ABSTRACT

COOK, ELIZABETH SUZANNE BOYETTE. Differential Phenotypes in Response to Developmental Leflunomide Exposure. (Under the direction of Dr. Carolyn Mattingly).

Zebrafish are a powerful animal model for toxicological studies; however, due to various factors, there is much genetic variation, which could lead to confounding results. This project investigated the effects of exposure to the pharmaceutical, leflunomide (lef), on zebrafish embryos. Lef is a drug commonly used to treat rheumatoid arthritis. Its therapeutic effects are attributed to its metabolite, teriflunomide (ter), which inhibits an enzyme important in *de novo* pyrimidine biosynthesis and T cell function. In addition, lef activates the aryl hydrocarbon receptor (AhR), a receptor important for drug metabolism but also implicated in the toxicity of some of its ligands. Due to neurological disturbances by other AhR ligands, we hypothesized that lef would induce behavioral changes in zebrafish larvae. After exposure to lef for 5 days, larvae were found to be hypoactive in the dark when compared to controls and this effect was not abrogated by a mutation of the aryl hydrocarbon receptor 2 (*ahr2*) gene. In the future, we will use fish with additional knockouts to determine whether the behavioral toxicity is mediated through other AhR paralogs.

Previous studies have shown that lef also interferes with neural crest cell function, leading to a lack of melanin. Concurrent with behavioral studies, we noticed that lef exposure delayed the onset of melanin in some, but not all, embryos within a clutch. This delayed onset of melanin was not recapitulated in ter-exposed embryos. In addition, lef-induced *cyp1a* mRNA expression was reduced significantly in

embryos lacking melanin vs. those with melanin. These data suggest that lef may interfere with melanin synthesis via the AhR pathway.

These findings provide novel insights into a) the role of the AhR pathway in the modulation of neural crest cell migration by lef, b) the complex and diverse effects of exposure to AhR ligands, c) a cautionary tale about potential neurological phenotypes of developmental exposure to lef, and d) the importance of considering genetic diversity when using zebrafish as a model for toxicological studies.

Differential Phenotypes in Response to Developmental Leflunomide Exposure

by
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DEDICATION

I would like to dedicate this thesis to my parents, David and Dianne Boyette. They have been instrumental in my educational success through their unconditional support and encouragement. I am thankful for their dedication and love.

BIOGRAPHY

Elizabeth Cook was born and raised in Raleigh, North Carolina. She attended Meredith College for her undergraduate degree where she received her Bachelor of Science in Biology. From there she worked one year at a pharmaceutical company, Hospira, where she was responsible for creating an organized system for receiving and distributing batch records for chemical testing of the drugs which were manufactured. After her brief stint at Hospira, she began her graduate degree at North Carolina State University where she hopes to receive her Masters of Science in Comparative Biomedical Sciences while working under the supervision of Carolyn Mattingly and Antonio Planchart.

ACKNOWLEDGMENTS

I would like to acknowledge my advisors, Carolyn Mattingly and Antonio Planchart. Through their guidance and direction, in addition to allowing me the freedom to think for myself, they have shaped my scientific mind. I am thankful to my committee for the time and effort they've given towards guiding my project. I would also like to thank the past and present members of the Planchart-Mattingly lab including D. Chris Cole, Stefanie Denning, Adrian Green, Carson Heck, Carson Lunsford, and Lindsey St. Mary.

I am also overwhelmingly grateful for Andrew Cook, my husband, and his inspiration, love, and comedic relief throughout this process.

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LITERATURE REVIEW

There are more than 80,000 chemicals that have been registered for use with the United States Environmental Protection Agency (EPA); however, very few of those have been tested for neurotoxic effects [1]. The list of neurotoxic chemicals includes categories such as heavy metals, endocrine disruptors, pesticides, and industrial waste products. Some well-known examples of neurotoxicants include lead, dichlorodiphenyltrichloroethane (DTT), phthalates, and benzo[α]pyrene (BaP) [2-7].

1.1 Aryl Hydrocarbon Receptor

Our lab has a long-standing interest in the aryl hydrocarbon receptor (AhR) and the effects of its aberrant activation during development. The AhR is classically known as a xenobiotic sensor because many of its ligands are environmental toxicants.

Upon ligand binding, AhR dissociates from its chaperone proteins and enters the nucleus where it binds to the aryl hydrocarbon receptor nuclear translocator (ARNT). Together, AhR and ARNT function as a transcription factor, initiating transcription of target genes including several that are important for drug metabolism, such as genes encoding cytochrome P450 enzymes (CYPs) [8, 9] (**Fig. 1**). AhR and ARNT are members of the basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) family of transcription factors, so named because of their similarity to the

Drosophila proteins, period circadian protein (PER) and single-minded protein (SIM) [10]. AhR is the only member yet to be identified as ligand-activated [11]. The AhR/ARNT complex binds the xenobiotic response element (XRE), consisting of a core pentanucleotide (5'-GCGTG-3') located upstream of promoters regulated by AhR/ARNT [12]. Binding of XREs by AhR/ARNT results in changes in expression of the associated gene.

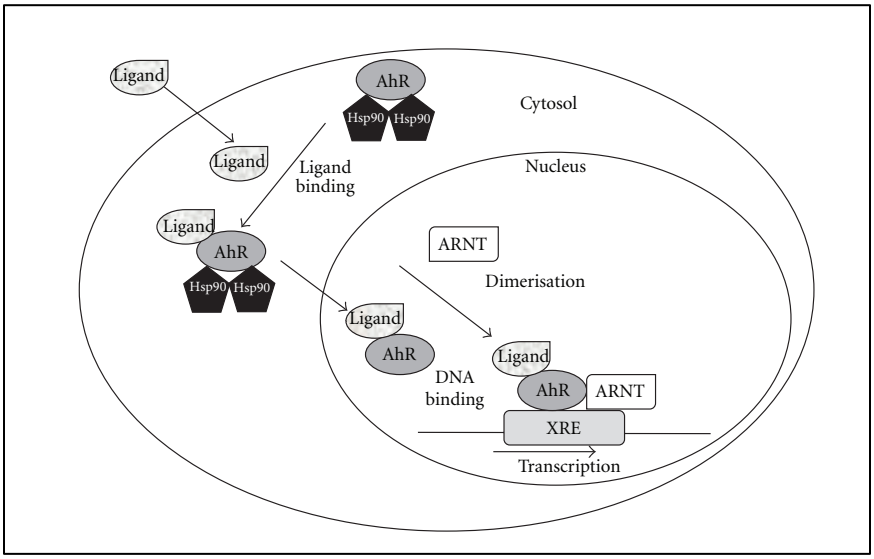


Figure 1. The aryl hydrocarbon receptor pathway activation by ligand.

CYPs are responsible for 95% of phase I metabolism of nearly all marketed drugs [13]. There are 57 human CYPs but only 5 of them are involved in drug metabolism. CYPs are either involved in detoxification of drugs, making them ready for excretion, or their conversion into an active form. One example of this is the CYP-mediated conversion of the carcinogen, BaP. BaP is metabolized in the human

liver into eight different metabolites by various CYPs. Some CYPs have the ability to activate BaP, leading to a toxic metabolite, and other CYPs detoxify BaP [14]. The amount of toxic metabolite of BaP is affected by the concentrations of the different CYPs within the liver; these concentrations can be affected by SNPs in the corresponding gene and their induction or inhibition by other exogenous or endogenous compounds. CYPs can be induced through mechanisms other than the AhR pathway. For example, in humans, CYP1A1 and CYP1A2 can be transcriptionally regulated through the constitutive androstane receptor [15].

