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Altered Reproductive Function and Amphibian Declines

by

Sherrie Gallipeau

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

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of the

University of California, Berkeley

Committee in charge:

Professor Tyrone B. Hayes, Chair

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## Abstract

### Altered Reproductive Function and Amphibian Declines

by

Sherrie Gallipeau

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor Tyrone B. Hayes, Chair

Agrochemical exposure is one of the factors that contributes to worldwide amphibian declines. Most studies that examine agrochemicals and amphibian declines focus on toxicity. However, declines are more likely caused by the sub-lethal effects of agrochemical exposure. Past emphases on the lethal effects of agrochemical exposure have overshadowed the contribution of decreased recruitment in amphibian declines. Additionally, studies that examine agrochemicals and reproductive function tend to focus on the effects of single chemical exposures instead of the effects of ecologically relevant mixtures. To address these issues, this dissertation examined the effects of ecologically relevant agrochemical exposures on the stress response and the reproductive endocrinology, morphology, and behaviors of male amphibians in the laboratory and the wild.

Chapter 1 provides a general review of the factors implicated in amphibian declines and provides an overview of the previous research conducted on the effects of agrochemical exposure on recruitment.

Chapter 2 is a field study that examined whether agricultural run-off alters the stress response and reproductive function of male bullfrogs (*Lithobates catesbeianus*). Bullfrogs were collected upstream and downstream of agricultural activity across three California river systems (Salinas, Sacramento and San Joaquin). Size, primary and secondary sex traits, sperm count, and corticosterone and testosterone levels were examined. Overall, bullfrogs living downstream of agricultural activity (i.e. exposure to agricultural run-off) were small and had elevated testosterone and corticosterone levels. In addition, downstream males from the Salinas and San Joaquin Rivers were also small in size and had elevated testosterone levels. However, only downstream males of the San Joaquin River had elevated corticosterone and exaggerated secondary sex traits. Together, these data suggest that living downstream of agriculture can alter size, hormone levels, and the expression of sexually dimorphic sex traits. Such changes to the reproductive endocrinology and morphology of male amphibians can be detrimental to the reproductive health and long-term reproductive success of amphibian populations.

In Chapter 3, I examined corticosterone, testosterone, and the reproductive clasping behaviors of adult male African clawed frogs (*Xenopus laevis*) exposed to field collected and simulated agricultural run-off. This experiment implemented a novel eco-relevant experimental design to mimic real-life agrochemical exposures. Male frogs were exposed to field water collected downstream (agricultural run-off) and upstream (negative control) of agricultural activity along the Salinas River, CA. In addition, a pesticide mixture containing the top agrochemicals used in the Monterey County was included to simulate agricultural run-off. Mating behavior was suppressed in males exposed to simulated agricultural run-off but enhanced in males exposed to field collected agricultural run-off. In addition, testosterone levels of clasping males were elevated in comparison to controls. Males immersed in simulated agricultural run-off had significantly lower testosterone levels than control males in 2010. These data suggest that agrochemical exposure (both field collected and simulated) can alter reproductive hormones and clasping behaviors. Altered sex hormones and behaviors in male amphibians may play a role in amphibian declines.

Lastly, this dissertation is summarized in Chapter 4. The applicability of this dissertation as a model for amphibian declines and other reproductive related human health concerns are also introduced.

This dissertation is dedicated to the animal nations.

“We send thanks to all the Animal life in the world. They have many things to teach us as people. We are glad they are still here and we hope it will always be so.”

– Excerpt from the Thanksgiving Address, Mohawk version –

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Always pass on what you have learned.  
Already know you that which you need.  
Try not. Do or do not. There is no try.  
– Yoda –



What we call the beginning is often the end. And to make an end is to make a beginning.  
The end is where we start from.  
– T. S. Eliot –

## Chapter 1



# The Impacts of Altered Reproductive Function on Global Amphibian Declines

## PURPOSE AND AIMS

This dissertation explores the role of agricultural contaminants (e.g. pesticides and fertilizers) as causes of decreased recruitment (via reproductive failure) in amphibian declines. A special emphasis was placed on examining the ability of agrochemicals to initiate a stress response, and how this stress response affects reproductive function in male amphibians. Field studies and laboratory simulations of ecologically relevant chemical exposures were conducted in order to gain a deeper understanding of agrochemical-induced stress and its impacts on male amphibian reproductive success.

## BACKGROUND

Although amphibian populations have been declining for over half a century (Alford and Richards, 1999; Beebee, 1973; Cooke, 1972; Houlahan et al., 2000), the magnitude of these declines was not realized until thirty years ago. In the early 1980's, researchers became aware of mass mortalities and disappearances of amphibian populations in both disturbed and pristine habitats around the world (Blaustein, 1994; Blaustein and Wake, 1990; Wake and et al., 1991; Wyman, 1990). Presently, worldwide declines are in the forefront of today's global biodiversity crisis. More than forty percent of amphibian species are in decline and an estimated one-third are on the brink of extinction (AmphibiaWeb; Stuart et al., 2004). In the wake of a sixth mass extinction (Wake and Vredenburg, 2008), much attention has focused on understanding why amphibian species are suffering from drastic declines.

The causes behind amphibian declines are context-dependent and are most often driven by the interaction of multiple stressors (Blaustein et al., 2011; Blaustein and Kiesecker, 2002; Boone and Bridges, 2003; Boone et al., 2003; Boone et al., 2007; Collins and Storer, 2003; Davidson and Knapp, 2007; Davidson et al., 2001; 2002; Hatch and Blaustein, 2003; Hayes et al., 2010a; Kiesecker et al., 2001; Reeves et al., 2010; Relyea, 2010; Richter et al., 1997; Rohr et al., 2004; Rohr et al., 2008). Habitat loss is the most documented cause of amphibian declines (reviewed in Alford and Richards, 1999). Other factors include climate change (Blaustein et al., 2010), introduced species (Adams, 1999; Boone et al., 2007; Bradford, 1991; Hayes and Jennings, 1986; Lawler et al., 1999; Vredenburg, 2004), over-exploitation (Gibbs et al., 1971; Jennings and Hayes, 1985), disease (Berger et al., 1998; Blaustein et al., 1994; Daszak et al., 1999; Daszak et al., 2003), and exposure to chemical contaminants (Blaustein et al., 2003; Boone et al., 2005; Collins and Crump, 2009). Of these factors, chemical contaminants (specifically those used in agriculture) have recently received more attention because of increasing evidence linking chemical exposure to amphibian declines.

In recent decades, agricultural pollution has been a suspected cause of amphibian declines (Beebee, 1973; Cooke, 1972; Frazer, 1964; Houlahan et al., 2000; Simms, 1969). Amphibian die-offs have been observed in populations living in areas of point-source agrochemical use since the late nineteen forties (Boyd et al., 1963; Fashingbauer, 1957; Herald, 1949; Mulla, 1963; Sanders, 1970; Vinson et al., 1963). Presently, prominent declines have occurred in contaminated ecosystems proximal to agrochemical use (Bishop et al., 1999; de Solla et al., 2002; Hamer et al., 2004; Houlahan and Findlay, 2003; Mann et al., 2009; Peltzer et al., 2008; Rouse et al., 1999; Russell et al., 1997) as well as habitats undisturbed by agricultural landscapes (Bradford et al., 2011; Davidson, 2004; Davidson et al., 2001; 2002; Fellers et al., 2007; Fellers et al., 2008; Hamer et al., 2004; Sparling and Fellers, 2009; Sparling et al., 2001;

Stallard, 2001). These disappearances have catalyzed widespread research efforts directed towards understanding the underlying mechanisms of agrochemical-associated amphibian declines. Of these investigations, the lethal effects of agrochemical exposure are the most documented. Numerous studies have confirmed the toxicity of agrochemical exposure and extensive data exists on the lethal effects of agrochemicals on amphibian species (reviewed in Pauli and Service, 2000; Sparling et al., 2010).

Although most studies that investigate the effects of agricultural chemicals and amphibian declines focus on toxicity, declines are more likely a result of the sub-lethal effects of agrochemical exposure (Davidson, 2004). Increased incidences of developmental aberrations such as retarded growth (Bridges, 2000; Carey and Bryant, 1995; Goleman et al., 2002; Hayes et al., 2006; Hecnar, 1995; Peltzer et al., 2008; Relyea, 2004a; Rohr et al., 2004) and limb deformities (Bridges, 2000; de Solla et al., 2002; Kiesecker, 2002; Ouellet et al., 1997; Rabinowitz et al., 2005; Reeves et al., 2010; Taylor et al., 2005) have been reported in animals subjected to agrochemical exposure. These malformations may hamper the ability to forage, which can lead to poor nutrition, increased susceptibility to predation and pathogens, as well as decrease reproductive success. As a result, long-term exposure at non-lethal doses can severely impact recruitment in amphibian populations and contribute to amphibian declines.

As more studies examine the impacts of non-lethal dose exposure on amphibian populations, the need for understanding the impact of sub-lethal effects on recruitment has been recognized (Bridges, 2000; Bridges and Semlitsch, 2000). However, few studies address impacts of agricultural chemicals on reproductive function. The paucity of studies in this area is concerning because endocrine disrupting agrochemicals are widespread in aquatic habitats that are home to amphibians which experience hormone regulated sexual development and reproductive cycles. Amphibians are especially vulnerable to agrochemicals because contaminants can easily traverse their highly permeable skin and unshelled eggs. Given these conditions, it is not surprising that sexual abnormalities such as hermaphroditism (Hayes et al., 2002a; McCoy et al., 2008; McDaniel et al., 2008; Reeder et al., 2005) and suppressed sex hormone levels (Mosconi et al., 2005) are linked to pesticide exposure in amphibian populations living in the wild.

In addition to observations in the field, laboratory models using relevant environmental concentrations have reported adverse effects on amphibian reproduction. Exposure to agrochemicals disrupts gonadal development (Hayes et al., 2002a; Hayes et al., 2002b), impairs gamete formation (Hayes et al., 2002a; Hayes et al., 2010b; Pickford and Morris, 2003; Stebbins-Boaz et al., 2004; Tavera-Mendoza et al., 2002a; b) and reduces sex hormone production (Hayes et al., 2002b; Hayes et al., 2010b; Hecker et al., 2005), as well as alters reproductive morphologies (Hayes et al., 2010b; van Wyk et al., 2003) and sexual behaviors (Hayes et al., 2010b; Park et al., 2001). Thus, exposure to these pollutants during reproductive development and sexual maturation and during breeding periods throughout adulthood can have adverse consequences on amphibian reproductive function and therefore negatively impact reproductive success.

In addition to direct effects, agricultural pollutants can inhibit reproduction function indirectly. For example, pesticide mixtures can increase stress hormone (corticosterone) levels in exposed adult amphibians (Hayes et al., 2006). Although moderate levels of corticosterone support reproductive activity by mobilizing energy stores (Moore and Jessop, 2003), chronically

elevated corticosterone (and stress in general) inhibits reproductive development and behavior and decreases fertility (Moore, 1983). Additionally, increased levels of corticosterone in amphibians can inhibit sex hormones (Burmeister et al., 2001) and breeding behaviors (Moore, 1983; Moore and Miller, 1984) necessary for reproductive success. Acute stress also decreases testosterone in male amphibians (Licht et al., 1983; Moore, 1983; Moore and Zoeller, 1985). Decreased testosterone is a concern because it regulates many functions necessary for reproduction, such as sperm production and breeding behaviors. Thus, agrochemical-induced stress may play a role in decreased reproduction in amphibians, thereby negatively impacting recruitment.

Chemical exposures (other than agrochemicals) can also induce stress responses in male amphibian species. Field studies of southern toads (*Bufo terrestris*) that resided in or transplanted from clean sites to areas contaminated with coal ash displayed elevated corticosterone (Hopkins et al., 1997). In the same study, exposed male toads also exhibited elevated testosterone levels in the presence of high plasma corticosterone during breeding and non-breeding months (Hopkins et al., 1997). Thus, the impact of chemical-induced stress on male amphibian reproductive function is complex. Firstly, many factors such as season, duration, and magnitude of stress hormone secretion, behavioral influence, and species-specific differences can also alter the dynamics of sex hormone suppression in reproduction (Wingfield and Sapolsky, 2003). Secondly, individuals that are exposed to enumerable chemical types and exposures can differ in duration and timing along an animal's life history. Lastly, the interactive effects of transient chemical mixtures on amphibian communities (in addition to other factors such as predation or disease) can have variable outcomes, and need to be examined in this context (Boone, 2008; Boone and Bridges, 2003; Boone and Semlitsch, 2001; Mann et al., 2009; Mills and Semlitsch, 2004).

## CURRENT RESEARCH

The decline of amphibian species can be attributed to many stressors that ultimately lead to death or decreased recruitment (Hayes et al., 2010a). However, emphases on catastrophic declines (i.e. rapid disappearances or massive die-offs) may overshadow the contribution of decreased recruitment in overall declining amphibian species (Hayes et al., 2010a). A broader research scope is needed to further understand amphibian declines. More reproductive studies examining the impact of decreased recruitment and amphibian declines are needed.

Additionally, studies that examine agro-pollutants and reproductive function tend to focus on the direct effects of single chemical exposures instead of the indirect effects of ecologically relevant mixtures. The direct and indirect effects of agricultural pollution raises concern because not only can agrochemicals act as individual stressors, they may also have synergistic effects when combined with other chemicals (Davidson and Knapp, 2007; Kiesecker, 2002). Moreover, the presence of environmental contaminants can exacerbate the effects of other co-existing stressors, thus increasing population declines (Blaustein et al., 2010; Relyea, 2004b).

Although there is a growing body of research implementing ecological relevancy in experimental design, such studies are still lacking. The shortage of ecologically relevant research may stem from a number of challenges associated with modeling the variability of real environmental chemical exposures under controlled conditions. Environmental chemical exposure is extremely variable because pollution sources are numerous and collectively

discharge a multitude of diverse chemical compounds (Alloway and Ayres, 1993). Environmental variables such as climate and topography create numerous exposure combinations of chemical types, concentrations, and duration of exposures (Farmer, 1997). Complexity is further increased as sensitivities to chemical exposure may differ across species (Bridges and Semlitsch, 2000; Hamer et al., 2004; Harris et al., 2000), between populations (Bridges and Semlitsch, 2000), and between individuals within a population (Bridges and Semlitsch, 2001).

Faced with such challenges, it is not surprising that the literature on amphibian reproductive health is primarily based on the impacts of a single or a few compounds. However, there is a great need for understanding the effects of agrochemical exposure on the reproductive function of declining amphibian populations within its true ecological context. Such research would provide a more holistic view of the underlying issues driving amphibian declines, which is necessary for understanding the true impact of agrochemicals on amphibian populations.

The role of agrochemical-induced stress in decreased reproductive function is not well understood. Although agricultural pollution has been proposed as a possible cause of amphibian declines, there is relatively little eco-toxicological research on agrochemical-induced stress and compromised reproductive ability. Despite a number of amphibian studies that examine capture-induced stress (Coddington and Cree, 1995; Licht et al., 1983; Moore and Zoeller, 1985; Mosconi et al., 1994; Mosconi et al., 2006) and pesticide exposure inhibition of reproductive hormones (Hayes et al., 2002b; McCoy et al., 2008; Mosconi et al., 2005), few studies have examined the repercussions of stress on other physiological and morphological amphibian reproductive characteristics.

Additionally, studies that have examined the effects of pesticides on reproductive traits or behaviors do not include analyses of the effects of stress on reproductive function. Pesticides can demasculinize male-specific traits such as coloration (McCoy et al., 2008) and breeding gland morphology (van Wyk et al., 2003), but corticosterone was not examined in these studies. Moreover, studies that examined the abatement of amphibian reproductive behaviors in the presence of corticosterone (Moore, 1983; Moore et al., 2005; Moore et al., 1994) did not specifically address the role of agrochemical-induced stress.

Although pesticides and stress both have consequences on amphibian reproduction, there is a dearth of information in regards to the effects of agrochemical-induced stress on reproductive function. Given the current state of the global biodiversity crisis and worldwide amphibian declines, the need for multi-factorial studies addressing massive declines in the context of reproductive function is paramount.

California is a hotspot for amphibian declines. Widespread agricultural pollution has been documented as a major factor contributing to dwindling populations. For over fifty years, California has been the nation's leading state in agriculture – and the number one user of pesticides in the United States. High pesticide use, resulting in ubiquitous non-point source pesticide contamination of California's waterways, provides an excellent opportunity to examine the relationship between agrochemical-induced stress and amphibian reproductive function.

## CHAPTER DESCRIPTIONS

This dissertation is divided into four chapters. The current chapter provides a general review of the factors contributing to worldwide amphibian declines and provides an overview of the previous research that has paved the way for this study. In Chapter 2 (a field study), I examined whether living downstream of agricultural activity alters the stress response and reproductive function of wild adult male American bullfrogs (*Lithobates catesbeianus*) of the Salinas, Sacramento and San Joaquin Rivers of California. To assess whether laboratory simulations predict situations in the wild (Chapter 3), I used field collected and laboratory-simulated agricultural run-off conditions along the Salinas River to assess the impacts of reproductive function on adult male African clawed frogs (*Xenopus laevis*). In Chapter 3 I describe the effects of simulated and field collected agricultural run-off on endogenous stress (corticosterone) and sex hormone (testosterone) levels. Continued investigations examine the results of altered stress and sex hormone levels by agrochemical exposure on the reproductive morphology, fecundity, and breeding behaviors of male *X. laevis*. In the concluding discussion (chapter 4) I introduce the applicability of this dissertation as a model for amphibian declines and other reproductive related human health concerns.

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## Chapter 2



Differences in Size, Corticosterone, Testosterone, and  
Secondary Sex Traits between Male Bullfrogs Living  
Upstream and Downstream of Agricultural Activity from the  
Salinas, Sacramento, and San Joaquin Rivers (CA)

## ABSTRACT

Agricultural pollution is one of the major factors contributing to amphibian declines worldwide. Exposure to agricultural run-off (which contains an innumerable amount of chemicals) can induce a stress response. Chronic stress, which is associated with elevated corticosterone levels, can suppress testosterone production. Decreased testosterone is a concern because it is the hormone responsible for the development and maintenance of sexual dimorphic traits in male amphibians. To test whether agrochemical exposure affected stress and reproductive function, I collected bullfrogs upstream and downstream of agricultural activity across three California river systems (Salinas, Sacramento, and San Joaquin) and examined primary and secondary sex traits, sperm count, and corticosterone and testosterone levels. Overall, exposure to agricultural run-off (i.e. residing downstream of agricultural activity) had a negative effect on body size and resulted in elevated corticosterone and testosterone levels. Although individual river analysis also revealed that downstream males from the Salinas and San Joaquin Rivers were small in size and had elevated testosterone levels, only downstream males of the San Joaquin had elevated corticosterone and exaggerated secondary sex traits. Together, these data suggest that living downstream of agriculture can alter size, hormone levels, and the expression of sexually dimorphic sex traits. Such changes to the reproductive endocrinology and morphology of male amphibians can be detrimental to the reproductive health and long-term reproductive success of amphibian populations.

## INTRODUCTION

Amphibian populations have been declining over the past several decades (Houlahan *et al.*, 2000). An estimated one-third of amphibian species are on the brink of extinction and more than forty percent of amphibian species are in decline (AmphibiaWeb; Stuart *et al.*, 2004). Agricultural pollution is one of the factors contributing to amphibian declines and extensive data exists on the lethal effects of agrochemicals on amphibian species (reviewed in Pauli *et al.*, 2000; Sparling and Fellers, 2009). Although numerous laboratory studies have confirmed the lethal effects of high dose exposures, amphibian declines more likely stem from the sub-lethal effects of agrochemical exposure (Davidson, 2004). With the increasing amount of studies that investigate the impacts of non-lethal dose exposure on amphibian populations, the need for understanding the impact of sub-lethal effects on recruitment has been recognized (Bridges, 2000; Bridges and Semlitsch, 2000). However, few studies address the impacts of agricultural chemicals on reproductive function. Endocrine disrupting agrochemicals are widespread in aquatic habitats, which is a concern because amphibians undergo hormone regulated sexual development and reproductive cycles. Amphibians are especially vulnerable because they have highly permeable skin, which contaminants can easily cross. Given these conditions, it is not surprising that low dose agrochemical exposure disrupts gonadal development (Hayes *et al.*, 2002a; Hayes *et al.*, 2002b), impairs gamete formation (Hayes *et al.*, 2002a; Hayes *et al.*, 2010; Pickford and Morris, 2003; Stebbins-Boaz *et al.*, 2004; Tavera-Mendoza *et al.*, 2002a; b) and reduces sex hormone production (Hayes *et al.*, 2002b; Hayes *et al.*, 2010; Hecker *et al.*, 2005), as well as alters reproductive morphologies (Hayes *et al.*, 2010; van Wyk *et al.*, 2003) and sexual behaviors (Hayes *et al.*, 2010; Park *et al.*, 2001). Thus, agrochemical exposure during reproductive development, sexual maturation, and adulthood can have adverse consequences on amphibian reproductive function and therefore negatively impact reproductive success.



In addition to direct effects, agricultural pollutants can inhibit reproductive function indirectly. For example, pesticide mixtures can increase stress hormone (corticosterone) levels in exposed adult amphibians (Hayes *et al.*, 2006). Although moderate levels of corticosterone support reproductive activity by mobilizing energy stores (Moore and Jessop, 2003), chronically elevated corticosterone (and stress in general) inhibits reproductive development and behavior and decreases fertility (Moore, 1983). Additionally, increased levels of corticosterone in amphibians can inhibit sex hormones (Burmeister *et al.*, 2001) and breeding behaviors (Moore, 1983; Moore and Miller, 1984) necessary for reproductive success. Acute stress also decreases testosterone in male amphibians (Licht *et al.*, 1983; Moore, 1983; Moore and Zoeller, 1985), and testosterone regulates many functions necessary for reproduction, such as sperm production and breeding behaviors. Thus, agrochemical-induced stress may play a role in decreased reproduction in amphibians, thereby negatively impacting recruitment and contributing to amphibian declines.

In the present study, I examined the effects of agrochemical exposure on the stress response and reproductive function of male American bullfrogs (*Lithobates catesbeianus*). To determine whether agrochemical exposure affects stress and reproduction, animals were collected upstream and downstream of agricultural activity across three California river systems (Salinas, Sacramento and San Joaquin). Size, primary and secondary sex traits, sperm count, and corticosterone and testosterone levels were examined in *L. catesbeianus*. I hypothesized that bullfrogs collected downstream of agricultural run-off would: (1) have elevated corticosterone levels (2) have lower testosterone levels as a result of elevated corticosterone levels and/or exposure to endocrine disrupting pesticides, and (3) be smaller in size, with less developed primary and secondary sex traits, and have lower sperm counts.

## MATERIALS AND METHODS

### *Field site selection*

I studied six field site locations along three California river systems (Salinas, Sacramento and San Joaquin). Field sites for each river system were chosen upstream (reference site) and downstream of agricultural activity. Areas of agricultural activity were determined by Google Earth Pro satellite images (Google Inc. Mountain View, CA).

### *Animal and plasma collection*

For the Salinas River, I collected upstream animals on 1 July 2009 in San Luis Obispo County, Santa Margarita (STM) (N 35°20.908', W 120°30.741') (males: n = 12; females: n = 11). Downstream Salinas River animals were collected on 27 June 2009 in Monterey County, Salinas (SAL) (N 36°38.828', W 121°42.161') (males: n = 14; females: n = 11). Upstream Sacramento River animals were collected on 7 July 2009 in Shasta County, Battle Creek (BC) (N 40°23.902' W 122°09.350') (males: n = 6; females: n = 10) and downstream animals were collected on 10 July 2009 in Sutter County, Sutter (SUT) (N 39°04.413' W 121° 44.913') (males: n = 10; females: n = 10). For the San Joaquin River, upstream animals were collected on 4 July 2009 in Stanislaus County, Del Puerto Canyon (DPC) (N 37°28.437', W 121°14.354') (males: n = 10; females: n = 6) and downstream animals were collected on 16 July 2009 in San Joaquin County, San Joaquin (SJ) (N 37°37.810' W 121°12.340') (males: n = 11; females: n = 2).

All bullfrogs (*Lithobates catesbeianus*) were collected at night between the hours of 22:00 and 04:30. Only adult bullfrogs were collected for this study. Adult status was determined by size and/or the presence of sexual dimorphic traits in males (e.g. yellow throat, enlarged tympanum, etc.). Additionally, small parasitic males (described in Howard, 1978; 1984) were also collected if they displayed sexually dimorphic traits. Animals were caught by hand and blood collected via cardiac puncture within five minutes of capture. Blood samples were immediately placed on ice. Plasma was collected by aspiration after low-speed centrifugation and stored frozen (-20°C) until analysis. Water temperature at the location of each animal was recorded. Animals were returned to the laboratory for morphometric analysis then euthanized by decapitation. Digital photographs were then taken of throats. After tissue dissection and identification of sex, animals were fixed in Bouin's fixative for 48 hours and preserved in 70% ethanol for future analysis.

#### *General measurements*

*Snout-vent length (SVL) & Body weight (BW).* Prior to euthanization, animals were weighed and (SVL) was measured from anterior of the lower jaw to vent.

#### *Primary sexual characteristics*

*Gonado-somatic index (GSI).* To evaluate gonad size in male bullfrogs, the left gonad was dissected and weighed before preservation. GSI was calculated using the following formula:

$$GSI = \left( \frac{\text{gonad weight}}{\text{body weight}} \right) \times 100$$

#### *Secondary sexual characteristics*

*Tympanum diameter & nuptial pad thickness.* Structures were measured to the nearest 1 mm with calipers graduated at 1 mm intervals. Tympanum diameters were measured horizontally, anterior to posterior. Nuptial pad measurements were taken of the thickest portion of the pad. For three-way, two-way, and one-way ANOVA analyses, tympanum diameter and nuptial pad thickness were normalized by SVL.

*Yellow throat coloration – Hue.* Animals were photographed immediately after euthanization. Digital images were taken of bullfrog throats using a Canon Powershot SX10 IS digital camera. Ambient light was standardized by keeping ambient light constant (uniform lighting) and all images were taken without flash. All photos were taken using the camera's portrait setting and the distance between the camera lens and white background was standardized at 71 centimeters. All digital images were saved as TIFF files (3648 x 2736 pixels, 32 bits per pixel: RGB) for yellow throat coloration analysis.

Hue values were obtained to quantify throat coloration in male bullfrogs. Hue, generally known as color (i.e. yellow, orange, green, etc.), is measured in degrees. Lower values indicate colors in the orange range and higher values in the yellow range. Throat images were analyzed using ImageJ, version 1.46 (NIH). Hue analysis of throat coloration was conducted using four methods. RGB (red, green, blue) values were recorded for the entire throat area, along the medial and lateral throat axis, and a patch of the darkest yellow area of the throat (Fig. 1, A-D).

The following formulas were applied to convert RGB values to hue (Gonzalez and Richard, 2002). First, RGB values were normalized using the following formula.

$$r = \frac{R}{R + G + B}, g = \frac{G}{R + G + B}, b = \frac{B}{R + G + B}$$

Second, normalized RGB values (rgb) were used to calculate hue (degrees).

$$h = \cos^{-1} \left\{ \frac{0.5 \cdot [(r - g) + (r - b)]}{[(r - g)^2 + (r - b)(g - b)]^{1/2}} \right\} \quad h \in [0, \pi] \text{ for } b \leq g$$

$$h = 2\pi - \cos^{-1} \left\{ \frac{0.5 \cdot [(r - g) + (r - b)]}{[(r - g)^2 + (r - b)(g - b)]^{1/2}} \right\} \quad h \in [\pi, 2\pi] \text{ for } b > g$$

*Yellow throat coloration – Yellow Index (YI).* YI of male throats was calculated to determine the area of yellow pigmentation of the throat region.

$$YI = \left( \frac{\text{yellow area}}{\text{total throat area}} \right) \times 100$$

Using ImageJ, the throat region was outlined and the total throat area (mm<sup>2</sup>) was recorded (Fig. 2, A). The following steps were taken to quantify the yellow area of the throat region. First, the area outside the throat region was deleted (edit → clear outside; (Fig. 2, B). Second, the original RGB image was converted to CMKY using the RGB to CMKY plug-in to obtain a yellow slice of the digital image (Fig. 2, C). Third, the yellow slice was converted to HSB and the brightness slice was obtained using the stack-to-images function (Fig. 2, D). Lastly, the brightness slice was then converted to binary (Fig. 2, E) and the threshold function was used to measure the area (mm<sup>2</sup>) selected (Fig. 2, F).

#### *Sperm count*

Sperm count was determined by measuring the histologically stained area of sperm in the largest tubule of the largest testicular cross-section. Preserved testes were dehydrated in graded alcohols and infiltrated with histoclear (National Diagnostics, Atlanta, GA) then paraffin (Fisher Scientific, Pittsburgh, PA). Sections were cut at 8 μm with a rotary microtome and stained in hematoxylin & eosin (H&E). The largest tubule from the largest cross-section was determined using a Nikon Optiphot-2 microscope (Technical Instruments). Photomicrographs were taken using a Nikon Digital Sight DS-U (Technical Instruments) and NIS Freeware 2.1 (NIS-Elements; Nikon Instruments). Digital images were saved as TIFF files (3648 x 2736 pixels) for sperm count analysis.

Methods for measuring sperm area were adapted as described in Murakami *et al.* (2005). In short, photomicrographs of the largest tubule were analyzed using Scion Image Software (NIH). A black and white image was inverted and objects of interest were separated from background using the threshold function in Scion Image. The black and white image was then converted to binary. For the color image, areas excluded from sperm analysis were removed and then the

image was converted into HSV (hue, saturation, value) format. Image math was used to combine the binary and HSV image. Lastly, the LUT (look-up table) toolbar isolated and highlighted the sperm within the largest tubule and sperm area was recorded ( $\text{mm}^2$ ).

#### *Radio-immuno assay (RIA)*

*Testosterone (T) & Corticosterone (CORT).* Plasma samples for T and CORT analysis were extracted with diethyl ether and dried under nitrogen gas. Samples were then reconstituted in phosphate buffered saline with gelatin (PBS-G). Plasma T and CORT levels were determined by RIA as described in (Licht *et al.*, 1983). Antibodies were obtained from Fitzgerald Industries (Acton, MA) for testosterone and MP biomedical (Solon, OH) for corticosterone and both were validated for use with *L. catesbeianus*.

*Bleed time and T.* Differences between blood collection times for upstream and downstream males within individual rivers were analyzed. Blood samples were obtained between the hours of 2300 and 0430. Reported hours (1 through 7) were assigned by first hour collected (2300 hours = 1) and last hour collected (0400 = 7). Minutes were converted into decimal time (minutes  $\div$  60). Correlation analysis was conducted to determine whether time bled affected testosterone levels.

#### *Statistical analysis*

All statistical analyses were conducted using Minitab 16.2.3 (Minitab Inc. State College, PA). Three-way ANOVA was used to examine whether animals differed in general measurements (SVL & BW), tympanum and nuptial pad size (normalized for SVL), and steroid levels (T & CORT) between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (upstream or downstream of agricultural activity), sex (male or female), and whether there was an interactive effect.

Two-way ANOVA was used to examine whether males differed in general measurements, primary and secondary sexual characteristics, sperm count, and steroid levels between rivers (Salinas, Sacramento and San Joaquin), regions (upstream or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p < 0.05$ ).

One-way ANOVA examined whether upstream and downstream males differed in general measurements, primary and secondary sexual characteristics, sperm count, bleed times, and steroid levels within individual river systems. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).

Analysis of covariance (ANCOVA) was used to examine the relationship between SVL (covariate) and the dependent variable (tympanum diameter or nuptial pad thickness) for each of the following groupings: (1) males and females (sex), (2) males collected from three different river systems (river), (3) upstream and downstream males (region), and (4) upstream and downstream males from each individual river system (site).

Pearson's correlation coefficients were used to examine correlations between general measurements, primary and secondary sexual characteristics, sperm count, and steroid levels of male bullfrogs. Correlation analysis of steroid levels and normalized measurements (GSI, tympanum, and nuptial pad) of males were also conducted, as well as male testosterone levels and blood collection times.

## RESULTS

**SVL.** Three-way ANOVA revealed that Salinas River animals were significantly larger than animals from the San Joaquin River (THREE-WAY ANOVA:  $F = 4.15$ ,  $df = 2$ ,  $p = 0.020$ ; Fig. 3). Overall, bullfrogs collected upstream of agricultural activity were larger than downstream animals (THREE-WAY ANOVA:  $F = 9.20$ ,  $df = 1$ ,  $p = 0.003$ ). There was no difference in SVL among male and female bullfrogs (THREE-WAY ANOVA:  $F = 0.58$ ,  $df = 1$ ,  $p = 0.448$ ). Bullfrogs collected upstream along the Salinas River were larger than animals collected upstream and downstream of agricultural activity along the Sacramento and San Joaquin Rivers (THREE-WAY ANOVA:  $F = 6.51$ ,  $df = 2$ ,  $p = 0.002$ ). There was no significant interaction between river and sex (THREE-WAY ANOVA:  $F = 0.39$ ,  $df = 2$ ,  $p = 0.681$ ). However, Salinas River females were significantly larger than San Joaquin River males ( $T = -3.155$ ,  $p = 0.027$ ). There was no significant effect of region and sex (THREE-WAY ANOVA:  $F = 0.27$ ,  $df = 1$ ,  $p = 0.605$ ). However, upstream females were significantly larger than downstream males ( $T = 3.013$ ,  $p = 0.0182$ ). There was no significant interaction between river, region, and sex (THREE-WAY ANOVA:  $F = 1.07$ ,  $df = 2$ ,  $p = 0.348$ ). However, upstream males and females from the Salinas River were significantly larger than San Joaquin downstream males (Fig. 3).

Two-way ANOVA revealed that males from the Salinas River were significantly larger than San Joaquin River males (TWO-WAY ANOVA:  $F = 6.05$ ,  $df = 2$ ,  $p = 0.005$ ; Fig. 4). Males collected upstream of agricultural zones were larger than males collected downstream of agricultural activity (TWO-WAY ANOVA:  $F = 6.25$ ,  $df = 1$ ,  $p = 0.016$ ). Upstream Salinas River males (STM) were significantly larger than downstream Salinas River males (SAL), upstream Sacramento males (BC), and upstream (DPC) and downstream (SJ) males of the San Joaquin River (TWO-WAY ANOVA:  $F = 9.49$ ,  $df = 2$ ,  $p < 0.001$ ; Fig. 4). Downstream males were smaller than upstream males along the Salinas (ANOVA:  $F = 21.5$ ,  $df = 1$ ,  $p = 0.001$ ) and San Joaquin (ANOVA:  $F = 13.1$ ,  $df = 1$ ,  $p = 0.002$ ) Rivers (Table 1).

**BW.** Three-way ANOVA revealed that Salinas River animals were significantly heavier than animals from the Sacramento and San Joaquin Rivers (THREE-WAY ANOVA:  $F = 10.88$ ,  $df = 2$ ,  $p < 0.001$ ; Fig. 5). Overall, bullfrogs collected upstream of agricultural activity were heavier than downstream animals (THREE-WAY ANOVA:  $F = 20.85$ ,  $df = 1$ ,  $p < 0.001$ ). There was no difference in BW among male and female bullfrogs (THREE-WAY ANOVA:  $F = 0.04$ ,  $df = 1$ ,  $p = 0.835$ ). Bullfrogs collected upstream along the Salinas River were heavier than animals collected upstream and downstream of agricultural activity along the Sacramento and San Joaquin Rivers (THREE-WAY ANOVA:  $F = 16.89$ ,  $df = 2$ ,  $p < 0.001$ ). There was no significant interaction between river and sex (THREE-WAY ANOVA:  $F = 0.23$ ,  $df = 2$ ,  $p = 0.789$ ). However, Salinas River males ( $T = -4.112$ ,  $p = 0.0013$ ) and females ( $T = -3.957$ ,  $p = 0.0022$ ) were significantly heavier than San Joaquin River males. There was no significant interaction between region and sex (THREE-WAY ANOVA:  $F = 0.23$ ,  $df = 1$ ,  $p = 0.631$ ). However, upstream males were significantly heavier than downstream males ( $T = 3.273$ ,  $p = 0.0085$ ) and downstream females ( $T = 3.1140$ ,  $p = 0.0135$ ). Likewise, upstream females were heavier than downstream males ( $T = 3.394$ ,  $p = 0.0059$ ) and downstream females ( $T = 3.2301$ ,  $p = 0.0096$ ). There was no significant interaction between river, region, and sex (THREE-WAY ANOVA:  $F = 1.03$ ,  $df = 2$ ,  $p = 0.360$ ). However, upstream males from the Salinas River were significantly heavier than downstream Salinas River animals, as well as upstream and downstream along the Sacramento and San Joaquin Rivers (Fig. 5). Similarly, upstream Salinas River females were

significantly heavier than all other groups except upstream Salinas River males (STM) and downstream Sacramento males (SUT) (Fig. 5).

Two-way ANOVA revealed that males from the Salinas River were significantly heavier than males from the San Joaquin River. (TWO-WAY ANOVA:  $F = 11.92$ ,  $df = 2$ ,  $p < 0.001$ ; Fig. 6). Upstream males were heavier than males collected downstream of agricultural activity (TWO-WAY ANOVA:  $F = 14.9$ ,  $df = 1$ ,  $p < 0.001$ ). Upstream Salinas River males (STM) were significantly heavier than downstream Salinas River males (SAL), upstream (BC) and downstream (SUT) Sacramento River males, and upstream (DPC) and downstream (SJ) males of the San Joaquin River (TWO-WAY ANOVA:  $F = 18.12$ ,  $df = 2$ ,  $p < 0.001$ ; Fig. 6). Downstream males had significantly lower body weights than upstream males along the Salinas River (ANOVA:  $F = 38.19$ ,  $df = 1$ ,  $p < 0.001$ ) and San Joaquin River (ANOVA:  $F = 6.23$ ,  $df = 1$ ,  $p = 0.022$ ) (Table 1).

*GSI*. There was no difference in GSI within river (TWO-WAY ANOVA:  $F = 0.11$ ,  $df = 2$ ,  $p = 0.893$ ; Fig. 7) and region (TWO-WAY ANOVA:  $F = 1.72$ ,  $df = 1$ ,  $p = 0.197$ ). Although the interaction between river and region was significant (TWO-WAY ANOVA:  $F = 4.18$ ,  $df = 2$ ,  $p = 0.022$ ), post-hoc analysis revealed that the difference between means were insignificant (Fig. 7). However, downstream males along the Salinas River (SAL) had significantly higher GSI values than upstream males (STM) (ANOVA:  $F = 4.80$ ,  $df = 1$ ,  $p = 0.053$ ; Table 1).

*Tympanum diameter*. Three-way ANOVA revealed there was no significant difference in normalized tympanum diameters across rivers (THREE-WAY ANOVA:  $F = 0.24$ ,  $df = 2$ ,  $p = 0.785$ ; Fig. 8) or region (THREE-WAY ANOVA:  $F = 20.85$ ,  $df = 1$ ,  $p = 0.257$ ). Normalized tympanum diameters were larger in males than females (THREE-WAY ANOVA:  $F = 145.08$ ,  $df = 1$ ,  $p < 0.001$ ; Fig. 8). There was no significant difference in normalized tympanum diameters between river and region (THREE-WAY ANOVA:  $F = 0.86$ ,  $df = 2$ ,  $p = 0.426$ ) and river and sex (THREE-WAY ANOVA:  $F = 0.26$ ,  $df = 2$ ,  $p = 0.774$ ). However, male normalized tympanums were significantly larger than females (Fig. 8). There was no significant interaction between region and sex (THREE-WAY ANOVA:  $F = 0.50$ ,  $df = 1$ ,  $p = 0.480$ ). However, male normalized tympanums were significantly larger than females (Fig. 8). There was a significant interaction between river, region and sex (THREE-WAY ANOVA:  $F = 3.62$ ,  $df = 2$ ,  $p = 0.032$ ; Fig. 8) stemming from the difference between male and female normalized tympanum diameters.

There was no difference in normalized tympanum diameters within river (TWO-WAY ANOVA:  $F = 0.02$ ,  $df = 2$ ,  $p = 0.985$ ; Fig. 9) or region (TWO-WAY ANOVA:  $F = 1.50$ ,  $df = 1$ ,  $p = 0.228$ ). Although the interaction effect was significant (TWO-WAY ANOVA:  $F = 4.20$ ,  $df = 2$ ,  $p = 0.021$ ), post-hoc analysis revealed that the difference between means was insignificant (Fig. 9). However, downstream males along the Salinas River had significantly smaller tympanum diameters than upstream males (ANOVA:  $F = 6.83$ ,  $df = 1$ ,  $p = 0.024$ ; Table 2). Additionally, downstream males along the San Joaquin River had larger tympanum diameters than upstream males (ANOVA:  $F = 3.20$ ,  $df = 1$ ,  $p = 0.090$ ; Table 2).

