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Energetic Cost and Physiological Trade-offs

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ENERGETIC COST AND PHYSIOLOGICAL TRADE-OFFS

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by

Heba Ahmed Khallaf Ali

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Heba Ahmed Khallaf Ali, and entitled Energetic Cost and Physiological Trade-offs, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2021

DEDICATION

This dissertation is dedicated to my lovely mother and role model, Prof. Dr. Saida Abdel-rejal and my father, Ahmed Khallaf. Your soothing words of encouragement, inspiration and emotional support got me through the rigors of the doctoral program. Your prayers and good wishes paid off in my excellence and accomplishments during the program.

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ABSTRACT OF THE DISSERTATION

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Professor Philip Stoddard, Major Professor

Understanding how organisms allocate limited resources across physiological systems is a major challenge in biology. My study revealed that high energetic demand of electric signals of male electric fish (*Brachyhypopomus gauderio*) is matched by a metabolic trade-off with other cellular functions. We used thyroxine (T4) to modulate the fish's signal metabolism, partitioned the energy budget pharmacologically, and measured energy consumption using oxygen respirometry. In males, total energy consumption was unchanged pre- and post-T4 treatment, while signal metabolism rose and the standard metabolic rate fell in an even trade-off. Total metabolism in females did the opposite. Under T4, the non-signal resting metabolism rose while the signal metabolism fell. These results reveal sex differences in metabolic trade-offs between signaling and cellular metabolism in electric fish and suggest that thyroid hormones regulate the allocation of energy between electric signals and somatic maintenance in favor of reproduction. To determine whether electric fish trade-off reproduction against innate immunity, as is common in vertebrates, we assessed changes in the bactericidal activity of plasma in *B. gauderio* challenged with bacterial lipopolysaccharide (LPS), before and after T4 treatment. Females did not modulate innate immunity with any of the treatments, while

males elevated bactericidal activity of plasma by about a third following LPS injections, T4 implants, or both together, relative to sham treatment. This outcome was unexpected given that T4 increases the energy consumed by the male's reproductive electric signals while lowering the rest of his metabolism. Thyroxine also increased expression of Na+K+ATPase pump mRNA in the electrogenic cells of males but not females, consistent with previous findings that T4 differentially regulates signal metabolism in the two sexes. This sex difference in gene regulation in my study suggests Na+K+ATPase underlies sexual dimorphism in electric signal energetics. The results provide further evidence that thyroid hormones play an essential role in the differential allocation of energy among metabolic functions. My work is the first to quantify an energetic trade-off between reproductive behavior and other metabolic functions. and implicates ion pumps, but not innate immunity, as molecular mechanisms underlying sex differences found in these energetic trade-offs.

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Atp1a2	ATPase Na+/K+ Transporting Subunit Alpha 2
BKA	Bacterial killing ability
cDNA	Complementary DNA
DEPC	Diethyl pyrocarbonate
DHT	Dihydrotestosterone
EOD	Electric organ discharge
gDNA	Genomic DNA
PCR	Polymerase Chain Reaction
Rpl13a	L13a ribosomal binding protein
RT	Reverse Transcription
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
T4	Thyroxine

CHAPTER 1: Introduction

"As a mother and PhD student, I have a lot going on in my life. So, I have to balance between the two lives without sacrificing one for the other".

Heba Ali "3MT"

1.1 Life-history trade-offs

Life-history strategies are complex and manifested by natural and sexual selections, which are constrained by trade-offs under conditions of resource restriction (Sinervo and Svensson, 1998). Organisms respond to environmental variations and stochastic stressors (e.g., pathogens) through their physiological, behavioral, and phenotypic plasticity (Apanius, 1998; Badyaev, 2005; Moore and Jessop, 2003; Whitman and Agrawal, 2009).

Many taxa respond to functional demand conflicts by trading off one activity or physiological function against another (Burness *et al.*, 2010; Congdon, 1989; Congdon *et al.*, 1982; Moore and Hopkins, 2009; Nespolo *et al.*, 2008). A trade-off exists when a constraint causes functional demands conflict with the quantity of energy, amount of time, and food availability (Clutton-Brock *et al.*, 1982; Sheldon and Verhulst, 1996b; Sinervo and Svensson, 1998; Smith and French, 2017; Stearns, 1989; Zera and Harshman, 2001).The differential allocation of energy or recourses is directed by endocrine regulatory mechanisms (Finch and Rose, 1995; Hau, 2007; Ketterson and Nolan Jr, 1992; Ricklefs and Wikelski, 2002).

Trade-off costs (cost of making a trade-off or that forces a trade-off) often vary among studies, leading to inconsistent reporting (Antonovics and van Tienderen, 1991; Leroi *et al.*, 1994; Reznick, 1985). In many studies, the trade-off is considered the result

of physiological fitness (e.g., survival) (Leroi *et al.*, 1994). While in others, the cost is used to define the cost that forces a trade-off (Reznick, 1985). The cost of function swaps can be referred to as either a pay-off or a penalty (current or imposed in the future) (Calow, 1979; Van Noordwijk and de Jong, 1986; Zera and Harshman, 2001). The pay-off of trade-offs can be in the form of energy (e.g., calories required to prioritize the function). Similarly, as an example, the cost of reproduction as the energy needed to maintain the function or the consequences of this reproduction, which can be a direct and current penalty or one imposed in the future.

On the other hand, competing for physiological processes can happen at the same or different life cycle times as a result of variations in ecological or behavioral factors. Considering the internal physiological factors relative to external environmental factor is vital to assessing the strategies and cost of trade-offs. Every physiological and behavioral performance requires energy, so energy has been considered a common currency in which important allocation decisions and trade-offs can be made and quantified (Careau *et al.*, 2008; Congdon *et al.*, 1982; Isler and van Schaik, 2006; Prestwich *et al.*, 1989). To our knowledge, most studies of trade-offs have included non-energetic currencies such as genetic, phenotypic, environmental, and hormonal mediated traits of resource allocation between the physiological functions (Congdon *et al.*, 1982; Reznick *et al.*, 2000; Rose and Bradley, 1998; Withers, 1992). Few studies have measured the actual energetic cost (calories or oxygen consumption) of physiological functions competing in a trade-off manner (Trillmich *et al.*, 2020) (Ali and Stoddard, *subm*). It is not easy to determine the energetic cost of a behavior or physiological function. Additionally, other currencies apart from energy, can contribute as a limitation invested in time, phosphate or even protein.

Numerous studies have been conducted to determine the energetic costs of breeding by determining the number, weight, or caloric value of eggs or neonates. Other studies measure reproductive costs by examining how breeding affects an individual's future survival and breeding potential. Whereas the optimal method is to quantify the energetic cost of reproductive effort itself and attempts to estimate the energetic reproductive costs directly are complicated (Clutton-Brock, 1984; Stoddard and Salazar, 2011).

Trade-off strategies are influenced by variation within and among species in resource availability and the ultimate amount of resource input. Increased nutrient or resource availability can substantially diminish or obviate trade-offs, and vice versa (Chippindale *et al.*, 2004; Kaitala, 1987; Zera *et al.*, 1998) (mostly in birds). Another critical aspect of trade-offs is resource acquisition timing and expenditure on physiological functions (Boggs, 1992; Doughty and Shine, 1997). Trade-offs can also vary and change during development and evolve over time (Leroi *et al.*, 1994; Leroi *et al.*, 2004).

Our objectives are to understand the physiological dimensions of life-history trade-offs and explore how different taxa have adjusted to the increase in one of the energetic physiological demands within a scope of limited resources, especially during the breeding season. Here also, we presented the interactions among life-history trade-offs, different physiological strategies of trade-offs, hormonal regulation of energetic processes and trade-offs, and the consequences of these trade-offs on reproductive performance. In addition, examples are given of animal systems used to study the physiology of life-history trade-offs, including crickets, electric fish, frogs, lizards, birds, and mice, chosen to highlight the physiological interactions of trade-offs from observational and experimental studies in the field and lab.

1.2 Trade-off assessment

Life history theory assumes that reproduction has a cost in terms of future survival, growth, mortality, fecundity, time and condition cost (Ball, 1986; Bryant, 1979; Calow, 1979). Assessment or measurements of life-history trade-off mechanisms (Reznick, 1985; Reznick *et al.*, 1990; Roff, 1993) can be tested using three different approaches: phenotypic correlations, calculation of genetic correlations by selection trials, and manipulative experiments as well as other methods to assess the trade-off between physiological functions is by measuring the observable characteristics of individuals (Partridge *et al.*, 1999; Partridge and Sibly, 1991; Reznick, 1985). Phenotypic correlations tend to have limitations caused by non-causal factors, which in turn can explain observable links (Chippindale *et al.*, 2004; Marler *et al.*, 1995; Murren *et al.*, 2015; Yi and Dean, 2016).

As a second approach, the genetic principle (quantitively genetic estimate or measure of pleiotropy degree that affect two traits) of trade-offs, particularly in insect studies, has been received much attention (Bell, 1980; Reznick, 1985; Rose and Bradley, 1998). Also, these studies depend only on the variation of genetic traits and need much more time and resources than other approaches (Moller *et al.*, 1989). *Drosophila* is a powerful genus example utilizing genetic approaches to investigate the life-history trade-off between reproduction and longevity (Luckinbill *et al.*, 1984; Partridge *et al.*, 1999; Rose, 1984). These physiological and genetic studies concluded that strains were characterized by longer-lived flies with decreased fecundity at an early age, and aging was a function of the damaging effects of earlier reproduction (Carlson *et al.*, 1998; Carlson and Harshman, 1999; Harshman, 1999). Experimental designs that apply genetics' quantative approach are

preferable as they investigate both the phenotypic and genetic basis of the trade-off (Crnokrak and Roff, 1998; Leroi *et al.*, 1994; Leroi *et al.*, 2004; Moller *et al.*, 1989).

The most successful experimental approach in life-history studies involves manipulating physiological functions (e.g., environmental or hormonal) and establishing very conclusively the existence of functional constraints (Partridge and Sibly, 1991; Reznick, 1985; Sinervo and Svensson, 1998). Such manipulations have proven powerful for studying the functional aspects of life-history trade-offs (Bribiescas and Ellison, 2008; Hau, 2007; Hou, 2013; Isler and van Schaik, 2006; Marler and Moore, 1989; Martin *et al.*, 2008; Schwarzkopf, 2014; Schwenke *et al.*, 2016; Wingfield, 1984; Zera *et al.*, 1998). Traits can be regulated/modified by endocrinological mediators such as sex and stress hormones, thus giving rise to altered trade-off functions (traits within those functions are altered). Similarly environmental mediators, such as nutrient availability or predation can generate and manipulate phenotypic trade-offs between physiological traits. An environmental factor can change the economic balance of the trade-off, but the choise to the animal itself to make the trade-off. Exploring trade-offs through physiological manipulation also has its limitations. For example, because many hormones serve as master regulators of multiple character suites (McGlothlin and Ketterson, 2008), hormone implantation can have unexpected side-effects on other physiological functions (Diamanti-Kandarakis *et al.*, 2009). Manipulative experiments can indicate the existence of a phenotypic trade-off, but cannot demonstrate that the focal trade-off has any evolutionary relevance (e.g. genetics, ecological, and physiological evolutionary prospective) (Partridge and Sibly, 1991; Reznick, 1985).

Studies on poikilothermic vertebrates such as fishes, amphibians, and reptiles have shaped our understanding of the life history of physiology. Over three decades, studies on lizards have examined endocrine aspects and manipulated competing physiological traits, while exploring physiological and energetic trade-offs between current and future reproduction (Huey and Stevenson, 1979; Landwer, 1994; Marler *et al.*, 1995; Schwarzkopf, 2014). A classic example of a physiological trade-off demonstrated in many lizard species is the negative relationship between the number and size of eggs produced (Doughty and Shine, 1997; Roff, 1992; Schwarzkopf, 2014; Sinervo and Svensson, 1998).

Intensive studies have documented the life-history energetic trade-offs between reproduction and immunocompetence in birds (Norris and Evans, 2000). Birds spend high amounts of energy during thermoregulation and reproduction (Burness *et al.*, 2010; Weathers and Sullivan, 1993) relative to other vertebrates. Thus, energy constraints in birds expose their physiological functions (reproduction, thermoregulation, growth) to competing demands (Elliott *et al.*, 2014; Griesser *et al.*, 2017; Gustafsson *et al.*, 1994; Isler and van Schaik, 2006; Norris and Evans, 2000; Santos and Nakagawa, 2012). Endocrinologically-mediated trade-off studies in birds have provided mechanisms whereby life-history traits are linked.

1.3 Optimal life-history strategies

Energy and nutrients are required by all living creatures to grow, maintain their bodies, and reproduce. Competition food and defenses mounted by potential food items both can limit nutrient availability. Thus, each organism will often lack sufficient resources to allocate among various physiological demands of cellular maintenance, growth, and

reproduction, needed to maximize their fitness (Williams, 1966; Williams, 2018; Williams and Burt, 1997). Resulting trade-offs are shaped in the landscape of selection.

Many trade-off models exist in life-history theory (Schaffer, 1974; Sibly and Calow, 1984; Van Noordwijk and de Jong, 1986). However, determining the shape of the trade-off curve is a monumental amount of work. Moreover, to determine the true cost of a characteristic, it is necessary to examine energetic expenditure in the context of the organism's life history, and in comparison, to the expenses that the organism incurs while expressing other life-history traits. According to the features of each species, as well as the habitat and other constraints that they face, the optimal life history strategy may differ. That is to say, a species, population, or sex may have no universal optimum.

Survival and reproduction are two physiological functions that are considered the most competitive for limited resources (Bårdsen *et al.*, 2010; Stearns, 1989; Zera and Harshman, 2001). The various sorts of reproductive costs have a significant effect on expected life-cycle optima. Thus, the actualization between life-history strategies is connected to the time at which reproduction costs are paid (Pierce and Ollason, 1987).

1.4 Cost of reproduction and its effect on optimal life-history strategies

"Direct-costing" organisms are those that pay for reproduction before the gametes are released into the environment. The cost is paid from the energy acquired prior to the commencement of the function itself, with the initial allocation going to viability and the residual budget going to fecundity (Sibly and Calow, 1984). Thus, organisms begin to pay reproduction-related expenses before the release of the first clutch of gametes (Hussell, 1972). Since the gamete synthesis has its own cost, it must be paid by the individual. As an example, males of many species invest more energy in the production of sperm and

courtship than other physiological functions. Before gamete release, reproduction costs may be incurred because of increased risks connected with altered behavior, for example, pertaining to the risks of courtship such as predators or aggressive behavior toward sexual rivals. Other behavioral costs include searching for food needed for gamete production or resources needed for nest preparation. It can be stated therefore, females of any species have the highest costs of gamete production. Finally, organisms might divert resources away from somatic tissues towards gametic tissues and processes (O'dor and Wells, 1978; Sibly and Calow, 1984).

On the other hand, the "absorption cost" of reproduction is paid after the release of gametes (Sibly and Calow, 1984). Absorption cost covers the allocation of resources to fecundity first, with the remaining going to subsequent survival. Parental care is considered as absorption costing. The effect of reproduction on adult survival and reproductive timing begin as soon as the organism has liberated the gametes. Therefore, survival after reproduction and the time it takes for an egg to reach independence are considered to be negative functions of expressing earlier fecundity (Askenmo, 1979). Consequently, costs might be incurred after gamete release that result from bodily physiological fluctuations, such as being vulnerable to predation and sickness, metabolic expenses or survival risks (Askenmo, 1979) result from caring for the offspring (Bryant, 1979). Additionally, metabolic stress results from a buildup of consequences that occur during the activities that lead up to the production of gametes. Moreover, individuals that mate-guard may be more vulnerable to attack by predators or conspecifics.

1.5 Survivorship specific reproductive output

An additional model of the life-history strategy of trade-off is the model that considers survival to be living after reproduction and optimization varies per age class (Schaffer, 1974). All species face the dilemma of being limited in their ability to maximize all aspects of fitness at the same time. Extrinsic time or energy restrictions frequently mediate such trade-offs, so that, for example, energy committed to reproduction detracts from energy available for survival and maintenance. Individuals overcome which conundrum by prioritizing certain features associated with primary energy allocation and fitness (such as growth rate, body size, stress response, reproductive timing, offspring number and quality, lifespan, and dispersal), at the expense of others (Lancaster *et al.*, 2017). The nature of trade-off functions among these traits can be quite complex, involving multidimensional allocation decisions or higher order properties of trait values.

The optimal reproductive strategy in stable environments is repeated breeding (iteroparity) (Bell, 1980; Charnov and Schaffer, 1973; Clutton-Brock, 1984; Ranta *et al.*, 2002; Young, 2010). Long-lived, iteroparous species have evolved strategies for energy allocation to maximize reproductive success over their lifetime. These strategies place increased emphasis on adult survival and less emphasis on any single reproductive event, favoring iteroparity (Drent and Daan, 1980). Individuals that die after a single breeding cycle are widely considered to be optimal only in a constant environment when the optimal reproductive expenditure is 100 percent (i.e., semelparity) (Oakwood *et al.*, 2001; Young, 2010).

Another essential aspect to understanding an organism's life history is the number of reproduction episodes over its lifetime. Evolutionary strategies in animal reproduction

usually vary among species. It is possible that for certain species, reproduction occurs once over a lifetime, as in semelparous species (e.g., antechinus, northern quoll, and salmon) (Young, 2010). Animals with this pattern spend most of their recourse budget on a single reproductive event and then die, risking their health to the point that they are unable to continue living (Groot, 1991; Oakwood *et al.*, 2001). In contrast, the opposite types of animals are iteroparous. As such, they do not devote all their resources to a single reproductive event. Instead, they reproduce several times to maximize fitness. Another case that is different from the others, animals with a seasonal estrus cycle only mate once a year, yet they can survive through numerous mating seasons (Rosa and Bryant, 2003). Estrus is a physiological condition that is regulated by hormones to prepare the body for a successful mating season. Similarly, females with (e.g., primates) monthly menstrual cycles make pregnancy possible only a few days per month during ovulation (Blomquist, 2009; Strum and Western, 1982). Organisms that utilize this pattern are free to attempt reproduction at any point.