The AhR pathway is regulated by diverse mechanisms. One mechanism is by induction of the aryl hydrocarbon receptor repressor (AhRR) by the AhR/ARNT complex. AhRR competes with ARNT for AhR but the AhR/AhRR complex is not transcriptionally active so the pathway is shut down [16-18]. An additional mechanism includes the AhR/ARNT-mediated upregulation of genes involved in drug metabolism, leading to a decrease in the amount of ligand present, acting as a negative feedback mechanism due to decreasing levels of ligand present to activate the AhR. Lastly, it has been reported that compounds such as TCDD are able to induce the degradation of AhR through its ubiquitination and proteosomal degradation [19].

The majority of compounds that activate AhR are polycyclic aromatic hydrocarbons (PAHs) [20]. The AhR pathway has been implicated in processes such as drug metabolism, immunity, development, and behavior [21-23]. The role of AhR in neurological processes has not been completely characterized.

Both BaP and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are agonists of the AhR that produce neurotoxic phenotypes, such as cytotoxicity of neurons [2, 24]. TCDD, BaP, and FICZ, the tryptophan metabolite that is one of a few identified endogenous AhR agonists, all induce a novelty-induced fear response towards food items in mice [25, 26]. BaP exposure in mice leads to decreased motor activity and sensitivity to sensory stimuli, such as tail pinch and click response [27, 28]. TCDD exposure in rats leads to increases in tyrosine hydroxylase (*Th*) mRNA expression and the number of dopaminergic neurons of the midbrain via AhR/ARNT binding to the XRE III site upstream of the *Th* promoter [29, 30].

After a battery of screening experiments we became interested in leflunomide (lef), a pharmaceutical, which is a known ligand of AhR [31-33].

1.2 Lef Background

Lef is a pharmaceutical used for the treatment of rheumatoid arthritis (RA), a disease characterized by inflammation in the synovial tissue of joints. Lef is a pro-drug that contains an isoxazole ring, which is cleaved by CYPs to produce the metabolite, teriflunomide (ter). This conversion is thought to be rapid and mediated by CYP1A2, CYP2C19, and CYP3A4 [34].

Lef is normally prescribed at a dose of 10-20 mg per day and has a half-life of approximately two weeks. The most frequent adverse events reported during clinical trials involving lef were diarrhea, elevated liver enzymes, alopecia, skin rash, respiratory infection, and nausea [35]. Lef is contraindicated in women who are or

may become pregnant due to its teratogenic effects [36, 37]. The teratogenic effects of lef include craniofacial malformations such as cleft palate, neural tube defects, tail deformities, and skeletal malformations [38].

Lef-induced toxicity has been correlated with single nucleotide polymorphisms (SNPs) in genes involved in the drug's metabolic pathway. One study reported an association between a SNP in the CYP1A2 gene and higher metabolic activity of the enzyme. It is unknown if this SNP, which is in a non-coding region, leads to differential binding of transcriptional regulatory proteins or if it is in linkage disequilibrium with other mutations that affect the inducibility of the enzyme. This SNP is associated with a 9.7-fold increased risk of toxicity, including diarrhea, nausea, abdominal pain, puritis, and transaminase elevation following treatment with lef [39, 40]. Another study showed the CYP2C19*2 loss-of-function allele correlated with an increased risk for lef toxicity [41]. This CYP2C19*2 allele was also associated with a lower ter plasma concentration indicating that the adverse effects may be due to lef, but not its metabolite ter [39, 41].

Furthermore, lef-induced hepatocytotoxicity was prevented by the pan-CYP inhibitor, 1-ABT; whereas, ter-induced toxicity in the same cells was not rescued by 1-ABT [42]. In addition, this study used liquid chromatography (LC)-mass spectrometry (MS) analysis to show that the levels of ter detected in hepatocytes after exposure to lef were lower than the levels of lef that were degraded, suggesting an additional metabolite(s) other than ter which could produce some of the known toxic side-effects [42]. Other experiments using mouse and human liver microsomes

and MS revealed two additional hydroxylated lef metabolites, M1 and M2. M1 was formed after incubation of microsomes with lef and ter; however, M2 was only formed after incubation with lef suggesting it is synthesized from lef, not ter [43] (**Fig. 2**).

The fact that some SNPs in CYPs lead to increased activity of the enzyme and others lead to loss-of-function while both may lead to lef-induced toxicity may be confusing; however, lef can be transformed into different metabolites, some of which are toxic and this may be regulated by which CYPs are more active.

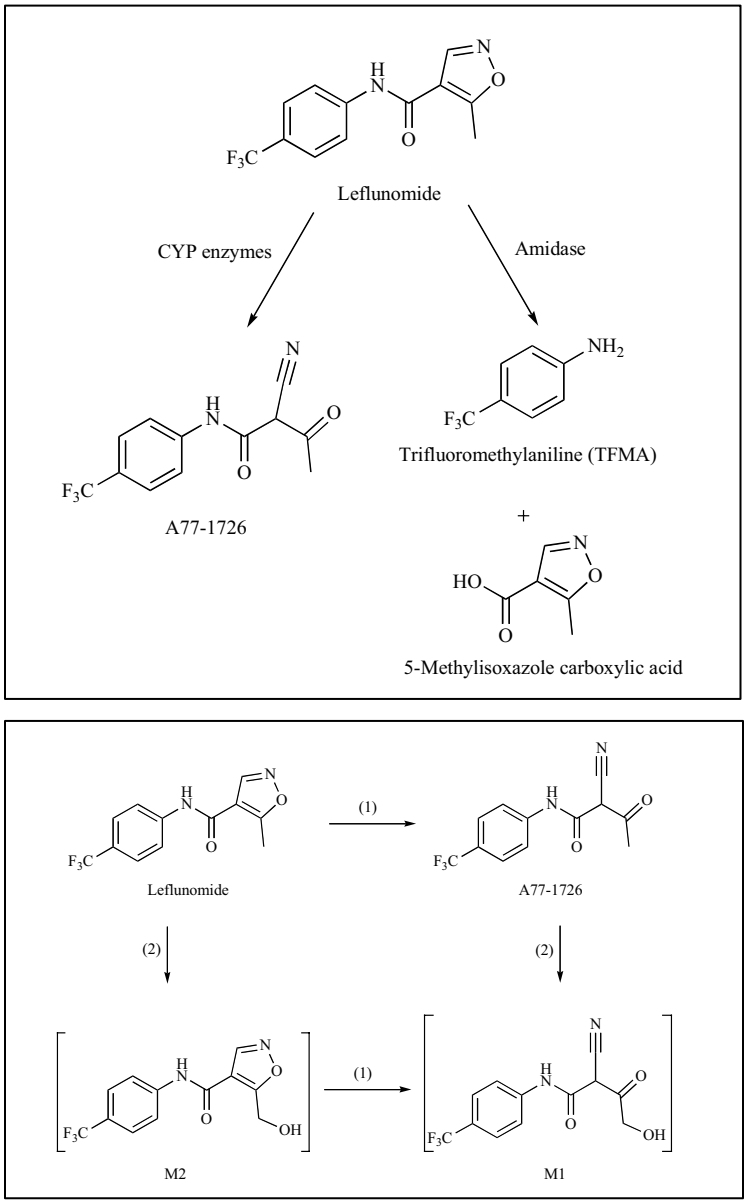


Figure 2. Diagrams reporting originally identified (top) and more recently discovered (bottom) lef metabolites.