ANCOVA showed that male tympanum size increases faster relative to SVL (ANCOVA:  $F = 19.08$ ,  $df = 1$ ,  $p < 0.001$ ) and males had larger tympanum diameters than females (ANCOVA:  $F = 5.14$ ,  $df = 1$ ,  $p = 0.026$ ) (Fig. 10A). There was no significant difference between tympanum growth relative to SVL (ANCOVA:  $F = 0.87$ ,  $df = 2$ ,  $p = 0.428$ ) and size (ANCOVA:  $F = 0.92$ ,  $df = 2$ ,  $p = 0.407$ ) of males across rivers (Fig. 11A). Tympanum growth relative to SVL (ANCOVA:  $F = 1.15$ ,  $df = 1$ ,  $p = 0.288$ ) and size (ANCOVA:  $F = 1.07$ ,  $df = 1$ ,  $p = 0.306$ ) did not differ between males collected upstream or downstream of agricultural activity (Fig. 12A).

Tympanum growth relative to SVL (ANCOVA:  $F = 0.67$ ,  $df = 1$ ,  $p = 0.435$ ) or size (ANCOVA:  $F = 0.57$ ,  $df = 1$ ,  $p = 0.486$ ) did not differ between upstream and downstream males from the Salinas River (Fig. 13A). Likewise, Sacramento River males did not differ in tympanum growth relative to SVL (ANCOVA:  $F = 0.21$ ,  $df = 1$ ,  $p = 0.657$ ) or size (ANCOVA:  $F = 0.40$ ,  $df = 1$ ,  $p = 0.538$ ) (Fig. 13B). Tympanum growth relative to SVL did not differ in males from the San Joaquin River (ANCOVA:  $F = 0.01$ ,  $df = 1$ ,  $p = 0.909$ ; Fig. 13C). However, tympanum diameters of downstream males were significantly larger than upstream San Joaquin males (ANCOVA:  $F = 5.55$ ,  $df = 1$ ,  $p = 0.030$ ) (Fig. 13C).

*Nuptial pad thickness.* Although three-way ANOVA revealed there was a significant difference in normalized nuptial pad diameters across river systems (THREE-WAY ANOVA:  $F = 3.18$ ,  $df = 2$ ,  $p = 0.047$ ), post-hoc analysis showed that the difference between means were insignificant (Fig. 14). Overall, upstream animals had significantly larger normalized nuptial pad diameters than downstream animals (THREE-WAY ANOVA:  $F = 4.11$ ,  $df = 1$ ,  $p = 0.046$ ). Normalized nuptial diameters were larger in males than females (THREE-WAY ANOVA:  $F = 113.55$ ,  $df = 1$ ,  $p < 0.001$ ). There was no significant difference in normalized nuptial diameters between river and region (THREE-WAY ANOVA:  $F = 1.70$ ,  $df = 2$ ,  $p = 0.190$ ). However, upstream animals from the Salinas River had larger nuptial diameters than downstream animals of the Sacramento River ( $T = 3.2523$ ,  $p = 0.0205$ ). There was no significant interaction between river and sex (THREE-WAY ANOVA:  $F = 0.58$ ,  $df = 2$ ,  $p = 0.560$ ). However, male normalized nuptial diameters were significantly larger than females (Fig. 14). There was no significant interaction between region and sex (THREE-WAY ANOVA:  $F = 0.11$ ,  $df = 1$ ,  $p = 0.742$ ). However, male normalized nuptial pads were significantly larger than females (Fig. 14). There was a significant interaction between river, region and sex (THREE-WAY ANOVA:  $F = 4.14$ ,  $df = 2$ ,  $p = 0.020$ ; Fig. 14) stemming from the difference between male and female normalized nuptial pad diameters.

There was no difference in normalized nuptial pad thickness within river (TWO-WAY ANOVA:  $F = 2.57$ ,  $df = 2$ ,  $p = 0.08$ ; Fig. 15) or region (TWO-WAY ANOVA:  $F = 1.46$ ,  $df = 1$ ,  $p = 0.233$ ). Upstream males along the Salinas River (STM) had larger nuptial pads than downstream Salinas River males (SAL), upstream (DPC) and downstream (SUT) Sacramento River males, and upstream San Joaquin River males (DPC) (TWO-WAY ANOVA:  $F = 2$ ,  $df = 5.13$ ,  $p = 0.010$ ; Fig. 15). Downstream males had significantly smaller nuptial pads (SAL) than upstream males (STM) along the Salinas River (ANOVA:  $F = 6.83$ ,  $df = 1$ ,  $p = 0.024$ ; Table 2).

ANCOVA showed that male nuptial pad size increases faster relative to SVL (ANCOVA:  $F = 8.73$ ,  $df = 1$ ,  $p = 0.004$ ) and males had larger nuptial pad diameters than females (ANCOVA:  $F = 100.67$ ,  $df = 1$ ,  $p < 0.001$ ) (Fig. 10B). There was a significant difference between nuptial pad growth relative to SVL across rivers (ANCOVA:  $F = 3.13$ ,  $df = 2$ ,  $p = 0.054$ ) but no difference in size (ANCOVA:  $F = 0.92$ ,  $df = 2$ ,  $p = 0.407$ ) of males across rivers (Fig. 11B). Upstream male nuptial pad size increased faster relative to SVL (ANCOVA:  $F = 9.90$ ,  $df = 1$ ,  $p = 0.003$ ) and had larger nuptial pads than downstream males (ANCOVA:  $F = 9.43$ ,  $df = 1$ ,  $p = 0.004$ ) (Fig. 12B). Nuptial pad growth relative to SVL (ANCOVA:  $F = 0.53$ ,  $df = 1$ ,  $p = 0.485$ ) or size (ANCOVA:  $F = 0.42$ ,  $df = 1$ ,  $p = 0.532$ ) did not differ between upstream and downstream males from the Salinas River (Fig. 16A). Likewise, Sacramento River males did not differ in nuptial pad growth relative to SVL (ANCOVA:  $F = 0.05$ ,  $df = 1$ ,  $p = 0.829$ ) or size (ANCOVA:  $F = 0.06$ ,  $df = 1$ ,  $p = 0.818$ ) (Fig. 16B). Nuptial pad growth relative to SVL did not differ in males from the San Joaquin River (ANCOVA:  $F = 0.14$ ,  $df = 1$ ,  $p = 0.713$ ; Fig. 16C). However, nuptial pad diameters of downstream

males were significantly larger than upstream San Joaquin males (ANCOVA:  $F = 4.29$ ,  $df = 1$ ,  $p = 0.053$ ) (Fig. 16C).

*Hue.* Hue analysis of yellow throat coloration was done in four ways. Hue was determined for the entire throat area, along the medial and lateral throat axis and a patch of the darkest yellow area of the throat (Fig. 1, A-D). For the first method, there was no difference in hue for the entire throat region within river (TWO-WAY ANOVA:  $F = 1.76$ ,  $df = 2$ ,  $p = 0.181$ ) and region (TWO-WAY ANOVA:  $F = 0.05$ ,  $df = 1$ ,  $p = 0.831$ ) (Table 3). Downstream Salinas River males (SAL) had significantly higher hue values than upstream Salinas River males (STM), downstream Sacramento River males (SUT) and downstream San Joaquin River males (SJ) (TWO-WAY ANOVA:  $F = 2$ ,  $df = 2$ ,  $p = 0.002$ ; Table 3). Upstream Salinas River males (STM) were more dark yellow to orange in color compared to the yellow pigmentation of downstream males (SAL) (ANOVA:  $F = 12.89$ ,  $df = 1$ ,  $p = 0.001$ ; Table 4).

The second method measured hue along the medial throat axis (Figure 1B). There was no difference in medial throat axis hue within region (TWO-WAY ANOVA:  $F = 1.96$ ,  $df = 1$ ,  $p = 0.167$ ; Table 3). Males of the Salinas River had significantly higher hue values than Sacramento River males (TWO-WAY ANOVA:  $F = 3.43$ ,  $df = 2$ ,  $p = 0.039$ ; Table 3). Downstream Salinas River males (SAL) had more yellow pigmentation than the orange pigment of downstream Sacramento River males (SUT) (TWO-WAY ANOVA:  $F = 5.69$ ,  $df = 2$ ,  $p = 0.006$ ; Table 3). Hue of upstream Salinas River males (STM) were more orange than downstream males (SAL) whose pigment was in the yellow range (ANOVA:  $F = 5.38$ ,  $df = 1$ ,  $p = 0.029$ ; Table 4).

Hue measurements taken along the lateral throat axis (Fig. 1C) revealed no difference within river (TWO-WAY ANOVA:  $F = 2.89$ ,  $df = 2$ ,  $p = 0.064$ ) or region (TWO-WAY ANOVA:  $F = 0.66$ ,  $df = 1$ ,  $p = 0.419$ ) (Table 3). The throats of downstream Salinas River males were more yellow along the lateral axis than downstream Sacramento River males (SUT) whose pigment was more orange (TWO-WAY ANOVA:  $F = 5.91$ ,  $df = 2$ ,  $p = 0.005$ ; Table 3). Upstream males of the Salinas River (STM) were more orange than downstream males (SAL) (ANOVA:  $F = 9.08$ ,  $df = 1$ ,  $p = 0.006$ ; Table 4).

The last method measured the hue of a patch outline taken of the darkest pigmented area of the throat (Fig. 1D). There was no difference in patch hue within region (TWO-WAY ANOVA:  $F = 1.61$ ,  $df = 1$ ,  $p = 0.209$ ; Table 3). Salinas River males had significantly higher patch hue values than Sacramento River males (TWO-WAY ANOVA:  $F = 8.35$ ,  $df = 2$ ,  $p = 0.001$ ; Table 3). The patch hue of downstream Salinas River males (SAL) were significantly higher than upstream males (STM), downstream Sacramento River males (SUT), and downstream San Joaquin River males (SJ) (TWO-WAY ANOVA:  $F = 11.56$ ,  $df = 2$ ,  $p < 0.001$ ; Table 3). Upstream Salinas River males (STM) were more orange than downstream males (SAL) (ANOVA:  $F = 22.01$ ,  $df = 1$ ,  $p < 0.001$ ; Table 4). In contrast, downstream San Joaquin River males (SJ) were more orange than yellow pigmented upstream males (DPC) (ANOVA:  $F = 5.48$ ,  $df = 1$ ,  $p = 0.030$ ; Table 4).

*Yellow Index (YI).* There was no difference in YI within region (TWO-WAY ANOVA:  $F = 0.00$ ,  $df = 1$ ,  $p = 0.987$ ). Salinas River males had significantly lower YI than Sacramento River males (TWO-WAY ANOVA:  $F = 4.30$ ,  $df = 2$ ,  $p = 0.018$ ; Fig. 17). Downstream Salinas River males (SAL) had significantly lower YI values than downstream Sacramento River males (SUT) and downstream San Joaquin River males (SJ) (TWO-WAY ANOVA:  $F = 4.23$ ,  $df = 2$ ,  $p = 0.019$ ; Fig. 17). Downstream San Joaquin River males (SJ) had significantly higher YI values than upstream males (DPC) (ANOVA:  $F = 4.47$ ,  $df = 1$ ,  $p = 0.048$ ; Table 5).



*Sperm count.* There was no difference in sperm count within region (TWO-WAY ANOVA:  $F = 0.35$ ,  $df = 1$ ,  $p = 0.559$ ) or the interaction between river and region (TWO-WAY ANOVA:  $F = 2.27$ ,  $df = 2$ ,  $p = 0.119$ ). Sacramento River males had significantly higher sperm counts than San Joaquin River males (TWO-WAY ANOVA:  $F = 3.39$ ,  $df = 2$ ,  $p = 0.045$ ; Fig. 18). Downstream Salinas River males (SAL) had significantly higher sperm counts than upstream males (STM) (ANOVA:  $F = 4.68$ ,  $df = 1$ ,  $p = 0.045$ ; Table 6).

*Corticosterone (CORT).* Three-way ANOVA revealed that animals from the San Joaquin River had significantly higher CORT levels than animals from the Salinas River (THREE-WAY ANOVA:  $F = 3.16$ ,  $df = 2$ ,  $p = 0.047$ ; Fig. 19). Downstream animals also had significantly higher CORT levels than upstream animals (THREE-WAY ANOVA:  $F = 11.78$ ,  $df = 1$ ,  $p = 0.001$ ). There was no difference in CORT levels between males and females (THREE-WAY ANOVA:  $F = 0.09$ ,  $df = 1$ ,  $p = 0.761$ ). Downstream animals from the San Joaquin River had higher CORT levels than upstream San Joaquin animals, as well as upstream and downstream animals from the Salinas and Sacramento River (THREE-WAY ANOVA:  $F = 5.00$ ,  $df = 2$ ,  $p = 0.009$ ). There was no significant interaction between river and sex (THREE-WAY ANOVA:  $F = 0.68$ ,  $df = 2$ ,  $p = 0.507$ ) or region and sex (THREE-WAY ANOVA:  $F = 0.84$ ,  $df = 1$ ,  $p = 0.361$ ). However, CORT levels of downstream females were nearly twice as high as upstream females ( $T = -2.728$ ,  $p = 0.0372$ ). There was no significant interaction between river, region and sex (THREE-WAY ANOVA:  $F = 0.47$ ,  $df = 2$ ,  $p = 0.625$ ; Fig. 19). However, San Joaquin downstream males had significantly higher CORT levels than upstream and downstream Salinas males, upstream Sacramento females, and upstream San Joaquin males (Fig. 19).

Two-way ANOVA revealed that males of the San Joaquin River had significantly higher CORT levels than Salinas River males (TWO-WAY ANOVA:  $F = 3.88$ ,  $df = 2$ ,  $p = 0.026$ ; Fig. 20). Downstream males had significantly higher CORT levels than upstream males (TWO-WAY ANOVA:  $F = 5.55$ ,  $df = 1$ ,  $p = 0.022$ ). Downstream San Joaquin males (SJ) had significantly higher CORT levels than downstream (SAL) and upstream males (STM) of the Salinas River, and upstream males (DPC) of the San Joaquin (TWO-WAY ANOVA:  $F = 6.35$ ,  $df = 2$ ,  $p = 0.003$ ; Fig. 20). Downstream San Joaquin males (SJ) had significantly higher CORT levels than upstream males (DPC) (ANOVA:  $F = 10.25$ ,  $df = 1$ ,  $p = 0.005$ ; Table 7).

*Bleed time and testosterone.* One-way ANOVA revealed that upstream males of the Salinas River ( $F = 7.98$ ,  $df = 1$ ,  $p = 0.009$ ), Sacramento River ( $F = 3.23$ ,  $df = 1$ ,  $p = 0.094$ ), and San Joaquin River ( $F = 23.76$ ,  $df = 1$ ,  $p < 0.001$ ) were bled earlier than downstream males (Fig. 21).

Correlation analysis of revealed that there was no significant difference between time bled and testosterone levels of upstream (STM:  $P = 0.309$ ;  $p = 0.328$ ) and downstream (SAL:  $P = 0.174$ ;  $p = 0.553$ ) Salinas River males (Fig. 22, A) and upstream (BC:  $P = -0.229$ ;  $p = 0.663$ ) and downstream (SUT:  $P = 0.320$ ;  $p = 0.367$ ) Sacramento River males (Fig. 22, B). Although there was no relationship between time bled and testosterone levels of upstream San Joaquin River males (DPC:  $P = -0.250$ ;  $p = 0.485$ ), there was a significant decline in testosterone levels with respect to time bled of downstream (SJ:  $P = -0.683$ ;  $p = 0.021$ ) San Joaquin males (Fig. 22, C).

*Testosterone (T).* Three-way ANOVA revealed that animals from the San Joaquin River had significantly higher T levels than animals from the Sacramento River (THREE-WAY ANOVA:  $F = 3.48$ ,  $df = 2$ ,  $p = 0.035$ ; Fig. 23). Downstream animals also had significantly higher T levels than upstream animals (THREE-WAY ANOVA:  $F = 4.84$ ,  $df = 1$ ,  $p = 0.030$ ). There was no difference in T levels between sex (THREE-WAY ANOVA:  $F = 0.00$ ,  $df = 1$ ,  $p = 0.968$ ), river and region (THREE-

WAY ANOVA:  $F = 0.61$ ,  $df = 2$ ,  $p = 0.545$ ), river and sex (THREE-WAY ANOVA:  $F = 0.16$ ,  $df = 2$ ,  $p = 0.856$ ), or region and sex (THREE-WAY ANOVA:  $F = 0.44$ ,  $df = 1$ ,  $p = 0.509$ ). There was no significant effect of river, region and sex (THREE-WAY ANOVA:  $F = 1.82$ ,  $df = 2$ ,  $p = 0.167$ ; Fig. 23). However, San Joaquin downstream males had significantly higher T levels than downstream Sacramento males, upstream Salinas and San Joaquin males, and upstream Salinas and Sacramento females (Fig. 23).

Two-way ANOVA revealed that males of the San Joaquin River had significantly higher T levels than Sacramento River males (TWO-WAY ANOVA:  $F = 6.62$ ,  $df = 2$ ,  $p = 0.003$ ; Fig. 24). Males collected downstream of agricultural activity had significantly higher T levels than upstream males (TWO-WAY ANOVA:  $F = 11.64$ ,  $df = 1$ ,  $p = 0.001$ ). Downstream San Joaquin males (SJ) had significantly higher T levels than upstream males (DPC), downstream (SAL) and upstream males (STM) of the Salinas River, and downstream (SUT) and upstream males (BC) of the Sacramento (TWO-WAY ANOVA:  $F = 7.31$ ,  $df = 2$ ,  $p = 0.001$ ; Fig. 24). Downstream San Joaquin males (SJ) had significantly higher T levels than upstream males (DPC) (ANOVA:  $F = 23.30$ ,  $df = 1$ ,  $p < 0.001$ ; Table 7).

*Correlation analysis.* Males were grouped and analyzed in the following grouping schemes: all males, upstream males, downstream males, Santa Margarita males (STM), Salinas males (SAL), Battle Creek males (BC), Sutter males (SUT), Del Puerto Canyon males (DPC) and San Joaquin males (SJ).

Correlation analysis of *all males* (Table 8) showed significant positive correlations between SVL & BW, SVL & GW, SVL & tympanum, SVL & nuptial pad, SVL & YI, BW & GW, BW & tympanum, BW & nuptial pad, BW & YI, GW & tympanum, GW & nuptial pad, GW & YI, tympanum & nuptial pad, tympanum & YI, nuptial pad & YI, YI & CORT, YI & T, and CORT & T. Significant negative correlations in *all males* were found between SVL & hue, SVL & T, BW & T, tympanum diameter & hue, nuptial pad & hue, hue & YI, and hue & CORT. Correlation analysis of testosterone and normalized measurements showed a significant positive correlation with GSI (Fig. 25, A) but no correlation with normalized tympanum diameters and nuptial pad thickness (Fig. 25, B-C). There were no significant correlations between corticosterone and normalized measurements (Fig. 26, A-C).

Correlation analysis of upstream males (Table 9) showed significant positive correlations between SVL & BW, SVL & GW, SVL & tympanum, SVL & nuptial pad, BW & GW, BW & tympanum, BW & nuptial pad, BW & YI, GW & tympanum, GW & nuptial pad, GW & YI, tympanum & nuptial pad, tympanum & YI, nuptial pad & YI, YI & T, and CORT & T. Significant negative correlations in upstream males were found between hue & YI. There were no significant correlations between normalized measurements and testosterone (Fig. 27, A-C) or corticosterone (Fig. 28, A-C).

Correlation analysis of downstream males (Table 10) showed significant positive correlations between SVL & BW, SVL & GW, SVL & tympanum, SVL & nuptial pad, BW & GW, BW & tympanum, BW & nuptial pad, GW & tympanum, GW & nuptial pad, GW & YI, tympanum & nuptial pad, tympanum & YI, nuptial pad & YI, YI & CORT, YI & T, and CORT & T. Significant negative correlations in downstream males were found between SVL & hue, SVL & T, BW & hue, tympanum & hue, nuptial pad & hue, hue & YI, and hue & CORT. There were no significant correlations between normalized measurements and T (Fig. 29, A-C). Correlation analysis of corticosterone and normalized measurements found a significant positive correlation with

normalized tympanum diameters (Fig. 28, B) but no correlation with GSI or normalized nuptial pad thickness (Fig. 30, A&C).

Correlation analysis of Santa Margarita males (STM) (Table 11) showed significant positive correlations between SVL & BW, SVL & YI, BW & tympanum, GW & tympanum, GW & YI, tympanum & nuptial pad, tympanum & YI, nuptial pad & YI, and CORT & T. There were no significant correlations between normalized measurements and testosterone (Fig. 31, A-C) or corticosterone (Fig. 32, A-C).

Correlation analysis of Salinas males (SAL) (Table 12) showed significant positive correlations between SVL & BW, SVL & GW, SVL & tympanum, SVL & nuptial pad, BW & GW, BW & tympanum, BW & nuptial pad, GW & tympanum, GW & nuptial pad, GW & YI, GW & CORT, GW & T, tympanum & nuptial pad, tympanum & YI, tympanum & CORT, tympanum & T, nuptial pad & YI, nuptial pad & CORT, nuptial pad & T, and hue & CORT. Correlation analysis of testosterone and normalized measurements showed a significant positive correlation with GSI (Fig. 33, A) but no correlation with normalized tympanum diameters and normalized nuptial pad thickness (Fig. 33, B-C). Correlation analysis of corticosterone and normalized measurements found a significant positive correlation with GSI (Fig. 34, A) and normalized tympanum diameters (Fig. 34, B) but no correlation with normalized nuptial pad thickness (Fig. 34, C).

Correlation analysis of Battle Creek (BC) males (Table 13) showed significant positive correlations between SVL & BW, SVL & tympanum, SVL & nuptial pad, SVL & sperm count, BW & tympanum, BW & nuptial pad, tympanum & nuptial pad, and tympanum & YI. Significant negative correlations in BC males were found between tympanum & hue. There were no significant correlations between normalized measurements and testosterone (Fig. 35, A-C). Correlation analysis of corticosterone and normalized measurements found a significant positive correlation with normalized nuptial pad thickness (Fig. 36, B) but no correlation with GSI or normalized tympanum diameters (Fig. 36, A&B).

Correlation analysis of Sutter (SUT) males (Table 14) showed significant positive correlations between SVL & BW, SVL & GW, SVL & tympanum, SVL & nuptial pad, BW & GW, BW & tympanum, BW & nuptial pad, GW & tympanum, GW & nuptial pad, tympanum & nuptial pad, and tympanum & YI. Significant negative correlations in SUT males were found between BW & hue, GW & hue, tympanum & hue, nuptial pad & hue, and hue & YI. There were no significant correlations between normalized measurements and testosterone (Fig. 37, A-C). Correlation analysis of corticosterone and normalized measurements found a significant negative correlation with GSI (Fig. 38, A) but no correlation with normalized tympanum diameters or normalized nuptial pad thickness (Fig. 38, B&C).

Correlation analysis of Del Puerto Canyon (DPC) males (Table 15) showed significant positive correlations between SVL & BW, SVL & GW, SVL & nuptial pad, BW & GW, BW & tympanum, BW & nuptial pad, BW & YI, GW & nuptial pad, tympanum & nuptial pad, tympanum & YI, nuptial pad & YI, and CORT & T. Significant negative correlations in DPC males were found between hue & YI. There were no significant correlations between normalized measurements and testosterone (Fig. 39, A-C) or corticosterone (Fig. 40, A-C).

Correlation analysis of San Joaquin (SJ) males (Table 16) showed significant positive correlations between SVL & BW, SVL & GW, SVL & tympanum, SVL & nuptial pad, BW & GW, BW & tympanum, BW & nuptial pad, GW & nuptial pad, tympanum & nuptial pad, tympanum &

YI, and nuptial pad & YI. Significant negative correlations in SJ males were found between tympanum & hue. There were no significant correlations between normalized measurements and testosterone (Fig. 41, A-C) or corticosterone (Fig. 42, A-C).

## DISCUSSION

In this field study, I examined the effects of agrochemical-induced stress on the reproductive function of male amphibians. Overall, bullfrogs living downstream of agriculture were smaller in size (SVL & BW) and had elevated corticosterone and testosterone levels. Although analysis of individual rivers also revealed that downstream males were small in size and had elevated testosterone levels, only downstream males of the San Joaquin had elevated corticosterone levels and exaggerated secondary sex traits. Together, these observations suggest that living downstream of agriculture can alter size, hormone levels and secondary sex traits of male amphibians. Altered hormone levels and secondary sex traits of male amphibians may play a role in amphibian declines.

Overall results indicate that frogs collected downstream of agriculture are smaller in size (SVL & BW) (Fig. 3-6; Table 1) and have elevated corticosterone (Fig. 19 & 20; Table 7) and testosterone levels (Fig. 23 & 24; Table 7) in comparison to reference animals. Decreased size resulting from pesticide exposure (Brodeur *et al.*, 2011; Spear *et al.*, 2009) and pesticide-induced stress (Christin *et al.*, 2013; Egea-Serrano *et al.*, 2012; Egea-Serrano *et al.*, 2009; Hayes *et al.*, 2006) has been demonstrated in previous studies. Exposure to agrochemicals can directly affect body weight by reducing food consumption (Egea-Serrano *et al.*, 2009) or increasing physiological stress (Egea-Serrano *et al.*, 2012; Hayes *et al.*, 2006). Physiological stress, leading to elevated stress hormone levels, has negative impacts on size. For example, exposure to exogenous glucocorticoids decreases body weight in tadpoles, which can lead to small metamorphic size, delayed reproductive maturity, and decreased size at first reproduction (Glennemeier and Denver, 2002).

The negative impact of pesticides on amphibian size is a concern because body size is strongly linked to fecundity (Halliday and Verrell, 1988). Animals with low body mass have less energy reserves which can impair reproductive output in amphibian species (Brodeur *et al.*, 2011). Thus, animals with reduced body size as a consequence of living in agricultural areas may have reduced fitness (Spear *et al.*, 2009) and impaired reproductive ability (Brodeur *et al.*, 2011).

Size differences between upstream and downstream animals may be attributed to the lack of older (assuming larger) individuals from downstream sites. Larger animals may be present downstream of agriculture but may reside outside of areas selected for field collection. Differences in body size may also stem from downstream males reaching sexual maturity at a smaller size than reference sites. In amphibians, there is a wide range of body size at first-breeding age (Halliday and Verrell, 1988). The minimal recorded SVL for breeding male bullfrogs is 8.5-8.9 mm (Wright and Wright, 1949). Whether downstream animals are smaller because they reached sexual maturity at a smaller size requires further study. Although animals of the same class size (small) were present at upstream sites, they were not collected because they were identified as juveniles and only sexually mature animals were included in this study.

Although downstream animals had elevated corticosterone levels and were smaller in size, correlation analysis revealed that corticosterone levels were not a good predictor for body size (SVL& BW) (Table 10). The lack of a significant correlation between corticosterone and body size was surprising because the negative effect of corticosterone on amphibian size is well documented. The lack of correlation may be because corticosterone levels at the time of capture may not reflect levels throughout an animal's life history. Elevated corticosterone levels during larval stages or metamorphosis can negatively impact adult size (Altwegg and Reyer, 2003; Denver, 2009; Glennemeier and Denver, 2002; Hu *et al.*, 2008; Janin *et al.*, 2011). Based on the current study, it is unclear whether the single blood sample taken at the time of capture is indicative of corticosterone levels during growth periods. Thus, further studies are needed to determine the relationship between elevated corticosterone levels and small size in downstream animals.

Interestingly, negative correlations were found between testosterone and SVL and BW in downstream males (Table 10). These results are consistent with a number of studies that suggest that elevations in testosterone levels shunt energy allocation to reproduction, resulting in less energy for metabolism and growth (Cox *et al.*, 2005; Hau, 2007; Miles *et al.*, 2007; Olsson *et al.*, 2000; Ryser, 1989). Although my results suggest that elevated testosterone has a negative effect on size, we cannot rule out the possibility that small size of downstream animals may be attributed to agrochemical exposure, elevated corticosterone, some unmeasured factor such as nutrition, or a combination of these factors.

I did not expect downstream animals to have elevated testosterone in the presence of elevated corticosterone levels. Although bullfrogs naturally have elevated corticosterone and testosterone during the breeding season (Romero, 2002) to mobilize energy for reproductive activities (Moore and Jessop, 2003; Moore *et al.*, 2000; Wingfield *et al.*, 1998), elevated corticosterone suppresses androgen secretion. For example, elevated corticosterone from capture-induced stress is associated with decreased sex hormone levels in bullfrogs (Licht *et al.*, 1983). Additionally, chorusing male bullfrogs have elevated corticosterone levels and depressed androgen levels in comparison to non-calling males (Hopkins *et al.*, 1997; Peterson *et al.*, 2009).

Although stress generally inhibits sex hormones, these negative effects are not universal (Moore *et al.*, 2000). Correlative studies have shown that animals with higher corticosterone also have higher testosterone levels (Emerson and Hess, 2001; Moore *et al.*, 2000; Schramm *et al.*, 1999). Moreover, treatment with exogenous testosterone elevates corticosterone in birds (Ketterson *et al.*, 1991; Schoech *et al.*, 1999). Concurrent elevations of both stress and sex steroids may suggest a positive relationship between these two hormones. In support of this hypothesis, significant positive correlations between corticosterone and testosterone were found in both upstream (Table 9) and downstream males (Table 10). Elevated corticosterone would make energy stores available to support elevated testosterone levels and for the maintenance sex traits, gametogenesis, and behaviors associated with elevated testosterone (Moore *et al.*, 2000).

Elevated levels of testosterone and corticosterone levels in downstream males may also be associated with mating behaviors such as clasping and chorusing. Clasping male marine toads (*Bufo marinus*) have concurrent elevations of androgens and corticosterone in both the laboratory and in the wild (Orchinik *et al.*, 1988). Additionally, actively calling males also have

elevated androgen and corticosterone levels (Emerson and Hess, 2001). Although elevated corticosterone and testosterone levels may be beneficial for reproduction (i.e. allocating energy for spermatogenesis and for the development of sexually dimorphic traits), simultaneous elevation of these steroids can impair immune function (Folstad and Karter, 1992; Hillgarth and Wingfield, 1997) and decrease body condition. It is not clear whether elevated corticosterone and testosterone in downstream males are agrochemical induced or stem from increased reproductive activity. Although both upstream and downstream males were chorusing throughout the time of collection, whether individuals were calling upon capture (and subsequent blood collection) was not recorded.

To analyze the overall effects of living upstream and downstream of agriculture on the reproductive endocrinology and morphology (or function) of male bullfrogs, each individual river system was treated as a replicate. However, the rivers used in this study are not true replicates. This is because agrochemical exposure can vary temporally because of differences in environmental conditions and concentrations of applied chemicals (Sprague and Greve, 2003) across rivers. Furthermore, agrochemical regimes vary depending on crop type, crop rotation, and weed species (Andersson and Milberg, 1996; 1998) which differ across the Salinas, Sacramento, and San Joaquin Rivers.

Because the Salinas, Sacramento, and San Joaquin Rivers are not true replicates, upstream and downstream animals were compared within each individual river system. In regards to the Sacramento River, large territorial males were not collected at the upstream site (BC) because these males were sequestered in tall reed beds that were inaccessible for collection. Because of this sampling bias, it is not surprising that upstream and downstream Sacramento River males did not differ in size, primary and secondary sex traits, or testosterone levels. However, I did not expect downstream Sacramento (SUT) animals to have comparable levels of corticosterone relative to reference site (Fig. 19 & 20; Table 7). These data suggest that downstream SUT animals did not incur increased corticosterone secretion as a result of agrochemical exposure.

Similar to SUT animals, downstream Salinas (SAL) animals also did not have elevated corticosterone levels in comparison to reference site (Fig. 19 & 20; Table 7). These results were surprising because I expected frogs from all downstream sites to have elevated corticosterone levels as a result of agrochemical exposure. Interestingly, elevated corticosterone levels found in overall downstream animals (combined river analysis) stem from plasma concentrations found in downstream San Joaquin (SJ) animals. Both male (Fig. 20; Table 7) and female SJ bullfrogs (Fig. 19) had twice the amount of plasma corticosterone than male and females from all other sites. These results suggest that SJ animals are more stressed than any other site.

Elevated corticosterone as a result of chemical exposure has been documented in other amphibian studies (Hayes *et al.*, 2006; Hopkins *et al.*, 1997; Larson *et al.*, 1998; Peterson *et al.*, 2009). For example, male toads collected from sites contaminated with coal ash had higher tissue levels of trace elements and elevated corticosterone levels than reference males (Hopkins *et al.*, 1997). Hopkins *et al.* (1997) proposed that stressful bi-products of coal ash (i.e. selenium, copper, cadmium, barium and arsenic), may interfere with the degradation and clearance of steroid hormones, resulting in elevated corticosterone. Other laboratory studies have also shown that selenium exposure induces a stress response, resulting in elevated corticosterone levels (Potmis *et al.*, 1993; Rasekh *et al.*, 1991).

Although chemical analysis was not performed in the current study, it is possible that elevated corticosterone levels in downstream SJ bullfrogs may be a result of selenium exposure. In the 1980s, the highly publicized selenium spill at Kesterson National Wildlife Refuge (approximately 40 miles upstream of the SJ site) resulted in the high mortality of waterfowl and fish (reviewed in Hamilton, 2004). Following the spill, high levels of selenium were found in the tissues of bullfrogs and their food sources collected upstream of the SJ site (Kesterson and San Luis National Wildlife Refuge) (Ohlendorf *et al.*, 1988). Since then, spikes of selenium levels in the San Joaquin River at Hills Ferry (approximately 30 miles upstream of the SJ site) have been documented (Littlefield, 2011). Although studies have documented the presence of selenium in the proximity of the downstream SJ site at the time of collection, the current study does not have direct evidence to support causation between selenium and elevated corticosterone levels. However, based on the severity of the spill, the relationship between selenium and elevated corticosterone cannot be ruled out. Selenium and corticosterone levels in SJ bullfrogs need to be examined to further test this conclusion.

Although SJ males had twice the amount of corticosterone levels than males from any other site, these levels are similar to reported levels of corticosterone in male bullfrogs during the breeding season (Mendonca *et al.*, 1985). Corticosterone in bullfrogs exhibit a seasonal rhythm, with levels reaching a maximum during the breeding season (Romero, 2002). Elevated glucocorticoids aid in mobilizing energy stores for the high energetic demands of reproduction (Romero, 2002). Thus increased corticosterone levels during the breeding season energetically support breeding behaviors such as fighting and amplexus (Mendonca *et al.*, 1985). At the downstream SJ site, one mating pair was recorded and no aggressive bouts were observed. Although males were actively chorusing at all field sites, I did not record whether individual males were calling at time of capture. Based on the current study, it is difficult to determine whether high corticosterone of SJ males relative to all other sites is from agrochemical exposure or normal breeding activity. Although highest recorded corticosterone levels for chronically stressed frogs are 50 – 75 ng/mL (Hopkins *et al.*, 1997), that study examined toads and not bullfrogs. Whether elevated corticosterone levels of SJ animals result from chemical exposure or increased reproductive activity requires further study.

Interestingly, elevated testosterone levels found in overall downstream animals (combined river analysis) also stemmed from plasma concentrations found in downstream San Joaquin (SJ) animals. Downstream SJ males had twice the amount of plasma testosterone than males from all other sites (Fig. 24; Table 7). These results were surprising because I expected testosterone levels to be depressed in the presence of elevated corticosterone. There are three likely scenarios that may explain elevated testosterone levels in downstream SJ males. First, testosterone levels may be elevated in response to heavy metal exposure. Male toads exposed to heavy metals from coal ash effluent had currently high levels of corticosterone and testosterone, whereas testosterone levels in reference males were depressed in the presence of corticosterone (Hopkins *et al.*, 1997). Authors concluded that elevated testosterone levels in male toads may be a result of exposure to trace elements such as cadmium, lead, and mercury which can increase androgen synthesis and/or impair steroid clearance. Heavy metals such as mercury and cadmium can inactivate enzymes responsible for steroid hormone degradation (Landis and Yu, 2003). Additionally, mercury exposure elevates adrenal androgens in male rats (Veltman and Maines, 1986). Although much of the literature has shown that mercury

decreases androgen levels in fish (reviewed in Crump and Trudeau, 2009), some fish studies have demonstrated that tissue mercury concentrations are positively correlated with androgen levels (Baldigo *et al.*, 2006; Friedmann *et al.*, 2002).

Whether elevated testosterone levels in SJ males can be attributed to heavy metal exposure requires further study. However, it is interesting to note that excessive mercury levels have been found in water and fish both upstream and downstream of the SJ field site (Foe C., August 2008; Lee and Jones-Lee, 2006). Additionally, cadmium levels were higher in juvenile striped bass at Crow's Landing (23 miles upstream of SJ site) in comparison to other watersheds (Saiki and Palawski, 1990). Future analysis of heavy metals is needed to determine whether it increases testosterone levels in male SJ bullfrogs.

Secondly, it is possible that downstream SJ males were in the peak of their breeding season and thus had higher testosterone levels. Androgen levels in male bullfrogs peak twice throughout an entire breeding season (Mendonca *et al.*, 1985; Oseen and Wassersug, 2002). It is possible that bimodal androgen peaks of SJ males and reference males did not overlap. Differences in bimodal peaks may be caused by significantly higher water temperatures found in downstream SJ in comparison to the reference site. Since the initiation of the breeding season in bullfrogs is primarily influenced by water temperature (Oseen and Wassersug, 2002), it is possible that SJ males began their breeding season earlier, resulting in an earlier peak, thus having higher testosterone levels than reference males. Although a shifted bimodal pattern of androgen secretion may explain elevated androgens in SJ males, tracking temperatures and testosterone levels throughout a breeding season is recommended for future studies.

Lastly, elevated androgen levels in downstream SJ males may be a result of sexual stimuli. Elevated androgens have been reported in amplexing male toads (*Bufo marinus*) in the laboratory and field (Orchinik *et al.*, 1988). Orchinik *et al.* (1988) also found that androgen levels increased in male toads 3 hours following clasping events and that there was a positive relationship between duration of clasping and androgen levels. In the current study, one out of the only two females collected at the downstream SJ site was caught in amplexus. This single clasping event was the only observation of amplexus in this field study. Although other amplexant events were not observed, significantly elevated testosterone levels of SJ males may stem from individuals who had recently copulated. However, in contrast to the findings of elevated androgen levels in copulating toads (Orchinik *et al.*, 1988), Mendonça *et al.* (1985) found that androgen levels of male bullfrogs engaging in amplexus do not have elevated androgen levels compared to other calling males. Based on the conflicting literature, I cannot determine whether elevated testosterone levels in SJ males can be attributed clasping behavior. Because the current study was not designed to address the behavioral consequences on testosterone secretion, future studies are needed to address this question.

Interestingly, there was a significant decline in testosterone with respect to time bled in downstream SJ males (Fig. 22, C). However, Mendonça *et al.* (1985) found no correlation between androgen levels and time of night in field collected male bullfrogs. The reason behind the significant decline in testosterone with respect to time in downstream SJ males remains unclear.

Although not significant, SAL males had nearly double the amount of testosterone levels than reference STM males (Table 7; Fig. 24). Elevated testosterone of SAL males is likely because small males were parasitic males. Parasitic males are small sexually mature males that



intercept and mate with females attracted to larger territorial males (Howard, 1984). Interestingly, parasitic males have larger testes (Emerson, 1997) and higher testosterone levels than larger territorial male bullfrogs (Emerson, 1997; Mendonça *et al.*, 1985).

With the exception of animals from the Sacramento River (sample bias), I hypothesized that downstream animals of the Salinas and San Joaquin Rivers would be smaller in size, have less developed primary and secondary sex traits, decreased sperm counts, elevated corticosterone levels and depressed testosterone levels. Although I observed commonalities in size and testosterone levels amongst downstream frogs, differences in primary and secondary sex traits, sperm count and corticosterone levels confirm that these rivers are not replicates and suggest that different factors (such as agrochemical exposure, habitat, temperature, and behaviors) may be affecting bullfrogs at any given point of time and space.

Small size of downstream SAL and SJ males in comparison to reference sites, as well as small size of downstream males in overall river analysis, supports the hypothesis that agrochemical exposure has a negative effect on size. In addition to agrochemical exposure, it is also possible that downstream SJ males are small because they had significantly higher corticosterone levels than all other sites. The negative effect of corticosterone on body size is well documented. However, correlation analysis found no relationship between corticosterone and body size in SJ males.

Though agrochemicals and glucocorticoids have been linked to decreased size in exposed amphibians, it is important to point out that other factors (independent of agrochemical exposure) may contribute to small size. For example, amphibian populations with small parasitic males or males that reach sexual maturity at a smaller size could skew size data. Parasitic males are small sexually mature males that intercept and mate with females attracted to larger territorial males (Howard, 1984). The presence of small parasitic males at the downstream SAL site and the lack of such males at the reference site may explain why downstream SAL males were significantly smaller. Identification of parasitic males were determined by size, light yellow throat coloration, and behaviors (described in Howard, 1978; 1984). Sexually mature parasitic males were collected along the periphery of larger territorial males at the downstream site. Of these parasitic males, sneaker-satellite males were the smallest (first years), were found motionless around leks of large males but occasionally displayed territorial behaviors (Howard, 1984). Opportunistic males (second years) were larger and also switched between parasitism and territoriality (Howard, 1984).

