

CORTISOL RELEASED DUE TO EXPERIMENTAL HANDLING AND UVB
RADIATION IN *XIPHOPHORUS* SPECIES

HONORS THESIS

Presented to the Honors Committee of
Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for Graduation in the Honors College

by

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San Marcos, Texas
May 2013

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ACKNOWLEDGEMENTS

I would like to acknowledge my advisors and research lab members who have assisted in my training and data collections for this project. I would like to thank Dr. Gabor and the GASP Lab from the Department of Biology for initiating my hormone research training. I would like to thank Dr. Chad Booth from the Department of Chemistry and Biochemistry for his guidance in my pursuit of research. Above all, I would also like to thank Dr. Walter and his lab for accepting me and integrating my research interests with interests of the Molecular Bioscience Research Group.

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ABSTRACT

CORTISOL RELEASED DUE TO EXPERIMENTAL HANDLING AND UVB RADIATION IN *XIPHOPHORUS* SPECIES

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May 2013

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Xiphophorus fish have been used as experimental models for UV-inducible melanoma for over 85 years. The aim of this study was to characterize the stress response (i.e., cortisol production) in *Xiphophorus* fish in skin after UV exposure. The primary stress response was characterized in *X. maculatus* Jp 163 B, *X. couchianus*, and F₁ interspecies hybrids (*Xm-Xh*) produced from the cross *X. maculatus* Jp 163 B (x) *X. couchianus*. The stress response in treated fish suggests experimental handling of the animals was the primary stressor and that UVB exposure effectively suppressed cortisol production rates. In *Xm-Xh* hybrids cortisol production rates were intermediate between parental species, but more similar to *X. maculatus* Jp 163 B. Thus, the cortisol stress responses appeared to be more species specific than treatment specific.

To eliminate species specific effects, we tested albino and wild type *X. hellerii* that only differed in pigment expression (i.e. were tyrosinase deficient). Although wild type fish exhibited a primary stress response to experimental handling the albino fish did not. This may suggest a role for tyrosinase in synthesizing catecholamines that provide substrate for the overall stress response. Analysis of stress recovery showed the fish returned to baseline cortisol production by 6 h, except for the albinos. This suggests albino skin is still repairing UV induced damage whereas wild type have repaired it by 6 h. Understanding the effects of stress and cortisol are important for elucidating the genetic interactions that lead to reduced immune function and/or increased susceptibility to tumorigenesis.

Chapter 1

Introduction

1.1 The Biological Response to Stress

Stress is described as a challenge or threat to homeostasis, to which an organism must respond and recover (Barton, 2002). Stress can be induced by physical, chemical, or perceptual stimuli. Physical stimuli often include handling, confinement, physical exertion, or pain. Chemical stimuli may include toxins, oxidizing agents, or carcinogens, and perceived stimuli can include predation or competition. The stress response is an adaptive mechanism with primary, secondary, and tertiary responses depending on the intensity and duration of stress (Barton, 2002).

The primary biological response to stress is initiated in the hypothalamus-pituitary-adrenal (HPA) axis and results in the production of cortisol (Barton, 2002). Circulating cortisol elevation can be detected within 30 s of a stressful event followed by a steady decline over several hours (Ellis et al., 2004). The hypothalamus initiates a response by producing corticotrophin releasing hormone (CRH) which induces cleavage of pro-opiomelanocortin (POMC), a peptide hormone precursor primarily located in the anterior pituitary gland (Slominski, 2010). POMC cleavage products include adrenocorticotrophic hormone (ACTH) that circulates in the bloodstream and stimulates adrenal tissues to produce cortisol (Barton, 2002; Canavello et al., 2011). ACTH and cortisol inhibit CRH

production in the hypothalamus to provide negative feedback as a means to recover homeostasis (Schreck, 2010). Feedback regulation occurs in all tissues of the HPA-axis and facilitates the adaptive nature of the stress response (Barton, 2002).

Cortisol is a corticosteroid which freely permeates cell membranes and may bind glucocorticoid receptors in the cytosol (GR; Staab and Maser, 2010). Upon binding with cortisol, the glucocorticoid receptors translocate from the cytosol to the nucleus where they may associate with GR response elements (GRE; Aluru and Vijayan, 2009). GRE binding may either inhibit or enhance expression of genes that provide physiological adjustments in response to stress (Aluru and Vijayan, 2009). This process then modulates the secondary and tertiary stress responses (below).

The secondary stress response corresponds with elevation in circulating cortisol that induces gene targets regulating immunity, metabolism, and the reproductive organs (Aluru and Vijayan, 2009). Acute or short-term exposures to stress may induce secondary responses. Through the release of anti-inflammatory cytokines, increased gluconeogenesis, and hormonal regulation, an organism may suppress inflammation from injuries and increase available energy to facilitate the fight-or-flight response (Canavello et al., 2011).

The tertiary stress response is brought about by long-term exposure to stress. Over time, chronic stress can reduce the immune response, suppress rates of growth, and decrease reproductive fitness through continuous secondary stress response signaling (Barton, 2002; Schreck, 2010). Chronic stress and inherently elevated cortisol production can result in constitutive immune feedback

responses that release pro-inflammatory cytokines and may lead to chronic inflammation and comprised immune functions (Robles et al., 2005). High concentrations of circulating pro-inflammatory cytokines often precede cardiovascular diseases and certain types of cancer (Robles et al., 2005). By elucidating the conditions that induce stress responses, criteria may be established to reduce or prevent susceptibility to tertiary stress responses and the resulting disease state.

In science and agriculture, it is an ethical duty to reduce animal stress and ensure their well-being. Potential sources of stress from facility conditions and animal treatment should always be under evaluation to help reduce secondary and tertiary effects from stress. Research animals may face stressors during experimentation, so assessing stress prior to testing is important to obtain consistent data. However, minimizing stress levels may provide the strongest results, as previous stressors from the facility would least affect animals. Due to biological variance between individual animals, this type of consistency is very important in biological research.

As the demand for food increases with a growing population, so too will the demand for more animal facilities in agriculture. Maintaining healthy animals in agriculture is necessary to sustain the quality of food, as well as animal growth and reproduction. As the volume of animals increases in agriculture facilities, it will become increasingly important to address stressors such as handling and confinement that most animals are exposed to. Understanding physical stressors and physiological responses to stress may provide insights to improve animal facility conditions and treatments while maximizing agricultural productivity.

1.2 Use of Fishes in Stress Research

Teleost fish species have been utilized to model and study the vertebrate stress response because the fish stress HPA-axis is very similar to humans. In addition, teleosts produce cortisol as the primary stress hormone, as opposed to murine models that produce corticosterone (Canvello et al., 2011). Furthermore, non-invasive sampling methods are imperative for stress research, and fish facilitate this necessity. Steroids can permeate cells and tissues, and circulating cortisol can be excreted through gill tissues and released into water (Ellis et al., 2004). By collecting and analyzing the water, teleost studies can be non-invasively performed, and this reduces primary and secondary stress responses due to the sampling protocol (Pankhurst, 2011). Reducing variability between states of stress is very important for observing specific stress responses to experimental treatments. Thus, teleost fish provide a tractable model for stress research, because they can be sampled with minimal stress.

Xiphophorus are live bearing teleost fishes that show complex behaviors. Their natural distribution extends from northern Mexico south along the Sierra Madre uplift and across the isthmus into Belize (Kallman and Kazianis, 2006). The *Xiphophorus* Genetic Stock Center (XGSC) at Texas State University currently maintains 23 of the 26 known species and can provide F₁ interspecies hybrids for experimental research (Walter and Kazianis, 2001). *Xiphophorus* are noted for their ability to produce fertile interspecies hybrids that exhibit increased susceptibility to induced tumorigenesis upon exposure to environmental carcinogenic agents such as ultraviolet light (UV) or alkylating agents. Thus, *Xiphophorus* interspecies hybrid models are used in many research protocols to

better understand the genes and biochemical modulations that make certain individuals susceptible to induced tumorigenesis.

As the primary line of defense against external environmental insult, skin may come in contact with UV radiation, oxidizing agents, and free radicals that induce cell and genetic damage. The skin is composed of several protective cell types such as melanocytes which produce melanin in response to UV light (Slominski and Pawelek, 1998). Melanin is also reactive with UV light and may produce oxidative intermediates that cause lesions and genetic mutations in melanocytes (Wood et al., 2006). Thus, melanin protects skin cells by absorbing UV radiation, but in response, produces harmful agents that can damage DNA. It is this duality of the melanocyte that contributes to many hypotheses of UV inducible melanoma.

Physical stress to the epidermis can induce pigmentation and synthesis of steroids like cortisol. Neuropeptides, such as POMC, can be expressed in the skin in response to stress and may also induce melanogenesis (Tsatmali et al., 2002). In teleosts, chronic social stress correlates with elevated circulating ACTH concentrations and darker pigmentation of the skin (Hoglund et al., 2000). POMC cleavage products activate melanocortin receptors (MCR) of the skin to induce melanogenesis, but they may also induce cortisol synthesis (Kittilsen, 2009). Many neuropeptides and hormones have been discovered in the skin and share similar regulation with the HPA-axis.

As a result, the skin has been recognized as a neuro-endocrine organ that is responsible for responding to numerous types of environmental stress (Slominski et al., 2007). As a secondary response to stress, the skin may also

suppress local cellular immune functions that may result in increased susceptibility to infection and tumorigenesis (Slominski et al., 1998, Roberts 1995). Genetic damage from UVB radiation (i.e., UVB, wavelengths from 280 – 315 nm) has been well characterized in *Xiphophorus* fish (Meador et al., 2000; Mitchell et al., 2001, 2004); however, the neuro-endocrine stress response to UV radiation remains unclear. *Xiphophorus* species offer a suitable model for UVB stress research, because there are genetic and molecular data available that may corroborate physiological observations of cortisol production.

The aim of this study was to elucidate the stress responses to experimental handling and UVB exposure in *Xiphophorus* fish treated according to previously published melanoma inducing protocols (Setlow et al., 1989, 1993; Nairn et al., 1996). These results provided stress profiles in four species and identified relationships between pigment phenotypes and stress responsiveness. These results may also provide evidence of a relationship between stress profiles and susceptibility to tumorigenesis in *Xiphophorus* interspecies hybrid melanoma models.

Chapter 2

Cortisol Production Rates of Pigment Varied *Xiphophorus* Species

2.1 Introduction

Pigment phenotypes can be influenced during development through regulation of melanocortin receptors (MCR) by circulating cortisol and neuropeptides (Yamada et al., 2011). Cleavage products of POMC, such as ACTH and alpha-melanocyte stimulating hormone (α -MSH), are known to play a key role in the pigmentation process. One way cortisol is associated with pigment phenotypes is through its ability to inhibit POMC cleavage (Tobin and Kauser, 2005). This relationship has been observed in adult salmon, where low cortisol production correlated with high ACTH and dark pigment phenotypes (Hoglund et al., 2000). Thus, high concentrations of cortisol may reduce POMC cleavage products and limit melanogenesis during development.

The skin has a stress axis similar to the HPA-axis that depends upon hormonal responses of melanocytes. Melanocytes originate from neural crest cells during development and have a key role in responding to cutaneous stress from heat and radiation by inducing synthesis of melanin and cortisol (Tobin and Kauser, 2005). In response to UVB radiation, melanocytes produce CRH that induces cleavage of POMC into α -MSH and ACTH (Slominski, 2010). This is the same primary response that occurs in the HPA-axis. Upon POMC cleavage,

α -MSH induces melanin dispersion and melanocyte proliferation while ACTH induces cortisol production (Eves et al., 2006; Slominski et al., 2005). These primary responses occur within the skin. Secondary stress responses may occur between melanocyte hormones, such as α -MSH or ACTH, and tissues expressing MCR, such as the skin, adrenal cortex and brain (Eves et al., 2006). Hormones from the HPA-axis and melanocytes may both regulate cortisol production and the primary stress response.

Xiphophorus fishes have a variety of pigment phenotypes that could reveal unique relationships between melanocyte dispersion and stress profiles. F₁ hybrids provide a comparison to parental fish strains as they correlate with pigment phenotypes and cortisol production. In this chapter we detail experiments investigating baseline cortisol production and the stress responses upon handling and UVB radiation in two parental species (*X. maculatus* Jp 163 B and *X. couchianus*) and F₁ hybrids produced from crossing these parental species [*X. maculatus* Jp 163 B (x) *X. couchianus*; herein designated *Xm-Xh*]. Because each species has unique pigment phenotypes (Fig. 3), it was expected that perhaps each may exhibit unique stress responses at genetic and/or physiological levels. However, our results support the hypothesis that stress profiles correlate more with differences between species than with pigment phenotypes.

2.2 Materials and Methods

Xiphophorus Species

Fish were maintained as pedigree lines according to standard protocols by the *Xiphophorus* Genetic Stock Center (XGSC) at Texas State University.

