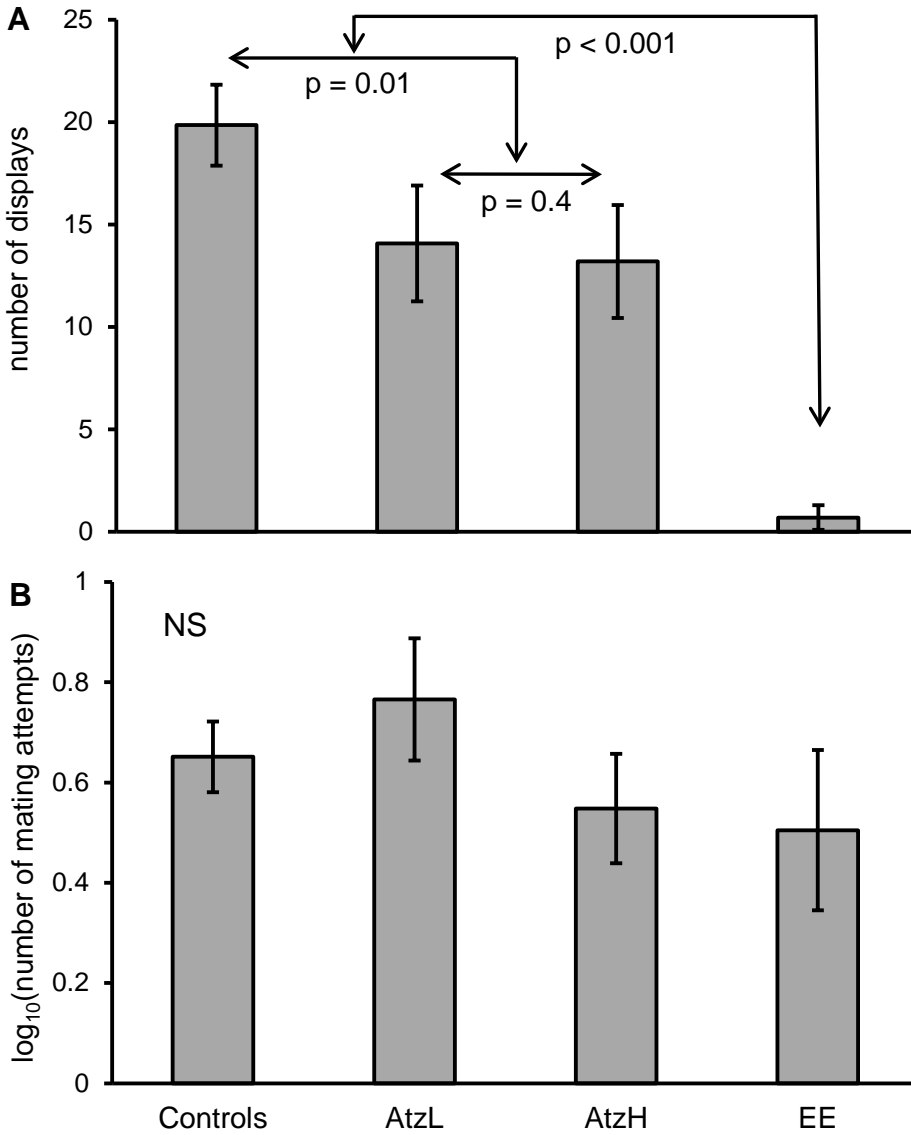
|                     | Proximity                                      | Courtship displays                            | Mating attempts              | Attacks                                       |
|---------------------|--|---|------------------------------|---|
| Courtship displays  | <b><math>r = 0.512</math></b><br>$P = 0.0002$  |   |                              |   |
| Mating attempts     | <b><math>r = 0.396</math></b><br>$P = 0.0059$  | <b><math>r = 0.398</math></b><br>$P = 0.0056$ |                              |   |
| Attacks             | $r = -0.129$<br>$P = 0.3874$                   | $r = -0.081$<br>$P = 0.5887$                  | $r = 0.134$<br>$P = 0.3676$  |   |
| Aggressive displays | <b><math>r = -0.368</math></b><br>$P = 0.0110$ | $r = 0.170$<br>$P = 0.2522$                   | $r = 0.0341$<br>$P = 0.8196$ | <b><math>r = 0.411</math></b><br>$P = 0.0041$ |

**Table 3.2.** Intercept and slope estimates of each treatment group from the ancova of the effects of treatments on mating behaviors in the presence of competing males. Intercepts and slopes of treatment groups that are significantly different ( $P < 0.05$ ) from those of the DMSO group are marked with an asterisk (\*).

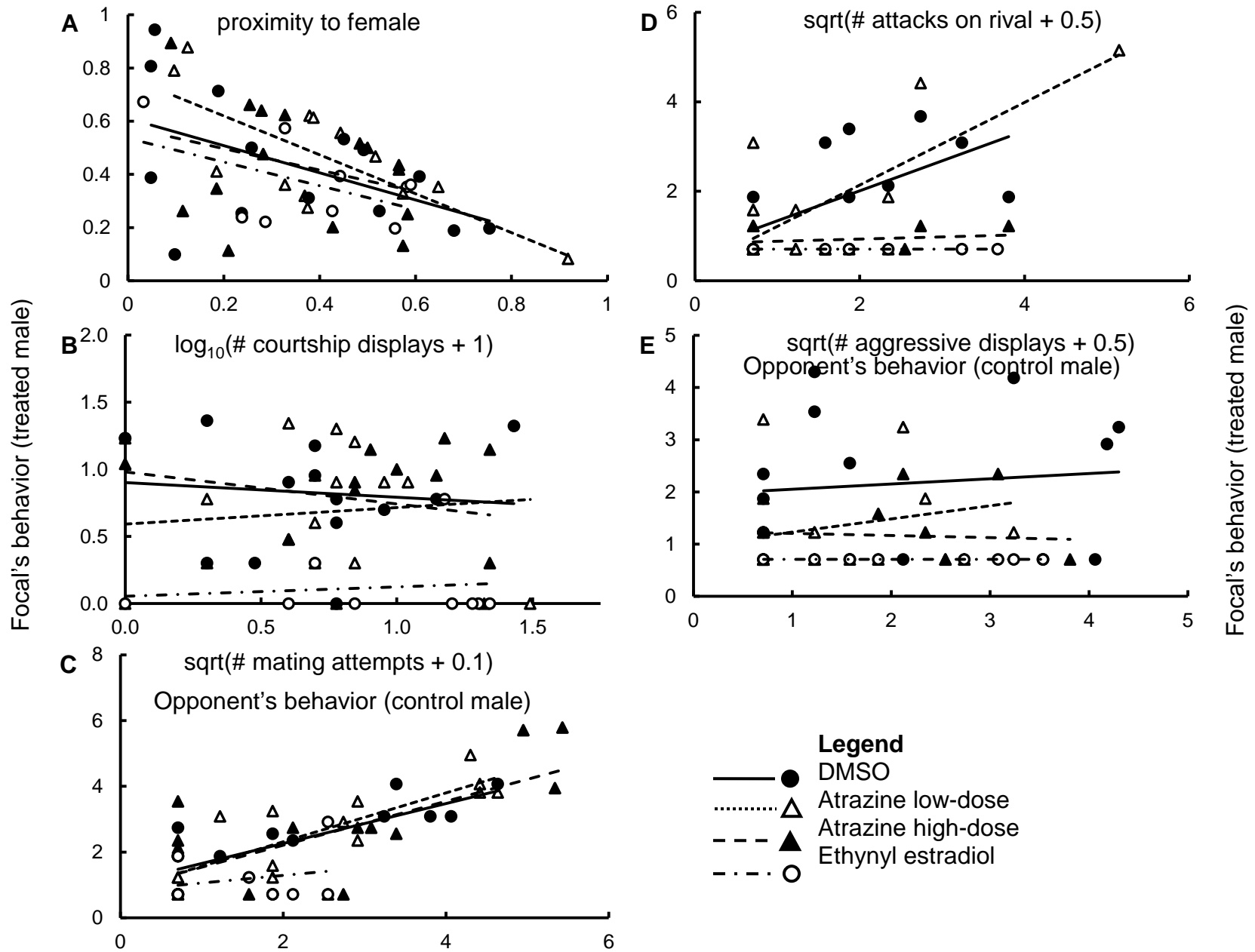
| Treatment group   | Intercept $\pm$ SE | Slope $\pm$ SE    |
|---|--------------------|-------------------|
| (A) Proximity   |                    |                   |
| DMSO  | 0.40 $\pm$ 0.061   | -0.42 $\pm$ 0.20  |
| Atrazine low-dose   | 0.53 $\pm$ 0.063   | -0.76 $\pm$ 0.30  |
| Atrazine high-dose  | 0.41 $\pm$ 0.060   | -0.46 $\pm$ 0.26  |
| Ethynyl estradiol   | 0.29 $\pm$ 0.071   | 0.05 $\pm$ 0.45   |
| (B) Number of courtship displays/ 10 min trial ( $\log_{10}$ transformed) |                    |                   |
| DMSO  | 0.79 $\pm$ 0.11    | -0.02 $\pm$ 0.27  |
| Atrazine low-dose   | 0.62 $\pm$ 0.11    | 0.17 $\pm$ 0.30   |
| Atrazine high-dose  | 0.76 $\pm$ 0.10    | -0.21 $\pm$ 0.29  |
| Ethynyl estradiol   | 0.13 $\pm$ 0.14*   | -0.02 $\pm$ 0.50  |
| (C) Number of mating attempts/ 10 min trial (square root transformed)     |                    |                   |
| DMSO  | 2.37 $\pm$ 0.24    | 0.63 $\pm$ 0.17   |
| Atrazine low-dose   | 2.77 $\pm$ 0.25    | 0.78 $\pm$ 0.18   |
| Atrazine high-dose  | 2.56 $\pm$ 0.23    | 0.72 $\pm$ 0.14   |
| Ethynyl estradiol   | 1.11 $\pm$ 0.30*   | 0.47 $\pm$ 0.45   |
| (D) Number of attacks/ 10 min trial (square root transformed)             |                    |                   |
| DMSO  | 1.77 $\pm$ 0.22    | 0.65 $\pm$ 0.15   |
| Atrazine low-dose   | 1.86 $\pm$ 0.23    | 0.87 $\pm$ 0.12   |
| Atrazine high-dose  | 1.07 $\pm$ 0.22*   | 0.06 $\pm$ 0.14*  |
| Ethynyl estradiol   | 0.96 $\pm$ 0.26*   | -0.17 $\pm$ 0.18* |
| (E) Number of aggressive displays/ 10 min trial (square root transformed) |                    |                   |
| DMSO  | 2.21 $\pm$ 0.27    | 0.12 $\pm$ 0.17   |
| Atrazine low-dose   | 1.38 $\pm$ 0.28    | 0.24 $\pm$ 0.34   |
| Atrazine high-dose  | 1.33 $\pm$ 0.27*   | 0.04 $\pm$ 0.26   |
| Ethynyl estradiol   | 1.02 $\pm$ 0.34*   | 0.15 $\pm$ 0.33   |



**Figure 3.1.** Treatment effects on change in proportion of body area covered by orange. Negative numbers suggest reduction in area of orange, while positive numbers suggest increase in area of orange. Treatments are labeled as follows: pooled control + DMSO group = “controls”, atrazine low-dose = “AtzL”, atrazine high-dose = “AtzH”, ethynyl estradiol = “EE”. Arrows between groups denote planned orthogonal contrasts.



**Figure 3.2.** Treatment effects on mating behaviors: (A) the number of courtship displays performed to a female per 10 minute trial, and (B) the number of mating attempts per 10 minute trial. Treatments are labeled as follows: pooled control + DMSO group = “controls”, atrazine low-dose = “AtzL”, atrazine high-dose = “AtzH”, ethynyl estradiol = “EE”. Arrows between groups in panel A denote planned orthogonal contrasts. These are not shown for panel B because none of the contrasts were significantly different.



**Figure 3.3.** Treated males' behaviors in relation to paired control males' behaviors for each response variable measured. Treated males--those belonging to DMSO, atrazine low-dose, atrazine high-dose and ethynyl estradiol groups--were the focals, while the paired male from the control group was the opponent. For each response variable, the x- and y-axes have the same measure and units. Results of the ANCOVA corresponding to each panel: (A) Proximity: treatment,  $F_{3, 36.7} = 2.71$ ,  $P = 0.059$ ; opponent's response,  $F_{1, 37.1} = 5.47$ ,  $P = 0.025$ ; treatment x opponent's response,  $F_{3, 36.9} = 0.65$ ,  $P = 0.59$ ; (B) number of courtship displays: treatment,  $F_{3, 39} = 4.71$ ,  $P = 0.007$ ; opponent's response,  $F_{1, 39} = 0.01$ ,  $P = 0.92$ ; treatment x opponent's response,  $F_{3, 39} = 0.23$ ,  $P = 0.88$ ; (C) number of mating attempts: treatment,  $F_{3, 39} = 5.63$ ,  $P = 0.0026$ ; opponent's response,  $F_{1, 39} = 19.97$ ,  $P < 0.0001$ ; treatment x opponent's response,  $F_{3, 39} = 0.18$ ,  $P = 0.91$ ; (D) number of attacks: treatment,  $F_{3, 28.9} = 8.25$ ,  $P = 0.0004$ ; opponent's response,  $F_{1, 33.1} = 14.41$ ,  $P = 0.0006$ ; treatment x opponent's response,  $F_{3, 28.4} = 10.37$ ,  $P < 0.0001$ ; (E) number of aggressive displays: treatment,  $F_{3, 30} = 4.10$ ,  $P = 0.015$ ; opponent's response,  $F_{1, 34.9} = 0.72$ ,  $P = 0.40$ ; treatment x opponent's response,  $F_{3, 32.4} = 0.06$ ,  $P = 0.98$ .

## CHAPTER FOUR. THE EFFECTS OF PRENATAL EXPOSURE TO ATRAZINE ON BROOD CHARACTERISTICS AND MALE SECONDARY SEXUAL TRAITS IN GUPPIES

### SUMMARY

Exposure to endocrine disrupting compounds during early life stages can have lasting effects in animals. Of particular interest are effects on the development of male sexual characteristics that have important implications for reproductive success. I examined the effects of prenatal exposure to the herbicide atrazine on male secondary sexual characteristics in guppies. Atrazine is estrogenic, possibly by inducing aromatization of testosterone to estradiol, though other mechanisms have also been suggested. I exposed mated adult female guppies to two environmentally relevant doses of atrazine (1 and 13  $\mu\text{g/L}$ ), a solvent control (DMSO), and a negative control; exposure lasted throughout the gestation period. I first tested for the effects of the treatment on survival, brood size and sex ratios of offspring produced. The offspring were raised to adulthood with no further treatment. Throughout development and at adulthood, I measured the following end-points: the age at which male specific morphologies appeared, including orange spots (sexual ornaments in guppies) and gonopodia (a modified anal fin used as a copulatory organ), and the size of these traits. Survival until the end of the experiment and sex ratio of broods were not affected by atrazine exposure. Exposure to the low dose of atrazine increased brood sizes significantly. The time taken to develop either trait was influenced by DMSO, but not by the atrazine treatments. The length of the gonopodium was not reduced by prenatal atrazine exposure, but the area of the orange spot was increased. I suggest that the increased fecundity may be a result of modest increases in estrogen levels. Increased brood sizes, though a seemingly positive effect at the organismal level, may have little effect at the population level if juvenile survival is density-dependent or if reproductive competence is compromised. The effects on gonopodium length were inconclusive, and I recommend more rigorous testing with larger sample sizes to determine whether atrazine exposure can affect this trait, as reported in other studies on xenoestrogens. The unexpected increase in the size of orange spots may be attributed to slight immune-enhancement as a result of small increments in estrogen levels; this may have allowed the fish to allocate more carotenoids to their ornament rather than to immunocompetence. I recommend studying a suite of end-points relating to reproductive success to completely realize the effects of a contaminant; behavioral and paternity assays would help understand whether the effects translate to loss of reproductive success and population changes.

### INTRODUCTION

Endocrine disrupting compounds (EDCs), which interfere with hormonal functioning in exposed animals, are ubiquitous in the environment and impact wildlife health in many ways (reviewed in: Colborn *et al.* 1993a; Fox 2001; Hamlin and Guillette 2011). Common types of EDCs include organochlorines, organophosphates, polychlorinated biphenyls (PCBs), phthalates, synthetic hormones and hormone-blockers, and phytoestrogens. These enter the natural environment through sources such as pesticides, industrial effluents, pulp mill effluents, plastics and sewage. Significant routes of exposure include direct exposures from living in contaminated soil or water, as well as indirect exposures through eating contaminated prey (Markman *et al.* 2007; Markman *et al.* 2008; Park *et al.* 2009; Walters *et al.* 2010). Some well documented effects of EDCs include gonadal malformations (Toft and Baatrup 2001; Hayes *et al.* 2002c, 2003; Kinnberg and Toft 2003; Hayes *et al.* 2006a), sex reversal and skewed sex ratios (Bayley *et al.* 2002; Teather *et al.* 2005; Örn *et al.* 2006; Pettersson *et al.* 2006), altered hormone concentrations (Guillette *et al.* 1999a; Hayes *et al.* 2002c; Saiyed *et al.* 2003; Toft *et*

al. 2003; Gunderson et al. 2004), reduced sperm counts (Baatrup and Junge 2001; Toft and Guillette 2005), reduced fecundity (Ankley *et al.* 2003; Kristensen *et al.* 2007), smaller copulatory organs (Batty and Lim 1999; Guillette et al. 1999a; Guillette et al. 1999b; Bayley et al. 2002; Gunderson et al. 2004), and altered sexual ornaments and behaviors (Baatrup and Junge 2001; Bayley et al. 2002; Arellano-Aguilar and Garcia 2008; Ottinger et al. 2008; Secondi et al. 2009; Shenoy 2012, Chapter 3). All of these effects have implications for reproductive success.

Many animal species are exposed to contaminants only during short time intervals corresponding to application of agrochemicals, heavy rains, outflux of effluents, and so forth. These exposure periods could coincide with crucial developmental periods in the animal's life, disrupting key physiological processes. Disruption of the endocrine system during development can delay sexual maturation and result in altered morphologies at adulthood, which can have serious effects on reproduction. In male vertebrates, androgens like testosterone, 5 $\alpha$ -dihydrotestosterone (DHT) and 11-ketotestosterone (KT) play important roles in the development and expression of secondary sexual characteristics (Borg 1994; Catz *et al.* 1995; Zuk *et al.* 1995; Gong *et al.* 1999; Jayasooriya *et al.* 2002; McGraw *et al.* 2006). Empirical evidence indicates that androgen levels can directly impact the timing of sexual maturation (Bayley et al. 2002; Saiyed et al. 2003), and the development and expression of male-specific morphologies, such as ornaments (Zuk et al. 1995; Potter *et al.* 2005; McGraw et al. 2006) and copulatory organs (Guillette et al. 1999b; Bayley et al. 2002; Gunderson et al. 2004; Ogino et al. 2004). Individuals that attain sexual maturity later than average incur loss of life-time reproductive success. In species with specific breeding seasons, delayed sexual maturity could lead to greatly reduced reproductive success due to loss of mating opportunities early in the breeding season. This could yield significant loss of life-time reproductive success. Mating signals and ornaments convey mate quality information about the bearer; in most species, it is the males that produce the signal, and females choose mates based on the strength of the signal. Males with weaker signals are less likely to obtain matings. In species with internal fertilization, the size of the male's intromittent organ may play a role in fertilization success. In some species the intromittent organ also acts as an indicator of mate quality (Langerhans *et al.* 2005; Kahn *et al.* 2010).

In this study, I focused on the effects of the triazine herbicide atrazine, the second most used pesticide in the US (Grube *et al.* 2011). Some research suggests that atrazine upregulates the enzyme aromatase (Sanderson *et al.* 2002; Hayes 2005; Fan *et al.* 2007), which is responsible for converting testosterone to estradiol, and androstenedione to estrone; however, some debate exists over this mechanism (Hecker *et al.* 2005). Alternative mechanisms suggested for the endocrine disruption by atrazine are direct inhibition of testosterone production from the Leydig cells (Friedmann 2002) and reduced levels of 5 $\alpha$ -reductase, the enzyme that converts testosterone to DHT (Kniewald *et al.* 1979; Babic-Gojmerac *et al.* 1989). Greater aromatase activity can increase estradiol levels in exposed individuals, and decrease testosterone levels either via aromatization to estradiol, or directly by the action of estradiol on the testes (Bartke *et al.* 1977; Kim *et al.* 2007). Lower testosterone levels will result in less production of DHT and KT, which are important for the expression of secondary sexual characteristics (Hews and Moore 1995; Angus *et al.* 2001). Atrazine has a half-life of 700 days in surface waters (Solomon *et al.* 1996; Comber 1999). The United States Environmental Protection Agency (USEPA) estimates the concentrations of atrazine in water bodies around agricultural fields to be in the range of 19-194 ppb over a 90 day period, depending upon the type of crop and application regime (USEPA 2006). Wildlife inhabiting water bodies close to agricultural fields are especially at risk for exposure to atrazine. Atrazine exposure has been shown to induce hermaphroditism and intersex, reduce testosterone levels, increase estradiol levels, and alter the expression of secondary sexual traits and mating behaviors (Hayes et al. 2002b; Hayes et al. 2002c, 2003; Hayes et al. 2010; Shenoy 2012, Chapter 3).



I tested the effects of prenatal exposure to atrazine on development of secondary sexual traits in male guppies (*Poecilia reticulata*). Guppies are a freshwater fish, native to Trinidad and parts of South America. The males have brightly colored spots on their body and fins; the orange colored carotenoid-based spots are ornaments that females use to assess mate quality and choose mates (Houde 1997). Because fertilization is internal, males have a modified anal fin called the gonopodium, which is used as an intromittent organ. Guppies are viviparous; I tested for the effects of atrazine exposure on response variables in the offspring of exposed mothers. I hypothesized that prenatal exposure to atrazine would affect (1) brood sizes, sex-ratios and survival of offspring, (2) the timing of appearance of male-specific morphologies, such as the gonopodium and orange spot, and (3) the size of these traits at adulthood.

## METHODS

**Study System:** I used guppies as a model organism to test my hypotheses. Guppies have distinct sexual dimorphism, their mating signals and behaviors have been well characterized (Houde 1997), and the role of sex hormones in the expression of these traits has been explored (Jayasooriya et al. 2002; Hallgren et al. 2006). Further, guppies have been used for testing similar questions in other ecotoxicological studies (Baatrup and Junge 2001; Toft and Baatrup 2001; Kristensen et al. 2005; Baatrup 2009). Guppies are small tropical fish native to Trinidad and parts of South America. They are especially useful for testing hypotheses related to sexual selection. Males have different colored spots on their body and fins (Houde 1997); they perform characteristic courtship displays (called “sigmoid” displays) and attempt forced copulations. Mating is predominantly through female mate choice: females respond to courtship displays and to males with larger and brighter orange spots (Houde 1997; Kodric-Brown and Nicoletto 2001), but avoid forced copulatory attempts (Houde 1997; Evans et al. 2003). The orange spots and sigmoid displays are considered honest mating signals, and correlate with immunocompetence (Houde and Torio 1992; Lopez 1998). Guppies are viviparous, and hence suited for testing the effects of maternal exposures.

**Animal care:** The experimental protocol for this study was approved by the University of Kentucky Institutional Animal Care and Use Committee (protocol number 2007-0137).

Adult female guppies used for this study were descendants of wild-caught guppies from Trinidad. Three populations—Aripo Upper River, Aripo Lower River, Guanapo Upper River—were equally represented in all treatment levels to account for geographic and genetic variation. During the period of the study, all fish were housed separately in individual glass bowls with 3 L of aged, pre-aerated, carbon filtered, conditioned water (conditioned with AmQuel® and NovAqua® by Kordon, LLC). Fish were fed with *ad libitum* quantities of tropical flake food once each day and brine shrimp nauplii once each day. Room temperature was maintained at an average of 25 °C; the light: dark cycle was 12:12 hours. Water was changed twice weekly with static renewal of chemical treatment. Mortality was recorded every day. Bowls were checked twice daily for offspring.

Offspring born to treated females were immediately removed from the mother's bowl. Each brood was housed together in a plastic tank with 6 L of aged, pre-aerated, carbon filtered, conditioned water, as before. All fish were fed with *ad libitum* quantities of tropical fish flake food four days per week, and *ad libitum* quantities of brine shrimp nauplii three days per week. All fish were checked daily for development of male specific morphologies: any fish that developed a gonopodium or color spot was immediately removed into another container to separate the sexes. Males and females of a brood were then housed in sex-specific tanks with not more than 4 fish per tank. All tanks contained gravel and were aerated continuously. Room temperature was maintained at an average of 25 °C; the light: dark cycle was 12:12 hours. Mortality was recorded every day.