Although possible, it is not likely that the parasitic male hypothesis explains small size of downstream SJ males for the following reasons. First, all males collected at the SJ site were small chorusing territorial males (field observation). Second, SJ females were also small (12.5 cm) and were releasing eggs upon capture. Lastly, there were no visible large males at the downstream SJ site. Though observations at the time of collection suggest that the SJ population consists of a single age group because of uniform body size, we cannot rule out the possibility that older territorial males migrated out of breeding ponds. Such a migration would lead to the lack of older territorial males, which would result in smaller parasitic males exhibiting territorial behaviors (Howard, 1978). However, the migration hypothesis does not explain the small size of sexually mature SJ females. In contrast to Howard (1978) observations that larger males migrate earlier in the breeding season, Emlen (1976) found that there is no

difference in age or size in reproductively active male bullfrogs at the end of the breeding season.

It is more likely that SJ males are small because they attained sexual maturity at a smaller size. There is a wide range in the body size of animals that have reached first breeding age (Halliday and Verrell, 1988). The minimal SVL for breeding male bullfrogs has been recorded as 8.5-8.9 mm (Schroeder, 1974; Wright and Wright, 1949). However, small individuals at sexual maturity will remain small throughout their lives (Halliday and Verrell, 1988) which can have negative effects on reproductive success. Body size is strongly linked to fecundity, especially in females (Halliday and Verrell, 1988). Additionally, body size at sexual maturity significantly affects mating strategy, mating success, and general fitness in male bullfrogs (Collins, 1979; Howard, 1978).

Larger GSI values of SAL males (Table 1) were surprising because SAL males were significantly smaller (SVL & BW) than reference males and SVL and BW have independent positive effects on testes weight (Hettyey *et al.*, 2005). These results support the hypothesis that parasitic males have relatively larger testes than large territorial males in a number of anuran species (Emerson, 1997). Additionally, parasitic males have higher testosterone levels than territorial males (Mendonca *et al.*, 1985). Although not significant, downstream SAL males had twice the amount of testosterone than reference males (Table 7;  $p = 0.160$ ). Elevated testosterone levels in downstream SAL males may also contribute to larger GSI values in comparison to reference males. This is because testis size is positively correlated with testosterone levels (Emerson, 1997). During spermatogenesis, which is promoted by androgens, the testes of anurans increase in size and weight (Duellman and Trueb, 1986). Additionally, SAL males were the only group to have significant positive correlations between testosterone and gonad weight (Table 12) and GSI (Fig. 33). These data suggest that testosterone has a positive effect on gonad size in downstream SAL males.

The difference in the development of secondary sex traits between downstream Salinas (SAL) and San Joaquin (SJ) River males were quite notable. Less developed sex traits in SAL males and more developed sex traits in SJ males in comparison to reference males suggest that SJ males may employ a terminal investment strategy. Candolin (1999) demonstrated that poor condition (induced by manipulating food intake) increased breeding coloration in male stickleback fish. Thus, males that are in poor condition (i.e. small SVL & BW) may allocate high amounts of energy to the exaggeration of traits because of a low probability of future reproduction (Candolin, 1999).

Larger tympanum and nuptial pad size (Figure 13C & 16C), dark yellow throats (Table 4), and higher YI values (Table 5) in downstream SJ males may stem from elevated testosterone levels. Although, the development and maintenance of these exaggerated traits from elevated testosterone levels may potentially increase breeding success, it may also have negative impacts on the immune system (Folstad and Karter, 1992). Though immune function was not examined in this study, multiple studies support the hypothesis that elevated testosterone results in fitness trade-offs (Miles *et al.*, 2007). Such trade-offs include decreased somatic growth, impaired immune function, and increased parasite loads which ultimately lead to decreased survival (reviewed in Miles *et al.*, 2007). Small size and exaggerated secondary sex traits in SJ males suggest that small size may be a consequence of maintaining these traits or males are engaged in a terminal investment strategy (Candolin, 1999; Schulte-Hostedde and

Schank, 2009). Thus, enhanced sex traits and small size in SJ males may have negative effects on their overall reproductive health and future success.

Interestingly, elevated sperm counts were only found in downstream SAL males (Table 6). One possible explanation is the number of parasitic males found at the SAL site. Parasitic males have relatively larger testes than larger territorial males and testes size is positively correlated with testosterone levels (Emerson, 1997). Additionally, amphibian spermatogenesis and spermiation are dependent on testosterone (Rugh, 1946). In addition to having larger gonad weights, SAL males had higher GSI values (Table 1) and testosterone levels (Table 7). These data suggest that higher sperm counts in SAL males stem from larger gonad size and higher testosterone levels in SAL males

Although I hypothesized corticosterone to have negative effects on reproductive health, I found that it had a positive relationship in GW, GSI, tympanum and nuptial pad size, and YI in downstream Salinas males only (Table 12; Fig. 34). These results suggest that corticosterone plays a supportive role in the maintenance of these structures. These results are interesting because higher corticosterone tend to have negative effects on sex traits such as coloration in birds (reviewed in Husak and Moore, 2008). Moreover, it is interesting that these positive trends are only found in males from the downstream SAL site. These data bring up the question of whether corticosterone is supportive in the maintenance of these structures in some populations but not in others. In addition, downstream SAL males were the only animals to have positive correlations between testosterone and GW, GSI, tympanum and nuptial pad size, and YI in comparison to males from all other sites (Table 12; Fig. 33). Overall, these data suggest that corticosterone may act directly on sexual dimorphic traits or indirectly through interactions with sex steroids such as testosterone (reviewed in Husak and Moore, 2008). It is an interesting question which requires further study.

## CONCLUSIONS

In conclusion, overall field observations across rivers suggest that elevated corticosterone and testosterone levels of downstream animals have a negative impact on size, which can be detrimental to the reproductive health and long-term reproductive success of amphibian populations. However, individual river analysis was not consistent with overall river analysis. Although downstream Salinas and San Joaquin males were small and had elevated testosterone levels, only downstream SJ males had elevated corticosterone levels and exaggerated secondary sex traits. These data suggest that the effects of agrochemical-induced stress on male amphibian reproductive function differ across river systems. Such differences are not surprising since factors such as habitat, agrochemical exposure, as well as responses to such factors can differ across populations as well as individuals.

Additionally, the reproductive strategies of small males may play a role in reproductive success. The reproductive strategies of small males such as downstream SAL parasitic males and the total investment reproductive strategy of downstream SJ males may not be affected by agrochemical exposure in the short term. However, the overall reproductive success of these males may be affected in the long term since size plays an important role in fecundity. Individuals who are reproductively active at a small size may result in reduced survival or lowered future fecundity or mating success (Ryser, 1989). Additionally, parasitic and total

investment strategies are associated with elevated androgen levels, which have shown to have negative effects on immune function.

Since atypical differences in secondary sex traits were most evident in male bullfrogs from the downstream SJ site, it is possible that selenium and mercury may play a role in elevated testosterone and their exaggerated androgen-dependent traits. Future field analysis should incorporate water and tissue chemical analysis in conjunction with morphological studies along with controlled lab studies to better interpret these conclusions.

Although hormones control the physiological, morphological, and behavioral aspects of reproduction, I could not use corticosterone or testosterone to consistently predict reproductive function across sites. This may largely stem from single blood sampling during the breeding season. The onset of reproductive morphology development begins before the onset of the breeding season. Blood samples taken during this time would better elucidate whether levels of these steroids are good indicators of reproductive health in amphibians.

The current field study provides ecologically relevant data in regards to agrochemical exposure and reproductive health in male amphibians. However, this field study has many limitations. I was unable to determine cause and effect relationships between real-life exposures and stress in part because the current study lacked pesticide analysis, but mostly because amphibians are exposed to a myriad of chemicals which vary in type and dose throughout an individual's life history. In addition to agrochemical exposure, reproduction may be affected by other unknown factors that could not be controlled for in this field study. Future analysis of monitoring corticosterone levels throughout different life stages (juveniles in addition to adults) in tandem with water and tissue agrochemical analysis need to be conducted throughout a population's life history to better understand if agrochemicals induce a stress response.

This study exemplifies the differences in the endocrine and morphological reproductive expressions in populations of male amphibians exposed to agrochemicals. Such differences demonstrate a great need for implementing ecological relevancy in experimental design. Future field studies tracking crop type and pesticide usage in conjunction with pesticide analysis throughout an amphibian's life history is needed to further our understanding of the effects of environmentally relevant agrochemical exposure on amphibian reproductive health. Furthermore, the effects of agrochemical exposure on breeding behavior are also needed.

Overall, our findings support the hypothesis that downstream animals are small in size and have elevated corticosterone. However, these results are not consistent throughout all downstream sites. These findings suggest that there are population-specific factors that can affect amphibian reproductive health and need to be examined in this context. More work is needed to elucidate the effects of real-life agrochemical exposure on corticosterone release (stress response) and reproductive health in male amphibians.

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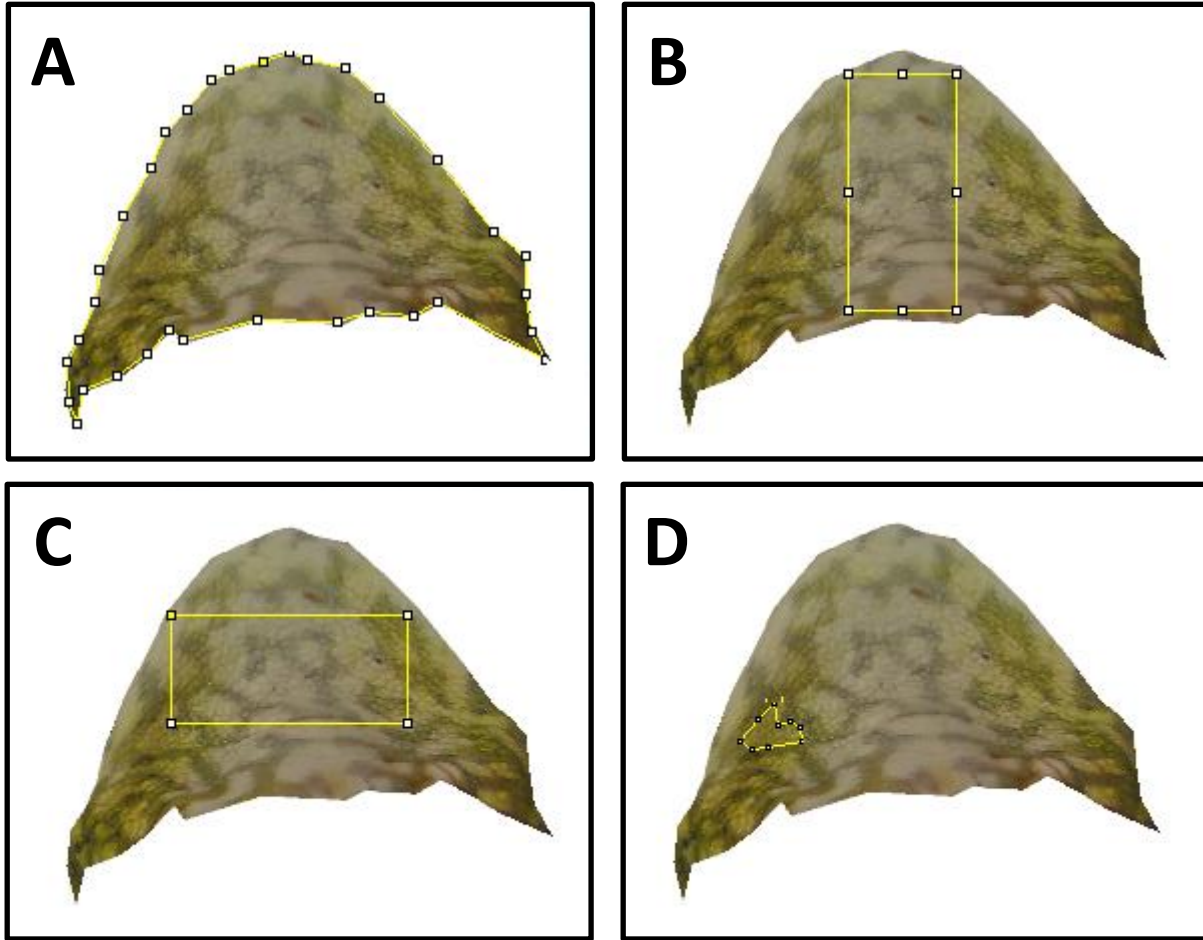
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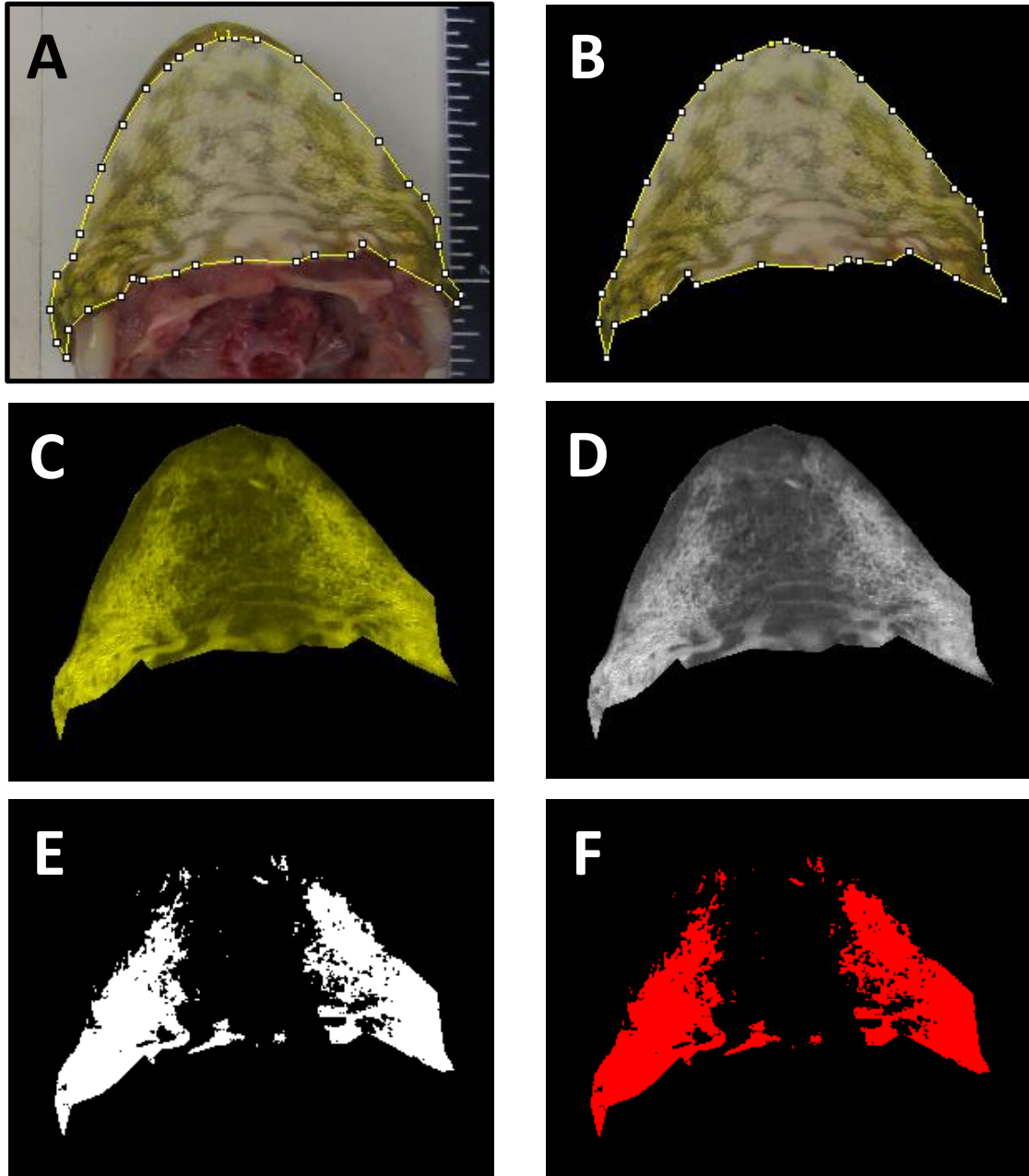


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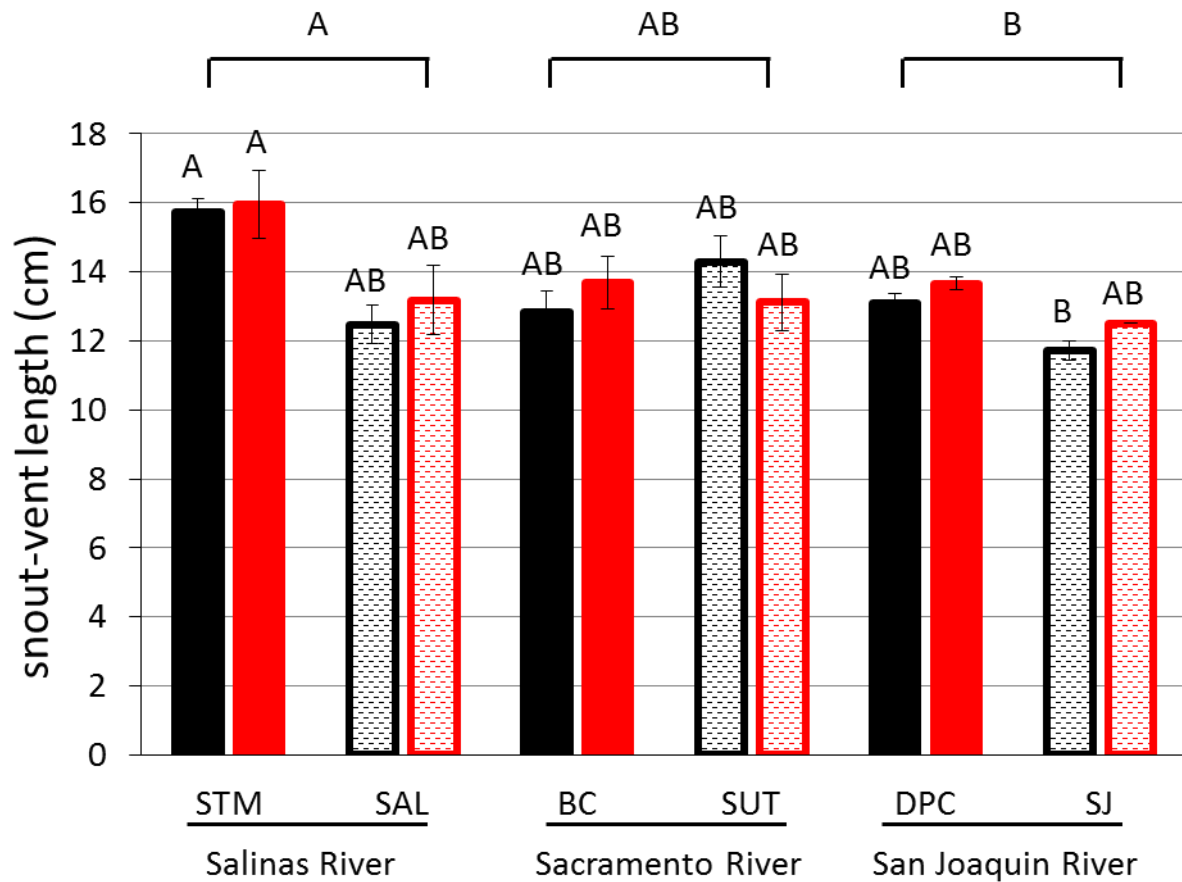
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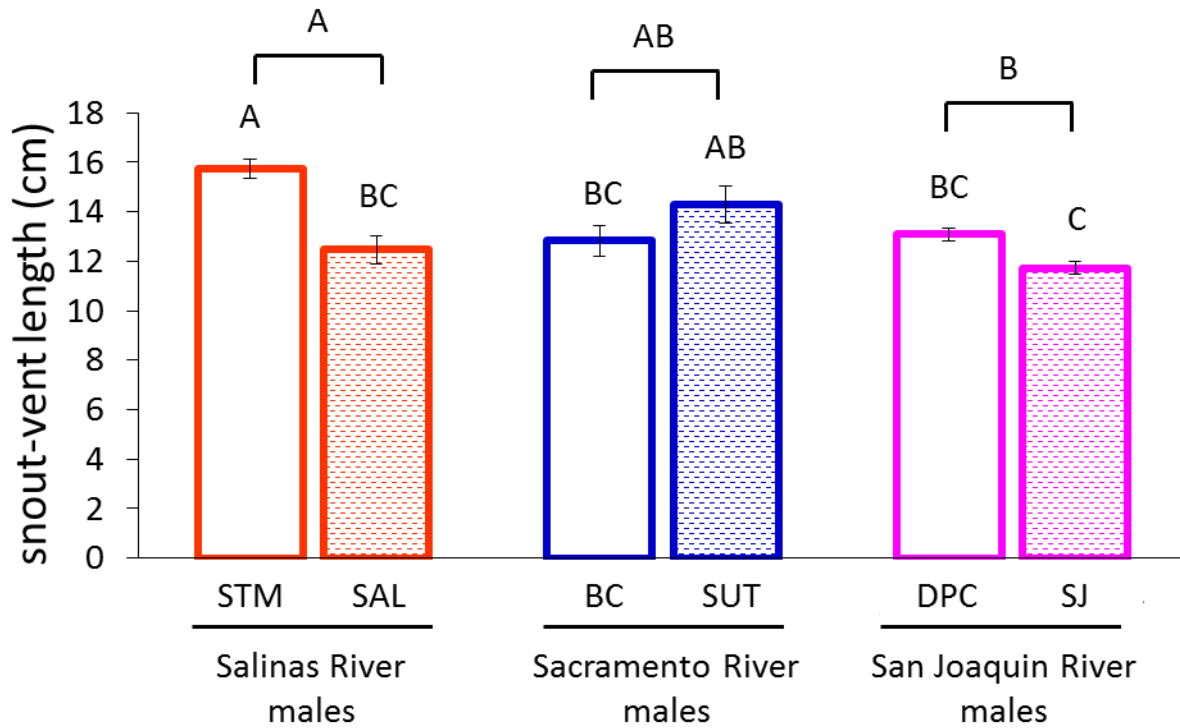
**Figure 1.** Four methods of hue analysis of yellow throat coloration in male American bullfrogs (*Lithobates catesbeianus*). Hue measurements were taken using the entire throat (A), along the medial throat axis (B), the lateral throat axis (C), and an outline of the darkest yellow area of the throat (patch) (D).



**Figure 2.** Yellow Index (YI) analysis of yellow throat coloration in male American bullfrogs (*Lithobates catesbeianus*) using ImageJ. (A) Original RGB image of outlined throat region. (B) RGB image of outlined throat region with background deleted. (C) Yellow slice of CMKY image. (D) Brightness slice of HSB image. (E) Binary image of brightness slice. (F) Image of area measured (in red).



**Figure 3.** Snout-vent length (SVL) for male (black) and female (red) American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas, Sacramento, and San Joaquin Rivers. Animals collected upstream of agricultural activity are denoted with solid bars (STM: Santa Margarita,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 5$ ; BC: Battle Creek,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 9$ ; and DPC: Del Puerto Canyon,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 6$ ) and animals collected downstream of agricultural activity have patterned bars (SAL: Salinas,  $n_{\text{males}} = 7$ ,  $n_{\text{females}} = 5$ ; SUT: Sutter,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 10$ ; and SJ: San Joaquin,  $n_{\text{males}} = 11$ ,  $n_{\text{females}} = 2$ ). Three-way ANOVA was used to examine whether animals differed in SVL between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity), sex (male or female) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

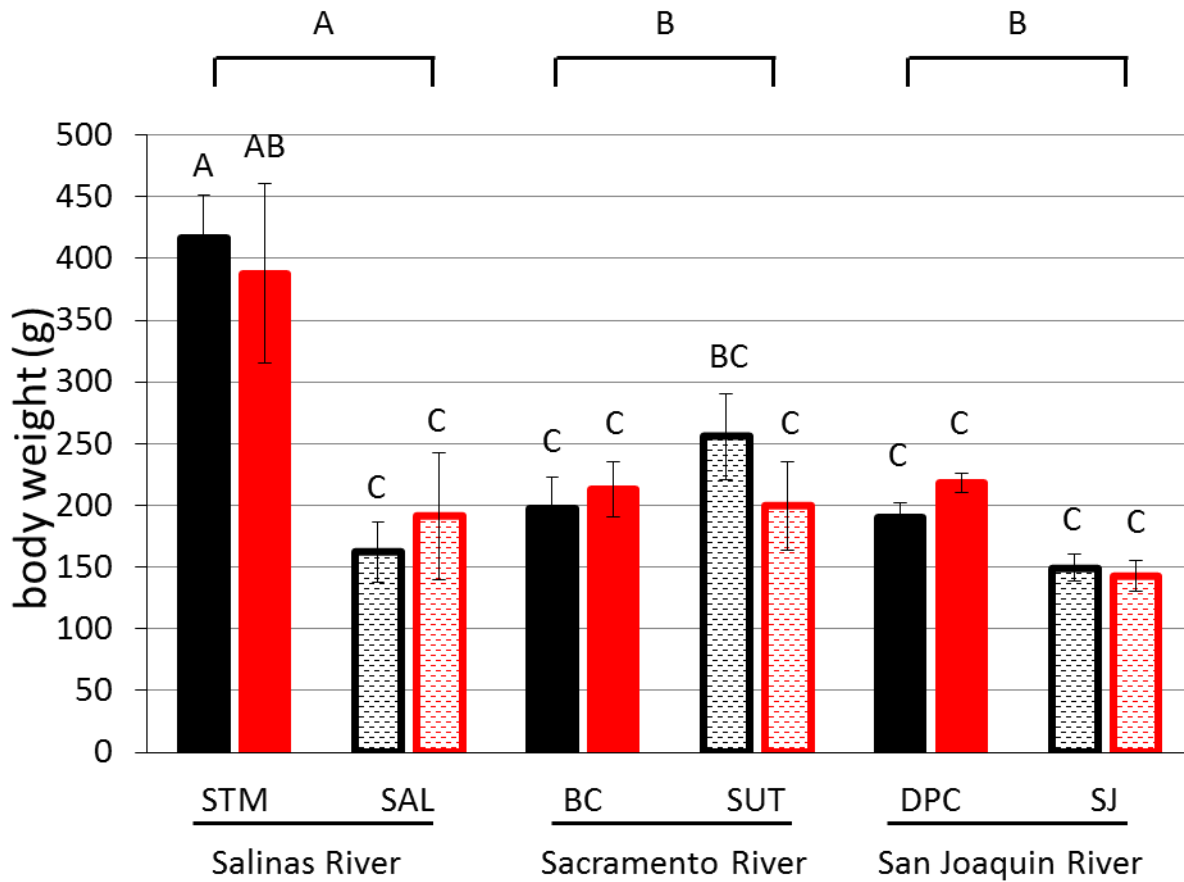


**Figure 4.** Snout-vent length (SVL) for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas River (orange), Sacramento River (blue) and San Joaquin River (pink). Numbers within bars represent sample size. Males collected upstream of agriculture are denoted with open bars (STM: Santa Margarita, n = 6; BC: Battle Creek, n = 6; and DPC: Del Puerto Canyon, n = 10) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 7; SUT: Sutter, n = 10; and SJ: San Joaquin, n = 11). Two-way ANOVA was used to examine whether males differed in SVL between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey’s honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

Site	Snout-vent length (cm)			Body weight (g)			Gonadal-somatic index								
	n	mean	df	F	p	n	mean	df	F	p	n	mean	df	F	p
<b>Salinas River</b>															
Santa Margarita (STM)	6	15.8±0.9**	1	21.50	0.001	6	416.7±85.3***	1	38.2	< 0.001	6	45.2±17.9*	1	4.80	0.053
Salinas (SAL)	7	12.5±1.5**				7	162.1±63.2***				6	68.6±19.0*			
<b>Sacramento River</b>															
Battle Creek (BC)	6	12.8±1.5	1	1.84	0.197	6	197.0±62.2	1	1.41	0.255	6	64.4±19.9	1	2.07	0.172
Sutter (SUT)	10	14.3±2.3				10	255.6±109.9				10	53.8±9.8			
<b>San Joaquin River</b>															
Del Puerto Canyon (DPC)	10	13.1±0.8**	1	13.09	0.002	10	189.7±38.7*	1	6.23	0.022	10	56.9±16.7	1	0.66	0.427
San Joaquin (SJ)	11	11.7±0.9**				11	149.4±35.3*				11	61.9±10.6			

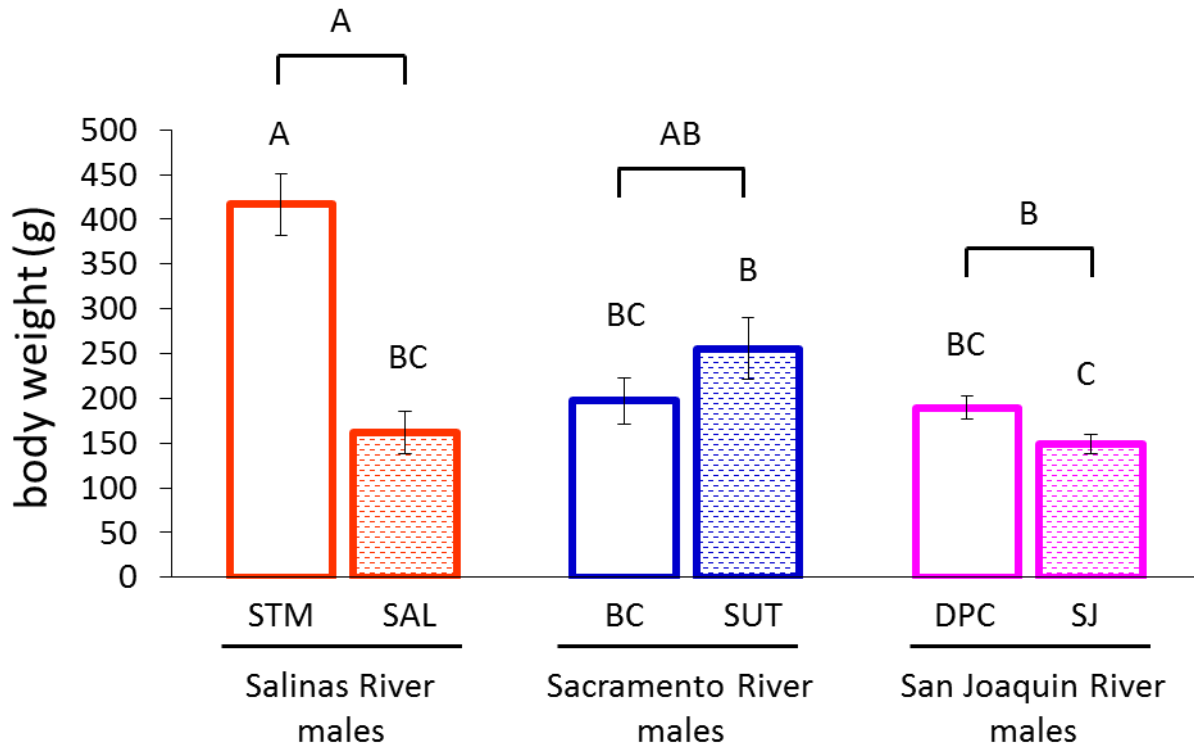
ONE-WAY ANOVA: significant at \*p≤0.05; \*\*p≤0.005; \*\*\*p≤0.001

**Table 1.** Snout-vent length (SVL), body weight (BW), and gonadal-somatic index (GSI) of male bullfrogs collected upstream (STM, BC & DPC) and downstream (SAL, SUT & SJ) of agricultural activity. One-way ANOVA was used to examine the differences of SVL, BW, and GSI of upstream and downstream males within each river system. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).

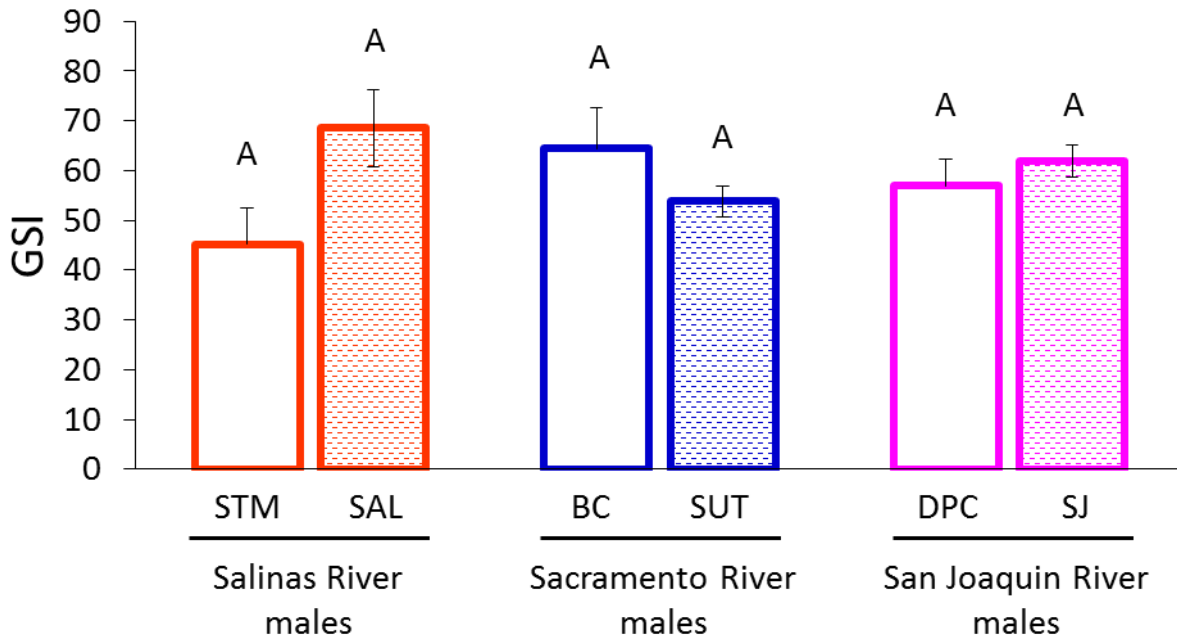


**Figure 5.** Body weight for male (black) and female (red) American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas, Sacramento, and San Joaquin Rivers. Animals collected upstream of agricultural activity are denoted with solid bars (STM: Santa Margarita,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 5$ ; BC: Battle Creek,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 9$ ; and DPC: Del Puerto Canyon,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 6$ ) and animals collected downstream of agricultural activity have patterned bars (SAL: Salinas,  $n_{\text{males}} = 7$ ,  $n_{\text{females}} = 5$ ; SUT: Sutter,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 10$ ; and SJ: San Joaquin,  $n_{\text{males}} = 11$ ,  $n_{\text{females}} = 2$ ). Three-way ANOVA was used to examine whether animals differed in BW between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity), sex (male or female) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

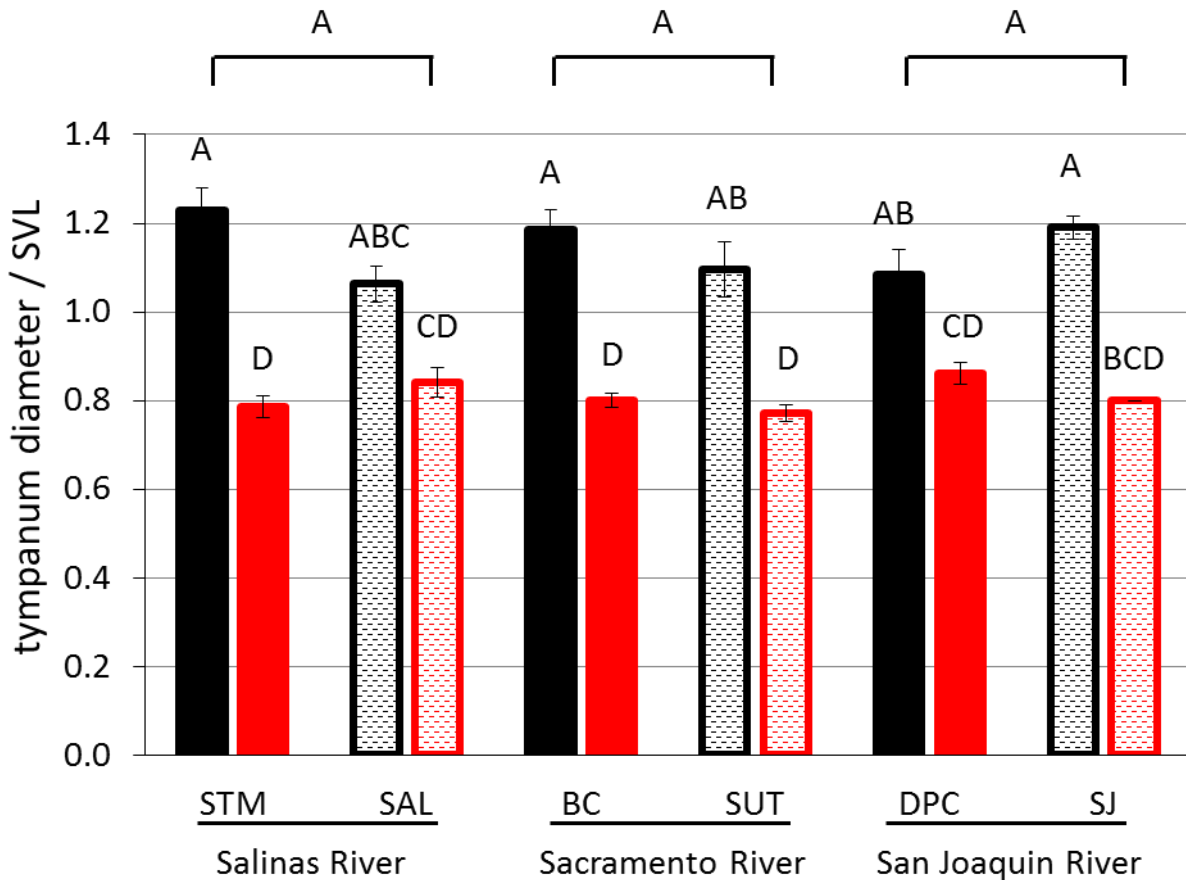




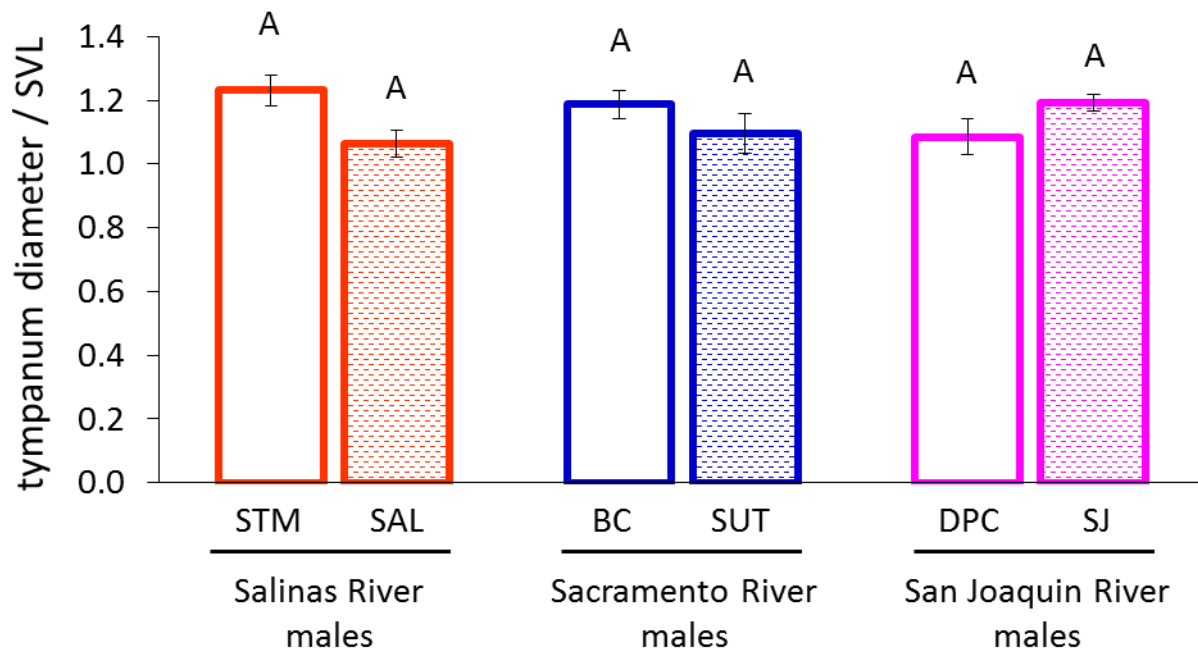
**Figure 6.** Body weight (BW) for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas River (orange), Sacramento River (blue) and San Joaquin River (pink). Males collected upstream of agriculture are denoted with open bars (STM: Santa Margarita, n = 6; BC: Battle Creek, n = 6; and DPC: Del Puerto Canyon, n = 10) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 7; SUT: Sutter, n = 10; and SJ: San Joaquin, n = 11). Two-way ANOVA was used to examine whether males differed in BW between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).