1.3 Lef and Dihydroorotate dehydrogenase (DHODH)

Ter is an inhibitor of DHODH [44], an enzyme important for *de novo* pyrimidine (Y) biosynthesis. Most cells can use the salvage pathway for Y biosynthesis, but T cells cannot. T cells infiltrate joints in patients with RA and cause inflammation. Inhibition of DHODH by ter prevents T cell proliferation and reduces inflammation. [45-47].

In addition to SNPs in CYPs being correlated with increased lef toxicity, the DHODH A40C polymorphism has been linked to increased risk of discontinuation of lef treatment due to adverse effects including diarrhea, nausea, weight loss, rashes, and increased transaminase levels [48].

Other potential applications of lef include its use in the treatment of melanoma. Melanocytes are derived from neural crest cells; when exposed to lef, self-renewal of neural crest progenitor cells is abrogated. It was hypothesized that DHODH inhibition by ter in these cells leads to reduced transcriptional elongation of genes important for neural crest development. Lef has been proposed as a treatment for melanoma due to its ability to inhibit DHODH and therefore inhibit neural crest cell proliferation [49]. However, in contrast to the hypothesis that lef may combat melanoma through DHODH inhibition, it was shown that AhR expression is important for lef-mediated inhibition of melanoma cell growth [50]. Lef inhibited growth of A375 melanoma cells and knockdown of AhR by short hairpin RNA rescued this effect. In addition, this study showed that the anti-proliferative effects of lef were not ameliorated by the addition of uridine, a precursor in the de novo

biosynthesis of pyrimidines, which would be expected if the effect of lef on melanoma proliferation were solely due to DHODH inhibition.

1.4 Behavior

Exposure-mediated behavioral changes can be used as an indicator of neurotoxic compounds. Numerous assays have been developed to assess specific types of behaviors. For example, the anxiolytic-like effects of a compound on mammals can be determined using behavioral assays such as the elevated plus maze (EPM) and hole-board test (HBT) [51]. EPM is a test that is used to examine the exploratory nature of rodents by quantifying how long they spend exploring an elevated, open, and bright space [52], whereas, HBT measures anxiety by quantifying how many times a rodent will dip its head in a hole [53]. Although there is a range of methods and longer history for measuring complex behavior in rodents, mammalian models are limited by the cost of upkeep and the numbers of chemicals needing to be tested.

1.5 Zebrafish

Zebrafish are an excellent complementary model to measure neurotoxic effects associated with chemical exposures [54]. Their many experimental advantages include high fecundity (200 eggs/female), external and transparent development, and ease of genetic manipulation. Around 70% of human genes have at least one orthologous gene in zebrafish [55].

Zebrafish have three paralogs of AhR, AhR1a, AhR1b, and AhR2, due to a genome duplication in the teleost fish lineage approximately 400 million years ago [56, 57]. Although TCDD-mediated toxicity is through AhR2, lef is able to activate all three paralogs [58].

Numerous features/characteristics make the zebrafish particularly valuable for studying neurodevelopment. To begin, the effects of chemicals on neurogenesis can be studied with temporal ease; neurulation is complete after 24 hours of development and the basic central nervous system (CNS) is formed by 5 days post-fertilization [59]. In addition, zebrafish posses many of the same neurotransmitters as mammals including dopamine, serotonin, noradrenaline, glutamate, histamine, and GABA [60]. The architecture of the zebrafish brain is conserved with humans and this model is being increasingly used to study neurodegenerative diseases such as Parkinson's [61] and Huntington's [62-66]. Finally, zebrafish present an excellent way to study neurogenesis or degeneration *in vivo* due to the many transgenic lines that express fluorescent proteins in specific cell types [67-70].

Moreover, zebrafish also exhibit many of the same behavioral phenotypes as mammals and these behaviors can be measured for toxicological purposes. Increasing interest in the use of the zebrafish model for determining the neurotoxic effects of xenobiotics has led to the compilation of a catalog describing many of the behaviors displayed by zebrafish [71]. The different behavioral endpoints that can be measured include locomotor changes, sensorimotor responses, and learning and memory [61, 72]. One of the most common, high-throughput ways to measure

developmental neurobehavioral changes is to evaluate locomotor response in zebrafish larvae at 5-6 days post fertilization (dpf) in response to alternating cycles of light and dark within an enclosed system [73]. Zebrafish typically exhibit increased movement during the dark periods.

EXPOSURE TO LEFLUNOMIDE INDUCES A HYPOACTIVE PHENOTYPE IN LARVAL ZEBRAFISH

2.1 Introduction

As was mentioned in section 1, ligands of the AhR, including TCDD and BaP, are known to produce neurotoxic side effects. There is an XRE in the tyrosine hydroxylase (Th) promoter and TCDD has been shown to upregulate *Th* through induction of the AhR pathway [30]. TH is an enzyme important for the synthesis of dopamine from tyrosine and alterations in this pathway lead to anxiety-like phenotypes [74].

Given that lef binds and activates the AhR and other AhR ligands have led to neurological defects, we hypothesized that lef-exposed zebrafish embryos would exhibit behavioral abnormalities at 5 dpf.

2.2 Methods

Zebrafish maintenance and strains.

Zebrafish breeding and maintenance were carried out under standard conditions at 28.5˚C in the embryos were housed in 0.5x E2 media [75]. Embryos were staged according to Kimmel et al. [76]. The *ahr2* knockout line, *ahr2*^{hu3335}, was developed by Targeting Induced Local lesions IN Genomes (TILLING) and was a generous gift of Robert Tanguay (Oregon State University, USA). The line contains a TTG to TAG point mutation in codon 534, which generates a Leu→STOP premature stop codon mutation in the transactivation domain of ahr2. The Tg(dat:EGFP)

zebrafish line was a gift from Marc Ekker (University of Ottawa, Canada) [77]. This transgenic fish expresses green fluorescent protein (GFP) under the control of the dopamine transporter promoter. The fluorescent cells in these fish were imaged with the Zeiss Light Sheet Z.1 microscope with a 488 nm laser and a 504-545 nm band pass emission filter. The AB strain of zebrafish was used as the wild-type fish [78].

Chemicals and exposures

Stock solutions of lef (75 mM; Sigma Aldrich Catalog #L5025) and ter (75 mM; Sigma Aldrich Catalog # SML0936) were prepared in dimethyl sulfoxide (DMSO). Working solutions for chemicals were prepared fresh on the day of exposure by dilution in DMSO from 1000X secondary stock concentrations. Stock solutions were stored at -20˚C.

Zebrafish adults were allowed to spawn and embryos were collected and scored for viability based on developmental stage. Exposure media was replenished daily. Embryos were checked daily for morphological differences and dead embryos were removed. For behavioral assays, zebrafish embryos were exposed from six hours post-fertilization (hpf) to five dpf with daily exchanges of exposure media.

Behavioral studies

At four dpf, after exposure replenishment, embryos were transferred, one per well, to a 96-well (round wells), flat-bottom plate (Olympus #25-104). On the day of the behavioral analysis, plates were transferred to the DanioVision observation chamber (Noldus Information Technology, Leesburg VA) and embryos were allowed to acclimate for 10 minutes. The DanioVision system is equipped with visible and

infrared light sources that allow measurement of activity during both light and dark conditions, respectively. Darkness is simulated by infrared light, which zebrafish cannot see. Behavior was monitored during five alternating 20-minute cycles of light (5.1 lux) and dark. Movement was sampled at a rate of 25 frames/second and quantified for distance moved using Ethovision XT 10.0 software. Following testing, larvae were examined under a dissecting microscope to identify fish that died or exhibited abnormalities; these larvae were excluded from all analyses.

