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Cortisol Release in Response to UVB Exposure in *Xiphophorus* Fish

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Abstract

Xiphophorus fishes are comprised of 26 known species. Interspecies hybridization between select species has been utilized to produce experimental models to study melanoma development. *Xiphophorus* melanoma induction protocols utilize ultraviolet light (UVB) to induce DNA damage and associated downstream tumorigenesis. However, the impact of induced stress caused by the UVB treatment of the experimental animals undergoing tumor induction protocols has not been assessed. Stress is an adaptive physiological response to excessive or unpredictable environmental stimuli. The stress response in fishes may be measured by assay of cortisol released into the water. Here, we present results from investigations of stress response during experimental treatment and UVB exposure in *X. maculatus* Jp 163 B, *X. couchianus*, and F1 interspecies hybrids produced from the mating *X. maculatus* Jp 163 B x *X. couchianus*. Overall, cortisol release rates for males and females after UVB exposure showed no statistical differences. At lower UVB doses (8 and 16 kJ/m²), *X. couchianus* exhibited 2 fold higher levels of DNA damage than either *X. maculatus* or the F1 hybrid. However, based on cortisol release rates, none of the fish types tested induced a primary stress response at the UVB lower doses (8 and 16 kJ/m²). In contrast, at a very high UVB dose (32 kJ/m²) both *X. maculatus* and the F1 hybrid showed a 5 fold increase in cortisol release rate. To determine the effect of pigmentation on UVB induced stress, wild type and albino *X. hellerii* were exposed to UVB (32 kJ/m²). Albino *X. hellerii* exhibited 3.7 fold increase in cortisol release while wild type *X. hellerii* did not exhibit a significant cortisol response to UVB. Overall, the data suggest the rather low UVB doses often employed in tumour induction protocols do not induce a primary stress response in *Xiphophorus* fishes.

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Keywords

Cortisol; Stress; Ultraviolet light; DNA damage; *Xiphophorus*; fishes

1. Introduction

Stress is an adaptive physiological response to unexpected or excessive environmental demand, otherwise known as stressors (Sorensen et al., 2013). Cortisol is a glucocorticoid that regulates physiological changes during a stress response (Barton, 2002). In the primary response to chemical, physical or perceived stressors, cortisol synthesis increases in interrenal tissues and enters circulation. The primary stress response is tightly regulated by negative feedback in the hypothalamus and pituitary gland. Cortisol circulates to target tissues throughout an organism and may alter physiology at the cellular level.

Glucocorticoid receptors (GRs) present in the cytosol of many tissues become active when bound to cortisol (Staab and Maser, 2010). Activated GRs translocate to the nucleus and bind to glucocorticoid response elements (GREs) that alter transcription of downstream genes (Aluru and Vijayan, 2009). A change in gene expression represents the secondary stress response and can remain for the time that cortisol continues to be elevated. However, chronic exposure to stressors can lead to tertiary stress response altering gene expression that may be disruptive to an organism's health (Barton, 2002). Many interleukins and cytokines are suppressed by elevated circulating cortisol that can lead to depressed immune functions (Robels et al., 2005) as well as effects to numerous aspects of reproductive health (Schreck, 2010).

Teleost fishes are frequently employed in stress research because the teleost primary stress response, via the hypothalamus-pituitary interrenal axis that regulates cortisol release, is conserved in vertebrates (Barton, 2002 and Sorensen et al., 2013). In addition, fish secrete cortisol through the gills into surrounding water (Ellis et al., 2004). Therefore, cortisol samples can be measured directly from the water the fish are in, circumventing invasive collection of blood or tissues. This is especially important for smaller fish that have very low amounts of blood available for sampling. Cortisol secreted into the water has been correlated with circulating blood cortisol concentrations in live bearing fishes and shown to provide an accurate relative measurement of released cortisol due to interaction with a primary stressor (Gabor and Contreras, 2012). The correlation between circulating and water-borne cortisol is due to free diffusion rates of steroids across gill tissues in which release rates are relative to the surface area of the gills. Since fish mass and length measurements are considered proportional to gill surface area both provide a useful value for normalizing cortisol release rates among fish of different sizes (Gabor and Contreras, 2012; Scott et al., 2008). Thus, teleost fish provide a reliable vertebrate model where cortisol release may be used to characterize the primary stress response.