**Figure 7.** Gonadal-somatic index (GSI) for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas River (orange), Sacramento River (blue) and San Joaquin River (pink). Males collected upstream of agriculture are denoted with open bars (STM: Santa Margarita, n = 6; BC: Battle Creek, n = 6; and DPC: Del Puerto Canyon, n = 10) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 6; SUT: Sutter, n = 10; and SJ: San Joaquin, n = 11). Two-way ANOVA was used to examine whether males differed in GSI between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).



**Figure 8.** Tympanum diameter measurements (normalized for SVL) for male (black) and female (red) American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas, Sacramento, and San Joaquin Rivers. Animals collected upstream of agricultural activity are denoted with solid bars (STM: Santa Margarita,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 5$ ; BC: Battle Creek,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 9$ ; and DPC: Del Puerto Canyon,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 6$ ) and animals collected downstream of agricultural activity have patterned bars (SAL: Salinas,  $n_{\text{males}} = 7$ ,  $n_{\text{females}} = 5$ ; SUT: Sutter,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 10$ ; and SJ: San Joaquin,  $n_{\text{males}} = 11$ ,  $n_{\text{females}} = 2$ ). Three-way ANOVA was used to examine whether animals differed in normalized tympanum diameters between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity), sex (male or female) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

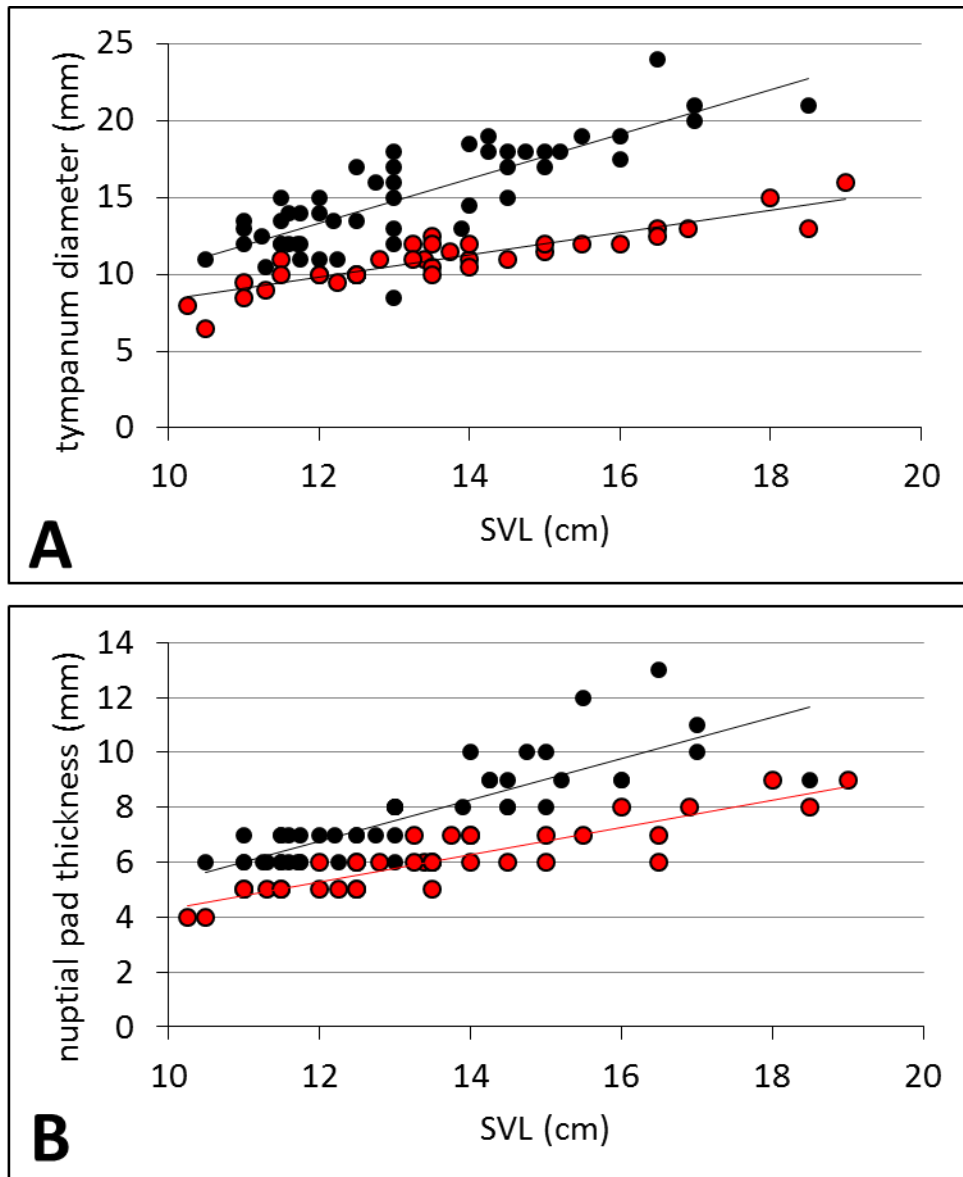


**Figure 9.** Tympanum diameter measurements (normalized for SVL) for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas River (orange), Sacramento River (blue) and San Joaquin River (pink). Males collected upstream of agriculture are denoted with open bars (STM: Santa Margarita, n = 6; BC: Battle Creek, n = 6; and DPC: Del Puerto Canyon, n = 10) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 7; SUT: Sutter, n = 10; and SJ: San Joaquin, n = 11). Two-way ANOVA was used to examine whether males differed in normalized tympanum diameters between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

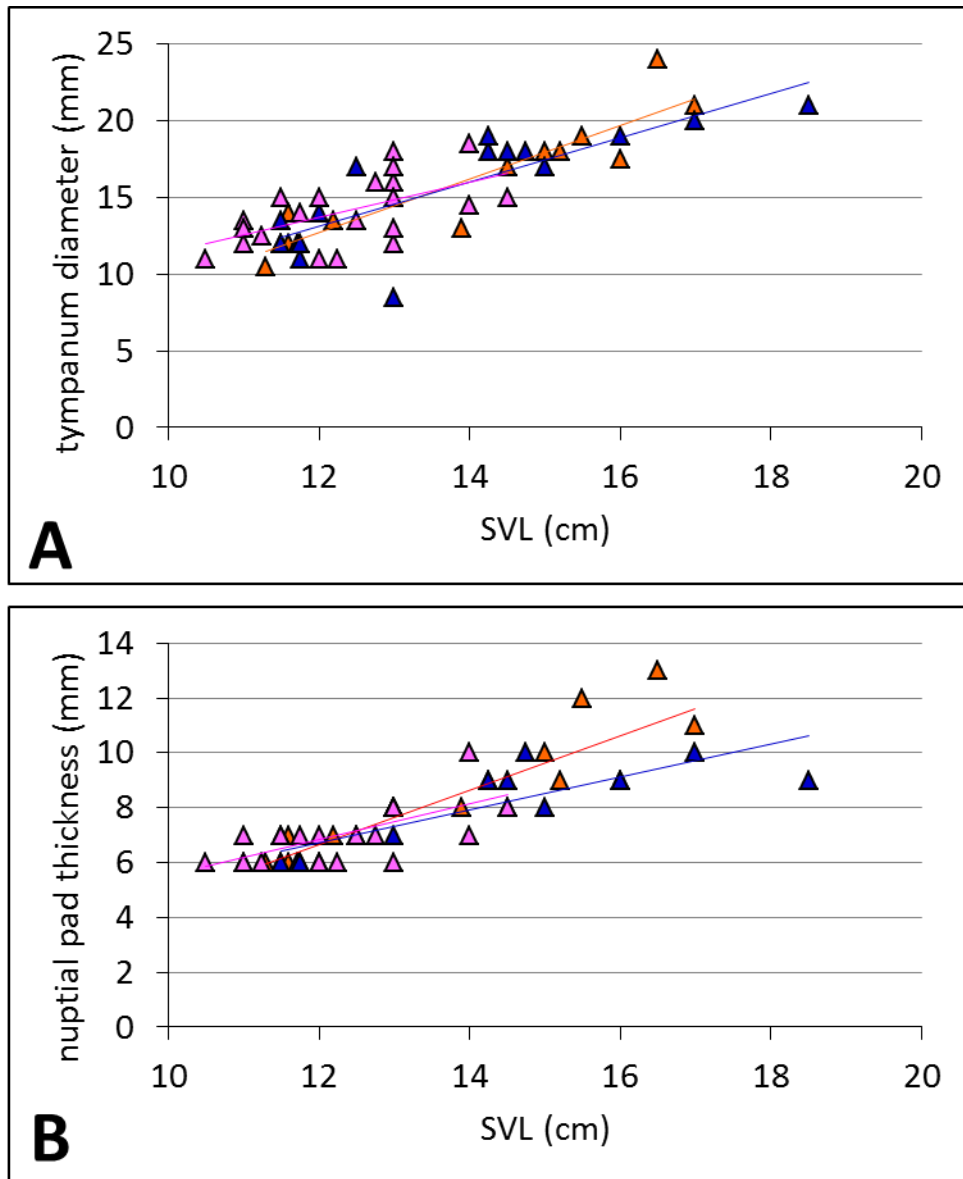
Site	n	Tympanum diameter / SVL				Nuptial pad thickness / SVL			
		mean	df	F	p	mean	df	F	p
<b>Salinas River</b>									
Santa Margarita (STM)	6	1.23±0.12*	1	6.83	0.024	0.067±0.010*	1	6.83	0.024
Salinas (SAL)	7	1.06±0.11*				0.056±0.004*			
<b>Sacramento River</b>									
Battle Creek (BC)	6	1.19±0.11	1	1.08	0.316	0.056±0.005	1	0.06	0.808
Sutter (SUT)	10	1.10±0.20				0.057±0.006			
<b>San Joaquin River</b>									
Del Puerto Canyon (DPC)	10	1.08±0.18	1	3.20	0.090	0.056±0.008	1	1.61	0.220
San Joaquin (SJ)	11	1.19±0.08				0.059±0.003			

ONE-WAY ANOVA: significant at \*p≤0.05; \*\*p≤0.005; \*\*\*p≤0.001

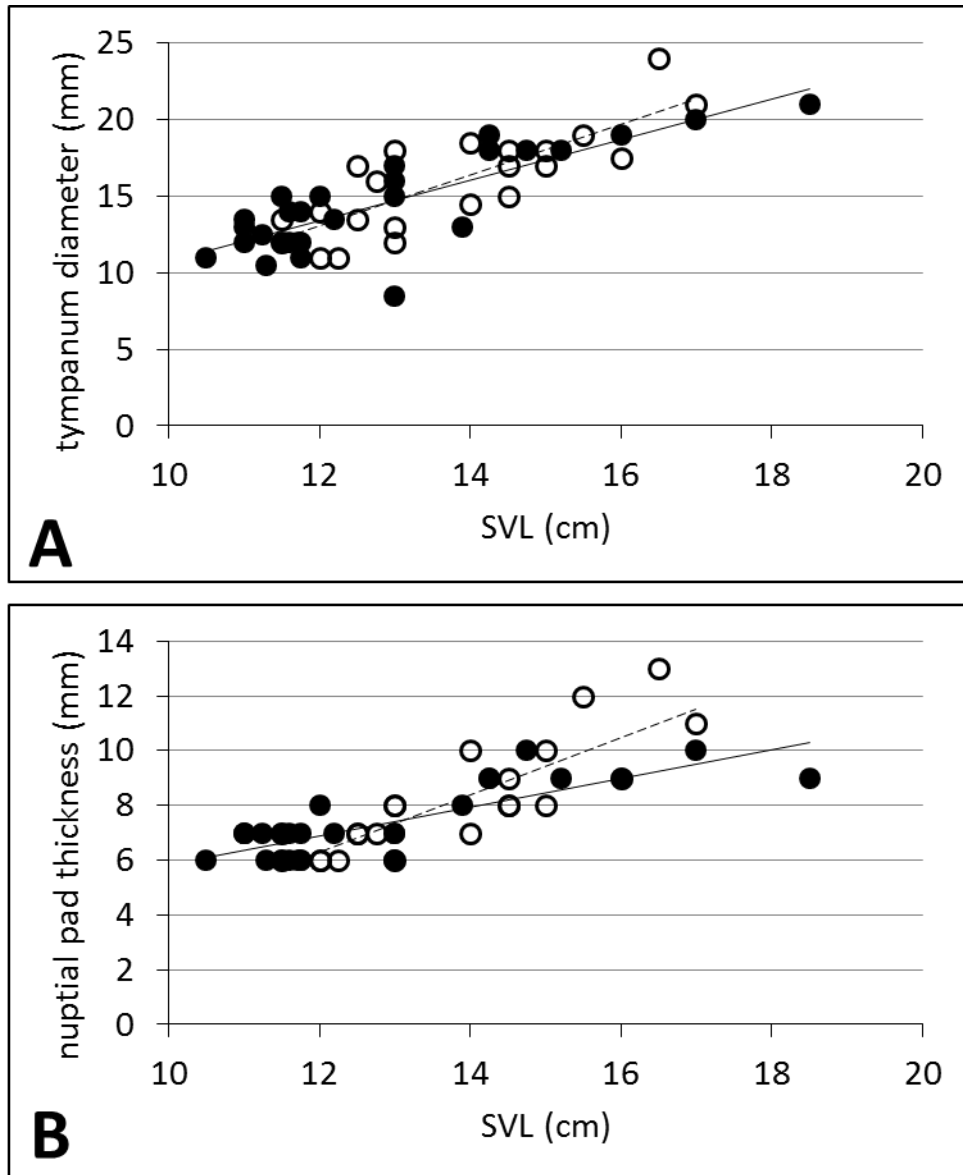
**Table 2.** Tympanum diameter and nuptial pad thickness (normalized for SVL) for males collected upstream (STM, BC & DPC) and downstream (SAL, SUT & SJ) of agricultural activity. One-way ANOVA was used to examine the differences of tympanum diameters and nuptial pad thicknesses within each river system. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).



**Figure 10.** Analysis of covariance (ANCOVA) of SVL (covariate), and dependent variable tympanum diameter (A) or nuptial pad thickness (B) for males and females (independent variable) from the Salinas, Sacramento, and San Joaquin Rivers. Black circles represent males and red circles represent females. (A) Male tympanum size increases faster relative to SVL (ANCOVA:  $F = 19.08$ ,  $df = 1$ ,  $p < 0.001$ ) and males have larger tympanum diameters than females (ANCOVA:  $F = 5.14$ ,  $df = 1$ ,  $p = 0.026$ ). (B) Male nuptial pad size increases faster relative to SVL (ANCOVA:  $F = 8.73$ ,  $df = 1$ ,  $p = 0.004$ ) and males have larger nuptial pad thickness than females (ANCOVA:  $F = 100.67$ ,  $df = 1$ ,  $p < 0.001$ ). Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).

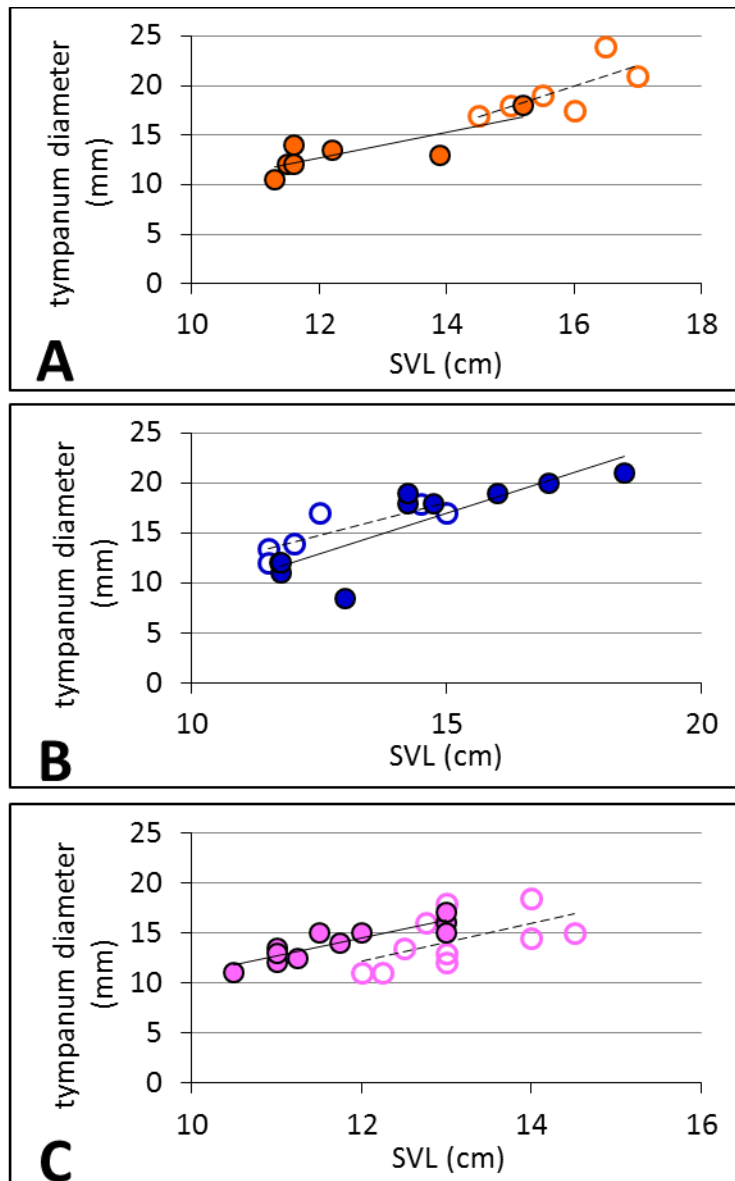


**Figure 11.** Analysis of covariance (ANCOVA) of SVL (covariate), and dependent variable tympanum diameter (A) or nuptial pad thickness (B) for males from the Salinas (orange), Sacramento (blue), and San Joaquin (pink) Rivers (independent variable). (A) Male tympanum growth relative to SVL (ANCOVA:  $F = 0.87$ ,  $df = 2$ ,  $p = 0.428$ ) and tympanum size (ANCOVA:  $F = 0.92$ ,  $df = 2$ ,  $p = 0.407$ ) did not differ across rivers. (B) Salinas River males' nuptial pad size increased faster relative to SVL (ANCOVA:  $F = 8.73$ ,  $df = 1$ ,  $p = 0.004$ ) but did not differ in size (ANCOVA:  $F = 0.92$ ,  $df = 2$ ,  $p = 0.407$ ) compared to males from the Sacramento and San Joaquin Rivers. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).

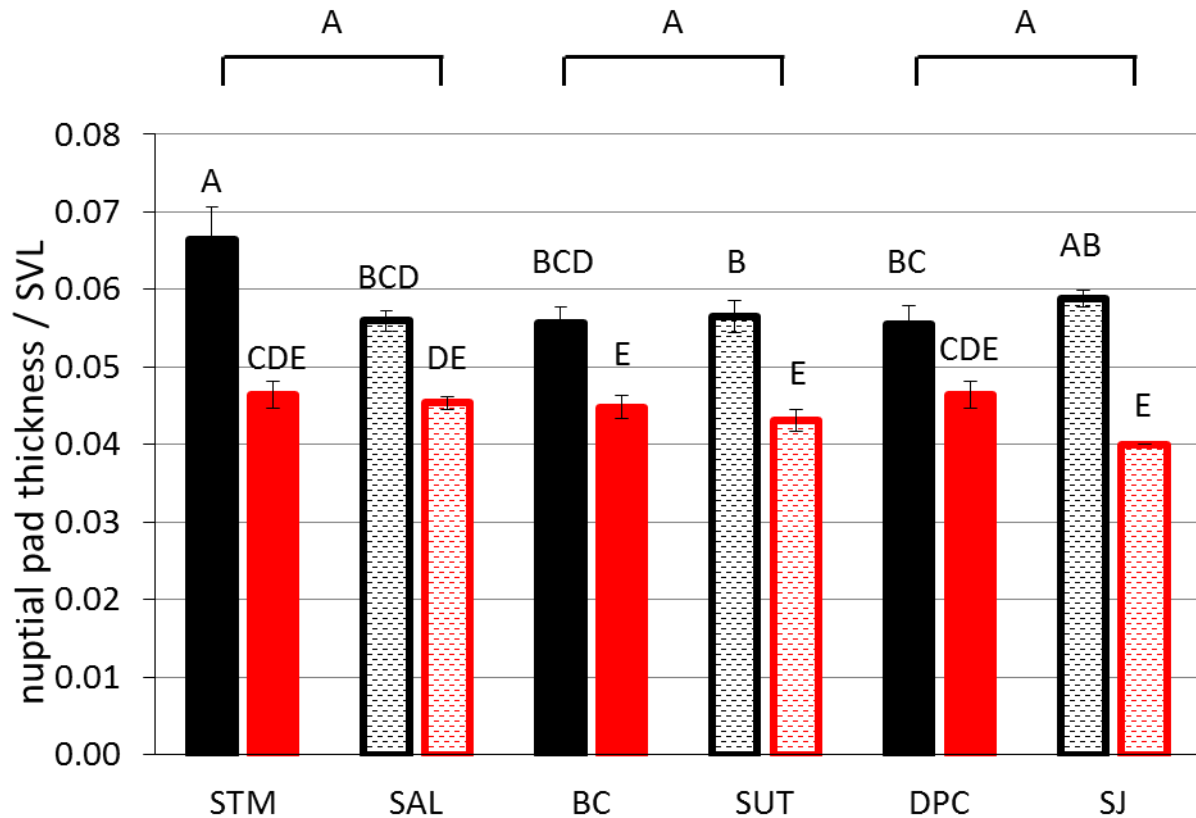


**Figure 12.** Analysis of covariance (ANCOVA) of SVL (covariate), and dependent variable tympanum diameter (A) or nuptial pad thickness (B) for region (independent variable). Upstream males represented by open circles and downstream males represented by black circles. (A) Male tympanum growth relative to SVL (ANCOVA:  $F = 1.15$ ,  $df = 1$ ,  $p = 0.288$ ) and tympanum size (ANCOVA:  $F = 1.07$ ,  $df = 1$ ,  $p = 0.306$ ) did not differ across region. (B) Upstream male nuptial pad size increased faster relative to SVL (ANCOVA:  $F = 9.90$ ,  $df = 1$ ,  $p = 0.003$ ) and had larger nuptial pads than downstream males (ANCOVA:  $F = 9.43$ ,  $df = 1$ ,  $p = 0.004$ ). Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).

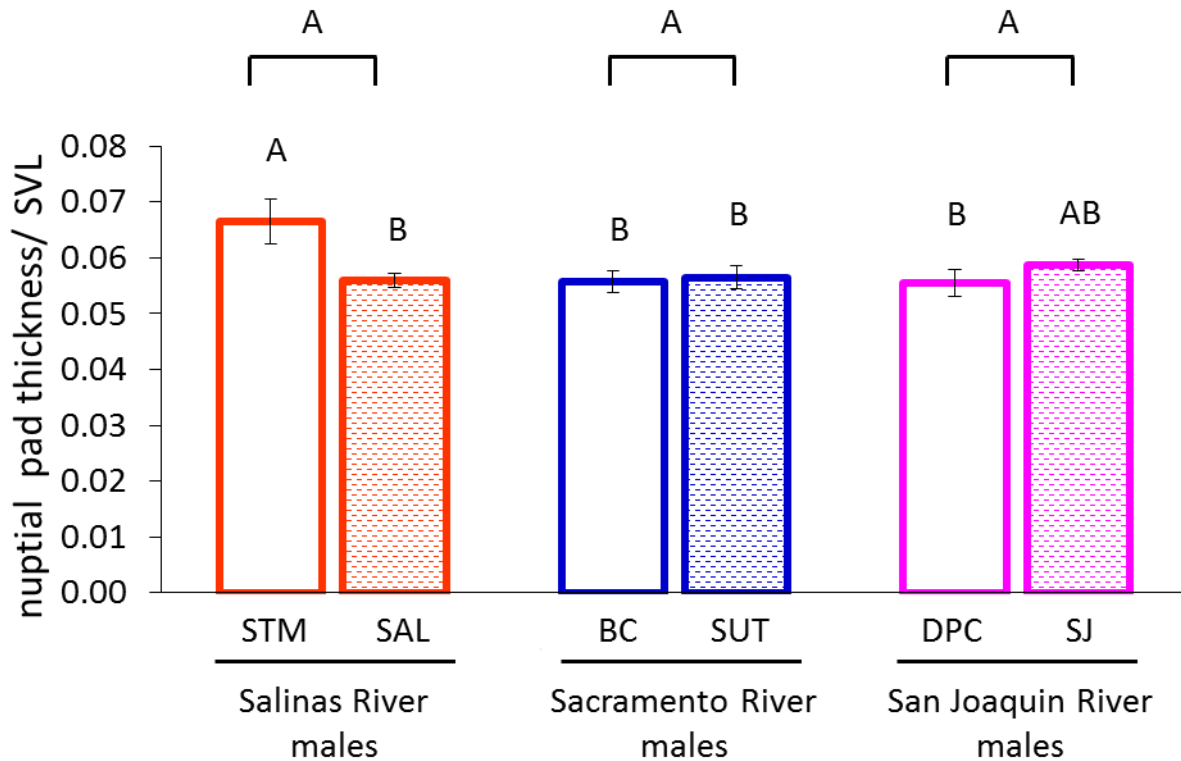




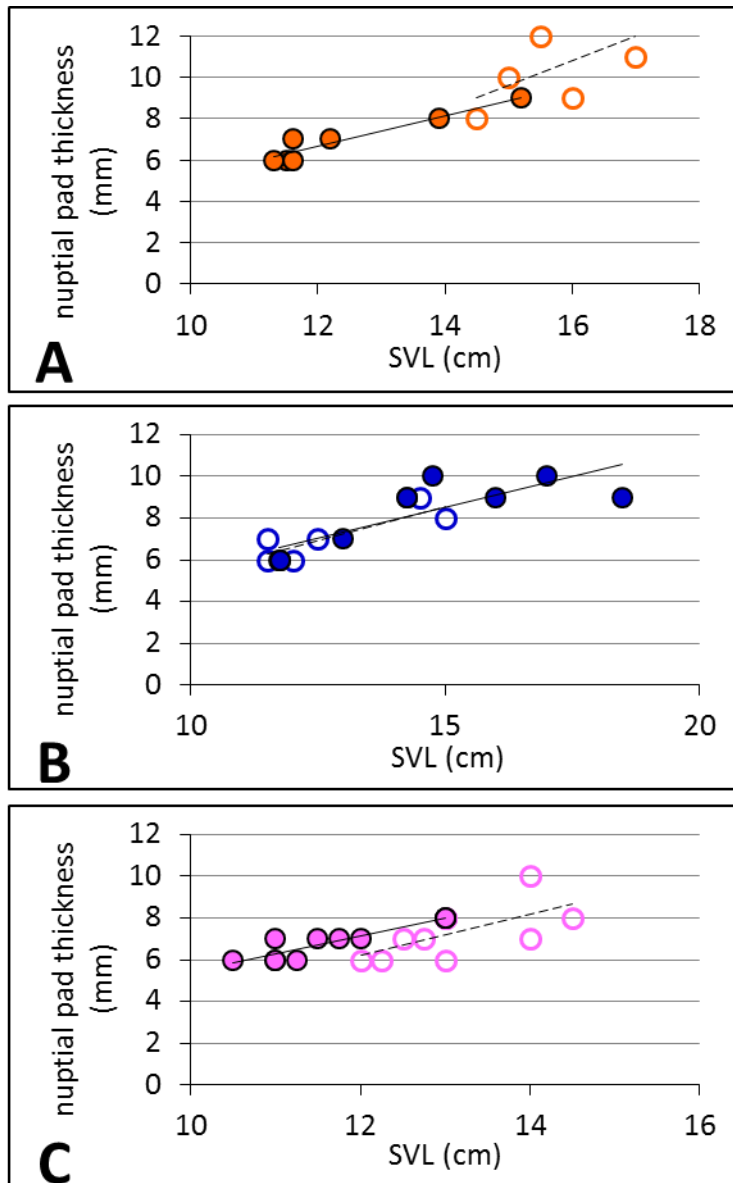
**Figure 13.** Analysis of covariance (ANCOVA) of SVL (covariate) and dependent variable tympanum diameter for region (independent variable). Upstream males represented by open circles and downstream males represented by closed circles for males from the (A) Salinas (orange), (B) Sacramento (blue), and (C) San Joaquin (pink) Rivers. (A) Tympanum growth relative to SVL (ANCOVA:  $F = 0.67$ ,  $df = 1$ ,  $p = 0.435$ ) or size (ANCOVA:  $F = 0.57$ ,  $df = 1$ ,  $p = 0.486$ ) did not differ between upstream and downstream males from the Salinas River. (B) Sacramento River males did not differ in tympanum growth relative to SVL (ANCOVA:  $F = 0.21$ ,  $df = 1$ ,  $p = 0.657$ ) or size (ANCOVA:  $F = 0.40$ ,  $df = 1$ ,  $p = 0.538$ ). (C) Tympanum growth relative to SVL did not differ in males from the San Joaquin River (ANCOVA:  $F = 0.01$ ,  $df = 1$ ,  $p = 0.909$ ; Fig. 13C). However, tympanum diameters of downstream males were significantly larger than upstream San Joaquin males (ANCOVA:  $F = 5.55$ ,  $df = 1$ ,  $p = 0.030$ ). Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).



**Figure 14.** Nuptial pad thickness (normalized for SVL) for male (black) and female (red) American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas, Sacramento, and San Joaquin Rivers. Animals collected upstream of agricultural activity are denoted with solid bars (STM: Santa Margarita,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 5$ ; BC: Battle Creek,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 9$ ; and DPC: Del Puerto Canyon,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 6$ ) and animals collected downstream of agricultural activity have patterned bars (SAL: Salinas,  $n_{\text{males}} = 7$ ,  $n_{\text{females}} = 5$ ; SUT: Sutter,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 10$ ; and SJ: San Joaquin,  $n_{\text{males}} = 11$ ,  $n_{\text{females}} = 2$ ). Three-way ANOVA was used to examine whether animals differed in normalized thumb pad thickness between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity), sex (male or female) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).



**Figure 15.** Nuptial pad thickness (normalized for SVL) for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas river (orange), Sacramento river (blue) and San Joaquin river (pink). Males collected upstream of agriculture are denoted with open bars (STM: Santa Margarita, n = 6; BC: Battle Creek, n = 6; and DPC: Del Puerto Canyon, n = 10) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 7; SUT: Sutter, n = 10; and SJ: San Joaquin, n = 11). Two-way ANOVA was used to examine whether males differed in normalized nuptial pad thickness between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).



**Figure 16.** Analysis of covariance (ANCOVA) of SVL (covariate), and dependent variable nuptial pad thickness for region (independent variable). Upstream males represented by open circles and downstream males represented by closed circles for males from the (A) Salinas River (orange), (B) Sacramento River (blue), and (C) San Joaquin River (pink). (A) Nuptial pad growth relative to SVL (ANCOVA:  $F = 0.53$ ,  $df = 1$ ,  $p = 0.485$ ) or size (ANCOVA:  $F = 0.42$ ,  $df = 1$ ,  $p = 0.532$ ) did not differ between upstream and downstream males from the Salinas River. (B) Sacramento River males did not differ in nuptial pad growth relative to SVL (ANCOVA:  $F = 0.05$ ,  $df = 1$ ,  $p = 0.829$ ) or size (ANCOVA:  $F = 0.06$ ,  $df = 1$ ,  $p = 0.818$ ). (C) Nuptial pad growth relative to SVL did not differ in males from the San Joaquin River (ANCOVA:  $F = 0.14$ ,  $df = 1$ ,  $p = 0.713$ ). However, nuptial pad thickness of downstream males were significantly larger than upstream San Joaquin males (ANCOVA:  $F = 4.29$ ,  $df = 1$ ,  $p = 0.053$ ).

Site	n	Throat hue (degrees)				Medial hue (degrees)				Lateral hue (degrees)				Patch hue (degrees)							
		mean	df	F	p	Tukey's	mean	df	F	p	Tukey's	mean	df	F	p	Tukey's	mean	df	F	p	Tukey's
<b>River</b>																					
Salinas	26	49.3±3.1				A	49.6±2.7				A	49.4±3.1				A	50.0±3.5				A
Sacramento	16	47.1±3.5	2	1.76	0.181	A	46.7±3.9	2	3.43	0.004**	B	46.4±4.0	2	2.89	0.064	A	44.5±4.7	2	8.35	0.001***	B
San Joaquin	21	48.1±2.9				A	48.8±2.8				AB	48.6±3.6				A	47.6±4.6				AB
<b>Region</b>																					
Upstream	28	48.2±2.9	1	0.05	0.831	A	46.9±4.1	1	1.96	0.738	A	46.5±4.3	1	0.66	0.419	A	48.2±3.9	1	1.61	0.209	A
Downstream	35	48.5±3.5				A	46.7±4.2				A	46.4±4.5				A	47.5±5.2				A
<b>River* Region</b>																					
<b>Salinas</b>																					
upstream	12	47.3±3.0				B	48.4±2.7				AB	47.6±3.1				AB	47.4±2.6				BC
downstream	14	50.9±2.1				A	50.6±2.2				A	50.8±2.3				A	52.2±2.6				A
<b>Sacramento</b>																					
upstream	6	48.7±4.3	2	7.12	0.002**	AB	48.9±4.7	2	5.69	0.002**	AB	48.2±4.9	2	5.91	0.005**	AB	47.1±5.9	2	11.56	<0.001***	ABC
downstream	10	46.2±2.8				B	45.3±2.8				B	45.3±3.2				B	42.9±3.1				C
<b>San Joaquin</b>																					
upstream	10	49.0±1.7				AB	49.7±2.0				A	49.9±2.0				A	49.8±3.7				AB
downstream	11	47.4±3.6				B	48.0±3.2				AB	47.5±4.4				AB	45.6±4.5				BC

TWO-WAY ANOVA: significant at \*p≤0.05; \*\*p≤0.005; \*\*\*p≤0.001

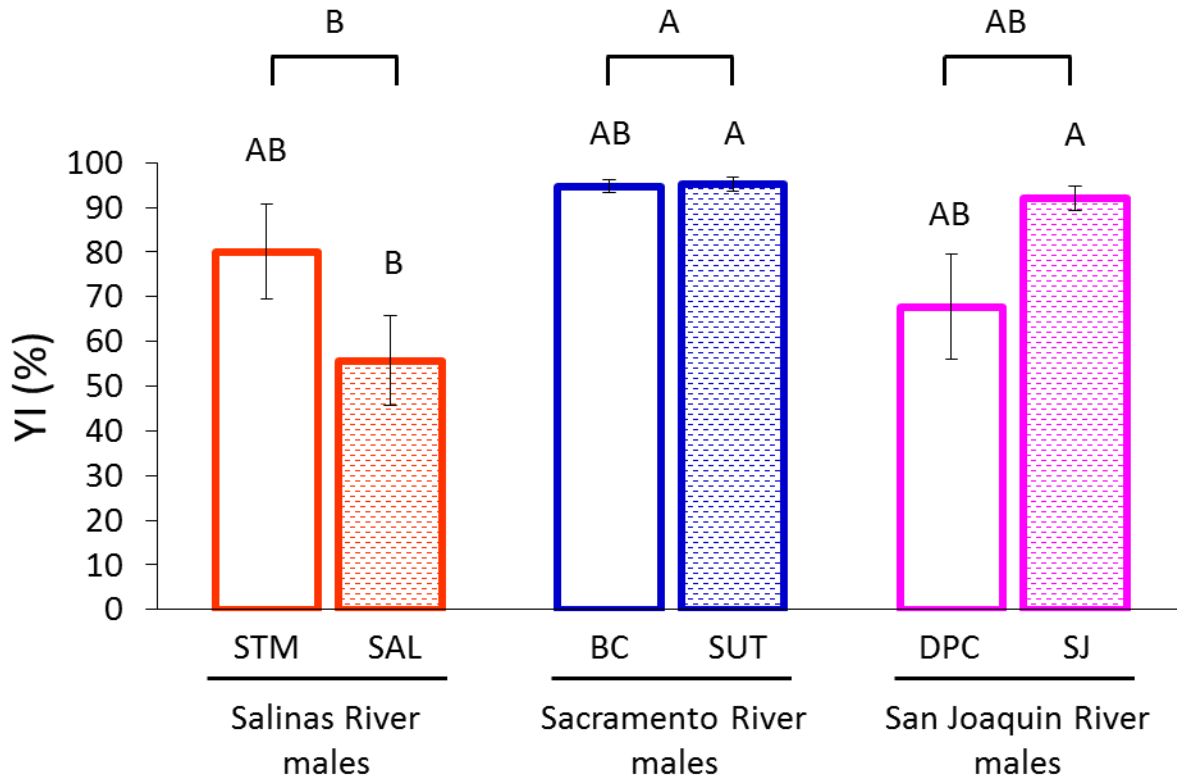
TUKEY'S HSD: means that do not share a letter are significantly different

**Table 3.** Hue analysis of yellow throat coloration for entire throat area (Throat hue), medial (Medial hue) and lateral (Lateral hue) throat axis, and outline of darkest yellow area of throat (Patch hue). A two-way ANOVA was used to examine whether males differed in yellow throat coloration between rivers (Salinas, Sacramento and San Joaquin), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance determined by Tukey's honestly significant difference (HSD) method. Means that do not share a letter are significantly different (p<0.05).

Site	n	df	Throat hue (degrees)			Medial hue (degrees)			Lateral hue (degrees)			Patch hue (degrees)		
			mean	F	p	mean	F	p	mean	F	p	mean	F	p
<b>Salinas River</b>														
Santa Margarita (STM)	12	1	47.3±3.0**	12.89	0.001	48.4±2.7*	5.38	0.029	47.6±3.1*	9.08	0.006	47.4±2.6***	22.01	<0.001
Salinas (SAL)	14		50.9±2.1**			50.6±2.2*			50.8±2.3*			52.2±2.6***		
<b>Sacramento River</b>														
Battle Creek (BC)	6	1	48.7±4.3	1.95	0.184	48.9±4.7	3.69	0.075	48.2±4.9	2.08	0.172	47.1±5.9	3.47	0.084
Sutter (SUT)	10		46.2±2.8			45.3±2.8			45.3±3.2			42.9±3.1		
<b>San Joaquin River</b>														
Del Puerto Canyon (DPC)	10	1	49.0±1.7	1.70	0.208	49.7±2.0	2.27	0.149	49.9±2.0	2.52	0.129	49.8±3.7*	5.48	0.030
San Joaquin (SJ)	11		11.7±0.9			48.0±3.2			47.5±4.4			45.6±4.5*		

ONE-WAY ANOVA: significant at \*p<0.05; \*\*p<0.005; \*\*\*p<0.001

**Table 4.** Hue analysis of yellow throat coloration for entire throat area (Throat hue), medial (Medial hue) and lateral (Lateral hue) throat axis, and outline of darkest yellow area of throat (Patch hue). One-way ANOVA was used to examine the differences of yellow throat coloration within each river system. Significance was determined by Tukey's honestly significant difference (HSD) method (p < 0.05).