**Experimental Treatment:** 40 adult female guppies were randomly assigned to one of the four treatment levels at 10 fish per treatment. Before the treatment was administered, each female was housed with a separate male on each of four consecutive days to improve brood quality (Evans and Magurran 2000). The treatment levels included a negative control (no manipulation), dimethylsulfoxide (DMSO, 83.3  $\mu\text{L/L}$ ) as the solvent control, atrazine low-dose (1  $\mu\text{g/L}$ ), atrazine high-dose (15  $\mu\text{g/L}$ ). A solvent control was used because atrazine and ethynyl estradiol were dissolved in DMSO; all treatment levels included the same concentration of DMSO. Atrazine concentrations used were based on USEPA estimated environmental concentrations (USEPA 2006b). Concentration of atrazine in the water column in three randomly selected jars per treatment was ascertained by liquid phase extraction with methylene chloride following an adaptation of USEPA Method 619 (USEPA 1993)—which produced 95% recovery of the target compound—and analyzed by gas chromatography/mass spectrometry. The average concentration at the end of 4 days was determined to be 13.56  $\mu\text{g/L}$  for the high-dose with negligible loss over 4 days. In previous experiments, I have found there to be negligible loss over 4 days for the low concentration as well (Shenoy 2012, Chapter 3). No atrazine was detected in the control samples. Atrazine (98% purity) was purchased from Chem Service, Inc., through Fisher Scientific. Treatment continued until a brood was produced to simulate a long-term exposure. Offspring born to treated females were raised to adulthood with no further treatment.

**Measurements:** Brood sizes and sex ratios of all broods were recorded. The date on which a fish developed a gonopodium and orange spot were also recorded to test for treatment effects on the age at which these male specific traits developed. At the average age of 13.7 months ( $\pm$  0.75 months, standard deviation), all fish were anesthetized in neutral buffered MS-222 solution and photographed with Canon Eos 5D Mark II camera, with a Canon 100mm macro lens. I used two White Lightning x 1600 studio lights placed opposite to each other, and dorso-ventral to the fish to minimize glare; the lights were diffused with translucent white scrims. ImageJ1.43u (Rasband 1997-2011) was used to measure the length of body and length of gonopodium in mm, and area of body and area of orange spots in  $\text{mm}^2$ .

**Data Analyses:** All data were analyzed with SAS 9.3 (SAS-Institute-Inc 2002-2009). Because males within a brood are correlated, and the number of males per brood varied from 1 to 7 (mean =  $2.96 \pm 1.7$  standard deviation), I used generalized estimating equations (GEE; PROC GENMOD in SAS) for all generalized linear models (GLMs) that used data from individual fish, by grouping individuals within broods (Hanley et al. 2003). GEE uses a quasi-likelihood approach to analyzing GLMs (Zeger and Liang 1986; Hanley et al. 2003), including ANOVAs, ANCOVAs, logistic regressions, and so forth. GEE focuses on within-cluster similarity of the residuals, and this estimated correlation is used to calculate regression parameters and standard errors (Hanley et al. 2003; Hubbard et al. 2010). Statistics reported for fixed effects are Wald  $\chi^2$  statistics for Type III GEE (the Wald statistic has a  $\chi^2$  distribution).

I tested for the effects of the treatment on the probability of a fish surviving until the end of the experiment using logistic regression (using GEE as described above), with brood identity as the cluster. Population of origin was included as a covariate, as it improved the fit of the model (as determined by QIC statistics). I also tested specific null hypotheses regarding the proportion of fish that were alive at the end of the experiment using *a-priori* planned orthogonal contrasts: (1) the control and DMSO groups will not differ from each other, (2) the two atrazine groups will not differ from each other, and (3) the atrazine groups will not differ from the control groups. The effect of the treatment on brood sizes was tested with a mixed model ANOVA, with population of origin as the random effect and treatment as the fixed effect. Population of origin did not significantly improve the fit of the model (as determined by QIC statistics), and so was removed. I also tested specific hypotheses using *a-priori* planned orthogonal contrasts as before. The effect of the treatment on sex ratio of each brood was tested with logistic regressions.

The effect of the treatment on the development of orange spots and gonopodium were analyzed using survival analyses: I used the Cox Proportional Hazards model to test the effects of the treatment on the time taken to produce each trait. The brood identity was included as a random effect. Population of origin was included as a covariate. To understand which treatment groups differed from which, I also used a GLM (using GEE as described above) to test for the effects of the treatment on the average age at which each trait developed. The age at gonopodium development (AGD) and the age at orange-spot development (AOD) were both log transformed to meet the assumptions of parametric statistics. I used *a-priori* planned orthogonal contrasts to test specific hypotheses as before. Both, the AGD and the AOD, were log transformed to meet the assumptions of parametric analyses.

The effects of the treatment on the adjusted gonopodium length (adjusted for body length), and adjusted orange-spot area (adjusted for body area), were also tested using GLM. Again, I used *a-priori* planned orthogonal contrasts to test specific hypotheses. Population of origin did not significantly affect either trait and so was not included in the models.

## RESULTS

**Mortality, Brood Size and Sex Ratios:** The treatment did not significantly affect the probability of a fish surviving until the end of the experiment ( $\chi^2 = 6.69$ ,  $df = 3$ ,  $P = 0.08$ ; Figure 4.1), though there was a non-significant trend for the atrazine groups to have a lower survival probability than the control groups ( $P = 0.055$ ), mainly driven by the atrazine low-dose group having a non-significantly higher mortality than the control group (*post hoc* comparisons, Tukey-Kramer adjusted  $P = 0.055$ ). The control groups did not differ from each other ( $P = 0.46$ ), and neither did the atrazine groups ( $P = 0.56$ ). Population of origin, though not significant in the model ( $\chi^2 = 2.29$ ,  $df = 2$ ,  $P = 0.32$ ), improved the fit as determined by QIC statistics, and so was retained in the model. At the end of the experiment, the proportion of fish that survived in each treatment group was as follows: control = 97.5%, DMSO = 88%, atrazine low-dose = 72%, atrazine high-dose = 82% (Figure 4.1).

The treatment had a significant effect on brood size ( $F_{3,34} = 3.23$ ,  $P = 0.03$ ; Figure 4.2). This effect was mostly driven by the atrazine low-dose group that had significantly larger broods than the control group (*post hoc* comparisons, Tukey-Kramer adjusted  $P = 0.027$ ). The planned contrasts showed that the two control groups had statistically indistinguishable brood sizes ( $P = 0.68$ ), but the atrazine low-dose group had a significantly higher broods size than the atrazine high-dose group ( $P = 0.02$ ). This effect was apparently the driver for the significant difference between the control groups and atrazine groups ( $P = 0.02$ ). The sex-ratios of broods did not differ between treatment groups ( $\chi^2 = 1.28$ ,  $df = 2$ ,  $P = 0.53$ ), and the average sex-ratio (proportion of males per brood) was 0.42.

**Development of male-specific traits:** The treatment significantly influenced the time taken to develop a gonopodium ( $\chi^2 = 8.78$ ,  $df = 1.4$ ,  $P = 0.006$ ), as did the brood identity ( $\chi^2 = 19.18$ ,  $df = 10.18$ ,  $P = 0.04$ ); the population of origin did not significantly affect the time taken to develop a gonopodium ( $\chi^2 = 3.0$ ,  $df = 0.87$ ,  $P = 0.07$ ). The DMSO group had a low hazard ratio (HR = 0.29,  $P = 0.03$ ), as did the atrazine low-dose group (HR = 0.23,  $P = 0.009$ ). Because the hazard ratio compares the instantaneous rate of development of the gonopodium of a treatment group compared with the control group, the survival analyses did not reveal whether either of the atrazine groups differed from the DMSO group. The results of the GLM testing the effects of the treatment on the average age at gonopodium development (AGD) brought out clearer results. The treatment had a significant effect on the AGD ( $\chi^2 = 9.06$ ,  $df = 3$ ,  $P = 0.03$ ; Figure 4.3A); the DMSO group had a significantly higher AGD than the control group ( $P = 0.01$ ), but AGD did not differ between the two atrazine groups ( $P = 0.16$ ), and was not significantly higher in the atrazine groups compared to the control groups ( $P = 0.43$ ).

The treatment significantly affected the time taken to develop an orange spot ( $\chi^2 = 9.7$ ,  $df = 3$ ,  $P = 0.003$ ), as did the brood identity ( $\chi^2 = 27.6$ ,  $df = 12.38$ ,  $P = 0.008$ ). The population of origin did not have a significant effect on the time taken to develop an orange spot ( $\chi^2 = 1.37$ ,  $df = 0.78$ ,  $P = 0.18$ ). Again, the DMSO group and the atrazine low-dose group had significantly low hazard ratios (DMSO: HR = 0.26,  $P = 0.02$ ; atrazine low-dose: HR = 0.18,  $P = 0.004$ ). However, the average age at orange-spot development (AOD) was not affected by the treatment ( $\chi^2 = 5.78$ ,  $df = 3$ ,  $P = 0.12$ ; Figure 4.3B), and population of origin did not have a significant effect on the model. Planned orthogonal contrasts showed that the controls had significantly different AOD ( $P = 0.049$ ), but AOD did not differ between atrazine groups ( $P = 0.12$ ), or between the atrazine and control groups ( $P = 0.13$ ).

**Size of gonopodium and orange spots:** The treatment did not significantly affect the adjusted gonopodium length ( $\chi^2 = 4.86$ ,  $df = 3$ ,  $P = 0.18$ ; Figure 4.4A). The two control groups did not differ from each other ( $P = 0.82$ ) nor did the two atrazine groups ( $P = 0.47$ ), as determined by the planned orthogonal contrasts. Also the atrazine groups did not differ from the control groups ( $P = 0.11$ ).

The treatment significantly affected the adjusted orange-spot area ( $\chi^2 = 8.93$ ,  $df = 3$ ,  $P = 0.03$ ; Figure 4.4B), though the patterns were opposite to what I had predicted. The *a-priori* planned orthogonal contrasts indicated that the atrazine groups had a non-significant trend for higher adjusted orange-spot area than the control groups ( $P = 0.06$ ); the two control groups did not differ from each other ( $P = 0.42$ ) nor did the two atrazine groups ( $P = 0.20$ ). Because the overall model showed a significant effect of the treatment, and the planned orthogonal contrasts did not clearly indicate whether the atrazine groups were significantly different from the control groups, I conducted all pair-wise *post hoc* comparisons: the atrazine high-dose group had a higher area of orange than the control groups (Tukey-Kramer adjusted  $P = 0.02$ ).

## DISCUSSION

Overall, the effects of prenatal exposure to atrazine were contrary to predictions: exposure to the low dose of atrazine increased brood sizes and exposure to the high dose of atrazine increased the area of orange spot (ornament size) in male guppies. There were no significant effects on mortality, development of male specific traits or the size of the copulatory organ. In most cases (all response variables except ornament size), a non-monotonic trend was observed, though this pattern could not be detected statistically.

In most studies of estrogenic compounds, exposure has an adverse effect on brood sizes (Ankley *et al.* 2003; Robinson *et al.* 2003; Nash *et al.* 2004; Fusani *et al.* 2007); increased estrogen levels causes the hypothalamus to reduce production of follicle stimulating hormone (FSH), which regulates ovulation, via a negative feedback loop. However, there have been a few cases of increased egg production as a result of EDC exposure (Edwards 2005; but see opposite trends in a follow-up study: Kristensen *et al.* 2007), and nonmonotonic responses (Giesy *et al.* 2000; Pawlowski *et al.* 2004), as in the current study. Some studies have indicated that estradiol can trigger meiosis and oogonial proliferation (Miura *et al.* 2007; reviewed by Jalabert *et al.* 2000; Lubzens *et al.* 2010), suggesting that estradiol, within a range of concentrations, can actually have a positive effect on fecundity (Weltje *et al.* 2005). While an increased brood size may be beneficial to individual fitness, unprecedented increases can disrupt population dynamics and have community-level effects by influencing species that are closely allied with the focal species. Further, long-term effects must also be considered, such as reproductive success, health and viability of these offspring.

Exposure to contaminants during early life stages has been linked to developmental disorders and abnormalities that appear at adulthood, including the following examples. African clawed frog (*Xenopus laevis*) exposed to atrazine throughout larval development had demasculinized larynxes at metamorphosis (Hayes *et al.* 2002b; Hayes *et al.* 2010). Male

American alligators living in contaminated lakes in Florida were found to have reduced phallus sizes (Guillette *et al.* 1999b; Pickford *et al.* 2000). Male guppies exposed to antiandrogenic chemicals during juvenile development had smaller orange spots and gonopodia, reduced sperm counts and suppressed courtship behaviors (Bayley *et al.* 2002). Copulatory behavior of male Japanese quails exposed to xenoestrogens during embryonic development was completely suppressed at maturation (Panzica *et al.* 2005).

In my study, the area of orange-spot responded to atrazine exposure in a manner contrary to predictions, albeit moderately, and this effect was more pronounced for the atrazine high-dose group than the low-dose group. The mechanism for this pattern is perplexing. It is possible that atrazine exposure may have increased estradiol, leading to upregulated immune function: small increases in estradiol have been shown to boost immune responses (Kenny *et al.* 1976; Bilbo and Nelson 2001; Knöferl *et al.* 2001), though higher concentrations of the hormone can be toxic (Herman and Kincaid 1988; Krisfalusi *et al.* 1998; Robinson *et al.* 2007). Individuals that can mount a better response to immune challenges can allocate more carotenoids to their ornaments (Hill 1991; Folstad and Karter 1992; Lozano 1994), and may thus produce a larger area of orange coloration. But this is not possible if the carotenoid allocation strategy is a non-plastic trait.

However, it is unclear whether the increased ornament size translates into better reproductive success. These same atrazine-exposed males performed fewer courtship displays and were not favored by females (Chapter 5). Most sexually selected traits are correlated, and this pattern has also been seen in guppies (Houde 1997; Kodric-Brown and Nicoletto 2001). Females have evolved to associate larger orange spots with higher display rates, and these traits correlate with better immunocompetence (Houde and Torio 1992; Lopez 1998). Edwards and Guillette (2007) reported a similar unexpected result of increased gonopodium lengths in mosquitofish inhabiting nitrate contaminated waters, while other testosterone-regulated traits were suppressed. The results of such studies underscore how contaminants can influence pathways differently producing contrasting outcomes. When there is a disconnect between the effects on different traits, and if this occurs across multiple generations through persistence of pollutants in the environment, or via maternal transfers, significant evolutionary changes can result in the mating systems of these populations (Shenoy and Crowley 2011, Chapter 2).

Prenatal exposure to atrazine did not reduce the size of gonopodium in this study, though there appeared to be a trend in this direction. Several studies have reported smaller intromittent organs as a result of exposure to an endocrine disrupting compound (Guillette *et al.* 1996; Guillette *et al.* 1999b; Toft *et al.* 2003; Gunderson *et al.* 2004). I believe that a larger sample size in my study would have produced more robust results, encouraging the need for more research on the effects of atrazine on copulatory organs. Reduced gonopodia can influence reproductive success in multiple ways. Females of some poeciliid species prefer to mate with males that have longer gonopodia (Langerhans *et al.* 2005; Kahn *et al.* 2010). The size of the intromittent organ can influence successful sperm transfer during forced copulations, when the male has a split second to deliver the sperm before the female escapes (Kelly *et al.* 2000). Also, in highly competitive environments, males with longer intromittent organs will have an advantage when two or more males are simultaneously attempting to force copulation with a female (Kelly *et al.* 2000). All this highlights the need for more robust testing on the effects of EDCs on copulatory organs.

Delayed sexual maturation has been observed in vertebrate species exposed to EDCs (Bayley *et al.* 2002; Stoker *et al.* 2002; Saiyed *et al.* 2003). I had hypothesized a similar phenomenon with the guppies, expecting a delayed development of the gonopodium and orange spot, but I failed to find such a trend. In fact, exposure to DMSO appeared to delay the development of these traits more than did atrazine, and this intriguing result highlights the importance of the effects of solvents and other inert ingredients in pesticide mixtures (Relyea 2011). Though mostly nontoxic (Brayton 1986; Máchová *et al.* 2009), DMSO has been shown to

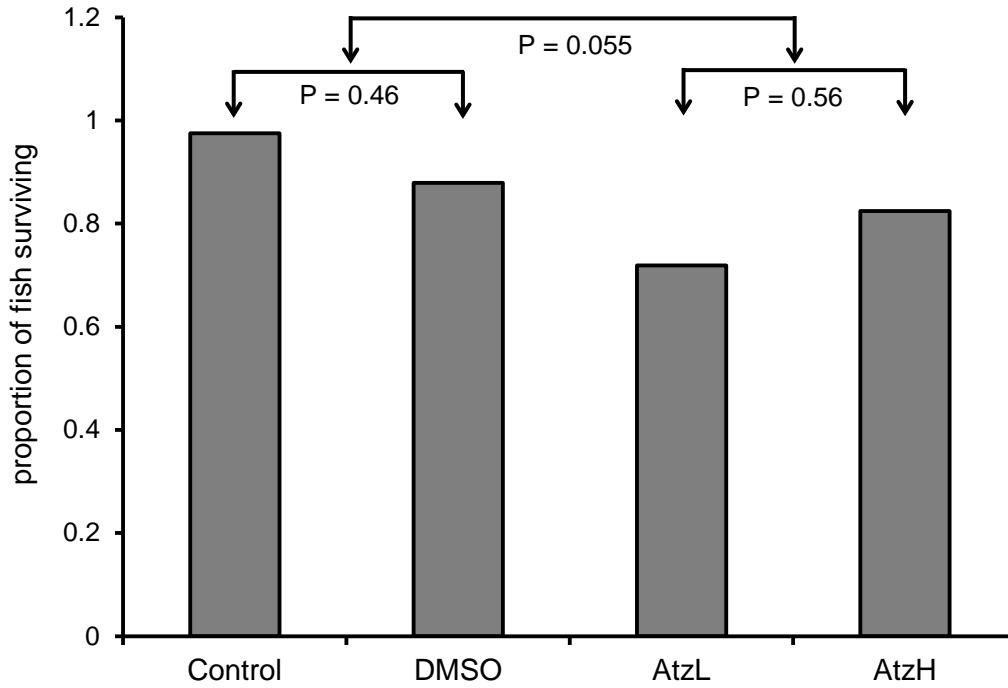
have some negative effects at the organismal (Máchová et al. 2009; Chen et al. 2011) and cellular (Pal *et al.* 2012) levels. However, I have failed to find an adverse effect of DMSO with regard to other end-points and exposures (Shenoy 2012, Chapter 3 and 5).

Prenatal exposure to atrazine did not significantly impact survival, and most fish in all treatment levels survived until the end of the experiment. Early exposure to xenoestrogens has been shown to affect survival (Nimrod and Benson 1998; Carlson *et al.* 2000). Both males and females were included in this data set, as many of the fish that died were too young to be sexed. Atrazine exposure also did not affect sex ratios of guppies in this study. Many studies have reported skewed sex ratios as a result of exposure to EDCs (Bayley et al. 2002; Teather et al. 2005; Örn et al. 2006; Pettersson et al. 2006). Sex determination, the development of sex-specific gonads from the bipolar primordial gonad, is genetically determined in many vertebrate taxa (amphibians: Hayes 1998; Wallace 1999; birds: Ellegren 2001; fish: Devlin and Nagahama 2002; mammals: Basrur 2006), but environmental cues can play an important role in some animal species (Bull 1980; Pieau *et al.* 1999). Xenobiotic interference during this process can cause sex-reversal or intersex (Reeder et al. 1998; Stoker et al. 2003; Hayes et al. 2006a). The gonads then produce sex-specific hormones important for the differentiation of somatic cells into sex-specific structures (Piferrer *et al.* 1993; Gray *et al.* 1994). The Leydig cells produce testosterone, and abnormal Leydig cells can produce insufficient levels of the hormone. Further exposure to EDCs during sex differentiation can produce phenotypic sex-reversal (Willingham and Crews 1999). Sex differentiation in guppies occurs postnatally, and begins about 5-6 weeks after birth (Houde 1997).

In the current study, exposure to atrazine was prenatal. Possibly, this early exposure did not alter gonadal hormone production enough to interfere with sex differentiation. Any atrazine stored in the body tissues was also probably not sufficient to disrupt the development of sex specific morphologies. However, it must be noted that wild guppies are not well suited for studying sex ratios; females from low predation sites have significantly smaller brood sizes than those from high predation sites (Reznick and Endler 1982), and in my study, brood sizes ranged from 1 to 16. Species that have consistently large brood sizes are more appropriate for testing predictions related to sex ratios.

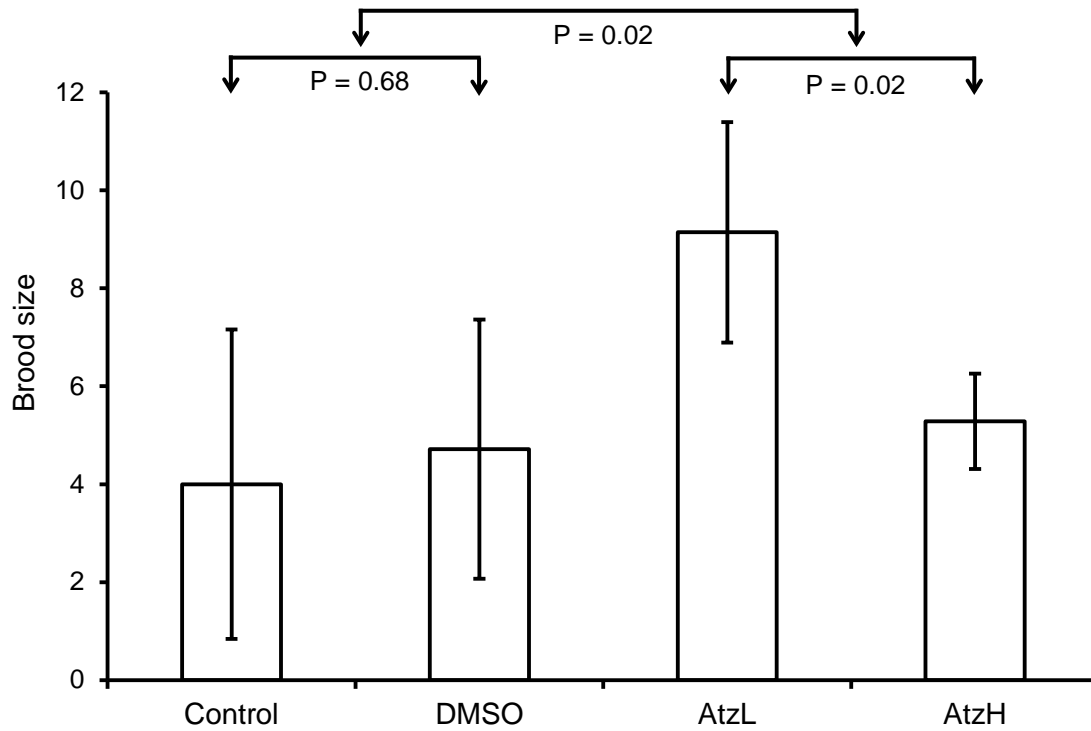
This study brings out the importance of low-dose exposures to atrazine during embryonic development on secondary sexual characteristics in guppies. In most cases (all variables except ornament size), a non-monotonic trend was observed. Such patterns, common in endocrinology and toxicology studies, are thought to be caused by oversaturation of hormone receptors (Welshons et al. 2003; Brodeur et al. 2009; Hamlin and Guillette 2011): increased hormone concentrations lead to reduced production of the hormones via a negative feedback loop, and this can lead to a removal of excess hormone receptors. These results can be extrapolated to other wildlife because steroid hormones are highly conserved across vertebrate taxa (Hamlin and Guillette 2011). Of interest are the unexpected results of increased brood sizes at low doses and increased area of orange spot at high doses. Ecotoxicology studies are fraught with conflicting results, where patterns are not consistent across species, or even within the same species (e.g. Edwards 2005 vs. Kristensen et al. 2007; reviewed in Solomon et al. 2008; Rohr and McCoy 2010). Timing of exposure, diet, age, strain of species, selection pressures, and so forth, could be important factors in influencing the measured end-points. In this study, population of origin had a significant effect on most end-points, indicating that genetic make-up has a considerable influence on the results. Because the mechanisms are complex and poorly understood, it is difficult to say why such inconsistencies arise. This only highlights the need for more in-depth research on the mechanisms of endocrine disruption. Also of importance are more studies across different taxa, and preferably in wild-caught species rather than inbred model organisms, and across a range of populations and genotypes, in order to extrapolate results confidently to other wildlife species.

I also point out with this study that the effects of contaminants cannot be declared unequivocally unless a suite of traits is examined. Studying only a small range of end-points may lead researchers to believe that a compound is non-toxic under the experimental conditions, while further examination of other end-points may reveal different patterns. The current study on the effects of prenatal atrazine exposure on morphological features showed no significant changes in gonopodium size and increased orange spot size, suggesting a nontoxic and perhaps beneficial effect of atrazine exposure. However, when taken in conjunction with results of a behavioral assay (Chapter 5), I find that prenatal exposure to atrazine can have definite adverse effects on reproduction of male guppies. Hence, I recommend that studies aim to use realistic exposures and measure all possible end-points that may be ecologically relevant to fitness.