Xiphophorus are a diverse group of live-bearing teleost fish consisting of 26 species with a variety of phenotypes and complex behaviors, yet little work has been done to characterize their cortisol physiology (Kallman and Kazianis, 2006). They are notable for producing fertile interspecies hybrids that are susceptible to UVB- and MNU-induced tumorigenesis (Setlow et al., 1989, 1993; Nairn et al., 1996; Kazianis et al., 2001a, 2001b). Parental strains

of these hybrids provide a powerful system to study complex heritable traits and gene interactions that underlie susceptibility to tumor induction. An often utilized UVB inducible melanoma experimental model employs interspecies hybrids between *X. maculatus* Jp 163 B and *X. couchianus*.

Here we report examination of the primary stress response after UVB exposure in *X. maculatus* Jp 163 B, *X. couchianus*, and within F1 hybrids produced from crossing these two parental lines in an attempt to relate UVB-induced DNA damage with cortisol release. We also report the primary stress response upon UVB exposure in wild type and albino *X. hellerii* to assess the effect of pigmentation on cortisol release rates. Our results indicate that UVB-induced DNA damage does not lead to a primary stress response until the UVB doses become quite high.

2. Materials and Methods

2.1 Xiphophorus Strains

Fish were obtained from the *Xiphophorus* Genetic Stock Center (XGSC) at Texas State University (San Marcos, TX, USA) where they are maintained as pedigreed lines (www.xiphophorus.txstate.edu). UVB-induced DNA damage and stress responses were analyzed in mature (≈ 9 mo.) female and male *X. maculatus* Jp 163 B, *X. couchianus*, and F1 hybrids ($n=2$). The *X. maculatus* Jp 163 B and *X. couchianus* used herein were highly inbred and stem from pedigrees that were in their 101st and 77th generation of inbreeding, respectively. The F1 hybrids were produced from crossing *X. maculatus* Jp 163 B (99th inbred generation) with *X. couchianus* (75th inbred generation). UVB stress response was also analyzed in mature (20 mo.) wild type *X. hellerii* (Lancetilla, pedigree 11325) and in albino *X. hellerii* (HeAlb, pedigree 11245) ($n=2$). Tyrosinase negative albino *X. hellerii* were a kind gift from Professor Manfred Scharl (Würzburg, Germany) and is maintained in the XGSC. All fish types used in this report are shown in Figure 1.

2.2 Cortisol Detection Range Assessment

A cortisol stock solution (0.1 mg/mL) provided with the EIA kit (Enzo, Farmingdale, NY, USA) was used to make a standard dilution series and absorbance readings were taken to plot a standard curve for all assays reported herein (Supplementary material, Fig. S2, Table S1). In addition, this standard curve was used to establish the verifiable range of absorbance and test cortisol background activity for filtered aquaria water used in each experiment (Table S1).

2.3 Cortisol Degradation Due to UVB Exposure

Cortisol (0.1 mg/mL) was provided in a Cortisol EIA kit (Enzo). A 1 L working solution was diluted in deionized water to 400 pg/mL. The working cortisol solution was stored in the dark overnight to mimic the overnight dark adaption as described for fish handling prior to UVB exposure (section 2.4). To assess degradation of cortisol by UVB exposure, 50 mL cortisol samples were transferred into fish exposure cuvettes (section 2.5) and exposed to 16 or 32 kJ/m² UVB. Water from cuvettes was collected immediately following UVB exposure and stored at -20°C until analyzed in the Cortisol EIA assay. Using the same protocol

described below for the cortisol release rate determination (see section 2.7), cortisol was extracted from the water and fluorescence measured at 405 nm using a plate reader (BioTech Inc., Winooski, VT, USA). This was done in duplicate runs for each UVB dose tested. Total cortisol from each sample was plotted using Excel (Microsoft, Redmond, WA, USA), the r^2 determined, and a best fit line added to determine exponential decay (Fig. 3). These data were used to apply standard corrections for cortisol degradation from UVB exposure where appropriate.