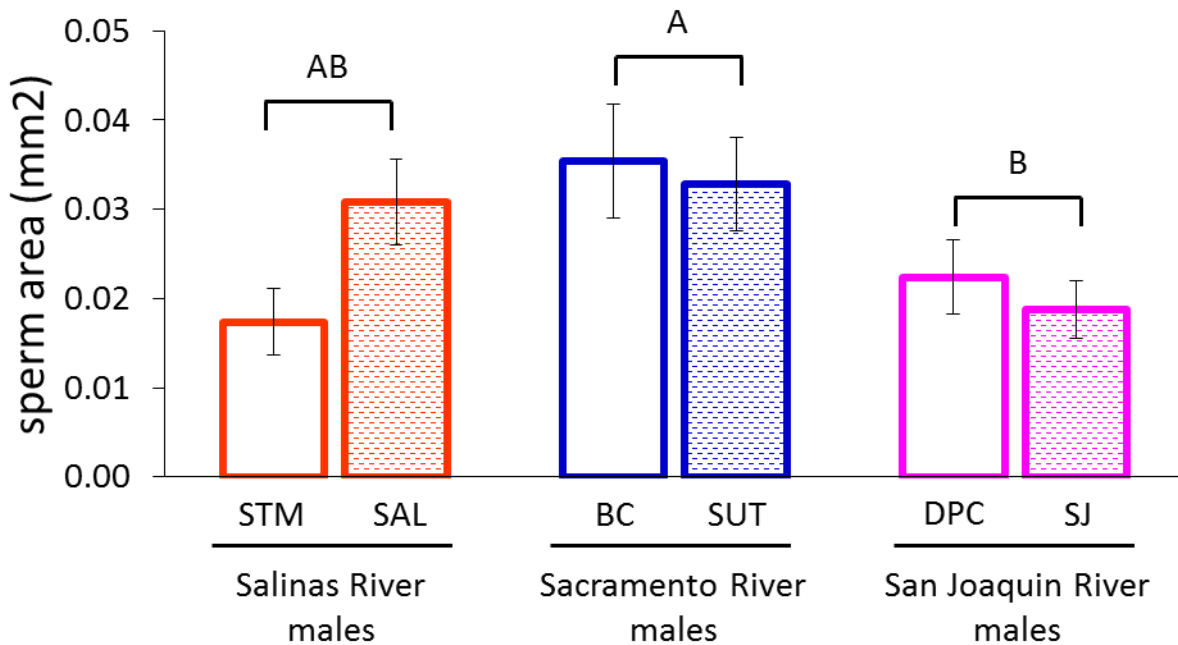
**Figure 17.** Yellow index (YI) for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas River (orange), Sacramento River (blue) and San Joaquin River (pink). Males collected upstream of agriculture are denoted with open bars (STM: Santa Margarita, n = 12; BC: Battle Creek, n = 6; and DPC: Del Puerto Canyon, n = 10) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 14; SUT: Sutter, n = 10; and SJ: San Joaquin, n = 11). Two-way ANOVA was used to examine whether males differed in YI between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

<i>Site</i>	<i>n</i>	<i>Yellow Index (%)</i>			
		<i>mean</i>	<i>df</i>	<i>F</i>	<i>p</i>
<b>Salinas River</b>					
Santa Margarita (STM)	12	80.1±36.9	1	2.80	0.107
Salinas (SAL)	14	57.7±37.4			
<b>Sacramento River</b>					
Battle Creek (BC)	6	94.7±3.4	1	0.04	0.840
Sutter (SUT)	10	95.2±4.7			
<b>San Joaquin River</b>					
Del Puerto Canyon (DPC)	10	67.7±37.2*	1	4.47	0.048
San Joaquin (SJ)	11	92.0±8.9*			

ONE-ANOVA: significant at \* $p \leq 0.05$ ; \*\* $p \leq 0.005$ ; \*\*\* $p \leq 0.001$

**Table 5.** Yellow index (YI) for males collected upstream and downstream of agricultural activity. One-way ANOVA was used to examine the difference of YI within each river system. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).



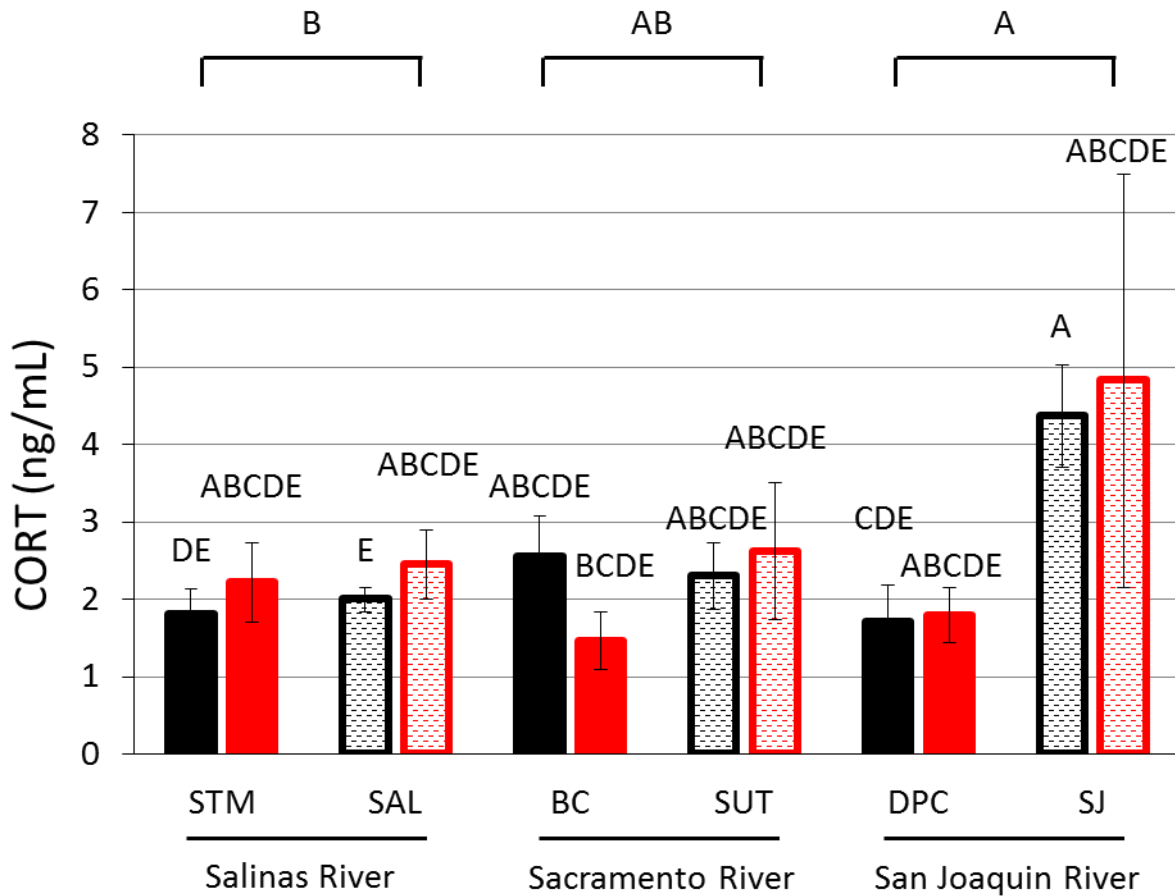


**Figure 18.** Sperm area for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas River (orange), Sacramento River (blue) and San Joaquin River (pink). Males collected upstream of agriculture) are denoted with open bars (STM: Santa Margarita, n = 9; BC: Battle Creek, n = 4; and DPC: Del Puerto Canyon, n = 5) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 10; SUT: Sutter, n = 6; and SJ: San Joaquin, n = 6). Two-way ANOVA was used to examine whether males differed in sperm area between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

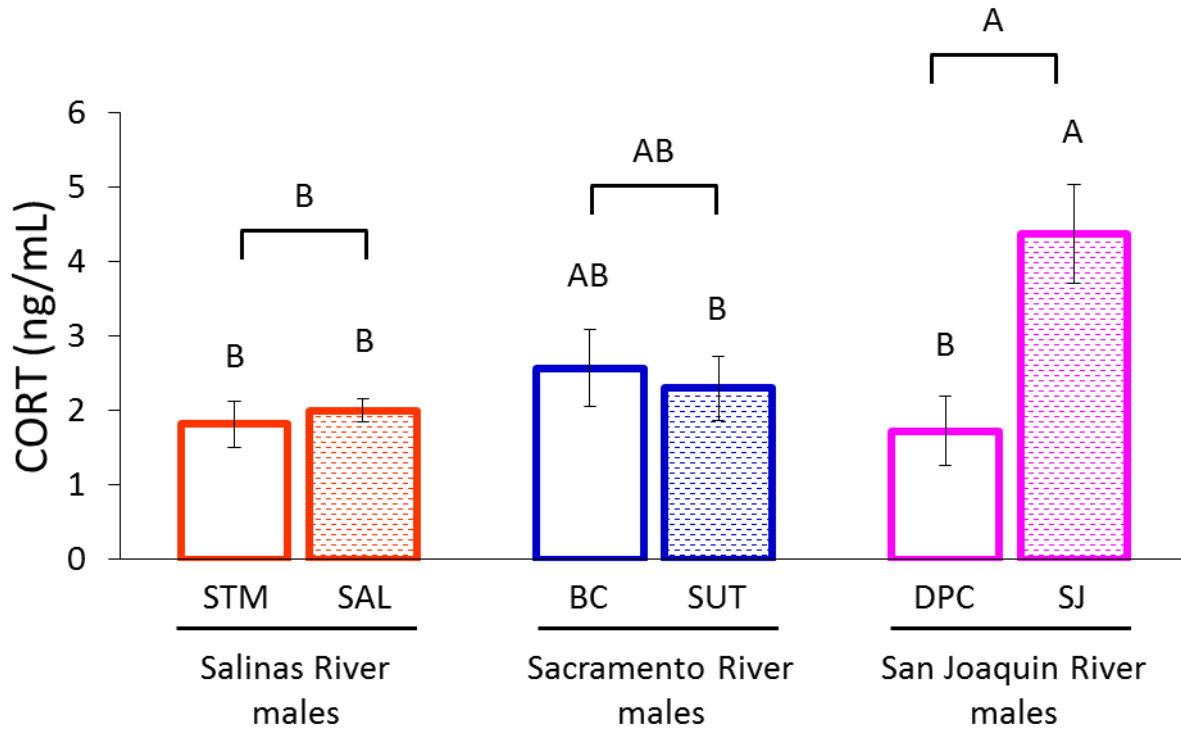
Site	n	Sperm area (mm <sup>2</sup> )			
		mean	df	F	p
<b>Salinas River</b>					
Santa Margarita (STM)	12	0.017±0.011*	1	4.68	0.045
Salinas (SAL)	14	0.031±0.015*			
<b>Sacramento River</b>					
Battle Creek (BC)	6	0.035±0.013	1	0.10	0.764
Sutter (SUT)	10	0.033±0.013			
<b>San Joaquin River</b>					
Del Puerto Canyon (DPC)	10	0.022±0.009	1	0.50	0.498
San Joaquin (SJ)	11	0.019±0.008			

ONE-ANOVA: significant at \*p≤0.05; \*\*p≤0.005; \*\*\*p≤0.001

**Table 6.** Sperm area for male bullfrogs collected upstream and downstream of agricultural activity. One-way ANOVA was used to examine the differences of sperm area of upstream and downstream males within each river system. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).



**Figure 19.** Corticosterone (CORT) levels for male (black) and female (red) American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas, Sacramento, and San Joaquin Rivers. Animals collected upstream of agricultural activity are denoted with solid bars (STM: Santa Margarita,  $n_{\text{males}} = 12$ ,  $n_{\text{females}} = 11$ ; BC: Battle Creek,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 10$ ; and DPC: Del Puerto Canyon,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 6$ ) and animals collected downstream of agricultural activity have patterned bars (SAL: Salinas,  $n_{\text{males}} = 14$ ,  $n_{\text{females}} = 11$ ; SUT: Sutter,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 10$ ; and SJ: San Joaquin,  $n_{\text{males}} = 11$ ,  $n_{\text{females}} = 2$ ). Three-way ANOVA was used to examine whether animals differed in CORT levels between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity), sex (male or female) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

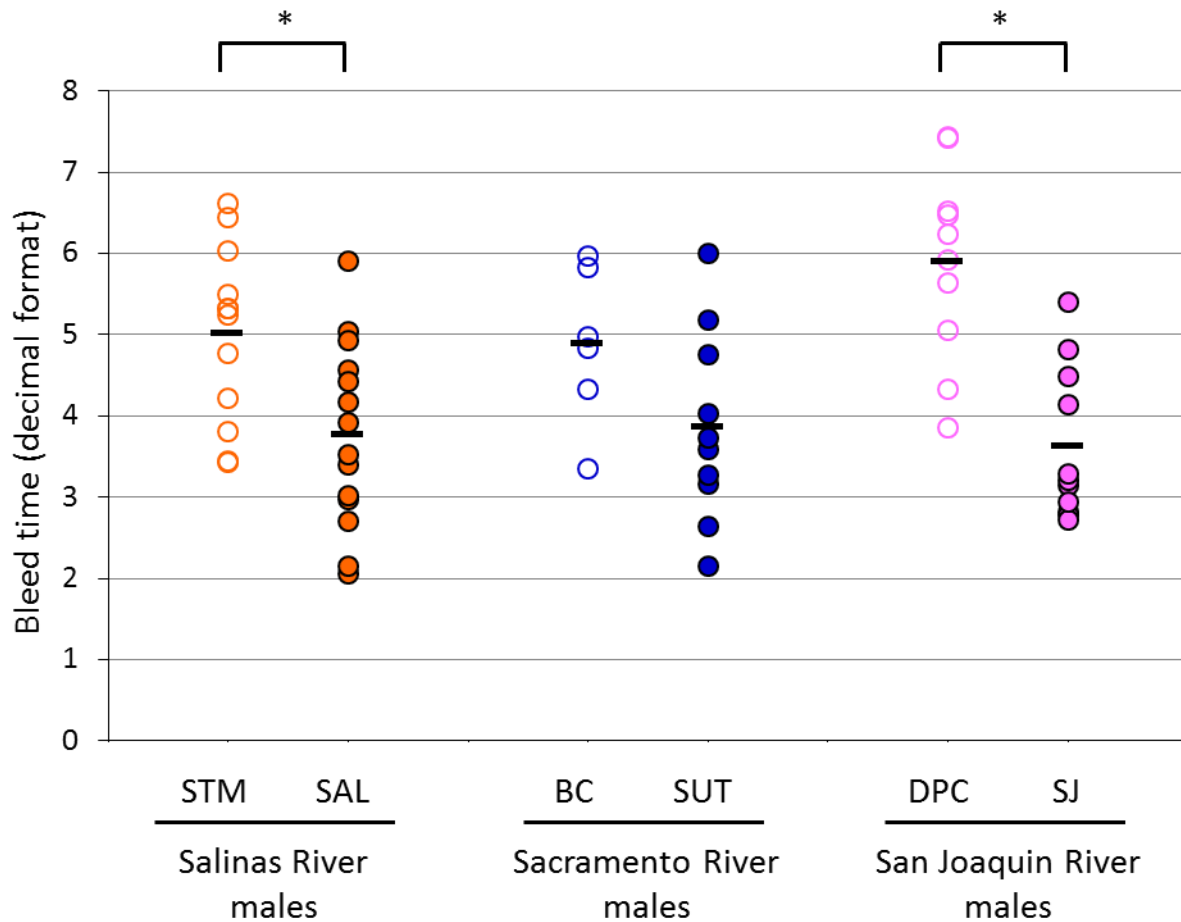


**Figure 20.** Corticosterone (CORT) levels for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas river (orange), Sacramento river (blue) and San Joaquin river (pink). Males collected upstream of agriculture) are denoted with open bars (STM: Santa Margarita, n = 12; BC: Battle Creek, n = 6; and DPC: Del Puerto Canyon, n = 10) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 14; SUT: Sutter, n = 10; and SJ: San Joaquin, n = 11). Two-way ANOVA was used to examine whether males differed in CORT levels between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

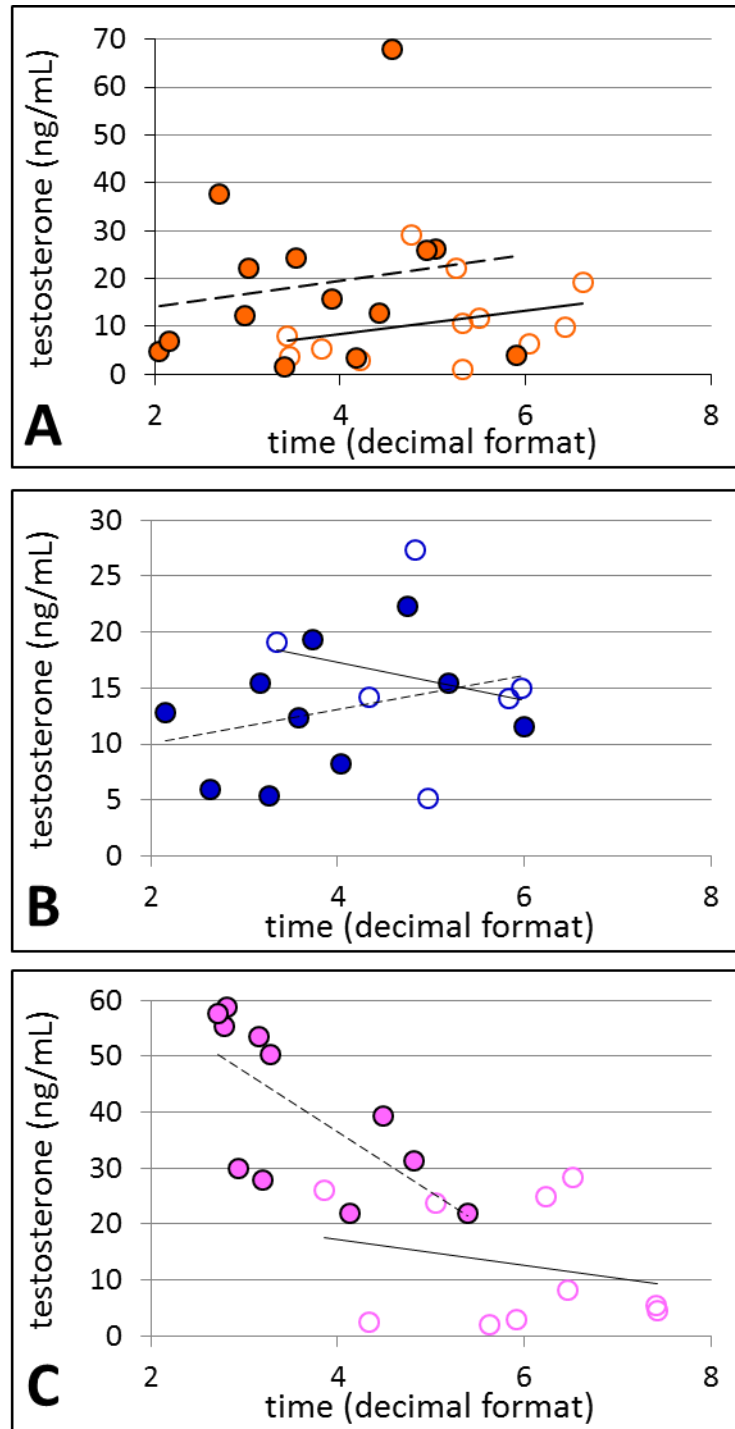
Site	n	df	Corticosterone (ng/mL)			Testosterone (ng/mL)		
			mean	F	p	mean	F	p
<b>Salinas River</b>								
Santa Margarita (STM)	12	1	1.82±1.07	0.29	0.596	10.84±8.54	2.11	0.160
Salinas (SAL)	14		2.00±0.59			19.00±17.75		
<b>Sacramento River</b>								
Battle Creek (BC)	6	1	2.57±1.27	0.15	0.701	15.79±7.27	0.84	0.376
Sutter (SUT)	10		2.30±1.35			12.87±5.48		
<b>San Joaquin River</b>								
Del Puerto Canyon (DPC)	10	1	1.72±1.48**	10.25	0.005	12.89±11.29***	23.3	<0.001
San Joaquin (SJ)	11		4.37±2.20**			40.71±14.69***		

ONE-ANOVA: significant at \*p≤0.05; \*\*p≤0.005; \*\*\*p≤0.001

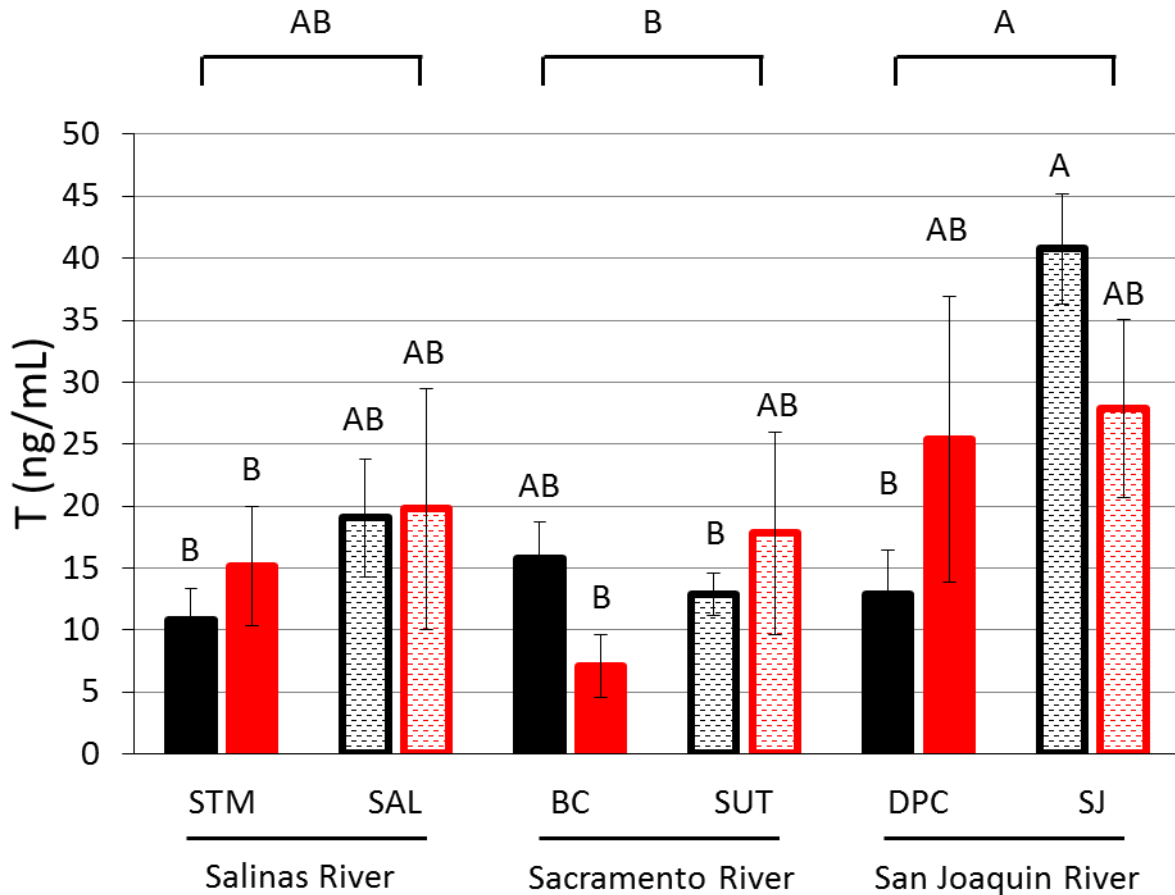
**Table 7.** Corticosterone (CORT) and testosterone (T) levels for male bullfrogs collected upstream and downstream of agricultural activity. One-way ANOVA was used to examine the differences of steroid levels between upstream and downstream males within each river system. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).



**Figure 21.** Blood collection times for males living along the Salinas River (orange), Sacramento River (blue) and San Joaquin River (pink). Males collected upstream of agriculture are denoted with open circles and males collected downstream of agricultural activity have closed circles. One-way ANOVA was used to examine the difference of bleed times within each river system. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ).

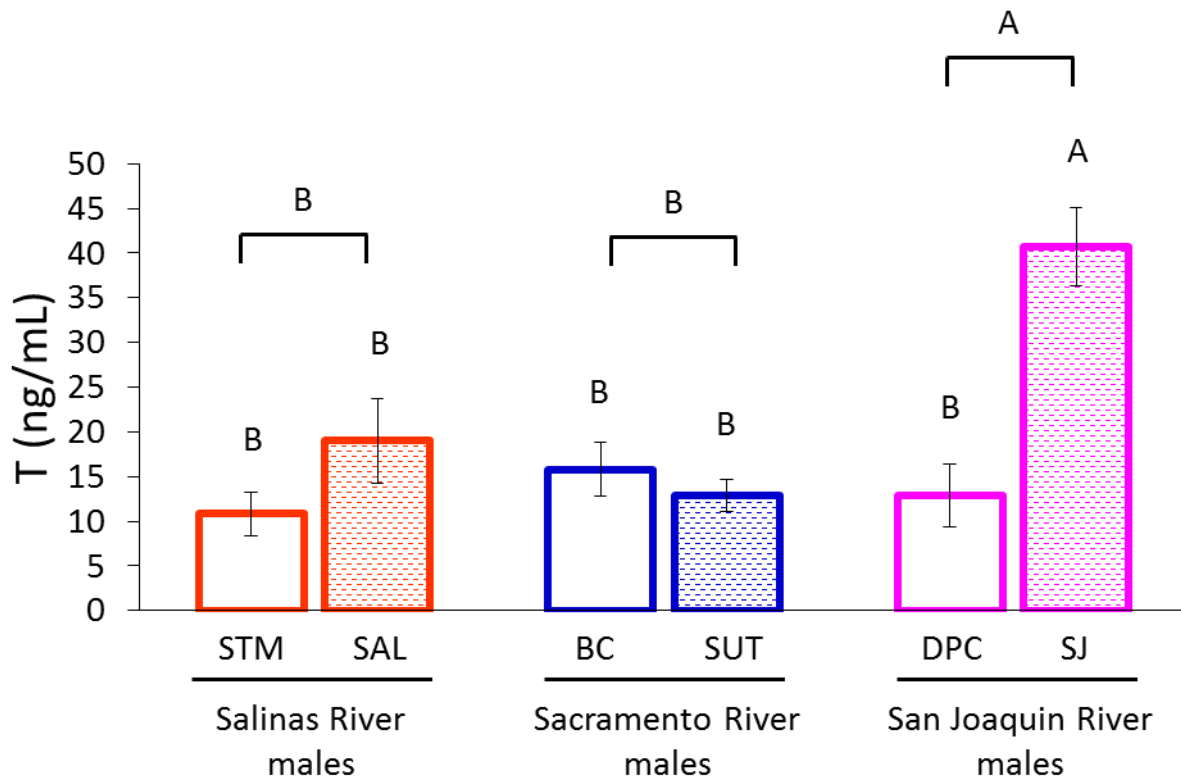


**Figure 22.** Correlation analysis of testosterone levels and blood collection times of males from the Salinas (orange), Sacramento (blue) and San Joaquin (pink) Rivers. Open circles and solid trendlines represent upstream males (STM, BC, DPC). Closed circles and dashed trendline represent downstream males (SAL, SUT, SJ). Salinas River (A) STM ( $P = 0.309$ ;  $p = 0.328$ ); SAL ( $P = 0.174$ ;  $p = 0.553$ ) Sacramento (B) BC ( $P = -0.229$ ;  $p = 0.663$ ); SUT ( $P = 0.320$ ;  $p = 0.367$ ) San Joaquin (C) DPC ( $P = -0.250$ ;  $p = 0.485$ ); SJ ( $P = -0.683$ ;  $p = 0.021$ )



**Figure 23.** Testosterone (T) levels for male (black) and female (red) American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas, Sacramento, and San Joaquin Rivers. Animals collected upstream of agricultural activity are denoted with solid bars (STM: Santa Margarita,  $n_{\text{males}} = 12$ ,  $n_{\text{females}} = 11$ ; BC: Battle Creek,  $n_{\text{males}} = 6$ ,  $n_{\text{females}} = 10$ ; and DPC: Del Puerto Canyon,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 6$ ) and animals collected downstream of agricultural activity have patterned bars (SAL: Salinas,  $n_{\text{males}} = 14$ ,  $n_{\text{females}} = 11$ ; SUT: Sutter,  $n_{\text{males}} = 10$ ,  $n_{\text{females}} = 10$ ; and SJ: San Joaquin,  $n_{\text{males}} = 11$ ,  $n_{\text{females}} = 2$ ). Three-way ANOVA was used to examine whether animals differed in T levels between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity), sex (male or female) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).



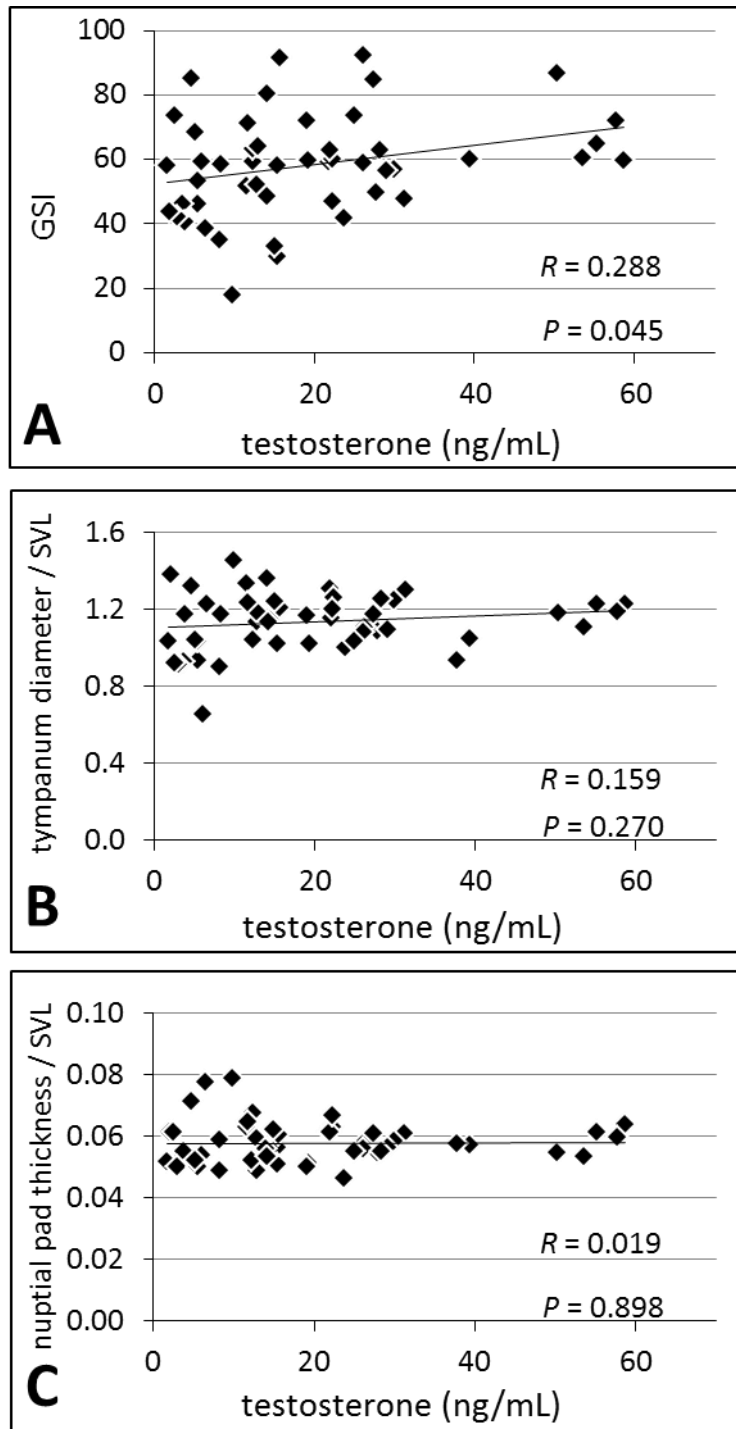


**Figure 24.** Testosterone (T) levels for male American bullfrogs (*Lithobates catesbeianus*) collected along the Salinas River (orange), Sacramento River (blue) and San Joaquin River (pink). Males collected upstream of agriculture are denoted with open bars (STM: Santa Margarita, n = 12; BC: Battle Creek, n = 6; and DPC: Del Puerto Canyon, n = 10) and males collected downstream of agricultural activity have patterned bars (SAL: Salinas, n = 14; SUT: Sutter, n = 10; and SJ: San Joaquin, n = 11). Two-way ANOVA was used to examine whether males differed in T levels between rivers (Salinas, Sacramento and San Joaquin Rivers), regions (up or downstream of agricultural activity) and whether there was an interactive effect. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ).

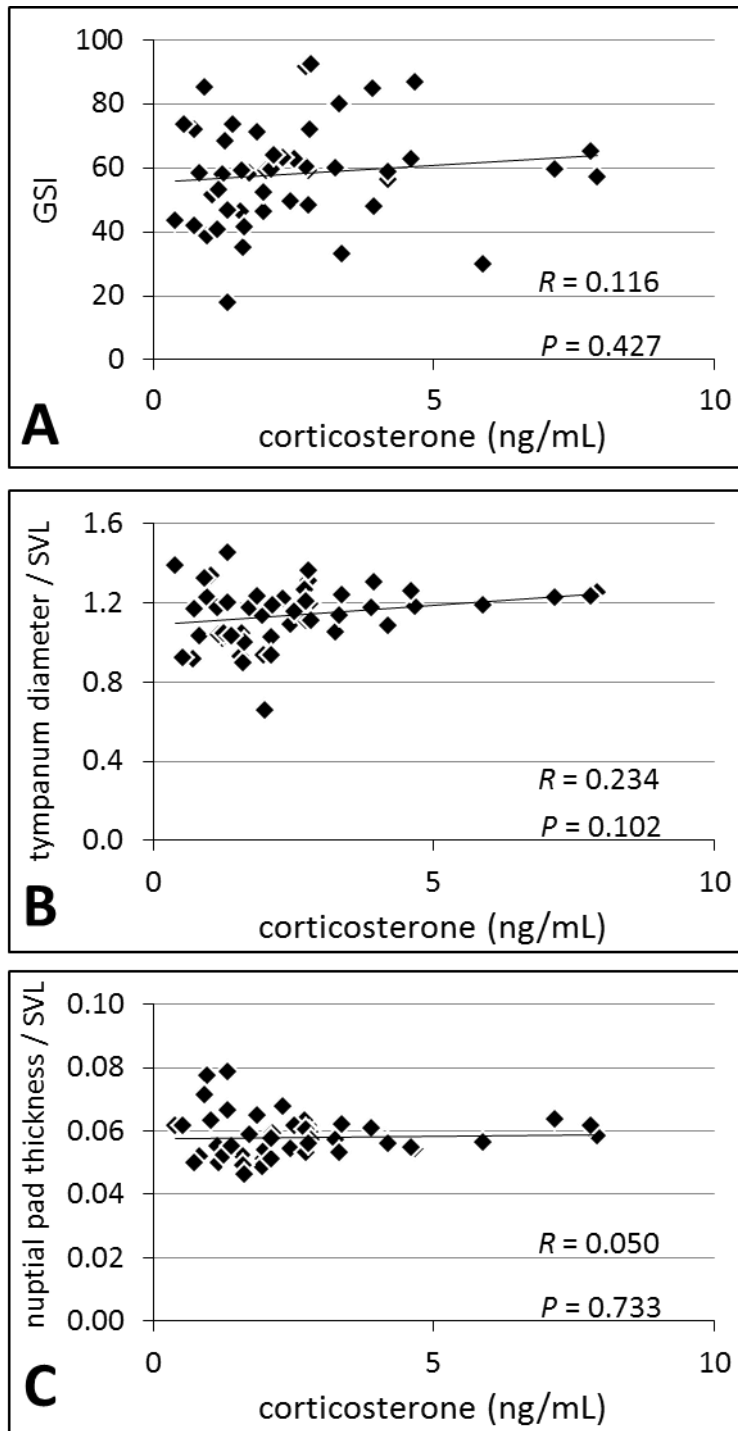
<i>all males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.920</b> ( <b>&lt; 0.001</b> )								
GW	<b>0.702</b> ( <b>&lt; 0.001</b> )	<b>0.759</b> ( <b>&lt; 0.001</b> )							
tympanum	<b>0.823</b> ( <b>&lt; 0.001</b> )	<b>0.841</b> ( <b>&lt; 0.001</b> )	<b>0.684</b> ( <b>&lt; 0.001</b> )						
nuptial pad	<b>0.823</b> ( <b>&lt; 0.001</b> )	<b>0.911</b> ( <b>&lt; 0.001</b> )	<b>0.719</b> ( <b>&lt; 0.001</b> )	<b>0.893</b> ( <b>&lt; 0.001</b> )					
hue-patch	<b>-0.275</b> ( <b>0.054</b> )	<b>-0.245</b> ( <b>0.086</b> )	<b>-0.167</b> ( <b>0.194</b> )	<b>-0.352</b> ( <b>0.005</b> )	<b>-0.282</b> ( <b>0.027</b> )				
YI	<b>0.299</b> ( <b>0.035</b> )	<b>0.366</b> ( <b>0.009</b> )	<b>0.473</b> ( <b>&lt; 0.001</b> )	<b>0.560</b> ( <b>&lt; 0.001</b> )	<b>0.492</b> ( <b>&lt; 0.001</b> )	<b>-0.391</b> ( <b>0.002</b> )			
sperm count	0.015 (0.935)	<b>-0.108</b> ( <b>0.549</b> )	<b>-0.037</b> ( <b>0.825</b> )	<b>-0.213</b> ( <b>0.188</b> )	<b>-0.172</b> ( <b>0.294</b> )	0.043 (0.791)	<b>-0.129</b> ( <b>0.429</b> )		
CORT	<b>-0.154</b> ( <b>0.287</b> )	<b>-0.158</b> ( <b>0.274</b> )	<b>-0.013</b> ( <b>0.918</b> )	<b>0.047</b> ( <b>0.713</b> )	<b>-0.048</b> ( <b>0.710</b> )	<b>-0.340</b> ( <b>0.006</b> )	<b>0.287</b> ( <b>0.023</b> )	<b>-0.031</b> ( <b>0.850</b> )	
T	<b>-0.329</b> ( <b>0.020</b> )	<b>-0.281</b> ( <b>0.048</b> )	<b>0.018</b> ( <b>0.890</b> )	<b>-0.055</b> ( <b>0.670</b> )	<b>-0.099</b> ( <b>0.445</b> )	0.011 (0.929)	<b>0.321</b> ( <b>0.010</b> )	<b>-0.129</b> ( <b>0.426</b> )	<b>0.621</b> ( <b>&lt; 0.001</b> )

p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 8.** Pearson correlation coefficients of general measurements and steroid levels for all males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p < 0.050$ ) in bold.