Quantitative reverse transcription polymerase chain reaction.

RT-qPCR analysis was performed on an Mx3000P Real-Time PCR system (Stratagene) using the Brilliant SYBR Green qPCR reagent (Stratagene) according to the manufacturer's protocol. The cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s. Threshold cycles (C_t) and dissociation curves were determined with MxPro software (Stratagene). Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which was invariant in every experiment performed regardless of the age of embryos or exposure condition. Fold-change (FC) was calculated using $FC = 2^{-\Delta\Delta C}{}_t$ where $\Delta\Delta C_t = \Delta C_{t,GOI} - \Delta C_{t,norm}$ (GOI, gene of interest; norm, *Gapdh*). All analyses were performed in triplicate. Primer sequences are as follows: *Tyrosine hydroxylase,* 5'-TGCTGTTCAGCCATACCAAG-3' (F) and 5'-CAGGCCTTTCTGGATCTTCA-3' (R); *gapdh,* 5'-TGGGCCCATGAAAGGAAT-3' (F) and 5'-ACCAGCGTCAAAGATGGATG-3' (R).

Statistics

Statistical analyses were performed using JMP Pro 11 statistical analysis software. The Shapiro-Wilk W test was used to determine the goodness-of-fit for the behavioral data. A p-value <0.05 indicated a non-normal data set. A t-test was used to determine statistical significance of the difference in means for the behavioral data. A p-value <0.05 was used to determine statistical significance. Effect size was represented by the Cohen's d coefficient. Cohen's d > 0.8 was used to consider the effect size to be large.

2.3 Results

Light-dark zebrafish behavioral assay analysis produces varying results.

In order to determine whether lef causes behavioral abnormalities, zebrafish embryos were exposed to either 0.1% DMSO or 500 nM lef and evaluated using the light-dark assay. In some circumstances, lef exposure lead to hypoactivity in the dark as compared to controls (**Fig.3a**); however, in other experiments this lack of movement was not seen (**Fig. 3b**). It became apparent that the results were not reproducible even when the methods were repeated exactly the same way each time To resolve the paradox, we pooled results across all experiments and plotted data as shown in Figure 4. As seen, the control data are distributed normally whereas the lef exposure data are bimodally distributed, indicating two different responses.

A new method of the light-dark zebrafish behavioral assay analysis shows reproducible results in WT larvae.

Due to the variability in lef-mediated movement in the dark, we decided to analyze these behavioral data differently. We previously noticed that some experiments showed lef-exposed larvae were hypoactive in the first dark cycle but responded similar to controls by the final dark cycle. This behavior was consistent with learning. Therefore, we increased our starting number of larvae (n=48/treatment) to obtain a better estimate of the population mean and only analyzed the first dark cycle to control for possible habituation. Using this approach, larvae exposed to lef were consistently hypoactive when compared with controls (**Fig. 5**). Furthermore, data from the first dark cycle were normally distributed. It is hypothesized that the non-normality determined by the previous analysis was due habituation.

A new method of the light-dark zebrafish behavioral assay analysis shows reproducible results in *ahr2*-null larvae.

Since AhR has been shown to be important in mediating neurotoxicity of other AhR ligands, we decided to see if lef produced a hypoactive phenotype in *ahr2*-null larvae. *Ahr2*-null larvae were exposed to either DMSO or 500 nM lef for 5 days and analyzed for behavioral changes as described above. Lef exposure caused hypoactivity in the first dark period in Ahr2 null larvae suggesting that the behavioral phenotype measured in wild type zebrafish was Ahr2-independent (**Fig. 6**).

Lef exposure affects dopaminergic biology.

TCDD is known to increase the number of th immunoreactive neurons in mice as well as induce the upregulation of *Th* mRNA [29]. We exposed our Tg(dat:EGFP) zebrafish to 500 nM lef at 6 hpf and imaged them at 96 hpf to characterize the effects of lef exposure on dopaminergic neurons. Lef exposure decreased the number of EGFP+ neurons in the ventral diencephalon (**Fig. 7 top**). In addition, we interrogated *th* mRNA expression at 48 hpf and found that there was a decrease in *th* mRNA at 250 nM (0.8 fold change) and 2 μM lef (0.3 fold change) (**Fig. 7 bottom**). Lef exposure interferes with dopaminergic biology in zebrafish, although the mechanism may be different than other AhR ligands due to lef-exposure causing downregulation of *Th,* whereas TCDD exposure causes upregulation of *Th*.

2.4 Conclusions

After acclimation and one light period, larvae that were exposed to lef were hypoactive in the dark as compared to control fish; however, by the 5th cycle of light and dark both treatment groups responded to the dark equally.

Habituation is a form of learning and refers to a decrease in the reaction to a single stimulus over time as the stimulus is administered repeatedly [79]. The lef-exposed larvae were hypoactive during the initial dark cycle but we hypothesize that they learn over time and begin responding to the dark in a similar way as the control.

Contrary to our expectations, a mutation in Ahr2 causing a premature stop codon did not rescue the lef-induced hypoactive phenotype. It is possible that this

hypoactivity could still be mediated through the activation of Ahr1a or Ahr1b instead of Ahr2. In the future, we will use fish with additional knockouts to determine whether the behavioral toxicity is mediated through other AhR paralogs.

In contrast to a TCDD-induced upregulation of *Th* in mice, lef exposure resulted in downregulation of *th* in zebrafish embryos. This could be attributed to the difference in models as well as the difference in the structure of the two AhR ligands. These data further prove the necessity to understand how activation of the AhR by distinct ligands can produce varying phenotypes.