2.4 Handling and Isolation

The day prior to testing, all fish were isolated in individual 250 mL Erlenmeyer flasks containing 100 mL of filtered home aquaria water (water from their home aquaria passed through 0.45 μ m filters). Fish were isolated at least 12 h in the dark (i.e., overnight) and remained unfed during isolation and testing periods the following day.

Handling and isolation methods were adapted from Gabor and Contreras (2012).

2.5 UVB Exposure

For each treatment, fish were poured from the overnight isolation flask into a net, rinsed with filtered aquaria water, and transferred into a single clear plastic cuvette ($9 \times 7.5 \times 1.5$ cm) containing 95 mL of filtered aquaria water under yellow incandescent light. Water (50 mL) from the overnight isolation flask was collected in a falcon tube and stored at -20°C for further analysis. The exposure cuvette, made from UV transparent plastic, was placed at the center of a radiation chamber ($77 \times 41 \times 36$ cm) and exposed to UVB for various time intervals (11, 22 or 44 min) to receive the appropriate UVB dose (8, 16, or 32 kJ/m^2 UVB, respectively). Sham treated fish were placed in the UVB exposure chamber as the 16 kJ/m^2 UVB treated fish (i.e., 22 min) but with the UVB lamps turned off. The UVB exposure chamber employed four Philips TL narrow band Ultraviolet B (20W/01 RS) bulbs with a constant fluence of 12.2 $\text{J/m}^2\text{s}$ and principal emission at 311 nm. The chamber has two lamps on each side so the exposure cuvette containing a fish hangs centrally between and 10 cm away from the UVB bulbs. Following light exposure, fish were poured from the cuvette into a net and the water collected. The fish were then rinsed with filtered aquaria water and transferred into individual 250 mL flasks containing 100 mL of filtered aquaria water then incubated 6 h in the dark before sacrifice and dissection. Water samples after 6 h post exposure incubation were collected and stored at -20°C until further use (Behrand et al., 2001; Gabor and Contreras, 2012). All fish were sacrificed and quickly dissected under yellow incandescent light. Dissected tissues were placed into centrifuge tubes containing 500 μL of RNeasy lysis buffer (Qiagen; Foster City, CA, USA) and placed at 4°C overnight; the next day the samples were moved to -80°C until used for analysis. Water sampling methods were adapted from Gabor and Contreras (2012) and UVB exposure methods as detailed in Yang et al. (2014).

2.6 DNA Damage Quantification by Radioimmunoassay

To assess the amount of DNA damage accrued in samples following UVB exposure, DNA was isolated from skin dissected from one side of each fish tested ($n=2$) following manufacturer protocols (Qiagen DNeasy Blood and Tissue kit, Qiagen, Chatsworth, CA,