**Figure 25.** Correlation analysis of testosterone levels and normalized measurements of all males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

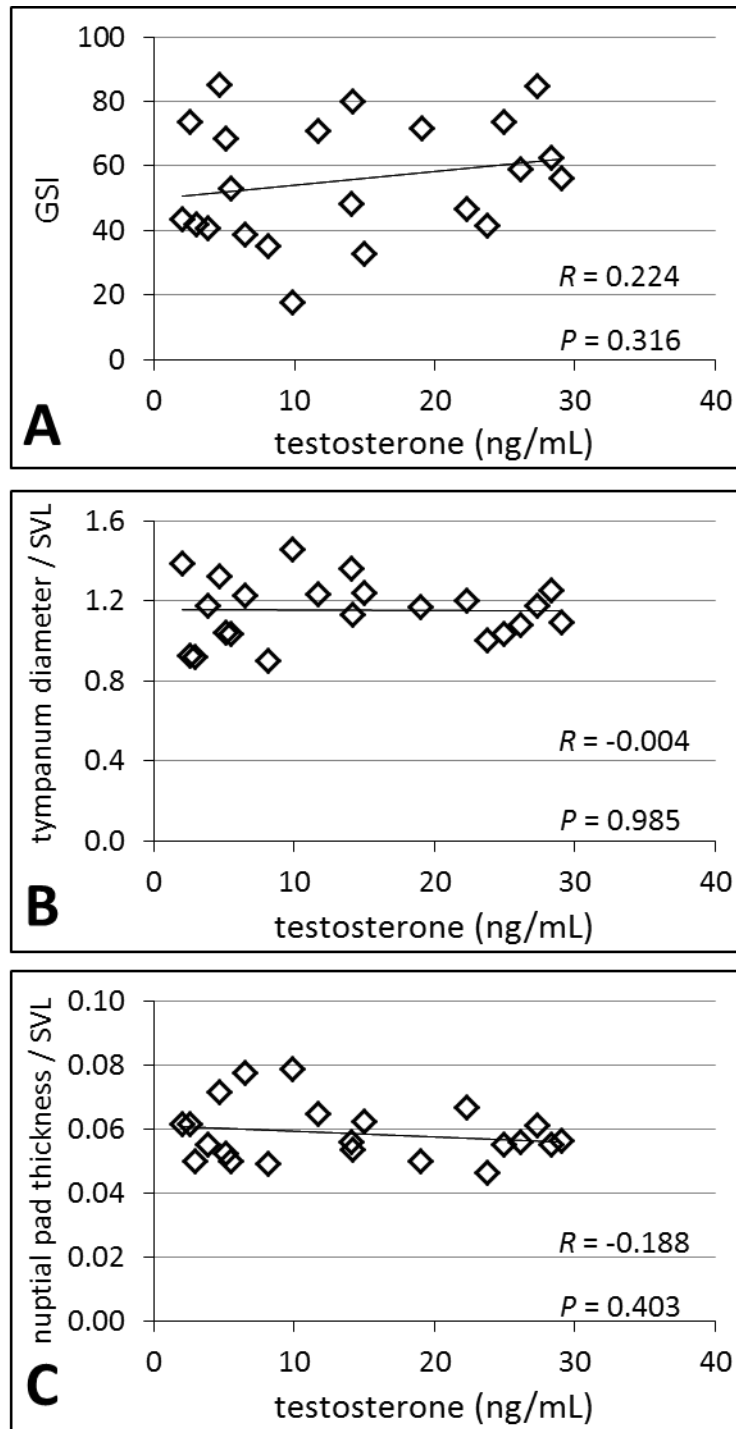


**Figure 26.** Correlation analysis of corticosterone levels and normalized measurements of all males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

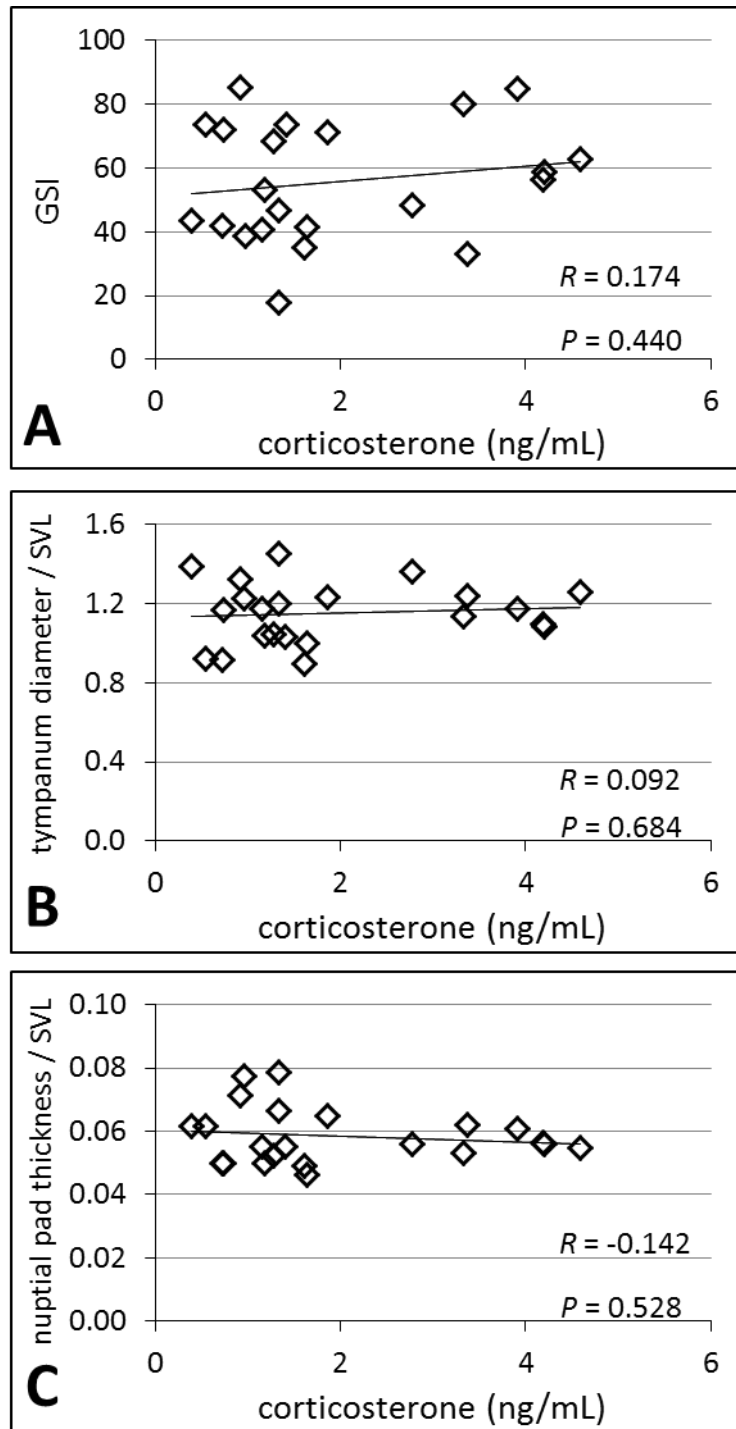
<i>upstream males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.923</b> ( <b>&lt; 0.001</b> )								
GW	<b>0.621</b> ( <b>0.002</b> )	<b>0.666</b> ( <b>0.001</b> )							
tympanum	<b>0.814</b> ( <b>&lt; 0.001</b> )	<b>0.846</b> ( <b>&lt; 0.001</b> )	<b>0.618</b> ( <b>&lt; 0.001</b> )						
nuptial pad	<b>0.846</b> ( <b>&lt; 0.001</b> )	<b>0.907</b> ( <b>&lt; 0.001</b> )	<b>0.630</b> ( <b>&lt; 0.001</b> )	<b>0.912</b> ( <b>&lt; 0.001</b> )					
hue-patch	-0.242 (0.277)	-0.207 (0.356)	-0.239 (0.222)	-0.335 (0.081)	-0.273 (0.168)				
YI	0.335 (0.127)	<b>0.423</b> ( <b>0.050</b> )	<b>0.506</b> ( <b>0.006</b> )	<b>0.620</b> ( <b>&lt; 0.001</b> )	<b>0.502</b> ( <b>0.008</b> )	<b>- 0.384</b> ( <b>0.044</b> )			
sperm count	-0.198 (0.497)	-0.429 (0.125)	-0.115 (0.649)	-0.121 (0.633)	-0.316 (0.217)	-0.027 (0.916)	0.142 (0.575)		
CORT	-0.005 (0.982)	-0.027 (0.903)	0.197 (0.315)	0.090 (0.648)	-0.031 (0.879)	-0.326 (0.090)	0.242 (0.214)	0.014 (0.958)	
T	-0.002 (0.992)	-0.021 (0.925)	0.213 (0.276)	0.060 (0.764)	-0.071 (0.724)	-0.120 (0.543)	<b>0.380</b> ( <b>0.046</b> )	0.005 (0.985)	<b>0.735</b> ( <b>&lt; 0.001</b> )

p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 9.** Pearson correlation coefficients of general measurements and steroid levels for upstream males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p \leq 0.050$ ) in bold.



**Figure 27.** Correlation analysis of testosterone levels and normalized measurements of upstream males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).



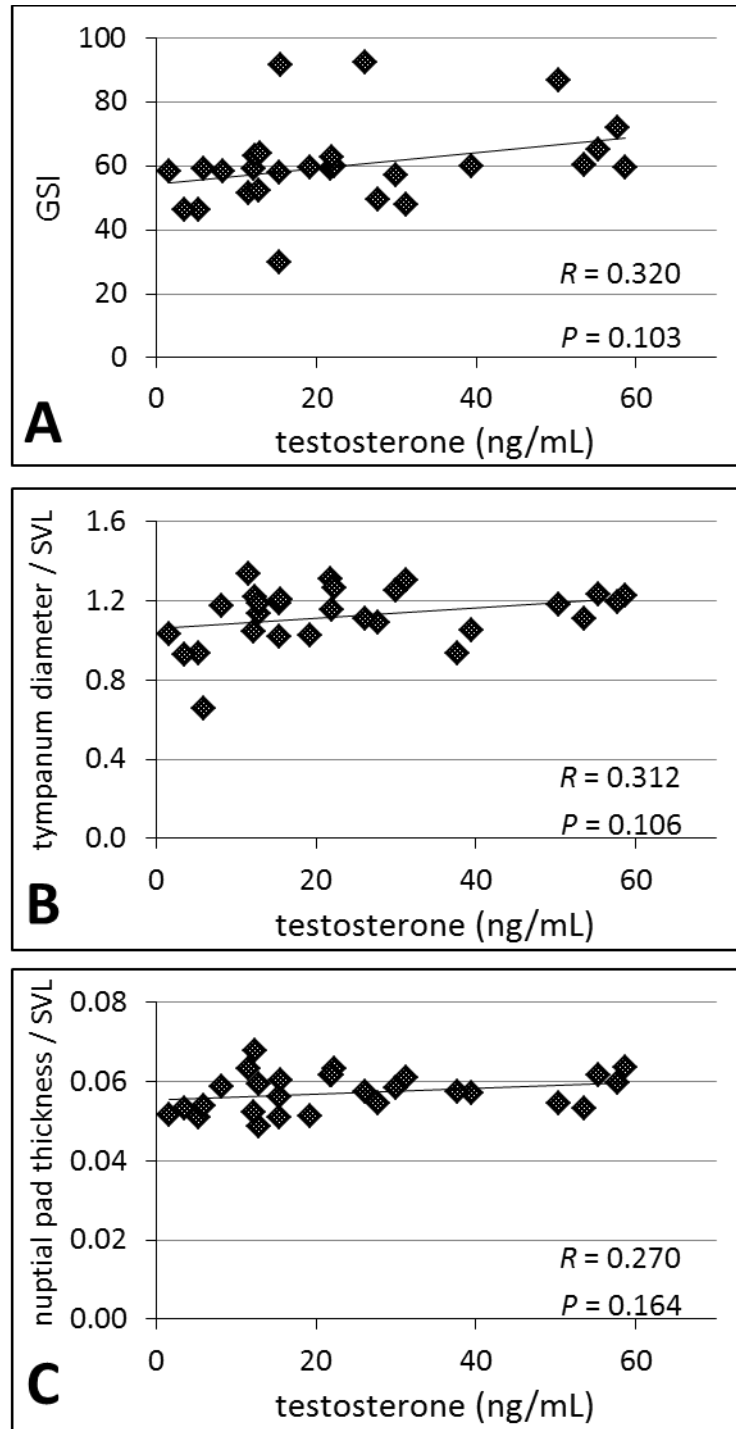
**Figure 28.** Correlation analysis of corticosterone levels and normalized measurements of upstream males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

<i>downstream males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.956</b> ( <b>&lt; 0.001</b> )								
GW	<b>0.817</b> ( <b>&lt; 0.001</b> )	<b>0.882</b> ( <b>&lt; 0.001</b> )							
tympanum	<b>0.821</b> ( <b>&lt; 0.001</b> )	<b>0.832</b> ( <b>&lt; 0.001</b> )	<b>0.765</b> ( <b>&lt; 0.001</b> )						
nuptial pad	<b>0.874</b> ( <b>&lt; 0.001</b> )	<b>0.913</b> ( <b>&lt; 0.001</b> )	<b>0.842</b> ( <b>&lt; 0.001</b> )	<b>0.873</b> ( <b>&lt; 0.001</b> )					
hue-patch	<b>-0.415</b> ( <b>0.028</b> )	<b>-0.477</b> ( <b>0.010</b> )	<b>-0.175</b> ( <b>0.324</b> )	<b>-0.437</b> ( <b>0.009</b> )	<b>-0.419</b> ( <b>0.012</b> )				
YI	<b>0.300</b> ( <b>0.121</b> )	<b>0.348</b> ( <b>0.070</b> )	<b>0.475</b> ( <b>0.005</b> )	<b>0.535</b> ( <b>0.001</b> )	<b>0.527</b> ( <b>0.001</b> )	<b>-0.410</b> ( <b>0.014</b> )			
sperm count	<b>0.167</b> ( <b>0.495</b> )	<b>0.232</b> ( <b>0.339</b> )	<b>0.145</b> ( <b>0.532</b> )	<b>-0.210</b> ( <b>0.349</b> )	<b>0.040</b> ( <b>0.860</b> )	<b>0.078</b> ( <b>0.731</b> )	<b>-0.262</b> ( <b>0.239</b> )		
CORT	<b>-0.123</b> ( <b>0.533</b> )	<b>-0.116</b> ( <b>0.558</b> )	<b>-0.086</b> ( <b>0.630</b> )	<b>0.156</b> ( <b>0.372</b> )	<b>0.096</b> ( <b>0.582</b> )	<b>-0.335</b> ( <b>0.049</b> )	<b>0.347</b> ( <b>0.041</b> )	<b>-0.158</b> ( <b>0.483</b> )	
T	<b>-0.367</b> ( <b>0.055</b> )	<b>-0.327</b> ( <b>0.089</b> )	<b>0.048</b> ( <b>0.789</b> )	<b>0.039</b> ( <b>0.825</b> )	<b>0.056</b> ( <b>0.751</b> )	<b>0.093</b> ( <b>0.596</b> )	<b>0.364</b> ( <b>0.031</b> )	<b>-0.301</b> ( <b>0.173</b> )	<b>0.546</b> ( <b>0.001</b> )

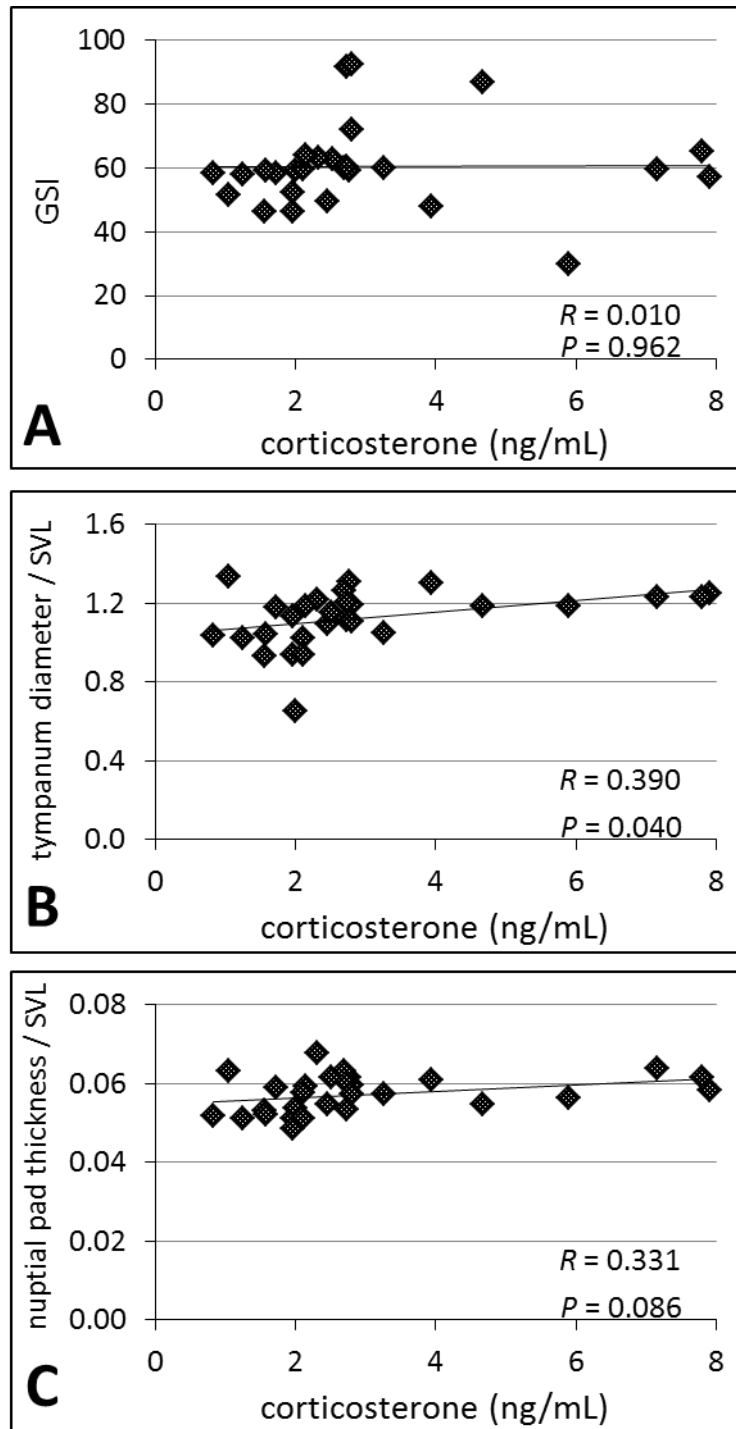
p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 10.** Pearson correlation coefficients of general measurements and steroid levels for downstream males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p \leq 0.050$ ) in bold.





**Figure 29.** Correlation analysis of testosterone levels and normalized measurements of downstream males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

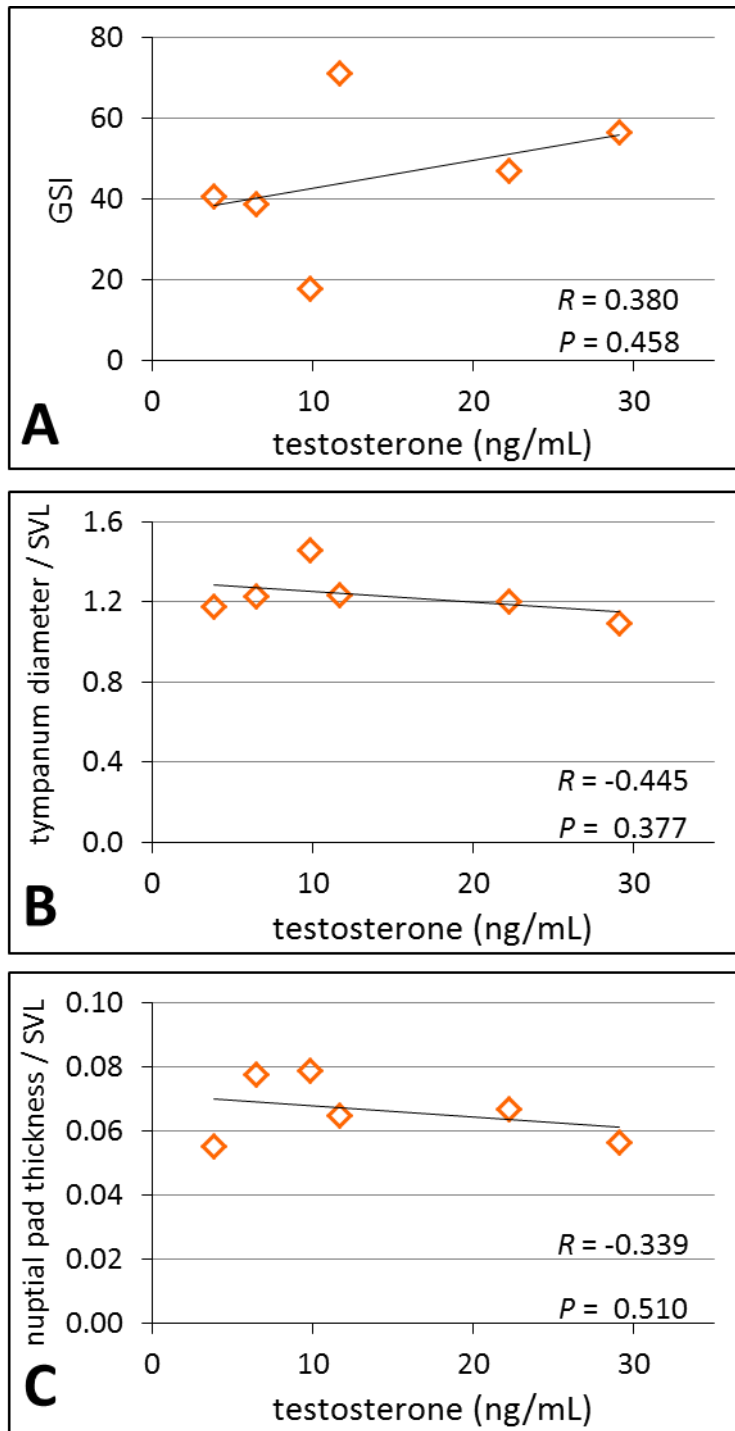


**Figure 30.** Correlation analysis of corticosterone levels and normalized measurements of downstream males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

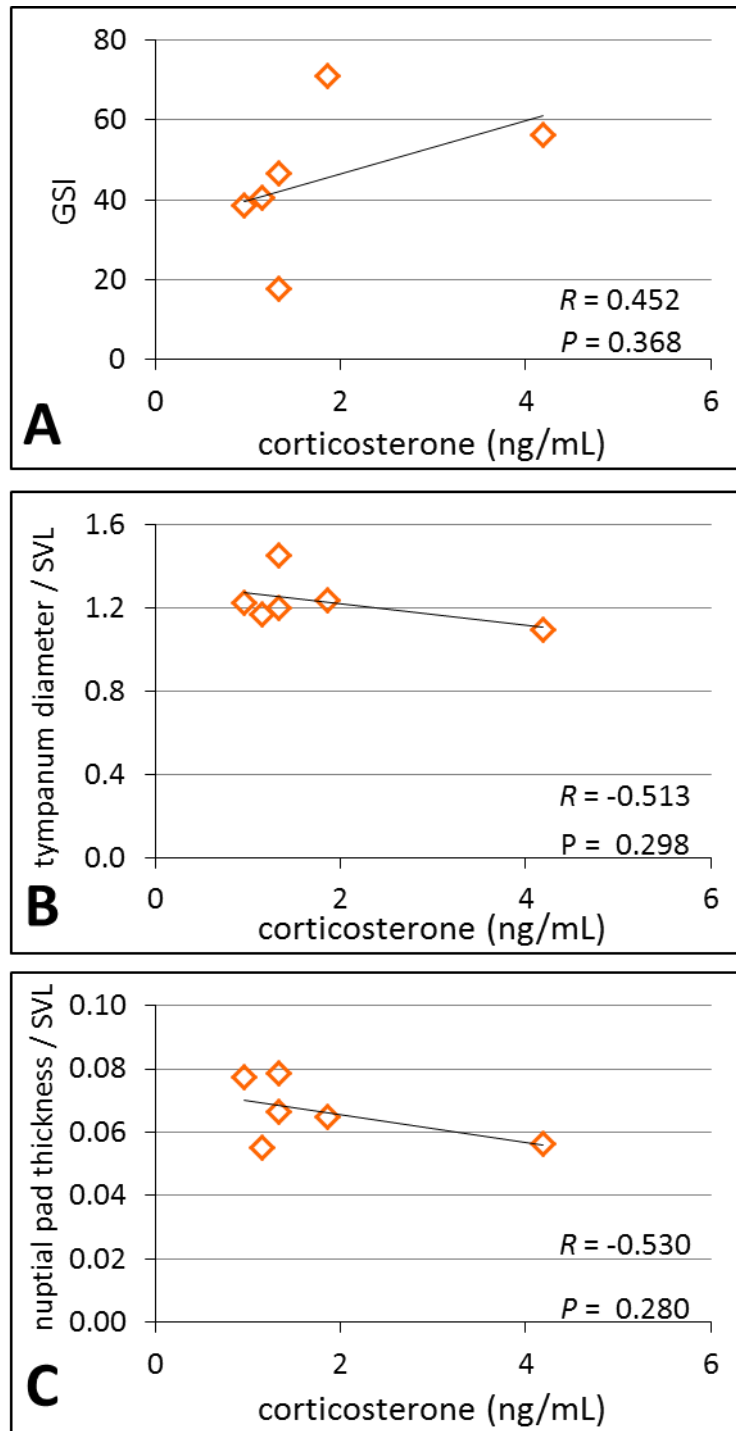
<i>STM males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.853</b> <b>(0.031)</b>								
GW	0.532 (0.277)	0.574 (0.234)							
tympanum	0.735 (0.096)	<b>0.799</b> <b>(0.057)</b>	<b>0.617</b> <b>(0.033)</b>						
nuptial pad	0.600 (0.208)	0.715 (0.110)	0.497 (0.120)	<b>0.952</b> <b>(&lt;0.001)</b>					
hue-patch	0.342 (0.507)	0.288 (0.579)	-0.091 (0.778)	0.066 (0.838)	-0.112 (0.743)				
YI	<b>0.848</b> <b>(0.033)</b>	0.740 (0.092)	<b>0.580</b> <b>(0.048)</b>	<b>0.783</b> <b>(0.003)</b>	<b>0.660</b> <b>(0.027)</b>	-0.164 (0.610)			
sperm count	-0.254 (0.680)	-0.725 (0.166)	-0.178 (0.647)	0.184 (0.636)	-0.150 (0.722)	0.080 (0.838)	0.296 (0.439)		
CORT	0.301 (0.562)	-0.194 (0.713)	0.394 (0.205)	0.087 (0.788)	0.006 (0.987)	-0.113 (0.727)	0.137 (0.671)	0.188 (0.629)	
T	0.135 (0.798)	-0.183 (0.729)	0.468 (0.125)	0.290 (0.360)	0.160 (0.639)	-0.239 (0.454)	0.405 (0.192)	0.048 (0.901)	<b>0.770</b> <b>(0.003)</b>

p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 11.** Pearson correlation coefficients of general measurements and steroid levels for Santa Margarita (STM) males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p \leq 0.050$ ) in bold.



**Figure 31.** Correlation analysis of testosterone levels and normalized measurements of Santa Margarita (STM) males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

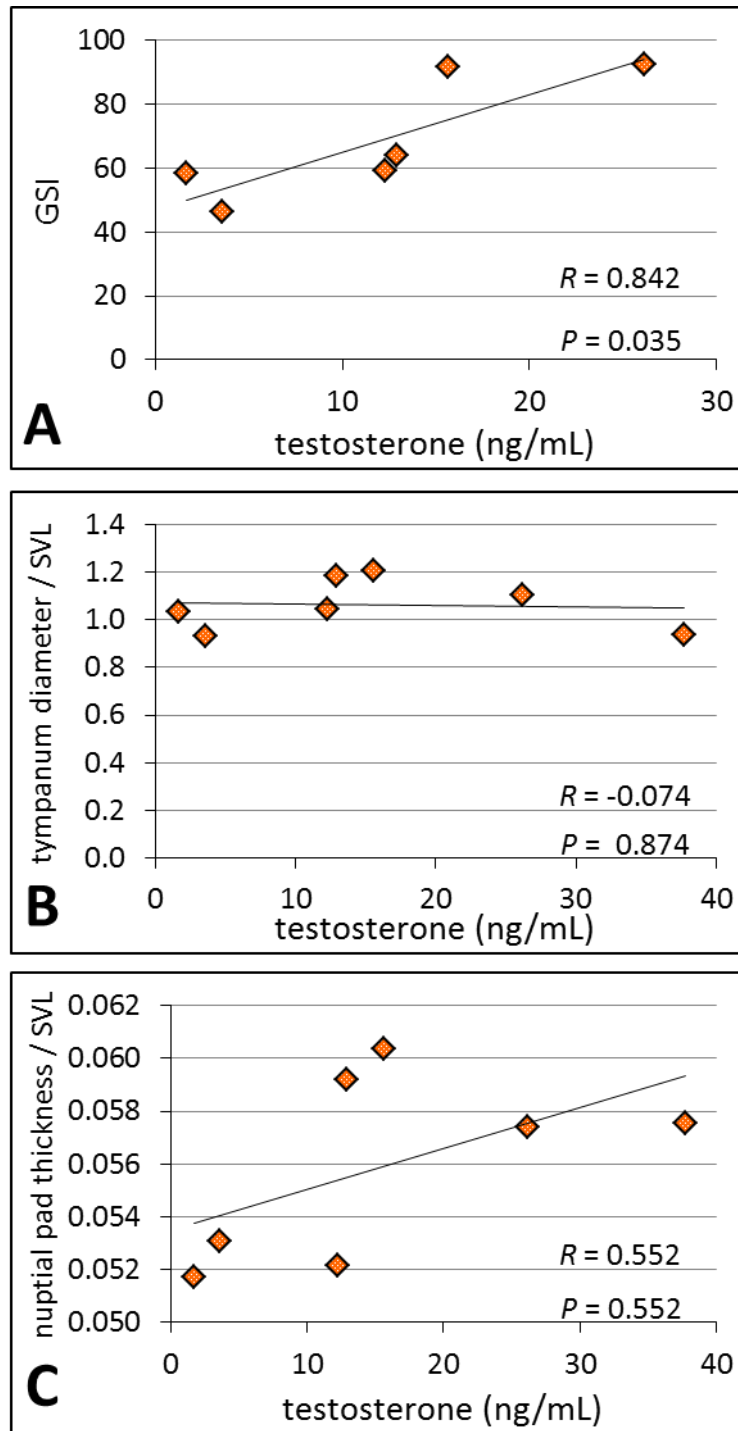


**Figure 32.** Correlation analysis of corticosterone levels and normalized measurements of Santa Margarita (STM) males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

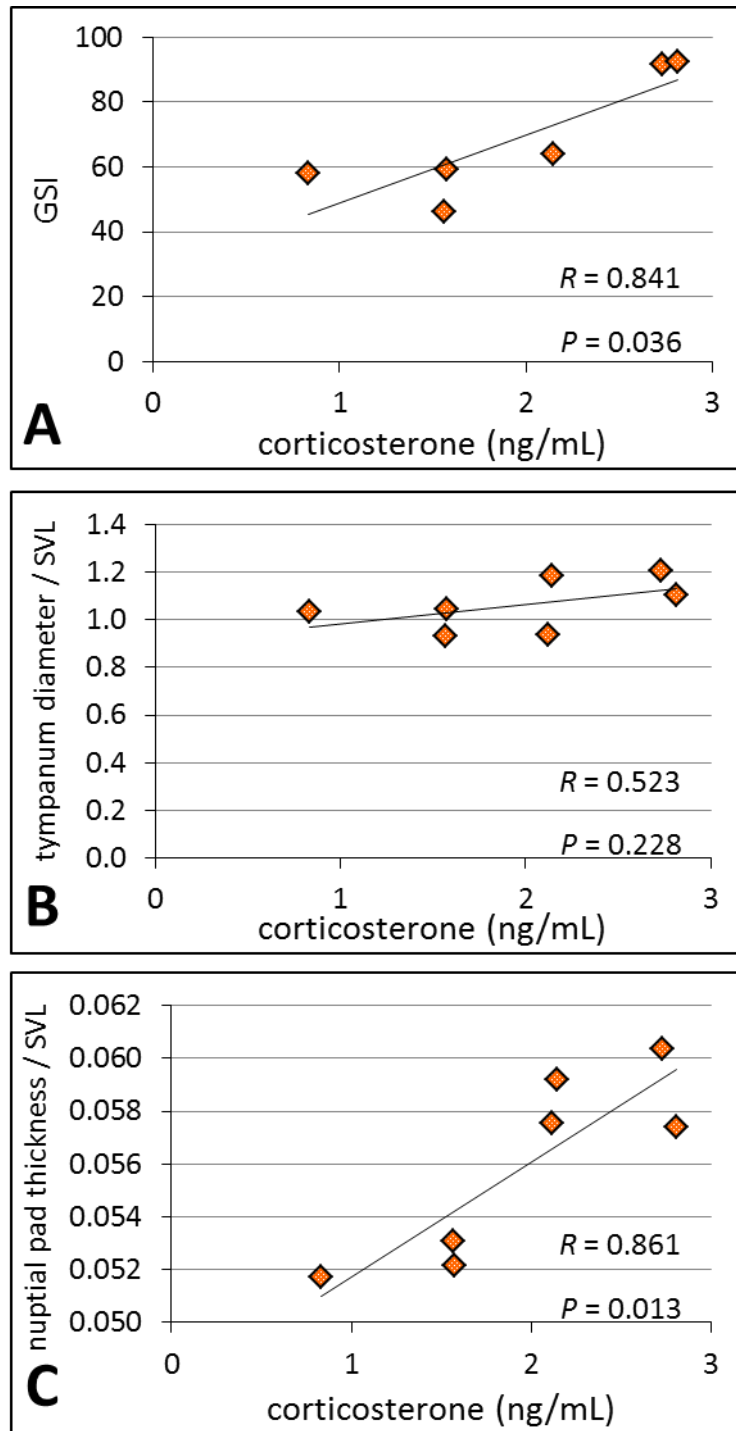
<i>SAL males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.988</b> ( <b>&lt;0.001</b> )								
GW	<b>0.831</b> ( <b>0.040</b> )	<b>0.838</b> ( <b>0.037</b> )							
tympanum	<b>0.817</b> ( <b>0.025</b> )	<b>0.776</b> ( <b>0.040</b> )	<b>0.912</b> ( <b>&lt;0.001</b> )						
nuptial pad	<b>0.956</b> ( <b>0.001</b> )	<b>0.959</b> ( <b>0.001</b> )	<b>0.879</b> ( <b>&lt;0.001</b> )	<b>0.855</b> ( <b>&lt;0.001</b> )					
hue-patch	-0.254 (0.583)	-0.223 (0.630)	0.520 (0.069)	0.307 (0.286)	0.294 (0.308)				
YI	<b>0.564</b> ( <b>0.188</b> )	<b>0.559</b> ( <b>0.192</b> )	<b>0.816</b> ( <b>0.001</b> )	<b>0.874</b> ( <b>&lt;0.001</b> )	<b>0.758</b> ( <b>0.002</b> )	<b>0.462</b> ( <b>0.096</b> )			
sperm count	-0.219 (0.636)	-0.156 (0.738)	-0.142 (0.716)	-0.341 (0.334)	-0.198 (0.584)	<b>0.436</b> ( <b>0.207</b> )	-0.266 (0.457)		
CORT	<b>0.261</b> ( <b>0.572</b> )	<b>0.292</b> ( <b>0.526</b> )	<b>0.652</b> ( <b>0.016</b> )	<b>0.517</b> ( <b>0.058</b> )	<b>0.562</b> ( <b>0.036</b> )	<b>0.636</b> ( <b>0.014</b> )	<b>0.474</b> ( <b>0.087</b> )	<b>0.369</b> ( <b>0.293</b> )	
T	<b>0.449</b> ( <b>0.312</b> )	<b>0.504</b> ( <b>0.249</b> )	<b>0.744</b> ( <b>0.004</b> )	<b>0.512</b> ( <b>0.061</b> )	<b>0.745</b> ( <b>0.002</b> )	<b>0.370</b> ( <b>0.192</b> )	<b>0.494</b> ( <b>0.073</b> )	<b>0.403</b> ( <b>0.249</b> )	<b>0.511</b> ( <b>0.062</b> )

p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 12.** Pearson correlation coefficients of general measurements and steroid levels for Salinas (SAL) males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p \leq 0.050$ ) in bold.



**Figure 33.** Correlation analysis of testosterone levels and normalized measurements of Salinas (SAL) males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).



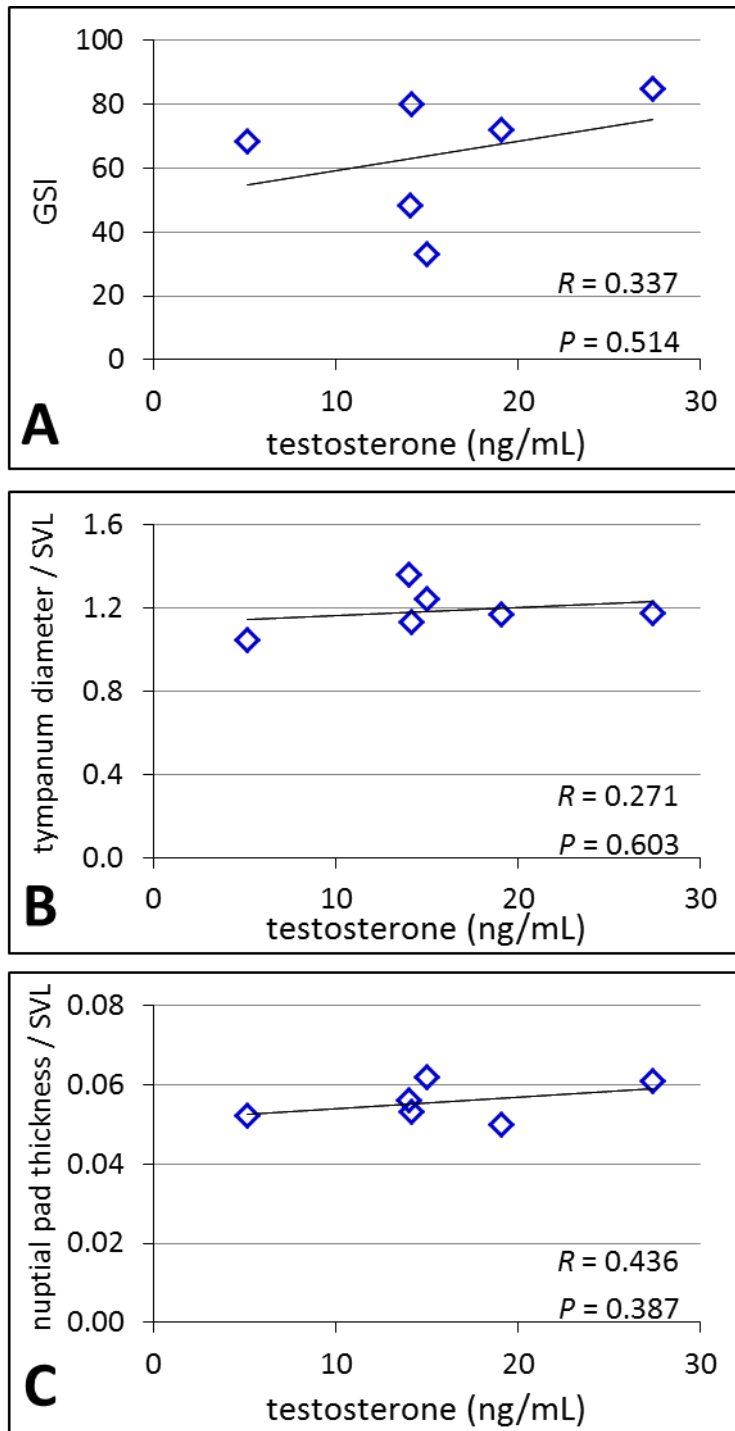
**Figure 34.** Correlation analysis of corticosterone levels and normalized measurements of Salinas (SAL) males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).



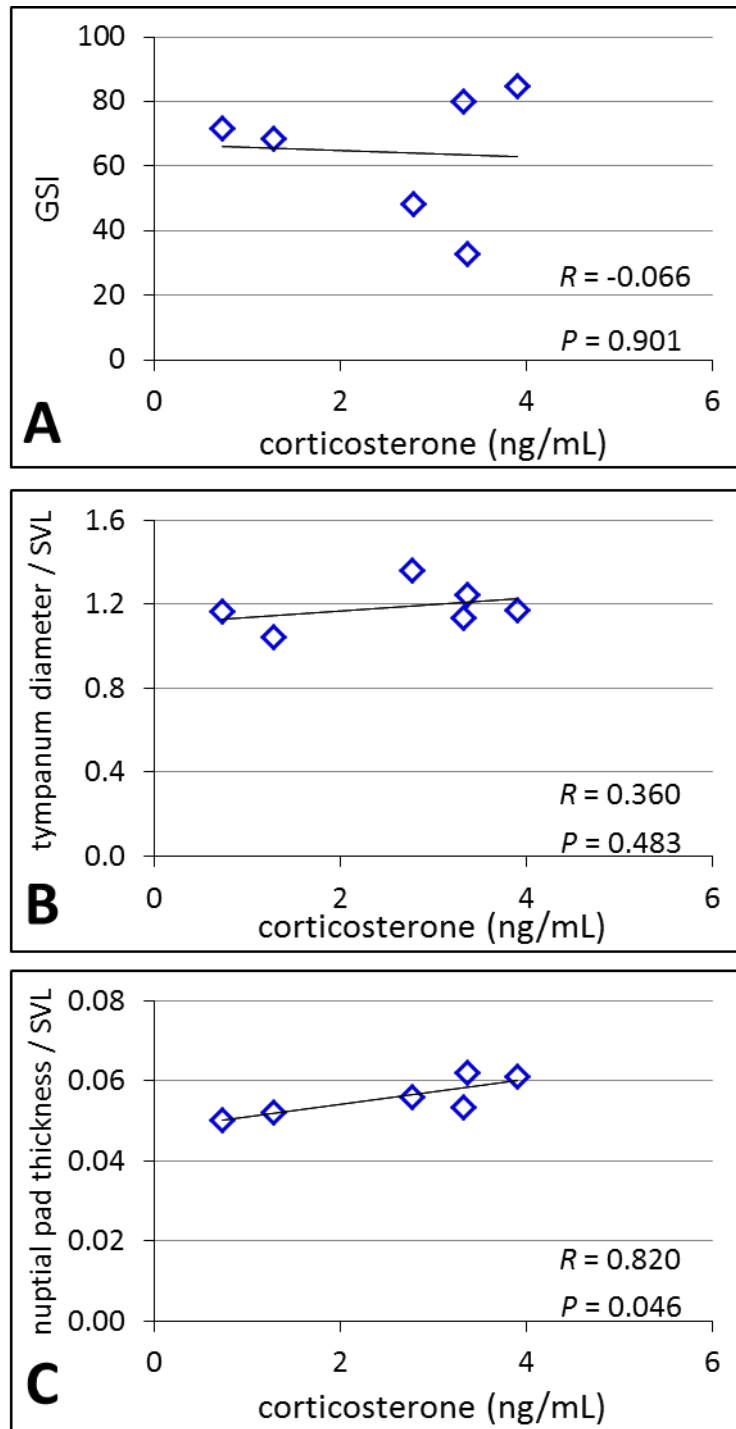
<i>BC males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.959</b> <b>(0.002)</b>								
GW	0.482 (0.333)	0.473 (0.344)							
tympanum	<b>0.839</b> <b>(0.037)</b>	<b>0.875</b> <b>(0.022)</b>	0.186 (0.725)						
nuptial pad	<b>0.853</b> <b>(0.031)</b>	<b>0.930</b> <b>(0.007)</b>	0.245 (0.640)	<b>0.836</b> <b>(0.038)</b>					
hue-patch	-0.770 (0.073)	-0.730 (0.099)	-0.450 (0.370)	-0.866 <b>(0.026)</b>	-0.617 (0.192)				
YI	0.510 (0.310)	0.553 (0.255)	-0.289 (0.578)	<b>0.820</b> <b>(0.046)</b>	0.710 (0.114)	-0.642 (0.169)			
sperm count	<b>0.975</b> <b>(0.025)</b>	0.867 (0.133)	0.780 (0.220)	0.888 (0.122)	0.683 (0.317)	-0.927 (0.073)	0.578 (0.422)		
CORT	0.447 (0.374)	0.598 (0.209)	0.387 (0.448)	0.526 (0.283)	0.738 (0.094)	-0.504 (0.308)	0.525 (0.285)	0.141 (0.859)	
T	-0.147 (0.781)	0.110 (0.836)	0.321 (0.535)	0.025 (0.963)	0.098 (0.854)	0.071 (0.894)	-0.145 (0.784)	-0.292 (0.708)	0.454 (0.365)

p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 13.** Pearson correlation coefficients of general measurements and steroid levels for Battle Creek (BC) males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p \leq 0.050$ ) in bold.



