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# A field-study of inducible molecular defenses, ultraviolet radiation, and melanomagenesis in natural *Xiphophorus* hybrids

**Seth W. Coleman · Zachary W. Culumber · Ashley Meaders · Jennifer Henson · Gil G. Rosenthal**

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**Abstract** Ultraviolet radiation—the primary natural pollutant affecting melanomagenesis—may represent a widespread ecological stressor for many fishes, and yet the relationship between UV-exposure and stress has not been investigated in natural fish populations. Recent lab-based studies have sought to characterize the relationship between tumorigenesis and the induction of molecular defenses, such as heat shock proteins. Here we show that ultraviolet radiation and heat shock protein gene expression explain a significant amount of the variation in hyper-melanization—the phenotypic precursor to melanoma—in wild hybrids of *Xiphophorus*, laboratory models in cancer research. Our results suggest exposure to UV radiation causes stress which induces molecular defense mechanisms, which in turn may facilitate tumorigenesis in natural fish populations. Studies of laboratory-based model organisms in natural settings, like this one, may provide important insights into ecological and evolutionary relationships obscured in controlled laboratory environments. We hope that ours is only the first of many studies to investigate the such relationships between environmental stress, stress-induced molecular defenses, and cancer in fishes.

**Keywords** Adaptation · Ecology · Stress · Cancer · Heat shock proteins

S. W. Coleman · Z. W. Culumber · A. Meaders · J. Henson · G. G. Rosenthal
Department of Biology, Texas A & M University, College Station, TX 77843, USA

S. W. Coleman · Z. W. Culumber · G. G. Rosenthal
Centro de Investigaciones Científicas de las Huastecas "Aguazarca", Calnali, Hidalgo, Mexico

S. W. Coleman (✉)
Biology Department, Gonzaga University, Spokane, WA 99258, USA
e-mail: colemans@gonzaga.edu

## Introduction

In many populations of fishes, individuals experience diverse and sometimes intense levels of environmental stress. Such stress may be anthropogenic—such as exposure to heavy metals or toxins—or natural—such as exposure to ultraviolet radiation, parasite infection, and temperature variation (all of which can be affected by human activities). Inducible molecular defenses, such as heat shock proteins (Hsps) and the genes that code for them (*hsp*s), are a highly conserved and evolutionarily ancient cellular response which can be engaged as a response to such stressors. The induction of Hsps promotes cell (and organism) survival in a stressful environment (Feder and Hofmann 1999; Mosser et al. 2000). It is suggested that because the clients of some key Hsps are in the apoptosis-inhibiting family, the induction of these proteins may facilitate tumorigenesis and tumor

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progression (Gething and Sambrook 1992; Pandey and Shukla 2000; Beere and Green 2001; Fortugno et al. 2003; Aghdassi et al. 2007; Seok et al. 2007). Moreover, Hsps have recently been recognized by ecologists as ideal "biomarkers" of environmental stress in natural populations (reviewed in Depledge et al. 1995; Di Pomerai 1996). Despite the appreciation of cancer researchers and ecologists, the evolutionary and ecological dynamics between tumorigenesis, inducible molecular defenses, and potential carcinogens remain unclear. Here we investigate these dynamics among several pure-breeding and hybrid populations of *Xiphophorus* fishes.

For more than 70 years, *Xiphophorus* hybrids have been a model system in cancer research (Schartl 1995), especially in the study of skin cancer, as they are susceptible to spontaneous and UV-induced melanomas that are histopathologically similar to human melanoma (Schartl 1995; Walter and Kazianis 2001; Mitchell and Nairn 2006). *Xiphophorus* hybrid melanomas are the result of a putative Dobzhansky-Muller incompatibility (Orr 2005; but see Schartl 2008) that results in overexpression of macromelanophore pigment patterns with a disproportionate number of melanocytes that actively proliferate without sufficient regulation (Mitchell and Nairn 2006). While these fish have greatly improved our understanding of melanomagenesis, to date, no studies have investigated the incidence of melanoma in naturally-occurring *Xiphophorus* hybrid populations.