USA). The radioimmunoassay (RIA) utilized to determine UVB induced cyclobutane pyrimidine dimers (CPDs) in DNA was conducted as detailed by Mitchell (2006). Briefly, heat-denatured DNA (2–5 µg) isolated from the skin following dissection was incubated with poly (deoxyadenosine):poly (deoxythymidine) (5–10 pg, labeled to $>5 \times 10^8$ cpm/mg by nick translation with ^{32}P -dTTP) in Tris buffer (1 mL total volume: 10 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl and 0.2% w/v gelatin, Sigma Aldrich, Kansas City, MO, USA). Antiserum was added at a concentration that yielded optimal binding to labeled ligand. After 3 h incubation at 37 °C, the immune pellet was precipitated for 2 d at 48 °C with goat anti-rabbit immunoglobulin (Calbiochem, Billerica, MA, USA) and normal rabbit serum (UTMDACC, Science Park/Veterinary Division, Bastrop, TX, USA). 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 mL tissue solubilizer (NCS, Amersham, Pittsburgh, PA, USA), mixed with 6 mL ScintiSafe scintillation fluor (Thermo Fisher Scientific, Waltham, MA, USA) containing 0.1% v/v glacial acetic acid and quantified using a liquid scintillation counter (Packard Instruments, Downers Grove, IL, USA). 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 CPDs per Mb/J/m². Data were calculated using Excel (Microsoft) and fitted curves made using SigmaPlot (Systat Software, San Jose, CA, USA). Statistical verification was performed using StatPlus version 5.8.0.0 for Mac (AnalystSoft Inc.) to run a two-way analysis of variance (ANOVA).

2.7 Cortisol Release Rate Determination

Water-borne hormones were extracted from all collected water samples representing before, during, and after UVB treatments using C18 Solid Phase System columns (Honeywell; Morristown, NJ, USA) with a 24 port vacuum manifold. The columns were primed with HPLC grade methanol followed by two water rinses before samples were added. Total hormone was eluted and collected with HPLC grade methanol (4 mL), dried under a stream of nitrogen in a 37 °C water bath and residues were stored at –20 °C. A competitive enzyme-linked cortisol immunoassay kit (Enzo Life Sciences Inc., Farmingdale, NY, USA) was utilized to assay cortisol according to the manufacturer's protocols. Briefly, hormone residues were re-suspended in 750 µL assay buffer (Tris-buffered saline, pH 7.5) and these 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 1 h. Then, absorbance readings of the EIA were analyzed at 405 nm on a plate reader (BioTech Inc.). When appropriate, total cortisol measured in the water samples was corrected for UVB degradation rates and then normalized to fish standard length (*L*_s; mm) and duration of sampling (h). Standard length correlated with fish mass and was used to normalize cortisol release rates to account for proportional variation between species (Gabor and Contreras, 2012; Fig. S1). Cortisol release rates (pg *L*_s^{–1} h^{–1}) were compared from each dose response.

Cortisol responses to UVB treatments were normalized to sham treated samples and fold change relative to sham determined (Fig. 4). To assess relative responses between

Xiphophorus strains to experimental handling, sham treatment cortisol release rates were compared to the overnight incubation rate (basal). Results were plotted using Excel (Microsoft) and standard error of the mean (SEM) was calculated for each group (n = 2 fish per sample or treatment and 2 technical repeats per assay). Statistical verification was performed using first StatPlus for Mac version 5.8.0.0 (AnalystSoft Inc.) to run a two-way ANOVA test ($P < 0.05$) for all samples and doses and then using a Student's *t*-test on all individual comparisons. In addition, standard curves for cortisol controls provided in the kit were run in triplicate on every plate (Table S1, Fig. S2) to ensure kit accuracy and reagent efficiency.

3. Results

3.1 Cortisol Degradation Under UVB

Cortisol solutions (400 pg/mL) were exposed to the same UVB doses used for fish treatment to determine rates of UV induced cortisol degradation (Fig. 3). As shown, UVB exposure reduced free cortisol in the water by 50% at about 22 kJ/m² (Fig. 3). The levels of UVB induced degradation from this experiment were used to normalize the post-UVB exposure cortisol levels obtained after fish treatment. In addition, standard curves were performed on each plate (Fig. S2) to assess the verifiable range of cortisol detection. Aquaria water used throughout the experiment had a reported absorbance of 0.65 ± 0.013 which is well below the detectable cortisol range of the standards tested (Table S1).