**Figure 35.** Correlation analysis of testosterone levels and normalized measurements of Battle Creek (BC) males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

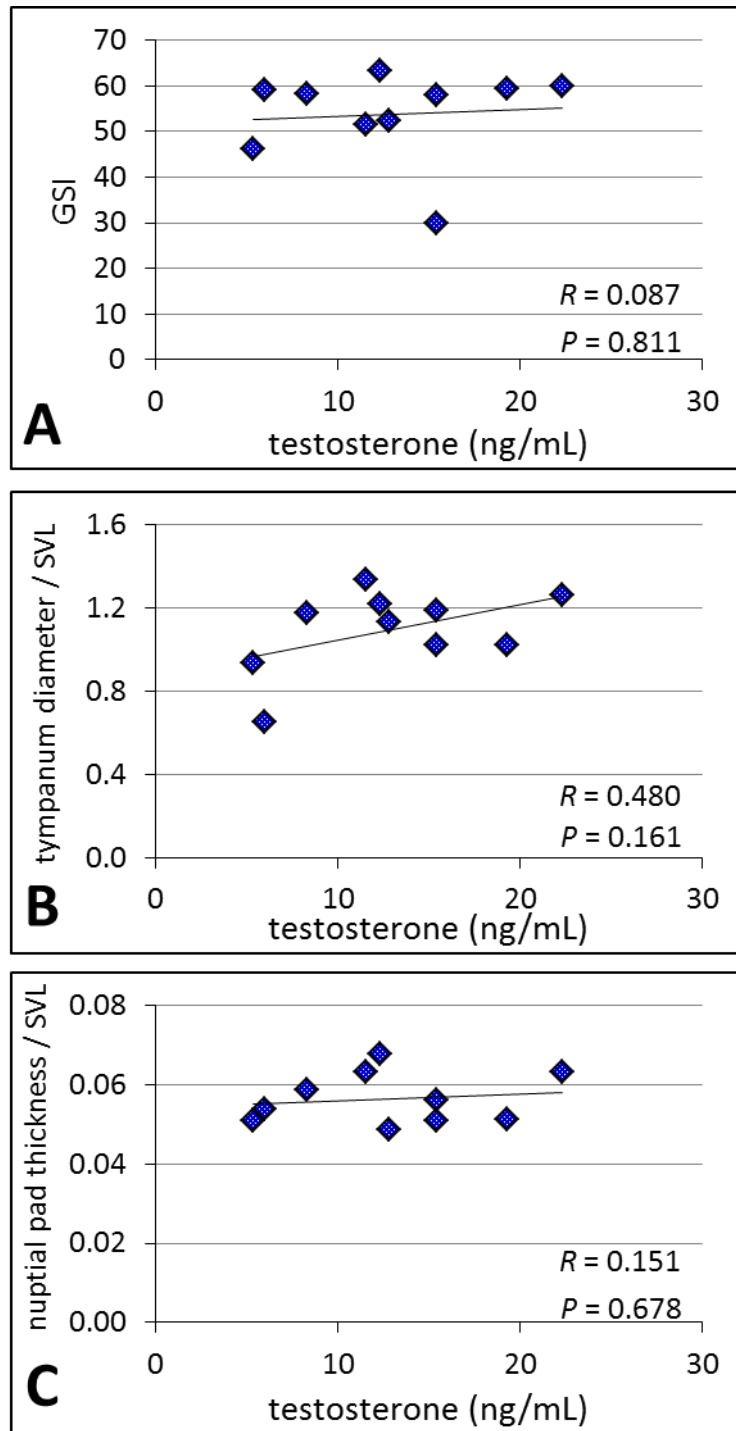


**Figure 36.** Correlation analysis of corticosterone levels and normalized measurements of Battle Creek (BC) males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

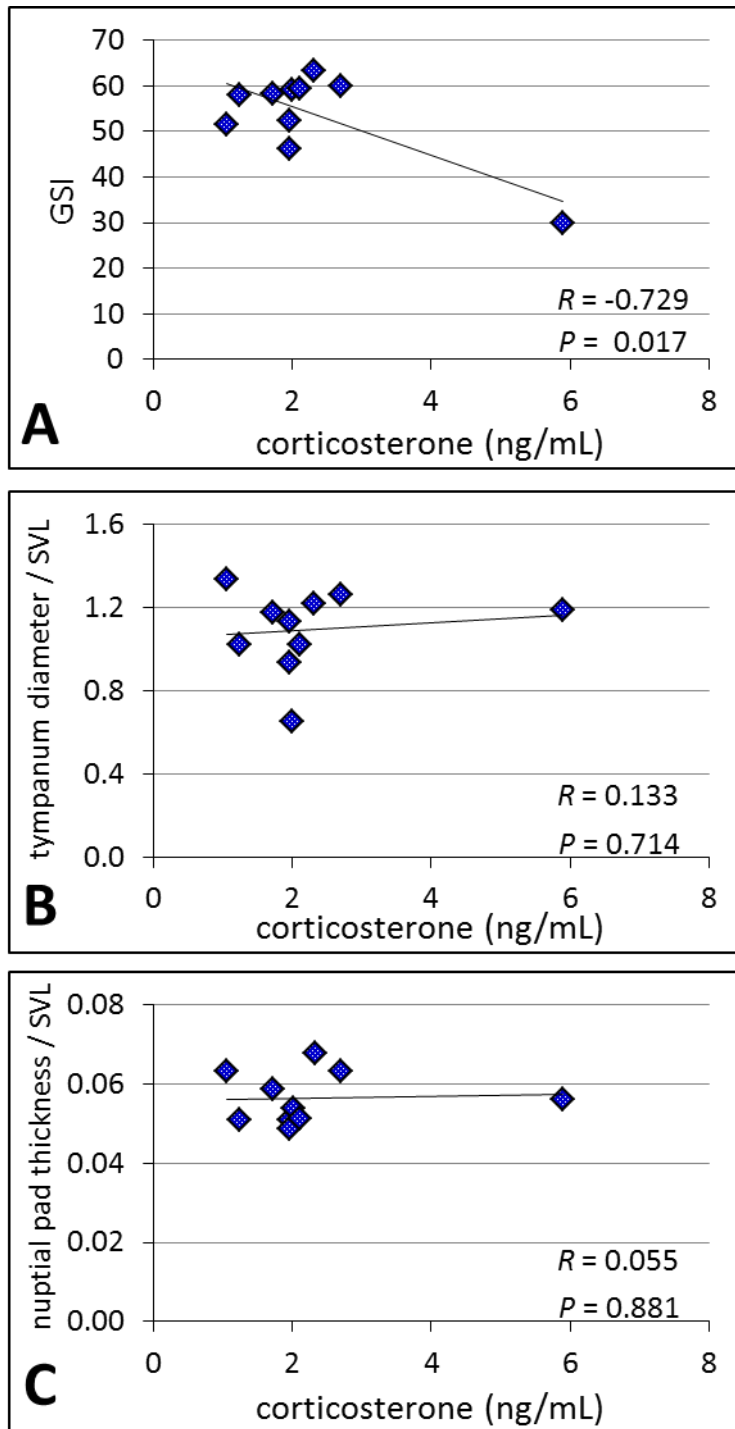
<i>SUT males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.960</b> ( <b>&lt; 0.001</b> )								
GW	<b>0.802</b> ( <b>0.005</b> )	<b>0.899</b> ( <b>&lt; 0.001</b> )							
tympanum	<b>0.855</b> ( <b>0.002</b> )	<b>0.891</b> ( <b>0.001</b> )	<b>0.746</b> ( <b>0.013</b> )						
nuptial pad	<b>0.820</b> ( <b>0.004</b> )	<b>0.919</b> ( <b>&lt; 0.001</b> )	<b>0.855</b> ( <b>0.002</b> )	<b>0.876</b> ( <b>0.001</b> )					
hue-patch	-0.499 (0.142)	-0.656 (0.039)	-0.758 (0.011)	-0.713 (0.021)	-0.856 (0.002)				
YI	0.434 (0.210)	0.502 (0.139)	0.412 (0.237)	<b>0.737</b> ( <b>0.015</b> )	0.588 (0.074)	-0.660 (0.038)			
sperm count	-0.212 (0.687)	-0.105 (0.843)	-0.007 (0.989)	-0.497 (0.316)	-0.078 (0.883)	0.238 (0.650)	-0.548 (0.261)		
CORT	0.267 (0.456)	0.146 (0.687)	-0.201 (0.577)	0.234 (0.515)	0.235 (0.514)	0.019 (0.958)	0.272 (0.446)	-0.218 (0.678)	
T	-0.033 (0.928)	-0.073 (0.840)	-0.090 (0.804)	-0.255 (0.477)	0.053 (0.884)	-0.173 (0.632)	0.451 (0.191)	-0.380 (0.458)	0.270 (0.451)

p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 14.** Pearson correlation coefficients of general measurements and steroid levels for Sutter (SUT) males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p \leq 0.050$ ) in bold.



**Figure 37.** Correlation analysis of testosterone levels and normalized measurements of Sutter (SUT) males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

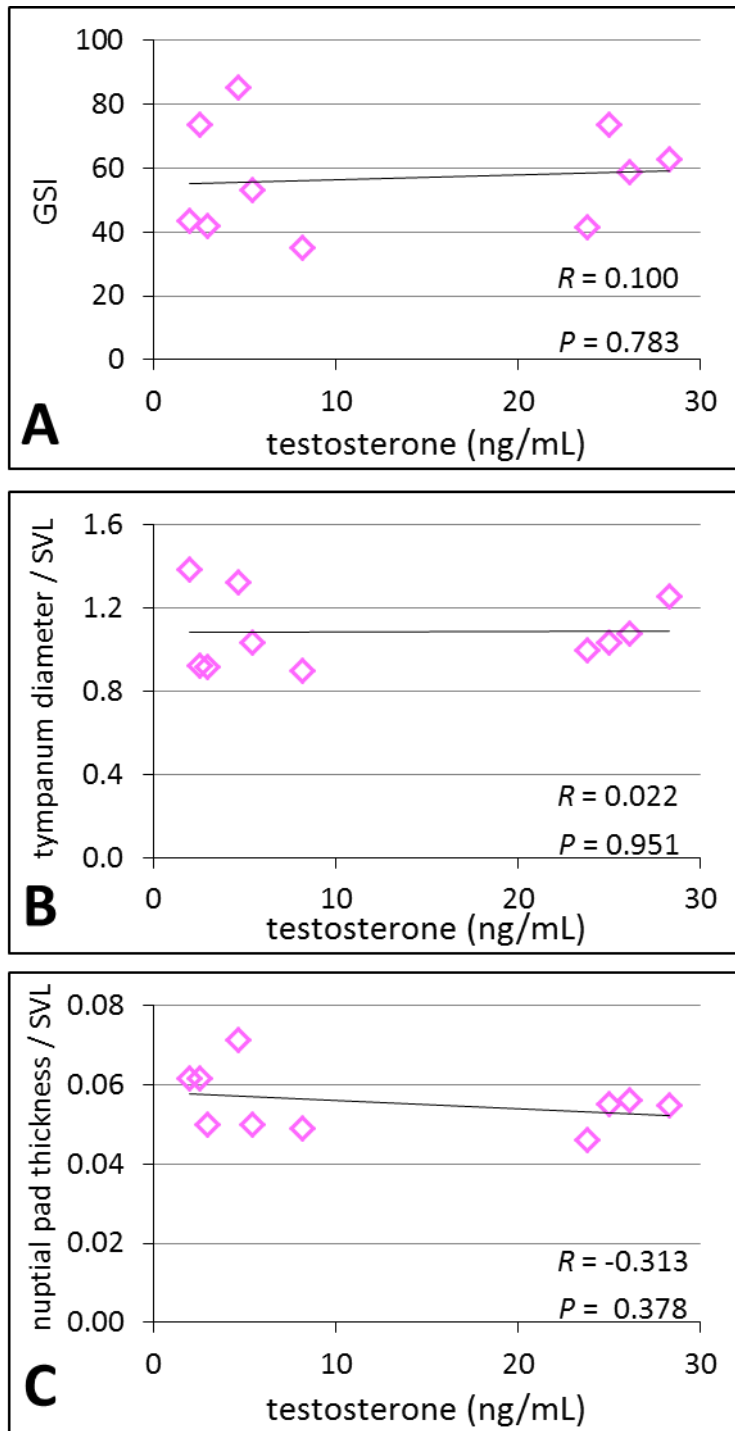


**Figure 38.** Correlation analysis of corticosterone levels and normalized measurements of Sutter (SUT) males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

<i>DPC males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.754</b> <b>(0.012)</b>								
GW	<b>0.686</b> <b>(0.028)</b>	<b>0.953</b> <b>(&lt; 0.001)</b>							
tympanum	0.572 (0.084)	<b>0.701</b> <b>(0.024)</b>	0.568 (0.087)						
nuptial pad	<b>0.646</b> <b>(0.044)</b>	<b>0.907</b> <b>(&lt; 0.001)</b>	<b>0.901</b> <b>(&lt; 0.001)</b>	<b>0.757</b> <b>(0.011)</b>					
hue-patch	0.080 (0.826)	- 0.273 (0.445)	- 0.295 (0.408)	- 0.326 (0.359)	- 0.328 (0.355)				
YI	0.361 (0.305)	<b>0.673</b> <b>(0.033)</b>	0.606 (0.063)	<b>0.634</b> <b>(0.049)</b>	0.605 (0.064)	- <b>0.669</b> <b>(0.034)</b>			
sperm count	0.052 (0.934)	- 0.314 (0.606)	- 0.444 (0.454)	- 0.387 (0.520)	- 0.223 (0.718)	0.534 (0.354)	- 0.348 (0.566)		
CORT	- 0.247 (0.491)	- 0.049 (0.893)	- 0.031 (0.932)	0.021 (0.954)	- 0.243 (0.499)	- 0.300 (0.400)	0.251 (0.484)	- 0.827 (0.084)	
T	0.066 (0.856)	0.010 (0.979)	0.016 (0.966)	0.021 (0.954)	- 0.237 (0.510)	- 0.199 (0.582)	0.409 (0.241)	- 0.449 (0.449)	<b>0.801</b> <b>(0.005)</b>

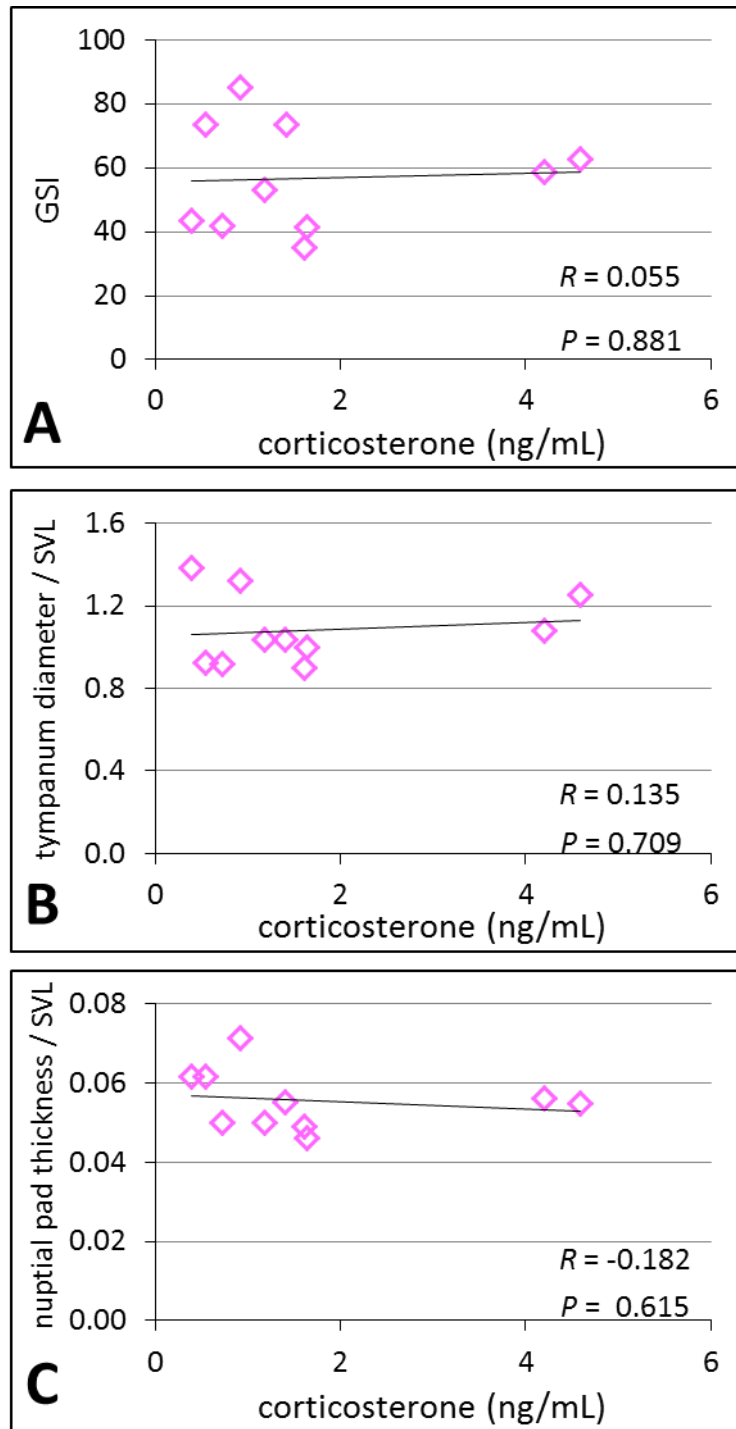
p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 15.** Pearson correlation coefficients of general measurements and steroid levels for Del Puerto Canyon (DPC) males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p \leq 0.050$ ) in bold.



**Figure 39.** Correlation analysis of testosterone levels and normalized measurements of Del Puerto Canyon (DPC) males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).



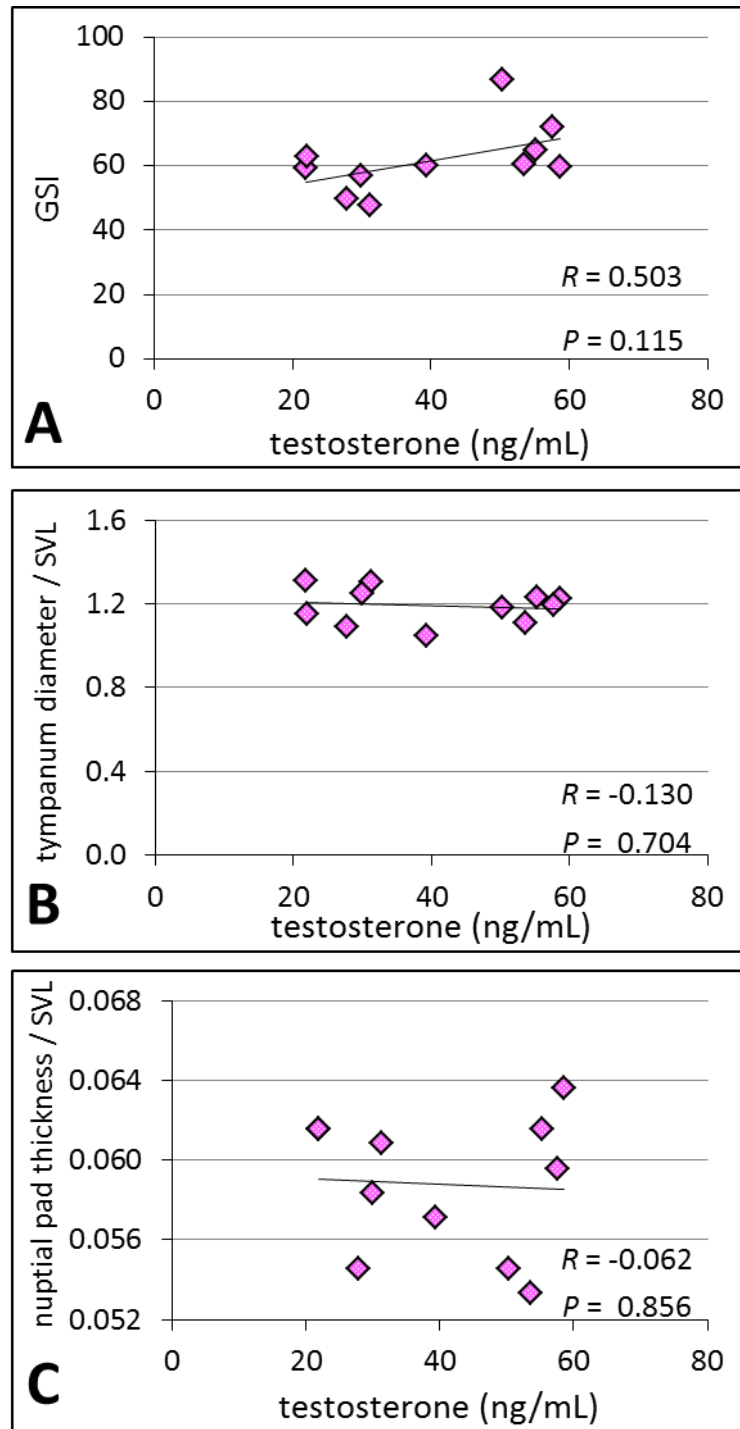


**Figure 40.** Correlation analysis of corticosterone levels and normalized measurements of Del Puerto Canyon (DPC) males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

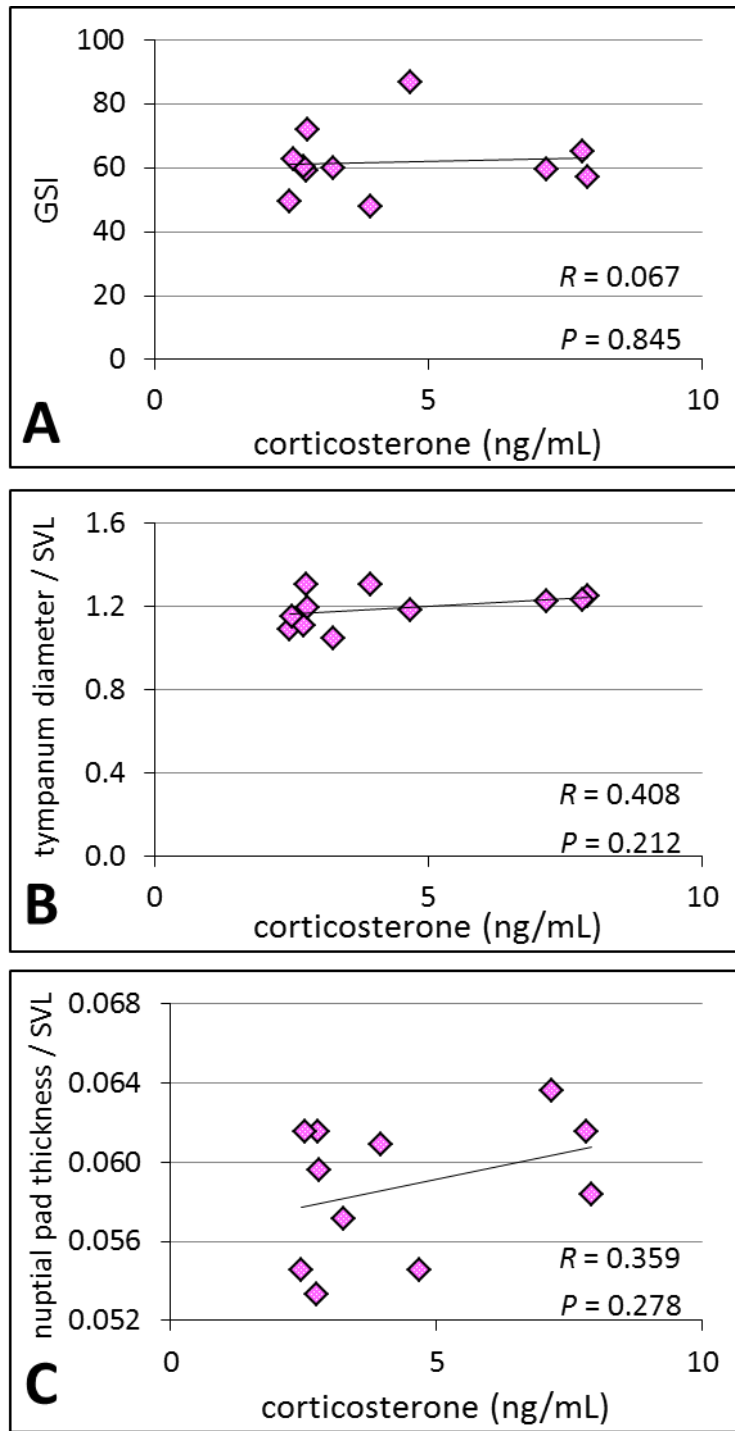
<i>SJ males</i>	SVL	BW	GW	tympanum	nuptial pad	hue-patch	YI	sperm count	CORT
BW	<b>0.749</b> ( <b>0.008</b> )								
GW	<b>0.621</b> ( <b>0.041</b> )	<b>0.822</b> ( <b>0.002</b> )							
tympanum	<b>0.890</b> ( <b>&lt; 0.001</b> )	<b>0.626</b> ( <b>0.039</b> )	0.477 (0.138)						
nuptial pad	<b>0.921</b> ( <b>&lt; 0.001</b> )	<b>0.798</b> ( <b>0.003</b> )	<b>0.611</b> ( <b>0.046</b> )	<b>0.901</b> ( <b>&lt; 0.001</b> )					
hue-patch	-0.468 (0.146)	-0.323 (0.332)	-0.070 (0.838)	-0.681 ( <b>0.021</b> )	-0.519 (0.102)				
YI	0.493 (0.123)	0.420 (0.198)	0.489 (0.127)	<b>0.738</b> ( <b>0.010</b> )	<b>0.632</b> ( <b>0.037</b> )	-0.396 (0.228)			
sperm count	-0.030 (0.956)	0.269 (0.606)	0.148 (0.779)	0.302 (0.560)	0.165 (0.754)	-0.577 (0.231)	0.295 (0.571)		
CORT	0.115 (0.737)	0.082 (0.812)	0.106 (0.757)	0.284 (0.398)	0.238 (0.482)	-0.573 (0.065)	0.404 (0.218)	0.505 (0.306)	
T	-0.335 (0.314)	-0.476 (0.139)	-0.110 (0.747)	-0.281 (0.403)	-0.256 (0.448)	0.415 (0.205)	0.166 (0.625)	-0.323 (0.532)	0.347 (0.296)

p-value for Pearson's Correlation Coefficient: significant at  $p \leq 0.05$

**Table 16.** Pearson correlation coefficients of general measurements and steroid levels for San Joaquin (SJ) males. Pearson correlation coefficient (top value) and corresponding p-value in parenthesis. Significant values ( $p \leq 0.050$ ) in bold.



**Figure 41.** Correlation analysis of testosterone levels and normalized measurements of San Joaquin (SJ) males. The relationship between testosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).



**Figure 42.** Correlation analysis of corticosterone levels and normalized measurements of San Joaquin (SJ) males. The relationship between corticosterone levels and (A) gonad weight normalized by body weight (GSI), tympanum diameter normalized by SVL (B), and nuptial pad thickness normalized by SVL (C). Significance was determined by ( $p \leq 0.05$ ).

## Chapter 3



The Effects of Agricultural Run-Off (Field Collected and Simulated) on  
the Reproductive Behavior of  
Male African-Clawed Frogs (*Xenopus Laevis*)

## ABSTRACT

Few studies have examined the effects of ecologically relevant pesticide exposures on the reproductive behaviors of male amphibians. To test whether agrochemical exposure had an effect on the clasping behaviors of male amphibians, adult male African-clawed frogs (*Xenopus laevis*) were exposed to field water collected downstream (agricultural run-off) and upstream (negative control) of agricultural activity along the Salinas River, CA. In addition, a pesticide mixture (positive control) containing the top agrochemicals used in the Monterey County, CA was included to simulate agricultural run-off (positive control). Mating behavior was suppressed in males exposed to simulated agricultural run-off but enhanced in males exposed to field collected agricultural run-off. In addition, testosterone levels of clasping males were elevated in comparison to non-clasping males. Moreover, males immersed in simulated agricultural run-off had significantly lower evening testosterone levels than control males in 2010. These data suggest that agrochemical exposure (both field collected and simulated) can alter reproductive hormones and clasping behaviors. Altered sex hormones and behaviors in male amphibians may play a role in amphibian declines.

## INTRODUCTION

In the previous chapter, I found that amphibians exposed to agricultural run-off were smaller in size and had elevated corticosterone levels. Although the endocrine and morphological data from the previous chapter suggests that agrochemical exposure can have negative effects on amphibian reproductive success, this study lacked behavioral evidence.

The current study focuses on endocrine and behavioral changes in adult male African clawed frogs (*Xenopus laevis*) exposed to field collected and simulated agricultural run-off. Although the American bullfrog (*Lithobates catesbeianus*) was the model organism for chapter 2, captive stress can abolish sex hormones and behaviors in captive bullfrogs. Instead, I chose *X. laevis* for the current study because they easily mate in captivity.

Few studies have examined the effects of ecologically relevant agrochemical exposure on the reproductive behaviors of male amphibians. In the present study, I examined the effects of agricultural run-off (collected field water) and simulated run-off (a pesticide mixture) on the clasping behavior in male *X. laevis*. I examined corticosterone levels, testosterone levels, and clasping behaviors in adult males exposed to agricultural run-off, field water collected upstream of agricultural activity (negative control), and simulated run-off (positive control) for 30 days. I predicted that males exposed to agricultural run-off (field water and pesticide mixture) would have elevated corticosterone levels, lower testosterone levels and decreased clasping behaviors.

## METHODS

### *Treatments*

Three treatments were used to test the effects of agricultural run-off on the reproductive behavior of male *X. laevis*. For the first and second treatment, I collected water upstream and downstream of agricultural activity along the Salinas River. Downstream water (agricultural run-off) was collected in Monterey County, Salinas (SAL) (N 36°38.828', W 121°42.161').

Upstream water (serving as a negative control) was collected at Santa Margarita Lake (STM) (N 35°19'0.99", W 120°27'21.36"), which is the drinking water reservoir for the city of San Luis Obispo, CA. Both upstream (STM) and downstream (SAL) water were collected in 5 gallon plastic buckets (Tap Plastics El Cerrito, CA) on May 8, 2009. After collection, each bucket was sealed, transported to UC Berkeley, and immediately frozen (-20°C) in a walk-in freezer. The third treatment served as a positive control. The following compounds were chosen to mimic chemicals that amphibians residing in Salinas Valley are likely exposed to. A mixture of 1 ppb each of maneb, glyphosate, fosetyl aluminum, dichloropropene, diazinon, chlorthal, chloropicrin, bensulide, acephate, propyzamide, oxydemeton methyl, metam sodium, methomyl, nitrates, and phosphates were dissolved in ethanol. These chemicals represent the top agrochemicals used in Monterey County, CA (SAL-mix) in 2009. Pesticide application information was obtained from the Pesticide Action Network (PAN) pesticide database (Kegley, 2011).

### *Animal Husbandry*

Adult male and female frogs were obtained from a long-term captive colony maintained at the University of California, Berkeley. Four one-year old virgin adult males (brothers) were housed in aquaria (16 x 25 x 20 cm) with 10 L of treatment water for a total of 30 days. Each treatment was replicated 4 times (4 adult males per replicate). Animals were fed and cages were cleaned, water changed, and treatments renewed every 6 days.

For upstream (STM) and downstream (SAL) river water treatments, buckets were thawed 3 days prior to use to insure that river water was thawed and equilibrated to room temperature. All control animals were housed in dechloraminated water.

For the Salinas pesticide mixture (SAL-mix), all pesticides/agrochemicals were predissolved in ethanol for a final concentration of 0.003 % ethanol and 1 ppb of each pesticide. Controls received an equal amount of ethanol only.

To determine whether treatments had an effect on overall corticosterone and testosterone levels, blood samples were obtained on the evening of day 25. Morning blood samples were taken on the morning of day 30 at the conclusion of the behavioral trials to determine whether testosterone levels were affected by the presence of females.

### *Experimental trials*

The current study was conducted twice; first in 2009 and repeated in 2010. The first round of this experiment was conducted on May 20, 2009. Both males and females used in these trials were virgins. This experiment was then repeated on July 5, 2010. Males used were 2 year old virgins (brothers of the 1-year old males used in 2009). However, females used for the 2009 behavioral mate choice trials were reused in 2010 (not virgins).

River treatment water collected on May 8, 2009 was used for both 2009 and 2010 trials. After river water was collected and frozen, half was apportioned for use in the 2009 trial. The remaining half of collected river water was stored frozen (-20°C) until its use in 2010.

### *Mate Choice*

A mate choice behavioral study (described in Hayes *et al.*, 2010) was used to compare the ability of control and treated (SAL, STM, and SAL-mix) males to attract females and achieve

amplexus (the male copulatory embrace). In brief, four control males and four treated males were placed in a circular pool with four hCG-injected females. On the next day, amplexant pairs were identified and blood was collected from all males via cardiac puncture. Blood samples were immediately placed on ice. Plasma was collected by aspiration after low-speed centrifugation and stored frozen (-20°C) until analysis. Behavioral trials were conducted four times for each treatment. However, data were obtained for only three trials for SAL-mix and controls in 2009. A fourth trial was not conducted because one of the SAL-mix males expired, which resulted in an unequal ratio between SAL-mix males and controls.

### *Hormone Analysis*

Plasma samples for testosterone and corticosterone analysis were extracted with diethyl ether and dried under nitrogen gas. Samples were then reconstituted in phosphate buffered saline with gelatin (PBS-G). Plasma testosterone and corticosterone levels were determined by RIA as described in Licht *et al.* (1983). Antibodies were obtained from Fitzgerald Industries (Acton, MA) for testosterone and MP Biomedicals (Solon, OH) for corticosterone and both were validated for use with *X. laevis*.

### *Statistics*

A replicated G-test of goodness of fit was used to examine the effects of treatment on the frequency of successful copulations amongst 4 trials (Sokal and Rohlf, 1981). A chi-square test for goodness of fit was employed to determine the goodness of fit between theoretical and experimental amplexant data.

All hormone statistical analyses were conducted using Minitab 16.2.3 (Minitab Inc. State College, PA). Two-way ANOVA was used to examine whether males differed in morning testosterone levels (post behavior trials) between clasping and non-clasping males within treatments and respective controls.

## RESULTS

*Hormone levels.* In 2009, ANOVA revealed that there was no difference in corticosterone levels between SAL (ONE-WAY ANOVA:  $F = 0.14$ ,  $df = 1$ ,  $p = 0.712$ ), STM (ONE-WAY ANOVA:  $F = 0.36$ ,  $df = 1$ ,  $p = 0.551$ ), and SAL-mix treatments (ONE-WAY ANOVA:  $F = 0.06$ ,  $df = 1$ ,  $p = 0.805$ ) and controls (Fig. 1A). Likewise, there was no difference in testosterone levels between SAL (ONE-WAY ANOVA:  $F = 0.03$ ,  $df = 1$ ,  $p = 0.857$ ), STM (ONE-WAY ANOVA:  $F = 3.31$ ,  $df = 1$ ,  $p = 0.080$ ) and SAL-mix (ONE-WAY ANOVA:  $F = 0.54$ ,  $df = 1$ ,  $p = 0.466$ ) and controls (Fig. 2A).

In 2010, ANOVA revealed that there was no difference in corticosterone levels between SAL (ONE-WAY ANOVA:  $F = 3.71$ ,  $df = 1$ ,  $p = 0.064$ ), STM (ONE-WAY ANOVA:  $F = 0.17$ ,  $df = 1$ ,  $p = 0.679$ ), and SAL-mix treatments (ONE-WAY ANOVA:  $F = 0.42$ ,  $df = 1$ ,  $p = 0.520$ ) and controls (Fig. 1B). Likewise, there was no difference in testosterone levels between SAL (ONE-WAY ANOVA:  $F = 0.03$ ,  $df = 1$ ,  $p = 0.857$ ) and STM (ONE-WAY ANOVA:  $F = 4.32$ ,  $df = 1$ ,  $p = 0.047$ ) and controls (Fig. 2B). However, SAL-mix males had significantly lower testosterone levels than controls (ONE-WAY ANOVA:  $F = 3.87$ ,  $df = 1$ ,  $p = 0.059$ ; Fig. 2B).

*Behavior trials.* In 2009 mate choice trials where control males and SAL-treated males competed for females, SAL males out-competed control males in all four trials examined (G test:  $G_T = 11.43$ ,  $df = 4$ ,  $p = 0.022$ ; Fig. 3A) and obtained 10 out of 11 observed amplexant



events (Chi-squared test:  $X^2 = 7.36$ ,  $df = 1$ ,  $p = 0.007$ ; Table 1A). Control and STM males won equal amount of trials (G test:  $G_T = 0.00$ ,  $df = 4$ ,  $p = 1.000$ ; Fig. 3A) and obtained amplexus the same number of times (Chi-squared test:  $X^2 = 0.00$ ,  $df = 1$ ,  $p = 1.000$ ; Table 1A). Control males out-competed SAL-mix males in all three trials examined (G test:  $G_T = 5.88$ ,  $df = 3$ ,  $p = 0.117$ ; Fig. 3A) and obtained 6 out of 7 observed amplexant events (Chi-squared test:  $X^2 = 3.57$ ,  $df = 1$ ,  $p = 0.059$ ; Table 1A).

There was no significant difference in overall testosterone levels in SAL (TWO-WAY ANOVA:  $F = 0.01$ ,  $df = 1$ ,  $p = 0.911$ ), STM (TWO-WAY ANOVA:  $F = 0.07$ ,  $df = 1$ ,  $p = 0.792$ ), and SAL-mix males (TWO-WAY ANOVA:  $F = 3.78$ ,  $df = 1$ ,  $p = 0.066$ ) in the presence of females in comparison to controls. However, two-way ANOVA revealed that males in amplexus have significantly higher testosterone levels than those who do not amplex between control males and SAL (TWO-WAY ANOVA:  $F = 8.68$ ,  $df = 1$ ,  $p = 0.006$ ), STM (TWO-WAY ANOVA:  $F = 33.81$ ,  $df = 1$ ,  $p < 0.001$ ), and SAL-MIX (TWO-WAY ANOVA:  $F = 17.32$ ,  $df = 1$ ,  $p < 0.001$ ) males (Fig. 4A).

In 2010, SAL males out-competed control males in three out of four trials examined (G test:  $G_T = 5.88$ ,  $df = 4$ ,  $p = 0.208$ ; Fig. 3B) and obtained 9 out of 13 observed amplexant events (Chi-squared test:  $X^2 = 1.92$ ,  $df = 1$ ,  $p = 0.166$ ; Table 1B). STM males won equal amount of trials as controls (G test:  $G_T = 1.39$ ,  $df = 4$ ,  $p = 0.847$ ; Fig. 3B) and obtained 8 out of 15 observed amplexant events (Chi-squared test:  $X^2 = 0.06$ ,  $df = 1$ ,  $p = 0.796$ ; Table 1B). Control males out-competed SAL-mix males in 2 out of 4 trials examined, tied in a single trial (G test:  $G_T = 2.43$ ,  $df = 4$ ,  $p = 0.657$ ; Fig. 3B) and obtained 9 out of 15 observed amplexant events (Chi-squared test:  $X^2 = 0.60$ ,  $df = 1$ ,  $p = 0.439$ ; Table 1B).

There was no significant difference in overall testosterone levels in SAL (TWO-WAY ANOVA:  $F = 0.11$ ,  $df = 1$ ,  $p = 0.747$ ) and STM males (TWO-WAY ANOVA:  $F = 0.09$ ,  $df = 1$ ,  $p = 0.767$ ) in the presence of females in comparison to controls. However, SAL-mix treated males had significantly higher testosterone levels (TWO-WAY ANOVA:  $F = 5.94$ ,  $df = 1$ ,  $p = 0.021$ ) in the presence of females in comparison to controls (Fig. 4B). Additionally, two-way ANOVA revealed that males in amplexus have significantly higher testosterone levels than those who do not amplex between control males and SAL (TWO-WAY ANOVA:  $F = 46.79$ ,  $df = 1$ ,  $p < 0.001$ ), STM (TWO-WAY ANOVA:  $F = 21.73$ ,  $df = 1$ ,  $p < 0.001$ ), and SAL-MIX males (TWO-WAY ANOVA:  $F = 72.36$ ,  $df = 1$ ,  $p < 0.001$ ) (Fig. 4B).