Trade-offs between survival and reproduction are most pronounced in long-lived species (Bohec *et al.*, 2007; Van Noordwijk and de Jong, 1986). Variation in post-breeding survival among adults favors increased investment in current reproduction. The long-lived species ages and costs of first reproduction, as well as their survival–reproduction trade-offs, are remarkable (Culina *et al.*, 2019).

Reproduction increases mortality risk for a variety of reasons. The quest for a mate increases the susceptibility to predators, particularly for males of polygynous species. Reproduction may incur physiological costs that reduce an individual's lifespan. Pregnant

or nursing females are vulnerable to predation because they are either more conspicuous or less mobile.

1.6 How do hormones mediate physiological trade-offs?

Hormones are the signal molecules that drive phenotypic plasticity in development, physiology, and behavior (Husak *et al.*, 2009). Here, we briefly review three main hormone groups (sex, stress, and metabolism (thyroid)) and their effects on physiological performance.

In vertebrates, sex hormones mediate numerous and various behaviors associated with reproduction, mate attraction, defense, and aggression (Wingfield and Sapolsky, 2003; Wingfield, 1984). Androgens and estrogens regulate behavioral performance traits associated with reproduction (Moore, 2007; Wingfield, 1984; Wingfield *et al.*, 1990). In both sexes, androgens and estrogens are essential mediators of aggression and reproductive behaviors (e.g., sexual interest) (Wingfield, 1984). The relationship between sex steroid peaks and reproductive behavior is associated and dependent on reproductive status, such as parental care, sexual signals, and mating system. Oxygenated androgens, including dihydrotestosterone (DHT) and 11-ketotestosterone, occur in higher concentrations in males than in females and regulate male-specific behavior.

Increased androgen levels in many vertebrates increase the myonuclei in muscle fibers, satellite cells and regulate the growth of skeletal muscle during development (Finch and Rose, 1995; Ketterson and Nolan Jr, 1992). Thus, androgens mediate endurance traits by acting on the skeletal muscles (increase in the size of motor neurons) (Herbst and Bhasin, 2004; Husak *et al.*, 2009).

Reduced androgens are often associated with significant investment in parental care, whereas naturally or experimentally elevated testosterone is often associated with a substantial investment in mating effort via ornaments, armaments, or aggressive behavior (Ketterson and Nolan Jr, 1992; McGlothlin *et al.*, 2007; Santos and Nakagawa, 2012; Wingfield, 1984).

Physiological links between the social environment, behavior, and health are established by hormones. Testosterone, for example, is a well-known regulator of aggressive behavior (Wingfield *et al.*, 1987), whereas corticosterone is a stress hormone that responds to a range of environmental and social stressors. Species and individuals within species vary in how they respond to environmental stressors and other stimuli, as well as in how these adjustments result in changes in behavior and physiology (Sapolsky, 1990). Individuals' behavior and physiological responses are influenced by the social environment (Sapolsky, 1990; Sapolsky, 1992).

Using the competing color morph of the Gouldian finch (*Erythrura gouldiae*) as an example, the hormonal and immunological reactivity of red- and black-headed males to their social milieu is drastically different. In a socially competitive environment, red-headed male Gouldian finches elevate their testosterone levels to levels higher than those found in isolation, resulting in an increased stress response. Consequently, red-headed birds are unable to maintain ideal levels of immunocompetence. The subservient black-headed birds, on the other hand, have the opposite behavior (Pryke *et al.*, 2007). As a result of the genetic differences in endocrine responsiveness and sensitivity to socially competitive environments, the red-headed finches face significant trade-offs and possible health risks on a regular basis. The difference in responsiveness could explain why black-

headed birds have a larger population than red-headed birds in the wild (Pryke and Griffith, 2007; Pryke *et al.*, 2007). We should consider the role of social context in shaping individual behavioral and physiological responses to split infinitive understand the physiological trade-off mechanisms that generate and maintain phenotypic variation (Sapolsky, 1992).

Glucocorticoids, also called glucocorticosteroids, (GCs) are released into general circulation in response to a stressful or energetically demanding situation (Moore and Jessop, 2003; Romero, 2004; Sapolsky, 1990). Accordingly, elevation of plasma glucocorticoids is the most widely used indicator of stress in vertebrates. Within-organism, circulating glucocorticoids have been linked with both reproductive output and survival.

Glucocorticoids play significant roles in energy mobilization and thus have an adverse effect on energetically expensive functions like reproduction (Greenberg and Wingfield, 1987; Moore and Jessop, 2003; Selye, 1936; Selye, 1956). In response to perceived stress, vertebrates activate the hypothalamic-pituitary-adrenal axis (hypothalamic-pituitary-interrenal axis in fish) and release glucocorticoids into the bloodstream circulation to mobilize energy and suppress unnecessary processes to facilitate immediate survival (Wingfield and Sapolsky, 2003).

Stress and sex steroids are positively associated in other situations with high energy demands. For instance, glucocorticoids can be elevated along with sex steroids during the mating season (Moore and Jessop, 2003; Moore *et al.*, 2000; Romero, 2002). Reproductive status and performance are likely to occur during the energy regulation of glucocorticoid hormones. A few studies have found that mate choice preferences may be made on the basis of a potential mate's low glucocorticoid levels. Indeed, the sexually-selected traits

suppressed by stress hormones often serve as honest signals of mate quality (Roberts *et al.*, 2007; Roulin *et al.*, 2008; Wada *et al.*, 2008). Glucocorticoids are also potent regulators of macromolecules (carbohydrate, protein, and lipid) that fuel the energy demands of stress responses and promote energy for other metabolic functions (Balsevich *et al.*, 2019). The stress response mediated by GCs appropriates other physiological processes for immediate survival, sometimes at the cost of reproductive success. In tree swallows (*Tachycineta bicolor*), glucocorticoids levels predicted the fitness of females. Females that maintained low levels of glucocorticoids showed the highest reproductive success across years. (Vitousek *et al.*, 2018).

Metabolic hormones such as thyroid hormones, THs (triiodothyronine, T3, and thyroxine, T4) are necessary for metabolism regulation, embryonic development, growth, and survival (Deal and Volkoff, 2020; Liu and Brent, 2010; Mullur *et al.*, 2014; Sestoft, 1980) (Norris, 1997). Therefore, the release of thyroid hormones is an integral part of the physiological response to oxygen consumption.Thyroid hormones are linked directly to the calorigenic effect (Ismail-Beigi and Edelman, 1971; Sestoft, 1980). Thyroid hormone can activate oxygen consumption, much of the action depending on activation of Na+/K+-ATPase in the cell membrane (Cheng *et al.*, 2010). Metabolic hormones such as thyroid hormones respond to environmental cues to release energetic resources such as glucose and fat (Deal and Volkoff, 2020; Sestoft, 1980).

An organism's performance is fundamentally determined by physiology. Thus, variation in performance has the potential to explain variation in an individual's physiology. Physiological function can affect performance as a function the selection type directly and indirectly (Huey and Stevenson, 1979; Irschick *et al.*, 2008). For example, sex

hormones can maintain the growth and maturation of gonads. Here, the selection effect is direct. However, sex hormones can also influence performance indirectly, as in song performance in birds (Brenowitz, 2002; Norris, 1997; Norris and Evans, 2000). Thus, understanding how selection acts on physiological functions is critical for comprehending how processes evolve. Hormones can produce correlated effects with potentially antagonistic fitness consequences, precisely the situation to clearly demonstrate the existence of trade-offs (Williams 1957). Interactions and trade-offs with other physiological processes influence a large number of elements of hormonal function.

Several common themes regarding sex and stress hormones regulate the differential allocation of resources between the competing functions, particularly those related to reproductive effort. Many anurans spend time and energy on vocalizations, which are among the most energetically demanding activities known in vertebrates, and this is mediated by androgens (Leary *et al.*, 2004; Prestwich *et al.*, 1989; Stoddard and Salazar, 2011; Taigen and Wells, 1985; Taigen *et al.*, 1985). Hormone manipulation studies documented that testosterone in males often regulates the trade-off between mating effort and caring for offspring. Testosterone decreases parental care while increasing intrasexual aggression (McGlothlin *et al.*, 2007). In many biparental species, the trade-off in physiological functions is critical since both competing functions require different use of space and time, such as parental care and territory defense. Thus, investment in one function usually requires reduced investment in the other. Interestingly, elevated testosterone in male Dark-eyed Juncos increased a male's paternity while decreasing his parental care; it came out even in terms of seasonal fitness (Ketterson and Nolan Jr, 1992;

Ketterson *et al.*, 1992; Ketterson *et al.*, 1991; Lynn *et al.*, 2000). Oxidative stress/damage might be higher, which can impose a long-term cost.

Elevated plasma hormone during any costly activity often serves as an underlying mechanism that drives these functions and other energy expenditures. For example, high testosterone promotes reproductive success in some cases, increases growth (Yanase *et al.*, 2008) and cellular metabolism (Sato *et al.*, 2008). For example, treatment of immature rainbow trout (*Oncorhynchus mykiss*) with 11-ketotestosterone resulted in increased ventricular mass relative to body mass and increased the cross-sectional area of lateral red muscle relative to the size of the same features in adult males (Thorarensen *et al.*, 1996). One of the proposed roles for androgens is to increase reproductive males' endurance, which would enhance display behaviors (Oliveira, 2009; Thorarensen *et al.*, 1996). Such changes in activity should be associated with an increase in energy costs. Other studies have conflicting results regardless of the elevated testosterone influence. In dark-eyed juncos, exogenous testosterone implants enhanced reproductive success opportunities by increasing frequency of song, daily energy expenditure on territorial defense and mate-guarding (Ketterson *et al.*, 1992; Ketterson *et al.*, 1991; Lynn *et al.*, 2000). But testosterone partially suppressed parental care (Ketterson *et al.*, 1992).

Many previous studies investigated the effect of endocrine regulatory mechanisms on the life history trade-offs, specifically in ectotherms (lizards). In male spiny lizards, *Sceloporus jarrovi*, elevated testosterone increased daily activity and conspicuousness to predators and decreased body lean (growth). Increased time and energy cost of territorial aggression mediated by testosterone led to a decrease in survivorship (Marler and Moore, 1989).

Studies of effects of stress hormones on expenditures of energy differ. Many perspectives rely heavily upon the finding that high levels of glucocorticoids negatively influence any energetic cost to the animal (McEwen and Wingfield, 2003; Wingfield *et al.*, 1998). Prolonged stress in mammals accompanied by elevated levels of glucocorticoids disturbs reproductive physiology and behavior (Wingfield and Sapolsky, 2003). In the Arctic Ground Squirrel, high corticosterone levels suppress the immune response in arctic ground squirrels by lowering white cell counts, whereas red squirrels resist its immunosuppressive effects during reproduction (Boonstra and McColl, 2000). Glucocorticoids can regulate energy allocation by downregulating discretionary processes such as immune response in favor of reproduction, in which reproduction is linked with the exhaustion of energy reserves.

1.7 Examples of animal models and different types of life history trade-off strategies

One significant tradeoff in life history strategies is the tradeoff between the number of offspring and the amount of investment as a parent in the offspring (total investment in offspring) (Ricklefs and Wikelski, 2002; Sinervo and Svensson, 1998; Stearns, 1989; Stearns, 2000; Zera and Harshman, 2001). When allocating resources to offspring, parents must trade-off quantity against quality. The greater an organism's fecundity, the less energy (or other nutrients) it can put into each offspring. Most organisms that have a large number of offspring make a relatively minimal energy investment in each of them, and they do not normally provide parental care for them (Clutton-Brock, 2019; Fabian and Flatt, 2012; Kölliker *et al.*, 2012; Partridge and Sibly, 1991; Santos and Nakagawa, 2012). The classic example is female Pacific salmon species. Because of the long river separating the Pacific ocean from the inland spawning grounds, Pacific salmon are both R-selected and

semelparous. After making the up-river migration, females invest most of their energy budget in laying thousands of eggs in one spawning, reserving no energy for somatic maintenance or the return downriver. As is typical of R-selected species, only a small percentage of progeny make it to maturity (Groot, 1991; Quinn *et al.*, 1995). Because not much energy goes into each individual offspring, they start life with limited energy reserves, leaving them vulnerable to predation and risk of disease. The same situation is true for the female North Pacific Giant Octopus (*Enteroctopus dofleini*), which lives only four years at most, and lays thousands of eggs in a single episode before dying (Conrath and Conners, 2014; Young, 2010).

Organisms that are K-selected produce a small number of offspring typically make a significant energy investment in each offspring and sometimes offer extensive parental care (Clutton-Brock, 2019; Fabian and Flatt, 2012; Kölliker *et al.*, 2012; Santos and Nakagawa, 2012). Progeny are larger and more energetic. This strategy employed by many birds and all mammals, and in sharks minus the parental care. In some of these species neonates are altricial, helpless at birth and requiring a substantial amount of care.

The timing of reproduction is another life history factor subject to trade-off strategies involving age of first reproduction and lifespan. The chance of leaving no offspring is lower in organisms that reproduce early, but this may come at the sacrifice of their growth and health. For example, small fish, such as guppies, devote all their energy to reproduction early in life. As a result, they never grow to the size that would allow them to defend themselves against predators (Reznick *et al.*, 1990; Reznick *et al.*, 2004). To put it another way, the relevance of predation (a factor that mediates competition between ecologically similar species) provides part of the foundation that shapes the life history of some species.

Thus, the disparities in age-specific survival will shape the evolution of life-history patterns.

In general, the age at which a species reproduces is related to the species' overall longevity. Species with short lifespans begin reproducing at early ages, whereas species with extended lifespans are more likely to postpone reproduction until later in their lifespan. Frogs and insects are thought to have evolved larval stages (how they were evolved during larval stages) as a result of the trade-off

The characteristics of trade-offs have been explored using quantitative-genetic and optimization models during the last decade. The majority of these models are sophisticated variants of the previously described conventional "Y" model of allocation. A key finding of this research is that a positive correlation can exist between characteristics that are connected in a functional trade-off for several reasons (Farahpour *et al.*, 2018; Garland, 2014). Positive correlations between traits that comprise a functional trade-off can occur if variability in nutrient input among individuals is greater than variability in nutrient allocation. As such, this phenomenon as described, can be as a result of either genetic variation in loci that control nutrient acquisition or environmental variation in available resources resulting in developed allocation tree models of trade-offs and involving successive dichotomous trade-offs.

The resource allocation model has been shown to be a valuable heuristic for understanding the evolution of life histories, although it is presently unclear to what degree the relevant evolutionary constraints adhere to the model's assumptions (Young, 2018). While several types of "absolute" restrictions will undoubtedly affect life-history evolution, the nature of the absolute constraints at work and their relative relevance is less obvious.

Furthermore, the significance of what is referred to as "mechanistic constraints" in life-history evolution (i.e., evolutionary constraints arising from aspects of an organism's existing genetic, developmental, and physiological mechanisms, which are themselves a product of phylogenetic history) is still up for debate.

1.8 Summary

The review entails the diverse nature of life-history trade-offs within our environment. As such, when a limitation causes functional demands to compete with the amount of energy, time, and food available, a trade-off occurs. It highlights the progress made in understanding the life history trade-offs among physiological determinants of performance and reproductive success. The goals of our review aim to gain a thorough understanding of the physiological aspects of life-history trade-offs and how many species have adapted to increased energetic physiological demands within constrained resources, particularly during mating seasons.

Additionally, we have also examined the interactions between life-history trade-offs, trade-off assessments- in terms of measuring observable characteristics of individuals, investigating the genetic principle of trade-offs, as well as observing the manipulation of physiological functions, different physiological strategies of trade-offs, hormonal regulation of energetic processes and trade-offs, together with the consequences of these trade-offs on reproductive performance. To add to the above, optimal life-history strategies were explained since energy and nutrients are essential to the growth, maintenance, and reproduction of species.

Furthermore, examples of vertebrate animal systems used to research the physiology of life-history trade-offs. These animals were chosen to show the physiological interplay of trade-offs discovered through field and laboratory observational and experimental studies. Current understanding of variation in hormonally regulated life-history trade-offs was also reviewed.

1.9 Dissertation objectives and organization

Chapter 1 reviews the progress made in understanding the life history trade-offs among physiological determinants of performance and reproductive success. Current understanding of variation in hormonally regulated life-history trade-offs was also reviewed.

Chapter 2 explains how electric fish *Brachhypopomus gauderio* trade-off energy between competing metabolic demands. Thyroid hormone appears to regulate the allocation of energy between electric signals and somatic maintenance in favor of reproduction in both sexes. This chapter has been submitted to the Proceedings of the Royal Society B. Our study is the first to quantify a direct energetic trade-off between reproductive behavior and other metabolic functions.

In Chapter 3, we determined whether an increase in males' signal metabolism is associated with reduced effectiveness of the innate immune response. Chapter 3 is the first to report sex differences in innate immunity in electric fish *B. gauderio*. Chapter 3 has also been submitted for publication to Proceedings of the Royal Society B.

Chapter 4 examines whether thyroxine allocates more energy to male signaling by elevating the Na+/K+ ATPase expression of electrocytes. The differential pattern of gene regulation in our data suggests that ATPase is potentially involved in the diversification

and sexual dimorphism of electric signals. The chapter 4 manuscript is currently being written and will be published soon.