Analyses were conducted for *X. maculatus* Jp 163 B, male and female *X. couchianus*, and F₁ hybrids (i.e., *Xm-Xh*). All fish were approximately two years old and of similar size. All fish utilized in these experiments and treatment protocols are covered under IACUC #'1102-0111-02 and protocol #01-040-122 (valid to 03-31-2014) and supported by NIH grant award R24-OD-024790.

DNA Damage Quantification by Radioimmunoassay

This protocol was taken directly from Mitchell 2006. Radioimmunoassay (RIA) was used to measure UV photoproducts (CPD and 6-4PDs) in purified DNA samples. Briefly, 2–5 µg DNA of heat-denatured sample DNA was incubated with 5–10 pg of poly (deoxyadenosine):poly (deoxythymidine) (labeled to $>5 \times 10^8$ cpm/mg by nick translation with ³²P-dTTP) in a total volume of 1 mL (10 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl and 0.2% w/v gelatin, Sigma). Antiserum was added at a dilution that yielded optimal binding to labeled ligand. After 3 h incubation at 37°C the immune pellet was precipitated for 2 days at 48°C with goat anti-rabbit immunoglobulin (Calbiochem) and normal rabbit serum (UTMDACC, Science Park/Veterinary Division, Bastrop, TX). The immune complex was centrifuged at ~3,700 rpm for 45 min at 10°C and the supernatant discarded. The pellet was dissolved in 100 µL tissue solubilizer (NCS, Amersham), mixed with 6 µL ScintiSafe (Fisher) scintillation fluor containing 0.1% v/v glacial acetic acid and quantified using liquid scintillation counter

(Packard Instruments). Sample inhibition was extrapolated through a standard (dose response) curve to determine the number of photoproducts in 10^6 bases (i.e., Mb). Rates of photoproduct induction were previously quantified using non-immunological enzymatic and biochemical techniques and determined to be 8.1 CPD and 1.56 P(6-4)PD per Mb/J/m², respectively. Data were calculated using Excel (v.9.0.2719, Microsoft), plotted and fitted to curves using SigmaPlot (v. 5.0, SPSS).

Handling and Isolation

The day prior to testing, groups of 8 fish were isolated by species and sex and placed into four separate 4 L beakers each containing 2.4 L of filtered home aquaria water. Isolation beakers were kept in the dark in an undisturbed room overnight. Feedings were not performed during isolation or testing periods the next day. Overnight isolation is defined as stage 1 of the experiment (Fig. 1).

UVB Radiation

For each treatment, two conspecifics were netted from the 4 L beaker and placed into a single clear plastic cuvette (9 x 7.5 x 1.5 cm) containing 75 mL of filtered tank water under yellow incandescent light. The cuvette was placed at the center of the radiation chamber (77 x 41 x 36 cm) and exposed to sham treatments or doses of 8, 16, or 32 kJ/m² UVB radiation. The radiation chamber used four Philips TL narrow band Ultraviolet B (20W/01 RS) bulbs with a constant fluence of 12.2 J/m²s, and thus doses were controlled by duration of exposure, up to 43 m 45 s for 32 kJ/m². For sham treatments, the UVB bulbs in the radiation chamber were not turned on, and the lid was propped open to ambient room lighting for the same duration as a dose of 8 kJ/m² UVB (10 m 56

s). Treatments to fish in the radiation chamber are defined as stage 2 of the experiment (Fig. 1). After treatment fish were removed from exposure cuvettes and isolated in the dark in separate 600 mL beakers containing 300 mL of filtered home aquaria water. Post-treatment isolation is defined as stage 3 of the experiment (Fig. 1).

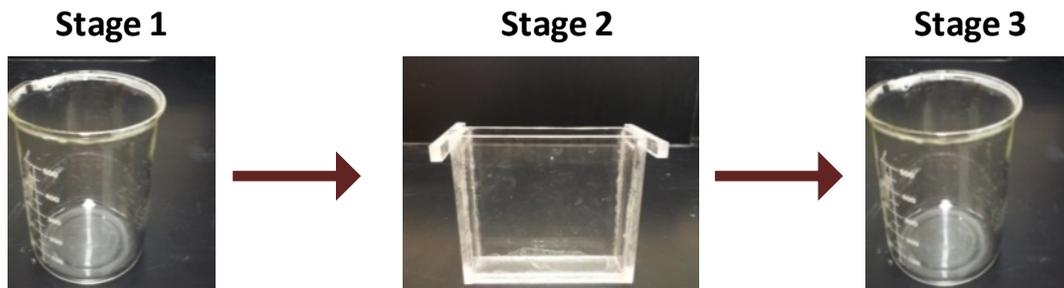


Figure 1. **Experimental Design.** There were three experimental stages when water was taken for cortisol analyses. Fish were isolated in the dark overnight in 600 mL beakers containing 300 mL water to establish baseline cortisol production rates (stage 1). Then, fish were transferred to cuvettes containing 75 mL water and exposed to sham treatments or UVB doses of 8, 16, or 32 kJ/m² (stage 2). After the sham or UVB treatments fish were isolated in the dark in 600 mL beakers containing 300 mL (post-treatment or stage 3). In the experiments described in Chapter 2, water samples were collected from stage 1 and stage 2 to analyze the primary stress response. In the experiments described in Chapter 3, water samples were collected from stage 1 and stage 2 to analyze primary stress responses and from stage 3 at 2 h and 6 h post-treatment to analyze recovery from the stress response.

Water Sampling

Immediately preceding treatments, stage 1 water samples of 100 mL were collected from overnight isolation beakers. Water was poured directly from isolation beakers after removing fish. Immediately following exposure to doses of UVB or sham treatment, stage 2 water samples of 75 mL were collected from the cuvettes by pouring contents into a beaker through a net. Netted fish were rinsed with fresh filtered aquaria water and immediately moved into their post-treatment isolation beaker. Previous data from the laboratory had established an optimal time of gene expression after UVB or MNU treatment to occur about 6 h post-treatment (Walter, R.B., unpublished results). Thus, at 6 h post-treatment, fish were sacrificed according to standard XGSC protocols and dissected under yellow incandescent light. Stage 3 water samples were not collected during this experiment. Skin from the fish was placed in a tube containing 250 μ L of RNeasy lysis buffer (Qiagen; Foster City, CA) and stored overnight at 4°C to allow absorption into the tissues. After overnight incubation in RNeasy lysis buffer at 4°C, all tissue samples were stored at -80°C for future RNA isolation. Water samples were stored frozen at -20°C until thawed and utilized for cortisol assay.

Hormone Analysis

Cortisol suspended in the water samples was extracted using C18 Solid Phase System columns (Honeywell; Morristown, NJ) with a 24 port vacuum manifold. The columns were primed with HPLC grade methanol followed by two water rinses before samples were added. Then, total hormone was eluted and collected with HPLC grade methanol. Eluted hormone was dried under a gentle

stream of nitrogen in a 37°C water bath and residues were stored at -20°C. A competitive immunoassay (EIA) kit from Enzo (Enzo Life Sciences Inc.; Farmingdale, NY) was utilized to assay cortisol, and protocols from the manufacturer were strictly followed. Briefly, hormone residues were re-suspended in 750 µL of assay buffer (Tris Buffered Saline, pH 7.5) supplied in the kit. Samples were loaded onto assay plates coated in goat anti-mouse IgG, followed by labeling with mouse monoclonal antibodies for cortisol, and p-nitrophenyl phosphate. Developed plates were washed and incubated with alkaline phosphatase for 60 min. Then, absorbance readings of the EIA were analyzed at 405 nm on a plate reader (BioTech Inc., Powerwave; Winooski, VT). Total cortisol present in the water samples was normalized by fish length (mm) and duration of sampling (h), and rates of cortisol production compared. Standard errors were calculated for each test group from four stage 1 water samples.

2.3 Results

Fish were exposed to various doses of UVB radiation as part of a large experiment in the host laboratory and DNA from the fish skin was isolated and RIA assayed for UV induced DNA damage (cyclobutane pyrimidine dimers or CPDs; Fig. 2) by Kevin Downs (MS thesis, 2013). *X. couchianus* incurred the most damage and had the lightest pigmentation, while *X. maculatus* Jp 163 B and F₁ hybrids incurred significantly less damage than *X. couchianus* and had darker pigmentation. In *X. couchianus*, 10.8 (± 1.70) CPDs per megabase DNA (CPDs/mb) were detected at 0 kJ/m², whereas 39.2 (± 4.62) CPDs/mb were detected at 8 kJ/m², 52.2 (± 9.19) CPDs/mb at 16 kJ/m², and 181.3 (± 10.4) CPDs/mb DNA were detected at 32 kJ/m². In *X. maculatus* Jp 163 B, 6.9 (± 1.0) CPDs/mb DNA were detected at 0 kJ/m², while 30.6 (± 4.04) CPDs/mb DNA were detected at 8 kJ/m², 46.5 (± 7.79) CPDs/mb at 16 kJ/m², and 78.5 (± 7.76) CPDs/mb at 32 kJ/m². *Xm-Xh* hybrids responded similarly to *X. maculatus* Jp 163 B, incurring 5.05 (± 1.0) CPDs/mb DNA at 0 kJ/m², 15.8 (± 1.65) CPDs/mb at 8 kJ/m², 27.6 (± 1.20) CPDs/mb DNA at 16 kJ/m², and 77.1 (± 10.0) CPDs/mb DNA were detected at 32 kJ/m².

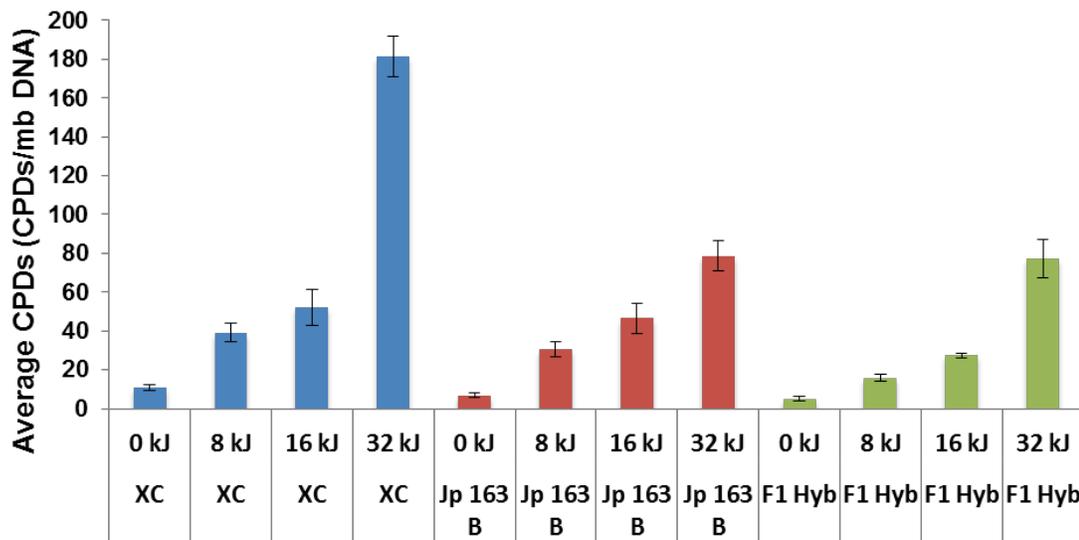


Figure 2. **RIA detection of CPDs in *Xiphophorus* DNA.** *X. couchianus* (XC), *X. maculatus* Jp 163 B (Jp 163 B), and F₁ hybrids produced from the mating [*X. maculatus* Jp 163 B (x) *X. couchianus*] (*Xm-Xh*) were exposed to various doses of UVB. These results show genetic damage incurred due to UVB for fish utilized in the cortisol testing protocols. Genetic damage increases with increasing doses of UVB and decreasing pigmentation (Data are courtesy of Kevin Downs, MS thesis, Walter laboratory, 2013).

Stage 1 water samples were analyzed to determine baseline cortisol production rates (Table 1, Column A). *X. maculatus* Jp 163 B produced cortisol at $2.0 (\pm 0.39) \text{ pg } L_s^{-1} \text{ h}^{-1}$. F_1 hybrids produced cortisol at $3.2 (\pm 0.41) \text{ pg } L_s^{-1} \text{ h}^{-1}$. Male *X. couchianus* produced cortisol at $17.0 (\pm 2.94) \text{ pg } L_s^{-1} \text{ h}^{-1}$ while female *X. couchianus* produced cortisol at $19.3 (\pm 4.20) \text{ pg } L_s^{-1} \text{ h}^{-1}$. *X. maculatus* Jp 163 B express the spotted side (*Sp*) pigment phenotype consisting of black spots (macromelanophores) on the flank of the animal. In *Xm-Xh* F_1 hybrids these black spots become much enhanced (Fig. 3). However, *X. couchianus* do not express the *Sp* phenotype or produce macromelanophores.

Table 1. Baseline Cortisol Production Rates Analyzed for *X. maculatus* Jp 163B, *Xm-Xh* F₁ Hybrids, and Male and Female *X. couchianus*. Fold change analyses were also conducted to compare stage 1 to stage 2 cortisol production rates and to compare stage 2 cortisol production rates from sham and UVB treatments.