## DISCUSSION

In the current study, I examined the effects of agricultural run-off (collected field water) and simulated run-off (pesticide mixture) on the reproductive clasping behaviors of male *X. laevis*. SAL-mix males had significantly lower evening testosterone levels than control males in 2010. Males exposed to Salinas River water (SAL) out-competed control males in mating choice study. In contrast, control males out-competed SAL-mix males. There was no effect of treatment on overall morning testosterone levels. However, there was a significant difference in morning testosterone levels between clasping and non-clasping males in the presence of females. These data suggest that agrochemical exposure (both field water and simulated) can alter reproductive hormones and behaviors. Disruption of normal hormonal secretion and decreased sex behaviors by agrochemical exposure can have negative impacts on the long-term reproductive success of amphibian populations.

Short term agrochemical exposure (field water and simulated) did not induce a stress response. Though some of the pesticides used in the SAL-mix can elevate corticosterone levels with individual exposures (Atanasov *et al.*, 2003; Cranmer *et al.*, 1977; Joshi and Rajini, 2009; Myers *et al.*, 2005; Pruett *et al.*, 2003; Sobotka *et al.*, 1972; Spassova *et al.*, 2000), corticosterone levels were not elevated in the current study. The lack of a stress response in pesticide treated males was surprising because exposure to pesticide mixtures elevated corticosterone levels in male *X. laevis* (Hayes *et al.*, 2006). Although exposure to pesticide mixtures resulted in elevated corticosterone levels in the study conducted by Hayes *et al.* (2006), different pesticides were used in this study and males were singularly housed. In the current study, four males were housed per tank. Group housing is preferred to individual housing as it reduces stress of laboratory *X. laevis* (Reed, 2005; Wolfensohn and Lloyd, 2013). Although it is possible that singular versus group housing may play a role in how *X. laevis* responds to stressors such as exposure to agrochemicals, this point requires further study.

It was not surprising that testosterone levels of SAL-mix males (2010) were significantly lower than controls. Though half of the pesticides used in SAL-mix decrease plasma testosterone levels (Abd *et al.*, 1994; Andersen *et al.*, 2008; Clair *et al.*, 2012; Fattahi *et al.*, 2009; Mahgoub and El-Medany, 2001; Ray *et al.*, 1992; Romano *et al.*, 2010; Shalaby *et al.*, 2010; Tsagué Manfo *et al.*, 2011), testosterone levels were not depressed in SAL-mix males (2009). The inconsistency between testosterone levels in 2009 and 2010 may stem from relatively low testosterone levels of first-year males in comparison to two year old male *X. laevis*. Reported testosterone values in *X. laevis* for younger males are much lower than older males (Kelley, 1996). It is possible that testosterone levels of first year males were low enough to not be affected by the inhibitory effects of SAL-mix. This finding is consistent with atrazine exposed male hatchling alligators whose testosterone levels do not differ from control males (Rey *et al.*, 2009).

Contrary to my hypothesis, evening testosterone levels of SAL males did not differ from controls (Fig. 2A & B). These data suggest that agrochemical exposure from field collected agricultural run-off had no effect on testosterone levels.

Although field collected agricultural run-off had no effect on evening testosterone levels, exposure to downstream Salinas River water had a positive effect on mating behaviors (Fig. 3, A & B). These results were surprising because pesticide exposure suppressed mating behaviors in male *X. laevis* (Hayes *et al.*, 2010). The mechanism behind the enhanced mating behaviors of males exposed to field collected agricultural run-off is not known and poses an interesting question. However, future studies should determine if agrochemical-induced mating behaviors occur during non-breeding periods. Reproductive activity during non-breeding periods (i.e. winter) could deplete energy stores, which may result in smaller body size and lower fecundity.

Though clasping behaviors in male *X. laevis* is controlled by testosterone and its metabolites (Kelley and Pfaff, 1976; Moore, 1983), testosterone levels of SAL males do not differ from controls. Testosterone levels alone may not be an accurate indicator of clasping behavior (Moore, 1983). For example, androgen levels in male rough-skinned newts (*Taricha granulosa*) do not differ between behaviorally (sexual) active and inactive males in the months of February and June (Moore and Muller, 1977). Thus, testosterone levels of SAL males may not correlate with reproductive behaviors (Moore, 1983). Additionally, exposure to downstream Salinas River water may have elevated other androgen metabolites that were not measured by our

polyclonal antibody. Such an increase in androgen levels would explain why SAL males out-competed control males. Moreover, blood sampling times used in this study may not have given us the most accurate indication of testosterone levels prior to clasping. Blood collection for overall testosterone levels were taken in the evening, five days prior to the mate choice assay to ensure that stress from blood collection did not interfere with breeding behaviors. However, it may be necessary to determine testosterone levels immediately prior to copulatory events. Monitoring testosterone levels prior to copulation is challenging as mating behaviors are easily disrupted and stresses of blood collection may abolish clasping behaviors.

The pattern of successful clasping events of SAL and SAL-mix males and their controls are similar across trials but more pronounced in 2009 and attenuated in 2010. The use of sexually experienced females (non-virgins) in the 2010 mating trials may have contributed to the attenuation of clasping data. In addition, older males used in the 2010 trial had higher testosterone levels than 1<sup>st</sup> year males (2009) which may have increased clasping behaviors in both treated and control males, leading to an increased number of amplexant events, thus attenuating the response.

The significant differences of testosterone levels between clasping and non-clasping males across groups support the findings that testosterone levels are elevated during amplexus (Orchinik *et al.*, 1988; Siboulet, 1981; Townsend and Moger, 1987). However, androgen levels are not positively correlated with clasping in all amphibian species (Itoh and Ishii, 1990; Mendonça *et al.*, 1985; Moore and Muller, 1977; Wada *et al.*, 1976). Although exogenous testosterone induces clasping behavior in *X. laevis* (Kelley and Pfaff, 1976), it is not known if testosterone levels immediately prior to initiating amplexus will predict whether a male will successfully clasp in a competitive arena. Whether elevated testosterone is the cause of successful amplexus or is the result of engaging in this behavior requires further study. In the case of marine toads, it is the clasping behavior that induces a rise in testosterone. But the role of testosterone levels just prior to amplexus is not known. Based on the minimal testosterone levels found in clasping males (current study; Hayes *et al.*, 2010), there seems to be some threshold level required to engage on sexual behaviors. Future examination is needed to determine whether testosterone levels are correlated with successful amplexus and whether there is a minimum amount of testosterone needed to induce copulatory behaviors.

## CONCLUSIONS

There is a paucity of data on the effects of agrochemical exposure on amphibian reproductive health and its contribution to amphibian declines. The present study provides evidence that reproductive behaviors such as clasping can be altered by agrochemical exposures. Changes to reproductive behaviors can be detrimental to the reproductive health and long-term reproductive success of amphibian populations.

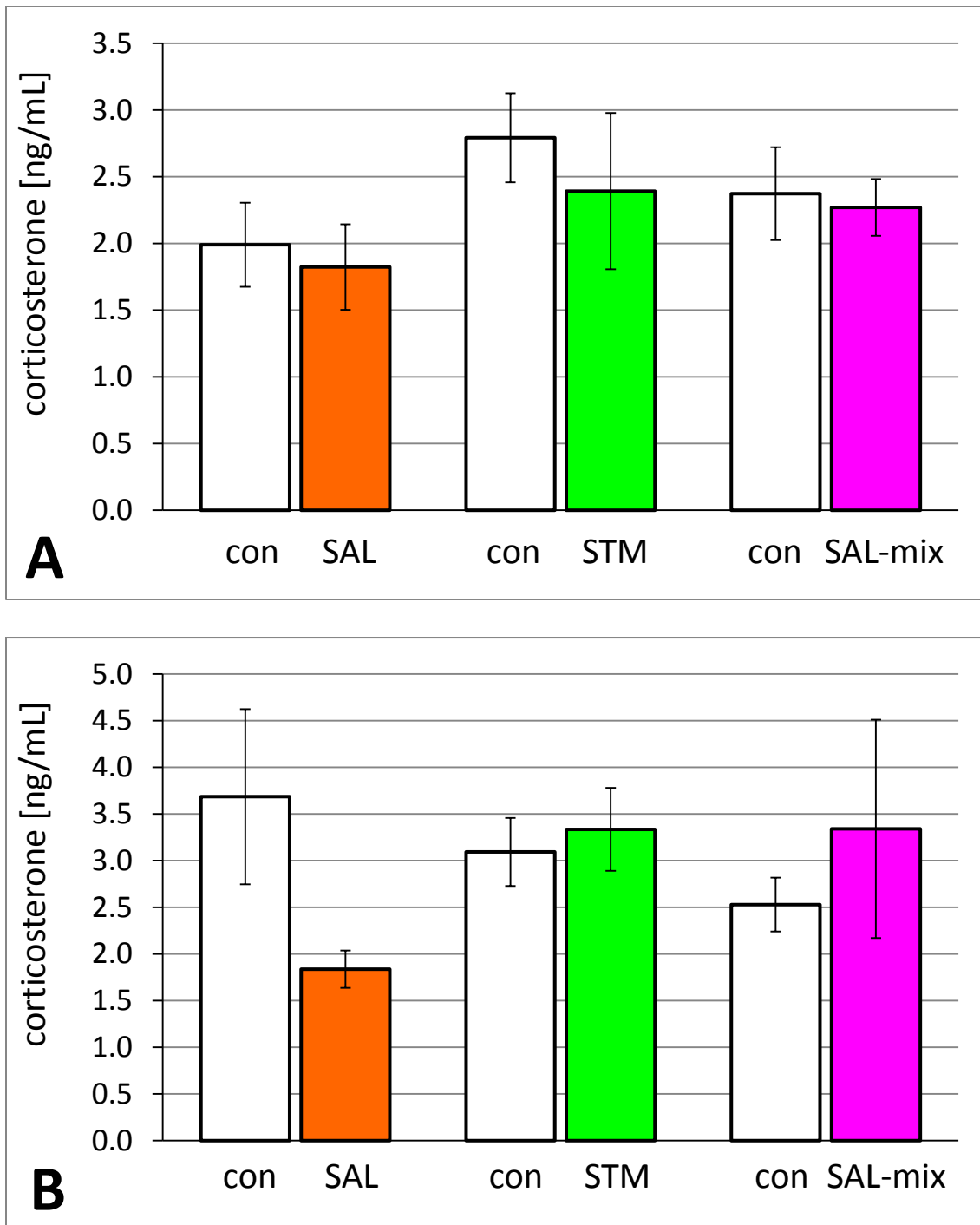
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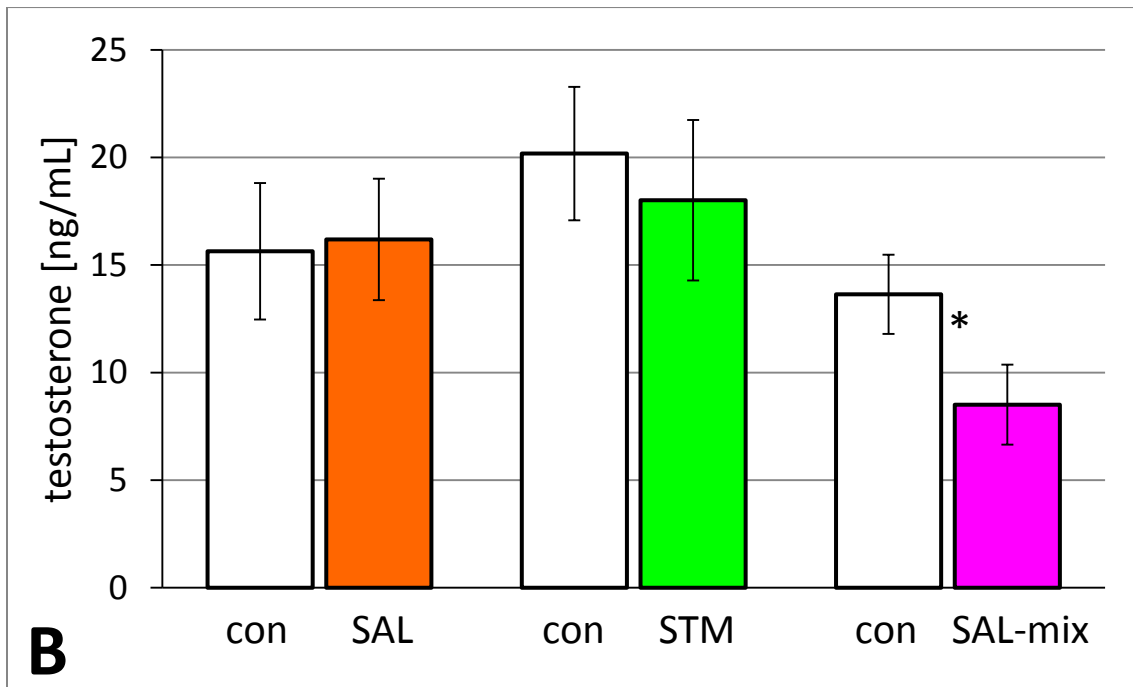
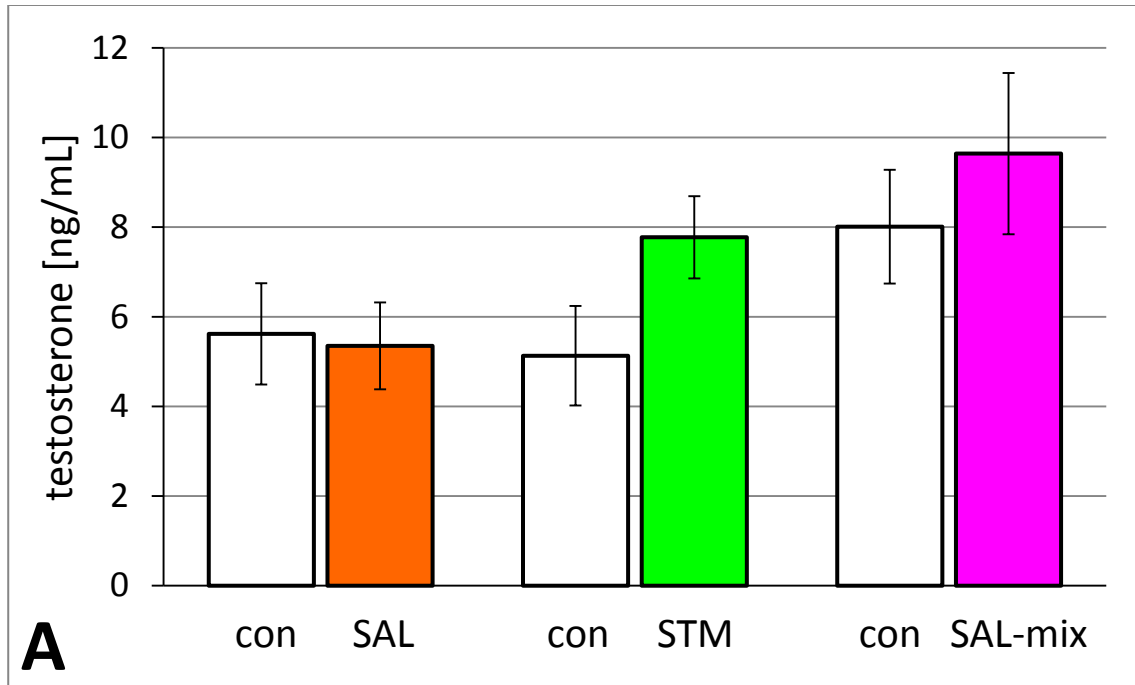
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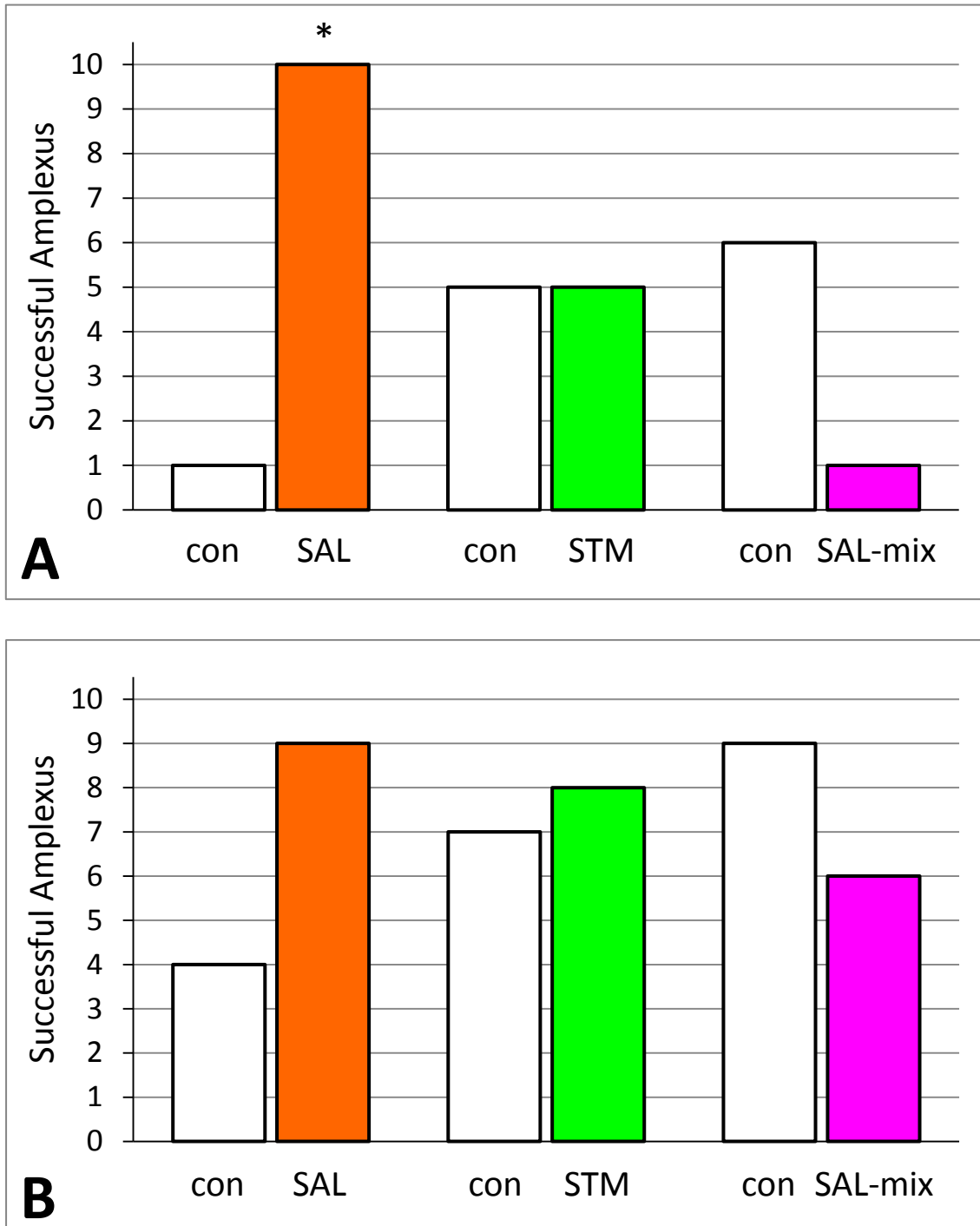


**Figure 1.** Evening corticosterone levels for control (con; white bars) and treated males (SAL = Salinas, orange bars; STM = Santa Margarita, green bars; SAL-mix = Salinas pesticide mixture, pink bars). One-way ANOVA was used to examine whether evening corticosterone levels differed between treatment (SAL, STM & SAL-mix) and control males. Significance was determined by Tukey’s honestly significant difference (HSD) method ( $p \leq 0.05$ ). This experiment was conducted twice. First in 2009 (A) and again in 2010 (B)



**Figure 2.** Evening testosterone levels for control (con; white bars) and treated males (SAL = Salinas, orange bars; STM = Santa Margarita, green bars; SAL-mix = Salinas pesticide mixture, pink bars). One-way ANOVA was used to examine whether evening testosterone levels differed between treatment (SAL, STM & SAL-mix) and control males. Significance was determined by Tukey's honestly significant difference (HSD) method ( $p \leq 0.05$ ). This experiment was conducted twice. First in 2009 (A) and again in 2010 (B)





**Figure 3.** Number of successful amplexus events from four mate choice trials for control (con; white bars) and treated males (SAL = Salinas, orange bars; STM = Santa Margarita, green bars; SAL-mix = Salinas pesticide mixture, pink bars). Replicated G-test of goodness of fit was used to examine whether the ability to amplex a female was affected by treatment ( $p < 0.05$ ). This experiment was conducted twice. First in 2009 (A) and again in 2010 (B)

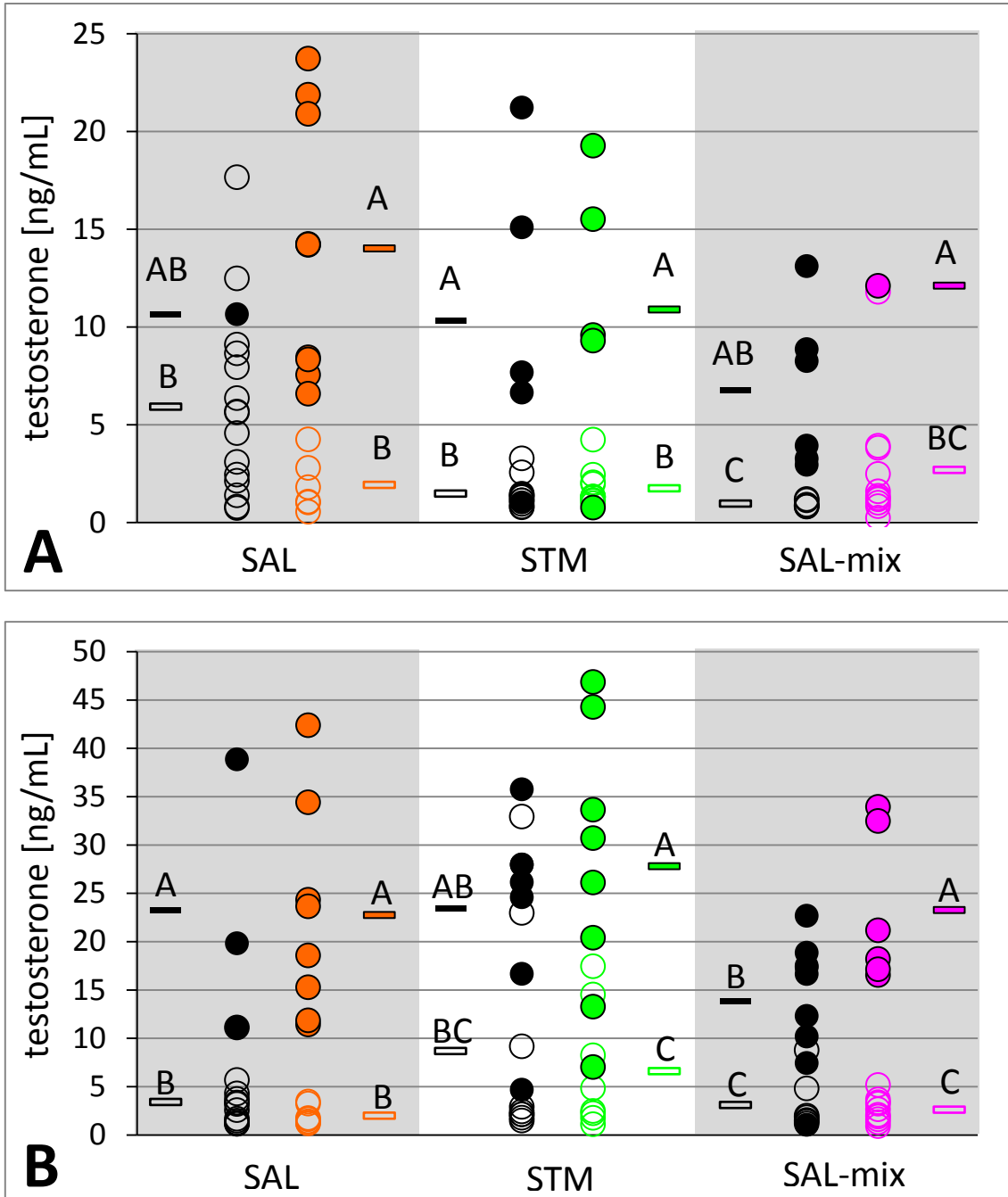
<b>A</b>	<i>Treatment</i>	# trials	# SA	<u>Replicated G-test of goodness of fit</u>		<u>Chi-square goodness of fit</u>		
				<i>df</i>	<i>G-total</i>	<i>p</i>	<i>df</i>	<i>Chi-sq</i>
<b>Salinas Site</b>								
	control		1					
	Salinas (SAL)	4	10	4	11.43	0.022*	1	7.36 0.007*
<b>Santa Margarita Site</b>								
	control		5					
	Santa Margarita (STM)	4	5	4	0.00	1.000	1	0.00 1.000
<b>Salinas Mix</b>								
	control		6					
	Salinas mix (SAL-mix)	3	1	3	5.88	0.117	1	3.57 0.059*

Significant at \* $p \leq 0.05$ ; \*\* $p \leq 0.005$ ; \*\*\* $p \leq 0.001$

<b>B</b>	<i>Treatment</i>	# trials	# SA	<u>Replicated G-test of goodness of fit</u>		<u>Chi-square goodness of fit</u>		
				<i>df</i>	<i>G-total</i>	<i>p</i>	<i>df</i>	<i>Chi-sq</i>
<b>Salinas Site</b>								
	control		4					
	Salinas (SAL)	4	9	4	5.88	0.208	1	1.92 0.166
<b>Santa Margarita Site</b>								
	control		7					
	Santa Margarita (STM)	4	8	4	1.39	0.847	1	0.06 0.796
<b>Salinas Mix</b>								
	control		9					
	Salinas mix (SAL-mix)	4	6	4	2.43	0.657	1	0.60 0.439

Significant at \* $p \leq 0.05$ ; \*\* $p \leq 0.005$ ; \*\*\* $p \leq 0.001$

**Table 1.** Results of replicated G-test goodness of fit and chi-squared goodness of fit analysis for three treatments (SAL, STM, and SAL-mix) and their controls ( $p < 0.05$ ). This experiment was conducted twice. First in 2009 (A) and again in 2010 (B)



**Figure 4.** Testosterone levels for control (black circles) and treated males (colored circles: SAL = Salinas, orange; STM = Santa Margarita, green; SAL-mix = Salinas pesticide mixture, pink). Filled circles represent successful males (achieved amplexus) and open circles represent unsuccessful males. Solid horizontal bars represent mean testosterone levels for successful males and open bars represent mean for unsuccessful males. Two-way ANOVA was used to examine whether testosterone levels differed between successful males within each treatment (SAL, STM & SAL-mix) and their controls. Significance was determined by Tukey's honestly significant difference (HSD) method. Letters above bars indicate statistical groupings ( $p < 0.05$ ). This experiment was conducted twice. First in 2009 (A) and again in 2010 (B)

## Chapter 4



## Conclusions

## INTRODUCTION

There is a dearth of information regarding the effects of eco-toxicological relevant agrochemical exposure on amphibian reproductive function. To address the need for more ecologically relevant studies, this dissertation examined the reproductive function of wild male amphibians living upstream and downstream of agricultural activity (chapter 2). Correlative lab studies were also conducted using field collected agricultural run-off and agrochemical mixtures to simulate exposure conditions relevant to the field (chapter 3). By conducting field and correlated lab studies, I was able to address how agrochemical exposure affects the reproductive endocrinology, morphology, and behaviors of male amphibians.

## SUMMARY

This dissertation had four specific aims. The first was to determine whether agrochemical exposure induced a stress response, which would result in elevated corticosterone levels. Second, I examined if testosterone levels were altered by either elevated corticosterone levels and/or exposure to agrochemicals. Third, I investigated whether agrochemical exposure altered the reproductive morphology of male amphibians. Lastly, I determined whether reproductive behaviors were altered by agrochemical exposure.

The studies presented in this dissertation examined the impact of ecologically relevant agrochemical exposure on the reproductive health of male amphibians. The main goal of chapter 2 was to determine whether living downstream of agricultural activity had any effects on the stress response and the reproductive endocrinology and morphology of male American bullfrogs (*Lithobates catesbeianus*). Bullfrogs were collected upstream and downstream of agricultural activity across three California river systems (Salinas, Sacramento and San Joaquin). Size, primary and secondary sex traits, sperm count, and corticosterone and testosterone levels were examined.

In addition to examining stress and sex steroids, chapter 3 focused on the effects of agrochemical exposure on the reproductive behaviors of adult male African clawed frogs (*Xenopus laevis*). To replicate field conditions, adult males were exposed to field water collected from the same downstream Salinas River site used in chapter 2. In addition, males were also exposed to upstream Salinas River water (negative control) and simulated agriculture run-off (positive control). After 30 days of treatment, the clasping behavior (male copulatory embrace) was examined. This experiment was conducted twice, once in 2009 and again in 2010.

### *Specific aim 1: Does agrochemical exposure induce a stress response?*

Results from chapter 2 support the hypothesis that living downstream of agriculture in some sites induces a stress response. That is, elevated corticosterone levels as a result of agrochemical exposure was only observed in wild male bullfrogs living downstream of agricultural activity along the San Joaquin River (SJ) (chapter 2). The lack of elevated corticosterone in the other pesticide exposed animals (chapter 2: downstream populations and chapter 3: laboratory exposures) were surprising because chemical exposure elevates corticosterone in other amphibians (Hayes *et al.*, 2006; Hopkins *et al.*, 1997; Larson *et al.*, 1998; Peterson *et al.*, 2009). Corticosterone levels may not be the best indicator for agrochemical-induced stress. Although corticosterone levels appear to be a good biomarker for acute

exposures (Billard *et al.*, 1981; Hopkins *et al.*, 1999), circulating corticosterone often decreases under chronic exposure conditions, thereby being a less useful indicator of stress (Adams, 1990; Bonga, 1997; Hopkins *et al.*, 1999).

*Specific aim 2: Does elevated corticosterone levels and/or agrochemical exposure alter testosterone levels?*

Data from chapters 2 and 3 support the hypothesis that agrochemical exposure alters testosterone levels. Interestingly, testosterone levels in downstream SJ male bullfrogs were elevated (chapter 2) and depressed in male *X. laevis* exposed to simulated agricultural run-off (chapter 3). The observed opposite effects of agrochemical exposure on testosterone levels suggest that the relationship between agricultural chemicals, corticosterone, and testosterone are complex.

Surprisingly, testosterone levels were elevated in the presence of high corticosterone in downstream SJ males (chapter 2). Although these results support the hypothesis that elevated corticosterone alters testosterone levels, I expected testosterone levels to be inhibited rather than elevated because corticosterone suppresses androgen secretion. Despite the inhibitory effect of corticosterone on testosterone, concurrent elevations of these steroids may be chemically induced. For example concomitant elevations in corticosterone and testosterone have been found in male toads exposed to coal ash (Hopkins *et al.*, 1997).

It is also possible that elevated corticosterone levels in SJ males may not be agrochemical-induced. Instead, elevated corticosterone may stem from high testosterone levels which are normally elevated during the breeding season. For example, treatment with exogenous testosterone elevates corticosterone in birds (Ketterson *et al.*, 1991; Schoech *et al.*, 1999). In addition, corticosterone has been shown to be positively correlated with testosterone in amphibians and reptiles (Emerson and Hess, 2001; Moore *et al.*, 2000; Schramm *et al.*, 1999). These findings are consistent with significant positive correlations found between corticosterone and testosterone levels in chapter 2. However, because the current study lacked pesticide analysis, correlation between corticosterone and agrochemical levels could not be tested. Pesticide analysis would elucidate whether the observed elevations in corticosterone and testosterone are normal or if they are agro-chemically induced.

In contrast to field observations, testosterone levels in male *X. laevis* exposed to simulated run-off experienced a decrease in testosterone levels in comparison to controls. Although decreased testosterone was in line with my hypothesis, these results were only observed in trials conducted in 2010 and not in 2009. It is possible that the use of younger males in 2009 may have not been as affected by pesticide exposure because their overall testosterone levels were half the amount of the older males used in 2010. This finding is consistent with atrazine exposed male hatchling alligators whose testosterone levels do not differ from control males (Rey *et al.*, 2009). The differences in testosterone levels between 2009 and 2010 suggest that the relationship between chemical exposure and hormone levels are complex. The use of 1 year and 2 year old male *X. laevis* in future studies may elucidate whether agrochemical exposure affects testosterone differently with regards to age.

*Specific aim 3: Does agrochemical exposure alter the reproductive morphologies of male amphibians?*

There are several lines of data supporting the hypothesis that agrochemical exposure alters the reproductive morphologies of male amphibians (chapter 2). Agrochemical exposure had a clear negative effect on bullfrog size (SVL & BW) in downstream animals across rivers. Additionally, downstream Salinas River males had larger GSI values and sperm counts but less developed tympana, nuptial pads, and throat coloration than upstream reference males. Moreover, downstream San Joaquin males had more developed tympana, nuptial pads, and throat coloration than reference males. Although these results support the hypothesis that agrochemical exposure alters reproductive morphologies in male amphibians, I was surprised to observe exaggerated traits in downstream males. Exaggerated sexually dimorphic traits of small downstream males suggest that these males may employ a terminal investment strategy. Candolin (1999) demonstrated that poor condition (induced by manipulating food intake) increased breeding coloration in male stickleback fish. Thus, males that are in poor condition (i.e. small SVL & BW) may allocate high amounts of energy to the exaggeration of traits because of a low probability of future reproduction (Candolin, 1999). Overall, these results suggest that adaptations to agrochemical exposure can be both inhibitory and stimulatory on reproductive morphology. However, enhanced sex traits and small size can have negative effects on overall reproductive health and future success. Future studies examining the immunological impact, fecundity, and fertility of terminal investment males is suggested to determine the impact of such males on population health and declines.

*Specific aim 4: Does agrochemical exposure alter reproductive behaviors of male amphibians?*

Results from behavioral studies in chapter 3 support the hypothesis that agrochemical exposure alters the reproductive clasping behaviors of male amphibians. Interestingly, clasping behaviors were enhanced in males exposed to downstream Salinas River water but depressed in males exposed to simulated agricultural run-off. I expected control males to outcompete males treated with simulated agricultural run-off. These results agree with previously reported data where control males outcompeted pesticide treated males in mate choice studies (Hayes *et al.*, 2010c). However, it was surprising that males treated with field collected agricultural run-off out-competed control males in both 2009 and 2010 trials. These results suggest that there is something in the collected Salinas River field water that is causing these males to out-clasp controls.

Although the simulated agricultural run-off mixture was designed to mimic field collected water, clasping results between males exposed to these treatments differed (were not similar). The differences in clasping behavior between field collected and simulated agricultural run-off illustrate the complex interaction between ecologically relevant chemical exposures and reproductive behaviors. More ecologically relevant studies are needed to understand the role of real-life agrochemical exposure on amphibian reproductive behaviors.

## LIMITATIONS & FUTURE STUDIES

Research using relevant ecological modeling is needed to increase our understanding of global amphibian declines. Such gains are important because diminishing populations most likely suffer from the direct and indirect effects, and complex interactions of multiple stressors (Boone and Bridges, 2003; Boone *et al.*, 2007; Collins and Storfer, 2003; Davidson and Knapp, 2007; Davidson *et al.*, 2001; 2002; Fleeger *et al.*, 2003; Hatch and Blaustein, 2003; Hayes *et al.*, 2010b; Mills and Semlitsch, 2004; Reeves *et al.*, 2010; Relyea, 2005; Relyea, 2010; Relyea and Mills, 2001; Rohr *et al.*, 2004; Rohr *et al.*, 2008a; Rohr *et al.*, 2008b). One limitation of the current study is it examined the role of isolated stressors (i.e. agrochemical exposure) on reproductive function. In addition to bringing the field back to the lab (via collected agriculture run-off), a mesocosm study would provide a more relevant and realistic approach by adding the stressors inherent in a natural agricultural landscape (Mann *et al.*, 2009).

Chronic activation of the stress response is a concern because it can potentially dampen reproductive function and thereby decrease recruitment in amphibian populations. However, circulating corticosterone often decreases under chronic exposure conditions, thereby being a less useful indicator of stress (Adams, 1990; Bonga, 1997; Hopkins *et al.*, 1999). Using other stress hormones such as CRH and ACTH as biomarkers may be useful in detecting whether animals are stressed and should be investigated.

## CONCLUSIONS

Overall, field and laboratory observations reveal that agrochemical exposure induces a stress response and alters the reproductive endocrinology, morphology, and behaviors of male amphibians. The present study suggests several ways that exposure to agrochemicals may lead to decreased recruitment. Firstly, field studies revealed that agrochemical exposure had a negative impact on size. Small size is a concern because body size is strongly linked to fecundity (Halliday and Verrell, 1988). Reproduction is a costly process. Animals with low body mass have less energy reserves which can impair reproductive output in amphibian species (Brodeur *et al.*, 2011). Thus, animals with reduced body size as a consequence of living in agricultural areas may have reduced fitness (Spear *et al.*, 2009) and impaired reproductive ability (Brodeur *et al.*, 2011).

Elevated corticosterone and testosterone can also negatively affect size. Elevated corticosterone at larval stages or metamorphosis can have a substantial impact on adult size (Altwegg and Reyer, 2003; Denver, 2009; Glennemeier and Denver, 2002; Hu *et al.*, 2008; Janin *et al.*, 2011). Inappropriate elevations in testosterone shunts energy allocation to reproduction, which results in less energy for metabolism and growth (Cox *et al.*, 2005; Hau, 2007; Miles *et al.*, 2007; Olsson *et al.*, 2000; Ryser, 1989). Additionally, elevated testosterone suppresses immune function (reviewed in Muehlenbein and Bribiescas, 2005). Impaired immune function can lead to increased disease rates and contribute to amphibian declines (Hayes *et al.*, 2010a).

Decreased testosterone production is a concern because testosterone levels are linked to fertility (Hayes *et al.*, 2010c). Reduced testosterone production can negatively impact sperm production (Rugh, 1946), development of sexual dimorphic traits (Duellman and Trueb, 1986; Greenberg, 1942), and inhibit sex behaviors (Kelley and Pfaff, 1976) in male amphibians.

The presence of exaggerated traits in small agrochemical exposed bullfrogs suggests that these males allocated resources to reproduction instead of survival. This reproductive tactic



(also known as terminal investments) is used to produce as many offspring as possible in situations where surviving to reproduce again is unlikely (Clutton-Brock, 1984; Velando *et al.*, 2006; Weil *et al.*, 2006). Terminal investment strategies, especially in small individuals, have a negative impact on reproductive success and overall survival (Clutton-Brock, 1984).

Lastly, the inability for males to compete for females would severely impact recruitment in amphibian populations and contribute to amphibian declines (Carey and Bryant, 1995). Although counterintuitive, enhanced clasping behavior can also have negative impacts on recruitment. Induced clasping at a younger age would result in reproductively active individuals that are small in size. If individuals are small at the onset of reproductive activity, they will remain small throughout their lives because energy resources are reallocated from somatic growth to reproduction (Halliday and Verrell, 1988). Consequently, small size would lead to decreased fecundity (Halliday and Verrell, 1988).

This dissertation suggests that decreased recruitment has a negative impact on amphibian declines. Amphibians are especially vulnerable to agrochemicals because contaminants can easily traverse their highly permeable skin and unshelled eggs. Thus, exposure to pollutants during reproductive development, sexual maturation, and during breeding bouts throughout adulthood can have adverse effects on amphibian reproductive function and therefore negatively impact reproductive success.

The impact of agrochemical use on amphibian declines has prompted concern for human health (Burggren and Warburton, 2007; Hopkins, 2007). Humans are also vulnerable to pesticides and can be exposed via inhalation, orally, and absorbing pesticides through their skin (EPA, 2014). Several parallels concerning agrochemical exposure and reproductive health can be made between the results of the current amphibian study and epidemiological investigations on humans. For example, pesticide exposure in utero results in reduced birth weight and body length of newborns (Whyatt *et al.*, 2004). Pesticide exposure is also associated with both early and delayed puberty, as well as less developed sex traits in boys (reviewed in Den Hond and Schoeters, 2006). Interestingly, male pesticide applicators that apply fungicides had lower testosterone levels but herbicide applicators had significant increases in testosterone levels in the fall compared to summer (Garry *et al.*, 2003). Furthermore, decreased sperm counts, diminished libido, and difficulties in erection and ejaculation were found in males working in a California pesticide factory (Whorton *et al.*, 1977). These parallels reveal that amphibians are a good model for understanding the possible effects of agrochemical exposure on human reproductive health.

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The end of THE END is the best place to begin THE END, because if you read THE END from the beginning of the beginning of THE END to the end of the end of THE END, you will arrive at the end.

— Lemony Snicket, The End —