All the chapters are summarized in chapter 5. Future directions and research gaps in the studies are also discussed.

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CHAPTER 2: Electric fish trade-off energy between competing metabolic demands

"Nature has invented reproduction as a mechanism for life to move forward".

Louis Schwartzberg

2.1 Abstract

Physiological trade-offs are driven by internal competition between two or more functional demands for a limited resource. Our study is the first to quantify a direct energetic trade-off between reproductive behavior and other metabolic functions. Males of the weakly electric fish *Brachyhypopomus gauderio* produce reproductive signals that are among the most energetically costly of any animal measured, consuming 11-22% of the daily energy budget of males. We determined that high energetic demand of male electric signals is matched by a metabolic trade-off with other cellular functions. We used thyroxine implants to modulate the signal metabolism, partitioned the energy budget pharmacologically, and measured energy consumption using oxygen respirometry. In males, total energy consumption was unchanged pre- and post-thyroxine treatment, while signal metabolism rose and the standard metabolic rate fell in an even trade-off. In contrast to males, total metabolism in females rose under thyroxine treatment and females traded off metabolic functions in the opposite direction from males, boosting their non-signal resting metabolism at the expense of their signal metabolism. These results reveal metabolic trade-offs between signaling and cellular metabolism in electric fish and suggest that thyroid hormones regulate the allocation of energy between electric signals and somatic maintenance in favor of reproduction, likely at the expense of survival.

2.2 Introduction

Limitations in resources, or resource processing capacity, constrain animal performance (Boratyński, 2020; Dukas, 1998; Moore and Hopkins, 2009). The principle of resource allocation is derived from evolutionary adaptive planning, in which allocation of limited resources is selected and shaped within a field of constraints (Cody, 1966; Levins, 1968; Sheldon and Verhulst, 1996a). Physiological resources allocated towards a specific function cannot be used for another function at the same time; for example, energy or amino acids invested in growth are unavailable for reproduction and vice versa. As a result of allocation constraints, organisms cannot fully support all useful physiological functions at the same time. Trade-offs occur when an increase in demand for one or more functions conflicts with the existing allocation to another function (Hirshfield and Tinkle, 1975; Roff, 1992; Stearns, 1989). Conflicts in resource allocation are commonly seen during reproduction where investment of energy and nutrients into courtship, gametes, offspring, or parental care trades off against somatic maintenance, often at the cost of longevity or survival (Blomquist, 2009; Potts *et al.*, 1980; Santos and Nakagawa, 2012; Strum and Western, 1982).

Life history literature has emphasized trade-offs between reproductive effort and survival. For example, female brown anoles (*Anolis sagrei*) increase survival frequency if they decrease reproductive output (Cox *et al.*, 2010). Similarly female tree lizards (*Urosaurus ornatus*) with reduced egg production experience higher growth and reduced mortality (Landwer, 1994). Conversely, some taxa favor reproduction over survival by downregulating their immune responses to pathogens, even when food is not limited (Cox *et al.*, 2010; Schwenke *et al.*, 2016; Smith and French, 2017). Male Arctic ground squirrels

(*Urocitellus parryii*) trade-off immunity for reproduction, with corticosterone stimulating reproduction and lowering the white blood cell count (Boonstra and McColl, 2000). These pioneering studies demonstrated fundamental trade-offs in life history functions but left for the future the technically difficult tasks of identifying the limiting resources and measuring their reallocation between physiological compartments.

Trade-offs are presumed to follow from absolute limits on key resources. Energetic tradeoffs might be forced by limitations on food availability, the ability to process food, or the degree to which cells can metabolize available energy ($VO_{2\ max}$). During times of high energy demand, such as reproduction, migration, or winter thermoregulation, animals are expected to trade off between different physiological processes to provide energy sufficient for the most critical needs (Broeckhoven *et al.*, 2017; Brönmark *et al.*, 2008; Folkvord *et al.*, 2014). Mammals and songbirds seasonally grow and shrink their gonads and the size and connectivity of brain circuits involved in reproductive behavior (DeVoogd and Nottebohm, 1981; Tramontin and Brenowitz, 2000; Woolley, 1998), presumably to lower energy costs when these metabolically active tissues are not in regular use (Piersma and Lindström, 1997; Tekumalla *et al.*, 2002). In scatter-hoarding birds, similar changes are seen in the hippocampus, the forebrain structure critical to memory-dependent seasonal food storage and retrieval (Sherry and Hoshooley, 2009).

Previously, seasonal trade-offs were attributed to changes in differential mass of various tissues rather than changes in their metabolic activity (Weber and Piersma, 1996). Only recently have scientists directly measured dynamic energetic trade-off between metabolic activity of the tissues themselves. In female mice, food restriction was shown to restrict energy allocated to lactation (Zhao *et al.*, 2020). Pregnant guinea pigs given an

immune challenge, but fed *ad-lib*, nonetheless restricted the energy they allocated to fetal growth (Trillmich *et al.*, 2020). Those authors postulated that females anticipated a future need for trade-offs, however reduction in fetal growth during immune challenge under *ad-lib* food conditions might also suggest a physiological limit on the ability to process or deploy critical nutrients. To our knowledge, that is the only study to measure trade-offs in energy allocated between physiological processes when food is not restricted. Presumably, however, such trade-offs are common.

Speakman (Speakman, 1997) suggested that animals divide their internal energy resources systematically between reproduction and basal metabolism (or standard metabolism in poikilotherms), subject to a constraint on the total amount of energy available for allocation, effectively a metabolic ceiling (Drent and Daan, 1980) (Figure 1). Under Speakman's model, once an animal's total metabolism reaches this ceiling, an inverse relationship ensues between the amount of energy allocated to various metabolic compartments. For instance, a metabolic increase in a structure or tissue involved in reproduction might be fueled by energy taken from the basal metabolism, or vice versa, creating an energetic trade-off (Speakman, 1997). Allocation of energy can be directed by regulatory mechanisms at different organizational levels, including physiological, environmental, and strategic trade-offs (Elliott *et al.*, 2015; Elliott *et al.*, 2014). Metabolism varies both among species and between individuals of the same species (Careau *et al.*, 2008; Pontzer *et al.*, 2021) so trade-offs should be species-typical and regulated dynamically at the individual level.

Electric organ discharges (EODs) of weakly electric fish are generated by excitable cells (electrocytes) located in the bilateral electric organs (Bennett, 1961; Szabo, 1974).

Because EODs can be measured quantitively and manipulated hormonally, electric fish are ideal models in which to study the evolution and physiology of communication and, in particular, the energetic cost of signals (Stoddard *et al.*, 2006). In reproductive female fish the standard metabolism cannot be separated readily from metabolism devoted to egg production (Chabot *et al.*, 2016). In our studies of metabolism in electric fish, we combine metabolic components under the moniker "residual metabolism," meaning what is left of the resting metabolism when electric signaling is suppressed. Residual metabolism and the metabolism of electrogenesis in the gymnotiform electric fish *Brachyhypopomus gauderio* are tightly and positively correlated across individuals (Salazar and Stoddard, 2008a). In reproductive males, however, an inverse relationship in energy allocation was found, in which those males with larger electric signals and higher metabolic expenditures on electrogenesis devoted less energy to the residual (standard) metabolism of other tissues, and vice versa (Stoddard and Salazar, 2011). While the electric organ discharge (EOD) is a multi-function sensory and communication signal, the fact that only males showed this inverse relationship is evidence that reproductive signaling function could be driving a metabolic trade-off rather than the EOD's function in active electrolocation. This inverse relationship, seen across multiple reproductive males, suggested an males might be making an energetic trade-off between reproductive signaling and general cellular maintenance such as immune system and cellular repair. Those authors speculated such a trade-off stemmed from an absolute limit on available energy as diagramed by Speakman (1997) (Speakman, 1997). The existence of such metabolic ceilings is well established (Drent and Daan, 1980; Elliott *et al.*, 2014), however direct evidence of an energetic trade-off around this ceiling has never been shown at the level of the individual. Such a demonstration would

be necessary to validate Speakman's model of the mechanism underling the energetic trade-offs at the metabolic level.

Energy expenditure resulting from metabolic activity can be measured either from the rates of carbon dioxide production or oxygen consumption as the animal oxidizes substrates to produce ATP and CO_2 (Brown *et al.*, 2004). Flow-through oxygen respirometry has demonstrated that the energetic cost of electric signals increases disproportionately with the EOD power (estimated as the median integral of the square of the waveform (Salazar and Stoddard, 2008a)) and EOD rate (the reciprocal of the median inter-pulse interval) (Lewis *et al.*, 2014); oxygen consumption per EOD increases as EOD power increases and as EOD rate increases. Males emit more powerful EODs at a higher rate than females and thus consume more ATP and oxygen to maintain membrane polarity while producing those signals.

We sought to determine whether the high energetic expense of electric signals in male *B. gauderio* can induce individuals to make a metabolic trade-off. If electric fish are operating at or near their metabolic limit, then forcing an increase in signal metabolism should drive a decrease in the residual metabolic rate (RMR), and vice versa. The EOD waveforms of many electric fish are plastic, under the regulation of hormones (Allee *et al.*, 2009; Bass and Hopkins, 1985; Dunlap and Ragazzi, 2015; Gavassa and Stoddard, 2012; Goldina *et al.*, 2011; Markham *et al.*, 2009; Markham and Stoddard, 2005), among them thyroid hormone, the master regulator of metabolism (Mullur *et al.*, 2014; Sestoft, 1980). We implanted *B. gauderio* of both sexes with the thyroid hormone thyroxine (T4) to stimulate their metabolisms, making the educated guess that only one or some energetic compartments would increase in activity, thereby forcing a decrease in others. We then

partitioned the energy budget pharmacologically and measured energy consumption of the signal metabolism and residual metabolism before and after thyroxine implant with flow-through oxygen respirometry, using established methods (Salazar and Stoddard, 2008a).

Our study tested two hypotheses. A key component of Speakman's metabolic trade-off model (Speakman, 1997) is that the total metabolism is constrained under a metabolic ceiling (Figure 1). If reproductive male *B. gauderio* are operating at their metabolic ceiling, then increasing signal metabolism through hormonal manipulation should not increase total oxygen consumption. The findings of Stoddard and Salazar (2011) were consistent with the hypothesis that sexually mature males are operating at their metabolic ceiling whereas females are not, but their data could not evaluate the hypothesis directly. On the basis of their findings, we predicted that driving one metabolic component higher with hormonal implants will not increase total metabolic output in males but might do so in females. If total metabolism is at the ceiling, an increase energy expended in one metabolic compartment will force a decrease in another. The trade-off should be seen in reproductive male *B. gauderio* implanted with a hormone that boosts energetic expenditure on electric signals. For males that have reached their metabolic ceilings, an increase in signal metabolism would be accompanied by a decrease in the residual metabolism. Conversely, females should not have reached their metabolic limits, so stimulating their metabolisms hormonally should elicit increases in total metabolic output and similarly signed slopes between metabolic compartments (Figure 1B).

2.3 Materials and methods

2.3.1 Subjects

Brachyhypopomus gauderio were raised and maintained in outdoor pools on the roof of the AHC1 building at FIU in Miami, Florida. Fish were kept in mixed sex social groups of 10-12 at 27-30° C, conductivity 70-120 µS/cm, and pH 6.5-7. The fish were fed oligochaetes ("blackworms") *ad libitum*, plus whatever chironomid larvae inhabit the pools naturally. Two weeks before an experimental procedure, sexually mature fish were selected at random from the colony and moved to the lab, where they were maintained in 26-liter polycarbonate aquaria filled with water conditioned to match the roof pools. In the lab, the fish acclimated in social groups of two males and one female. Subject males were 130-188 mm in length and weighed 4-8 g. Subject females were 115 to 145 mm in length and weighed 3-10 g. Indoor fish were fed blackworms every other day but were fasted 24 h prior to each respirometry run to assure the oxygen we were measuring was metabolized from a single stored fuel (lipids).

2.3.2 Pharmacological partitioning of the energy budget

We used an established pharmacology procedure to separate the energetic cost of electric signal generation from the remainder of the cellular metabolism in resting fish (Salazar and Stoddard, 2008a).We applied two pharmacological agents to suppress different aspects of metabolism. The tranquilizer (+) metomidate HCl (Aquacalm^{tm}, Western Chemical, Inc.) is a GABA binding enhancer that suppresses swimming, ventilation, and muscle tone without silencing EOD generation. The paralytic drug flaxedil (gallamine triethiodide, G8134, Sigma-Aldrich) is a curare analog that binds with moderate

affinity to the nicotinic acetylcholine receptors on the electric organ, reversibly silencing the electric signal of a fish already tranquilized with metomidate (Figure S1). We measured oxygen in the water exiting the respirometry tube (described below) with no fish present ($VO_{2\ Water}$) and with the fish at rest under three pharmacological states (Figure 2):

(1) Fish at rest with no drugs ($VO_{2\ Total}$) produced our oxygen measurement of the total metabolism.

(2) Fish on metomidate alone ($VO_{2\ Met}$), which includes the electric signal ($VO_{2\ EOD}$) + residual metabolism ($VO_{2\ RMR}$), but no motion or muscle tone ($VO_{2\ Motor}$).

(3) Fish on metomidate + flaxedil, provided measurements of just the residual metabolism ($VO_{2\ RMR}$).

Subtracting measurements #2 from #1 yields the metabolic rate from motor activity at rest, $VO_{2\ Motor}$. *B. gauderio* is nocturnal, so the measurement reflects the fish's normal daytime state. Subtracting measurements #3 from #2 yields the instantaneous oxygen consumption rate from electrogenesis. The instantaneous rate is adjusted to the mean daytime EOD rate of 23 Hz (Salazar and Stoddard, 2008a), yielding $VO_{2\ EOD}$, the daytime oxygen consumption rate attributable to electrogenesis (Figure 2). Subtracting measurement #3 from the measurement of water in the tube with no fish present yields the residual metabolic rate ($VO_{2\ RMR}$).

2.3.3 Respirometry

Oxygen consumption measured with pass-through respirometry was used as proxy for energy consumption (Salazar and Stoddard, 2008a).A 280-liter tank (122 cm long x 46 cm wide x 50 cm tall) was filled with filtered, deionized water adjusted to match

temperature and conductivity in the outdoor pools and conditions in nature. The fish was held in the geometric center of the tank inside an unglazed ceramic tube with an acrylic inspection window. Air-saturated water was delivered into the rostral end of the ceramic tube at 30 mL • min^{-1} by a peristaltic pump (Masterflex L/S, Model 77200-62, Cole-Parmer Instrument Co.) and flowed out the caudal end of the tube. Oxygen concentration of water flowing out of the tube was measured with an optical oxygen probe (FOSPOR-R NeoFox, Ocean Optics, Inc.), calibrated to zero in water deoxygenated with nitrogen gas. When the fish was tranquilized by metomidate, a mesh and foam cradle apparatus held the fish in the center of the tube and assured that water flowed over the gills. Before the start of each experiment, we waited until the oxygen measurement was stable for 7–10 min before recording oxygen concentration and the fish's EOD.

Oxygen concentration of water (ppm) was monitored with the fish at rest with no pharmacological treatment ($V\dot{O}_{2\ Total}$). The fish was induced for 15 min with a solution of metomidate in tank water 0.20 g/liter, approximately 15-20 minutes until oxygen consumption stabilized ($V\dot{O}_{2\ Met}$). The fish's EOD was silenced with an IM injection of flaxedil (3 µg/g). Oxygen consumed by the residual metabolism ($V\dot{O}_{2\ RMR}$) was recorded when the EOD had completely disappeared from the electric field recordings and the $\dot{O}_2$ recordings had stabilized. At the end of the run we relocated the fish to the recovery tank and recorded the oxygen concentration of water exiting the respirometry tube in the measurement tank to record system background respiration ($V\dot{O}_{2\ Water}$). Water was pumped over the fish's gills in the recovery tank until it had recovered from the flaxedil, usually 10-20 hours, whereupon it was returned to the social tank.

2.3.4 EOD measurements

The electric signal was measured through the walls of the ceramic tube with a pair of nichrome wires fixed at opposite ends of the tank, amplified with a differential bioamplifier (World Precision Instruments Inc., Sarasota, FL USA, AC-coupled, low-pass filter corner 0.1 Hz, high-pass filter corner 10 kHz), and digitized at 16 bits, 50 kHz (National Instruments, USB-6216). The EOD recording methods followed Franchina and Stoddard (1998). The EOD records (1 s) were digitized three times during each experiment (resting, metomidate, and metomidate + flaxedil). From the metomidate recording we calculated EOD rate as the reciprocal of the median inter-pulse interval. The EOD power was estimated as the median integral of the square of the digitized waveform values, following Salazar and Stoddard (2008a). Data acquisition and calculations were performed using custom software written in MATLAB (Mathworks).

2.3.5 Hormone implantation

At least eight days after the baseline recordings, we implanted 11 males and 10 females with small silastic tube containing the thyroid hormone L-thyroxine (T4) (T2376; Sigma-Aldrich) following methods of (Dunlap and Ragazzi, 2015), or with a saline control (7 males, 8 females). Each silastic tube (0.51 mm ID x 0.94 mm OD x 0.23 mm wall, ~1.60 mm long) contained 0.5 mg of T4 or saline for the control. We anesthetized the fish by immersion in a 0.6 ml/liter clove oil (eugenol) solution and inserted the implant intraperitoneally, ventral to the lateral line and dorsal to the electric organ, through a hole between two ribs made with a sterile 18-gauge hypodermic needle, then sealed with surgical glue (Vetbond^{TM}, 3M). Implantation took less than three minutes, after which the

fish was returned to its aquarium. On the basis of studies with goldfish, *Carassius auratus* (Hurlburt, 1977), our T4 implants should have elevated plasma T4 levels by ~1 ug/ml. We repeated the respirometry procedure three days after implantation to measure changes in the metabolic compartments induced by T4.