		UVB Exposures													
		Cortisol (pg L ⁻¹ h ⁻¹)					Fold Change								
Stage 1		Stage 2					Stage 1 to Stage 2					Stage 2 sham to Stage 2 UVB			
Avg.	sham	8 kl/m ²	16 kl/m ²	32 kl/m ²	SE ¹	sham	8 kl/m ²	16 kl/m ²	32 kl/m ²	8 kl/m ²	16 kl/m ²	32 kl/m ²	8 kl/m ²	16 kl/m ²	32 kl/m ²
	B	C	D	E	F	G	H	I	J	K	L	M			
2.0	58.9	25.2	16.8	18.7	±0.39	29	12	8	9	0.43	0.29	0.32			
3.2	161.6	65.4	21.0	29.2	±0.41	51	20	7	9	0.40	0.13	0.18			
17.0	255.1	83.4	74.5	18.9	±2.94	15	5	4	1.1	0.33	0.29	0.07			
19.3	208.5	167.5	103.6	37.5	±4.20	11	9	5	2	0.80	0.50	0.18			

Column
X. maculatus Jp 163 B
Xm-Xh F₁ Hybrid
X. couchianus (m)
X. couchianus (f)

1. SE, standard errors were calculated per test group from four stage 1 measurements

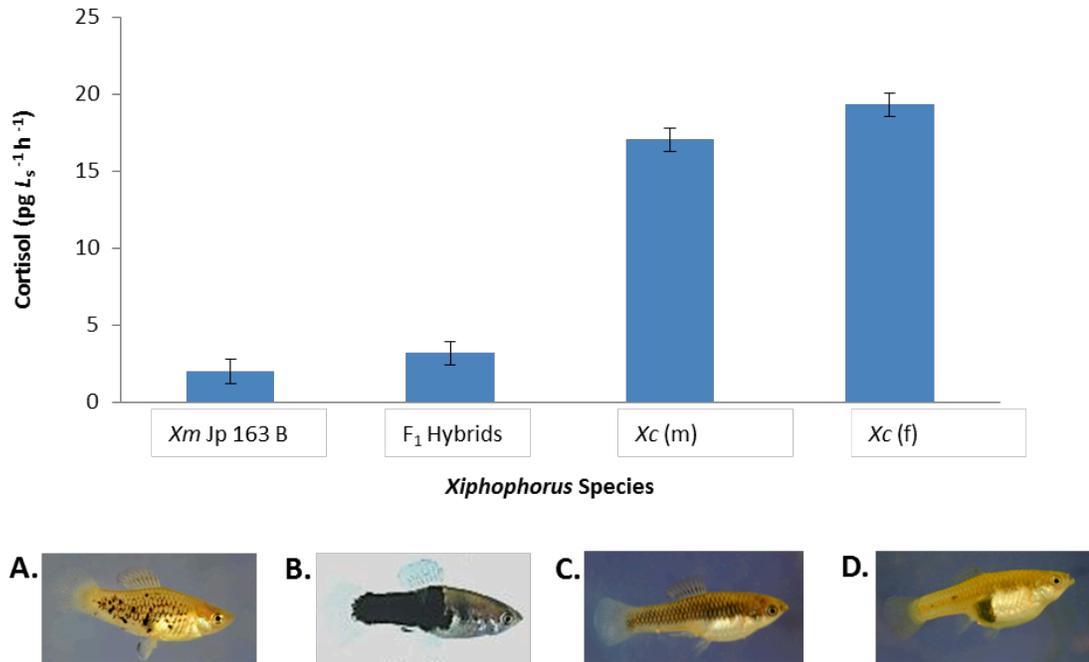


Figure 3. **Baseline Cortisol Production Rates in *Xiphophorus* Fish.**

Stage 1 water sample analyses revealed *X. maculatus* Jp 163 B (A) produced cortisol at $2.0 (\pm 0.39) \text{ pg } L_s^{-1} \text{ h}^{-1}$. *Xm-Xh* F_1 hybrids (B) produced cortisol at $3.2 (\pm 0.41) \text{ pg } L_s^{-1} \text{ h}^{-1}$. Male *X. couchianus* (C) produced cortisol at $17.0 (\pm 2.94) \text{ pg } L_s^{-1} \text{ h}^{-1}$ and female *X. couchianus* (D) produced cortisol at $19.3 \text{ pg } L_s^{-1} \text{ h}^{-1} (\pm 4.20) \text{ pg } L_s^{-1} \text{ h}^{-1}$.

Stage 2 water samples were collected and analyzed to determine the primary stress response to UVB exposure in *X. maculatus* Jp 163 B (Fig. 4; Table 1, Column A-M). In response to sham treatments cortisol production rates increased from stage 1, 29-fold (Column G) to $58.9 (\pm 0.39) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 8 kJ/m^2 UVB, cortisol production rates increased from stage 1, 12-fold (Column H) to $25.2 (\pm 0.39) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 16 kJ/m^2 UVB, cortisol production rates increased relative to stage 1, 8-fold (Column I) to $16.8 (\pm 0.39) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 32 kJ/m^2 UVB, cortisol production rates increased from stage 1, 9-fold (Column J) to $18.7 (\pm 0.39) \text{ pg } L_s^{-1} \text{ h}^{-1}$. Cortisol production rates were lower in response to doses of UVB radiation than to sham treatments. Cortisol production rates were at 43% of the observed response to sham treatments in response to 8 kJ/m^2 UVB (Column K). Cortisol production rates were at 29% of the observed response to sham treatments in response to 16 kJ/m^2 UVB (Column L); and cortisol production rates were at 32% of the observed response to sham treatments in response to 32 kJ/m^2 UVB (Column M).

Stage 2 water samples were collected and analyzed to determine primary stress responses to doses of UVB radiation in F_1 hybrids (Fig. 4; Table 1, Column A-M). In response to sham treatments cortisol production rates increased from stage 1, 51-fold (Column G) to $161.6 (\pm 0.41) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 8 kJ/m^2 UVB, cortisol production rates increased from stage 1, 21-fold (Column H) to $65.4 (\pm 0.41) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 16 kJ/m^2 UVB, cortisol production rates increased from stage 1, 7-fold (Column I) to $21.0 (\pm 0.41) \text{ pg } L_s^{-1} \text{ h}^{-1}$.

h^{-1} . In response to doses of 32 kJ/m^2 UVB, cortisol production rates increased from stage 1, 9-fold (Column J) to $29.2 (\pm 0.41) \text{ pg } L_s^{-1} \text{ h}^{-1}$. Cortisol production rates were lower in response to doses of UVB radiation than to sham treatments. Cortisol production rates were at 40% of the observed response to sham treatments in response to 8 kJ/m^2 UVB (Column K). Cortisol production rates were at 13% of the observed response to sham treatments in response to 16 kJ/m^2 UVB (Column L); and cortisol production rates were at 18% of the observed response to sham treatments in response to 32 kJ/m^2 UVB (Column M).

Stage 2 water samples were collected and analyzed to determine primary stress responses to doses of UVB radiation in male *X. couchianus* (Fig. 4; Table 1, Column A-M). In response to sham treatments cortisol production rates increased from stage 1, 15-fold (Column G) to $255.1 (\pm 2.94) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 8 kJ/m^2 UVB, cortisol production rates increased from stage 1, 5-fold (Column H) to $83.4 (\pm 2.94) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 16 kJ/m^2 UVB, cortisol production rates increased from stage 1, 4-fold (Column I) to $74.5 (\pm 2.94) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 32 kJ/m^2 UVB, cortisol production rates did not significantly increase from stage 1, 1.1-fold (Column J) to $18.9 (\pm 2.94) \text{ pg } L_s^{-1} \text{ h}^{-1}$. Cortisol production rates were lower in response to doses of UVB radiation than to sham treatments. Cortisol production rates were at 33% of the observed response to sham treatments in response to 8 kJ/m^2 UVB (Column K). Cortisol production rates were at 29% of the observed response to sham treatments in response to 16 kJ/m^2 UVB (Column L); and cortisol production

rates were at 7% of the observed response to sham treatments in response to 32 kJ/m² UVB (Column M).

Stage 2 water samples were collected and analyzed to determine primary stress responses to doses of UVB radiation in female *X. couchianus* (Fig. 4; Table 1, Column A-M). In response to sham treatments cortisol production rates increased from stage 1, 11-fold (Column G) to 208.5 (± 4.20) pg L_s⁻¹ h⁻¹. In response to doses of 8 kJ/m² UVB, cortisol production rates increased from stage 1, 9-fold (Column H) to 167.5 (± 4.20) pg L_s⁻¹ h⁻¹. In response to doses of 16 kJ/m² UVB, cortisol production rates increased from stage 1, 5-fold (Column I) to 103.6 (± 4.20) pg L_s⁻¹ h⁻¹. In response to doses of 32 kJ/m² UVB, cortisol production rates increased from stage 1, 2-fold (Column J) to 37.5 (± 4.20) pg L_s⁻¹ h⁻¹. Cortisol production rates were lower in response to doses of UVB radiation than to sham treatments. Cortisol production rates were at 80% of the observed response to sham treatments in response to 8 kJ/m² UVB (Column K). Cortisol production rates were at 50% of the observed response to sham treatments in response to 16 kJ/m² UVB (Column L); and cortisol production rates were at 18% of the observed response to sham treatments in response to 32 kJ/m² UVB (Column M).

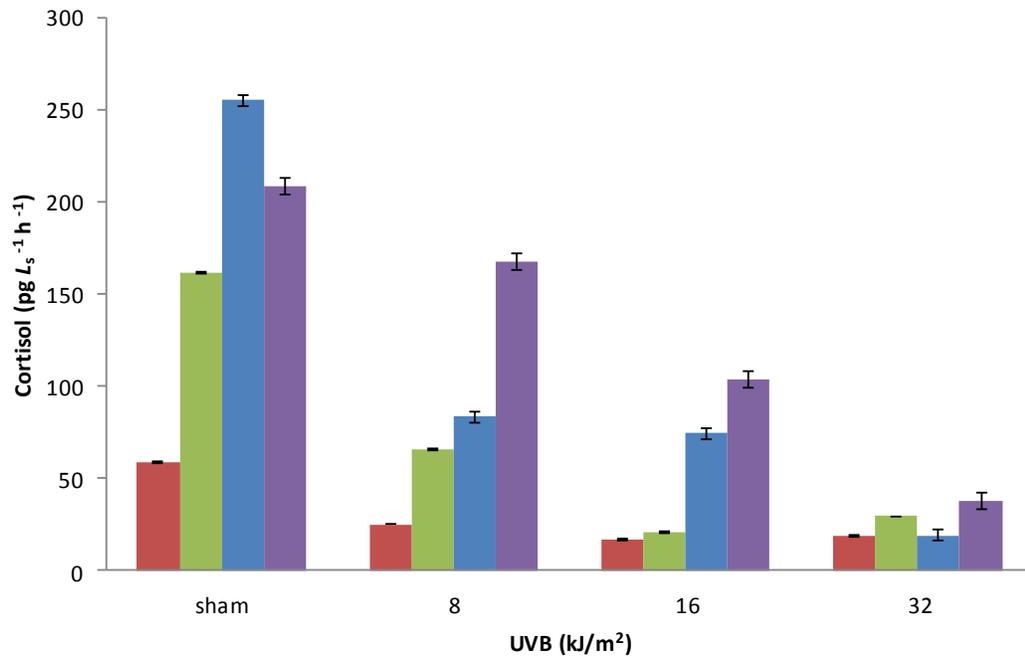


Figure 4. **Cortisol Production Rates in Response to UVB.** Stage 2

water analyses revealed primary stress responses to sham treatments and doses of UVB. Cortisol production rates were significantly higher in response to sham treatments than to doses of UVB. Dose of 32 kJ/m² UVB induced the lowest rates of cortisol production. *X. maculatus* Jp 163 B (red) produced cortisol at rates between 16.8 and 58.9 (± 0.39) pg $L_s^{-1} h^{-1}$. *Xm-Xh* F₁ hybrids (green) produced cortisol at rates between 21.0 and 161.6 (± 0.41) pg $L_s^{-1} h^{-1}$. Male *X. couchianus* (blue) produced cortisol at rates between 18.9 and 255.1 (± 0.39) pg $L_s^{-1} h^{-1}$. Female *X. couchianus* (purple) produced cortisol at rates between 37.5 and 208.5 (± 0.39) pg $L_s^{-1} h^{-1}$.

Comparing the fold-change of cortisol production rates between stage 1 and stage 2 revealed the greatest primary stress response in sham treatments of F₁ hybrids, a 51-fold increase (Column G). The least fold-change was in response to 32 kJ/m² UVB in male *X. couchianus*, a 1.1-fold change (Column J). Male *X. couchianus* revealed the greatest difference in cortisol production between sham treatments and 32 kJ/m² UVB, by producing cortisol at 7% of the rate observed in sham treatments in response to 32 kJ/m² UVB (Column M). No significant change was observed at doses of 8 kJ/m² UVB for female *X. couchianus*.