3.2 UVB-Induced DNA Damage

Mature male fish were individually exposed to various doses of UVB and the DNA from the fish skin was isolated and subjected to an RIA assay of UVB-induced cyclobutane pyrimidine dimers (CPDs; Fig. 2 and Table S2). *X. maculatus* Jp 163 B skin incurred significantly less CPD DNA damage than the *X. couchianus* skin DNA at higher UVB doses ($P < 0.01$). The F1 hybrids sustained about the same levels of CPD damage, or perhaps less at the lower doses, than did *X. maculatus* ($p > 0.05$). In all fish, the dose of UVB received differentially altered the gene expression profiles in exposed skin (Yang et al., 2014). Indeed, at the highest dose (i.e., 32 kJ/m²) RNA-Seq analysis of fish skin showed down regulation of repair responses and concurrent enhanced expression of genes involved with cell death (apoptosis and necrosis, not shown).

3.3 Stress Responses to UVB

Cortisol release rates, for all fish types tested, were calculated from water samples before and after UVB exposure (Table 1, and Fig. 4). The cortisol release response among males and females of the same fish type was remarkably similar with no substantial difference ($P > 0.05$) between sexes observed (Table 1, Fig. 4). In general, *X. couchianus* and the F1 hybrid females showed more similar basal levels of cortisol release after 12 h dark incubation ($p > 0.05$), while *X. maculatus* females produced statistically lower basal levels of cortisol (2–3 fold, $P < 0.01$) and hybrid males produced statistically higher basal levels of cortisol (~2 fold, $P < 0.05$). The *X. maculatus* males and females and the F1 hybrid females were less responsive to handling, showing little increase in cortisol release after sham treatment (± 2 fold or less, $p > 0.05$); however, *X. couchianus* males increased cortisol release

rates upon sham treatment about 2.6 fold (Table 1, Fig. 4, $P<0.01$). The *X. hellerii* species exhibited a large cortisol response to sham treatment with albino swordtails showing about a 4 fold increase ($P<0.01$).

The effect of UVB exposure on cortisol release, relative to the sham treatment is rather muted for all fish at the lower dose (8 kJ/m^2). After 8 kJ/m^2 UVB, *X. maculatus*, *X. couchianus*, and the F1 hybrids all show very little difference from the sham levels in regard to cortisol release (less than 2 fold, $p>0.05$). After 16 kJ/m^2 UVB, *X. couchianus* males showed a 3 fold increase in cortisol release rate compared to sham ($P<0.01$). In contrast, *X. maculatus* and F1 hybrid males failed to exhibit an induced cortisol release response at 16 kJ/m^2 . However, at the high dose, 32 kJ/m^2 UVB, *X. maculatus* females and both F1 hybrid males and females showed a 5 fold increase in cortisol release (range 4.8 to 6.1 fold; $P<0.05$). Overall, the F1 hybrid tended to mirror the *X. maculatus* parent in regard to cortisol release response (i.e., fold change) over the higher two (16 and 32 kJ/m^2) UVB doses (Table 1, Fig. 4). A two-way ANOVA test revealed that UVB dose ($P=0.017$), and not species or sex ($p>0.05$), was responsible for the change in cortisol release rates.

The genetic background of the species or interspecies hybrids directs rather large phenotypic differences in skin pigmentation patterns as are apparent among *X. maculatus*, *X. couchianus* and the F1 hybrid animals (Fig. 1). To further assess the impact of pigmentation on the resulting stress response, we exposed lines of *X. hellerii* (Lancitila) that either expressed the wild type pigment pattern or were albino mutant (Fig. 1, tyrosinase deficient) to UVB. As shown in Table 1, *X. hellerii* wild type did not exhibit an induced cortisol release response after 32 kJ/m^2 (1.4 fold, $p>0.05$) while albino *X. hellerii* exhibited a lower basal and sham response, but modest 3.7 fold increase ($P<0.05$) in cortisol release rate after 32 kJ/m^2 UVB exposure.