Initially, we tested 16 males implanted with 5α-dihydrotestosterone (DHT), which, along with other androgens, has previously been found to masculinize the EOD (Allee *et al.*, 2009; Hagedorn and Carr, 1985). However, we found the masculinizing effects of DHT on EOD power were inconsistent (Figure S4), as previously noted for testosterone and 11-ketotestosterone (Goldina *et al.*, 2011), as were effects on the metabolism of the electric organs. Implants of L-thyroxine (T4) did not masculinize the EOD but did prove highly consistent at modulating the metabolic compartments in both sexes, including that of the electric organ.

2.3.6 Data analysis

Distribution of metabolic differences before and after implantation were skewed upwards, as indicated by Kolmogorov-Smirnov tests. Unless otherwise specified, p values are reported from non-parametric 2-tailed Wilcoxon sign-rank tests. The two one-sided test for equivalence (TOST) was done on data log-transformed to normal. Statistics were performed in MATLAB R2020b.

2.4 Results

Following T4 implants, male signal metabolism ($\dot{V}O_{2\ EOD}$) increased by 47% ($p = 0.002$; Figure 3A) while residual metabolic rate ($\dot{V}O_{2\ RMR}$) fell by 50% ($p = 0.002$; Figure 3C), a striking metabolic trade-off between the males' signals and their other cellular

functions (Fig. 4A). The males' total oxygen consumption remained unchanged after receiving T4 implants (Wilcoxon $p = 0.85$; TOST < 20% difference, $p = 0.03$; Figure 3E), consistent with the metabolic ceiling prediction of Speakman's model. Saline control implants induced no significant change in the metabolic compartments (Figure S2) and no consistent trade-off between signal metabolism and residual metabolism (Figure S3). DHT implants in males produced inconsistent results (Figure S4).

The T4 had precisely the opposite effect in females. Following T4 implants, female signal metabolism fell by 47% (Wilcoxon $p = 0.002$; Figure 3B) while residual metabolic rate rose by 67% ($p = 0.001$; Figure 3D). Thus, T4 caused females to trade-off signal metabolism and residual metabolism in the opposite direction as males (Figure 4B). In females, the total oxygen consumption rose after T4 implant ($p = 0.04$; Figure 3F) consistent with the model that females are functioning below the metabolic ceiling. As in males, saline control implants induced no significant change in the metabolic compartments (Figure S2) and no consistent trade-off between signal metabolism and residual metabolism (Figure S3).

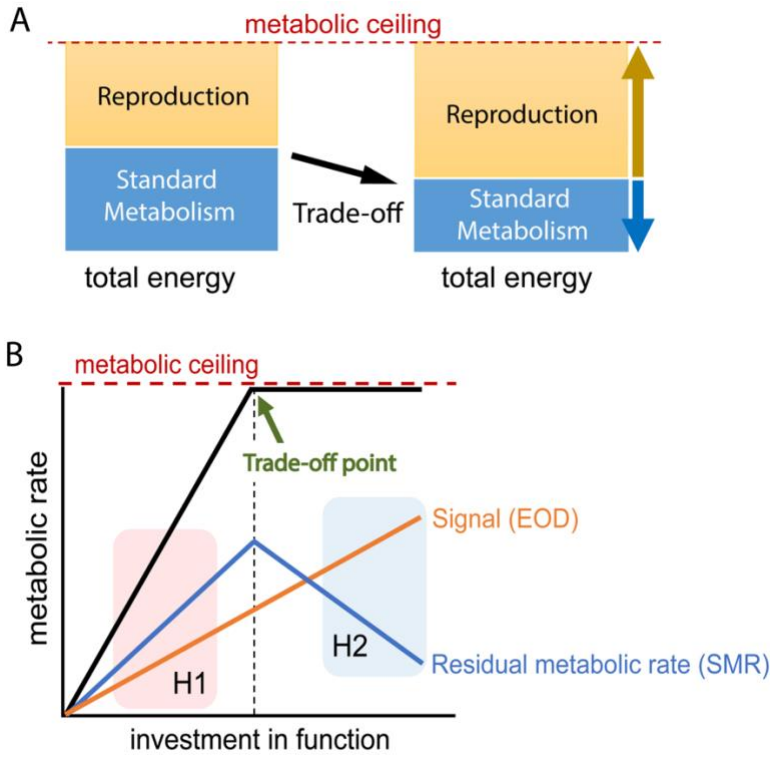

Figure 1. (A) Energy allocation model (following Speakman 1997) in which total available energy is divided between two functions, reproduction and standard metabolism. Increase in energy use by one function will lead to a decrease in the amount of energy used by the other function and vice versa. (B) Predictions from Speakman's (1997) metabolic trade-off model. In the pink-shaded patch, H1, two female metabolic components are positively correlated. Their sum falls below the metabolic ceiling such that neither component is constrained by the other. In the blue-shaded box, H2, males are operating at the metabolic ceiling. As the signal metabolism (gold) rises, the standard metabolism (blue) must fall to keep the total within the metabolic ceiling, effecting a trade-off between the two metabolic functions.

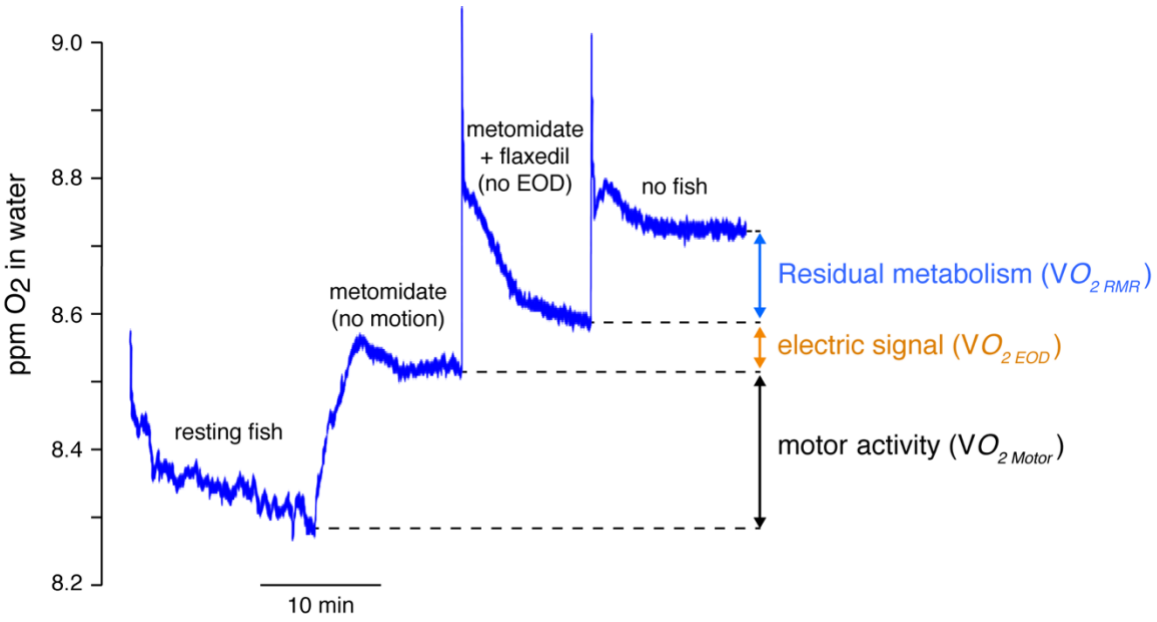

Figure 2. Oxygen respirometry recording, showing the energy budget of *B. gauderio* as an additive function that can be partitioned by subtracting oxygen measurements obtained under different pharmacological treatments.

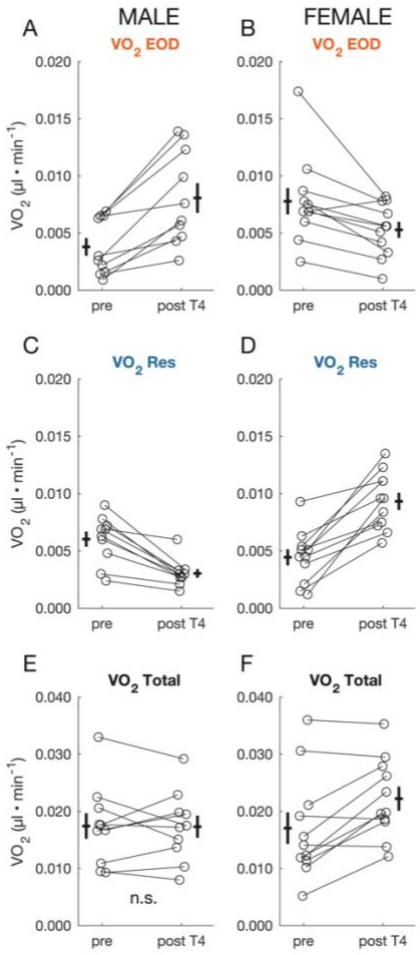

Figure 3. (A-F) Oxygen consumption by the different metabolic compartments in both sexes of mature *B. gauderio*, measured before and after T4 implantation. Means and standard errors are shown next to the raw data. (A) Males' electric signals consume more oxygen post T4 than before. (B) Females' electric signals consume less oxygen post T4 than before. (C) Males consumed less oxygen for residual metabolism post T4, whereas females **(**D) consumed more. (E) Total oxygen consumption by males is not affected by thyroxine, consistent with a metabolic ceiling predicted by the model. (F) Females consume more oxygen after T4 implant, indicating that they were not at a metabolic ceiling prior to T4 treatment.

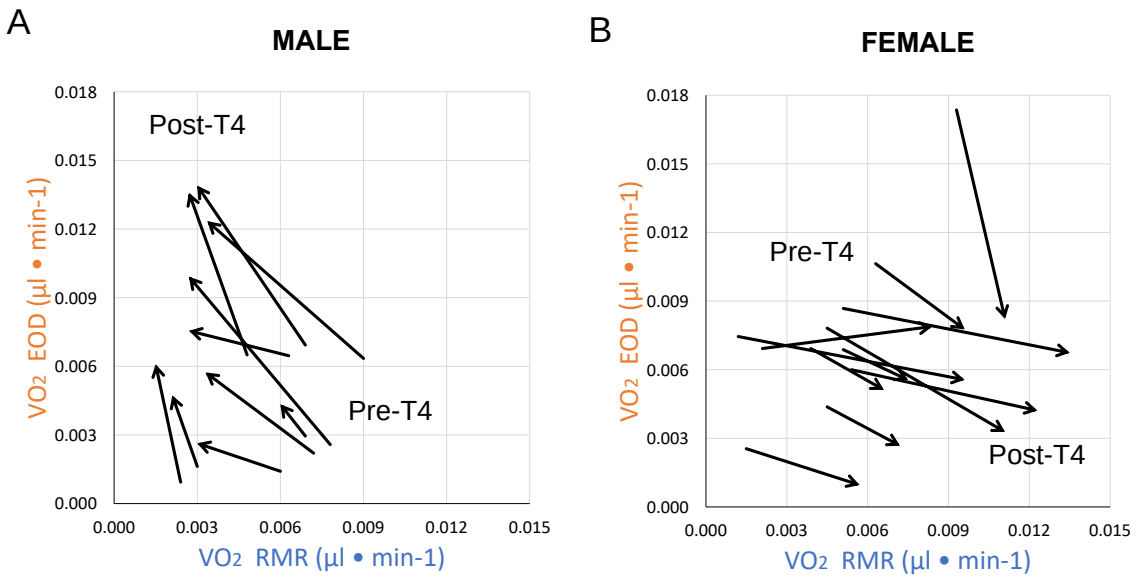

Figure 4. Effect of T4 on oxygen consumed by the residual metabolism versus signal production. (A) After the T4 implant, males consume more energy in signal production and less in cellular metabolism. (B) T4 causes females to direct the energy into the residual metabolism and away from electric signals. Arrows show the direction of change in oxygen consumption after the T4 implant.

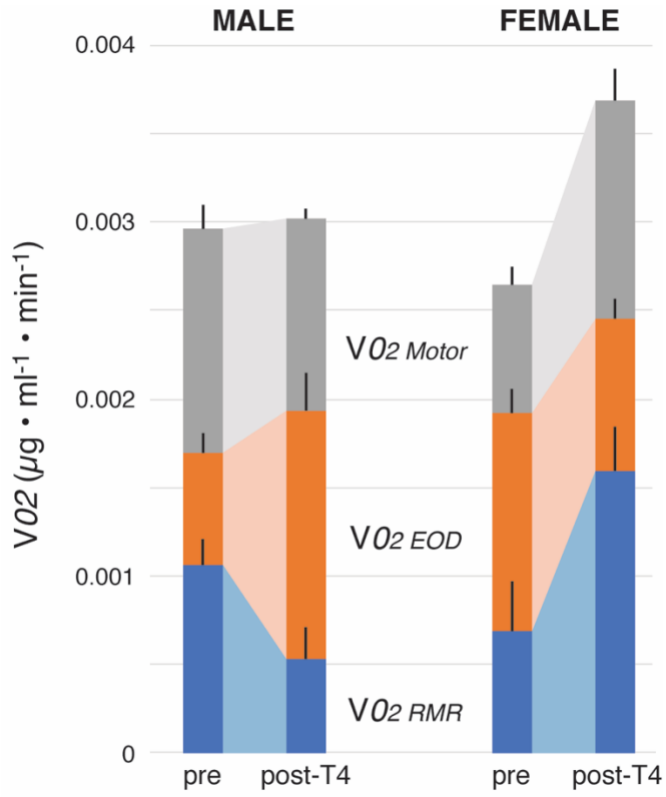

Figure 5. Summary of T4-mediated trade-offs, showing the means and standard errors for the two sexes. All the key effects of T4 are evident: in males VO2 EOD increases while VO2 RMR decreases and VO2 Total remains unchanged. In females VO2 EOD decreases, while VO2 RMR increases and VO2 Total rises.

Supporting Figures and Tables

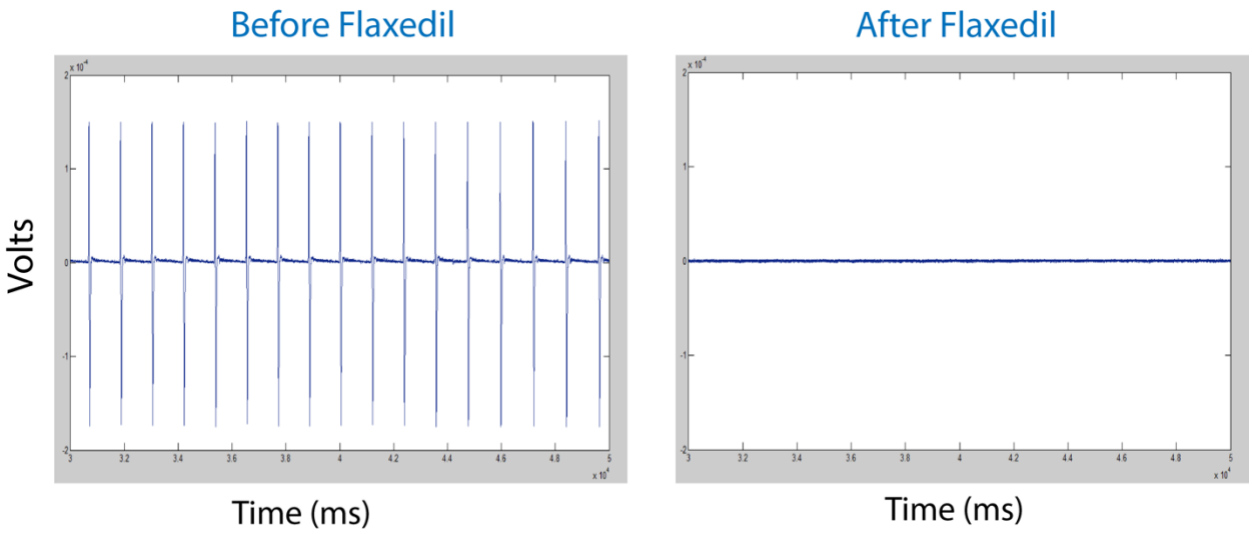

Figure S1. EOD waveform recordings showing that flaxedil successfully suppressed the electric signals.

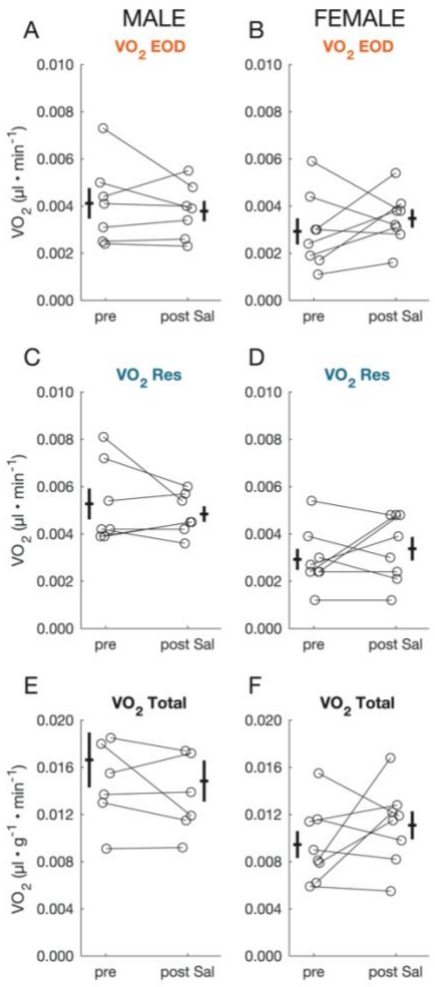

Figure S2. Saline implants had no significant effect on oxygen consumption of *B. gauderio* for EOD production (A, B), residual metabolism (C, D), or total oxygen consumption (E, F). Means and standard errors are shown next to the raw data.