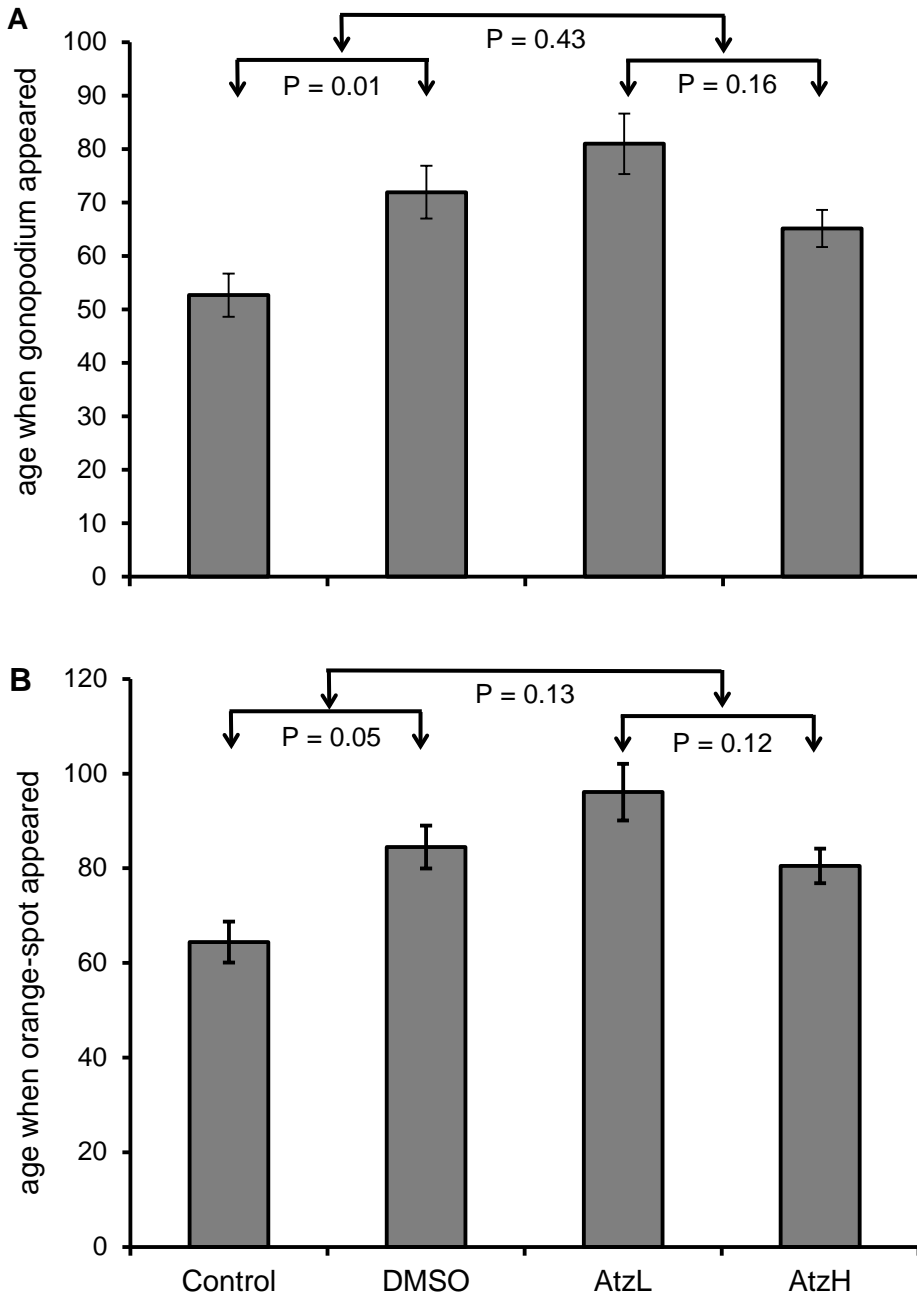


**Figure 4.1.** Proportion of guppies surviving until the end of the experiment in each treatment group. Arrows between groups with P-values denote *a-priori* orthogonal contrasts. AtzL = atrazine low-dose, AtzH = atrazine high-dose.

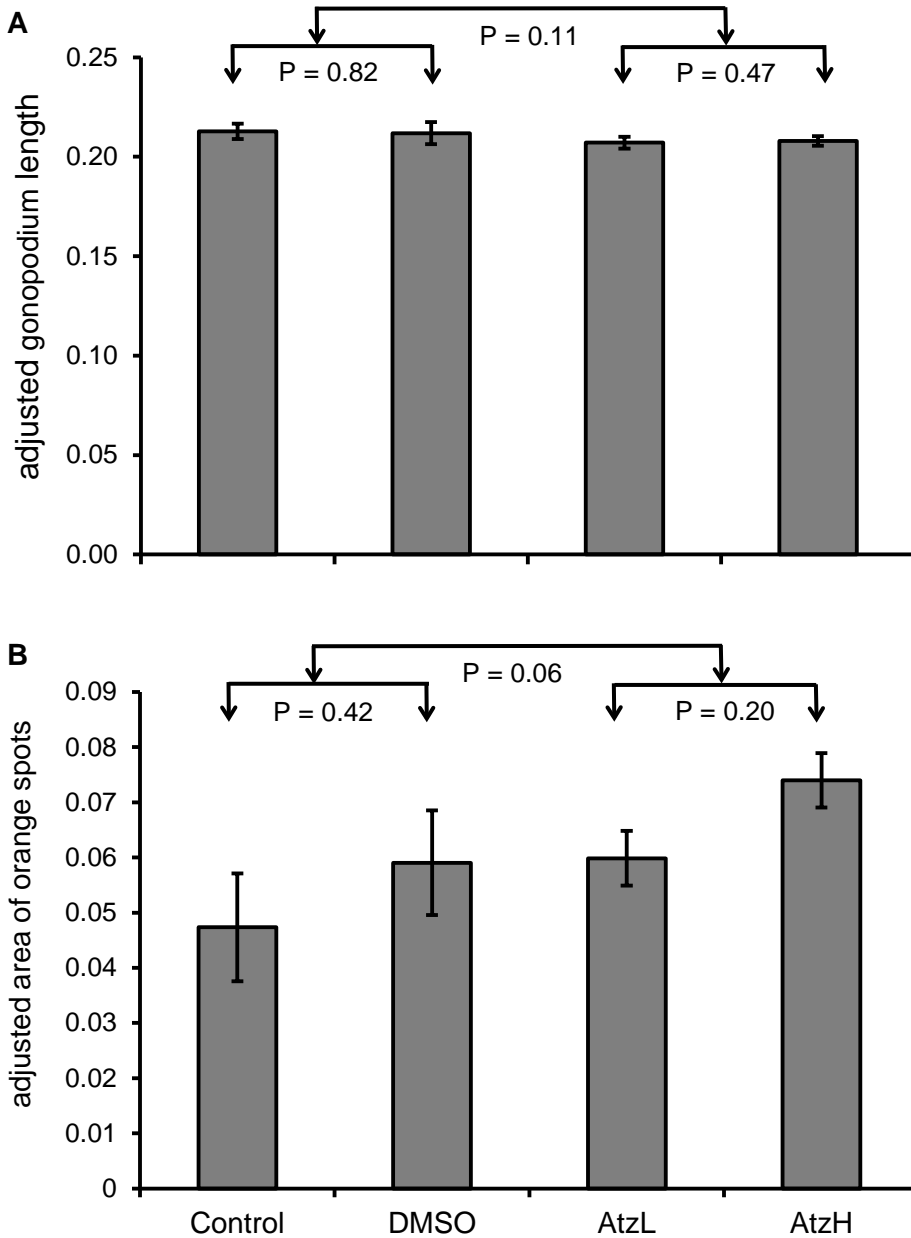




**Figure 4.2.** Brood sizes of adult female guppies exposed to the treatment. Arrows between groups with P-values denote *a-priori* orthogonal contrasts. AtzL = atrazine low-dose, AtzH = atrazine high-dose.



**Figure 4.3.** Average age at development of secondary sexual characteristics of male guppies that were prenatally exposed to the treatment. The secondary sexual characteristics measured were (A) gonopodium, and (B) orange spot. Arrows between groups with P-values denote *a priori* orthogonal contrasts. AtzL = atrazine low-dose, AtzH = atrazine high-dose.



**Figure 4.4.** Size of secondary sexual characteristics of male guppies that were prenatally exposed to the treatment. The secondary sexual characteristics measured were (A) gonopodium, and (B) orange spot. Arrows between groups with P-values denote *a-priori* orthogonal contrasts. AtzL = atrazine low-dose, AtzH = atrazine high-dose.

## CHAPTER FIVE. PRENATAL EXPOSURE TO LOW DOSES OF ATRAZINE AFFECTS MATING BEHAVIORS IN MALE GUPPIES

### SUMMARY

Performing appropriate mating behaviors is crucial to male reproductive success, especially in species where mating is predominantly via female mate choice. In most cases, mating behaviors are hormonally regulated and may be sexually selected traits: courtship displays are selected via mate choice, while forced copulations and aggressive behaviors are selected for via intrasexual competition. Endocrine disrupting compounds (EDCs) interfere with proper hormonal functioning in exposed animals. Organisms living in water bodies around agricultural areas are at risk of exposure to such contaminants during part or all of their life. Exposures during developmentally crucial life stages can have irreversible effects lasting through adulthood. I tested the effects of prenatal exposure to environmentally relevant doses of a commonly used herbicide, atrazine (1 and 13.5 µg/L) on mating behaviors in male guppies. Guppies were used as a model organism to test the effects of atrazine exposure on wildlife reproductive health. Adult female guppies were mated and exposed to the treatments throughout the gestation period, and offspring born to these females were raised without any further treatment. At adulthood, the males were tested for the effects of prenatal exposure on their mating behaviors such as courtship displays, gonopodium swings, forced copulatory attempts, and competitive and aggressive behaviors towards rivals who were not exposed to atrazine. I also tested female preference for treated males compared to control males. Atrazine-exposed males were less likely to perform the mating behaviors, and performed them less frequently, than control males. Atrazine exposure also made males less aggressive towards rivals. Females preferred untreated males over atrazine-treated males. These results have obvious implications for reproductive success. In all cases, a non-monotonic pattern was seen, highlighting the significance of low-dose exposures, which may often be neglected, or incorrectly considered safe for human or wildlife health.

### INTRODUCTION

Mating behaviors can be characterized as any behaviors that influence an individual's reproductive success. In many animal species, females choose mates by assessing courtship behaviors (Lopez 1998; Sargent et al. 1998; Edvardsson and Arnqvist 2000; Rebar et al. 2009). Males may also gain some fertilizations by employing other tactics like forced copulations (Clutton-Brock and Parker 1995). In species with high male-male competition, aggression between competing males may be a factor influencing access to females and monopoly of fertilizations (Brown et al. 2007; Hurtado-Gonzales and Uy 2010; Bertram et al. 2011), and females may assess males by watching male-male contests (Doutrelant and McGregor 2000; Mennill et al. 2002). Males performing sub-optimally in any of these mating behaviors will suffer reduced mating and reproductive success. In most cases, mating behaviors are hormonally regulated, and are especially under the control of gonadal hormones (Balthazart et al. 1990; Bell 2001; Ball and Balthazart 2004). Altered levels of gonadal hormones lead to altered expression of mating behaviors (Chapter 2, Shenoy and Crowley 2011).

Endocrine disrupting compounds (EDCs) are a class of compounds that interfere with proper hormonal functioning in exposed animals. Common types of EDCs include organochlorides, organophosphates, polychlorinated biphenyls (PCBs), phthalates, synthetic hormones and hormone-blockers, and phytoestrogens; common sources of EDCs include pesticides, industrial effluents, pulp mill effluents, plastics and sewage. Animals may be exposed directly by living in contaminated soil or water, or indirectly by eating contaminated prey (Markman et al. 2007; Markman et al. 2008; Park et al. 2009; Walters et al. 2010). EDC

concentrations can differ temporally within a region, thus exposing animals only during certain life stages. Often, this can coincide with developmentally crucial periods, and the effects of disruption during these stages can be manifested at later life stages. Such sublethal and latent effects can often go undocumented, yet their effects on population dynamics can be significant.

I tested the effects of prenatal exposure to atrazine on mating behaviors at adulthood in male guppies (*Poecilia reticulata*). Atrazine is the second most commonly used pesticide in the US (Grube et al. 2011). Its half-life in surface waters can be over 700 days (Solomon et al. 1996; Comber 1999), an interval that can span a part or all of the life of many aquatic and semi-aquatic animals. Depending on the type of crop and application rate, atrazine concentrations in water bodies around agricultural fields are expected to be in the range of 19-194 ppb over a 90 day period (USEPA 2006). It has been hypothesized that atrazine induces aromatization of testosterone to estradiol (Hayes 2005; Fan et al. 2007), but this mechanism has been debated (Hecker et al. 2005). Several studies have demonstrated the feminizing effects of atrazine in amphibians (Hayes et al. 2002b; Hayes et al. 2002c, 2003; Hayes et al. 2010) and fish (Shenoy 2012, Chapter 3), yet the number of studies with ambiguous and conflicting results (Solomon et al. 2008; Rohr and McCoy 2010) contributes to preventing policy changes regarding the use of this pesticide.

I hypothesized that prenatal exposure to environmentally relevant doses of atrazine would (1) reduce the likelihood and frequency of males performing mating behaviors such as courtship displays, gonopodium swings and forced copulatory attempts (these were considered behaviors related to mating effort); (2) in the presence of competing males, reduce the frequency of behaviors related to mating effort and those related to male-male aggression; and (3) reduce the attractiveness of exposed males in the context of female mate choice. The second hypothesis was tested because male-male competition is high in many animal species, including guppies: examining behaviors in the context of mate competition is ecologically relevant. Contaminants are often differentially distributed in the landscape, and different individuals in a population may be exposed unequally. Often, populations that are migratory or that converge at breeding sites would contain differentially exposed individuals. Since individuals impacted to varying degrees would be competing with each other within a population, and females may choose between such individuals, I tested the second and third hypotheses by pairing treated males with those that were not exposed to the contaminants. This also standardized the condition of each experimental male's opponent. These hypotheses test the effects of low dose exposures at early life stages on wildlife reproductive health.

## METHODS

**Study System:** I used guppies (*Poecilia reticulata*) as a model organism to test my hypotheses. Guppies have distinct sexual dimorphism, their mating signals and behaviors have been well characterized (Houde 1997), and the role of sex hormones in the expression of these traits has been explored (Jayasooriya et al. 2002; Hallgren et al. 2006). Further, guppies have been used for testing similar questions in other ecotoxicological studies (Baatrup and Junge 2001; Toft and Baatrup 2001; Kristensen et al. 2005).

Guppies are especially useful for testing hypotheses related to sexual selection. Males have different colored spots on their body and fins (Houde 1997); they perform characteristic courtship displays (called "sigmoid" displays) and attempt forced copulations. Mating is predominantly through female mate choice: females respond to courtship displays and to males with larger and brighter orange spots (Houde 1997; Kodric-Brown and Nicoletto 2001), but avoid forced copulatory attempts (Houde 1997; Evans et al. 2003). Fertilization is internal, and males have a modified anal fin called the gonopodium, which is used as a copulatory organ. Males frequently swing their gonopodium forward, and this appears to increase in frequency during mating or aggressive interactions. Displaying the copulatory organ as an ornament is common

in poeciliids (Langerhans et al. 2005, Schlosberg et al. 1949, Basolo 1995). Females are viviparous and hence suited for testing the effects of maternal exposures.

Guppies are small tropical fish native to Trinidad and parts of South America. Adult female guppies used for this study were descendants of wild-caught guppies from Trinidad. Three populations—Aripo Upper River, Aripo Lower River, Guanapo Upper River—were equally represented in all treatments to account for geographic and genetic variation.

**Treatments:** 40 adult female guppies were randomly assigned to one of the four treatment levels at 10 fish per level. Before the treatment was administered, each female was housed with a separate male on each of four consecutive days to improve brood characteristics (Evans and Magurran 2000). The treatment levels included a negative control (no manipulation), dimethylsulfoxide (DMSO, 83.3  $\mu\text{L/L}$ ) as the solvent control, atrazine low-dose (1  $\mu\text{g/L}$ ), and atrazine high-dose (15  $\mu\text{g/L}$ ). A solvent control was used because atrazine and ethynyl estradiol were dissolved in DMSO; all treatments received the same concentration of DMSO. Atrazine concentrations used were based on USEPA estimated environmental concentrations (USEPA 2006). Concentration of atrazine in the water column in three randomly selected jars per treatment was ascertained by liquid phase extraction with methylene chloride following an adaptation of USEPA Method 619 (USEPA 1993)—which produced 95% recovery of the target compound—and analyzed by gas chromatography/mass spectrometry. Water was changed twice a week with static renewal of chemicals. The average concentration at the end of 4 days was determined to be 13.56  $\mu\text{g/L}$  for the high-dose with negligible loss over 4 days. In other experiments (Shenoy 2012, Chapter 3), I have found there to be negligible loss over 4 days for this concentration as well. No atrazine was detected in the control samples. Atrazine (98% purity) was purchased from Chem Service, Inc., through Fisher Scientific. Treatments continued until a brood was produced to simulate a long-term exposure. Offspring born to treated females were raised to adulthood with no further treatment.

**Animal Care:** During the period of the study, all fish were housed separately in individual glass bowls with 3 L of aged (for at least 24 hours), pre-aerated, carbon filtered, conditioned water (conditioned with AmQuel® and NovAqua® by Kordon, LLC). Fish were fed with tropical fish flake food once each day and brine shrimp nauplii once each day in *ad libitum* quantities. Room temperature was maintained at an average of 25 °C; the light: dark cycle was set to 12:12 hours. Mortality was recorded every day. Bowls were checked twice daily for offspring.

Offspring born to treated females were immediately removed from the mother's bowl. Each brood was housed together in a plastic tank with 6 L of aged, pre-aerated, carbon filtered, conditioned water, as before. All fish were fed *ad libitum* quantities of tropical fish flake food four days per week, and *ad libitum* quantities of brine shrimp nauplii three days per week. All fish were checked daily for development of male-specific morphologies: any fish that developed a gonopodium or color spot was immediately transferred to another container to separate the sexes. Males and females of a brood were then housed in sex-specific tanks with no more than 4 fish per tank. All tanks contained gravel and were aerated continuously. Room temperature was maintained at an average of 25 °C; the light: dark cycle was set to 12:12 hours. Mortality was recorded every day.

The experimental protocol for this study was approved by the University of Kentucky Institutional Animal Care and Use Committee (protocol number 2007-0137).

**Behavior trials:** At an average age of 13 months ( $\pm 0.75$  months, standard deviation), the fish were subjected to three sets of behavior trials: (1) to assess behavior of the males towards a female in the absence of competition from another male, (2) to assess mating behaviors in the presence of a competing male, and (3) to assess male attractiveness to a female. All trials were conducted within the first four hours after lights turned on and during the last four hours before lights turned off. All trials were conducted blind: the observer did not know the treatment that any of the fish had received and identified males by their color patterns only. Data were recorded in real time. Trial tanks were illuminated with full-spectrum light including ultra violet

wavelengths to ensure that all colors were perceived naturally by the other fish in the trial (Endler 1991, 1993). The observer sat in darkness, 1 m away from the tank, to avoid startling the fish; the fish did not appear to notice or be disturbed by the presence of the observer. All behavioral observations were made using JWatcher v1.0 (Blumstein et al. 2000-2006).

*Trials without competing males:* each male was placed in a trial tank of dimensions 30 x 20 x 15 cm (height x length x width) and 7.5 L of water, with one virgin female from the same population. The water used was aged, pre-aerated, carbon filtered, and conditioned, as before, and water temperature was maintained between 23-25 °C. Three walls of the tank were lined with black landscape fabric on the inside to prevent reflections. After a 5 minute acclimation period, the fish were observed for 10 minutes. The total number of sigmoid courtship displays, gonopodium swings and copulatory attempts were recorded throughout the trial period. Only gonopodium swings greater than 90° were counted.

*Trials with competing males:* These trials were conducted to test whether treatments altered male behaviors compared to an untreated male in the context of competition. Males were paired in the following fashion—each pair consisted of one male from the control group (opponent) and one male (focal male) from one of the other three treatment groups: DMSO, atrazine low-dose, or atrazine high-dose. Control-group males were used in multiple pairs, as there were not enough males to be used only once. Control-group males were paired with each of the different treatment group males in random order. As far as possible, focal males of a single brood were paired with control males of a single brood. Males of a pair belonged to the same population. Pairs could not be size-matched after matching for population and brood. Each pair was placed in a trial tank as described above, with a virgin female from the same population. After a 5 minute acclimation period, behaviors were recorded for 10 minutes. At each 10s point, the observer recorded which male was closer to the female. A male had to be more than one body length ahead of the other male to be “closer”, and received 1 point in such cases. If both males were within one body length of each other, and within at least two body lengths of the female’s vent, they were both recorded as being equally close; in such cases both males received 0.5 points. If both males were further than two body lengths from the female’s vent, they were both recorded as being far from the female and received 0 points for that event. At the end of the 10 minute trial period, each male’s “closeness” points were summed and its ratio to the total number of events gave a measure of proximity. Throughout the whole trial period, the observer counted for each male the total number of sigmoid courtship displays, forced copulatory attempts, aggressive displays to the rival male, and attacks on the other male. The number of gonopodium swings was not recorded, as these happened in quick succession, and the observer could not keep a reliable count for both males.

*Attractiveness to females:* These trials were conducted to test whether females had a stronger preference for control males when paired with a treated male. Males were paired as above. The trial tank 25.4 x 40.6 x 20.3 cm (height x length x width) was physically divided into three compartments with transparent plastic dividers; the compartments were adjacent to each other along the length of the tank: two compartments of 5 cm length on either end of the tank, and a central compartment where the virgin female was placed. Each male of a pair was randomly placed in one of the two end compartments. Males were identified by their color patterns and the observer did not know which treatment either male had received. The central compartment was further divided into three compartments by marking on the walls of the tank with a marker. The areas immediately adjacent to the male compartments were considered the “preference zone” where the female would spend time if she was interested in the male; these areas were 5 cm in length. Preliminary trials found that females interested in mating (determined by vigorous swimming against the partition or steadily watching the male) spent time in this region. The central portion of the tank was considered the “non-preference zone” where the female spent time if she was not interested in mating (again, determined from preliminary trials).

After 10 minutes of acclimation, observations of time spent by the female in each compartment were made for 10 minutes.

**Data Analyses:** All data were analyzed with SAS 9.3 (SAS-Institute-Inc 2002-2009). Because males within a brood are correlated, and the number of males per brood varied from 1 to 7 (mean =  $2.96 \pm 1.7$  standard deviation), I used generalized estimating equations (GEE; PROC GENMOD in SAS) for all analyses, by grouping individuals within broods (Hanley et al. 2003). GEE uses a quasi-likelihood approach to analyzing generalized linear models (GLMs), such as ANOVAs, ANCOVAs, logistic regressions, and so forth (Zeger and Liang 1986; Hanley et al. 2003). GEE focuses on within cluster similarity of the residuals, and this estimated correlation is used to calculate regression parameters and standard errors (Hanley et al. 2003; Hubbard et al. 2010). Statistics reported for fixed effects are Wald  $\chi^2$  statistics for Type III GEE (the Wald statistics follows a  $\chi^2$  distribution).

*Mating behaviors without competition:* The response variables analyzed were the number of displays, the number of gonopodium swings, and the number of forced copulatory attempts, performed within the 10 minutes trial period. Pearson product-moment correlations between the variables were analyzed; the correlation coefficients indicated that the number of gonopodium swings was strongly correlated with the number courtship displays performed ( $r = 0.66$ ,  $P < 0.0001$ ) and moderately with the number of forced copulatory attempts ( $r = 0.5$ ,  $P < 0.0001$ ), while the number of courtship displays and the number of copulatory attempts were more weakly correlated ( $r = 0.32$ ,  $P = 0.003$ ). No variables were eliminated from the analysis, because none of the correlations were extremely strong, and each was biologically meaningful.

I tested for the effects of treatments on (i) the likelihood of performing mating behaviors using logistic regressions (with binary responses), and (ii) the frequency of mating behaviors performed using GLMs. For the GLMs I assumed negative-binomial error (number of courtship displays and number of gonopodium swings) or Poisson error (number of forced copulatory attempts), determined by lower quasi-likelihood under the independence model criterion (QIC) values. Since I had specific null hypotheses, I used *a-prior* planned orthogonal contrasts to test whether (1) the two control groups were similar to each other, (2) the two atrazine groups were similar to each other, and (3) the atrazine groups were similar to the control groups, with respect to the likelihood of performing the behaviors and the frequency of the behaviors performed. Population of origin and the number of days that the fish received treatment gestationally were included as fixed effects, but these did not have a significant effect on the responses and so were removed. Because the data were over-dispersed to some extent, a scale correction factor was applied to the parameter covariance matrix and likelihood function (courtship displays: negative binomial dispersion parameter,  $k = 5.15$ ; gonopodium swings:  $k = 3.67$ ; forced copulation attempts: scale parameter,  $\phi = 2.54$ ).

*Mating behaviors in the presence of competition:* Pearson product-moment correlations between all variables were analyzed. The correlations between some of the response variables were moderate to strong (proximity and courtship displays:  $r = 0.43$ ,  $P = 0.0007$ ; proximity and forced copulatory attempts:  $r = 0.64$ ,  $P < 0.0001$ ; courtship displays and forced copulatory attempts:  $r = 0.38$ ,  $P = 0.003$ ; aggressive displays and attacks:  $r = 0.48$ ,  $P < 0.0001$ ), and low among others ( $r$  and  $P$  values not shown). I did not eliminate any variables from the analyses as none of the correlations were extremely strong, and all the variables were biologically meaningful. Since males were paired, and their behaviors were dependent on each other, an ANCOVA was performed to analyze the effect of the treatments on the focal male's behavior in response to his paired opponent's behavior, which was included as the covariate. Covariates were mean-centered within treatments so that mean estimates for each treatment corresponded with the mean value of the covariate. I specifically tested for differences between treatment means (seen by a significant effect of the treatment) and slopes (seen by a significant interaction of treatment by covariate). I performed *a-prior* planned orthogonal contrasts to test



whether (1) the means and slopes of the two atrazine groups were similar, and (2) the atrazine groups had similar means and slopes to the DMSO group.