2.4 Discussion

UVB radiation can induce genetic damage in the form of cyclobutane pyrimidine dimers (CPDs; Mitchell et al. 2004). RIA results revealed that CPDs increased in a dose dependent manner in experimental UVB exposures (Fig. 2). The highest levels of CPDs were detected in *X. couchianus* after exposure to 32 kJ/m² UVB. *X. maculatus* Jp 163 B skin incurred less damage than did *X. couchianus* at any given dose, and the and F₁ hybrid showed less damage than the *X. maculatus* JP 163 B or *X. couchianus*. These differences in damage per UVB exposure appeared enhanced at 32 kJ/m² UVB where *X. couchianus* incurred over 2-fold more CPD damage per megabase of genomic DNA (Fig. 2). The *Xm-Xh* F₁ hybrids showed the least damage across all doses of UVB perhaps indicating the protective effects of melanin in regard to direct UVB induced DNA damage. The results presented (Fig. 2) are consistent with melanin's proposed function, since fish showing the highest degree of melanin pigmentation (i.e., F₁ hybrids) also show the lowest levels of induced genetic damage in the form of CPDs.

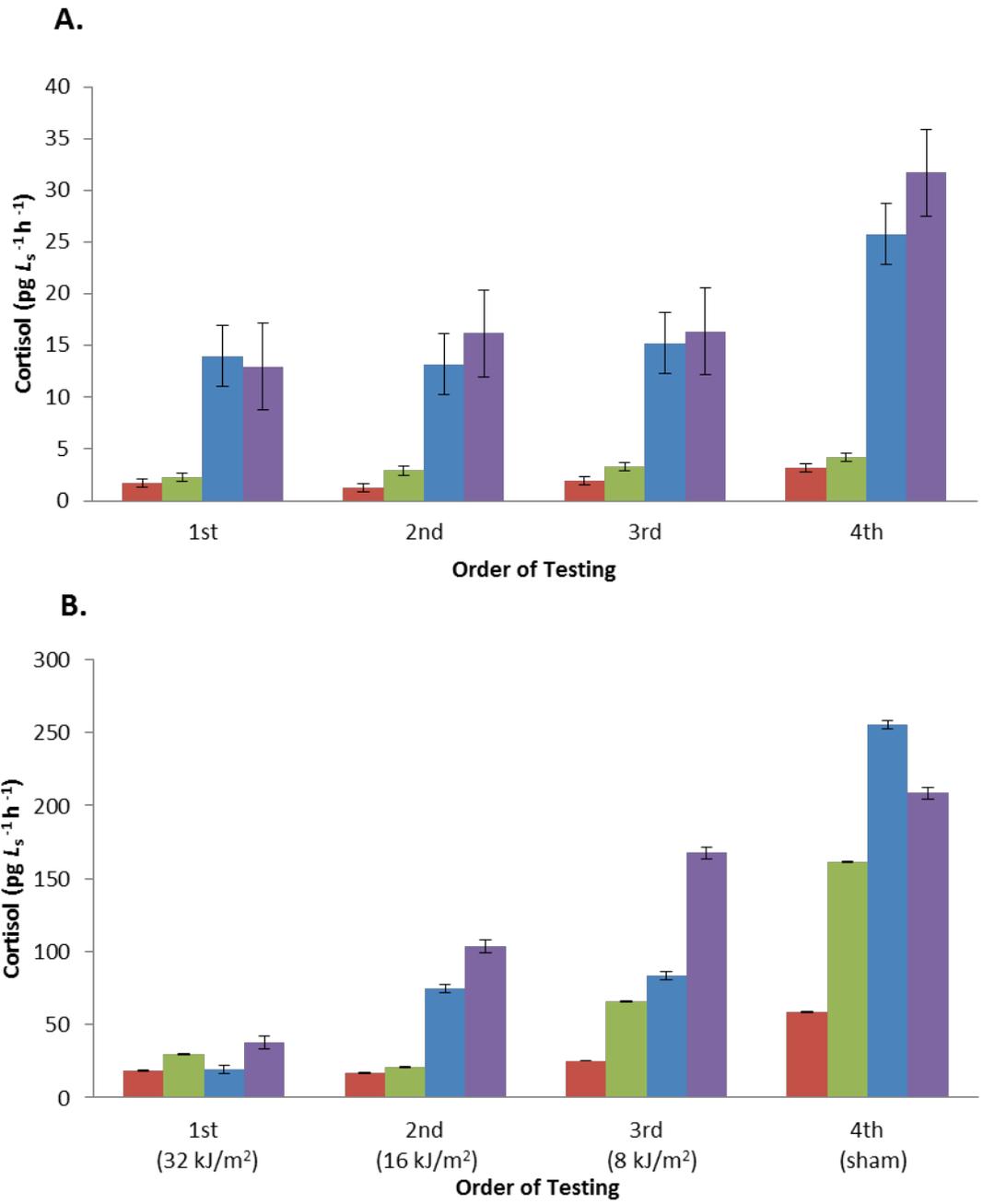
Water samples from stage 1 were analyzed to establish baseline cortisol production rates in the tested *Xiphophorus* fish types (Fig. 3). Female *X. couchianus* produced baseline cortisol at 19.3 (±4.2) pg L_s⁻¹ h⁻¹ and males at 17.0 (±2.9) pg L_s⁻¹ h⁻¹. Both *X. maculatus* Jp 163 B and F₁ hybrids had similar cortisol production rates in stage 1, producing about 1/4th the cortisol (less than 5 pg L_s⁻¹ h⁻¹) upon overnight incubation in the dark as did the *X. couchianus* (see Table 1). Macromelanophores are a special cell type derived from the embryonic neural crest and appear as black pigmented spots in some fish. *X. maculatus* Jp 163 B

carries the *Sp* pigment pattern resulting in punctate macromelanophores compartmentalized on the flank of the animal; in contrast, *Xm-Xh* hybrids express a very much enhanced *Sp* pigment phenotype where the black pigment is becomes established and covers over half of their body (Fig. 3; Walter et al., 2006). In contrast, *X. couchianus* do not possess the *Sp* pigment pattern and thus do not express macromelanophore derived pigment patterns. It is unclear why less pigmented fish (i.e., *X. couchianus*) would show higher cortisol production than pigmented ones after a long incubation in the dark. Perhaps this is simply due to differences between species that are 4-6 million years diverged. In this case *X. couchianus* produces a constitutive gene expression level for stress response genes that is higher than *X. maculatus* and the *Xm-Xh* hybrids assume the *X. maculatus* pattern (Fig. 3). This is consistent with past observations on complex trait phenotypes such as DNA repair capability (Walter et al., 2001), where interspecies hybrids take on the phenotype of one or the other parental line. Due to these findings, an interesting avenue for further studies would be to determine stress responses in the next generation of hybrids (i.e., the *Xm-Xh* hybrid backcrossed to the *X. couchianus* parent). Analysis of the stress response in the backcross hybrids may allow one to determine which genes lead to the species specific stress constitutively high stress hormone levels in *X. couchianus*. Another interesting avenue would be to utilize albino mutant and wild type fishes from the same species to further investigate the relationship between cortisol production and pigment phenotype.

Cortisol production rates in stage 2 were significantly lower in response to UVB exposure compared to the sham treatment (Fig. 4). Over the range of UVB exposures, *X. couchianus* consistently produced cortisol at the highest rate compared to the F₁ hybrid and *X. maculatus* Jp 163 B that produced cortisol at the lowest rate. Surprisingly, the greatest increase in cortisol production was observed in response to sham treatments, while 32 kJ/m² UVB induced the least increase from stage 1 cortisol production rates. To address this, we re-evaluated the data and organized it with regard to the time and position the fish were taken for UVB or sham treatments. The manner this experiment was performed is opposite to the order shown in Figure 4, that is; all fish were stored in the same cabinet overnight in the dark, then the 32 kJ/m² UVB exposure fish were removed from the cabinet, exposed to UVB and placed into post-UV incubation. Following this, the 16 kJ/m² UVB exposure fish were removed from the cabinet, and treated, followed by the 8 kJ/m² UVB and sham treated fishes. If we express the data based on the order in which the fish were removed from overnight incubation, it appears that cortisol production rates increase in a time or experimental treatment dependent manner (Fig. 5). This was likely due to the experimental set-up; eight fish were isolated in each beaker for overnight incubation, from this beaker two fish were periodically removed for each treatment in the order listed above. Thus, fish were exposed to the potentially stressful stimuli of netting two animals prior to being exposed to the experimental stimulus, UVB. Prior to the 4th test, there was a significant increase in stage 1 cortisol production rates indicating a stress response had occurred.

Figure. 5. Cortisol Production Rates in Response to Testing Order.

Unexpected observations required an evaluation of cortisol production rates in regards to order of testing. Stage 1 (A) cortisol production rates were not consistent and revealed increases prior to the 4th test. Stage 2 (B) revealed increases in cortisol production rates relative to order of testing. *X. maculatus* Jp 163 B (red) *Xm-Xh* F₁ hybrids (green) male *X. couchianus* (blue) and female *X. couchianus* (purple) produced cortisol at the lowest rates in response to the 1st, 2nd and 3rd test but produced cortisol at the highest rates in response to the 4th test.



UVB radiation was not a clear source of stress as reflected by stage 2 cortisol production rates. Rather, UVB radiation may have reduced circulating cortisol by enhancing degradation or impeding synthesis. The stress response to sham treatments suggests that experimental handling and confinement were the primary stressors and not the insult of UVB induced DNA damage. Our working hypothesis correlates the development of macromelanophores in the skin to low rates of cortisol production (less than $5 \text{ pg } L_s^{-1} \text{ h}^{-1}$), as observed in *X. maculatus* Jp 163 B and F₁ hybrids. However, stage 1 and stage 2 cortisol production rates may be regulated and influenced by other physiological differences between species. These results underscore the need to fully consider and minimize fish handling in experimental protocols unless changes in the gene and protein level that result from the general stress response are of no consequence to the experimental goal; however, an experiment where stress considerations would not be important to a result is considered to be quite rare.

Chapter 3

Effects of Pigmentation on Cortisol Production

3.1 Introduction

Results in Chapter 2 indicated that fish were responding to stress prior to experimental UVB treatments. To attempt to reduce stressful stimuli we employed a new experimental protocol wherein fewer fish were isolated on days before testing, and fewer fish were tested daily. Only one fish was isolated per beaker during overnight dark incubation (stage 1) and only two fish were tested per day. Additional changes to the experiment included stage 3 water sampling. Stage 3 water samples were collected to analyze the recovery of baseline cortisol production rates. Furthermore, the experiment was designed to test the hypothesis that pigment phenotypes correlated with stress response. Thus, *X. hellerii* Lancetilla (*La*) line of swordtails was chosen for these experiments because there are isogenic wild type and albino phenotypes available in the XGSC that could provide a control for pigmentation.

Different cell types of the skin can produce hormones, but melanocytes are the primary source of hormones that respond to cutaneous stress from heat and radiation. Albinos do not produce melanin, and this is often due to a loss of tyrosinase function (Fu et al., 2000). Tyrosinase, also known as tyrosine

hydrogenase, is an oxidoreductase responsible for melanogenesis and reducing reactive oxygen species (ROS) generating compounds produced by UV radiation, such as tyrosyl radicals (Garcia-Molina, 2005). In the absence of tyrosinase, melanin cannot be synthesized to provide cellular protection from UV radiation. Tyrosinase is also important for producing catecholamines (e.g., dopamine and serotonin) that have significant effects on neurological activities such as learning and HPA-axis stimulation (Kvetnansky et al., 2009). In the absence of tyrosinase, an irregular low concentration of these catecholamines could dysregulate neurological responses to environmental stimuli, including potential threats to homeostasis.

Pigmented fish are protected from UVB radiation, but albinos lacking melanin are susceptible to more genetic and cellular damage (Garcia-Molina, 2005). Because albinos are more susceptible to damage from UVB radiation, it was expected that they would have a greater stress response to UVB radiation than to sham treatments. It was also expected that albinos would reveal a greater stress response than wild type fishes in response to UVB treatments due to the greater amount of damage incurred.

3.2 Material and Methods

***Xiphophorus* strains and handling**

Fish were maintained as pedigreed lines according to standard protocols by the *Xiphophorus* Genetic Stock Center (XGSC) at Texas State University. Analyses were conducted for wild type and albino *X. hellerii* (La).

Fish Handling and Isolation

The day before testing, individual fish were isolated by phenotype into separate 600 mL beakers containing 300 mL of filtered home aquaria water. Isolation beakers were kept in the dark near the radiation chamber in an undisturbed room, and trials began the following morning. Feedings were not performed during isolation or the morning of testing. Overnight isolation defined stage 1 of the experiment (Fig. 1).