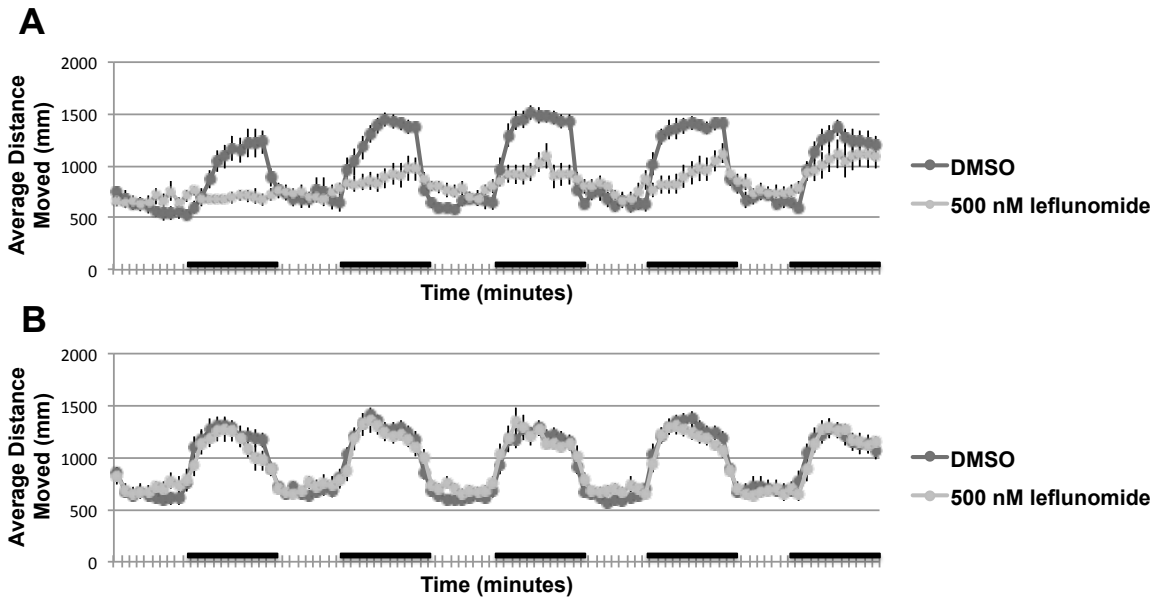
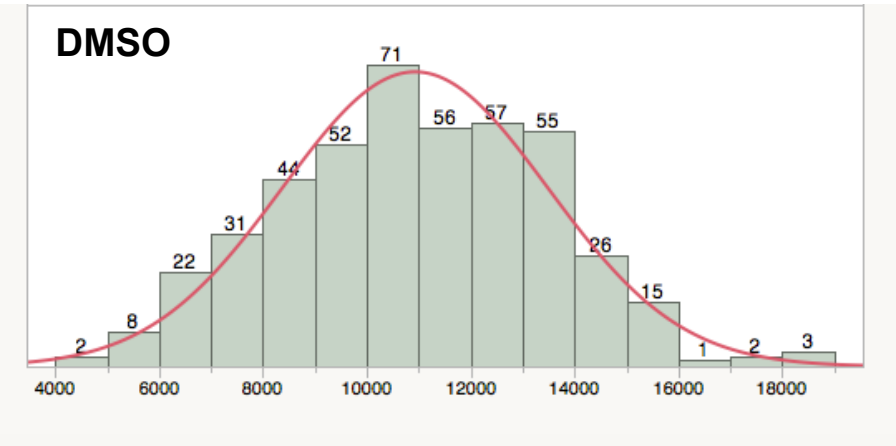


Figure 3. Lef exposure alters behavior. Lef (500 nM) reduced larval (5 dpf) response to the dark when compared with control in one experiment (A) but not the other (B). Both experiments used AB wild-type embryos (n=12 per experiment) with the same exposure regimen and assay parameters. Black bars represent dark intervals in a light-dark cycle. Ticks on x-axes represent 1-minute marks.



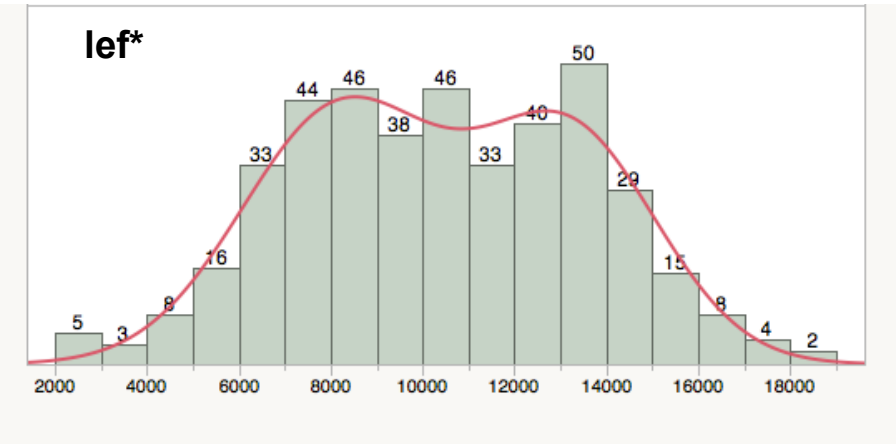


Figure 4. Lef exposure produces two different behavioral responses. Graphs represent histograms of average distance moved for DMSO-exposed (top) and lef-exposed (bottom). Numbers on top of bars represent total fish that swam in the corresponding interval for average distances moved as represented on x-axis. Histogram of average distance move for DMSO-exposed was normally distributed whereas histogram for lef-exposed was not normally distributed (*p-value <0.05, Shapiro-Wilk W-test).

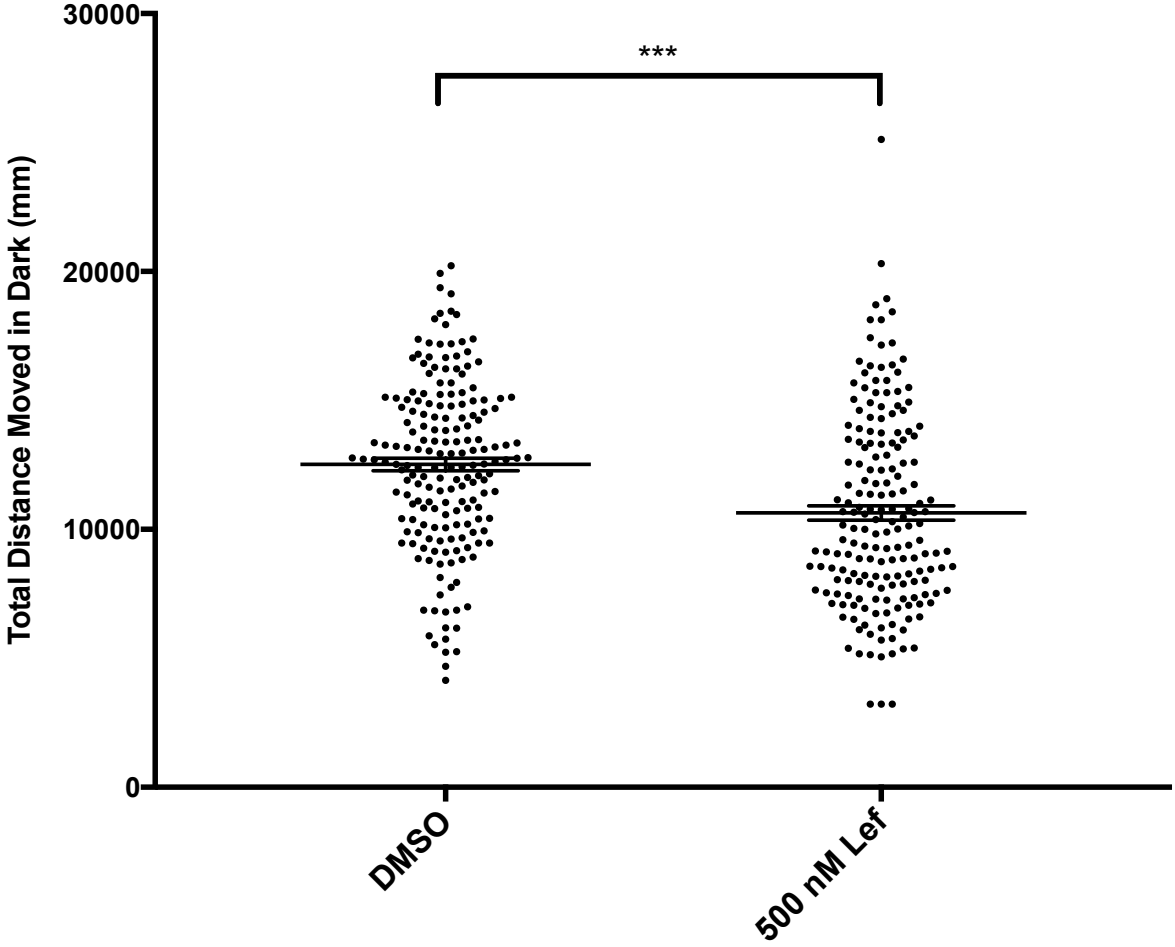


Figure 5. Total distance moved by lef-exposed wild type larvae in the first dark period of a light-dark behavioral assay. The lef-exposed larvae swam significantly less in the first dark period than the DMSO controls. n=184 larvae/treatment, *** p-value <0.001, t-test. Black lines represent mean +/- SEM. Cohen's d = 0.54.