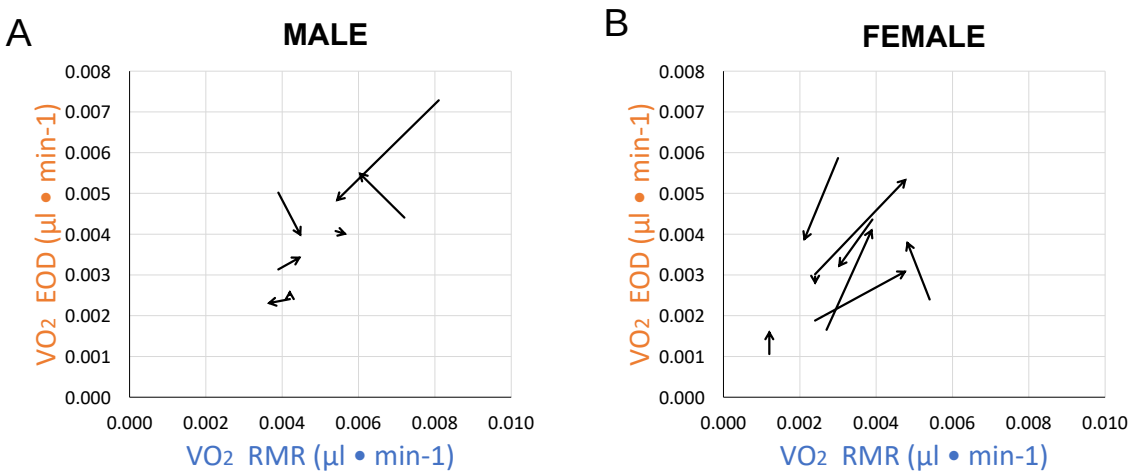

Figure S3. Saline implants in both sexes of *B. gauderio* did not produce consistent tradeoffs between oxygen consumed by the residual metabolism and signal production.

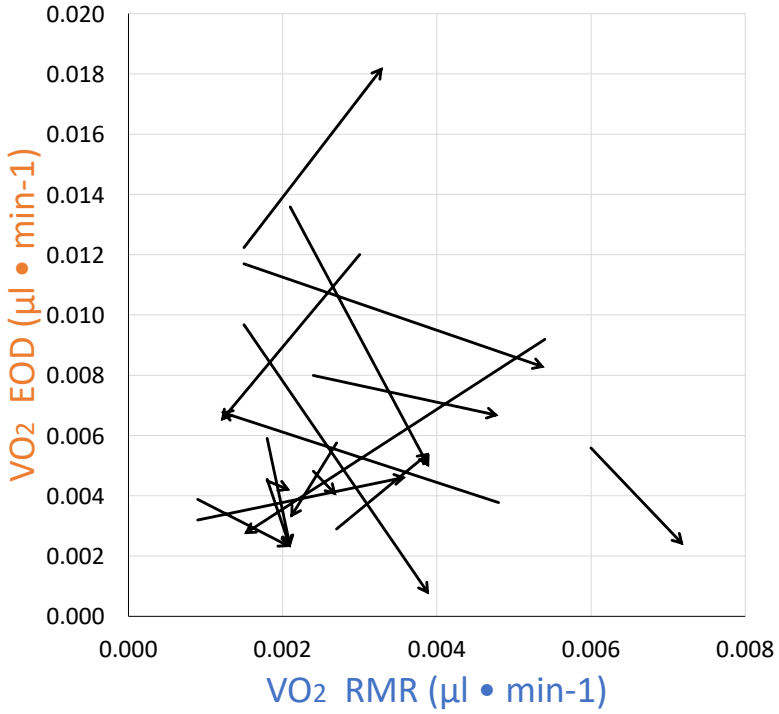

Figure S4. DHT implants in male *B. gauderio* produced no consistent trade-offs between oxygen consumed by the standard metabolism and signal production.

	Male (Pre-T4)	Male (Post-T4)	Female (Pre-T4)	Female (Post-T4)
EOD	3.8E-03± 7.8E-04	8.1E-03± 1.3E-03	7.8E-03± 1.2E-03	5.3E-03± 7.0E-04
RMR	6.0E-03± 6.6E-04	3.0E-03± 3.7E-04	4.4E-03± 7.0E-04	9.3E-03± 7.5E-04
Other	7.6E-03± 1.4E-03	6.2E-03± 1.1E-03	4.8E-03±2.8E-03	7.6E-03± 1.7E-03
Total	1.7E-02± 2.2E-03	1.7E-02± 1.9E-03	1.7E-02± 2.8E-03	2.2E-02± 2.2E-02

Table S1. Males and females *B. gauderio* signal, cellular and total oxygen consumption. Summary showing the male and female electric fish oxygen consumption (µl/ min) (residual metabolic rate (RMR), signals (EOD) and total oxygen consumption pre and post thyroxine. Values (oxygen consumption, µl/ min) are mean ± SEM. Sample size are 10 males and 11 females. Asterisks denote significantly different values after thyroxine implantation determined by a paired t-test, 2-tailed, alpha=0.01.

2.5 Discussion

2.5.1 Trade-offs found, both expected and unexpected

On the basis of a previously reported inverse correlation between the signal metabolism and residual metabolism in male *B. gauderio* (Stoddard and Salazar, 2011), we had predicted that individual males would trade off between these compartments as the signal metabolism of individuals was pushed upwards by hormonal treatment. We found that both sexes trade off between signal metabolism and residual metabolism under the influence of T4, but in opposite directions (Figure 4 & 5). Following T4 implantation, males boosted metabolic expenditure on electric signals at the expense of the residual metabolism (standard metabolism) while females increased their residual metabolism (standard metabolism + egg production) and reduced signal metabolism. Sex steroids likely regulate the direction of the trade-off driven by T4. Both sexes are likely using this trade-off to favor reproduction in a sex-specific manner. Males use their enhanced electric signals when competing with other males and attracting and courting females (Franchina *et al.*, 2001; Gavassa *et al.*, 2013b), whereas females increase reproductive success by producing more eggs, a metabolic cost that falls within the residual metabolism. In amphibians, the thyroid hormone T3 (the more bioactive conversion product of T4) is known to upregulate transcription of vitellogenin, a key egg protein (Duarte-Guterman *et al.*, 2014).

2.5.2 A metabolic ceiling?

As we predicted, total energy consumption in males was unchanged by T4, in keeping with the proposed metabolic ceiling in Speakman's model (Figure 1B). However, in females, the total metabolic oxygen consumption increased about 40%, driven largely by

a 130% increase in residual metabolism (Figure 3 & 5). These findings are consistent with the idea that females started the experiment below their metabolic ceiling, but T4 drove their RMRs upwards until a metabolic limit forced the signal metabolism downwards. The metabolic ceiling could be mechanism that senses total metabolic expenditure and exerts negative feedback on select metabolic compartments when a threshold is reached. Conversely, total metabolism may be a more open circuit, with each metabolic compartment independently programmed to regulate itself according to circulating molecular signals. Resolution of this uncertainty would benefit from finer temporal resolution of the metabolic dynamics as thyroid hormones drives the trade-offs documented above and respirometry of the isolated cell types hormonally manipulated in culture.

During times of high energy demand, organisms are expected to trade off between different physiological processes to provide sufficient energy for the most critical needs. Many taxa down-regulate immune response to pathogens during the reproductive season (Ahtiainen *et al.*, 2005; Cox *et al.*, 2015; Cox *et al.*, 2010; Schwenke *et al.*, 2016; Smith *et al.*, 2017). In the Brown Anole, reproduction causes marked reduction in immunity, a likely trade-off between reproduction and self-maintenance (Cox *et al.*, 2015). Pregnant viviparous snakes challenged with LPS endotoxin, simulating a bacterial infection, increased their lymphocyte counts, but reduced the litter mass (Lind *et al.*, 2020), demonstrating not only the high physiological cost of immunity, but the trade-off between immunity and other critical functions. We speculate that the reduction in residual metabolic rate seen in our male *B. gauderio* could have been largely a reduction in some aspect of the immune system. The specific organs or systems where cellular functions enhanced by

thyroxine in females were not revealed by our pharmacological partitioning but can be investigated with different methods in the future.

2.5.3 Differential regulation of metabolic compartments by thyroid hormone

Electric signal waveforms of electric fish are regulated at the subcellular level by steroids and peptide hormones (Stoddard *et al.*, 2006). Electrocytes can dynamically regulate the number of ion channels in the plasma membrane, increasing ion flux in electrocytes (Markham *et al.*, 2009). Androgens have also been shown to cause hypertrophy of the electric organ and changes in the electric waveform as well (Hagedorn and Carr, 1985). Thyroid hormones have been previously shown to increase the frequency of the signals of the gymnotiform *Apteronotus leptorhynchus* (Dunlap and Ragazzi, 2015). The hormone T4 was sufficient to increase the energy expended by electrogenesis in males, but not to boost the EOD power measured at a distance from the fish, an effect driven instead by androgens and melanocortins acting in tandem(Goldina *et al.*, 2011). Thyrotropin releasing hormone (TRH) causes both an increase in circulating thyroid-stimulating hormone (TSH) that stimulates T4 release, as well as a release of circulating melanocortins that boost the power of the EOD (Markham *et al.*, 2009; Rotllant *et al.*, 2000). While T4 did not increase the male's signal power measured at a distance, it likely increased amount of energy consumed by the electrocytes in making signals. The energetic demand of electric fish signals is set by the number of Na+ ions that flow into electrocytes during the action potentials that make up the electric organ discharge (Salazar *et al.*, 2013). The energetic cost of action potentials occurs when Na+/K+ ATPase hydrolyzes ATP to restore the Na+ and K+ gradients across the excitable cell membrane (Attwell and

Laughlin, 2001; Hasenstaub *et al.*, 2010; Laughlin *et al.*, 1998; Lennie, 2003). Electrogenic cells in *B. gauderio* make EODs from pairs of inward Na+ fluxes on opposing posterior and anterior membrane surfaces, one current headward and one tailward, offset in time by 30-100 µs (Markham and Stoddard, 2005; Markham and Stoddard, 2013). Temporal offset of these directionally opposed Na+ fluxes is regulated by melanocortins acting in concert with androgens (Goldina *et al.*, 2011; Markham and Stoddard, 2005). That T4 raised the EOD's energetic expense for males while lowering their EOD power output suggests that T4 acting alone boosts the Na+ flux of action potentials in the males' electrocytes without maintaining or increasing the temporal offset of the flux pair.

The mechanism by which thyroid hormone exerts opposite effects on metabolic components in the two sexes of electric fish could be through differential effects of androgens and estrogens on expression of thyroid receptor isoforms or co-repressors. While thyroid hormone typically increases cellular metabolism, it can also lower metabolism in particular cell groups through differential expression of receptor isoforms and other regulators. For instance, the TRa2 isoform does not bind T3, but rather acts to reduce its action, while co-repressors, such as NCoR and SMRT, decrease transcription of T3-regulated genes (Mullur *et al.*, 2014). Our data indicate that the metabolism rates of some cell groups are differentially regulated by thyroxine according to sex. Whether those changes occur through regulation of cell number or per-cell metabolic activity remains to be determined, and both mechanisms are possible.

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CHAPTER 3: Sex differences in innate immunity in the electric fish *B. gauderio*

"The cells in your body react to everything that your mind says"

Bruce Lipton

3.1 Abstract

Trade-offs between physiological functions are a universal feature of life history strategies. Mounting an immune response can be metabolically costly, so animals sometimes restrict immune responses when reproduction demands a higher investment of the body's resources. In the gymnotiform electric fish *Brachyhypomus gauderio* thyroid hormone appears to regulate the allocation of energy between energetically expensive signaling behavior and somatic maintenance in ways that favor reproduction in both sexes. We previously found that thyroxine (T4) treatment induces large sex differences in the trade-off between the signal metabolism and the rest of the metabolism. The T4 causes males to increase signal metabolism and females to decrease it, while the rest of the metabolism in each sex changes in the opposite direction. To determine whether these trade-offs involve the innate immune system, we assessed changes in the bactericidal activity of plasma in mature *B. gauderio* challenged with lipopolysaccharide (LPS) before and after T4 treatment. Females did not modulate bactericidal activity of plasma following any of the treatments, while males elevated bactericidal activity by about a third following LPS injections, T4 implants, or both together, relative to controls. The outcome was unexpected given that T4 also increases the energy consumed by the male's reproductive electric signals while lowering the rest of his metabolism. Upregulation of humoral innate

immunity may be a short-term survival strategy for males, while possible changes to acquired immunity remain unexplored.

3.2 Introduction

The energetically demanding processes of reproduction and immune defense have been observed to trade-off against one another in a variety of animals (Ahtiainen *et al.*, 2005; Demas, 2004; French *et al.*, 2007a; Sandland and Minchella, 2003; Sköld-Chiriac *et al.*, 2019). A trade-off in resource allocation arises when physiological functions compete for one or more limiting resources (Lind *et al.*, 2020; Lochmiller and Deerenberg, 2000; Martin *et al.*, 2008). A trade-off between reproductive effort and immunity can occur when resource allocation for reproduction forces the individual to reduce constitutive and/or induced immunity, but can also occur reciprocally when reallocation of the resources required to mount a response to infection forces the individual to reduce reproductive output (Ahmed *et al.*, 2002; Contreras-Garduño *et al.*, 2014; Deerenberg *et al.*, 1997; French *et al.*, 2007b; Nordling *et al.*, 1998).

Several studies have demonstrated that stress and resource scarcity impair immunocompetence during the reproductive season (Demas, 2004; Demas *et al.*, 1997; Folstad and Karter, 1992; Hanssen *et al.*, 2004). This evidence of the cost of immune defenses may imply that suppressing immune responses is adaptive when the risk of incurring such costs exceeds the predicted expenses associated with the infection. As a result, we can forecast an adaptive immunosuppressive strategy during times of stress and resource scarcity.

Reproductive functions, including courtship, egg production, litter size, and lactation, have been associated with compromised immunity in many taxa (Ahtiainen *et al.*, 2005;

French *et al.*, 2013; Künkele, 2000; Smith and French, 2017; Trillmich *et al.*, 2020; Uller *et al.*, 2006a). Accordingly, trade-offs between reproductive effort and immunocompetence have been documented in a wide variety of taxa. Under limited recourses, increased reproductive effort in female tree lizards (*Urosaurus ornatus*) resulted in immunosuppression (French *et al.*, 2007a). In another study, the immune challenge (exposure to bacterial lipopolysaccharide) led to a decline in egg mass of the female dragon lizard, *Ctenophorus fordi*. However, no effect was observed on the offspring, except that juvenile lizards showed a decrease in growth rate (Uller *et al.*, 2006a). Immune competence is shown to shape reproduction, growth, and survival strategies in different taxa.

Negative effects of reproductive effort on parental health have been shown across avian taxa (Lochmiller and Deerenberg, 2000; Nordling *et al.*, 1998; Rauw, 2012). Females of the Common Eider (*Somateria mollissima*), a capital breeder, show signs of immunosuppression indicated by low lymphocyte counts during reproduction at the cost of increased abandonment of their ducklings (Hanssen *et al.*, 2003). In zebra finches, *Taeniopygia guttata*, artificially increasing the brood size for reproducing females or increasing the activity workload for non-reproducing females suppresses the humoral immune response, specifically antibody production (Deerenberg *et al.*, 1997). In mammals, lactation lowers the mother's immune defense against infection. Lactating female spotted hyenas (*Crocuta crocuta*) were significantly more infected with hookworm *Ancylostoma* compared with non-lactating females (East *et al.*, 2015; Wiehn *et al.*, 1997; Zylberberg *et al.*, 2015).

Trade-offs have even been found between two antagonistic immune functions. The immune system consists of innate and adaptive immune arms (Martin *et al.*, 2008). The

innate immune system is the first line of defense, much quicker to respond than the specific immune mechanisms (Medzhitov, 2007). In contrast, the adaptive immune effects are slower to develop and more expensive to build. During periods of limited resources, such as in the reproductive seasons, the innate and adaptive responses can be up- or downregulated, potentially creating an energetic trade-off between the two arms (Martin *et al.*, 2008; Medzhitov, 2007). As an example, pregnancy and lactation in guinea pigs, even under *ad libitum* food conditions, correlated with a decreased response in the antibody-mediated adaptive immunity. However, serum from pregnant and lactating females showed a higher bacteria-killing ability (Trillmich *et al.*, 2020).

Although physiological trade-offs between reproduction and immune function have been documented, the regulation of these trade-offs remains unknown. Endocrine mediators often orchestrate energy allocation among different physiological functions (Boonstra and McColl, 2000; Martin *et al.*, 2008; Nelson *et al.*, 2002; Zera and Harshman, 2001). Many hormones exert a strong effect on reproduction, growth, immunocompetence, and survival. Endocrine systems manage energy allocation by downregulating discretionary processes such as immune response in favor of reproduction (Apanius, 1998; Boonstra and McColl, 2000; Wingfield and Sapolsky, 2003).

Sexual signals used to attract mates, often impose costs from increased predation and energy consumption (Bradbury and Vehrencamp, 1998; Grafen, 1990a; Grafen, 1990b; Grafen and Johnstone, 1993; Zahavi, 1975; Zahavi and Zahavi, 1999). The metabolic cost of courtship sexual signals has been suggested to serve as the physiological basis of parasite-mediated sexual selection (Zuk and Kolluru, 1998). For instance, the investment in courtship drumming in wild populations of the wolf spider *Hygrolycosa rubrofasciata*

decreases immunocompetence measured as lytic activity, compared to that of non-reproducing males (Ahtiainen *et al.*, 2005).