UVB Radiation

For each treatment, fish were transferred into a single clear plastic cuvette containing 75 mL of filtered tank water under yellow incandescent light. The cuvette was placed at the center of the radiation chamber and exposed to a sham or specified dose of UVB radiation as previously described. Treatments to fish in the radiation chamber defined stage 2 of the experiment (Fig. 1). Post-treatment, fish were removed from exposure cuvettes and isolated in the dark in separate 600 mL beakers containing 300 mL of filtered home aquaria water. Post-treatment isolation defined stage 3 of the experiment (Fig. 1).

Sampling and Hormone Analysis

Immediately preceding treatments, stage 1 water samples of 100 mL were collected from overnight isolation beakers. Water was poured directly from isolation beakers after removing fish. Immediately following exposure to UVB or sham treatments, stage 2 water samples of 75 mL were collected from the cuvettes by pouring contents into a beaker through a net. Netted fish were immediately moved into post-treatment isolation beakers in the dark. At 2 h and 6 h post-treatment, stage 3 water samples of 100 mL were collected by pouring water directly from isolation beakers. No lights were used during stage 3 isolation, except for the yellow incandescent light during dissections. At 6 h post-treatment fish were sacrificed and dissected for skin RNA isolation as previously described (Chapter 2). Tissue samples were stored at -80°C , and water samples were stored frozen at -20°C . Hormone extraction and sample analyses were performed as previously described (Chapter 2).

3.3 Results

This experimental protocol was adjusted to allow testing of two fish per day to reduce disturbance during stage 1. Stage 3 was included in this protocol to assess recovery of baseline cortisol production rates at 2 h and 6 h post-UVB treatment. Wild type and albino *X. hellerii* (*La*) were used in these experiments to assess the contribution of melanin pigmentation to the stress response. *X. hellerii*, like *X. couchnianus*, do not produce macromelanophores. The wild type and albino *X. hellerii* are isogenic and thus may be considered to differ only in their ability to express tyrosinase, a requirement for melanogenesis.

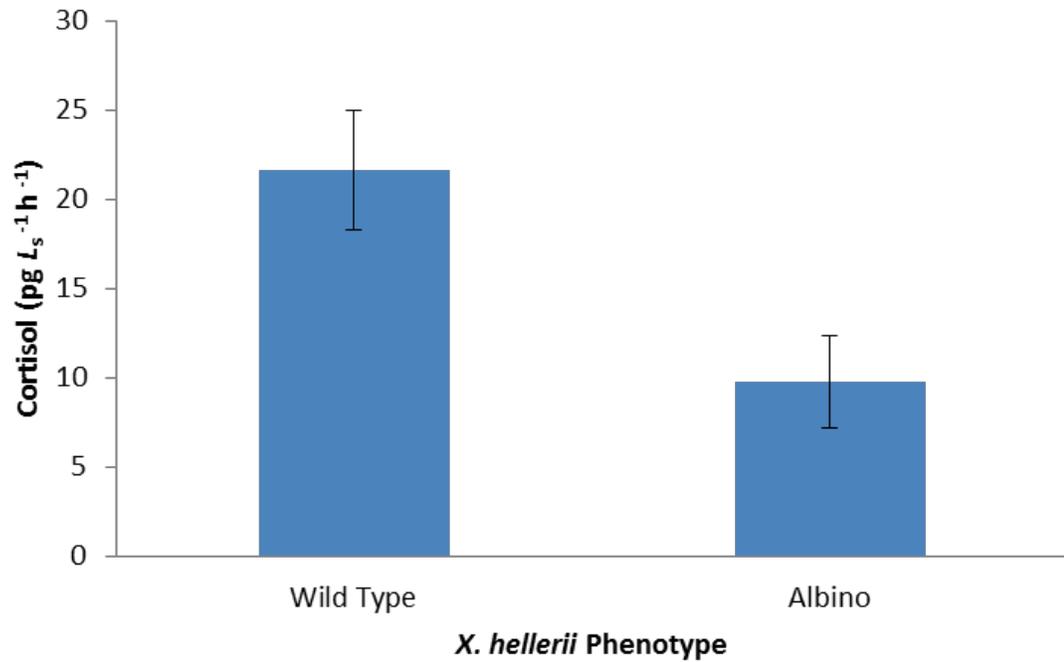
Stage 1 water samples were collected and analyzed to determine baseline cortisol production rates (Fig. 6; Table 2, Column A). Wild type *X. hellerii* produced cortisol at $21.6 (\pm 3.34) \text{ pg } L_s^{-1} \text{ h}^{-1}$, and albino *X. hellerii* produced cortisol at $9.8 (\pm 2.58) \text{ pg } L_s^{-1} \text{ h}^{-1}$.

Table 2. Cortisol Production Rates Analyzed for Wild Type and Albino *X. hellerii* Lancelilla. Fold change analyses were conducted to compare stage 1 to stage 2 cortisol production rates and to compare stage 2 cortisol production rates from sham and UVB treatments. Cortisol production rates were also analyzed during recovery and compared to stage 2 cortisol production rates.

		UVB Exposures				Recovery								
		Cortisol ($\text{pg L}_s^{-1} \text{h}^{-1}$)		Fold Change		Cortisol ($\text{pg L}_s^{-1} \text{h}^{-1}$)				Fold Change from Stage 2				
Stage 1	Stage 2	Stage 2		Stage 1 to Stage 2	Stage 2 sham to Stage 2 UVB	Stage 3 T=2 h	Stage 3 T=6 h	Stage 3 T=2 h	Stage 3 T=6 h	Stage 3 T=2 h	Stage 3 T=6 h	Stage 3 T=2 h	Stage 3 T=6 h	
Avg.	sham	32 kJ/m ²	SE ¹	sham	32 kJ/m ²	sham		sham		sham		32 kJ/m ²		
	B	C	D	E	F	G	H	I	J	K	L	M	N	O
21.6	166.9	60.7	±3.34	8	3	0.36	75.3	23.0	45.1	14.3	2	7	1.3	4
9.8	7.7	33.1	±2.58	0.8	3	4	9.37	5.21	24.5	14.6	0.8	1.5	1.4	2

1. SE, standard errors were calculated per test group from six stage 1 measurements

Column
X. hellerii (WT)
X. hellerii (Albino)



A.



B.



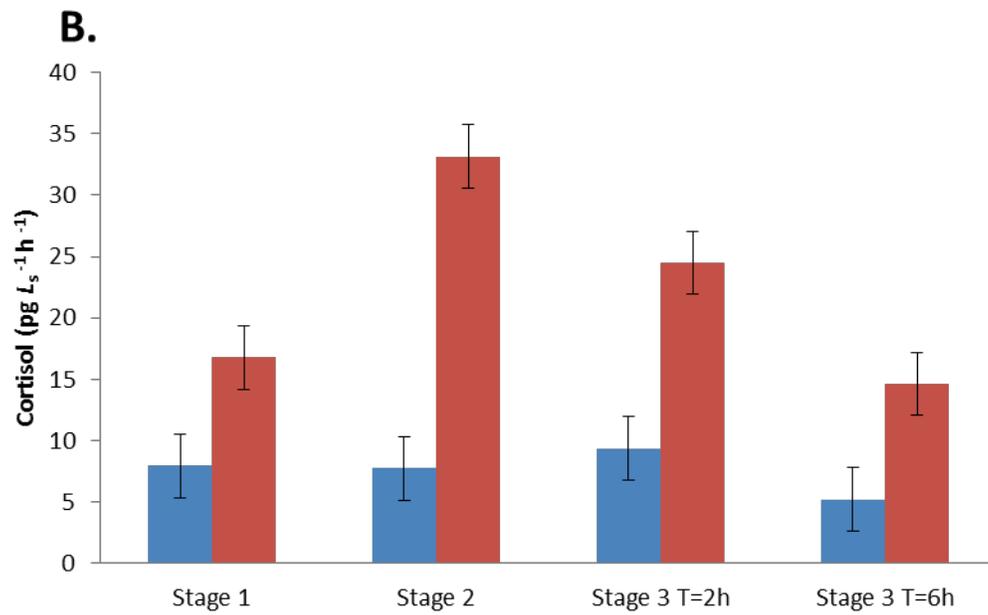
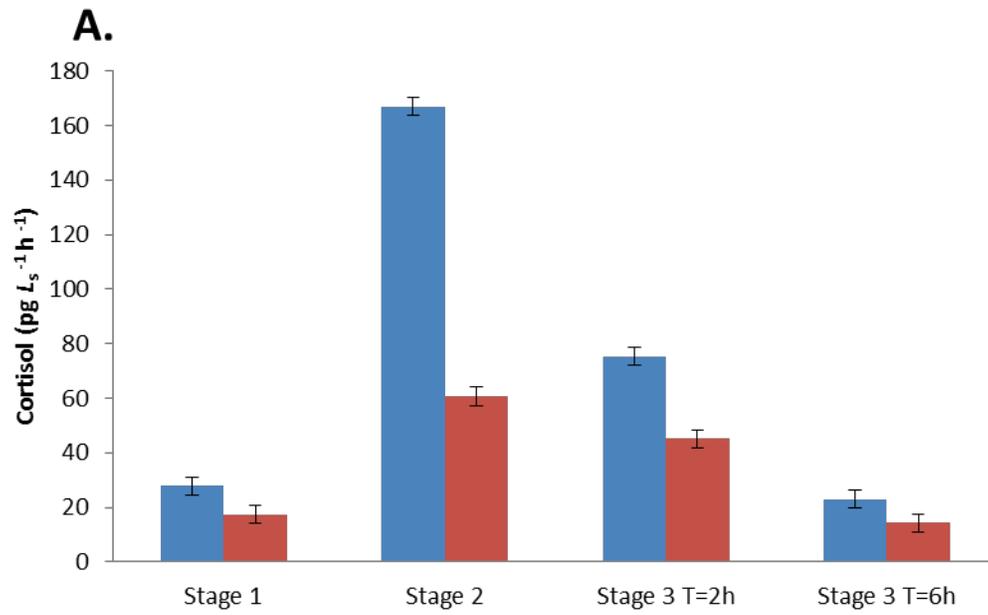
Figure 6. **Baseline Cortisol Production Rates in *Xiphophorus hellerii*.**

Wild type (A; cortisol at $21.6 (\pm 3.34) \text{ pg } L_s^{-1} \text{ h}^{-1}$), and albino *X. hellerii* (B; cortisol at $9.8 (\pm 2.58) \text{ pg } L_s^{-1} \text{ h}^{-1}$).

Stage 2 water samples were collected and analyzed to determine primary stress responses to doses of UVB radiation in wild type *X. hellerii* (Fig. 7; Table 2, Column A-K). In response to sham treatments, cortisol production rates increased relative to stage 1, 8-fold (Column E) to $166.9 (\pm 3.34) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to doses of 32 kJ/m^2 UVB, cortisol production rates increased relative to stage 1, 3-fold (Column F) to $60.7 (\pm 3.34) \text{ pg } L_s^{-1} \text{ h}^{-1}$. Cortisol production rates were significantly lower in response to various UVB doses than to sham treatments. Doses of 32 kJ/m^2 UVB induced cortisol production rates at 36% of the rate observed in sham treatments (Column G).

Stage 2 water samples were collected and analyzed to determine biological response after various UVB exposures for albino *X. hellerii* (Fig. 7; Table 2, Column A-K). In response to sham treatments, cortisol production rates did not significantly change from stage 1 (Column E), producing cortisol at $7.7 (\pm 2.58) \text{ pg } L_s^{-1} \text{ h}^{-1}$. In response to 32 kJ/m^2 UVB, cortisol production rates increased relative to stage 1, 3-fold (Column F) to $33.1 (\pm 2.58) \text{ pg } L_s^{-1} \text{ h}^{-1}$. Cortisol production rates were significantly higher in response to UVB exposure than to sham treatments. Doses of 32 kJ/m^2 UVB induced cortisol production rates 4-fold greater than rates induced by sham treatments (Column G).

Figure 7. **Cortisol Production Rates in Response to UVB.** Wild type (A) and albino *X. hellerii* (B) revealed unique responses. Stage 1, 2, and 3 cortisol production rates revealed baseline cortisol production rates, primary stress responses, and recovery from sham treatments (blue) and after doses of 32 kJ/m² UVB (red). Wild type *X. hellerii* (A) produced cortisol at 166.9 (± 3.34) pg L_s⁻¹ h⁻¹ in response to sham treatments and 60.7 (± 3.34) pg L_s⁻¹ h⁻¹ in response to 32 kJ/m² UVB. Wild type *X. hellerii* significantly increased cortisol production in response to both treatments. Albino *X. hellerii* (B) produced cortisol at 7.7 (± 2.58) pg L_s⁻¹ h⁻¹ in response to sham treatments and 33.1 (± 2.58) pg L_s⁻¹ h⁻¹ in response to doses of 32 kJ/m² UVB. Albino *X. hellerii* significantly increased cortisol production in response to 32 kJ/m² UVB and did not respond to sham treatments. Recovery was not observed in stage 3 at 2 h, but seemed to return to baseline cortisol production rates by 6 h post-treatment.