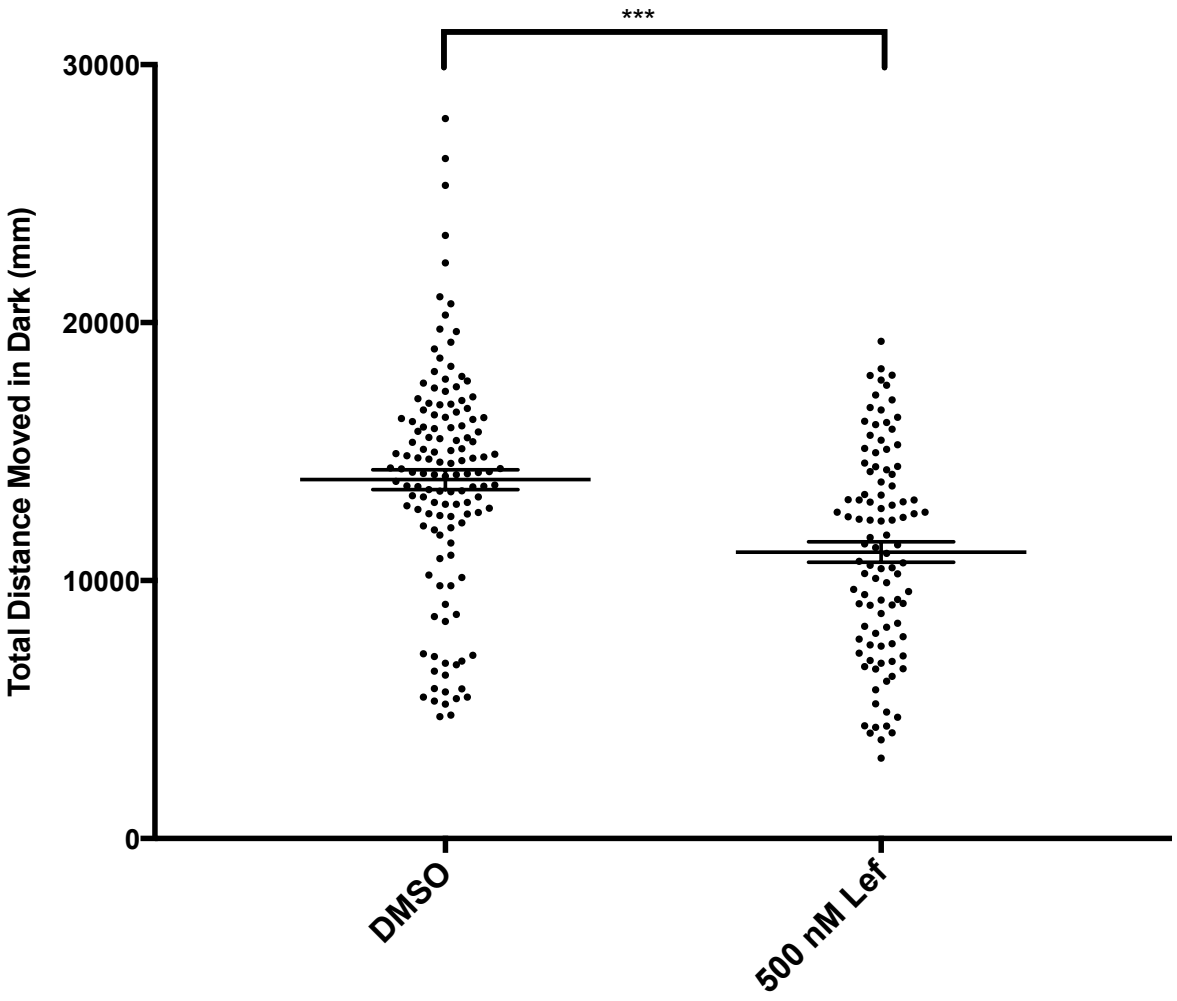


Figure 6. Total distance moved by lef-exposed AhR2-null larvae in the first dark period of a light-dark behavioral assay. The lef-exposed larvae swam significantly less in the first dark period than the DMSO controls. These data were normally distributed and analyzed using a t-test (n=133 larvae/treatment). *** p-value <0.001. Black lines represent mean +/- SEM. Cohen's d = 0.67.

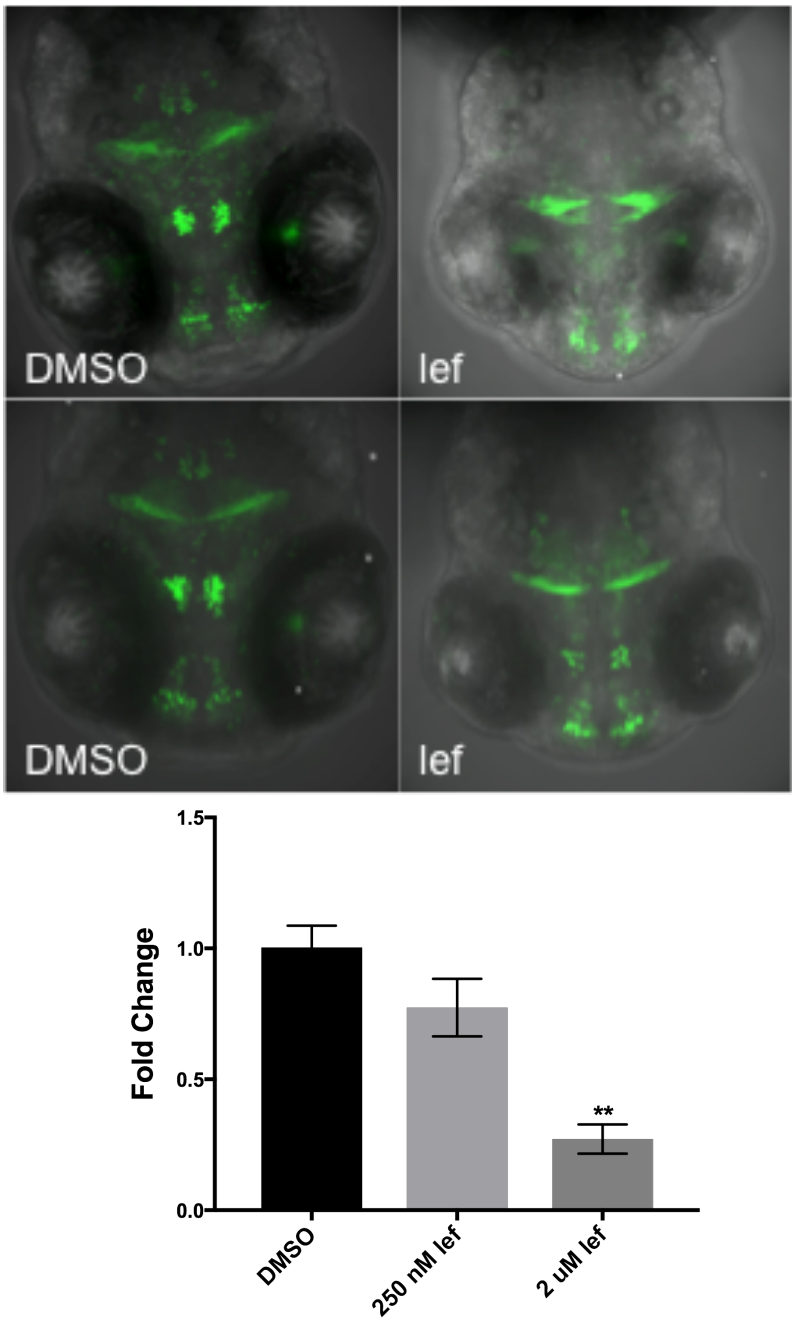


Figure 7. Lef exposure affects dopaminergic cells. Tg(dat:EGFP) larvae (96 hpf) develop fewer EGFP+ cells when exposed to lef than when exposed to DMSO control (top). *Th* mRNA expression is decreased in 48 hpf WT embryos after exposure to lef (250 nM and 2 μM) (bottom). Bars represent mean +/- SEM, * p-value <0.05, t-test.