4. Discussion

In all fish types utilized, production of UVB induced CPDs in DNA isolated from skin increased in a dose dependent manner (Fig. 2). However, as the dose increased (i.e., 16 and 32 kJ/m^2) the levels of UVB induced CPDs in *X. couchianus* skin exceeded those observed in *X. maculatus* and the F1 hybrids (i.e., 2 fold at 32 kJ/m^2). The F1 hybrid appeared more resistant to CPD production than the *X. maculatus* parental line at the lower doses, but exhibited similar levels of CPDs at 32 kJ/m^2 (Fig. 2). The differences in DNA damage levels observed among these three fish types is consistent with previously published reports showing melanin pigmented fish skin incurs about 50% less damage per UVB dose than does non-melanized *Xiphophorus* fish skin (Mitchell et al, 2009). Thus, a relative lack of melanin pigment in *X. couchianus* skin compared to *X. maculatus* and F1 hybrids (Fig. 1) would be expected to result in more damage per unit dose, as observed. However, the very heavy melanization observed in the F1 hybrids, compared to *X. maculatus*, might lead one to expect much lower CPD induction; and such an effect is not observed for any UVB dose analyzed. Thus, relative levels of DNA damage produced are complex and may be in flux with a concurrent ability of each fish type to repair CPD DNA damage (e.g., light repair) during the exposure. This complexity may result in strain specific differential CPD/Mb end points that do not directly reflect relative pigmentation levels.

The cortisol release rates for female F1 hybrids appeared to reflect the *X. maculatus* parental line and show a dose dependent increase in cortisol released at the higher exposures (i.e., $\approx$ 2–3 fold at 16 kJ/m² and $\approx$ 5–6 fold at 32 kJ/m²). In contrast, exposure of female *X. couchianus* to UVB failed to induce a primary stress response as indicated by cortisol release rates of only $\approx$ 2 fold above sham. The similarity in cortisol release pattern for female F1 hybrids and *X. maculatus* is also observed in males for cortisol response at the highest UVB dose (32 kJ/m², 4.8 and 5.2 fold, respectively). However, male *X. couchianus* show a marginal increase in cortisol release after 16 kJ/m² (3.4 fold) or 32 kJ/m² (2.9 fold) without apparent dose dependency. These observations are reminiscent of previous reports involving assessment of both base or nucleotide excision repair capabilities within *Xiphophorus* parental lines and interspecies F1 hybrids. In these studies, it was shown that F1 hybrids may exhibit DNA repair capabilities that reflect one parent or the other, as we see here for cortisol release between *X. maculatus* and the F1 hybrids, or that in some cases the hybrids may present an entirely new phenotype different from either parent (Walter et al., 2001; Mitchell et al., 2004; David et al., 2004).

The production of cortisol is known to be under control of the hypothalamus-pituitary-interrenal (HPI) axis and release of cortisol from the blood stream is dependent on *de novo* synthesis within the interrenal tissue (Mommensen et al., 1999; Neelakanteswer and Vijayan, 2004). Use of water-borne hormone (i.e., cortisol) collection methods in small live-bearing fishes, such as *Xiphophorus*, has been well studied, correlated with plasma cortisol levels, and validated as an accurate measure of teleost primary stress responses (Gabor and Contreras, 2012; Scott et al., 2008). Nine additional teleost species support the correlative nature between circulating blood cortisol levels and cortisol released into the water the fish are in (Scott et al., 2008).