The swordtail species *Xiphophorus malinche* and *X. birchmanni* are ideal for evaluating the evolutionary and ecological dynamics of melanomagenesis at multiple molecular, phenotypic, and environmental levels. *X. birchmanni* is broadly distributed over lowland areas (elevation 161–300 m) of the southern Río Pánuco drainage of the Atlantic slope of central Mexico (Rauchenberger et al. 1990), while *X. malinche* are restricted to highland tributaries (658–1499 m). Hybrid populations are found at intermediate elevations (272–1188 m). The best-characterized hybrid zone, the Río Calnali (Rosenthal et al. 2003) effectively represents an upstream to downstream gradient of distinct hybrid populations, each subjected to a distinct combination of environmental stressors that may contribute to melanomagenesis. Here we focus on UV radiation, a "physical pollutant", which is the primary environmental factor inducing melanomagenesis in humans (Autier et al. 1994; Marks 2002) and *Xiphophorus* hybrids (Schartl 1995; Walter and Kazianis 2001; Mitchell and Nairn 2006; Wood et al. 2007). Also, exposure to acute UV radiation induces molecular defenses, such as Hsps (Ennamany et al. 2007). Thus, under natural conditions, UV radiation may be a salient source of environmental stress for many organisms (Lin and Fisher 2007). To investigate the natural dynamics between UV radiation, melanomagenesis, and Hsp gene expression, for each population we characterized (i) the local UV photic environment, (ii) melanization patterns, and (iii) *hsp*70 and *hsp*90 expression profiles.

## Materials and methods

All field work was conducted out of the Centro de Investigaciones Científicas de las Huastecas "Aguazarca" (CICHAZ), a field research station on the Río Calnali, in the municipality of Calnali, Hidalgo, Mexico. Using a StellarNet EPP2000 spectrometer (Stellarnet, Inc.), at each site, three downwelling irradiance measures were collected on a single day in rapid succession (within 120 s) between the hours of 11:00 and 14:00, between 1 and 10 June 2006. Downwelling irradiance was measured at approximately 0.5 m depth—a depth where we observed typical feeding and courtship behavior in each population prior to our measurements. On the same days as light measurements were taken, fish were collected using baited traps, and photographs were taken of many (>20) fish in each populations; some of these photographs were used to quantify macromelanophore phenotypes (see below). Immediately after collection, six individuals from each population were sacrificed via rapid decapitation, and their heads were placed in RNAlater (Ambion, Inc.). RNA was isolated from brain tissue using a TRIzol reagent (Invitrogen) isolation. Superscript III First-Strand Synthesis System (Invitrogen) was used to create cDNA. Hsp qPCR primers were designed using Primer3 primer design software[1] based on *Poecilia reticulata hsp*70 coding sequence (GenBank accession number: AB298594) and *Danio rerio hsp*90 coding sequence (GenBank accession number: AF068773); *hsp*70 forward primer: ACAGAG AGGCTCATCGGAGA; reverse primer: CCACC

[1] http://frodo.wi.mit.edu/

AGGTCATCAAACCTT; *hsp*90 forward primer: GGTGGACTCTGAGGATCTGC; reverse primer: CGATGGGCTCGATCATGTAG. Samples were sent to the Center for Functional Genomics (CFC) at the University at Albany with blind reference codes such that the technicians at the CFG had no knowledge of sample origin. For analysis, a single reference sample was designated to which all samples were then compared; for each sample QuantumRNA Universal 18 s RNA (Ambion, Inc.) was used as an internal reference gene to normalize each sample; this technique allowed us to make comparative quantifications by the delta delta Ct method. Each reaction (template + primer pair) was run in triplicate. There were some occasions in which one member of a triplicate was discarded as an outlier. All of qPCR assays were run in a 384-well block, with 20 ul reactions in each well. In each assay, a single reference sample was designated; the expression levels of the samples were then quantified relative to this sample. Dissociation curves were analyzed following every assay. With the qPCR results sent from the CFG, we used ANOVA to investigate population differences in opsin expression patterns. ANOVA revealed no among-population differences in the expression of the 18 s qPCR internal control (*hsp*70 reactions: $F_{1,4}$=0.39, $P$=0.83; *hsp*90 reactions: $F_{1,4}$=0.34, $P$=0.85).