Comparison of wild type and albino *X. hellerii* revealed two types of responses. The wild type responses to sham treatments revealed an 8-fold increase in cortisol production rates from stage 1, but albino responses revealed no significant fold change in cortisol production rates (Table 2, Column E). Wild type responses to doses of 32 kJ/m² UVB reveal a 3-fold increase in cortisol production rates from stage 1, similar to the 3-fold change observed in albino responses (Table 2, Column F). Doses of 32 kJ/m² UVB induced wild type cortisol production rates at 36% of the rate observed in response to sham treatments; in contrast 32 kJ/m² UVB increased albino cortisol production rates 4-fold.

Stage 3 water samples were collected at 2 h and 6 h post-UVB treatment in wild type *X. hellerii* and compared to stage 2 cortisol production rates (Fig. 7; Table 2, Column H-K). At 2 h post-sham treatment cortisol production rates had decreased 2-fold to 75.3 (± 3.34) pg L_s⁻¹ h⁻¹ (Compare column H and B). At 6 h post-sham treatments cortisol production rates had further decreased 7-fold to 23.0 (± 3.34) pg L_s⁻¹ h⁻¹ (Compare column I and B). At 2 h post-UVB treatments cortisol production rates had decreased 1.3-fold to 45.1 (± 3.34) pg L_s⁻¹ h⁻¹ (Compare column J and C). Also, at 6 h post-UVB treatment cortisol production rates further decreased 4-fold to 14.3 (± 3.34) pg L_s⁻¹ h⁻¹ (Column K and C).

Stage 3 water samples were collected at 2 h and 6 h post-treatment for albino *X. hellerii* and compared to stage 2 cortisol production rates (Fig. 7; Table 2, Column H-K). At T=2 h post-sham treatments, cortisol production rates had increased 1.2-fold to 9.4 (± 2.58) pg L_s⁻¹ h⁻¹ (Compare column H and B). At 6 h

post-sham treatments, cortisol production rates had decreased 1.5-fold to 5.2 (± 2.58) $\text{pg L}_s^{-1} \text{h}^{-1}$ (Compare column I and B). At 2 h post-UVB treatments, cortisol production rates had decreased 1.4-fold to 24.5 (± 2.58) $\text{pg L}_s^{-1} \text{h}^{-1}$ (Compare column J and C). At 6 h post-UVB treatments, cortisol production rates had further decreased 2-fold to 14.6 (± 2.58) $\text{pg L}_s^{-1} \text{h}^{-1}$ (Compare column K to C).

Comparison of recovery cortisol production rates revealed that wild type *X. hellerii* recovered more quickly from primary stress responses than albinos (Table 2, Column L-O). At T=2 h post-sham treatments, wild type fish had decreased 2-fold from stage 2 cortisol production rates, though albinos had not yet decreased (Column L). Rather, a 1.2-fold increase in their cortisol production rate was detected. At T=6 h post-sham treatments, wild type fish had further decreased 8-fold, though albinos had only decreased 1.5-fold from stage 2 cortisol production rates (Column M). At T=2 h post-UVB treatments, wild type and albino fish had recovered in a similar manner, with a 1.3-fold and 1.4-fold decrease in cortisol production rates, respectively (Column N). However, at T=6 h post-UVB treatments, wild type cortisol production rates had decreased 4-fold, though albinos had only decreased cortisol production rates 2-fold (Column O).

Stage 1 cortisol production rates were compared to analyze the consistency of baseline cortisol production between *X. hellerii* biological duplicates (Fig. 8). In wild type fish, baseline cortisol production rates were similar on the first day of testing at 20.1 and 20.36 (± 3.34) $\text{pg L}_s^{-1} \text{h}^{-1}$, but there was a difference of 35.5 and 14.4 (± 3.34) $\text{pg L}_s^{-1} \text{h}^{-1}$ between fish on the second

day of testing. In albino fish, there was a difference of 8.8 and 19.8 (± 2.58) $\text{pg L}_s^{-1} \text{h}^{-1}$ on the first day of testing and a difference of 7.1 and 13.7 (± 2.58) $\text{pg L}_s^{-1} \text{h}^{-1}$ on the second day of testing.

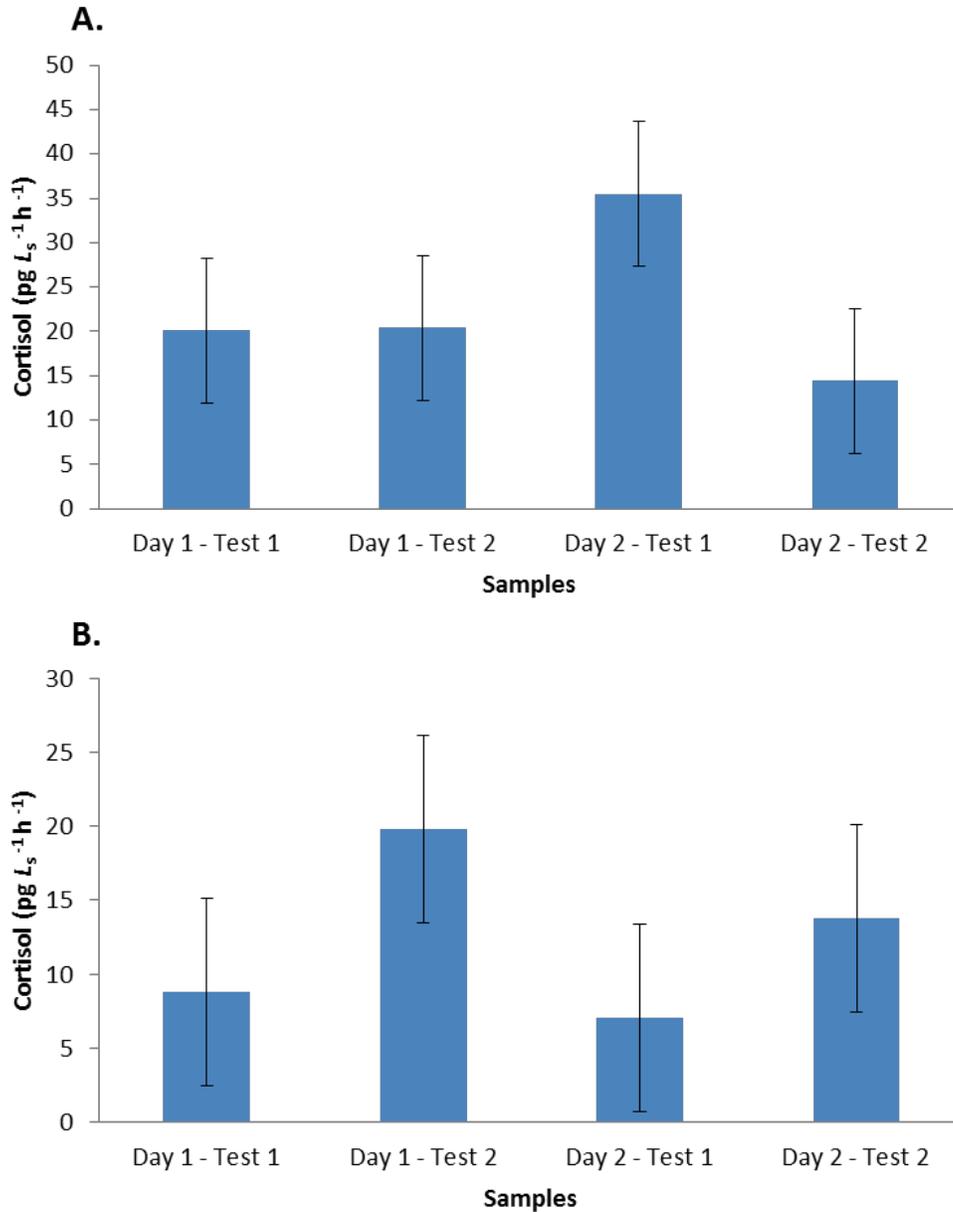


Figure 8. Cortisol Production Rates in Response to Testing Order.

Stage 1 cortisol production rates were compared in wild type (A) and albino (B) *X. hellerii* to evaluate preliminary stress responses relative to the order of testing. Baseline cortisol production rates were not significantly different between test fish except in wild type *X. hellerii* day 2-test 2.

A summary of stage 2 cortisol production rates provided a comparison between responses to sham treatments and 32 kJ/m² UVB to illustrate the two types of responses (Fig. 9). Wild type *X. hellerii* cortisol production rates were lower in response to 32 kJ/m² UVB compared to sham treatments, whereas *X. hellerii* albino cortisol production rates were higher in response to 32 kJ/m² UVB compared to sham treatments. The wild type response was comparable to responses observed in Chapter 2. However, the albino response was not comparable to previous observations.

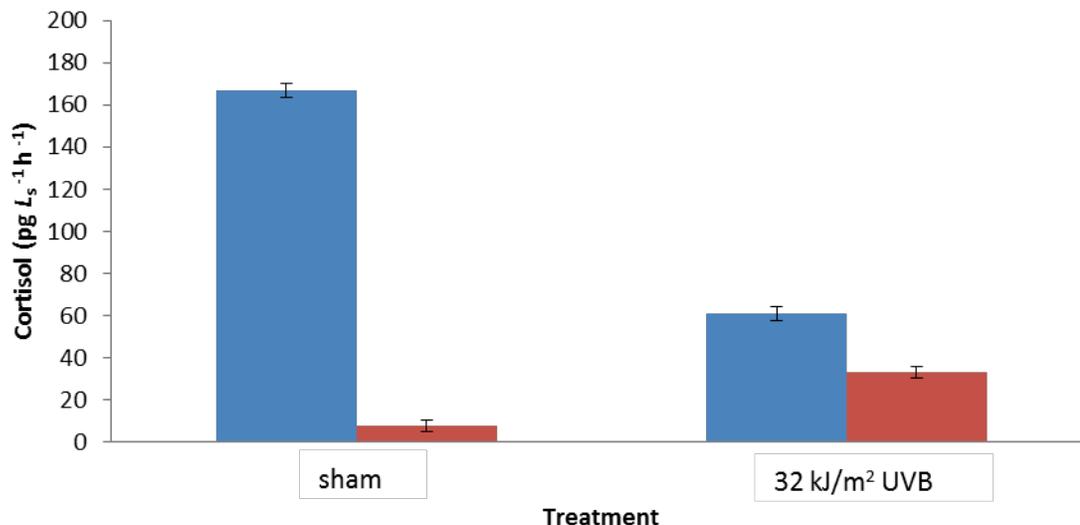


Figure 9. Compared Cortisol Production Rates in Response to UVB.

A scaled comparison of cortisol production rates in response to sham treatments and 32 kJ/m² UVB in wild type (blue) and albino (red) *X. hellerii* facilitated the evaluation of stress responses. Wild type *X. hellerii* (A) produced cortisol at 166.9 (± 3.34) pg $L_s^{-1} h^{-1}$ in response to sham treatments and 60.7 (± 3.34) pg $L_s^{-1} h^{-1}$ in response to 32 kJ/m² UVB. Wild type *X. hellerii* significantly increased cortisol production in response to both treatments but produced significantly less cortisol in response to 32 kJ/m² UVB than to sham treatments. Albino *X. hellerii* (B) produced cortisol at 7.7 (± 2.58) pg $L_s^{-1} h^{-1}$ in response to sham treatments and 33.1 (± 2.58) pg $L_s^{-1} h^{-1}$ in response to 32 kJ/m² UVB. Albino *X. hellerii* significantly increased cortisol production in response to doses of 32 kJ/m² UVB but did not significantly respond to sham treatment. Wild type *X. hellerii* produced significantly more cortisol than albinos in response to sham treatments or 32 kJ/m² UVB.

Cortisol production rates were analyzed in response to sham treatments and doses of 8, 16 and 32 kJ/m² UVB (Fig. 10). However, duplicate trials were run for sham treatments and doses of 32 kJ/m² UVB only. Fish were tested at doses of 8 and 16 kJ/m² UVB to observe trends in their stress responses, but these trials were unable to be duplicated due to lack of appropriate animals. Wild type *X. hellerii* cortisol production rates were lower in response to all doses of UVB compared to sham treatments. However, little can be said of the albino *X. hellerii* stress responses to UVB, because cortisol production rates were greater in response to 8 kJ/m² but lower in response to 16 kJ/m² UVB. Duplicate trials will be necessary to validate these suggested trends in the albino response to UVB.