Proximity to the female was square-root transformed (for the focal males and paired control males), and the data fit a normal distribution. The underlying distributions for all variables (except proximity to female) were specified as Poisson or negative binomial, depending on which distribution improved the fit of the model. Population of origin and control male's trial number (because males from the control group were used in multiple pairs) were included as additional fixed effects wherever they improved the fit of the model. The data were under- or over-dispersed to some extent, so a correction factor was applied to the parameter covariance matrix and likelihood function (proximity:  $k = 0.19$ ; forced copulation attempts:  $k = 2.35$ ; aggressive displays:  $k = 2.44$ ; attacks:  $\varphi = 4.43$ ).

The number of courtship displays had a large number of zeroes and could not be analyzed quantitatively. Qualitatively, I reported for each treatment level, the proportion of pairs in which the focal male performed fewer displays than the paired control male, from amongst the pairs where at least one male performed at least one courtship display.

**Attractiveness to females:** The strength of the female's preference (henceforth SFP) for the treated male (belonging to DMSO, atrazine low-dose or atrazine high-dose group) over the paired control male was measured by the difference between time spent in the treated male's preference zone and the time spent in the paired control group male's preference zone, as a proportion of total time spent in either preference zone. A positive SFP would indicate a preference for the treated male over the paired control male, while a negative SFP would indicate a preference for the paired control male over the treated male. Preliminary analysis showed that there was no significant difference in time spent with a DMSO male compared to the paired control male (paired t-test,  $P = 0.51$ ). I tested for the differences in SFP between groups using GLM, and the data fit a normal distribution. Population of origin was input as a fixed effect as it had a significant effect on the SFP. The data were somewhat under-dispersed, so a scale correction factor was applied to the parameter covariance matrix and likelihood function ( $\varphi = 0.46$ ).

## RESULTS

**Behaviors in the absence of competitors:** The treatment had a significant effect on the likelihood of performing any of the mating behaviors (courtship displays:  $\chi^2 = 13.65$ ,  $df = 3$ ,  $P = 0.0034$ ; gonopodium swings:  $\chi^2 = 9.18$ ,  $df = 3$ ,  $P = 0.027$ ; forced copulatory attempts:  $\chi^2 = 8.59$ ,  $df = 3$ ,  $P = 0.035$ ; Figure 5.1). The atrazine low-dose group was significantly less likely to perform courtship displays compared to the control group (Tukey-Kramer *post-hoc* test,  $P = 0.0035$ ), and there was a tendency for a lower likelihood of performing gonopodium swings (Tukey-Kramer *post-hoc* test,  $P = 0.06$ ) or forced copulatory attempts (Tukey-Kramer *post-hoc* test,  $P = 0.08$ ). The atrazine high-dose group did not differ from the control group in their likelihood to perform any of the mating behaviors (Tukey-Kramer *post-hoc* test, courtship displays:  $P = 0.14$ ; gonopodium swings:  $P = 0.09$ ; forced copulatory attempts  $P = 0.37$ ). The lack of power from performing all pair-wise comparisons prevented the detection of significant differences, which were brought out by planned orthogonal contrasts (Figure 5.1). The DMSO group was equally likely to perform mating behaviors compared to the control group (planned orthogonal contrasts: courtship displays:  $P = 0.81$ ; gonopodium swings:  $P = 0.86$ ; forced copulatory attempts:  $P = 0.92$ ). The atrazine groups were also indistinguishable from each other (planned orthogonal contrasts: courtship displays:  $P = 0.17$ ; gonopodium swings:  $P = 0.89$ ; forced copulatory attempts:  $P = 0.2$ ), but the atrazine groups were significantly less likely to perform any of the behaviors compared to the control groups (planned orthogonal contrasts: courtship displays:  $P = 0.002$ ; gonopodium swings:  $P = 0.006$ ; forced copulatory attempts:  $P = 0.003$ ).

The treatment had a significant effect on the frequency of some of the behaviors performed within the 10 minute trial period (courtship displays:  $\chi^2 = 5.73$ ,  $df = 3$ ,  $P = 0.13$ ; gonopodium swings:  $\chi^2 = 10.55$ ,  $df = 3$ ,  $P = 0.014$ ; forced copulatory attempts:  $\chi^2 = 16.93$ ,  $df = 3$ ,  $P = 0.0007$ ; Figure 5.2), and the significant effect was driven by reduced behaviors of males from the atrazine low-dose group (significantly lower than control group, Tukey-Kramer *post-hoc* test: gonopodium swings,  $P = 0.03$ ; forced copulations,  $P = 0.02$ ). For all three behaviors, the two control groups responded similarly (planned orthogonal contrasts: courtship displays,  $P = 0.97$ ; gonopodium swings,  $P = 0.91$ ; forced copulations,  $P = 0.09$ ), as did the two atrazine groups (planned orthogonal contrasts: courtship displays,  $P = 0.36$ ; gonopodium swings,  $P = 0.25$ ; forced copulations,  $P = 0.15$ ). The atrazine groups together responded lower than the control groups with respect to gonopodium swings (planned orthogonal contrasts,  $P = 0.018$ ) and forced copulations (planned orthogonal contrasts,  $P = 0.007$ ), but not courtship displays (planned orthogonal contrasts,  $P = 0.06$ ).

**Behaviors in the presence of competitors:** These data were analyzed using ANOCOVAs with the focal male's behavior as the response variable and the paired control male's behavior as the covariate; I tested for treatment effects on the mean behaviors (detected by a significant effect of the treatment) and the slopes of the behaviors in response to the covariate (detected by a significant interaction of the treatment and the covariate). The slopes indicate the focal male's behavior in response to his paired control male's behavior, which is indicative of competitiveness.

Proximity to the female (Table 5.1) was not influenced by the treatment ( $\chi^2 = 0.86$ ,  $df = 2$ ,  $P = 0.65$ ), or the interaction of treatment and paired control male's behavior ( $\chi^2 = 3.36$ ,  $df = 2$ ,  $P = 0.19$ ), but was influenced by the paired control male's proximity ( $\chi^2 = 17.74$ ,  $df = 1$ ,  $P < 0.001$ ). The atrazine groups had similar means (planned orthogonal contrasts,  $P = 0.91$ ) and slopes (planned orthogonal contrasts,  $P = 0.2$ ), and did not differ from that of the DMSO group (planned orthogonal contrasts: mean,  $P = 0.38$ ; slope,  $P = 0.37$ ).

The number of forced copulations (Table 5.1) was not influenced by the treatment ( $\chi^2 = 1.06$ ,  $df = 2$ ,  $P = 0.59$ ), and only slightly by the paired control male's behavior ( $\chi^2 = 3.24$ ,  $df = 1$ ,  $P = 0.07$ ), but was strongly influenced by the interaction of treatment with the paired control male's behavior ( $\chi^2 = 11.84$ ,  $df = 2$ ,  $P = 0.0027$ ). The means of the two atrazine groups were similar (planned orthogonal contrasts,  $P = 0.78$ ), and did not differ from the mean of the DMSO group (planned orthogonal contrasts,  $P = 0.33$ ). The slopes of the atrazine groups were also similar (planned orthogonal contrasts,  $P = 0.12$ ) but were significantly lower than the slope of the DMSO group (planned orthogonal contrasts,  $P = 0.005$ ).

The number of aggressive displays to the rival male (Table 5.1) was influenced by the treatment ( $\chi^2 = 6.19$ ,  $df = 2$ ,  $P = 0.045$ ), but not by the paired control male's behavior ( $\chi^2 = 2.20$ ,  $df = 1$ ,  $P = 0.14$ ) or the interaction of the treatment with the covariate ( $\chi^2 = 3.98$ ,  $df = 2$ ,  $P = 0.14$ ). The atrazine groups had similar means (planned orthogonal contrasts,  $P = 0.51$ ) and slopes (planned orthogonal contrasts,  $P = 0.26$ ), but together had significantly lower mean (planned orthogonal contrasts,  $P = 0.02$ ) and slope (planned orthogonal contrasts,  $P = 0.049$ ) compared to the DMSO group.

The number of attacks on the rival male (Table 5.1) was significantly influenced by the treatment ( $\chi^2 = 10.39$ ,  $df = 2$ ,  $P = 0.0055$ ), but not by the paired control male's behavior ( $\chi^2 = 1.99$ ,  $df = 1$ ,  $P = 0.16$ ) or the interaction of the treatment with the covariate ( $\chi^2 = 0.5$ ,  $df = 2$ ,  $P = 0.78$ ). The atrazine groups did not differ from each other in means (planned orthogonal contrasts,  $P = 0.57$ ) or slopes (planned orthogonal contrasts,  $P = 0.62$ ), but together they had a significantly lower mean (planned orthogonal contrasts,  $P = 0.0017$ ) than the DMSO group, though not a lower slope (planned orthogonal contrasts,  $P = 0.68$ ).

Population of origin had a significant effect on proximity to the female ( $\chi^2 = 6.47$ ,  $df = 2$ ,  $P = 0.04$ ), the number of forced copulation attempts ( $\chi^2 = 9.28$ ,  $df = 2$ ,  $P = 0.0097$ ), and the number of attacks ( $\chi^2 = 7.4$ ,  $df = 2$ ,  $P = 0.02$ ), while the paired control male's trial number ( $\chi^2 = 9.05$ ,  $df =$

1,  $P = 0.0026$ ) significantly influenced the number of attacks. The latter result may be the result of winner-loser effects influencing a male's propensity to attack his rival (Hsu and Wolf 1999; Dugatkin and Druen 2004).

A qualitative examination of the number of courtship displays performed revealed that the proportion of pairs wherein the focal male performed fewer number of courtship displays than the paired control was approximately equal in all treatment levels (DMSO = 0.67, atrazine low-dose = 0.67, atrazine high-dose = 0.75) suggesting that the treatment probably did not affect the frequency of courtship displays performed.

**Attractiveness to females:** The strength of the female's preference for the control male over the treated male (SFP) was significantly influenced by the treatment ( $\chi^2 = 8.46$ ,  $df = 2$ ,  $P = 0.015$ ; Figure 5.3), and this effect was mainly driven by the atrazine low-dose group (Tukey-Kramer *post-hoc* test,  $P = 0.02$ ). Planned orthogonal contrasts brought out differences in the SFP of the atrazine groups ( $P = 0.03$ ), yet together they had a lower SFP than the DMSO group ( $P = 0.035$ ). Population of origin also influenced SFP ( $\chi^2 = 13.44$ ,  $df = 2$ ,  $P = 0.0012$ ).

## DISCUSSION

Prenatal exposure to atrazine reduced the likelihood that a male courted, the frequency of gonopodium swings, and the number of forced copulatory attempts performed. In the presence of a competing male, atrazine-exposed males performed fewer courtship displays, aggressive displays and attacks on the rival; their competitiveness, indicated by the slope of their behavior in response to the rival's escalating behavior, was also reduced with regard to forced copulatory attempts and aggressive displays. Females had a greater strength of preference (SFP) for the control males over the atrazine low-dose males, compared to their SFP for control males over DMSO males or the atrazine high-dose males.

The effects of atrazine on male sexual characteristics such as gonadal morphology, hormone production and sperm counts have been explored in amphibians and fish (Hayes et al. 2002b; Hayes et al. 2003; Hayes et al. 2006b; Abarikwu et al. 2010; Hayes et al. 2010; Tillitt et al. 2010). Evidence for the effects of atrazine on sexual signals and behaviors are fewer. Effects of atrazine on larynx size (Hayes et al. 2002b) and structure (Hayes et al. 2010) of African clawed frog larvae exposed to atrazine have been reported. The larynx produces vocalization in anurans, their primary mating signal. Smaller larynxes produce suboptimal calls. Shenoy (2012) found that adult male guppies exposed to atrazine had a tendency toward reduced orange coloration and performed fewer courtship displays. This study adds to the literature on the effects of atrazine on sexual signaling, especially mating behaviors.

Atrazine has been postulated to upregulate the production of the enzyme aromatase that is necessary for the conversion of testosterone to estradiol (Hayes 2005; Fan et al. 2007). This mechanism has been debated (Hecker et al. 2005). Alternative mechanisms suggested for the endocrine disruption by atrazine are direct inhibition of testosterone production from the Leydig cells (Friedman 2002) and reduced levels of 5 $\alpha$ -reductase, the enzyme that converts testosterone to DHT (Kniewald et al. 1979; Babic-Gojmerac et al. 1989). If atrazine does upregulate aromatase production, then the increased rate of conversion of testosterone to estradiol would reduce available testosterone and increase estradiol concentrations. Increased estradiol can directly reduce testosterone production (Bartke et al. 1977; Kim et al. 2007). Further, a reduction in testosterone levels will affect the production of dihydrotestosterone and, in teleost fish, 11-ketotestosterone, hormones that are necessary for the development of secondary sexual traits (Ankley et al. 2002; Ankley et al. 2005). Aromatase also converts androstenedione to estrone; testosterone is produced from androstenedione, while estradiol is produced from estrone. Hence, increased aromatase can further alter testosterone:estradiol ratios. All of this would be instrumental in the development of sexual traits that are dependent on testosterone and estradiol balances.

The regions of the brain controlling sexual behaviors are larger in males and rich in androgen receptors (Ball and Balthazart 2004). Castration can shrink these regions with corresponding reduction in sexual behaviors, but implanting testosterone can restore behaviors to pre-castration levels (Ball and Balthazart 2004). 11-ketotestosterone is required for courtship behavior in male mosquitofish (Toft and Guillette 2005) and guppies (Baatrup and Junge 2001; Bayley et al. 2002) and for female recognition and courtship in male goldfish (Thompson et al. 2004a). In this study, endocrine disruption during embryonic development may have impeded the development of the brain regions that respond to sexual stimuli or disrupted the development of the gonads, thereby affecting hormone production.

Interestingly, the low dose of atrazine had consistently stronger effects on guppy behavior than the high dose of atrazine (though the differences were not always statistically significant); in many cases the effects of the high dose of atrazine were indistinguishable from the controls. Such nonmonotonic (inverted U-shaped) response curves are common in endocrinological studies and have been reported for EDCs (Welshons et al. 2003; Brodeur et al. 2009; Hamlin and Guillette 2011). This is mainly due to saturation of hormone receptors, and hence, the saturation of responses (Welshons et al. 2003): increased hormone concentrations can lead to removal of a negative feedback loop shutting down further hormone production, which in turn can lead to the removal of excess hormone receptors. This highlights the fact that low concentrations of contaminants are not negligible and can have profound effects on wildlife health, often more so than higher doses. However, higher doses of EDCs might affect other physiological systems and must not be considered harmless.

**Implications of altered mating behaviors:** In this study, males that had been exposed to atrazine during embryonic development showed reduced interest in mating, as seen by a lower likelihood and frequency of performing mating behaviors. Altered mating behaviors as a result of contaminant exposure have been documented (Baatrup and Junge 2001; Toft and Baatrup 2001; Bayley et al. 2002; Bayley et al. 2003; Bortolotti et al. 2003; Nakayama et al. 2004; Arellano-Aguilar and Garcia 2008; Bernhardt and von Hippel 2008; Larsen et al. 2008; Saaristo et al. 2009). In species where female mate choice predominates, performing appropriate mating behaviors is crucial to securing fertilizations. Male guppies that are unlikely to court females, or that perform few courtship displays, will not be favored by females (Houde 1997), and this is often the case in other species as well (Gerhardt 1994; Reynolds 1996; Talyn and Dowse 2004; Baird et al. 2007). Courtship displays are regulated by sex hormones in guppies (Baatrup and Junge 2001; Bayley et al. 2003; Hallgren et al. 2006; Baatrup 2009) and other vertebrates (Ball and Balthazart 2004; Adkins-Regan 2005, 2007); exposure to atrazine appears to have disrupted this pathway in the present study. Males of many species use multiple traits simultaneously or sequentially to attract females (Candolin 2003). The gonopodium swing appears to be a form of display; this variable was correlated with courtship displays in this study, and similar correlations have been found in previous studies (Schlosberg et al. 1949; Shenoy 2012, Chapter 3). If courtship displays are regulated by sex hormones, it is reasonable to expect gonopodium swings to also be similarly controlled.

Although females try to avoid forced copulations, males do attain some fertilizations through this strategy. In a highly competitive environment, males that do not attempt forced copulations are compromising their chances of obtaining fertilizations. So far there is little evidence to support the hypothesis that forced copulations are controlled by sex hormones (Davis 2002). Shenoy (2012, Chapter 3) found that adult male guppies exposed to atrazine performed fewer courtship displays, but the exposure did not affect the number of forced copulations attempts. It is possible that the timing of exposure may have influenced the difference in these results.

Mating signals in guppies consist of the orange colored spots on their bodies as well as courtship displays (Houde 1997) and possibly other behaviors like gonopodium swings. Females choose mates by assessing all these traits, which are often correlated (Nicoletto 1993)

(Kodric-Brown and Nicoletto 2001). Males with brighter and larger orange spots are preferred by females (Houde 1997; Rodd et al. 2002), as are males that display frequently (Kodric-Brown 1993). Unexpectedly, atrazine exposure increased the area of orange spots in these males (Chapter 4), yet females showed a clear lack of preference for the atrazine treated males. Since these males performed courtship displays and gonopodium swings less frequently, it is reasonable to assume that females based their assessment on the behavioral traits. In guppies, as in many other animal species, males that are not preferred by females have little chance of obtaining matings except via sneaker strategies.

Courtship displays in most animal species are considered signals of mate quality that females use to assess potential mates (Lopez 1998). If male mating signals are rendered unreliable as a result of contaminant exposure, females may not be able to accurately assess mate quality. If these effects last for multiple generations, either via persistence of the contaminants in the environment or through maternal transfer, it is possible that females may not rely on these signals leading to a loss of the signal from the population (Shenoy and Crowley 2011, Chapter 2). Altered mating signals reduce the availability of attractive mates in a population, thereby altering the effective sex ratio. Altered sex ratios can influence population dynamics, extra-pair copulation rates (Kempnaers et al. 1997) and subsequently, offspring sex ratios and qualities (Quader 2005). Altered mating signals as a result of contaminant exposure may not reflect mate quality, leading to "incorrect" female choices and resulting in lowered offspring number and quality (Shenoy and Crowley 2011, Chapter 2). Quader (2005) reviews other ecological implications of altered female mate choice.

Male-male competition is high in many animal species, including guppies. EDCs are known to affect competitive behaviors in exposed animals (Jaeger et al. 1999; Bell 2001; Majewski et al. 2002; Palanza et al. 2002). When competing with a rival male that was not exposed to treatments, the atrazine exposed males escalated less in response to the paired control male's behavior compared to the DMSO treated males. These males are clearly at a mating disadvantage in the face of competition. When competition is high, males will indulge more in forced copulations (Jirotkul 1999b) and aggressive behaviors, and focus less on courtship; this was apparent from the low numbers of males that performed courtship displays in the presence of a rival male in this study.

Aggression between rival males is an important factor in gaining fertilizations: first, winning contests allows access to females (Brown et al. 2007; Hurtado-Gonzales and Uy 2010; Bertram et al. 2011), and second, females often choose mates by watching contests (Doutrelant and McGregor 2000; Mennill et al. 2002). More aggressive males will also guard mates to prevent sperm competition (Bateman and Toms 1998; Watts 1998; van Dongen 2008). Hence reduced aggression towards rival males can lead to lowered reproductive success. In this study, atrazine exposed males performed fewer aggressive displays and attacks on rivals, which could result in significantly reduced mating success.

Population of origin affected some of the behavioral variables. Guppy mating behaviors vary depending on the predation regime they originate from (Luyten and Liley 1985; Godin 1995; Houde 1997). Males from high predation sites are less likely to perform courtship displays and attempt more forced copulations, while males from low predation sites will invest in courtship displays (Luyten and Liley 1985). Further, males from high predation sites may spend less time in aggressive acts as this can attract predators (Luyten and Liley 1985). The strength of female's preference (SFP) was also influenced by population of origin. This could either be an artifact of the difference in male behavior patterns based on population of origin or a diminished inclination for female guppies from high predation sites to associate with very active males to reduce predation risk (Godin and Briggs 1996). Future studies testing the interactive effects of EDC-exposure and predation regimes would be important to understand how selection against a trait can be compounded by novel disruptors like contaminants.

**Timing of exposure:** An important highlight of the current study is the effects of exposure to a contaminant during embryonic development being manifested during adulthood, 13 months post parturition. Maternal exposure to contaminants has been shown to affect reproductive development in wildlife (reviewd in: Colborn et al. 1993a; Hamlin and Guillette 2011). Laboratory studies have shown that prenatal exposure to various contaminants affects reproductive behaviors at adulthood (e.g. Dalsenter et al. 1997, Veeramachaneni et al. 2001, Halldin et al. 1999, Palanza et al. 1999). Atrazine is lipophilic (Matsui et al. 1995) and hence, can be stored in the yolk. Since the mothers of the exposed fish were living in atrazine-contaminated water throughout gestation, the embryos developing within them were also constantly exposed to atrazine. Disruption during key developmental stages has been shown to irreversibly interfere with hormone production (Guillette et al. 1994; Bigsby et al. 1999). Atrazine may also be stored in the developing embryos' fat tissues, and be released gradually during its lifetime.

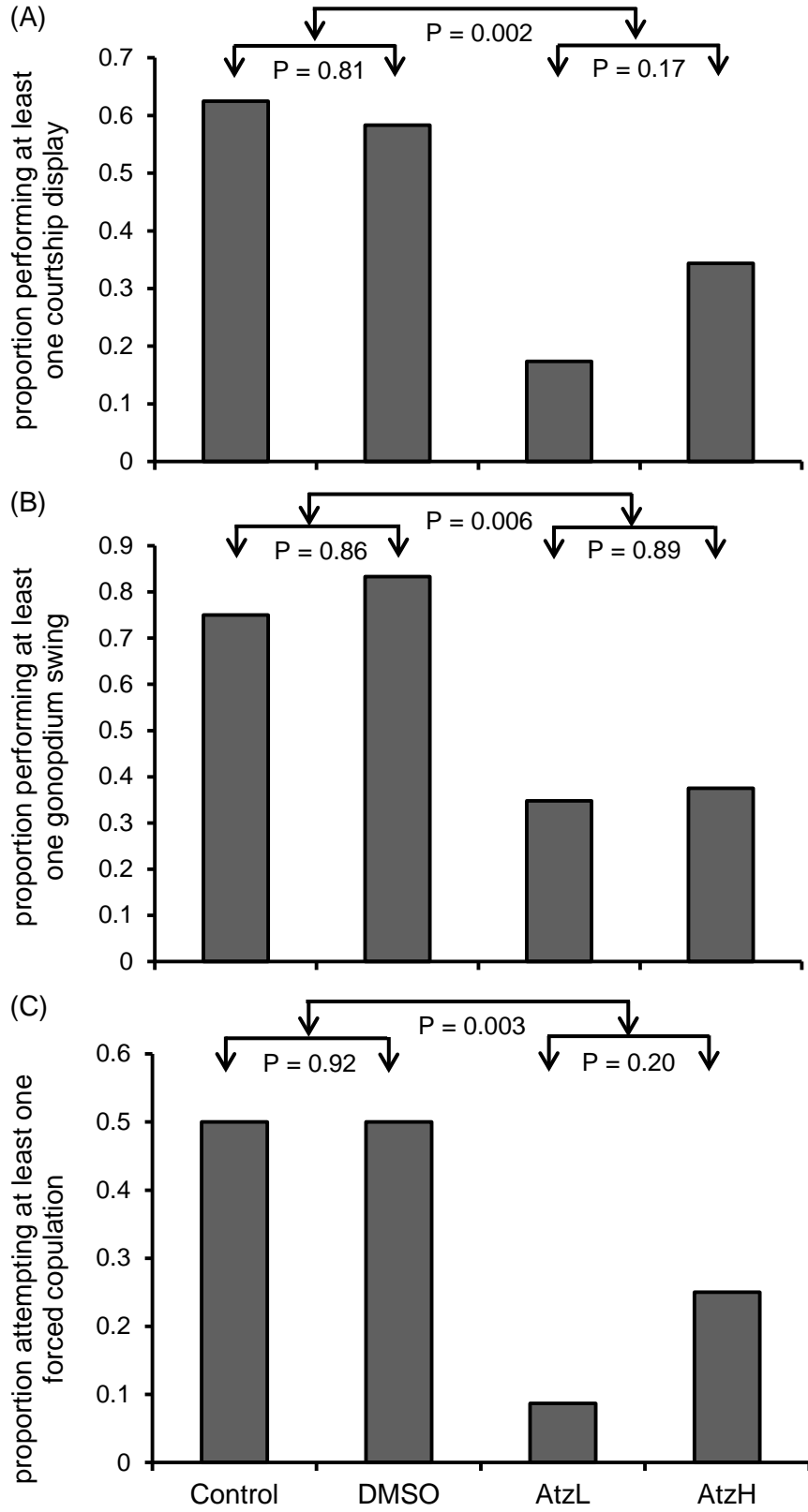
Contaminants may appear in environments in pulses during application periods or through run-off during heavy rains (Heckmann and Friberg 2005; Dueri et al. 2009). Many animals living in these environments may be exposed to the contaminants only during short periods, and this may coincide with developmentally crucial periods. As seen in this study and others (Guillette et al. 1994; Markey et al. 2005; Gore 2008; reviewed in Damgaard et al. 2002; Hamlin and Guillette 2011), no further exposure is required for clear reproductive abnormalities to be apparent at adulthood. These results have significant implications to wildlife reproduction as well as human health.