THERE IS A DIFFERENTIAL SUSCEPTIBILITY TO THE LEFLUNOMIDE-MEDIATED DELAY IN ONSET OF MELANIN IN ZERBAFISH POPULATIONS

3.1 Introduction

Similar to other lef-induced phenotypes, exposure resulted in a significant reduction in melanin in some, but not all, embryos. In addition to binding and activating the AhR, lef inhibits DHODH [44] and it is thought that this inhibition is what leads to inhibition of neural crest cell self-renewal, and therefore loss of melanocytes. However, in our preliminary studies, ter exposure had no effect on the number and distribution of differentiated melanocytes as lef exposure. This observation led us to believe that, contrary to a previous report [49], DHODH may not be involved in the inhibition of neural crest cell self-renewal.

In addition to our preliminary observation, another lab has published that the AhR is required for lef-mediated inhibition of melanoma cell growth [50]. This group used a melanoma cell line with an inducible short hairpin RNA for AhR to show that, when AhR expression was knocked down, lef was not able to inhibit T cell proliferation.

We hypothesized that the variation in melanin phenotype in lef-induced fish may be due to underlying genetic variation in the zebrafish population and is mediated, at least in part, by lef-induced activation of AhR.

3.2 Methods

Zebrafish maintenance and strains

See maintenance methods from chapter 2. The [Tg(-4.9*sox10*: EGFP)^{ba2}] (also known as Tg(sox10: EGFP)) line of zebrafish was generated by injecting 4.9 kB of DNA upstream of the *sox10* transcriptional start site into the 1-cell stage of a zebrafish embryo. These fish express EGFP under the control of the *Sox10* promoter. This transgenic line was a gift from Thomas F. Schilling [80]. Epifluorescent images were taken using a LEICA MZFLIII microscope equipped with an arc lamp and appropriate filters.

Chemicals and exposures

Refer to chapter 2 for chemical information.

Zebrafish adults were allowed to spawn, and embryos were collected, scored for viability, and sorted by developmental stage. For all exposures, except for the Tg(*sox10*:EGFP) exposures, exposure medium was replenished daily. The Tg(*sox10*:egfp) embryos were exposed for 24 hours, washed with E2 medium, and then reared in an incubator until 96 hpf. Embryos were checked daily for morphological differences and dead embryos were removed.

Melanin scoring

The effect of a chemical on the production of melanin was scored by exposing the embryos to the chemical at six hpf. Treatment was replenished at 24 hpf and at 32 hpf the number of embryos with and without melanin were counted and relative percentages were calculated. This time point was chosen due to the fact that all

DMSO control embryos have melanin by 32 hpf so comparisons could be made with the chemical of interest. Images were taken with a LEICA MZFLIII microscope for a visual representation of melanin at 48 hpf. This time point was chosen due to robust melanin expression.

Quantitative reverse transcription polymerase chain reaction.

qRT-qPCR analysis was performed as described in Chaper 2. Primer sequences are as follows: *Tyr*, 5'-CGGACACAACGACGGATACT-3' (F) and 5' CCAGATACGGCGTCAGGAA-3' (R), *Cyp1a*, 5'-GGATATCAACGAACGCTTCA-3' (F) and 5'-TTCTCATCGGACACTTGCAG-3' (R), *Gapdh* 5'-TGGGCCCATGAAAGGAAT-3' (F) and 5'-ACCAGCGTCAAAGATGGATG-3' (R).

Statistics

Refer to chapter 2.

3.3 Results

Lef exposure delays the onset of melanin in some, but not all, embryos of a clutch.

Lef exposure has been shown to impede neural crest cell self-renewal [49]. Pigment-producing melanocytes are a lineage derived from the neural crest and lef exposure causes a lack of melanin that can be easily visualized and quantified. Consistent with variability observed in lef-mediated behavior, we noticed that lef-exposed embryos exhibited varying amounts of melanin at 48 hpf while their aged-

matched DMSO-exposed counterparts had normal pigmentation. The presence of melanin at 48 hpf inversely correlated with morphological toxicity at 5 dpf (**Fig. 7**).

Next, we exposed the embryos to increasing levels of lef and saw that as the dose of lef increased, the amount of melanin decreased. The number of embryos with melanin were counted at 32 hpf and presented as percent of embryos in a clutch with melanin (**Fig. 8 bottom**) and the embryos were imaged at 48 hpf (**Fig. 8 top**). To rule out the possibility that in a group exposure design, embryos would be exposed to varying concentrations of lef, we repeated the experiment, except each embryo was exposed individually in a 96-well plate. The same effect was observed, which allowed us to conclude that the variation seen in a group exposure was not due to some embryos being exposed to more lef than others as a function of location in the petri dish (data not shown).

A previous report used a chemical screen and structural similarity analysis to conclude that lef could interfere with neural crest cell migration and differentiation via DHODH inhibition by its active metabolite, ter (Reference). To determine whether lef-mediated reduction in melanin was due to ter, we exposed zebrafish embryos at concentrations equivalent to our lef exposures as well as at a much higher dose (10 μM). Notably, ter exposure had no effect on melanin production at either concentration (**Fig. 8**).

Exposure of Tg(*sox10*:EGFP) embryos to lef reveals craniofacial malformations in zebrafish larvae.

In order to tease out the mechanism through which lef-exposure delays melanin synthesis, we decided to characterize the effects of lef exposure on sox10 expressing neural crest cells. The *sox10* gene codes for a transcription factor that is a common marker of neural crest cells [81]. Sox10 is expressed in multipotent neural crest cells and is downregulated as the cells begin to differentiate [82].

Tg(*sox10*:EGFP) embryos were exposed to 0.1% DMSO, 2 μM lef, or 2 μM ter. Embryos were visualized at 24 hpf and imaged at 96 hpf to look for neurocristopathies (define this…). No major differences were seen at 24 hpf (data not shown); however, while ter-exposed larvae were indistinguishable from DMSO-exposed controls, lef-exposure caused aberrant jaw patterning (**Fig.9)**. These data show that lef exposure causes craniofacial malformations, most likely as a result of interfering with neural crest cell proliferation.

Variability in melanin is reflected in expression levels of *tyr* and *cyp1a*.