To determine whether differences in hypermelanization patterns and *hsp* expression reflect species-specific genetic effects, four species-specific single nucleotide polymorphism (SNP) markers were designed for use in this hybrid system (GG Rosenthal, manuscript in preparation): mitochondrial control region D-loop (CR), ligase (Lig), DNA polymerase beta (PolB) and tumor protein 53 (Tp53) (Walter et al. 2004). Genotyping of 12 hybrid animals was conducted with TaqMan SNP Custom Genotyping Assays (Applied Biosystems). Polymerase chain reactions (PCR) were carried out with 8 ng of genomic DNA in 25 μL reactions. The 25 μL reactions contained 12.5 μL TaqMan genotyping Master Mix (Applied Biosystems), 1.25 μL custom SNP assay mix (20×), 2 μL of 4 ng/μL genomic DNA and 9.25 μL dd$H_2$0. Samples were run and analyzed on a 7500 Fast Real-Time PCR System (Applied Biosystems) by performing a post-read of fluorescence following amplification at 95°C for 10 min followed by 40 cycles of 92°C for 15 s then 60°C for 1 min.

Later, the bodies of the ethanol-preserved hybrids (the same fish for which we calculated *hsp* expression) were photographed for quantification of melanization; the *X. birchmanni* and *X. malinche* bodies were used for other applications not associated with the present study, and thus were not available for photographing. Instead, for these two species, we quantified macromelanophore patterns from the pictures taken of fish on the same day as irradiance measures were collected. We used Image-J image processing and analysis software[2] (NIH) to quantify the proportion of each individual's body where macromelanophores were overexpressed (see the hypermelanized male in Fig. 1D). We used ANOVA to investigate population differences in mean melanization and Hsp expression patterns. If an ANOVA revealed significant ($P$<0.05) differences, then we used Fisher LSD tests to investigate differences among specific means.

## Results

Among populations, we found significant differences in the amount of body area characterized by hypermelanization ($F_{2,215}$=84.33, $P$<0.001; Fig. 1A), and in *hsp*70 ($F_{2,23}$=6.53, $P$=0.017) and *hsp*90 ($F_{2,23}$= 8.17, $P$=0.013) expression (Fig. 1B). Consistent with UV radiation being both a carcinogen and an environmental stressor, the intensity of UV radiation explained a large, though non-significant amount of the variation in hypermelanization ($R^2$=0.67, $F_{1,3}$= 6.08, $P$=0.087; Fig. 1C), and a significant amount of the variation in *hsp*70 ($R^2$=0.78, $F_{1,3}$=11.00, $P$=0.045) and *hsp*90 ($R^2$=0.73, $F_{1,3}$=10.21, $P$=0.049) expression. Supporting the hypothesis that the upregulation of *hsp*s facilitates tumorigenesis and tumor progression, among hybrids, *hsp*70 and *hsp*90 expression levels explained a significant amount of the variation in hypermelanization (*hsp*70: $R^2$=0.80, $F_{1,12}$=54.14, $P$<0.001; *hsp*90: $R^2$=0.77, $F_{1,12}$=44.23, $P$<0.001; Fig. 1D). Overall, our results suggest that exposure to natural doses of UV radiation may induce the Hsp stress response, which in turn may facilitate the development and progression of melanoma in *Xiphophorus* hybrids.