**Table 5.1.** Means and slopes for the focal males' behaviors regressed against the paired control group males' behaviors.

|   | Mean <sup>1</sup> ± SE | P <sup>2</sup> | Slope ± SE       | P <sup>2</sup> |
|---|------------------------|----------------|------------------|----------------|
| (A) Proximity to female (square root transformed) |                        |                |                  |                |
| Atrazine high dose                                | 0.351 ± 0.032          | 0.505          | 0.6776 ± 0.2525  | 0.974          |
| Atrazine low dose                                 | 0.3562 ± 0.0426        | 0.359          | 0.3719 ± 0.1828  | 0.089          |
| DMSO  | 0.3182 ± 0.0609        |                | 0.6854 ± 0.0959  |                |
| (B) Forced copulation attempts                    |                        |                |                  |                |
| Atrazine high dose                                | -1.0855 ± 0.8288       | 0.305          | -0.0778 ± 0.0372 | 0.001          |
| Atrazine low dose                                 | -1.3664 ± 0.8668       | 0.424          | 0.0576 ± 0.0765  | 0.076          |
| DMSO  | -2.4697 ± 1.2612       |                | 0.2424 ± 0.0831  |                |
| (C) Aggressive displays to rival                  |                        |                |                  |                |
| Atrazine high dose                                | 0.2892 ± 0.3836        | 0.034          | -0.0731 ± 0.0659 | 0.172          |
| Atrazine low dose                                 | -0.3456 ± 0.8864       | 0.065          | -0.3114 ± 0.2019 | 0.085          |
| DMSO  | 1.4343 ± 0.3813        |                | 0.0545 ± 0.0662  |                |
| (D) Attacks on rival                              |                        |                |                  |                |
| Atrazine high dose                                | -1.9597 ± 0.8334       | 0.002          | 0.0179 ± 0.0495  | 0.510          |
| Atrazine low dose                                 | -1.574 ± 0.6155        | 0.010          | 0.0637 ± 0.0737  | 0.928          |
| DMSO  | 0.6708 ± 0.7141        |                | 0.0725 ± 0.0663  |                |

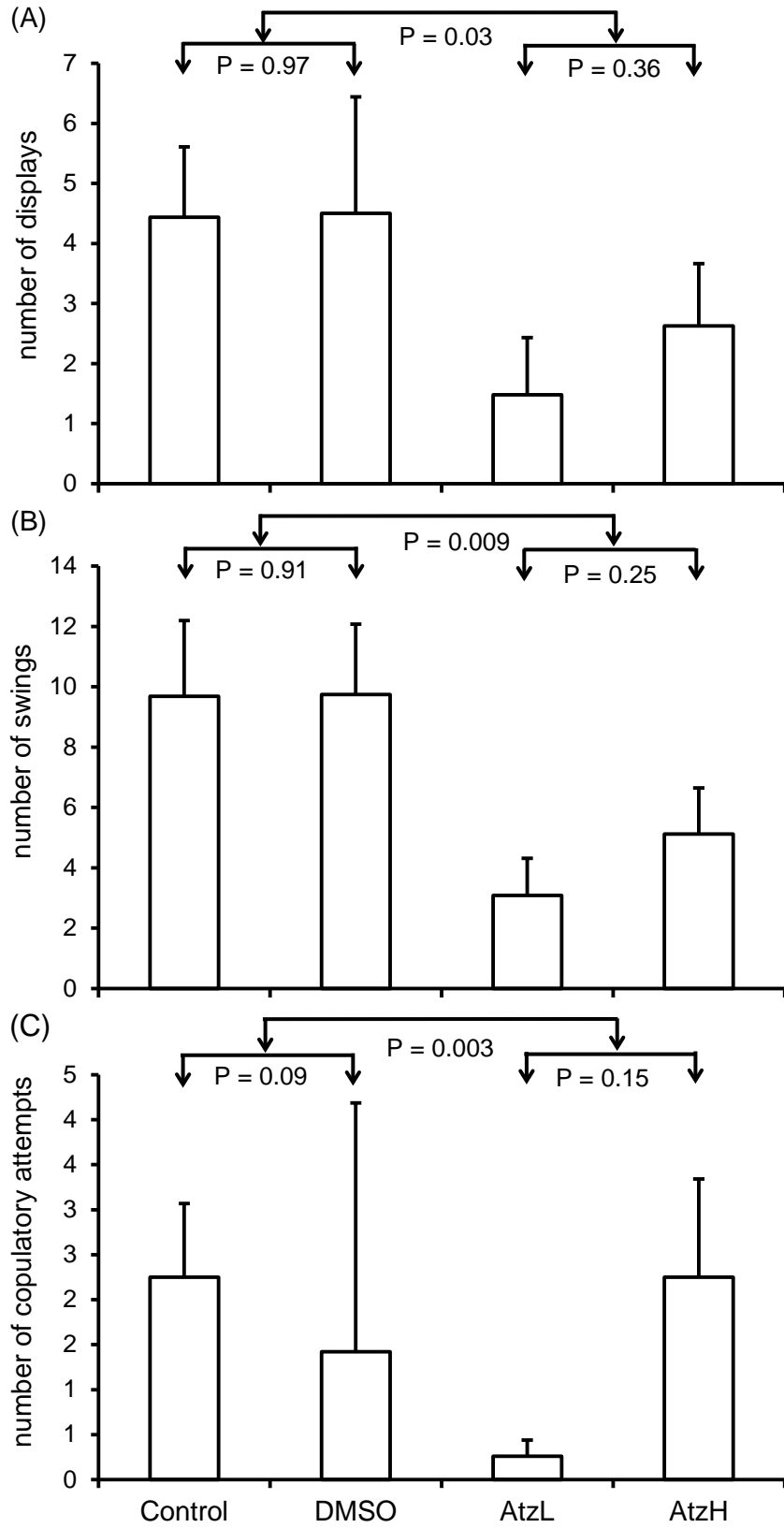
<sup>1</sup>Means are least-squares estimates of the mean

<sup>2</sup>P-values for the difference between the treated males and DMSO males

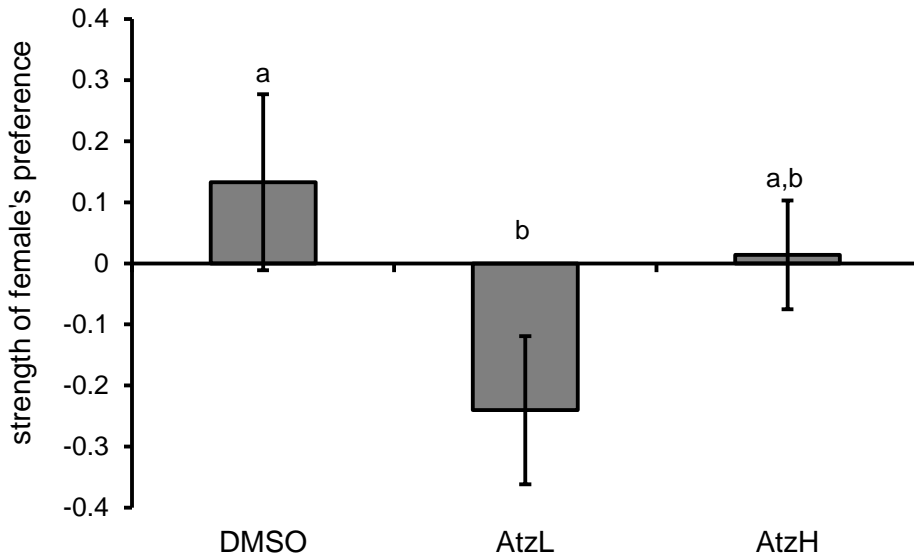




**Figure 5.1.** Likelihood of performing mating behaviors, depicted by the proportion of fish that performed at least one event of the mating behavior. Mating behaviors include (A) courtship displays, (B) gonopodium swings, (C) forced copulatory attempts. Arrows between bars indicate *a-priori* planned orthogonal contrasts.



**Figure 5.2.** The effect of treatments on the number of mating behaviors performed. Mating behaviors include (A) courtship displays, (B) gonopodium swings, (C) forced copulatory attempts. Arrows between bars indicate *a-priori* planned orthogonal contrasts.



**Figure 5.3.** Strength of the female's preference (SFP) for treated male compared to the paired control male. SFP was the ratio of the difference in time associated with the treated male and the time associated with the paired control male, to the total time associated with either male. Groups with similar letters were not significantly different from each other ( $P > 0.05$ ), as determined by Tukey-Kramer *post-hoc* tests.

## CHAPTER SIX. ENDOCRINE DISRUPTION AND THE CAROTENOID MODULATION OF THE IMMUNOCOMPETENCE HANDICAP HYPOTHESIS: AN EVOLUTIONARY MODEL

### SUMMARY

Carotenoids are pigments that produce yellow, orange, and red colors. Animals obtain carotenoids from their diet to make colorful ornaments important for mate attraction. Males with elevated circulating testosterone levels to enhance ornament expression produce high levels of free radicals that damage cells of the immune system and cause oxidative stress. Carotenoids can be used to trap free radicals and thus enhance immunocompetence. Carotenoids are often a limited resource: allocation to ornament versus immunocompetence poses a trade-off. Endocrine disrupting compounds (EDCs) are ubiquitous in the environment and interfere with proper hormonal functioning in exposed animals. EDC-exposure can affect carotenoid-based ornaments and immunocompetence by disrupting the hormonal pathways involved in their expression. A mathematical model was used to simulate the carotenoid allocation strategy--which is considered to be a non-plastic and heritable trait--that maximizes lifetime reproductive success by maximizing both mating success (via ornament expression) and survival (via immunocompetence). This model was used to ask (1) is there a loss of signal reliability in the presence of EDCs, (2) will there be an evolutionary shift in the allocation strategy under multi-generational EDC-exposure, (3) will signal reliability be restored under the shifted allocation strategy, and (4) how is signal reliability impacted in the presence of additional selective pressures like predation, and its interaction with EDC-exposure? The model produced lower signal reliability in the presence of EDCs. Allocation to ornament was somewhat increased when populations adapted to EDCs under multi-generational exposure, but ornament expression, immunocompetence and signal reliability were not restored. Predation pressure further suppressed signal reliability and caused an increased allocation to immunocompetence.

### INTRODUCTION

Mating signals are conspicuous morphological and behavioral traits used by animals to attract members of the opposite sex. In many animal species, males produce signals and females assess the signals to choose mates; in some species, females also produce mating signals – either females alone, or both sexes together (Amundsen 2000). Here, I focus only on male mating signals, specifically ornaments, which are color patterns or morphological traits that have arisen through sexual selection. Pigments used for coloration may be synthesized *de novo* or obtained through diet. Carotenoids are pigments that produce yellow, orange or red coloration and cannot be synthesized *de novo* by animals (Goodwin 1984), yet these pigments have very important roles in sexual signaling. Males of many species display carotenoid based ornaments using dietary carotenoids (e.g. guppies: Endler 1980; Houde 1997; red jungle fowl: Zuk et al. 1990; three-spined sticklebacks: Milinski and Bakker 1990; northern cardinals: Wolfenbarger 1999, zebra finches: McGraw and Ardia 2003), which may often be scarce in the environment (Grether et al. 1999). Hence, the size and color intensity of the ornament is often an indicator of the bearer's foraging ability (Endler 1980; Karino et al. 2005).

Mating signals are reliable indicators of quality if the strength of the signal correlates with the measure of quality (Maynard Smith and Harper 2003). I use the term "quality" to indicate a trait that is beneficial to the mate, directly or indirectly. To reduce ambiguity, I use the term "mate quality" to indicate an individual's quality as a mate. Mate quality can differ according to the species and the mating system peculiar to that species and could refer to direct benefits that

the male confers upon the female, such as nuptial gifts or access to high quality territory, or indirect benefits such as better parental care, more viable or more attractive offspring (Jennions and Petrie 2000). Foraging ability has been used to define quality in some species (Endler 1980; Karino et al. 2005); this trait has obvious implications for fitness, is believed to be heritable, and is hence desirable in a mate. Signals such as carotenoid-based ornaments are obvious indicators of this measure of mate quality, as the ornament is partly dependent on the amount of carotenoids obtained through diet.

Immunocompetence is another commonly used measure of mate quality (Hamilton and Zuk 1982). Males with higher immunocompetence are less likely to have pathogens to transfer to the female mate and will pass on their immunocompetence to offspring. In many species, the expression of the ornament is closely associated with immune response to a challenge (Hamilton and Zuk 1982; Lozano 1994; Blount et al. 2003). The Immunocompetence Handicap Hypothesis (ICHH: Folstad and Karter 1992) states that increased testosterone levels required for breeding and maintaining ornaments create high levels of free radicals that are immunosuppressors. Individuals with especially effective immune systems are able to bear the burden of the enhanced testosterone levels and can thus employ more exaggerated mating signals (Figure 6.1). Carotenoids can neutralize the free radicals and thus alleviate the oxidative stress on the immune system (Lozano 1994; Alonso-Alvarez et al. 2008), but carotenoids must be allocated to the ornament to attract mates. This poses a trade-off in the optimal allocation of carotenoids between mating signal and immunocompetence (Figure 6.1). Again, individuals that can mount better immune responses will be able to allocate more carotenoids towards the mating signal rather than the immune system. Thus, individuals that mount higher immune responses generally have brighter carotenoid signals, while those that are unable to mount strong responses to immune challenges will display dull ornaments, thereby maintaining the honesty of the signal.

Predation is an important selection pressure that also influences ornament expression ((Reznick and Endler 1982; Godin and McDonough 2003). Populations that experience high predation pressures tend to have more modest ornaments compared to populations living under relatively low predation risk (Endler 1980; Endler 1993). However, within a population, the expression of an honest signal will correlate with mate quality, and females generally tend to show a preference for males with better signals (Jennions and Petrie 1997).

The role of androgens in the expression of mating signals has been studied extensively. Carotenoid-based ornaments are no exception, and there is evidence indicating that the expression of the ornament is directly related to testosterone levels (e.g. red jungle fowl, combs: Zuk et al. 1995; superb fairy-wren, plumage: Peters et al. 2001; house sparrow badges: Gonzalez et al. 2001; Strasser and Schwabl 2004; guppy coloration: Jayasooriya et al. 2002; zebra finch beaks: McGraw et al. 2006; cichlid nuptial coloration: Dijkstra et al. 2007). Increased testosterone has been shown to increase circulating carotenoid levels (Blas et al. 2006), as well as lipoproteins required to transport the carotenoids (McGraw et al. 2006). Any disruption in the production or functioning of androgens has been shown to hamper the expression of carotenoid based ornaments.

Endocrine disrupting compounds (EDCs) are a group of compounds, natural or synthetic, that interfere with proper hormonal functioning in exposed animals. Common types of EDCs include organochlorines, organophosphates, polychlorinated biphenyls (PCBs), phthalates, synthetic hormones and hormone-blockers, and phytoestrogens, and their sources include pesticides, industrial effluents, pulp mill effluents, plastics and sewage. Significant routes of exposure include direct exposures from living in contaminated soil or water, or indirect exposures through eating contaminated prey (Markman et al. 2007; Markman et al. 2008; Park et al. 2009; Walters et al. 2010). Many EDCs can be persistent in the environment, lasting for several years in low concentrations (USEPA 2009). Many animals are exposed to contaminants throughout their lives, while others are exposed during some life stages only, with significant

effects on reproductive characteristics (e.g. Porter and Wiemeyer 1969; Baatrup and Junge 2001; Toft and Baatrup 2001; Bayley et al. 2002; Kristensen et al. 2005; Kristensen et al. 2007; Saaristo et al. 2009; Secondi et al. 2009; Hayes et al. 2010; Shenoy 2012).

Most of the common EDCs are either estrogenic or anti-androgenic or both. Estrogenic EDCs either mimic estrogens or upregulate the production of estrogens, such that exposed animals have higher levels of circulating estradiol. Estrogenic EDCs can also suppress testosterone production (Blake and Boockfor 1997; Takao et al. 1999; Mills et al. 2001; Saiyed et al. 2003; Kim et al. 2007; Hayes et al. 2010; Nakamura et al. 2010; Victor-Costa et al. 2010) by influencing the expression of steroidogenic enzymes (Bartke et al. 1977; Thompson et al. 2004a; Kim et al. 2007; Murugesan et al. 2008) and altering cholesterol metabolism and transport (Thompson et al. 2004a; Kim et al. 2007) in Leydig cells. Anti-androgens prevent the binding of androgen with the androgen receptor (AR), thereby inhibiting transcription of AR-dependent genes (Quigley et al. 1995; Zhou et al. 1995), or prevent AR binding to DNA or the initiation of transcription (Truss et al. 1994; Kelce and Wilson 1997).

In addition to their effects on ornaments, EDCs can reduce immunocompetence, directly or indirectly (Ndebele et al. 2003; Hayes et al. 2006a; Brodtkin et al. 2007; Filby et al. 2007; reviewed in Ahmed 2000; Inadera 2006; Milla et al. 2011). However, it is unlikely that both immunocompetence and ornament expression will be impacted proportionately such that the correlation between them holds even after disruption. The disproportionate disruption of the two traits can prevent the ornament from indicating immunocompetence. It is unclear whether EDCs can impact foraging rates, but after a reduction ornament expression may no longer correlate with foraging ability. Thus, EDC-exposure has the potential to disrupt the relationship between ornaments and measures of mate quality, with implications for signal reliability and female mate choice (Shenoy and Crowley 2011).

Here, I use a mathematical model to simulate the optimal allocation of carotenoids to a carotenoid-based ornament and to immunocompetence, and I ask the following questions: (1) does EDC-exposure reduce the reliability of a signal, determined by the correlation coefficient between signal size and immune response, (2) does the optimal allocation shift (evolutionarily or physiologically) under multi-generational EDC-exposure, and (3) is signal reliability restored under the new shifted allocation strategy. Since predation is an important selection pressure in many animal species, I then ask: (4) does the interaction of predation pressure and EDC-exposure impact signal reliability, and (5) is there a shift in the allocation strategy that can restore signal reliability under long-term predation pressure and EDC-exposure?

I used the guppy (*Poecilia reticulata*) as the study organism to conceptualize and parameterize the model. With this model, I make predictions that will encourage empirical studies to test whether long-term exposure to EDCs can alter evolutionary trajectories by altering signal reliability, especially for carotenoid-based ornaments, and whether allocation strategies are non-plastic or plastic, or can evolve.

## METHODS

**The Guppy System:** The model was based on the guppy, a sexually dimorphic freshwater fish species native to Trinidad and parts of South America. Male guppies have different colored spots on their bodies and fins, which are used in conjunction with sigmoidal courtship displays to attract females (Houde 1997; Kodric-Brown and Nicoletto 2001). The expression of guppy mating signals, including sigmoid courtship displays and orange spots, have been associated with different traits such as immunocompetence (Houde and Torio 1992; Lopez 1998), foraging ability (Karino et al. 2005), and sperm characteristics (Evans and Magurran 2001; Pitcher et al. 2007). Of chief importance are the orange colored spots which correlate with, and hence carry information about, dietary carotenoid intake (Grether et al. 1999; Karino et al. 2005) and

parasite resistance (Houde and Torio 1992). Both males and females of this species mate promiscuously, and males do not provide parental care or any form of direct benefits to females.

Androgen levels have been linked to the expression of the mating signals, that is the size and intensity of orange spots (Baatrup and Junge 2001; Bayley et al. 2002; Jayasooriya et al. 2002), and the frequency of courtship displays (Baatrup and Junge 2001; Bayley et al. 2003). Guppies are livebearers, and females can store sperm for several months. They are receptive to mating for a period of 3-5 days after producing a brood (Houde 1997). When receptive, females will respond to male courtship (Houde 1997) and show preference for males with larger and brighter orange spots, and who display more frequently (Houde 1997; Kodric-Brown and Nicoletto 2001). Males frequently attempt forced copulations, but are rarely successful (Evans et al. 2003). Sperm competition is high, and brighter males' sperm tends to have precedence over that of dull males via cryptic female choice (Pitcher et al. 2003; Pilastro et al. 2004; Locatello et al. 2006). I used guppies as the model organism because their mating system has been well studied and characterized (Houde 1997), and the link between carotenoids, immune responses, androgens and signals have been explored (Houde and Torio 1992; Baatrup and Junge 2001; Toft and Baatrup 2001; Jayasooriya et al. 2002; Grether et al. 2004; Kristensen et al. 2005; Kolluru et al. 2006).

**Model Overview:** I used MATLAB® to develop and run the model. I used an optimality model to find the optimal allocation of carotenoids to ornaments. The model was built around the concept of a trade-off in allocation of carotenoid between the ornament and immunocompetence, assuming that all carotenoids consumed through diet must be allocated to one component or the other and cannot be stored (see Table 6.1 for a comprehensive list of assumptions made in the model). Allocation to the ornament will enhance mating success, while allocation to immunocompetence will enhance survival. The optimal allocation strategy maximized fitness, defined as lifetime reproductive success, reflecting survival and mating success. I assumed that the allocation strategy is genetically determined and is an evolutionarily stable strategy (ESS), because the success of any given strategy depends on the frequency of other strategies in the population. The ESS allocation varies in a population depending upon an individual's quality, because low quality individuals may need to allocate more carotenoids to immunocompetence compared to better quality individuals.

The term quality may refer to different traits in different contexts. Here, two types of quality were defined: foraging ability and immunocompetence quality, which were assumed to be heritable and non-plastic traits. Foraging ability (expressed as the foraging exponent) was defined as the extent of an individual's approach to the maximum foraging intake per unit increase in resource availability. To define immunocompetence quality, we must first define condition index and immune response. Condition index was defined as the proportion of fatty stores for an individual, measured as the mass of lipids stored per body mass. Immune response was defined as the resistance to an immune challenge. Because the model is based on the guppy system, the immune challenge was assumed to be infection by *Gyrodactylus turnbulli*, a ubiquitous ectoparasite that infects guppies in their natural habitat. Immunocompetence quality (expressed as the immunity exponent) determined how rapidly an individual approached the highest possible immune response per unit increase in condition. All pair-wise combinations of 15 levels each of the two quality types were used.

A suite of physiological relationships ultimately determined fitness; this is outlined diagrammatically in Figure 6.1. Here I briefly describe some key causal relationships. An individual's condition index determined his immune response (Houston et al. 2007; Martin II et al. 2008) and testosterone concentrations (Marler and Ryan 1996; Volek et al. 1997; Lovern and Adams 2008). Testosterone impacted condition index (Ketterson et al. 1991; Oppliger et al. 2004) and immune response (Folstad and Karter 1992; Mougeot et al. 2004; Dijkstra et al. 2007; Kurtz et al. 2007) negatively, but enhanced circulating carotenoids (McGraw et al. 2006)



which were obtained from resources consumed. Mounting an immune response was energetically costly and diminished the condition index (Eraud et al. 2005; Amat et al. 2007). Carotenoids could be allocated partly to enhancing the immunocompetence (Lozano 1994; Alonso-Alvarez et al. 2004) or to enhancing the ornament (McGraw et al. 2006; Mougeot et al. 2007; Peters et al. 2007). Being able to mount a sufficient immune response determined whether the individual survived to the next day or not, and if condition index dropped to zero or below the individual was considered to have died of starvation. Testosterone was involved in ornament size via two pathways: (1) indirectly, through the bioavailability of plasma carotenoids, and (2) directly, by limiting the binding of androgen receptors necessary for signal production (Figure 6.1). The size of the ornament determined mating success (Houde 1997) and cryptic female preference (Pitcher et al. 2003; Pilastro et al. 2004). A preferred male fertilized a larger proportion of the female's brood compared to other individuals siring a brood. Using the ornament expression and immune response values corresponding to the optimal allocation strategies, I determined signal reliability as the correlation between the two traits.

I then introduced an estrogenic EDC into the environment. I assumed the EDC to be Ethynylestradiol (EE), as this is one of the most common contaminants of water bodies, especially around urban areas. EE disrupts hormonal pathways by mimicking estrogen and binding to estrogen receptors, thereby increasing estrogenicity in exposed individuals. In the model, EE reduced testosterone levels and suppressed immune functioning, thus impacting ornament expression, mating success and survival. I tested whether the exposure to the EDC reduced signal reliability. As I was interested in how populations might shift their allocation strategies through evolutionary or physiological (plastic) responses to multi-generational exposure to persistent EDCs, I developed a version of the model that incorporated EDC-exposure into finding the optimal allocation and related variables of fitness. This allowed me to test whether signal reliability was restored under the shifted allocation strategy.

Because predation is an important selection pressure in most environments, predation risk was incorporated into the model by making the probability of dying a function of immune response and ornament size. I used the model to predict how optimal allocation might shift under dual selection pressures (predation and EDC-exposure) and how signal reliability might be affected.

### Details of the model

*Quality:* The definition of foraging ability (i.e. the foraging exponent) was based on the relationship in which resources consumed increased with diminishing returns to an upper asymptote with increasing resource availability (Appendix 6.1). In this relationship, the upper asymptote (the maximum amount of resources an individual can eat) could also be thought of as a measure of foraging ability, but I believe that this parameter depends on body size, which is assumed to be constant for all individuals in a population in this model. For simplicity, the resource landscape was assumed to be homogenous, and resources are replenished at the same rate that they are depleted. Individuals range from poor foragers (foraging exponent =  $0.05 \text{ mg}^{-1}$ ) to good foragers (foraging exponent =  $2.0 \text{ mg}^{-1}$ ); the values used for this parameter encompass a range that produced reasonable variation in resources consumed for different amounts of resources available.