The weakly electric fish *Brachyhypopomus gauderio* senses its environment and communicates by generating electric signals, which are inexpensive in females but energetically costly in males (Salazar and Stoddard, 2008b; Stoddard and Salazar, 2011; Stoddard *et al.*, 2006). The electric organ discharges of weakly electric fish (EODs) are created by the myogenic excitable cells (electrocytes) located in the electric organs (Bennett, 1961; Szabo, 1974). Electric organs in gymotiform fish run bilaterally from the pectoral fin to the tip of the tail. Electric fish are ideal models to study the evolution and physiology of communication and examine the energetic cost of signals because their EODs can be quantitively measured and hormonally manipulated (Stoddard *et al.*, 2006).

Male *B. gauderio* produces reproductive signals that are among the most energetically costly of any animal measured, consuming 11-22% of the daily energy budget (Salazar and Stoddard, 2008b). Ali and Stoddard (in submission) explored sex differences in the trade-off between the signal metabolism and the rest of the metabolism, using T4 implants to initiate trade-offs. In males, the signal metabolism rose after the T4 implant and the residual metabolism decreased, while total energy consumption remained unchanged. In females, T4 implants increased total energy consumption, lowering the signal metabolism and raising the residual metabolism that includes both egg production and the standard metabolic rate (SMR). These results suggest that thyroid hormone regulates the allocation of energy between electric signals and somatic maintenance in favor of reproduction in both sexes.

Which organs or metabolic processes are deprived of energy when males increase energy allocation to their reproductive signals? Do males reallocate energy from the whole body or from a more restricted, discretionary compartment such as the immune system?

For decades, it has been recognized that the neuroendocrine system influences the immune system's developmental and functional activities. Many recent studies have investigated the bidirectional relationship and communication between the neuroendocrine and immune responses in vertebrates (Deal and Volkoff, 2020; Klecha *et al.*, 2000; Lam *et al.*, 2005; Lam, 1994; Montesinos and Pellizas, 2019; van der Spek *et al.*, 2018).

The interplay between the hypothalamus-pituitary-thyroid axis and the immune system has yet to be fully characterized. However, the interaction between the two systems plays an important role in the maintenance of homeostasis during the course of infections and inflammatory processes (Jara *et al.*, 2017; Klein, 2006). Thyroid hormones (THs) can induce responses in a variety of immune cells, including monocytes, macrophages, natural killer cells, and lymphocytes, thereby impacting a variety of inflammatory processes (such as phagocytosis and reactive oxygen species generation). In mice, thyroxine (T4) treatment in vivo has been found to increase the antibody titers. Reduced thyroid hormone levels, on the other hand, have a detrimental effect on humoral and cellular immunological responses (Klecha *et al.*, 2000). Thyroid hormones also induce the phagocytic activity of macrophages (Montesinos and Pellizas, 2019). Thyroid hormones have been shown to play a critical role in innate immune cells (e.g., neutrophils) in zebrafish (Lam *et al.*, 2005; van der Spek *et al.*, 2018) and represent potential target cells in mice (van der Spek *et al.*, 2017).

For electric fish, we do not know how thyroid hormones affect immunity, either between or within the sexes. Since immunocompetence can follow adaptive reallocation of

resources in response to increased energetic demand of reproduction, we predicted that thyroid hormones downregulate the immunocompetence of reproductive *B. gauderio* to free-up energy for reproductive signaling.

We explored whether thyroid hormone alters the humoral immune response in a manner consistent with the energetic trade-offs already documented in male and female *B. gauderio.* We triggered immune responses with injections of lipopolysaccharide (LPS), then measured the bactericidal ability of their plasma against *E. coli.* The procedure was conducted before and afterT4 implantation.

3.3 Materials and methods

3.3.1 Experimental animals

Our subjects were captive-reared weakly electric fish *Brachyhypopomus gauderio,* native to southern South America. We raised and maintained the fish in outdoor pools on the roof of our laboratory building at FIU in Miami, Florida. Fish were kept in mixed sex social groups of 10-12 at 27-30° C, conductivity 70-120 µS/cm, and pH 6.5-7. The fish were fed oligochaetes ("blackworms") *ad libitum*, plus whatever chironomid larvae inhabit the pools naturally. We selected 50 mature individuals of each sex at random and relocated them to the laboratory where they resided temporarily in 26-liter polycarbonate aquaria filled with water conditioned to match the roof pools. In the lab, the fish were acclimated to social groups of two males and three female for two weeks before any experimental procedures. These fish were fed blackworms every other day.

Fish were randomly assigned to four groups: control (untreated or saline-injection), lipopolysaccharide (LPS) injection, T4 (T4) implant, and LPS injection + T4 implant. Male

subjects were 12.5-22.5 mm in length and weighed 3-13 g. Females were 11-18.5 mm in length and weighed 4-10 g.

Twenty fish of each sex were implanted with T4 (T4) and 20 with a saline-filled sham, and 10 were left untreated. After 48h, half of each group received an LPS injection or a saline injection, and after another 24h were returned to their social aquariums. After an additional 24 h, blood was collected to extract plasma for assessing changes in the bactericidal ability induced by the LPS injection.

3.3.2 Hormone implantation

The T4 hormone has previously been found to boost the signal metabolism of the male electric fish (*Ali & Stoddard, subm.*). Implants were constructed of silastic tube (0.51 mm ID x 0.94 mm OD x 0.23 mm wall, ~1.60 mm long) containing either 0.5 mg L-T4 (T2376; Sigma-Aldrich) or saline (sham) and sealed with silicone adhesive following Dunlap & Ragazzi (2015). We anesthetized the fish by immersion in a 0.6 ml/liter clove oil (eugenol) solution, then used a sterile 18-gauge hypodermic needle to cut a small hole between two ribs ventral to the lateral line and dorsal to the electric organ. We inserted the implant intraperitoneally and sealed the hole with Vetbond^{tm} (3M) surgical adhesive. Implantation took less than 3 min, after which the fish was returned to its aquarium. On the basis of results with goldfish, *Carassius auratus* (Hurlburt, 1977), our T4 implants should have elevated plasma T4 levels by ~1 µg/ml.

3.3.3 Immune challenge (Lipopolysaccharide injection)

Lipopolysaccharide (LPS), an endotoxin, was used to elicit the immune response. Fish were injected intraperitoneally with LPS (2.5 µg/g body mass; L3129 Sigma- Aldrich, St. Louis, MO, USA) from *E. coli*, diluted in phosphate buffer saline (0.35 mg LPS / 2 ml

PBS) or saline control. The amount of LPS injected was similar to levels shown to stimulate the immune response in terrestrial ectotherms (Deen and Hutchison, 2001; Do Amaral *et al.*, 2002; López *et al.*, 2009; Smith *et al.*, 2017; Uller *et al.*, 2006b).

3.3.4 Blood (plasma) collection

Ten fish of each sex from treated and control groups were netted from their social pools, anesthetized by 1.5-2 min immersion in a 0.6 ml/liter clove oil (eugenol) solution, bled from the subvertebral sinus, and returned to their pools. Blood (~15 µl) was collected into a 1 ml polypropylene tube containing 5 µl of heparin solution and centrifuged for 15 min at 7200 rpm using an Eppendorf MiniSpin centrifuge. Plasma (supernatant) was transferred into a separate polypropylene tube and stored at -20°C for bactericidal assay application (plasma methods followed Dunlap et al. (2002) and Salazar and Stoddard (2009).

Timeline of procedures for blood collection:

Untreated fish:	Blood collected directly from each fish
Treated fish:	
Day 1	T4 or sham implant
Day 3	LPS or saline injection
Day 4	Blood collection

3.3.5 Assessment of the immune response

To determine the effects of T4 and LPS injection on innate immunocompetence, a bactericidal assay was performed to measure the fish's overall capacity to fight against bacterial infection. Methods followed Lind et al. (2020), and Smith & French (2017). Briefly, agar plates were inoculated with *E. coli* (ATCC NO. 25922) and grown for 12 hours. Bacteria were then seeded at 10^4 producing units of fresh inoculated media and cultured in a 96-well plate. Bactericidal ability of fish plasma was tested by adding 2 µl

plasma from each treatment group to 6 µl bacterial culture in 125 µl LB broth and incubated at 37°C for 8 h. A positive control containing 6 µl bacteria with LB broth and no plasma was used to determine the bacterial growth potential. A negative control contained LB broth without plasma or bacteria. All experiments were performed in technical triplicates for each biological sample, with the mean value being used for calculations. Bacterial growth under different treatments was determined by measuring the absorbance at 570 nm (OD_{570}) with a BioTek^{tm} microplate reader at 0 h and 8h incubation. The bactericidal ability (BKA) was calculated using the following equation:

$$BKA = 1 - [(OD_{570} \text{ at } 8 \text{ h} - OD_{570} \text{ at } 0 \text{ h})/OD_{570} \text{ of positive control})] \times 100$$

3.3.6 Statistical analyses

All statistical tests were performed using the GraphPad Prism 9 software package (GraphPad Software, San Diego, CA, USA). Data are presented as arithmetic means with standard error (mean ± SE) and all significance tests are 2-tailed. Details of statistical statistical tests used, number of trials (n), number of animals, and how significance was determined can be found in the figure legends.

3.4 Results

The treatment groups, T4, LPS, and T4+LPS all significantly elevated bactericidal ability of male plasma relative to untreated and saline controls (Fig. 6A). Plasma from females, however, showed no significant changes in bactericidal ability regardless of treatment (Fig. 6B). Likewise, in controls (untreated and saline), plasma from the two sexes showed no significant sex differences in bactericidal ability ($p=0.76$; $p=0.22$, 9 d.f., t-test, 2-tailed). However, in all three treatment groups (T4, LPS, and T4+LPS) bactericidal ability of male plasma became significantly greater than that of females (Fig. 7).

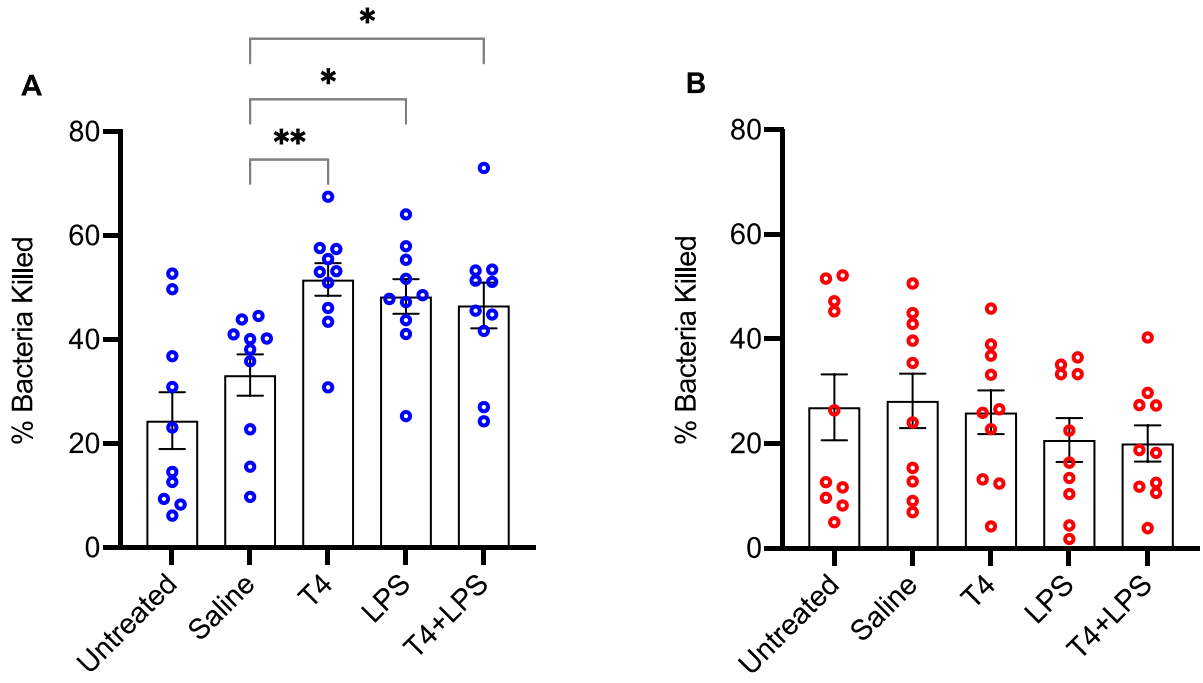

Figure 6: Bactericidal activity of electric fish plasma under 8-hour incubation. Bars represent mean (±SEM), n = 10 in each treatment group. **A**, bacterial killing ability is significantly higher in plasma of males treated with T4 implant, LPS injection and T4 implant plus LPS injection (P= 0.004, P=0.02, P=0.04; one-way ANOVA, Dunnett Test) compared to the control (saline). **B**, there were no significant differences in the bactericidal percentage in the plasma of treated females compared to the control. Significant differences are designated with asterisks (* P<0.05, ** P<0.01; one-way ANOVA, Dunnett Test). Untreated group is included for comparison purposes but does not differ statistically from the saline treatment. Red (female) and blue (male) dots represent the different biological individuals.

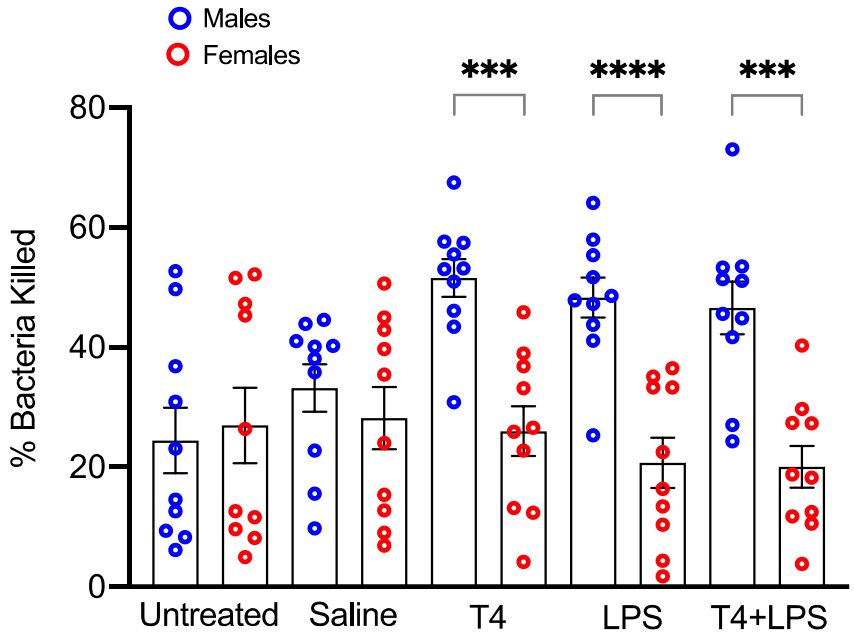

Figure 7: Sex differences in the bactericidal ability of male and female electric fish plasma under 8-hour incubation. Bars represent mean (±SEM), n = 10 in each treatment. Significant differences (*** P<0.001, **** P<0.0001; an unpaired t-test, 2-tailed) designated with asterisks. Red (female) and blue (male) dots represent the different biological individuals.

3.5 Discussion

The previous finding that T4 induces males to elevate their signal metabolism and lower their residual (non-signal) metabolism (Ali & Stoddard subm.), led to the prediction that T4 would reduce innate immunity in males. Unexpectedly, males mounted an increased innate immune response following T4 implant, LPS injection, or both. The increased BKA levels in treated males may reflect the net fitness benefit of fighting a bacterial challenge for just a brief period. Male *B. gauderio* disappear just a few weeks after reaching sexual maturity, whereas females survive and reproduce for the entire Austral summer (Miranda *et al.*, 2008). Our unexpected findings that males responded to LPS challenges or T4 implants by elevating the humoral immune response suggest that innate immunity is a short-term survival strategy for males.

Previous findings showed that T4 elevates the residual metabolism of females (Ali & Stoddard subm.), leading to the prediction that T4 would either elevate the female's innate immunity or, at least, not lower it. Females did not modulate BKA with any of the treatments.

Instead of modulating innate immunity, females may reallocate energy for egg production, a possibility we have not tested directly. Thyroid signaling in fishes is known to allocate energy to egg production and immunity in females (Deal and Volkoff, 2020; Lam, 1994). Thyroid signaling may hold a central position in the allocation of resources required for both reproduction and all components of the immune response, except perhaps humoral innate immunity.

Together, these findings indicate that energetic trade-offs documented in the two sexes of *B. gauderio* do not involve the humoral side of the innate immune response.

Combining these results with the lowered residual metabolic rate of males induced by T4 suggests males trade-off a different metabolic component against the signal metabolism instead, perhaps cellular immunity or somatic repair. Further studies on electric fish are required to identify the cellular basis of energy reallocation between signaling, reproduction, and maintenance.

Trade-offs involving the immune system are common. Pregnant guinea pigs increased their innate immune response against bacterial infection while decreasing specific humoral adaptive immunity (Trillmich *et al.*, 2020). Far more work has been done examining sex differences in immune responses, which have been documented in lizards, birds, and mammals. In most cases, males have been shown to have lower innate and adaptive immunity than females. The phagocytic activity of macrophages is greater in female lizards than in males because of the androgen repressed effects on macrophage activity in males (Mondal and Rai, 1999). Female birds during mating seasons exhibit higher cell-mediated immune responses to immune challenge compared to males (which have high levels of testosterone during the mating seasons) (Fargallo *et al.*, 2007; Pap *et al.*, 2010). Male Covid-19 patients have higher plasma levels of innate cytokines than females, while female patients have more robust specific T-cell activation than males (Takahashi *et al.*, 2020).