[2] http://rsb.info.nih.gov/ij/

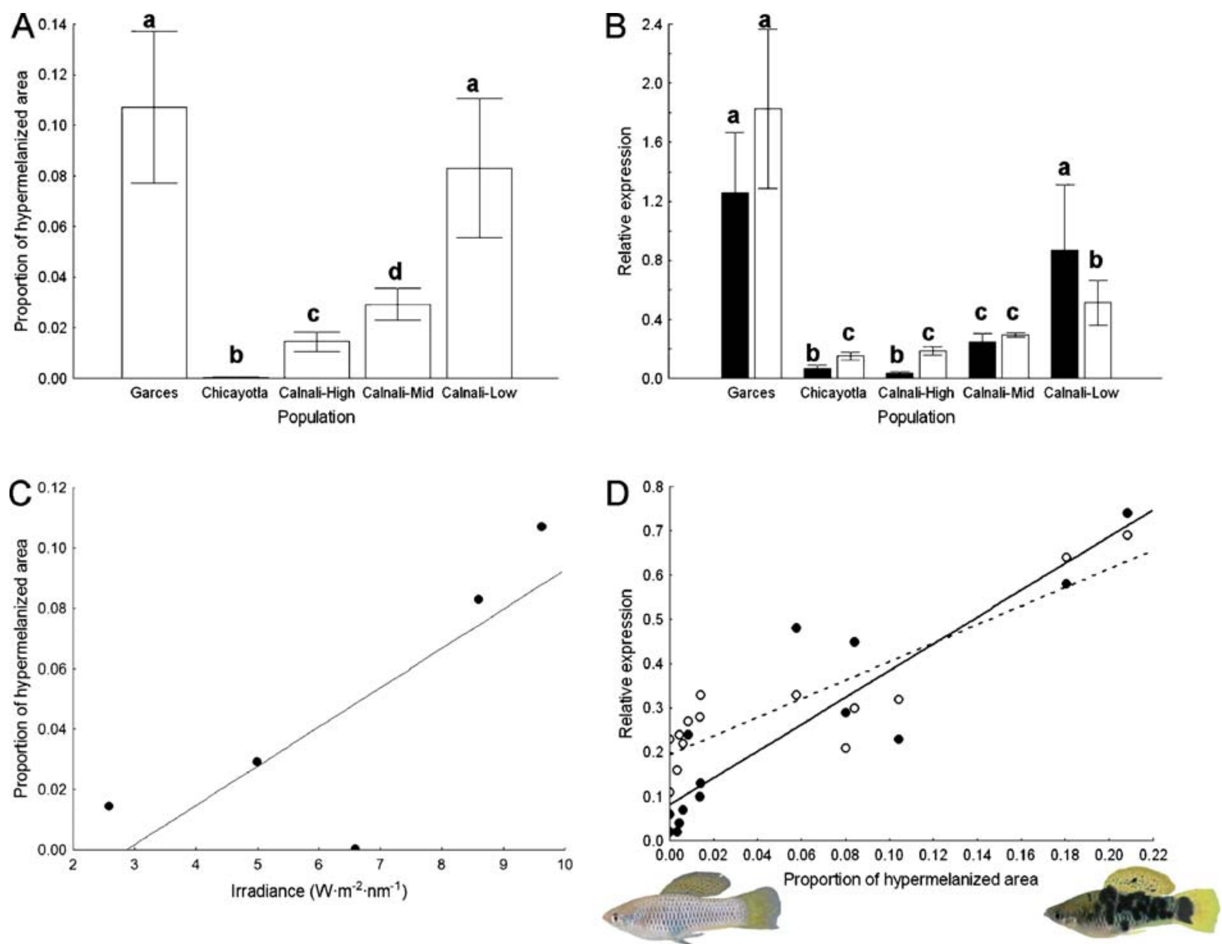


**Fig. 1** Relationships between hypermelanization, *hsp* expression, and UV radiation among populations of *X. birchmanni* (Garces), *X. malinche* (Chicayotla), and three populations of *X. birchmanni* / *X. malinche* hybrids (Calnali-Low, -Mid, -High). (**A**) The proportion (mean ± SE) of body area characterized by hypermelanized tissue. Bars with different letters above are significantly different ($P<0.05$). (**B**) The relative expression levels (mean ± SE) of *hsp*70 (*black bars*) and *hsp*90 (*white bars*). Within genes, bars with different letters above are significantly different ($P<0.05$). (**C**) The relationship between incident solar irradiance across the UV range (units are divided by the interval—101 nm) and melanization among populations. (**D**) The relationship between *hsp*70 (*black circles/solid line*) and *hsp*90 (*white circles/broken line*) expression and the proportion of an individual hybrid's body characterized by hypermelanized tissue. The Calnali-Mid males pictured below the x-axis illustrate some of the variation in macromelanophore expression within populations of hybrids