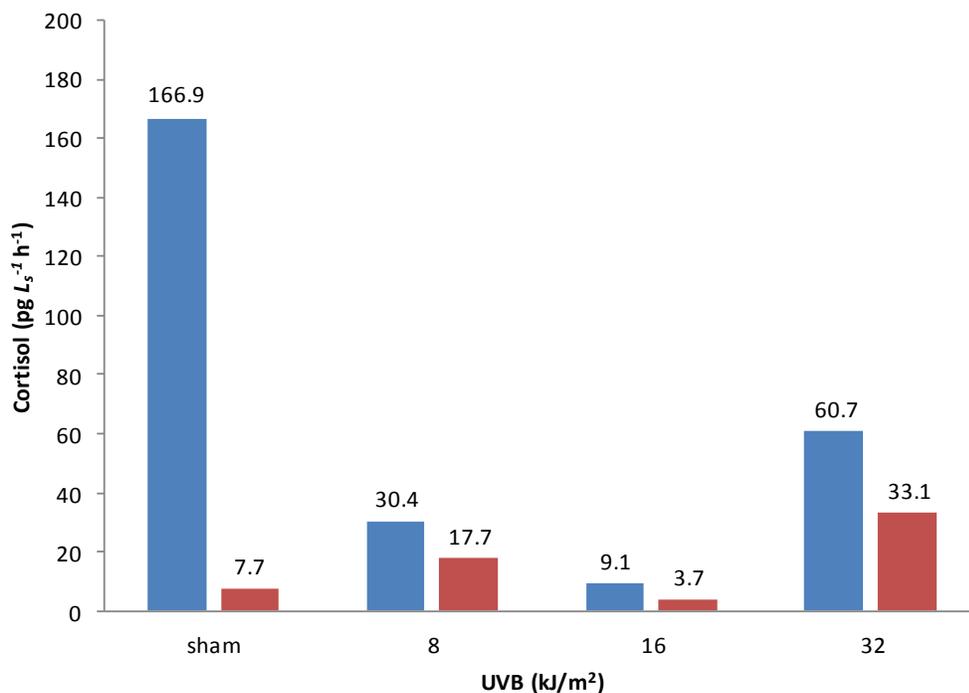


Figure 10. **Cortisol Production Rates in Response to Other Doses of UVB.** Stage 2 cortisol production rates decreased with increasing doses of UVB in wild type *X. hellerii* (blue) but were inconsistent in albinos (red). Duplicate trials were run for sham and 32 kJ/m² UVB only. Fish tested at doses of 8 and 16 kJ/m² UVB were unable to be duplicated due to lack of appropriate animals. Cortisol production rates determined from these trials are labeled in figure. Wild type *X. hellerii* cortisol production rates were lower in response to all doses of UVB compared to sham treatments. Albino *X. hellerii* cortisol production rates increased in response to 8 kJ/m² but decreased in response to 16 kJ/m² UVB as compared to sham treatments. Stress responses to sham treatments and doses of 32 kJ/m² UVB were presented in Figure 9.

3.4 Discussion

In Chapter 2, we concluded the experimental protocol in practice had induced a stress response during stage 1. Stress responses were detected between the 3rd and 4th test, but not between the 1st and 2nd or the 2nd and 3rd test. Stress in the previous experimental design may have been induced by cohousing of fish in the same beaker for overnight dark incubation resulting in having to go into the same beaker repeatedly to retrieve fish for each test. Thus, we decided to assess this by repeating the experiment, but in this new protocol, fish were isolated individually (one fish/beaker) for the dark incubation instead of by species group. This method of isolation reduced the netting process and handling time. Fish could be poured directly from beakers into a net, rinsed, and transferred to the exposure chambers all rather quickly and without having to disturb fish in the cohabiting group. In addition, in these experiments we analyzed recovery by observing cortisol production rates 6 h following the primary stress response. Results presented in this chapter investigated stress responses in the swordtail, *Xiphophorus hellerii* (La) that was utilized since we had available isogenic wild type and albino (i.e., tyrosinase negative) fish lines and therefore were able to observe the relationship between pigment phenotype and cortisol production rates. In these experiments, duplicate trials were run for sham treatments and 32 kJ/m² UVB exposures.

During stage 1, wild type *X. hellerii* cortisol production rates were 2-fold greater than albinos (Fig. 6). Chapter 2 results suggested that perhaps pigmentation correlates with stress related cortisol production. Wild type *X.*

hellerii do not produce macromelanophores and so cannot be directly compared to *X. maculatus* Jp 163 B or F₁ hybrids from the results presented in Chapter 2 (Fig. 3 and 6). However, pigmentation of wild type *X. hellerii* may be comparable to *X. couchianus* since neither species produce macromelanophores. Here we see that wild type *X. hellerii* cortisol production rates are comparable to *X. couchianus*, producing cortisol at 21.6 (± 3.34) pg L_s⁻¹ h⁻¹ and 19.3 (± 2.58) pg L_s⁻¹ h⁻¹ during stage 1, respectively (Fig. 11).

Wild type fish revealed a primary stress response that can be observed in sham and UVB treatments (Fig. 7). Wild type fish revealed a greater primary stress response to sham treatments than to UVB. This may be due to the melanogenic response to UVB that leads to POMC cleavage into α -MSH and ACTH (Slominski, 2010). Because POMC cleavage products inhibit activities of the HPA-axis, UVB could modulate negative feedback on the HPA-axis of pigmented fish thereby reducing cortisol production (Slominski et al., 2005). Thus, fishes that express higher levels of POMC would be expected to have a greater reduction in cortisol production rates due to the feedback response.

Albinos do not express tyrosinase, which is necessary to synthesize melanin. Thus, it was expected, as observed, that albinos would have a primary stress response to UVB (Fig. 7). However, a primary stress response to sham treatments could not be detected in albino fish. It is unlikely this is due to the short duration of the sham treatments (10 m 56 s) since an equivalent time was adequate to produce a stress response in wild type fish. However, it has been

noted in other species that different phenotypes may require more time for inducing a stress response (Pankhurst, 2011).

Stage 3 water samples were analyzed to observe cortisol production rates during recovery from stage 2 treatments (Fig. 7). At 2 h, cortisol production rates began to decline in wild type fish and they had recovered to stage 1 cortisol production rates (baseline) by 6 h post-exposure. Sham treated wild type fish showed the most rapid stress recovery and the greatest reduction in cortisol production at 6 h post-treatment (e.g., a 7-fold decrease from stage 2 cortisol production rates; Table 2, Column M). Wild type *X. hellerii* exposed to 32 kJ/m² UVB exhibited the slowest recovery, with little decline at 2 h (1.3-fold decrease from stage 2). However, the recovery of wild type fish was more efficient than that observed in albinos. Although we did not measure the DNA damage induced by UVB in this experiment we may assume from previous results (Chapter 2) that albino fish received more damage per UVB dose than wild type fish. If UVB induced damage in the albinos resulted in greater levels of cell death and a higher inflammatory response this may account for their slower recovery after UVB.

As stated, it is expected that albinos acquire more UVB-induced DNA damage and thus require more recovery time compared to wild type fish. However, we observed the albinos show a rather small or negligible increase, rather than decrease, in stage 3 cortisol production rates at 2 h post-sham treatments from 7.73 to 9.37 (± 2.58) pg L_s⁻¹ h⁻¹. This could be the result of a delayed stress response to stimuli from cell death that occurs in a delayed

timeframe after initial UVB damage (1.4-fold from stage 2 cortisol production rates at 2 h; Table 2, Column N). This was slower but similar to the wild type response. However, consistent with the delayed cell death, albinos did not appear to fully recover from 32 kJ/m² UVB even after 6 h post-treatment (Table 2, Compare column A to K).

In contrast to UVB treatments, albinos did not appear to have a primary stress response to sham treatments, as cortisol production rates did not increase from stage 1 to stage 2. Because pigment phenotypes provide variable levels of protection from the physical stress of UVB, albinos were expected to have primary stress responses that exceeded wild type response (i.e., higher rates of cortisol production). This was not the case. Wild type stage 2 cortisol production rates detected post-UVB treatments exceeded albino cortisol production rates. Albino stage 2 cortisol production rates detected post-sham provided additional evidence that cortisol physiology was affected in these tyrosinase-negative albinos (Baron, 2002). Tyrosinase is necessary to synthesize melanin, but it is also necessary to synthesize catecholamines, which are the primary neurotransmitters produced in a stress response (Kobayashi et al., 2000; Kvetnansky et al., 2009). Catecholaminergic neurons, that release catecholamines, innervate the hypothalamus and provide the first signal to release CRH in the primary stress response (Kvetnansky et al., 2009). In addition, disrupting catecholamine activity in the brain stem is known to block primary responses to physical stress (Kvetnansky et al., 2009). Thus, albinos may have delayed or blocked responses to the physical stress of confinement

and handling that were not detectable in the time frame of sham treatments. However, the duration of 32 kJ/m² UVB treatments provided sufficient time to respond to the physical stress of the experiment or begin responding to non-physical (perceived) stress.

We can conclude that UVB increased the albino cortisol production rates as it did with wild type fish. Albinos express POMC and can produce its cleavage products such as ATCH and α -MSH and thus their cells would not appear to contain melanin to initiate this UVB response. However, if higher levels of DNA damage led to increased levels of cell death, release of cellular components and apoptotic signals from dead cells may serve to initiate the stress response.

Due to uncontrolled stress responses observed during stage 1 in Chapter 2, obtaining consistent measurements for stage 1 cortisol production became an important goal in the experiments of Chapter 3. Changes to the experimental protocol employed resulted in more consistent baseline cortisol production rates. Stage 1 cortisol production rates were most consistent on Day 1 (Fig. 8). On Day 2, there was a significant difference observed between wild type *X. hellerii* experimental duplicates. The first fish produced cortisol at 35.5 (± 3.34) pg L_s⁻¹ h⁻¹ and the second test fish produced cortisol at 14.4 (± 3.34) pg L_s⁻¹ h⁻¹. This could have been an effect of the set-up or perhaps just variation between individual fish physiology. Obtaining consistent baseline cortisol measurements is an important challenge in stress research that may require repeat sampling beyond a single duplicate trial. Duplicate measurements help establish a mean baseline for cortisol production rates in a species. However, cortisol production rates may

occupy a broad range, especially in social species where dominant males will often produce cortisol at higher rates while subordinates produce cortisol at significantly lower rates (Hoglund et al., 2000; Kittlesin et al., 2009). Social order and stress behaviors often correlate to baseline cortisol production rates.

Our fish were housed and maintained by the XGSC. In these limited populations, one may question how the social order has been affected. For example, salmonid fish can diverge into stress-response phenotypes within a population, high-response and low-response. Salmonids in these populations have evolved different stress-coping mechanisms in which subordinate low-response fish produce cortisol at lower rates and dominant high-response fish produce cortisol at higher rates (Overli et al., 2002). It may be of value to identify individual fish as low-response or high-response fish prior to designing experiments or preparing for analyses of their stress response.

Identifying different stress-coping mechanisms could provide an in-depth characterization of various stress responses in *Xiphophorus* fishes. As the stress-responses in these species become better defined, it may become clearer which parental alleles contributed towards which stress-coping mechanisms in hybrids and backcrosses. Characterizing these contributions will be a key part in understanding the stress response in UVB-inducible melanoma model. A molecular genetic analysis of the primary and secondary stress response in *Xiphophorus* fish may further elucidate the genetic activities that lead to genome instability and susceptibility to UVB-inducible melanomas.

Chapter 4

Conclusions

4.1 Transcriptomic Analysis of *X. maculatus* Jp 163 B Skin

During the course of the experiments detailed in Chapter 2, skin samples were collected for RNA isolation and Illumina based high-throughput sequencing (i.e., RNAseq). This type of RNAseq data gives a snapshot of all RNA transcripts that exist in cells at the time of RNA isolation and thus, may be used to assess relative transcript expression levels. The RNAseq data were analyzed for transcript RNA levels of known stress pathways as a measure of transcript expression in *X. maculatus* Jp 163 B. RNAseq data was normalized to total RNA reads to obtain a base mean expression value, and transcripts were mapped to the *X. maculatus* Jp 163 A transcriptome. RNA expression levels were analyzed in a pairwise comparison between sham and UVB exposed fish skin using DeSeq. A minimum read count cut-off of 50 reads was applied to the data; thus, transcripts that did not have at least 50 reads mapped in the transcriptome were not analyzed between groups. All data sets were compared for \log_2 (fold changes) with a significant cut-off p -value of <0.01 . Relevant transcripts that did not meet the cut-off criteria were filtered out from the final set of transcripts. In sham compared to 8 kJ/m² UVB, 348 transcripts were significantly differentially expressed, in sham compared to 16 kJ/m² UVB, 350 transcripts were significantly

differentially expressed and in sham compared to 32 kJ/m² UVB, 212 transcripts were significantly differentially expressed. These transcripts were then further analyzed to determine if they were differentially expressed and whether or not expression correlated with HPA stress and cortisol production rates.

Genes such as POMC, GR, MCR, 3 β -HSD and 11 β -HSD are some of the primary hormones, receptors, and enzymes responsible for initiating a primary stress response to synthesize and release cortisol into circulation. The transcriptome data set contained several of these genes (Table 3). However, many of them were not represented by 50 reads that was a statistical cut-off. This shows a well-known caveat of RNAseq data that may show infinite up-regulation but can only limit down-regulation from the higher level of transcript present in the samples. Therefore, it was concluded that the primary stress response transcripts are expressed in the skin, but too low a level to be analyzed in our dataset. Correlating their activity, would require more sensitive measurements using much higher read coverage. Differential expression of the primary response genes could not be concluded although differential read counts of their transcripts did appear in the raw data.