It remains something of a scavenger hunt to delineate the allocation of resources between competing functions and to resolve the cellular mechanisms that regulate differential allocation. Identifying these mechanisms will be necessary to understand how traits trade-off and how trade-offs evolve.

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CHAPTER 4: Differential expression of Na+, K+-ATPase alpha 2 subunit in electrocytes of electric fish *Brachyhypopomus gauderio* by thyroid hormone

"The distance between insanity and genius is measured only by success".

Bruce Feirstein

4.1 ABSTRACT

In gymnotiform weakly electric fish, electric organ discharges (EODs, electric signals) are used to detect objects in the environment and communicate with other fish. The shape and kinetics of electrocyte action potentials are regulated directly by hormones, including steroids and melanocortin peptides. Reproductive signals produced by male *Brachyhypopomus gauderio* are among the most energetically costly of any animal measured. We documented in a previous study that thyroid hormone drives a trade-off between male signal metabolism and the rest of the resting metabolism. An opposite trend is manifested in females, where thyroid hormone causes the signal metabolism to fall and the residual metabolism (which includes egg production) to rise.

Here, we report that thyroid hormone induces increased expression of an ATPase pump in the electrogenic cells of males but not females, consistent with (matched by parallel) previous findings that thyroid hormone differentially regulates signal metabolism in the two sexes. This differential pattern of gene regulation suggests that ATPase is potentially involved in the diversification and sexual dimorphism of electric signals. The results provide further evidence that thyroid hormones play an essential role in the differential allocation of energy among metabolic functions.

4.2 Introduction

Trade-offs are universal in the biological kingdom, while the details may be taxon-specific (Stearns, 1989; Zera and Harshman, 2001). Many taxa resolve competing metabolic demands by trading off energy allocated to different physiological processes (Burness *et al.*, 2010; Congdon *et al.*, 1982; Moore and Hopkins, 2009; Stearns, 1989). Organisms are forced to make trade-offs when functional demands conflict, caused by constraints on limited resources such as time, energy, or nutrients (Blomquist, 2009; Potts *et al.*, 1980; Strum and Western, 1982). In the life-history literature, trade-offs are reported between investment in current reproduction and survival for future reproduction, for instance, investment in offspring versus somatic maintenance (Blomquist, 2009; Griesser *et al.*, 2017; Ohgushi, 1996; Santos and Nakagawa, 2012; Zera and Harshman, 2001). Hormonal signaling mediates these energetic functions and may play a central position in the allocation of resources between competing metabolic functions (Boonstra and McColl, 2000; Chinn *et al.*, 2018; Crnokrak and Roff, 1998; Fargallo *et al.*, 2007; Moore and Jessop, 2003; Muehlenbein and Bribiescas, 2005; Wingfield and Sapolsky, 2003). For example, glucocorticoids, essential mediators of energy mobilization, sometimes suppress other physiological functions in favor of survival (Boonstra and McColl, 2000; Wingfield and Sapolsky, 2003).

The high energetic demand of signaling by males of the weakly electric gymnotiform fish *Brachyhypopomus gauderio* is accompanied by a metabolic trade-off with other cellular functions (Stoddard and Salazar, 2011). We recently found that this trade-off can be invoked experimentally by the administration of the thyroid hormone thyroxine (T4) (Ali and Stoddard, *subm*). Electric organ discharges (EODs) of many electric fish species

are regulated by steroid hormones that control entire suites of correlated characters. Androgens and glucocorticosteroids are the best-characterized regulators of the EOD waveform (Allee *et al.*, 2009; Bass and Hopkins, 1985; Dunlap and Ragazzi, 2015; Gavassa *et al.*, 2013a; Gavassa and Stoddard, 2012; Goldina *et al.*, 2011; Markham *et al.*, 2009; Markham and Stoddard, 2005).

The EODs of the weakly electric fish are created by myogenic (muscle-derived) electrocytes which are located in the electric organ (EO) (Bennett, 1971), and are used for communication, navigation, defense, and predation (Bullock and Heiligenberg, 1986; Moller, 1995). These signals are under the control of the pacemaker nucleus in the hindbrain (Heiligenberg, 1991; Heiligenberg and Dye, 1982; Heiligenberg *et al.*, 1981; Kawasaki and Heiligenberg, 1989; Metzner, 1999). The pacemaker command nucleus in the hindbrain sends synchronous action potentials down to relay cells projected into the spinal cord where they activate the spinal motor neurons, the axons of which innervate the electric organ (Bennett, 1971; Bennett, 1961; Heiligenberg *et al.*, 1981; Kawasaki and Heiligenberg, 1989). The efferent action potential in the motor neurons depolarizes electrocytes, rich in voltage-gated sodium channels on the posterior and anterior ends (Stoddard, 2006). When depolarized, the firing of the posterior face (head-positive phase) depolarizes the anterior (head-negative phase), and a signal is produced (Bennett, 1971; Bennett, 1961).

The Na+/K+-ATPase is an integral membrane protein that functions as an ion pump, hydrolyzing one molecule of ATP to pump three Na+ out of the cell in exchange for two K+ entering the cell per pump cycle (Hodgkin, 1951; Hodgkin and Huxley, 1952; Skou, 1957). The enzyme Na+/K+-ATPase is responsible for maintaining the polarization of cells

with excitable membranes such as neurons, myocytes, and electrocytes. The cost of action potential generation in neurons and electrocytes arises primarily from the Na/K ATPase (Attwell and Laughlin, 2001; Laughlin *et al.*, 1998; Lennie, 2003). Na^+/K^+ATPase is an abundantly expressed protein that accounts for approximately 30% of the total energy consumed by mammals, 50% of the energy consumed by the brain, and 80% of the energy consumed by the kidney (Astrup *et al.*, 1981; Clausen *et al.*, 1991; Howarth *et al.*, 2012; McBride and Milligan, 1985; Tomsic *et al.*, 2011).

Flow-through oxygen respirometry has been used to measure the cost of electric signals (Salazar and Stoddard, 2008a) (Ali and Stoddard, *in subm*). We used thyroxine (T4**,** thyroid hormone) implants to increase the signal metabolism, partitioned the energy budget pharmacologically, and measured energy consumption through oxygen respirometry. In males, the signal metabolism ($\dot{V}O_{2\ EOD}$) increased, and the residual metabolic rate ($\dot{V}O_{2\ RMR}$) (effectively the standard metabolic rate for males) decreased in a one-to-one trade-off. Females showed the opposite trade-off in which the signal metabolism dropped while the residual metabolism rose; in females, the residual metabolism includes both the standard metabolism and egg production. These results reveal metabolic trade-offs between signaling and cellular metabolism in electric fish and suggest that thyroid hormones regulate energy allocation between electric signals and somatic maintenance in favor of reproduction. Thyroid hormones stimulate the activity of the Na^+/K^+ ATPase by increasing the number of pump molecules (Ewart and Klip, 1995; Ismail-Beigi, 1988; Lin and Akera, 1978).

Given that thyroid hormone regulates electric signal metabolism of the two sexes of the electric fish *B. gauderio* in opposite directions and that sodium/potassium ATPase is

the energy-consuming molecule in electric signal production, the current study explored whether gene expression of sodium/potassium ATPase is likewise regulated by thyroid hormones. We hypothesized that thyroxine upregulates Na^+/K^+ ATPase quantity in electrocytes of males and downregulates it in females. Using quantitative reverse transcription PCR (RT-qPCR) (**Figure 1**), we analyzed the expression of the Na^+/K^+ ATPase subunit 2-alpha *(atp1a2)* in electrocytes of both sexes of *B. gauderio* before and after thyroxine treatment.

4.3 Materials and methods

4.3.1 Experimental animals

Our subjects were captive-reared weakly electric fish *Brachyhypopomus gauderio,* native to southern South America. The fish were raised and maintained in outdoor pools on the roof of the lab building (AHC1) at FIU in Miami, Florida. Fish were kept in mixed-sex social groups of 10-12 at 27-30° C, conductivity 70-120 µS/cm, and pH 6.5-7. The fish were fed oligochaetes ("blackworms") *ad libitum*, plus whatever chironomid larvae inhabit the pools naturally. Forty mature individuals of each sex were selected at random and relocated to the lab where they resided temporarily in 26-liter polycarbonate aquaria filled with water conditioned to match the roof pools. In the lab, the fish were acclimated to social groups of two males and three females for two weeks before any experimental procedures. Fish were fed blackworms every other day. Fish were randomly assigned to two groups; thyroxine (T4) implant and sham control. Male subjects (n=20) were 13.5-19.5 cm in length and weighed 3-8 g. Females (n=20) were 10.8-16 cm in length and weighed 3-8 g.

4.3.2 Hormone implantation

At least ten days after the fish acclimation in the lab, ten males and ten females were implanted with small silastic tubes containing the thyroid hormone L-thyroxine (T4) (T2376; Sigma-Aldrich) following the methods of (Dunlap and Ragazzi, 2015) or with saline control (10 males and ten females). Each silastic tube (0.51 mm ID x 0.94 mm OD x 0.23 mm wall, ~1.60 mm long) contained 0.5 mg of T4 or saline for the control. Fish were anesthetized by immersion in a 0.6 ml/liter clove oil (eugenol) solution. The implant was inserted intraperitoneally, ventral to the lateral line and dorsal to the electric organ, through a hole between two ribs made with a sterile 18-gauge hypodermic needle, then sealed with surgical glue (Vetbond^{TM}, 3M). Implantation took less than two minutes, after which the fish was returned to its aquarium. Following studies of goldfish *Carassius auratus* (Hurlburt, 1977), our T4 implants should have elevated plasma T4 levels by ~1 ug/ml. Implants of L-thyroxine (T4) have proved highly consistent at modulating the metabolic compartments in both sexes, including that of the electric organ (*Ali & Stoddard, subm*).

4.3.3 Tissue dissection and electrocytes collections

To quantify the activity of Na+/K+-ATPase in males and females *B. gauderio* electrocytes before and after thyroxine implant, a 2 cm section of the tail tip was removed and dissected to harvest electrocytes. The skin was peeled and removed with forceps and the electric organ tissue immersed for 5 to 7 hours in electric fish saline (Ferrari and Zakon, 1993) with Worthington type IV collagenase (10%) until the electrocytes were loosened from the connective tissue on both sides of the tail. Electrocytes were immediately

transferred and weighted in sterilized 1.5 ml centrifuge tubes followed directly by RNA extraction, or were stored at −80 C until processed.

4.3.4 RNA isolation, quantification, and digestion of genomic DNA

Total RNA was extracted using the Trizol®LS reagent (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) (**Figure 2**). Fish electrocytes (30-50 mg) were homogenized and lysed in 300-500 µl of Trizol reagent. Cell lysates were then subjected to centrifugation at 12,000 g for 30 min at 4ºC. The supernatant was collected and transferred into a new 1.5 ml microcentrifuge tube and incubated at room temperature for 5 minutes, allowing dissociation of the nucleoprotein complex. Subsequently, 60-100 µl of chloroform (J.T. Baker, Pennsylvania, USA) was added to the samples. Samples were vortexed for 20 sec and centrifuged at 12,000 g for 30 mins at 4ºC. The upper aqueous phase was transferred into a new microcentrifuge tube. Total RNA in the aqueous phase was precipitated with ethanol precipitation. To every 100 µl aqueous solution was added 10 µl of 3M sodium acetate pH 5.2, 300 µl cold ethanol (100%) and 3 µl linear acrylamide (7.5 µg/ µl). To increase the yield of precipitated RNA of electrocytes, samples were kept at −80 °C for 24 to 48 hours. Samples were then centrifuged at 12,000 g for 1 h at 4ºC. The RNA pellets were washed with cold 75% ethanol (J.T. Baker, Pennsylvania, USA), air-dried at room temperature for 10 min. The RNA was resuspended in 20 µl DEPC-treated Tris EDTA buffer and incubated for 5 min at 37ºC. The concentration of total RNA was measured by NanoDropND-1000 UV-Vis Spectrophotometer (Thermo Scientific, MA, USA). Genomic DNA was eliminated from the RNA via digestion using the TurboDNase kit.

4.3.5 Primer design and validation by conventional PCR

As a result of the absence of genomic resources for gymnotiform species, we used the DNA sequences from PCR products generated from the reference genes for *B. gauderio* reported by Ivey et al. (manuscript in prep). Forward and reverse primers were designed to amplify the gene, *atp1a2*, ATPase Na+/K+ transporting subunit alpha two, and the reference gene, *rpl13a*, ribosomal binding protein. The sequences of the primers are listed in Table 1. The sizes of the PCR products were validated using 1% agarose gel electrophoresis (Figure 2). The sequences were validated using PCR-direct sequencing (Florida International University DNA Sequencing Core Facility). Amplicon base pair size was validated by sequencing our forward and reverse PCR products. The PCR amplifications were carried out using 12.5 µl Promega Go Taq™ Master Mixes, 10.5 µl DEPC water and 1µl of each primer (10 µM) with the PCR program: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s; and a final extension step of 72 °C for 7 min. The PCR products were checked by 1% agarose gel electrophoresis. PCR products were visualized and imaged using the Bio-Rad Doc Imaging System and Bio-Rad ChemiDoc™ XRS software (Figure 1).

4.3.6 Reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the change in expression of the atp1a2 gene and reference gene rpl13a before and after thyroxine treatment, RT-qPCR reactions were performed on electric organ tissue extracted from male and female electric fish *B. gauderio*. The real-time quantitative PCR (qPCR) assays were performed using two steps, reverse transcription and real-time

PCR amplification reaction. Two µg of total RNA from male and female electric fish electrocytes (T4-treated or control) were used as templates for reverse transcription reaction in a reaction mixture (20 µl) containing oligo dT_{20}, primer (0.5 µM), dNTPs (500 µM), reverse transcriptase (50 U) (Promega, USA), RNase Inhibitor (20 U) (Promega, USA), and 1x reverse transcriptase buffer. Reverse transcription reaction was performed with the incubation at 25 °C for 5 min, followed by incubation at 42°C for 30 min and 4°C for 24 min. cDNA was then subjected to quantitative real-time PCR (qRT-PCR) using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) and MJ Research Real-time-PCR system and MJ Research Opticon Monitor software, version 3.1.

In brief, the qRT-PCR reaction was performed in a reaction mixture (25 µl) containing 3 µl cDNA, 10 µl of DEPC water, 10 µl PowerUp SYBR Green Master Mix, and 1 µl of each primer (10 µM). The cycling conditions for qRT-PCR were as follows: 95ºC for 10 min 1 cycle, followed by 40 cycles at 95ºC for 20 sec, 48.4 ºC for 30 seconds, and 72 ºC for 30 sec. The relative mRNA level of the ATPase was calculated using cycle threshold (Ct) values using the equation: $2^{-\Delta\Delta Ct}$. To check reproducibility, experiments were performed in technical triplicate for each of the ten biological replicates of each group, with the mean values for Ct values being used for the calculation**.**

4.3.7 Statistical analysis

Normalized gene expression levels (ΔCT) were calculated using the mean of the reference genes *rpl13a* (CT Reference − CT Gene of interest). The fold change was presented as mean ± standard error (SE) of the ten biological individuals. The relative transcript levels of *atp1a2* and *rpl13a* were calculated for each individual using the $2^{-\Delta\Delta Ct}$

method (Schmittgen and Livak, 2008). Independent Student's t-test with GraphPad Prism 9 was performed to compare the treated and control groups with significance reported at $p < 0.05$.

4.4 Results

The *atp1a2* and *rpl13a* genes were confirmed to be expressed in adult male and female electrocytes (Fig.2). Comparisons between the relative *atp1a2* mRNA level in the electric organ showed a significantly higher expression in males after thyroxine treatment ($p = 0.01$, 9 d.f.; t-test, 2-tailed) (Fig. 3a) compared to the control (by 82 % much higher than male control), indicating thyroxine likely increases the amount of Na+/K+-ATPase in electrocytes. In contrast, female *atp1a2* RNA expression showed no upregulation in the electric organ after thyroxine implant compared to control females ($p = 0.20$, 9 d.f.; t-test, 2-tailed) (Fig. 3b). Females showed a non-significant tendency towards lower expression of *atp1a2* in the electric organ after thyroxine treatment than the control females.

In saline controls, no sex difference was apparent in the *atp1a2* gene expression of electrocytes ($p=0.19$, nine d.f., t-test, 2-tailed) (Fig. 4a), however, following thyroxine implant, male electrocytes increased *atp1a2* mRNA expression by 75% ($p= 0.01$, 9 d.f., t-test, 2-tailed) relative to females (Fig. 4b).

Gene	Protein	Primer 5`-3`	Amplicon size (bp)
ATPase (Na+/K transporting)			
atp1a2	ATPase Na+/K+ Transporting Subunit Alpha 2	F-GGTATGGTAGTGGAAGCGGTTCTG R-CATCACGGATCACCATGGCTTG	471
Reference gene			
rpl13a	L13A ribosomal binding protein	F-CGTCCTCCAGAAGATACGGC R-GTGAGGGCATCAACATCTCTGG	137

Table 1. Primer sequences pairs and amplicon size of candidate and reference genes used for qPCR experiments with samples of male and female electrocytes before and after thyroxine treatment.

Figure 8. Na+/K+-ATPase RNA quantification steps of electric fish *B. gauderio* electrocytes.