Immune response was measured as susceptibility to the ectoparasite *G. turnbulli*. Parasite load was a measure of the individual's susceptibility. In the lab, guppies can harbor >100 parasites (Scott 1985), but these heavily infected individuals do not survive (Scott 1985, KS personal observation). So I set the maximum parasite load to 100, at which an individual was considered to have zero resistance. I used a linear relationship to convert parasite load to resistance:  $I = I_{P0} - P$ , where  $I_{P0}$  was the immune response when susceptibility was zero, and  $P$  was the parasite load or susceptibility (Appendix 6.1), resulting in resistance values in the range 0 - 100. Immune responses are known to depend upon condition (Martin II et al. 2008). Here,

immune response, a dimensionless variable, increased with diminishing returns to an upper asymptote with increasing condition (Appendix 6.1). Immunocompetence quality ( $q_i$ , the immunity exponent, Table 6.2) was the exponential coefficient that determines how rapidly an individual approaches the highest possible immune response per unit increase in condition. Individuals ranged from poor immune responders (immunity exponent = 15) to good immune responders (immunity exponent = 50); the parameter values for immunity exponent spanned a range that produced natural variation in  $I$ .

I incorporated this second influence via the exponential growth to a maximum of ornament size in response to increased carotenoid allocation; individuals with high concentrations of testosterone produced larger signals for a unit increase in carotenoid availability compared to individuals with lower concentrations of testosterone (see Appendix 6.1 for details).

*Finding the optimal allocation strategy:* For each quality combination, a hill climbing technique was used to find the evolutionarily stable strategy (ESS) for  $x$ , the proportion of carotenoids allocated to the signal, such that  $1-x$  was the ESS proportion of carotenoids allocated to immune function. The hill climbing technique found the optimal value of  $x$  ( $x^*$ ) in the following way: starting at any value of  $x$ , ranging from 0 to 1, the focal male's fitness was calculated (as described below). The population and the focal male were assumed to be wildtype individuals who allocated the proportion  $x_w$  of carotenoids to the ornament. A mutant male whose allocation proportion ( $x_m$ ) was very slightly different from that of the focal by an amount  $dx$  was introduced into the population. The mutant could take over the population and become the new wildtype if his fitness exceeded that of the original wildtype male. The hill climbing technique continued in this way to find the ESS allocation strategy (i.e. ESS value of  $x$ ) that is non-invasible by a mutant allocating any other proportion of carotenoids to his signal. This optimal  $x$  value (henceforth  $x^*$ ) was then used to calculate the optimal ornament size and the fitness achieved at this  $x$  value. Allocation strategy was genetically inherited and non-plastic. Hence individuals that allocated suboptimally incurred fitness losses.

*Baseline runs of the model:* For each combination of foraging ability and immunocompetence quality, I ran 100 simulations. Because of stochasticity introduced by the way I determined survival (i.e. if the probability of dying at the end of each day was lower than a random number, the focal lived to the next day), I obtained slightly different values for each variable in consecutive runs; hence, I used the mean values of  $x^*$ , fitness, immune response and ornament size, averaged over 100 runs. The model generated values of  $x^*$ , immune response, ornament size, and fitness at each combination of foraging ability and immunocompetent quality value. The correlation coefficient between immune response and ornament size was a measure of signal reliability, and this was also calculated after the introduction of the EDC. The mean difference in ornament size, immune response and fitness after the introduction of the EDC was computed.

*EDC:* Using the already computed  $x^*$ , the model incorporated the altered testosterone and immunocompetence value to obtain the altered ornament size and fitness values.

I again used the hill climbing technique to find a new  $x^*$ , and corresponding ornament size, immune response and fitness. The model predicted whether signal reliability could be restored under the shifted allocation strategy.

*Predation:* Predation risk was incorporated into the model by making the probability of dying a function of immune response and ornament size. To simulate different predation regimes, I used two values of  $D_{OU}$ , the probability of dying due to predation alone for an individual expressing the largest possible ornament:  $D_{OU} = 0.1$  and  $D_{OU} = 0.2$ . The  $x^*$ , corresponding fitness and ornament size in the absence and presence of EDCs, and the shift in  $x^*$  and corresponding variables as a result of physiological or evolved responses to EDC exposure, are calculated for each value of  $D_{OU}$ .

**Calculating fitness:** Fitness ( $W$ ) was measured as lifetime reproductive success, which was the accumulation of daily reproductive success ( $F$ ) over an individual's life time. The probability of dying ( $D$ ) determined whether an individual continued to live to the next day, up to a maximum lifetime of 365 days.  $D$  depended upon immunocompetence ( $I$ ) and ornament size ( $O$ ) and was calculated each day as:

$$D = 1 - [(1-D_i)(1-D_o)] \quad (1)$$

where  $D_i$  was the probability of dying as a function of immune response alone, and  $D_o$  was the probability of dying as a function of ornament size alone.  $D_i$  and  $D_o$  were mutually independent.  $D$  was set to 1 if the condition index  $C \leq 0$ . The relationships are described in Appendix 6.1, and parameters included in Table 6.2.

Daily reproductive success ( $F$ ) depended upon the proportion of body area covered by the orange spots (ornament size), which determined the success of courtship displays (Appendix 6.1). Individuals with larger ornaments achieve greater mating success ( $d$ ), defined as the proportion of all females courted that responded positively by mating with the focal individual (Table 6.3). The average number of receptive females ( $n_f$ ) in a population on any given day was 5. This was based on the assumption that, on average, 10% of the females in a population were sexually receptive on any given day. The focal male could potentially court all receptive females. Ornament size influenced mating success and fertilization success of the focal male's sperm, as described in equation 4 below. The focal male's sperm competed with the sperm of  $n_m$  other males for fertilization of the brood. The number of competitors was calculated based upon a diminishing returns function increasing to an upper asymptote for the relationship between the number of males siring a brood and the number of males in a population (Appendix 6.1). Under the simplifying assumption that all males' sperm have equal fertilization efficiency (Table 6.1), and that fertilization is proportional to the amount of sperm present, the proportion of a brood that the focal male's sperm could fertilize was a proportion,  $p_i$ , of the total number of males fertilizing a brood. In the following equations, the focal male's phenotype is denoted by the subscript  $i$ , and the wildtype male's phenotype is denoted by the subscript  $j$ . The focal male might be mutant or wildtype, but all males in the population were wildtype individuals.

Daily reproductive success was given by:

$$F_i = d_i n_f p_i b \quad (2)$$

where  $d_i$  was the success of courtship displays for the focal male (described above and in Appendix 6.1),  $n_f$  was the number of receptive females,  $b$  was the average brood size, and  $p_i$  was the proportion of the brood that the focal male sired, in competition for fertilizations with  $n_m$  other males of phenotype  $j$ . The proportion of the brood sired by the focal male was given by:

$$p_i = \frac{w_i}{w_i + n_m w_j} \quad (3)$$

where  $w_i$  was the focal male's sperm precedence and  $w_j$  was the competitors' sperm precedence; the sperm precedence added a preference weight to that individual's sperm. The sperm precedence depended on ornament size ( $O$ ) in the manner described below (equation 4), because female guppies use cryptic mate choice to give precedence to the brighter male's sperm (Pitcher et al. 2003; Pilastro et al. 2004). For the sake of simplicity, I assumed that the order of mating does not affect sperm precedence (but see Pitcher et al. 2003).

$$w_i = s/(s + n_m) \text{ and } w_j = 1/(s + n_m) \quad \text{if } O_i > O_j \text{ and } w_i = s w_j \quad (4)$$

where  $s$  was the value by which sperm precedence of the focal phenotype differed from that of the competing phenotype. The focal phenotype may be favored over the competitor ( $s > 1$ ), less preferred ( $0 < s < 1$ ), or equally preferred ( $s = 1$ ). Note that sperm precedence weights of all males sum to 1, such that  $w_i + n_m w_j = 1$ .

Carotenoids were sequestered to the ornament and the signal size refreshed each day (Table 6.1). Daily reproductive success accrued over an individual's life to determine lifetime reproductive success.

## RESULTS

**Optimal  $x^*$ :** The optimal proportion of carotenoids allocated to the ornament ( $x^*$ ) ranged from 0.24 for the lowest quality males to 0.56 for the highest quality males (Figure 6.2A). Shifting the allocation strategy under long-term EDC-exposure produced a new  $x^*$  of 0.22 for the lowest quality males and 0.58 for the highest quality males, increasing  $x^*$  by an average of 3.4%. Although the values of  $x^*$  changed very slightly, the change in the allocation pattern was more apparent, with increasing  $x^*$  along the foraging-ability gradient being more striking than the increase along the immunocompetence axis. (Figure 6.2B).

**Ornament size:** The ESS ornament size corresponding to  $x^*$  ranged from 0.15 to 0.38 (Figure 6.3A); this reflects the natural range of variation in ornament size for guppies (Houde 1997). EDC-exposure reduced the ornament size to 0.023 for the lowest quality individual to 0.083 for the highest quality individual (Figure 6.3B); this was an overall reduction in ornament size of 80.5%. Empirical experiments using similar levels of EE produced a reduction of 55.7% in the ornament size of adult male guppies (Shenoy 2012, Chapter 2). Shifting the allocation strategy after multi-generational EDC-exposure only increased ornament size slightly (by 3.1%) compared to ornament size in the presence of EDCs; this is mainly because the presence of the EDC suppresses its expression. Ornament size under the shifted allocation was 0.021 for the lowest quality male and 0.086 for the highest quality male (Figure 6.3C), which is very close to the ornament size of males exposed to EDCs under the original allocation strategy. The patterns in ornament size along the two quality axes mirror the patterns in  $x^*$  (Figure 6.3).

**Immune response:** The immune response corresponding to  $x^*$  ranged from 73.03 for the lowest quality individuals to 84.06 for the highest quality individuals (Figure 6.4A). EDC-exposure reduced immune response by a modest 9% (Figure 6.4B). Interestingly, after EDC-exposure, males that had lower immunocompetence quality produced higher responses than males with higher immunocompetence quality. Immune response under the shifted allocation only improved by 2.2% compared to the immune response of EDC-exposed males under the original allocation (Figure 6.4C), and was lower than immune responses in the absence of EDCs.

**Signal reliability:** Signal reliability, measured by the correlation coefficient ( $r$ ) between ornament size and immune response, was 0.63 (Figure 6.5). EDC-exposure reduced the signal reliability to  $r = -0.14$ , such that males that had higher immune responses had slightly smaller ornament sizes than males that mounted lower immune responses (Figure 6.5). This relationship was driven by the higher immune responses of lower quality males compared to higher quality males in the presence of EDC. Under the shifted allocation, signal reliability was increased to  $r = 0.14$  and was not completely restored (Figure 6.5).

**Fitness:** Fitness, measured by lifetime reproductive success, ranged from 1825 to 2817 offspring sired in a lifetime (Figure 6.6A). EDC-exposure reduced fitness by 67% of the original number of offspring (Figure 6.6B). Shifted allocation increased fitness by 1.9% compared to the fitness in the presence of EDC (Figure 6.6C). It must be kept in mind that most fish species, especially those that do not invest in parental care, such as guppies, have high juvenile mortality. The number of offspring generated by the model only represents the number born; the majority of these are unlikely to survive past the juvenile stage, and even the adults are susceptible to mortality through predation.

**Predation pressure:** Including predation risk into the model changed  $x^*$  such that males with lower foraging ability allocated a greater proportion of carotenoids to the ornament than the males with better foraging ability. But immunocompetence quality did not affect  $x^*$ . This was true for both predation regimes. However, ornament size ( $D_{OU} = 0.1$ : lowest quality male  $O = 0.15$ , highest quality male  $O = 0.20$ ;  $D_{OU} = 0.2$ : lowest quality male  $O = 0.14$ , highest quality male  $O = 0.19$ ) and immune response ( $D_{OU} = 0.1$ : lowest quality male  $I = 73.03$ , highest quality male  $I = 96.56$ ;  $D_{OU} = 0.2$ : lowest quality male  $I = 74.6$ , highest quality male  $I = 96.9$ ) corresponded to

both types of quality. Signal reliability decreased with increasing predation pressure ( $D_{OU} = 0.1$ :  $r = 0.22$ ;  $D_{OU} = 0.2$ :  $r = 0.26$ ). The reduced reliability was due to increased allocation to immunocompetence and a corresponding reduction in ornament size.

Exposure to EDC further decreased ornament size ( $D_{OU} = 0.1$ : lowest quality male  $O = 0.023$ , highest quality male  $O = 0.031$ ;  $D_{OU} = 0.2$ : lowest quality male  $O = 0.021$ , highest quality male  $O = 0.028$ ), but had a negligible effect on immune response ( $D_{OU} = 0.1$ : lowest quality male  $I = 72.19$ , highest quality male  $I = 93.18$ ;  $D_{OU} = 0.2$ : lowest quality male  $I = 73.5$ , highest quality male  $I = 93.7$ ). Interestingly, for both predation regimes, predation pressure did not produce the pattern of higher immune responses from lower quality males in the presence of EDC-exposure as was seen in the model with no predation risk. Signal reliability was not much affected, and in fact was slightly improved for the lowest predation regime ( $D_{OU} = 0.1$ :  $r = 0.25$  and  $D_{OU} = 0.2$ :  $r = 0.18$ ).

An evolutionary shift in allocation strategy as a result of multi-generational EDC exposure increased  $x^*$ , making the shifted optimal allocation under predation pressure very similar to the shifted allocation without predation pressure. This was true for both predation regimes. However, this did not translate to as dramatic an increase in ornament size ( $D_{OU} = 0.1$ : lowest quality male  $O = 0.021$ , highest quality male  $O = 0.075$ ;  $D_{OU} = 0.2$ : lowest quality male  $O = 0.02$ , highest quality male  $O = 0.09$ ). The altered allocation of carotenoids to ornament affected immune responses, such that immune response no longer corresponded to quality in any obvious pattern. Signal reliability was not improved under the higher predation regime ( $r = 0.10$ ) but was restored under the lower predation regime ( $r = 0.46$ ).

**Important parameters:** Changing EDC to  $1 \text{ ng ml}^{-1}$  and to  $2.5 \text{ ng ml}^{-1}$  produced patterns consistent with the default model. When I used  $E = 1 \text{ ng ml}^{-1}$ , signal reliability was  $-0.12$  in the presence of EDC, and  $0.21$  under the shifted allocation. When  $E = 2.5 \text{ ng ml}^{-1}$ , signal reliability was  $-0.15$  in the presence of EDC, and  $0.48$  under the shifted allocation. I feel that my model reflects estrogenic EDC-exposure reliably.

Because the default value of  $s$ , the value by which the preferred male's sperm had a greater precedence over the less preferred male's sperm, had been chosen arbitrarily ( $s = 2$ ), I changed the values of  $s$  to 3 and 5 to test if this affected any patterns. All patterns remained consistent with the default model. For  $s = 3$ , signal reliability in the absence of EDC was  $0.71$ , signal reliability in the presence of EDC was  $-0.09$ , and signal reliability under the shifted allocation was  $0.19$ . For  $s = 5$ , signal reliability in the absence of EDC was  $0.75$ , signal reliability in the presence of EDC was  $-0.096$ , and signal reliability under the shifted allocation was  $0.36$ . I feel that using the lowest value,  $s = 2$  as the default value is reasonable in the absence of better data on sperm precedence.

## DISCUSSION

It has been well established empirically with a number of species that exposure to EDCs can reduce the expression of a sexual ornament (Baatrup and Junge 2001; Toft and Baatrup 2001; Hayes et al. 2002b; Arellano-Aguilar and Garcia 2008; Secondi et al. 2009; Hayes et al. 2010; Shenoy 2012). The overall reduction in immune response as a result of EDC-exposure is also empirically supported (Christin et al. 2003; Brodtkin et al. 2007; reviewed in Ahmed 2000), although low concentrations of estradiol can enhance immunocompetence (Kenny et al. 1976; Bilbo and Nelson 2001; Knöferl et al. 2001). This model reflects the patterns seen in laboratory and natural experiments testing the effects of EDCs on sexually selected traits and immune responses. However, what is unknown empirically is whether EDC-exposure has the potential to disrupt signal reliability. It is also unknown how animals allocate resources, particularly carotenoids, to different physiological functions. My model predicts the optimal allocation of carotenoids between two competing functions—sexual ornamentation and immunocompetence. Further, my model predicts how individuals of different quality respond to EDC-exposure. Most

importantly, my model predicts how allocation strategies might shift under long-term exposure to EDCs and the corresponding changes in ornamentation, immune response and lifetime reproductive success. The shift in allocation strategy may be an evolutionary shift if there was selection on the strategy itself, which can produce ecotypes that are especially suited for inhabiting contaminated habitats. Such evolutionary shifts have not been documented, but it is known that genetic divergence due to selection on other traits, such as resistance to pesticides, has occurred in populations exposed to contaminants over multiple generations (e.g. Bard 2000; Hemingway and Ranson 2000; Twigg et al. 2002; Lopes et al. 2008; Brausch and Smith 2009; Arzuaga and Elskus 2010).

**Patterns in immunocompetence:** The model brought out some interesting patterns in the immune response of different quality individuals to EDC-exposure. Low quality individuals, defined by lower immunity exponent, mounted a higher immune response in the presence of EDCs than did the higher quality individuals (although this response was lower than the response mounted in the absence of EDCs). This was mainly because of assumption about the effect of EDCs on immune response: due to a lack of empirical evidence to the contrary, I assumed a linear and proportional decrease in immune response in the presence of EDCs. Hence, the higher-quality individuals mounting higher responses had a larger reduction in immune response than the low quality individuals. Whether this pattern might occur in nature must be tested, and such information is important to the understanding of how immunocompetence responds to EDC-exposure.

**Signal reliability:** Reduced reliability of a signal has profound implications for the evolution of mating systems. When a mating signal ceases to correlate with a measure of mate quality, the signal no longer transmits information about the bearer. Male mating signals and female responses to the male signals have coevolved (Arnqvist and Rowe. 2005), such that females can adjust their response to varying levels of signal expression. Female responses to male signals can be a strong driving force for genetic divergence (Basolo 1998a), (Seehausen et al. 1997; Rodd et al. 2002; Rosenthal et al. 2002). In a population where sufficient numbers of individuals produce mating signals that do not correspond to their true quality, females should rapidly lose the preference across generations (Shenoy and Crowley 2011). The loss of female preference can lead to the loss of the ornament from the population. However, in many animal species, female preferences are based on pre-existing sensory biases (Rodd et al. 2002; Maynard Smith and Harper 2003), and hence are unlikely to be lost. In such cases females may make "incorrect" mating choices and incur fitness losses (Ryder and Somppi 1979; Fox 1992; Kokko and Brooks 2003; Quader 2005). The reduction in signal reliability as a result of EDC-exposure predicted by the model can be tested empirically. However, it would be more difficult to test whether long-term exposure to EDCs at the population level can lead to loss of the signal or of female preference for the signal in the populations. Field comparisons of animals living in habitats that have been contaminated for many years with conspecifics from pristine regions would allow us to understand whether signal reliability can be reduced in polluted habitats. But this would not inform us whether evolutionary shifts in allocation strategy, that can potentially improve signal reliability, have occurred. Long-term mesocosm experiments using species with relatively short reproductive cycles might help provide insights into these mechanisms.

**Shift in allocation strategy:** It is possible that the shift in allocation strategy could be a physiological response. Plasticity of the allocation strategy is possible in populations that have experienced stochastic exposure to EDCs over multiple generations. In such populations, individuals that are able to shift their allocation in response to pollution will be at an advantage. I consider a strategy to be non-plastic or plastic for a given immune challenge. Greater immune challenges would have different allocation strategies to compensate for greater oxidative stress. Alonso-Alvarez and colleagues (2008) found that testosterone treatment of male red-legged partridges increased oxidative stress and this was presumably compensated for by increased allocation of carotenoids to anti-oxidative functions, such that signal coloration was

compromised. This suggests that perhaps animals can shift the allocation strategy flexibly. However, it is not clear whether low-quality and high-quality males compensated equally or not. McGraw and Toomer (2010) have quantified the concentrations of various carotenoids in the zebra finch ornaments and internal tissues. Similar approaches can be employed to understanding whether animals exposed to EDCs can shift the optimal allocation of carotenoids, and to understand the ways in which this trade-off is compromised under disturbance.

The shift in optimal allocation of carotenoids to the ornament under multigenerational exposure to EDC increased the importance of foraging ability as a measure of quality; that is, allocation increased substantially along the foraging gradient, while the increase was much more gradual along the immunocompetence gradient. This is possibly because of the constraint on immunocompetence in the presence of EDCs causing fitness to be more dependent on the ability to acquire carotenoids. In such a situation, resource availability will become an important factor in ornament expression and immunocompetence, more than in the absence of EDCs.

**Predation:** As expected, predation risk reduced the optimal allocation of carotenoids to the ornament, because males with larger ornaments incurred costs due to predation. Hence, these individuals were able to allocate more carotenoids to their immune system and mounted better immune responses. van Oosterhout and others (2003) found that male guppies from the low predation site, Upper Aripo drainage, were more susceptible to parasite infections than those from the high predation site, Lower Aripo drainage. A similar pattern was found for innate immunity, though not acquired immunity, in a follow up study by the same group (Cable and van Oosterhout 2007). But Martin and Johnsen (2007) found an opposite trend, with guppies from low predation sites harboring lower parasite loads than those from high predation sites. This was a field study, and various other factors might influence parasite loads in the field. Based on the ICHH, my model predicts that males from high predation sites will allocate more carotenoids towards supporting immune health, because allocating carotenoids to the ornament is counter-productive in a predatory environment. In the context of benefits of allocating carotenoids towards immune health, I note that very high carotenoid levels can actually be detrimental to health (Kolluru et al. 2006). For the sake of simplicity, the model has not included this effect, and I have used carotenoid levels within the natural range available in diet to assess effects on ornaments and immune responses.

Under predation pressure, males that were good foragers allocated less carotenoids towards the ornament than males who were poorer foragers. This is because males who are obtaining more carotenoids in their diet must nevertheless de-emphasize ornamentation, because enhanced ornaments would attract predators. On the other hand, poor foragers are obtaining fewer carotenoids and will not be colorful enough to attract predators. Hence, their allocation to ornaments will not be suppressed. Since there is already mortality stress due to poor immunocompetence, and a very low level of predation risk compounding it, these poor foragers have a shorter life span and must allocate as many carotenoids as possible towards ornamentation to attract mates.

With these patterns in ornamentation and immune response under predation pressure, the reduction in signal reliability produced by the model seems rather intuitive. However, since there is no empirical evidence to support this, I recommend such studies to add to our understanding of the ICHH and its consequences. A corollary to this might be the effects of this reduced reliability on female mate choice: are females less choosy in high-predation sites in response to the lower reliability of signals? In general, female guppies from high-predation sites have a lower preference for brighter males than their counterparts from low predation sites (Stoner and Breden 1988). However, it is difficult to distinguish the effect of predation on female preference (e.g. an inclination to refrain from associating with highly ornamented males that may attract predators: Breden and Stoner 1987; Godin and Briggs 1996; Rosenthal et al. 2002) from the predation-risk effect of reduced signal reliability on female preferences.

An evolutionary shift in the allocation strategy under multi-generational exposure to EDC reduced signal reliability under the higher predation regime but was restored under the lower predation regime. In the higher predation regime, the pattern is mainly driven by the somewhat similar immune responses of all quality males, though they roughly followed the pattern of  $x^*$ . In the lower predation regime, the immune response pattern was more similar to that of  $x^*$  and ornament size distribution. The increased allocation to the ornament reduced the immune response under the new allocation strategy, tightening the correlation between ornament expression and immune response. The increased allocation is possibly driven by an attempt to improve reproductive success in a much shortened lifespan; this results from the increased mortality from predation and reduced immunocompetence in the presence of EDCs. The increased allocation, however, did not increase ornament size beyond the original ornament size in the absence of EDCs; this is because of the effect of the EDC on testosterone levels.

Overall, the results of the model indicate that whenever there is a constraint on ornament size, there is an increased allocation to immunocompetence in a compensatory manner, thus reducing signal reliability. But reliability is somewhat restored under multi-generational EDC-exposure because of a reduced spread in the range of immune responses. Will mating signals that have been disrupted by EDC-exposure become reliable to some extent in populations adapting to persistent EDC exposure? This is not known, and will be an important question to address in the field of evolutionary ecotoxicology.

The ICHH has been debated for many years with many authors supporting it as well as refuting it (reviewed in Cotton et al. 2004; Roberts et al. 2004). Some field studies have failed to find a relationship, or found only a weak association, between ornament expression and some components of immunocompetence (Westneat et al. 2003; Martin and Johnsen 2007). It must be remembered that multiple selection pressures are often acting in tandem in natural populations, and it can be difficult to identify a particular suite of relationships dependent on a single source of selection pressure. Predation is a classic selection pressure that has been explored by ecologists in relation to the evolution of many traits. EDCs have been introduced into our environment very recently in evolutionary history, yet the impacts of EDCs are profound and far reaching (Colborn et al. 1993b; Fox 2001; Hayes 2004; Hamlin and Guillette 2011). Most importantly, we have a poor understanding of the actual biochemical trade-offs involved, and researchers are still trying to resolve this (Hill and Johnson 2012). Also yet to be considered, are other pigments occurring along with carotenoids in the ornaments, such as pterins, pteridines and melanin that can be synthesized *de novo* and are also antioxidants (Grether et al. 2001; McGraw 2005).