Glucocorticoid stress hormones, such as cortisol, are released within minutes of environmental stress stimuli (i.e., thermal stress, confinement, exposure to toxicants, salinity, etc.) and have been shown to affect many physiological functions (Barton, 2002). Indeed, for carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) UVB exposure has been shown to cause profound effects on immune capability (Salo et al., 2000; Markkula et al., 2005, 2006). Interestingly, it has been suggested that fish inhabiting clear water may have evolved more tolerance to UVB exposure and thus a lower cortisol response capability, compared to bottom dwelling fish that inhabit more turbid waters (Markkula et al., 2006). This reasoning may have relevance to the data presented here since *X. maculatus* is derived from southern Mexico and may be found inhabiting stagnant and turbid waters, but also clear water streams as well. The type location for *X. couchianus* is described as “a spring pool” in northern Mexico around Monterrey (Kallman and Kazianis, 2006). Herein, we show *X. couchianus* receives more CPD damage per UVB dose and shows a lesser UVB induced primary stress response (cortisol release rate) than does *X. maculatus* Jp 163 B, consistent with the above reasoning.

There are several reports suggesting either direct DNA damage produced by UVB (Mitchell et al., 2010), and/or indirect oxidative radical production upon UVB exposure (Wood et al., 2006), are initiating events leading to melanoma induction in *Xiphophorus* backcross hybrids. However, reports in other fish systems indicating immunomodulation is caused by

UVB exposure may lead one to question whether UVB induced stress might also be playing a role in UVB tumor induction experiments using *Xiphophorus* backcross hybrid melanoma models. The UVB doses analyzed here are quite high compared to those used in most *Xiphophorus* melanoma induction protocols (Setlow et al., 1989; Nairn et al., 2001). However, our lowest UVB dose, 8 kJ/m², is close to that reported in protocols used to induce tumors (6.2 kJ/m²). In addition, separate studies have been performed that employ RNA-Seq methodology to assess global changes in gene expression within *X. maculatus* skin after 8 or 6.2 kJ/m². The genes modulated at these two doses of UVB were found to be very closely correlated indicating one may expect both treatments to illicit very similar physiological response (Yang et al., 2014). Thus, a somewhat surprising overall lack of the three fish types tested (*X. maculatus*, *X. couchianus*, or F1 hybrids) to exhibit a primary stress response after 8 kJ/m², and rather muted response even after 16 kJ/m², may suggest immunomodulatory effects due to UVB likely play little substantive role on the UVB induced carcinogenesis in experiments using the *Xiphophorus* hybrid models.

The increase in cortisol release exhibited by *X. maculatus* and the F1 hybrid, compared to *X. couchianus*, may lead one to suggest heavier pigmentation (Fig. 1) is somehow related to the increased response at high UVB dose (32 kJ/m²). However, assessment of UVB induced stress in wild type and albino *X. hellerii* at the higher UVB dose (32 kJ/m²) showed the albino more prone to stress induction (3.7 fold increase) then wild type (1.4 fold). Thus, the effect of pigmentation on stress induction after UVB insult may be complex.

5. Conclusions

Although cortisol is known to play important roles in modulating physiological processes and is utilized as a biomarker in aquaculture, there is much less attention given to its use as an added measure in experimental biology protocols. Here, we establish cortisol release rates after UVB exposure, present species specific differences for two *Xiphophorus* species, and show these to be reflected in F1 interspecies hybrids.

Overall, UVB was shown to be a rather poor inducer of the primary stress response as confinement of the fishes in the UVB exposure chamber elicited a cortisol response about equal to a UVB dose of 8 kJ/m². The rather muted UVB induced cortisol response of *Xiphophorus*, compared to that of other teleosts, suggest the primary stress response is not a large factor in experimentation where UVB is employed to induce melanoma among cohorts of *Xiphophorus* interspecies backcross hybrids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. *Xiphophorus* fish types used in the experiments detailed herein

(Top left) *X. maculatus* Jp 163 B (female), (Top right) *X. couchianus* (male), (Center) F1 interspecies hybrid (male; *X. maculatus* Jp 163 B $\times$ *X. couchianus*), (bottom left) *X. hellerii* (female; wt), (bottom right) *X. hellerii* (female; albino).