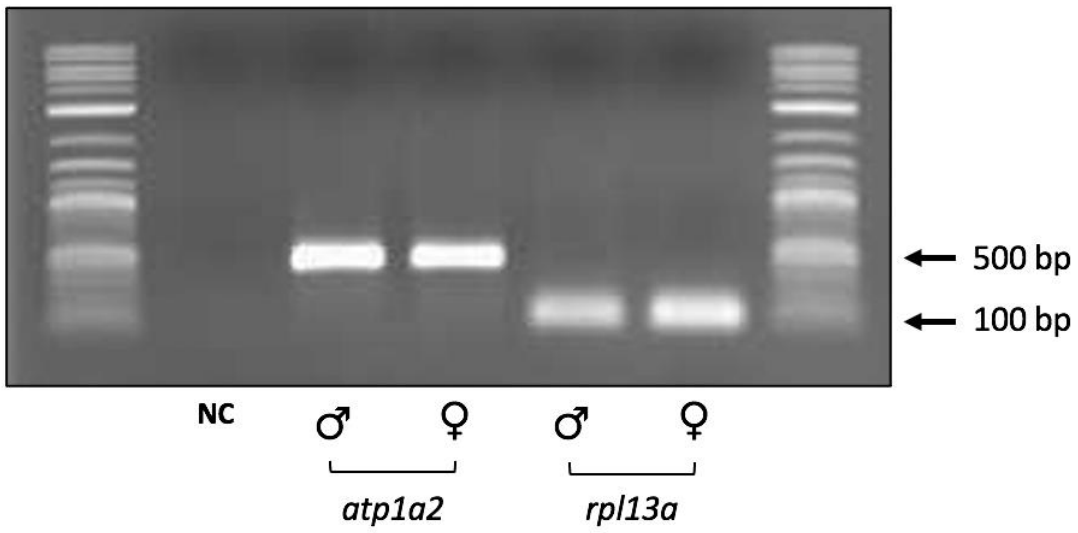

Figure 9. Validation of cDNA template results by conventional PCR analysis shows the differential expression of the target gene *atp1a2* and housekeeping gene *rpl13a* in male and female electrocytes with negative controls (NC).

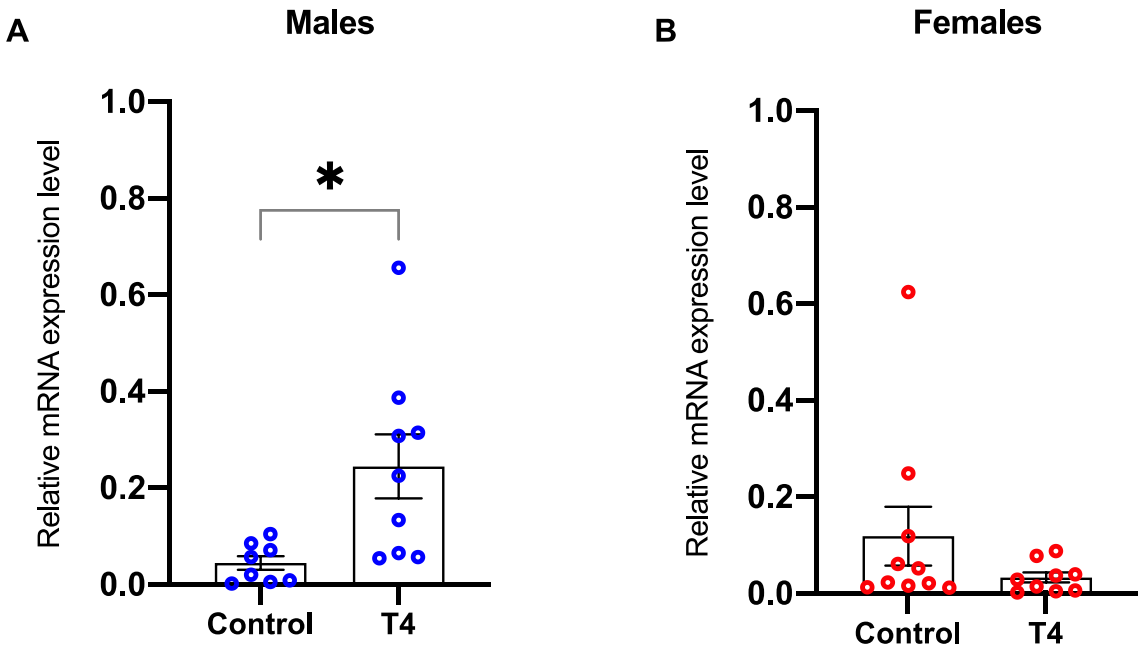

Figure 10. Relative fold expression of *atp12a* in male (n=10) and female (n=10) electrocytes pre- and post-thyroxine implant (T4). Male electrocytes have significantly higher *atp12a* expression after thyroxine treatment compared to the control. *atp12a expression* in electrocytes of females did not change significantly following thyroxine treatment. Normalized atp1a2 expression is higher in male electrocytes post-thyroxine compared to females post-thyroxine. The data represent the normalized target gene amount relative to control. Asterisk indicate p values: $*p < 0.05$, $**p < 0.01$; t-test, 2-tailed. Blue (male) and red (female) dots represent the different biological individuals.

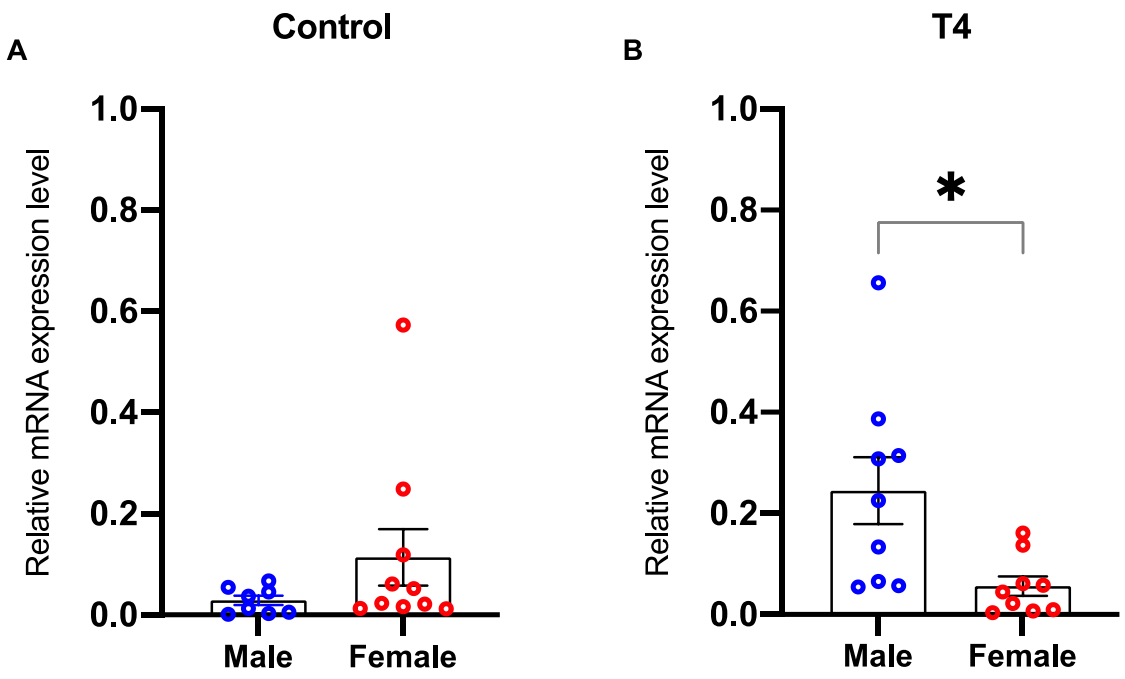

Figure 11. Sex differences in expression of ATPase Na+/K+ ion pump *atp1a2* mRNA of male and female electrocytes before and after thyroxine treatment. The data are presented as mean ± standard error (SE) of the 10 biological subjects in each group.

4.5 Discussion

4.5.1 Sexual dimorphism in the quantification of ATPase pump gene expression

We found a significantly higher expression of the Na+/K+ transporting pump *atp12a* in the electric organ in thyroxine-treated males respective to control males. Females treated with thyroxine did not show any significant difference in the electrocytes' Na+/K+-ATPase expression compared to controls. We expected the quantity of ATPase expression of the electrocytes in females to decrease, however, females showed no significant expression difference between pre-and post-thyroxine treatment. The observed upregulation of the Na+/K+-ATPase in males after thyroxine treatment suggests that the concentration of voltage-gated ion channels influences the electric organ function consistent with our previous finding that males (but not females) expend more energy producing electric signals following thyroxine treatment (Ali and Stoddard, *in subm*). To the best of our knowledge, ours is the first study addressing the expression of the Na+/K+-ATPase gene of the electric organ in *B. gauderio*.

4.5.2 Possible regulation of Na+/K+-ATPase by thyroid hormone

The shape and kinetics of electrocyte action potentials are regulated by steroid hormones and melanocortins (Allee *et al.*, 2009; Bass and Hopkins, 1985; Gavassa *et al.*, 2013a; Goldina *et al.*, 2011; Markham *et al.*, 2009). Thyroid hormone (Triiodothyronine, T3) have been shown to elicit rapid and sustained changes in Na+/K+ pump activity (Ewart and Klip, 1995; Li and Langhans, 2015). In *B. gauderio*, thyroid hormone (T4) appears to sustain an increase in signal metabolism by increasing Na+/K+ ATPase.

Thyroid hormones could induce an increase in the transmembrane Na+ and K+ electrochemical gradient or increase the permeability of the electrocytes' cell membrane to Na+ and K+. The effect of thyroid hormone may comprise a prior increase in intracellular sodium ions. It is likely that thyroid hormone (T4) treatment elicits the cellular Na/K ATPase enzymatic activity and the permeability to Na+ and K+ ions in males' electrocytes. Stimulation of either pathway separately or both together could increase the active Na, K transport to higher rates in the electrocytes.

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CHAPTER 5: Conclusions and Future Directions

"Let me tell you the secret that has led me to my goal. My strength lies solely in my tenacity".

"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world".

Louis Pasteur

5.1 Conclusions

Understanding how organisms allocate finite resources across physiological systems is a major challenge in biology. Physiological functions such as reproduction, self-maintenance and immunity signficiantly influence fitness, but frequently compete for limited resources. Using the weakly electric fish as a model organism, my dissertation research explored how vertebrates control trade-offs in competing metabolic demands.

The experimental results reveal metabolic trade-offs between the signaling and cellular metabolism in electric fish and suggest that thyroid hormones regulate the allocation of energy between the electric signals and somatic maintenance in favor of reproduction, likely at the expense of survival. We present evidence that the total energy consumption remained unchanged pre- and post-thyroxine treatment, though the signal metabolism increased and the standard metabolic rate fell in an even trade-off.

In contrast to males, the total metabolism in females was increased under the thyroxine treatment. The females traded off metabolic functions in the opposite direction to that of the males, boosting their non-signal resting metabolism at the expense of their signal metabolism.

To determine whether these trade-offs involve the innate immune system, we assessed changes in the bactericidal activity of plasma in mature *B. gauderio* challenged with lipopolysaccharide (LPS) before and after the T4 treatment. Females did not modulate innate immunity with any of the treatments. On the other hand, an ~1/3 elevation in bactericidal activity in the males' plasma followed the LPS injections, T4 implants, or both together, relative to the sham treatment. The outcome was unexpected given that T4 also increases the energy consumed by the male's reproductive electric signals while lowering the rest of its metabolism. Upregulation of humoral innate immunity may be a short-term survival strategy for males, while the changes to cellular immunity remain unexplored.

Furthermore, we report that the thyroid hormone induces an increased expression of mRNA encoding the Na+K+ATPase pump in the electrogenic cells of males but not females, consistent with the previous findings that thyroid hormone differentially regulates signal metabolism in the two sexes. This differential pattern of gene expression suggests that regulation of Na+K+ATPase is part of the mechanism behind sexual dimorphism of electric signal energetics. These results provide further evidence that the thyroid hormones play an essential role in the differential allocation of energy among metabolic functions, holding a central position in the allocation of resources required for both reproduction and other metabolic functions.

The trade-off between reproduction and other metabolic functions has been detected in electric fish *B. gauderio* (Figure 12). The energetic requirement of male reproductive signals and other metabolic functions suggests that the reallocation of a common resource may be the base for the trade-off between the traits.

5.2 Future Directions

The respirometry data before and after thyroxine treatment was pivotal for evaluating the original hypothesis proposed by Stoddard & Salazar (2011) that male and female *B. gauderio* trade off energy in the signal and the rest of the metabolism in opposite directions. The hurdle of repeating respirometry and pharmacological experiments in the same individuals (chapter 2, DHT and T4 data) was difficult to accomplish because of the stressful nature of the treatments. All procedures of the study experiments were eventually mastered and an adequate sample size selected to ensure reasonable statistical power. The oxygen consumption study discloses the energy allocation strategies and assists in building a better understanding of the communication system evolution. However, we do not know exactly the mechanistic details of the evolutionary trade-offs between reproduction (sexual signals in males and egg production in females) and other metabolic functions mediated by thyroid hormones. Because the work was done *in vivo* thyroid hormone effects could be direct actions on the target tissues or involve an interaction with other hormones such as androgens or cortisol. Are these mechanisms shared across other electric fish taxa? A useful avenue to be addressed is understanding how the endocrine system regulates trade-offs at the level of hormone receptors, and eventually how the endocrine system controls gene expression involved in these trade-offs.

The humoral innate immune response did not show any downregulation in male electric fish, but was readily upregulated in males. So, the humoral innate immunity might be a short-term survival strategy for males. Further studies are necessary to uncover how reproduction could affect other cellular components of immunity in the two sexes.

Another potential study could look at whole genome expression related energetics, perhaps turning up differential regulation of metabolic genes such as hexokinase, a potentially rate limiting enzyme in the Krebs Cycle. Extension of molecular genetic studies is required to understand the gene regulation of the model organism in terms of transcription factors and regulatory DNA elements. Extension of molecular genetics will contribute to understanding the gene regulation of organismal trade-offs.

Additional studies need to be conducted to achieve better understanding of the cellular repair energetics in *B. gauderio*. A good experiment would be to investigate the cellular regeneration of the electric fish tail before and after thyroxine treatment. Another significant area that could be examined is the oxidative stress. Resistance to oxidative stress may induce a trade-off with life-history characteristics.

The incorporation of genetics, physiology, and development, into observational and experimental studies will increase our understanding of the evolutionary life history of trade-offs.

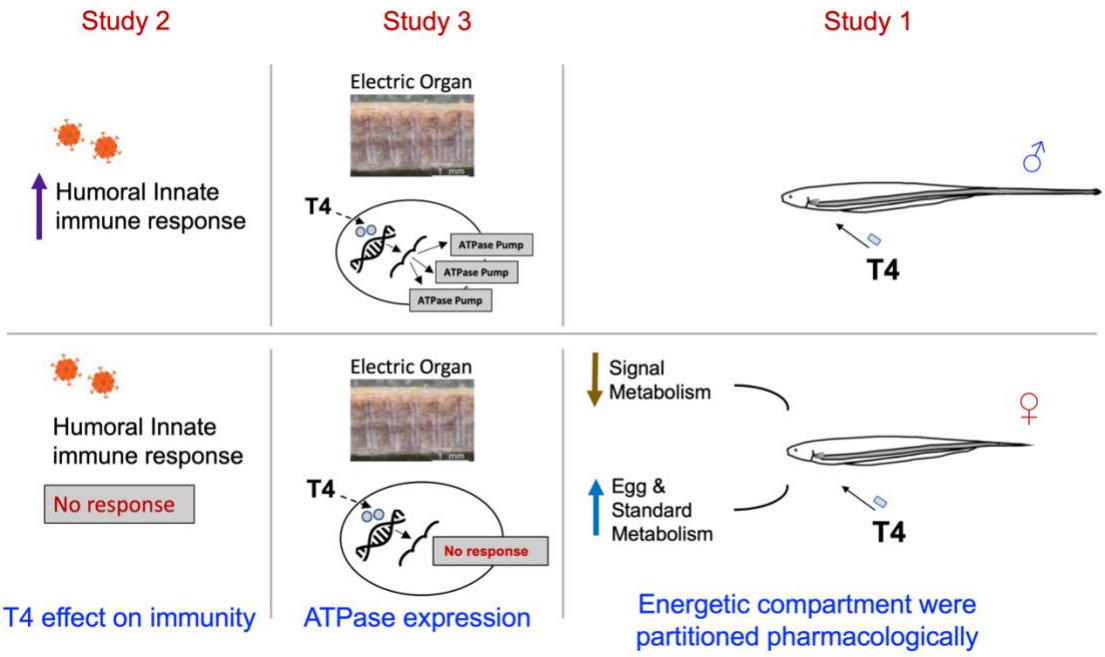

Figure12. Schematic representation of the three typical studies of the dissertation. Study 1, *B. gauderio* males and females were implanted with thyroxine to modulate the signal metabolism, partitioned the energy budget pharmacologically, and measured energy consumption using oxygen respirometry. In study 2, we assessed changes in the bactericidal activity of plasma in mature *B. gauderio* challenged with lipopolysaccharide (LPS) before and after T4 treatment. In study 3, we determined the quantification of ATPase pump expression before and after T4 treatment.

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PUBLICATIONS RECORD/CONFRENCES/ORAL/POSTER PRESENTATIONS

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Heba Ali (April 1st, 2019) "Energetic cost and physiological trade-offs" Heba Ali. Three minutes thesis competition. University Graduate School, FIU.

Heba Ali (April 1st, 2019) "How do electric fish regulate trade-offs in competing metabolic demands?". Graduate symposium, University Graduate School, FIU.

Heba Ali (February 2019) "Energetic cost and physiological trade-offs". Three minutes thesis competition. Biological Sciences, FIU.

Heba Ali (February 2019) "Energetic cost and physiological trade-offs". Three minutes thesis competition. College of Arts, Sciences and Education, CASE, FIU.

Heba Ali (February 2019), Poster presentation "How do electric fish regulate trade-offs in competing metabolic demands?". Annual Biosymposium, Biological Sciences, FIU.

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