To my knowledge this is the first attempt to examine the carotenoid modulation of the immunocompetence handicap hypothesis in the context of EDCs using a mechanistic model. By no means is this model a complete representation of the biochemical pathways and selection pressures involved in the complex tug-of-war between sexually selected traits and naturally selected traits; it is an initial attempt to address some basic questions about these complex relationships. But I hope that the predictions from this model will encourage a host of experiments, natural and controlled, to provide answers to many perplexing issues in the field of evolutionary ecotoxicology.



## APPENDIX 6.1. RELATIONSHIPS BETWEEN VARIABLES.

All variables have been defined in Table 6.3, and parameter values provided in Table 6.2. The functions described below are based primarily on reasonable estimates of the shapes of the relationships when depicted graphically.

1. The relationship between resources consumed ( $r$ ) and available resources ( $R$ ): The more resources that are available to the animal, the more it can consume, but with diminishing returns, increasing from zero to an upper asymptote:

$$r = r_{max}(1 - e^{-q_f R})$$

where  $r_{max}$  is the maximum foraging intake of an individual, and  $q_f$  is the foraging exponent.

2. The influence of resources consumed ( $r$ ) on condition index ( $C$ ): Consuming resources will increase the individual's condition in a sigmoid fashion (Kieffer and Tufts 1998; Cook et al. 2000).

$$C = \frac{C_{max}}{1 + \left(\frac{C_{max}}{C_{min}} - 1\right)^{\left(1 - \frac{r}{r_{C/2}}\right)}}$$

where  $C_{max}$  is the maximum condition index,  $C_{min}$  is the minimum condition index, and  $r_{C/2}$  is the amount of resources consumed to produce  $C = C_{max}/2$

3. The relationship between resources consumed ( $r$ ) and plasma carotenoid concentration ( $K$ ): since animals obtain carotenoids through their diet, the more an individual eats, the higher will be the circulating carotenoid concentrations. I expect this relationship to be a diminishing returns curve increasing from zero to an upper asymptote.

$$K = K_{max}(1 - e^{-kr})$$

where  $K_{max}$  is the maximum plasma carotenoid concentration,  $k$  is the carotenoid exponent.

4. The influence of condition ( $C$ ) on testosterone concentrations ( $T$ ): An individual can have high testosterone levels only if his body is in good condition (Marler and Ryan 1996; Volek et al. 1997; Lovorn and Adams 2008). A linear increasing relationship through the origin is assumed.

$$T = \varepsilon C$$

where  $\varepsilon$  is the condition-dependent testosterone increase.

5. The impact of EDC ( $E$ ) on testosterone concentrations ( $T$ ): EE reduces testosterone levels exponentially (Watanabe et al. 2009).

$$T = T_{E0}e^{-\alpha E}$$

where  $T_{E0}$  is the testosterone concentration before EDC-exposure, and  $\alpha$  is the testosterone decay coefficient.

6. Influence of testosterone concentrations ( $T$ ) on condition ( $C$ ): raising testosterone levels is energetically costly, and individuals maintaining high testosterone concentrations suffer from decreased condition (Ketterson et al. 1991; Oppliger et al. 2004). The relationship is assumed to be linear decreasing.

$$C = C_{T0} - \sigma T$$

where  $C_{T0}$  is the condition index before the impact of testosterone, and  $\sigma$  is the testosterone-dependent condition coefficient.

7. The relationship between condition (C) and immune response (I): Animals in poor condition cannot mount a sufficient immune response (Houston et al. 2007; Martin II et al. 2008). The relationship is assumed to increase from zero up to an upper asymptote.

$$I = I_{max}(1 - e^{-q_i C})$$

where  $I_{max}$  is the maximum immune response mounted by an individual in high condition, and  $q_i$  is the immunity exponent.

8. The impact of mounting an immune response (I) on condition (C): Mounting an immune response is energetically costly (Eraud et al. 2005; Amat et al. 2007). I assumed this relationship to be linear decreasing.

$$C = C_{I0} - \theta I$$

where  $C_{I0}$  is the condition index when no immune response is mounted, and  $\theta$  is the immunity-dependent condition decline.

9. The influence of testosterone concentrations (T) on plasma carotenoids (K): Testosterone upregulates lipoproteins associated with carotenoid transfer (McGraw et al. 2006). This relationship is assumed to increase from  $K_{T0}$  towards an upper asymptote at  $K_{T0} + \psi$ .

$$K = K_{T0} + \psi(1 - e^{-jT})$$

where  $K_{T0}$  is the plasma carotenoid concentration before the influence of testosterone,  $\psi = K_{max} - K_{T0}$ , and  $j$  is the testosterone-dependent carotenoid increase.

10. The influence of plasma carotenoids (K) on ornament size (O): There is a direct relationship between increased concentrations of circulating carotenoids and the individuals carotenoid-based ornament expression (McGraw et al. 2006; Mougeot et al. 2007; Peters et al. 2007). I expect this relationship to increase from zero, with diminishing returns, towards an upper asymptote, passing through the origin.

$$O = O_{max}(1 - e^{-\Omega x K})$$

where  $O_{max}$  is the maximum ornament size,  $\Omega$  is the carotenoid-dependent increase in ornament size,  $x$  is the proportion of plasma carotenoids allocated to the ornament.

11. The influence of testosterone concentrations (T) on carotenoid-dependent increase in ornament size ( $\Omega$ ): ornament expression is limited by testosterone concentrations because T must bind with androgen-receptors to make the ornament. To simulate this, I used a linear increasing relationship, passing through the origin, between T and  $\Omega$ , which is involved in the relationship between ornament size and plasma carotenoid concentrations in 10 above.

$$\Omega = ZT$$

where  $Z$  (ornament increaser) is the slope of the relationship between  $\Omega$  and T.

12. The relationship between testosterone concentrations (T) and immune response (I): Testosterone suppresses immunocompetence (Folstad and Karter 1992; Mougeot et al. 2004; Dijkstra et al. 2007; Kurtz et al. 2007). I assume a linear decreasing function here.

$$I = I_{T0} - \rho T$$

where  $I_{T0}$  is the immune response that is unimpacted by testosterone,  $\rho$  is the testosterone-dependent immunity decline.

13. The impact of EDC (E) on immune response (I): estrogens can suppress immune functioning (Herman and Kincaid 1988; Robinson et al. 2007). I assume a linear decreasing relationship.

$$I = I_{E0} - \beta I_{E0}$$

where  $I_{E0}$  is the immune response unimpacted by EDC-exposure, and  $\beta$  is the proportion by which  $I$  is reduced.

$\beta = PE$ , where  $P$  is the slope of the relationship between  $\beta$  and  $P$  such that at  $E = 3 \text{ ng ml}^{-1}$ ,  $P$  is maximum, i.e.  $P = 0.99$ .

14. The relationship between plasma carotenoid concentrations ( $K$ ) and immune response ( $I$ ): Carotenoids are immuno-enhancers and can be allocated towards immunocompetence and antioxidative functions (Lozano 1994; Alonso-Alvarez et al. 2004). I expect a sigmoid increasing relationship here.

$$I = \frac{I_{max}}{1 + \left(\frac{I_{max}}{I_{min}} - 1\right)^{\left(1 - \frac{(1-x)K}{K_{1/2}}\right)}}$$

where  $I_{max}$  is the maximum immune response,  $I_{min}$  is the immune response when no carotenoids are allocated to immunocompetence,  $K_{1/2}$  is the carotenoid concentration when  $I = I_{max}/2$  and  $x = 0$ , and  $(1-x)$  is the proportion of carotenoids allocated to immunocompetence.

15. The relationship between success of courtship displays ( $d$ ) and ornament size ( $O$ ): Males that express larger and more colorful ornaments are more successful at achieving matings (Houde 1997), but this relationship should have diminishing returns. So I assume an increasing relationship from zero with diminishing returns towards an upper asymptote.

$$d = d_{max}(1 - e^{-\delta O})$$

where  $d_{max}$  is the maximum success of a courtship display where all females that were courted responded positively, and  $\delta$  is the display exponent.

16. The influence of ornament size ( $O$ ) on sperm precedence ( $w$ ): this is described in detail in the Methods section.

17. Daily reproductive success ( $F$ ) was calculated as:

$$F = dn_fpb$$

where  $n_f$  is the number of receptive females in the populations,  $p$  is the proportion of the brood that is fertilized by the focal male, and  $b$  is the average brood size. The calculation of  $p$  is provided in the Methods section.

18. Calculating the number of males competing with the focal to sire a brood ( $n_m$ ): the number of males siring a brood is dependent upon the density of the population (Soucy and Travis 2003), in a diminishing returns relationship towards an upper asymptote.

$$n_m = S_{min} - S(1 - e^{-VM(N-1)})$$

where  $S_{min}$  is the minimum number of males siring a brood (i.e. 1),  $S = S_{max} - S_{min}$ ,  $S_{max}$  is the maximum number of males that could sire a brood,  $V$  is the paternity exponent,  $M$  is the sex ratio and  $N$  is the population size.

18. Relationship between immunocompetence ( $I$ ) and probability of dying ( $D$ ): Individuals that can mount a high immune response are unlikely die from a pathogen challenge, while those that are poor immune responders are more likely to die. I assume that all individuals in the population are challenged with the same level of pathogen infection. The relationship is assumed to be reverse sigmoid.

$$D_I = D_I - \frac{D_{IU}}{1 + \left(\frac{D_{IU}}{D_{IL}} - 1\right)^{\left(1 - \frac{I}{I_F}\right)}}$$

where  $D_{IU}$  is the probability of dying when  $I = 0$ ,  $D_{IL}$  is the probability of dying when  $I$  is maximum,  $I_F$  is the value of  $I$  corresponding to the inflection point of the sigmoid curve.

19. The relationship between ornament size ( $O$ ) and probability of dying ( $D$ ): Males that have larger and more colorful ornaments are more likely to attract predators (Endler 1980; Godin and McDonough 2003). I expect this relationship to be sigmoid increasing.

$$D_O = \frac{D_{OU}}{1 + \left(\frac{D_{OU}}{D_{OL}} - 1\right)^{\left(1 - \frac{O}{O_{D/2}}\right)}}$$

where  $D_{OU}$  is the probability of dying when  $O$  is maximum,  $D_{OL}$  is the probability of dying when  $O = 0$ ,  $O_{D/2}$  is the ornament size that corresponds to a probability of dying of  $0.5D_{OU}$ .

20. Probability of dying was calculated as follows:

$$D = 1 - (1 - D_I)(1 - D_O)$$

where  $D_I$  is the immunocompetence death probability described in 18 above,  $D_O$  is the predation death probability described in 19 above.

**Table 6.1.** Assumptions made in the model.

|   |
|---|
| Population demography   |
| <ul style="list-style-type: none"><li>• All individuals are sexually mature; they are of the same age and size.</li></ul>   |
| Females   |
| <ul style="list-style-type: none"><li>• All females produce broods of the same size.</li><li>• The number of receptive females per day is constant.</li><li>• Females do not use stored sperm.</li></ul>  |
| Pathogens   |
| <ul style="list-style-type: none"><li>• All individuals in the population have had similar infection histories.</li><li>• Pathogen environment is constant: all individuals are challenged by a ubiquitous pathogen to the same extent every day.</li></ul>   |
| Condition index and other physiological variables   |
| <ul style="list-style-type: none"><li>• The condition index does not affect foraging rate.</li><li>• The condition index from the previous day carries over to the next day.</li><li>• All variables, except the condition index, are refreshed at the start of each day to account for dynamic changes in physiology throughout the day.</li></ul>   |
| Carotenoids and signal  |
| <ul style="list-style-type: none"><li>• All available carotenoids are used either for signal or immunocompetence.</li><li>• The intensity of color in the ornament does not change, only the area of orange color changes.</li><li>• Ornament size is refreshed each day. Ideally, carotenoids are added to and lost from the signal in a dynamic fashion. To simplify this, the model refreshes signal size each day, depending on resources consumed, testosterone levels, immune response and other physiological variables.</li></ul> |
| Other male reproductive traits  |
| <ul style="list-style-type: none"><li>• All males have the same sperm count, regardless of other physiological variables.</li><li>• Testosterone and other physiological variables do not affect the frequency of courtship displays.</li><li>• Females choose mates only based on signal size; all males display at the same frequency.</li><li>• Males do not indulge in forced copulations, or if they do, these are unsuccessful.</li></ul>   |

**Table 6.2.** Parameter names, symbols and definitions with units and default values. Parameters include derived parameters.

| Symbol     | Parameter                                 | Definition   | Default value with units               |
|------------|---|--|--|
| R          | Resources available                       | The amount of resources available to a fish at the start of each day.  | 8 mg                                   |
| $q_f$      | Foraging exponent                         | Exponential coefficient that determines how close an individual is to consuming his maximal foraging intake per unit resources available; a measure of foraging ability.           | $(0.05 - 0.19 \text{ mg}^{-1})^*$      |
| $r_{\max}$ | Maximal foraging intake                   | The maximum resources that an individual can consume.  | $(8 \text{ mg})^2$                     |
| $C_{\max}$ | Maximal condition                         | Highest condition index of an individual in excellent health and vigor. See Table 5.3 for the definition and measurement of condition.   | $(0.08)^3$<br>dimensionless            |
| $C_{\min}$ | Minimal condition                         | Lowest body condition of an individual at the point of starvation; the individual is considered dead at this point. See Table 5.1 for the definition and measurement of condition. | 0.001<br>dimensionless                 |
| k          | Carotenoid exponent                       | Exponential coefficient that determines how close an individual is to accumulating his maximum possible carotenoids per unit resources available.                                  | $(0.3 \text{ mg}^{-1})^*$              |
| $K_{\max}$ | Maximal plasma carotenoids                | Maximum possible carotenoid concentration that an individual can accumulate.   | $(0.8 \text{ } \mu\text{g ml}^{-1})^4$ |
| $\epsilon$ | Condition-dependent testosterone increase | Increase in testosterone concentration per unit increase in condition.   | $(3500 \text{ ng ml}^{-1})^*$          |
| E          | EDC                                       | Ethynyl estradiol concentration in water.  | $(2 \text{ ng ml}^{-1})^5$             |
| $\alpha$   | Testosterone decay coefficient            | Exponential decay in testosterone concentration per unit increase in EDC concentration.  | $(1 \text{ ml ng}^{-1})^*$             |
| $\sigma$   | Testosterone-dependent condition decline  | Decrease in condition per unit increase in testosterone concentration.   | $(0.00005 \text{ ng}^{-1})^*$          |

\*These parameter values generate the range of values of the dependent variable corresponding to the range of values of the independent variable in the relationship it is associated with. Ranges of values for variables are provided in Table 3.

<sup>2</sup>KS personal observation

<sup>3</sup>Neff B, Cargnelli L, 2004. Relationships between condition factors, parasite load and paternity in bluegill sunfish, *Lepomis macrochirus*. *Environmental biology of fishes* 71:297-304.

<sup>4</sup>Amar EC, Kiron V, Satoh S, Watanabe T, 2004. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. *Fish & Shellfish Immunology* 16:527-537.

<sup>5</sup>Shenoy K, 2012. Environmentally realistic exposure to the herbicide atrazine alters some sexually selected traits in male guppies. *PLoS ONE* 7:e30611.

Table 6.2, continued

|            |  |   |  |
|------------|--|---|--|
| $q_i$      | Immunity exponent                              | Exponential coefficient that determines how close an individual is to mounting the highest immune response possible per unit increase in condition; this is a measure of immunocompetence quality.  | (15 - 50)*<br>dimensionless                      |
| $I_{\max}$ | Maximal immune response                        | The maximum immune response mounted to completely clear a challenge infection. See Table 5.3 and Methods for the definition and measurement of immune response.   | 100<br>dimensionless                             |
| $\theta$   | Immunity-dependent condition decline           | Decrease in condition index per unit increase in immune response.   | (0.0003)*<br>dimensionless                       |
| $j$        | Testosterone-dependent carotenoid increase     | Exponential coefficient that determines how close an individual is to the maximum concentration of carotenoids per unit increase in testosterone.   | (0.001 ml ng <sup>-1</sup> )*                    |
| $\psi$     | Plasma carotenoid difference                   | $K_{\max} - K_i$ , where $K_i$ is the plasma carotenoid concentration if testosterone concentration was 0 ng ml <sup>-1</sup>   | Derived;<br>$\mu\text{g ml}^{-1}$                |
| $\Omega$   | Carotenoid-dependent increase in ornament size | The exponential coefficient that determines how close an individual is to expressing the largest possible ornament per unit increase in plasma carotenoids allocated to the ornament. Because ornament expression also depends on testosterone concentration, as testosterone must bind to androgen receptors, $\Omega$ is dependent on testosterone concentrations in a linear manner. | $\Omega = ZT$<br>(0 - 4 ml $\mu\text{g}^{-1}$ )* |
| $Z$        | Ornament increaser                             | increase in $\Omega$ per unit increase in T   | (0.012 ml <sup>2</sup> $\mu\text{g}^{-2}$ )*     |
| $O_{\max}$ | Maximum ornament size                          | Maximum proportion of the body area covered by the ornament.  | (0.5) <sup>6</sup><br>dimensionless              |
| $\rho$     | Testosterone-dependent immunity decline        | Decrease in testosterone concentration per unit increase in immune defense.   | (0.2 ml ng <sup>-1</sup> )*                      |
| $P$        | Proportional immunity decreaser                | increase in the immunity decrease proportion per unit increase in EDC concentrations.   | (0.33 ml ng <sup>-1</sup> )*                     |
| $\beta$    | Disruption-related immunity decline            | proportion by which immune response decreases as a result of EDC-exposure; $\beta = PE$   | derived;<br>dimensionless                        |
| $I_{K0}$   | Unenhanced immunocompetence                    | The immune response when no carotenoids are allocated to immunocompetence   | dimensionless                                    |

<sup>6</sup>Houde A, 1997. *Sex, color, and mate choice in guppies*. Princeton, New Jersey: Princeton University Press.

Table 6.2, continued

|            |  |   |                                   |
|------------|--|---|-----------------------------------|
| $d_{\max}$ | Maximum display success                | The maximum proportion of displays that result in successful copulation   | 1<br>dimensionless                |
| $\delta$   | Display exponent                       | The exponential coefficient that determines how close to maximum display success an individual reaches per unit increase in ornament size.  | (7)*<br>dimensionless             |
| $b$        | Brood size                             | Average brood size  | (5) <sup>7</sup><br>dimensionless |
| $p$        | Brood proportion                       | Proportion of a brood fertilized by the focal male  | derived;<br>dimensionless         |
| $M$        | Sex ratio                              | Proportion of males in population   | 0.5<br>dimensionless              |
| $w$        | Sperm precedence                       | Weighting that defines the focal male's sperm precedence; $w_i$ (sperm precedence of focal male) and $w_j$ (sperm precedence of competing males) is defined in the Methods section. | derived;<br>dimensionless         |
| $N$        | Population size                        | Number of individuals in the population   | 100<br>dimensionless              |
| $n_f$      | Receptive females                      | Number of receptive females available per day; $n_f = y(1-M)N$  | Derived;<br>dimensionless         |
| $y$        | Receptive female ratio                 | Proportion of females that are receptive per day  | 0.1<br>dimensionless              |
| $n_m$      | Competing males                        | number of males competing with the focal male for fertilizations of a brood   | derived;<br>dimensionless         |
| $S_{\max}$ | Sire maximum                           | maximum number of sires per brood   | (8) <sup>8</sup><br>dimensionless |
| $S_{\min}$ | Sire minimum                           | minimum number of sires per brood   | 1<br>dimensionless                |
| $V$        | Sire exponent                          | The exponential coefficient that determines how close the number of sires is to the sire maximum per unit increase in population size.  | (0.004)*<br>dimensionless         |
| $D_{IU}$   | Zero-immunity mortality probability    | Probability of death when $I = 0$   | 1<br>dimensionless                |
| $D_{IL}$   | Maximum-immunity mortality probability | Probability of death when $I$ is maximum  | 0.001<br>dimensionless            |
| $D_{OL}$   | Zero-ornament mortality probability    | Probability of death when $O = 0$   | 0.001<br>dimensionless            |
| $D_{OU}$   | Maximum-ornament mortality probability | Probability of death when ornament expression is maximum  | 0.1<br>dimensionless              |

<sup>7</sup> KS personal observation<sup>8</sup>Neff BD, Pitcher TE, Ramnarine IW, 2008. Inter-population variation in multiple paternity and reproductive skew in the guppy. *Molecular ecology* 17:2975-2984.



**Table 6.3.** Variables, definitions, and their range of values.

| Variable                           |                | Definition  | Range and Units                               |
|------------------------------------|----------------|---|---|
| Carotenoid allocation              | x              | proportion of carotenoids allocated to the ornament, such that 1-x is the proportion allocated to immunocompetence              | 0 - 1<br>dimensionless                        |
| Resources consumed                 | r              | Amount of resources consumed per day. For simplicity, I assume that the individual consumes all resources at start of each day. | (0 - 8 mg) <sup>9</sup>                       |
| Condition index                    | C              | Proportion of fatty stores per unit body mass; measured as mass (g) of lipids per mass (g) of body tissue                       | (0.001 - 0.08) <sup>10</sup><br>dimensionless |
| Plasma carotenoids                 | K              | Plasma concentration of carotenoids   | (0 - 0.8 µg ml <sup>-1</sup> ) <sup>11</sup>  |
| Testosterone concentrations        | T              | Plasma concentration of testosterone  | (0 - 280 ng ml <sup>-1</sup> ) <sup>12</sup>  |
| Immune response                    | I              | Resistance to parasite infection, a linear response to increasing susceptibility or parasite load                               | 1-100<br>dimensionless                        |
| Ornament size                      | O              | Proportion of body area covered by carotenoid-based ornament  | (0 - 0.5) <sup>13</sup><br>dimensionless      |
| Display success                    | d              | Success of courtship displays measured as the proportion of matings achieved out of all the females courted                     | 0 - 1<br>dimensionless                        |
| Daily reproductive success         | F              | The number of offspring sired at the end of each day  | 0 - ∞<br>dimensionless                        |
| Fitness                            | W              | lifetime reproductive success, calculated as the total reproductive success summed over the days that the individual lived      | 0 - ∞<br>dimensionless                        |
| Immunocompetence death probability | D <sub>I</sub> | Probability of dying at the end of the day because of insufficient immune response to a challenge                               | 0 - 1<br>dimensionless                        |
| Predation death probability        | D <sub>O</sub> | Probability of dying at the end of the day because of predation   | 0 - 1<br>dimensionless                        |

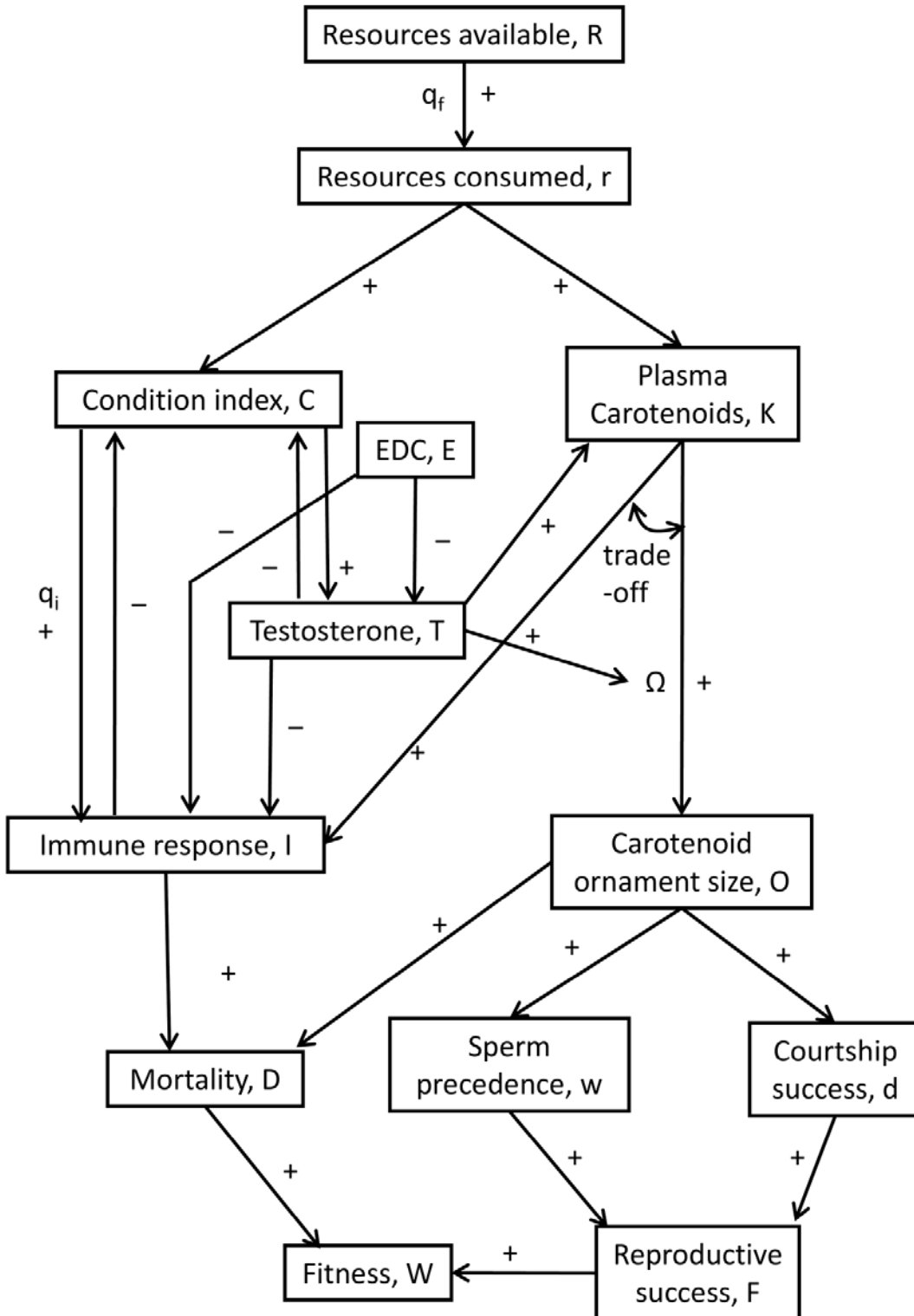
<sup>9</sup>KS personal observation

<sup>10</sup>Neff B, Cargnelli L, 2004. Relationships between condition factors, parasite load and paternity in bluegill sunfish, *Lepomis macrochirus*. *Environmental biology of fishes* 71:297-304.