Figure 2. RIA detection of CPDs in *Xiphophorus* DNA

X. maculatus Jp 163 B (red), *X. couchianus* (blue) and F₁ hybrids (green) were exposed to various doses of UVB. UVB-induced CPDs for each species were present in a UVB dose dependent manner. Melanin pigmentation in *X. maculatus* and F₁ hybrids appears to be associated with lower levels of CPDs (ANOVA, p.0.05).

Figure 3. Cortisol degradation under UVB

A 50 mL cortisol solution of 400 pg/mL was stored overnight (0 kJ/m²) then exposed to 16 or 32 kJ/m² UVB and remaining cortisol (pg) was plotted in Excel to determine approximate rates of decay. The equation for the best fit line is $y = -233.5x + 10499$ with an $r^2 = 0.94$. (n = 1 and 2 technical repeats per assay).

Figure 4. Relative Stress Response

Cortisol release rates (A) were measured in female (light bars) and male (dark bars) *X. maculatus* (blue), *X. couchianus* (red) and the F1 interspecies hybrid (green) produced from their mating. Samples were measured following a 12 h overnight dark isolation, sham treatment, or doses of UVB light (8, 16, or 32 kJ/m², respectively). Mean fold change (B) was calculated for UVB exposed fish versus sham to observe effects of UVB after experimental stress. Values were corrected for UVB degradation and normalized to fish standard length (Ls, mm) and duration of sampling (h) (see Table 1, $\pm$ SEM of individual fold changes).

Cortisol release rates

Table 1

Cortisol response to 12 h overnight dark incubation, sham treatment, and UVB doses of 8, 16, and 32 kJ/m². Cortisol release was analyzed in *X. maculatus* Jp 163 B, *X. couchianus*, and F₁ hybrids produced from crossing these two parental line fish. In addition, cortisol release was assessed in wild type *X. hellerii*, and an albino *X. hellerii*. Absorbance values from cortisol readings were corrected for UVB degradation and normalized to fish standard length (*L*_S; mm) and duration of sampling (h) ±SEM. Wt =Wild Type. Alb = Albino. ND = Not Determined.

	<i>L</i> _S (mm)	Cortisol release rates (pg <i>L</i> _S ⁻¹ h ⁻¹)				
		12 h dark	Sham	8 kJ/m ²	16 kJ/m ²	32 kJ/m ²
Female						
<i>X. maculatus</i>	28.5 ± 1.1	7.0 ± 1.5	7.1 ± 0.4	9.2 ± 0.4	22.2 ± 9.8	43.0 ± 9.8
<i>X. couchianus</i>	27.7 ± 0.5	39.1 ± 4.1	65.3 ± 38.8	72.6 ± 17.9	150.2 ± 55.2	76.0 ± 24.0
F ₁ hybrid	32.1 ± 0.9	35.3 ± 2.0	23.9 ± 4.7	12.3 ± 0.4	56.8 ± 6.2	135.8 ± 31.4
Male						
<i>X. maculatus</i>	23.3 ± 1.6	7.3 ± 1.0	7.4 ± 0.8	9.2 ± 2.8	11.1 ± 2.0	35.6 ± 18.3
<i>X. couchianus</i>	22.6 ± 0.4	11.9 ± 2.3	30.5 ± 1.7	36.9 ± 13.3	102.6 ± 6.9	87.6 ± 10.4
F ₁ hybrid	23.7 ± 0.9	23.3 ± 2.4	42.0 ± 6.1	34.1 ± 14.1	56.5 ± 14.2	220.4 ± 28.0
<i>X. hellerii</i> (wt)	33.7 ± 2.1	22.8 ± 4.5	195.1 ± 75.7	ND	ND	265.7 ± 19.5
<i>X. hellerii</i> (alb)	41.7 ± 3.4	12.6 ± 2.8	40.9 ± 1.7	ND	ND	150.3 ± 31.6