To determine if differences among populations in hypermelanization (Fig. 1A) and *hsp* expression (Fig. 1B) were due to species-specific genetic effects, we identified four species specific single nucleotide polymorphisms (SNPs). We genotyped 12 hybrid individuals at these loci, and evaluated how their genetic complement of *X. malinche*-specific versus *X. birchmanni*-specific SNPs was related to their hypermelanization patterns and *hsp* expression levels. Suggesting that the differences in these variables are not due to hybrid genetic architecture, we found no differences in mean hypermelanization patterns ($F_{2,8}=1.17$, $P=0.36$), or *hsp*70 ($F_{2,8}=1.67$, $P=0.25$) or *hsp*90 ($F_{2,8}=0.44$, $P=0.66$) expression between hybrids that were typed as "malinche-like" (all SNPs malinche, $n=2$), "birchmanni-like" (all SNPs birchmanni, $n=6$), or "hybrid" (markers from both parent species, $n=4$) for the four SNPs.

## Discussion

Interactions between environmental stressors—such as UV radiation—and inducible molecular defenses—

such as Hsps—likely reflect both current ecological conditions as well as evolutionary history. Here we show, in multiple natural pure-breeding and hybrid populations of *Xiphophorus* fishes, correlations between UV radiation levels, heat shock protein gene expression, and the occurrence and degree of hypermelanization—the precursor to melanoma in these animals (Schartl 1995; Walter and Kazianis 2001; Mitchell and Nairn 2006). Among populations, both UV radiation levels (Fig. 1B) and Hsp gene expression explained a significant amount of the variation in hypermelanization. Among individual hybrids, *hsp* gene expression levels explained a significant amount of the variation in the proportion of individuals' bodies characterized by hypermelanized tissue (Fig. 1D). Together, these results support the hypothesis that the induction of the heat shock protein defense in response to environmental stress may facilitate tumorigenesis and tumor progression.

We are not arguing against the paradigm that stress related to exposure to UV radiation is the primary factor causing melanoma (e.g. Autier et al. 1994; Marks 2002). It is possible that exposure to UV radiation is directly causing the hypermelanistic patterns we observed in our populations, and that the correlated increase in *hsp* expression (Fig. 1D) reflects stress on an animal based on its hypermelanized phenotype. While hypermelanization is the precursor to malignant melanoma in these fishes (Mitchell and Nairn 2006), it is unclear whether hypermelanized tissue is itself harmful to the individual or whether it is simply a benign hyperproliferation of melanocytes (Schartl 1995; Walter and Kazianis 2001). Regardless of the direction of the relationship (UV radiation → hypermelanization → stress → *hsp* induction; UV radiation → stress → *hsp* induction → hypermelanization), our results show that there are interesting predictive patterns within and between populations of *Xiphophorus* between environmental stressors, inducible molecular defenses, and melanomagenesis.

The remarkably high evolutionary conservation of the stress response and of the genes involved (Feder and Hofmann 1999) suggests that this is an evolutionarily ancient adaptive mechanism critical for successful survival of eukaryotes and prokaryotes under hostile environmental conditions. Clearly, this stress response grossly predates the introduction of many particularly salient and carcinogenic pollutants, thus setting up the scenario where the pathways involved in Hsp induction and the pathways involved in tumorigenesis may interact (Beere and Green 2001), having deleterious results that natural selection has not had sufficient time to purge. This hypothesis emphasizes the urgency of more studies like ours, investigating the ecological interactions between environmental stressors—both natural and anthropogenic—stress responses, and cancer. Such studies should provide both (1) a better understanding of how, and whether, species can adapt to rapidly changing environmental stress regimes, and (2) future directions for effective cancer treatment and prevention strategies.

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