<sup>11</sup>Amar EC, Kiron V, Satoh S, Watanabe T, 2004. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. *Fish & Shellfish Immunology* 16:527-537.

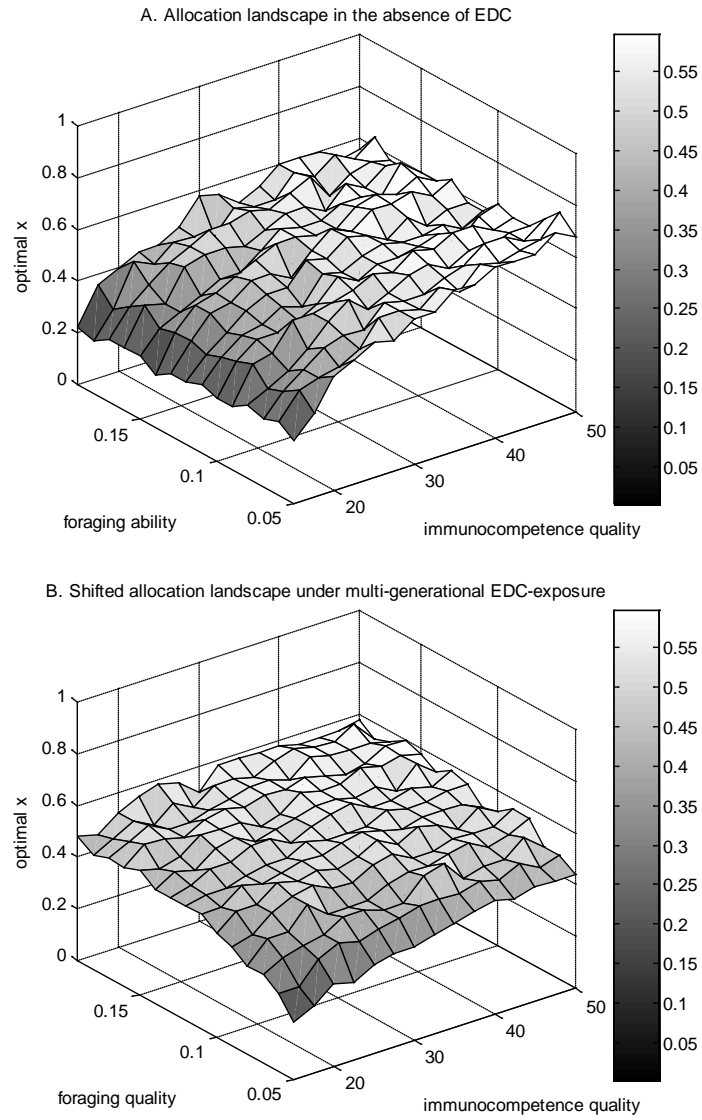
<sup>12</sup>Toft G, Edwards TM, Baatrup E, Guillette LJ, 2003. Disturbed sexual characteristics in male mosquitofish (*Gambusia holbrooki*) from a lake contaminated with endocrine disruptors. *Environmental Health Perspectives* 111:695-701.

<sup>13</sup>Houde A, 1997. *Sex, color, and mate choice in guppies*. Princeton, New Jersey: Princeton University Press.

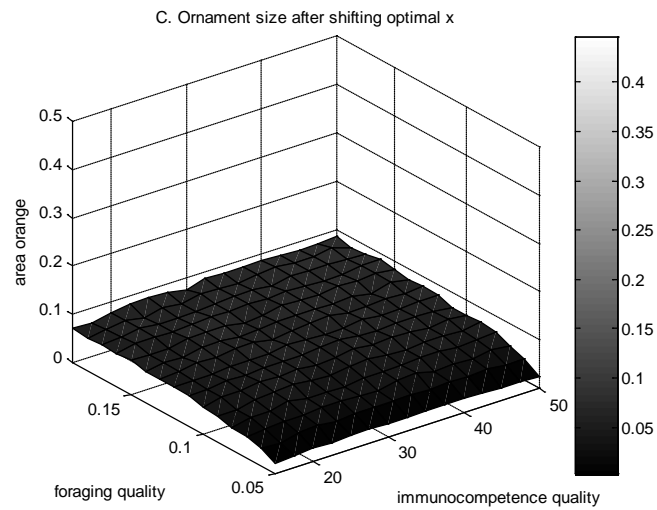
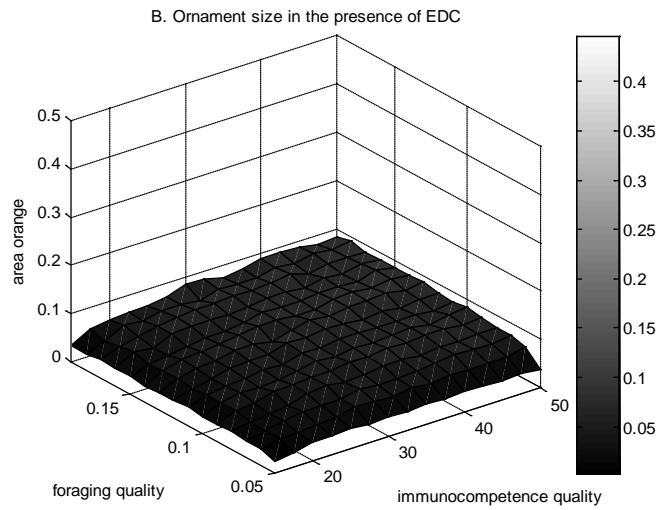
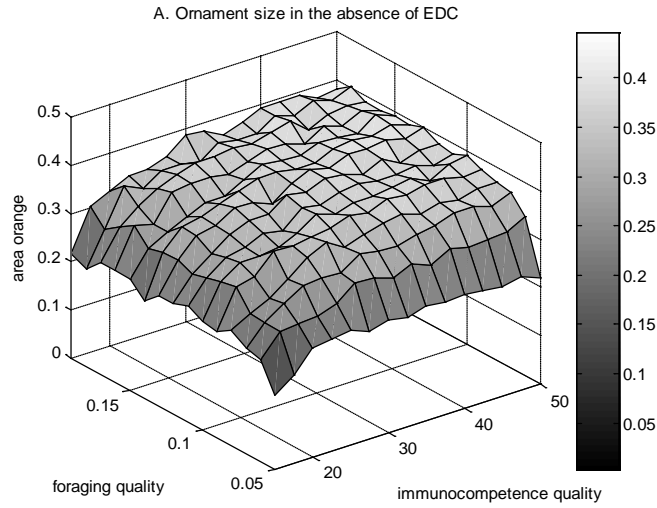


**Figure 6.1.** The causal relationships between the variables that ultimately affect fitness. Symbols (+ or -) on arrows denote the qualitative nature of the relationship between the two variables. The amount of resources consumed depends upon the amount of resources available, and the efficiency of this relationship depends on foraging ability,  $q_f$ . The amount of

resources consumed influences the concentration of plasma carotenoids,  $K$ , and the individual's condition index,  $C$ .  $C$  influences testosterone concentrations,  $T$ , and immune response,  $I$ , via immunocompetence quality,  $q_i$ . Both of these variables negatively impact condition index, because mounting an immune response and raising testosterone levels are energetically costly.  $T$  also suppresses  $I$  but increases  $K$  by mobilizing carotenoids from stores. The concentration of EDC,  $E$ , negatively impacts  $T$  and  $I$ .  $K$  can be allocated to increasing ornament size,  $O$ , or enhancing immune response,  $I$ , and this poses a trade-off.  $T$  also influences the parameter  $\Omega$ , which influences the relationship of  $O$  with  $K$ .  $O$  is involved in courtship success and sperm precedence, which directly influence reproductive success.  $O$  increases the probability of dying,  $D$ , as does  $I$ . The probability of dying and the reproductive success,  $F$ , together add to the individual's fitness,  $W$ . All relationships are described in Appendix 6.1, parameters are defined in Table 6.2, and variables are defined in Table 6.3.



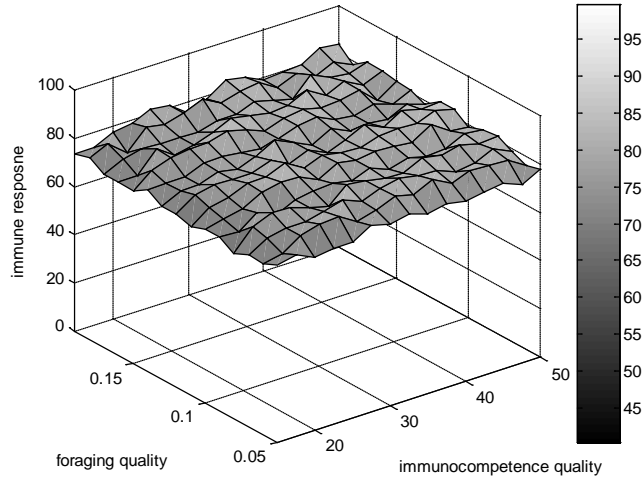
**Figure 6.2.** Optimal proportion of carotenoids ( $x$ ) allocated to the ornament. (A) Optimal allocation under default conditions, and (B) shifted optimal allocation under long-term EDC-exposure.



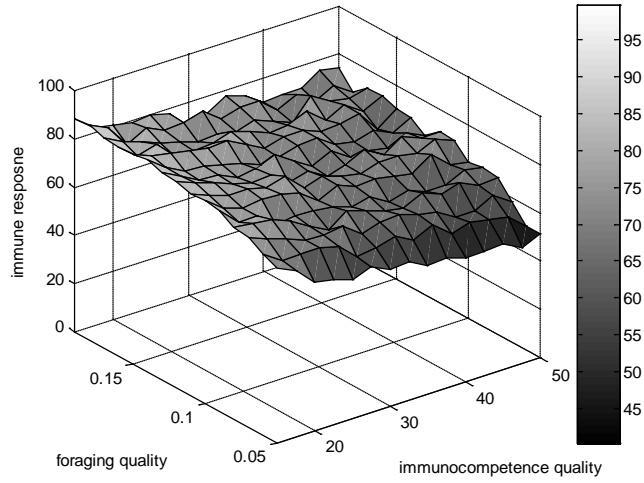
**Figure 6.3.** Ornament size, defined as the proportion of body area covered by orange spots, corresponding to the optimal allocation of carotenoids. (A) Ornament size under default

conditions, (B) ornament size after EDC-exposure, and (C) ornament size corresponding to shifted optimal allocation under long-term EDC-exposure.

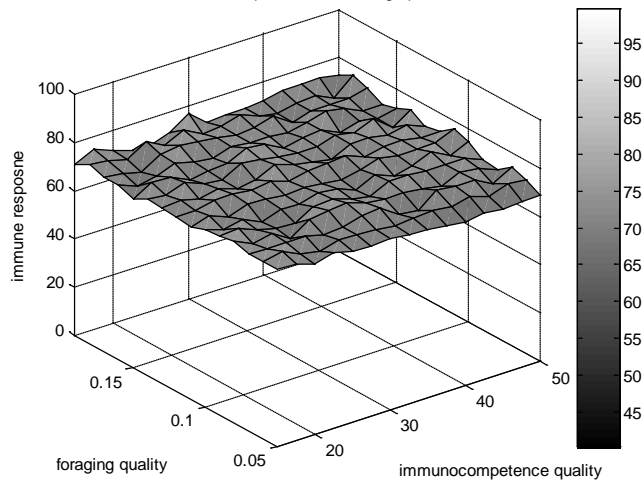
A. Immune response in the absence of EDC



B. Immune response in the presence of EDC

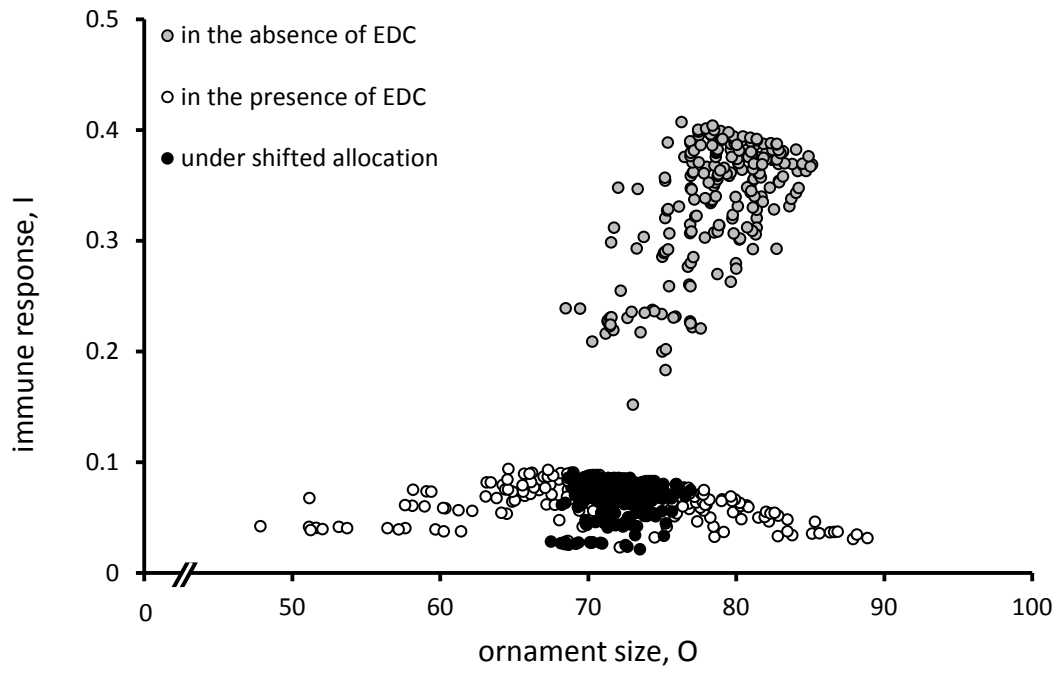


C. Immune response after shifting optimal x



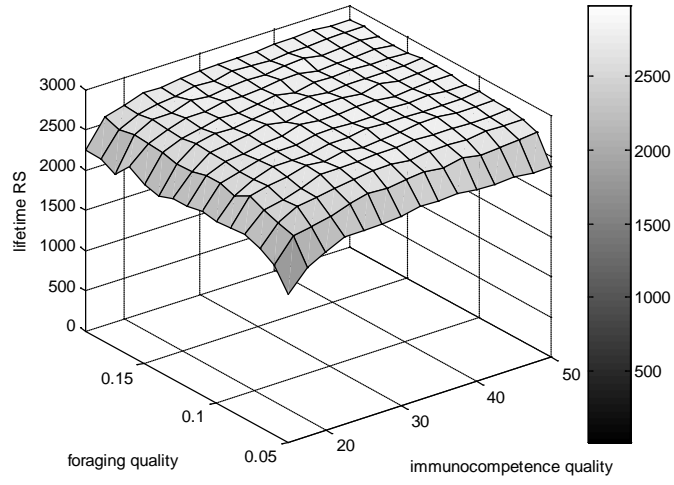
**Figure 6.4.** Immune response corresponding to the optimal allocation of carotenoids. (A) Immune response under default conditions, (B) immune response after EDC-exposure, and (C) immune response corresponding to shifted optimal allocation under long-term EDC-exposure.



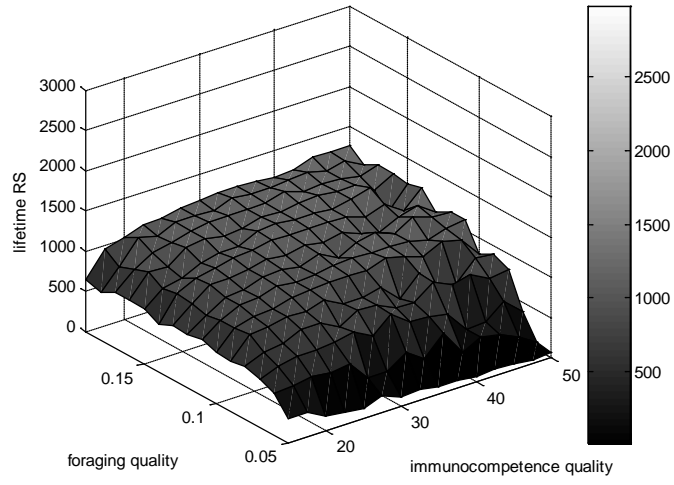


**Figure 6.5.** Signal reliability, measured by the correlation coefficient between ornament size and immune response.

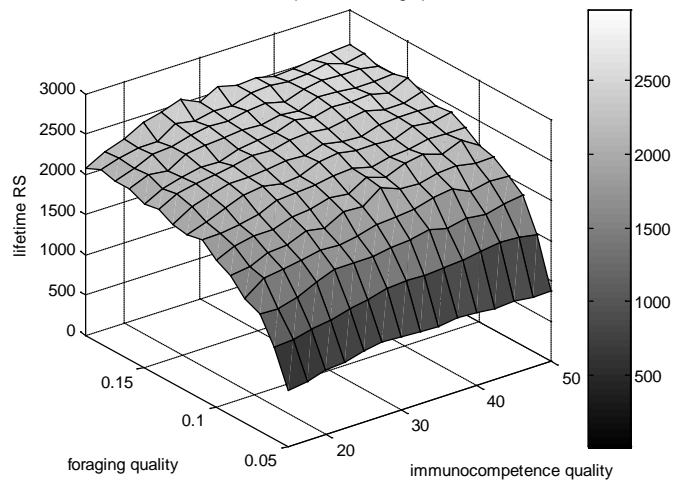
A. Fitness landscape in the absence of EDC



B. Fitness landscape in the presence of EDC



C. Fitness landscape after shifting optimal x



**Figure 6.6.** Fitness, as measured by lifetime reproductive success, corresponding to the optimal allocation of carotenoids. (A) Fitness under default conditions, (B) fitness after EDC-exposure, and (C) fitness corresponding to shifted optimal allocation under long-term EDC-exposure.

## CHAPTER SEVEN. FUTURE DIRECTIONS

My dissertation has laid the groundwork for a future career at the interface of ecotoxicology and sexual selection. Several intriguing hypotheses have been proposed and await testing. Here I describe some empirical studies that would test a few notable hypotheses and predictions that stem from this dissertation. I believe these studies will help further the field of evolutionary ecotoxicology.

The most exciting hypothesis from this dissertation is the predicted loss of signal reliability as a result of EDC-exposure. I had designed an experiment to test this hypothesis, and had hoped to complete it as part of my dissertation. Unfortunately, the experiment failed because of unprecedented mortality in the study animals. In this study, signal reliability in male guppies was defined as the correlation between ornament size (area of orange spots) and acquired immunity to a common ectoparasite, *Gyrodactylus turnbulli* (parasite load was a measure of resistance), because literature indicated that the ornament size in guppies reflected immunity (Houde and Torio 1992). I predicted that control males would show a strong correlation between the ornament size and the immunity, while males exposed to the EDC would show a lower correlation between these two traits.

In the unsuccessful study, adult male guppies were exposed to the treatment, and at the end of the exposure-period, all fish were challenge infected with *G. turnbulli* (following methods by Kolluru et al. 2006) to trigger an immune response, cleared of infection with Clout by Aquarium Products, and then re-infected after a recovery period. Unfortunately, 75% of the experimental fish died after the first infections. This was possibly because the fish were housed individually in 2 L jars without aeration, and the medication may have severely depleted oxygen. Having previously used Clout in 10 L aquaria with aeration, I had not anticipated the problem of oxygen depletion in the smaller containers as I had conducted all my experiments in such jars without negative effects. Despite the low samples sizes, I was able to detect a significant difference in the number of parasites on control fish versus atrazine-exposed fish (controls: mean =  $2.06 \pm 0.96$ , atrazine groups: mean =  $6.2 \pm 1.3$ ,  $F_{1,24} = 6.75$ ,  $P = 0.016$ ). I also measured variables related to ornament expression such as area of orange spots (with a digital still camera), and spectral properties of the orange spots (with a spectrometer). These variables have not yet been analyzed. I plan to complete the color analyses so that I have preliminary information whether EDC-exposure can reduce signal reliability as predicted.

I hope to conduct this experiment again in a more robust manner. In retrospect, I do not believe that acquired immunity is a better measure of immunocompetence. Guppies acquire immunity to the parasite after an infection, but lose this resistance as quickly as 6 weeks after the infection (Scott 1985). Perhaps measuring innate immunity to the parasite may be more relevant; some authors have tested innate immunity rather than acquired immunity (Houde and Torio 1992; Kolluru et al. 2006). In either case, it would be imperative to determine what measure of immunocompetence is truly reflected by the area and intensity of the carotenoid-based orange spots, because several authors have reported conflicting results (Houde and Torio 1992; Lopez 1998; Kolluru et al. 2006; Martin and Johnsen 2007). Perhaps this question could be tested better with an organism whose signal reliability is better established in the literature such as zebra finches.

Also of interest is the proportion of carotenoids and other pigments allocated to the ornament, and the effect of EDC-exposure on these proportions. In particular, the orange spot ornament of guppies is composed of two pigments--carotenoids, which must be obtained by foraging on unicellular algae, and drosopetrin, which is synthesized *de novo*. Grether and colleagues (2001) hypothesized that perhaps guppies can cheat by synthesizing more drosopetrins in habitats low in algal densities and thus appear equally colorful. However, they found that the proportions of the two pigments were correlated, and guppies in habitats lacking

sufficient algae did not produce drospterins in larger concentrations to counter the lack of carotenoids.

It is unclear what limits the production of drospterins and other pigments, but this appears to be genetically determined (Grether et al. 2005). McGraw (2005) reviewed a host of pigments that animals use in their ornaments and suggested that they all act as antioxidants to relieve the body of oxidative stress. I believe that the fish in low carotenoid habitats must use up the synthesized drospterins for antioxidative purposes and as immuno-enhancers and are thus unable to dishonestly signal their true condition. I propose testing whether EDC-exposure can alter pigment production by disrupting biochemical pathways involved in maintaining signal honesty, in light of the oxidation handicap hypothesis (Alonso-Alvarez et al. 2007). Guppies may be a suitable organism to test this if it can be established that integumentary pigment proportions are correlated to immune responses (a proxy for oxidative condition). However, other colorful animals like zebra finches and fighting fish could also be good candidates for this study.

My dissertation focused mainly on the effects of EDC-exposure on male signals. However, in populations exposed to EDCs, females are also impacted, and their hormonal pathways are being disrupted as well. Female mating behaviors, such as receptivity and choosiness are modulated by gonadal hormones such as estradiol (Kelley 1982; Lynch et al. 2006; Vyas et al. 2008; Chakraborty and Burmeister 2009; Vyas et al. 2009). Disrupting the production and functioning of key hormones can alter female mating behaviors, with important ecological and evolutionary implications.

I conducted an experiment to test whether prolonged exposure to atrazine could alter the strength of a female guppy's preference between bright and dull guppies. Adult virgin female guppies were exposed to the treatment for a period of 8 weeks. Negligible mortality was recorded during the exposure period. At the end of the exposure, each female guppy was put through a choice test to choose between a brightly colored male and a dull male. I recorded the female's movements in the tank to record where she spent the longest time: close to either male's compartment, or in the central neutral compartment indicating a lack of interest in either male. The data have not yet been analyzed. I plan to test for treatment effects on the strength of the female's preference for the bright male versus the dull male, and the degree of lack of interest in mating. I predict that atrazine and ethynylestradiol treated females will show a weaker strength of preference and greater lack of interest in mating compared to females in either control group. Females have been euthenized and preserved. I also plan to test for treatment effects on gonadosomatic index.

In the future, some experiments worth conducting would be long-term EDC-exposures of fish or amphibian populations in mesocosms to understand population level effects of multi-generational exposure to contaminants. Will there be overall loss of signal reliability in males and reduced choosiness in females? Can signal reliability and female choosiness be restored by removing the EDC? Are life-history traits such as brood sizes and sex ratios altered and is the overall population size reduced? Field studies comparing animal populations in contaminated habitats with those in pristine habitats would allow us to ascertain whether the effects seen in semi-natural set-ups can be extrapolated to natural populations. It would be especially interesting if the populations in contaminated habitats showed trends for altered mating systems and if these changes appeared to be non-plastic. These and other questions will shed light on the consequences of exposure to persistent contaminants to wildlife and ecosystem health.

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**Professional Publications**

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