The UVB response involves expression of melanogenic receptors and hormones derived from POMC that coincide with cortisol synthesis. It was expected that these genes and peptide hormones could be differentially expressed in the skin during a stress response (Slominski et al., 2007; Slominski et al., 2005). In the presence of UVB, it was expected that cellular stress could also induce differential expression of melanogenic pathways. However, the data

did not indicate significant differential expression of melanogenic or secondary stress response genes. Many stress-research scientists have quantified expression of such genes from the brain and adrenal tissues, because these organs constitute the primary initiators of the HPA-axis (Hoglund et al., 2000; Johansen et al., 2011). Secondary stress response genes with metabolic and immune functions were also searched within the pairwise comparison data set. Metabolic stress genes were not differentially regulated in the skin transcriptome, but these genes are most commonly expressed in the liver (Aluru and Vijayan, 2009) and not the skin. There are constitutive immunological defenses of the skin that respond to changing environments. However, the immunological stress genes were not differentially expressed in response to UVB treatments.

Secondary stress responses may not have been induced in sham and UVB treatments, if the primary HPA and cellular responses are sufficient. Secondary stress response genes may be up- or down-regulated beyond a certain threshold of cellular damage. However, none appeared to be differentially expressed in the filtered data. It is possible that transcript expression was also affected at low transcript levels that could not be detected in our data or was discarded during read filtration. The expression of these genes may be modulated by other cellular conditions and the state of HPA stress. Secondary stress responses may not be necessary in an organism that has managed cellular stress through other means.

Table 3. List of Primary and Secondary Stress Response Genes Analyzed for Differential Expression in the Skin of *X. maculatus* Jp 163 B.

<u>Primary Stress Response Genes</u>			<u>Associated with Primary Stress Response</u>		
Gene	Abreviations	Differentially Expressed?	Gene	Abreviations	Differentially Expressed?
Pro-Opiomelanocortin	POMC	N	Heat Shock Protein	Hspa4	N
Melanocortin receptor	MCR	N	Heat Shock Protein	Hsp90aa1	N
Mineralcorticoid Receptor	MR	N	GR Chaparones	Dnaja2	N
Glucocorticoid Receptor	GR	N	Prosteglandin Synthase	Ptges3	N
11 β Hydroxylase	Cyp11b1	N	Synaptotagmin Interacting Protein	Stip2	N
3 β Hydroxylase	Cyp3b	N	GR Chaparones	Fkbp4	N
21 α Hydroxylase	Cyp21a2	N	Peptidyl Prolyl isomerase D	Ppid	N
17 α Hydroxylase	Cyp17a1	N	Protein Phosphatase	Ppp5c	N
			GR Activators	Smarca4	N
			Lysine Acetyl Transferase	KAT2B	N
			CREB Binding Protein	Crebbp	N
			Nuclear Receptor Co-Activator 1	Ncoa1	N
			Prohormone convertase	PC2	N
<u>Secondary Stress Response Genes</u>					
Gene	Abreviations	Differentially Expressed?			
Interleukin 1B	IL-1B	N			
Interleukin 6	IL-6	N			
Interleukin 8	IL-8	N			
Tumor Necrosis Factor	TNF-α	N			
Nuclear Factor κ B	NF-κB	N			
Mouse Mammary Tumor Virus	MMTV	N			
Tyrosine Aminotransferase	TAT	N			
Glucocorticoid Induced Leucine Zipper	GILZ	N			
I κ B α	Iκ-Bα	N			
Phenyl-N-methyl-transferase	PNMT	N			
Phosphoenolpyruvate Carboxykinase	PEPCK	N			
Glucokinase	GK	N			

4.2 Xm-Xc F₁ Hybrids and Parental Responses to UVB

In a related study of light-induced damage and repair, an RIA for detecting UVB-induced CPDs revealed a relationship between DNA damage incurred in skin from UVB with levels of pigmentation in *X. maculatus* Jp 163 B, *X. couchianus*, and *Xm-Xh* F₁ interspecies hybrids. In this study, our goal was to characterize changes in cortisol production rates and differentially expressed stress transcripts in the skin upon UVB exposure. Stress responses to UVB were expected to correlate with CPDs and pigment levels due to the associated levels of DNA damage. We expected cortisol production to increase relative to UVB exposure in a dose dependent manner. We also expected to see a response in the skin transcriptome associated with cortisol production or secondary stress response genes.

X. maculatus Jp 163 B, *X. couchianus*, and *Xm-Xh* F₁ hybrids were exposed to sham and UVB treatments to observe primary stress responses. Stage 1 (baseline) cortisol production rates were determined (Fig. 11). *X. maculatus* Jp 163 B produced cortisol at the lowest rate. We found that *Xm-Xh* F₁ hybrids produced cortisol in a range between the parental species, but more similar to *X. maculatus* Jp 163 B. Male and female *X. couchianus* produced cortisol at the highest rate in these experiments, but there was no significant difference in cortisol production rates between sexes. The stage 2 stress response to treatments revealed that handling, not UVB, was the primary stressor, and that UVB affectively reduced the stress response to experimental handling.

In response to sham and UVB treatments, stage 2 cortisol production rates increased from stage 1 (baseline) in all fish. That is to say that every experiment induced some level of stress. However, cortisol production rates increased with the greatest fold change in response to sham treatments (Fig. 4). Stage 2 cortisol production rates maintained the order of male and female *X. couchianus* as the highest producer, *X. maculatus* as the lowest producer, and *Xm-Xh* F₁ hybrids producing cortisol between parental rates. UVB treatments increased cortisol production rates; however, the fold change was much lower at higher doses of UVB. Because *Xm-Xh* F₁ hybrids were the heaviest pigmented, but not the lowest producers of cortisol, it appeared that the stress response was species specific, not pigment specific. However, this was not conclusive. To further address pigment effects, we tested wild type and albino *X. hellerii*.

4.3 X. hellerii (La) Wild Type and Albino Responses to UVB

Albinos and wild type *X. hellerii* fish were exposed to sham and UVB treatments to observe primary stress responses. Stress recovery was also analyzed in *X. hellerii* to further characterize the stress response. Stage 1 (baseline) cortisol production rates were determined (Fig. 11). Albinos produced cortisol at one half the rate of wild type *X. hellerii*. The stage 2 stress responses to treatments in wild type *X. hellerii* was similar to previous responses that indicated handling, not UVB, was the primary stressor. Albinos revealed that UVB was the primary stressor, but this appeared to be an albino specific response.

Albinos produced cortisol at unexpectedly low rates (i.e., baseline) and in response to all treatments. Surprisingly, albino cortisol production rates did not increase from stage 1 in response to sham treatments as observed in all other fish. These unexpected results may be dependent upon tyrosinase or a downstream effect of its function. Albinos do not produce tyrosinase, which is needed for melanin and catecholamine synthesis. Without melanin, albinos are subject to more UVB damage than wild type fish, and the cellular stress response is probably quite different. However, cellular stress alone does not induce an HPA stress response. Initiating an HPA stress response to physical stress requires nerve stimulation and catecholamine release to signal the HPA-axis. Tyrosinase-negative albinos may lack the catecholamine concentrations necessary to induce an HPA stress response to the physical stress of handling, as observed in wild type fish. In response to UVB, a primary stress response

was induced. However, cortisol production rates were significantly lower than wild type. Because tyrosinase affects melanogenesis and the HPA stress response, albinos did not clarify the effects of pigment on the stress responses. Rather, they have revealed the inseparable effects of pigment and tyrosinase on stress.

Stage 3 recovery responses to stress could indicate the state of cellular damage or physical and psychological stress incurred from treatments. Wild type *X. hellerii* recovered baseline cortisol production rates in stage 3 suggesting that any stress was managed by the HPA-axis and cellular responses by 6 h post-treatment. An elevated concentration of circulating cortisol should correlate to the induction of secondary stress response genes that function to coordinate stress recovery. However, because cortisol production rates had returned to baseline, secondary stress response genes were not likely being induced.

Albino *X. hellerii* did not fully recover baseline cortisol production rates by 6 h post-UVB. Albinos were not protected by melanin and very likely had more genetic and cellular damage from UVB than did wild type fish. A cellular damage threshold may exist where UVB-induced cell death (apoptosis) induces the HPA primary stress response. This type of response would be imperative to evade further cellular damage and induce the secondary stress response genes that facilitate cellular and organismal recovery from stress.

We have analyzed the differential expression of secondary stress response transcripts in *X. maculatus* Jp 163 B skin exposed to sham and UVB treatments. *X. maculatus* Jp 163 B stress recovery was not analyzed. However,

they have the lowest cortisol production rate at baseline and in response to treatments. It would be expected that *X. maculatus* Jp 163 B recovery would occur by 6 h, because *X. hellerii* had higher cortisol production rates at stage 2 and recovered by 6 h post-treatment in stage 3. Differential expression of secondary stress response transcripts were not observed in any comparison between treatments. At the time of RNA isolation, circulating cortisol concentrations were not likely different from their baseline. One may expect to observe differential expression of secondary stress response genes in fish that are dissected at a time point when cortisol production rates are significantly higher than the baseline. In this scenario, secondary stress responses would be more likely induced and detectable in the skin transcriptome.

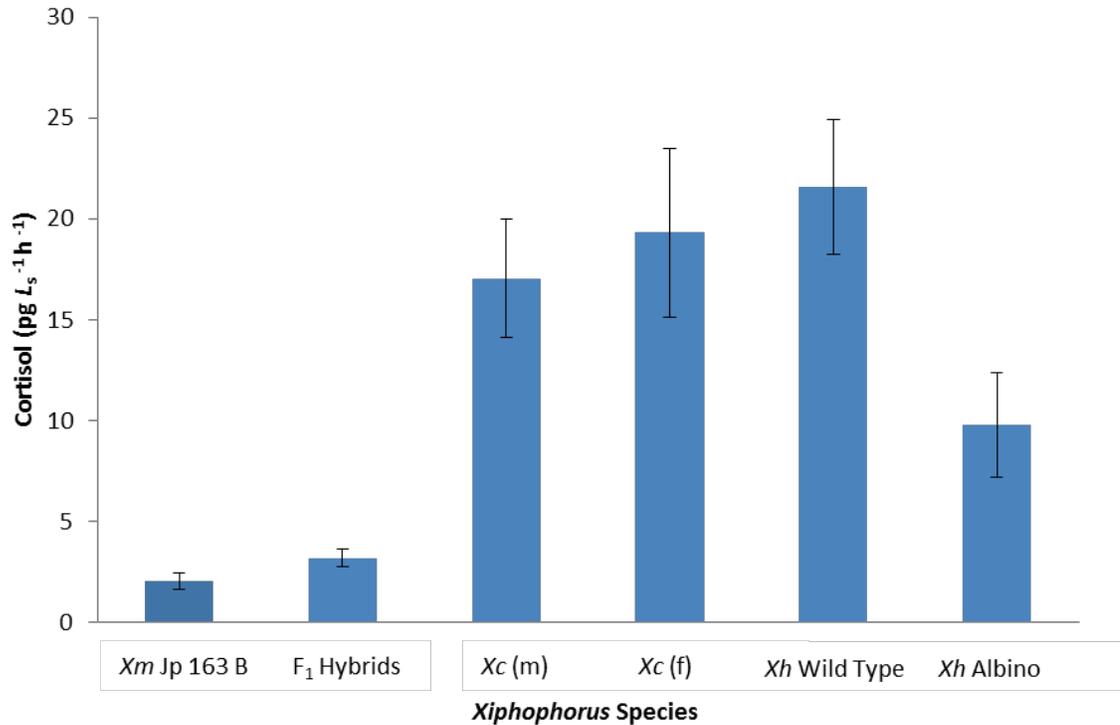


Figure 11. **Baseline Cortisol Production Rate Summary.** In total, three *Xiphophorus* species, an *Xm-Xh* hybrids and an *X. hellerii* albino phenotype were analyzed for baseline cortisol production rates from stage 1 water samples. *X. maculatus* Jp 163 B produced cortisol at $2.0 (\pm 0.39) \text{ pg } L_s^{-1} \text{ h}^{-1}$; *Xm-Xh* *F*₁ hybrids produced cortisol at $3.2 (\pm 0.41) \text{ pg } L_s^{-1} \text{ h}^{-1}$; male *X. couchianus* produced cortisol at $17.0 (\pm 2.93) \text{ pg } L_s^{-1} \text{ h}^{-1}$, female *X. couchianus* produced cortisol at $19.3 \text{ pg } L_s^{-1} \text{ h}^{-1} (\pm 4.20) \text{ pg } L_s^{-1} \text{ h}^{-1}$; *X. hellerii* (*La*) wild type produced cortisol at $21.6 (\pm 3.34) \text{ pg } L_s^{-1} \text{ h}^{-1}$, and albino *X. hellerii* (*La*) produced cortisol at $9.8 (\pm 2.58) \text{ pg } L_s^{-1} \text{ h}^{-1}$.

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