Tyrosinase (tyr) is an enzyme that catalyzes the production of melanin from tyrosine. We explored whether the variability in melanin reflected changes in *tyr* transcription expression levels. Embryos were exposed to DMSO or lef at 500 nM or 2 μM from six to 48 hpf. The percentage of embryos exhibiting pigmentation was concentration-dependent, resulting in 100% of 500nM lef-exposed with melanin and 70% of 2 uM lef-exposed with melanin. We harvested RNA from pooled control embryos, 500 nM lef-exposed embryos with melanin, and from embryos exposed to

2 uM with and without melanin. Similar to pigmentation, *tyr* levels correlated with the degree of pigmentation. *Tyr* expression was downregulated 0.7 fold in the 2 uM lef-exposed melanin-positive embryos and 0.4 fold change in the 2 uM lef-exposed ; however, *tyr* downregulation was only significant in the 2 uM lef-exposed melanin-negative embryos (**Fig. 11a**). Despite knowing that lef exposure results in a downregulation of *tyr*, we still do not know if this is due to transcriptional regulation of the gene or the lack of melanocytes altogether.

In addition, we investigated whether AhR activation, as measured by *cyp1a* expression, was similarly variable. At 48 hpf *cyp1a* was upregulated in 500 nM (25 fold change) and 2 μM lef-exposed embryos with (100 fold change) and without melanin (80 fold change); however, upregulation was significantly greater in the 2 μM lef-exposed embryos with melanin than without (**Fig. 11b**).

The link between the two remains unclear; however, there are differences in expression of genes involved in the melanin synthesis pathway and the AhR activation pathway.

A mutation in the *ahr2* gene does not lead to increased susceptibility to lef-mediated delay in onset of melanin.

After discovering a difference in *cyp1a* upregulation between lef-exposed embryos with or without melanin at 48 hpf, we hypothesized that the AhR pathway is involved in regulating this phenotype. Since AhR2 is important for the conversion of lef into ter through the upregulation of CYPs, AhR2 null fish would be less efficient at converting lef to ter. Higher concentrations of lef would lead to increased

percentages of embryos without melanin after exposure to lef. The *ahr2*-null line, *ahr2*^{hu3335}, was exposed to DMSO or 1 μM lef and scored for the presence of melanin. The average percentage of AhR2 null embryos with melanin after exposure to lef was not significantly different than the percentages of WT embryos (**Fig. 12**). These data suggest that the AhR2 is not necessary for the lef-mediated delay in the onset of melanin.

The leflunomide-induced lack of melanin is a heritable trait.

Since the 2 μM lef-exposed group that did not have melanin also had changes in the expression of *cyp1a* and *tyr* when compared to the group that did have melanin, we hypothesized that there was a genetic basis in susceptibility to lef resulting in a variable melanin phenotype. We exposed embryos to 1 uM lef and at 48 hpf, we recorded the percentage of embryos with melanin, removed them, rinsed them multiple times, and reared them to sexual maturity in the absence of lef. Next, we intercrossed these individuals (F0) to determine whether we had selected for resistance to lef-mediated reduction in melanin. Their offspring (F1) were then exposed to 1 uM lef and at 32 hpf the percentage of embryos with melanin was recorded and compared to the F0 generation. Among the F0 embryos, 72% were melanin positive at 32 hpf compared to 92% in the F1 generation (**Fig. 13**). These findings suggest that the resistance to lef-induced delay in melanin is heritable.

Scoring system for presence of melanin designed for future genomic sequencing assays.

In order to further characterize the genetic alterations between embryos that are resistant to lef and the ones that are susceptible, we decided to sequence their genomes. So far, the embryos had been classified as “with melanin” or “without melanin”. However, for the purpose of discovery of genetic differences, we developed a more granular scoring system for determining five levels of lef-mediated delay in the onset of melanin. At 32 hpf, lef-exposed embryos are split into three groups including “unpigmented”, “partially pigmented”, and “pigmented”. At 48 hpf, the “partially pigmented” and “pigmented” groups are left alone due to their general lack of change in pigment status. The “unpigmented” group from 32 hpf are split into three groups again including “unpigmented”, “partially pigmented”, and “pigmented”. This results in 5 distinct groups of melanin-based responses to lef; representative images for each of these groups can be seen in **Fig. 14**.

3.4 Conclusions

Through this work we have shown that lef, not ter, causes a delay in the onset of melanin in zebrafish. Ter, not lef, is able to inhibit DHDOH so these findings suggest that the inhibition of DHODH by ter is not responsible for the delay in the onset of melanin by lef.

One caveat to our research is that given that ter is structurally distinct from lef, it still needs to be confirmed that ter is getting into the developing embryo. Ter

has been shown to inhibit the proliferation of $CD4^{+,}$ $CD8^{+}$, and $CXCR5^{+}$ T-cells [83]. In the future, embryos could be exposed to lef and ter and stimulated with lipopolysaccharide to illicit an immune response. Theses embryos could then be homogenized to form a single cell suspension and flow cytometry could be used to count the numbers of T-cells present in lef and ter when compared with controls.

Supporting the hypothesis that the AhR pathway is involved in the lef-mediated delay in the onset of melanin, our work has shown that the embryos, which were sensitive to the lef-induced delay in melanin, exhibited lower induction of *cyp1a* than the ones that were resistant to the lef-induced delay in the onset of melanin. Even though levels of *cyp1a* seem to correlate to the amount of melanin present after exposure to lef, the mechanistic role of this enzyme in this phenotype is unclear. The diminished levels of *cyp1a* in the lef-exposed embryos without melanin could be due to SNPS in the *cyp1a* promoter in that group of embryos.

Furthermore, the delay in the onset of melanin due to lef exposure was not exacerbated in Ahr2 null embryos as was expected. In the future, we will use fish with additional knockouts to determine whether the melanin phenotype is mediated through other AhR paralogs.

Using our Tg(*sox10*:EGFP) larvae we showed that lef exposure caused craniofacial malformations in the jaw at 96 hpf. Similarly, TCDD causes these craniofacial malformations [84] and it is mediated through activation of AhR [85]. These data suggest that the lef-induced delay in the onset of melanin is due to aberrations in the neural crest, which are melanocyte precursors, and not due to

alterations in the biosynthesis of melanin. Additionally, the AhR-mediated TCCD-induced craniofacial malformations provides more evidence that the effects of lef exposure on the neural crest is mediated through AhR, not inhibition of DHODH by ter. In the future, we will repeat the Tg(*sox10*:EGFP) experiment, but inhibit AhR activation during exposure, and look for craniofacial malformations at 96 hpf. We will use mutants for all three of the different paralogs for zebrafish AhR to determine which AhR is responsible.

Not all embryos of a clutch were equally susceptible to lef-mediated delay in the onset of melanin. We attribute this to genetic diversity in zebrafish. Zebrafish are an outbred animal model and have significant inter-individual genetic variation. One study has examined the genomes of several different zebrafish strains (AB, Tu, and WIK, n=80 per strain) and found high levels of intra- and inter-strain copy number variants (CNVs) [86]. They also confirmed that the CNVs led to changes in the gene's expression. To confirm that the genetic differences between zebrafish might contribute to the variation in the melanin phenotype, we intercrossed fish that had been embryonically exposed to lef and found to be resistant to the lef-mediated delay in onset of melanin. The percentage of embryos exposed to lef with melanin was higher in the offspring of this intercross than in the offspring of naïve, wild-type fish. These results led us to conclude that there are alleles segregating in some of our fish that influence an embryo's susceptibly to lef and we plan to sequence the DNA to look for genomic alterations.

Leflunomide is in clinical trials to be used as a treatment for melanoma. Further studies on the genetic differences in zebrafish that make them differentially susceptible to lef-mediated alterations in the neural crest could provide insight into the effectiveness of lef as a drug to treat melanoma in humans.

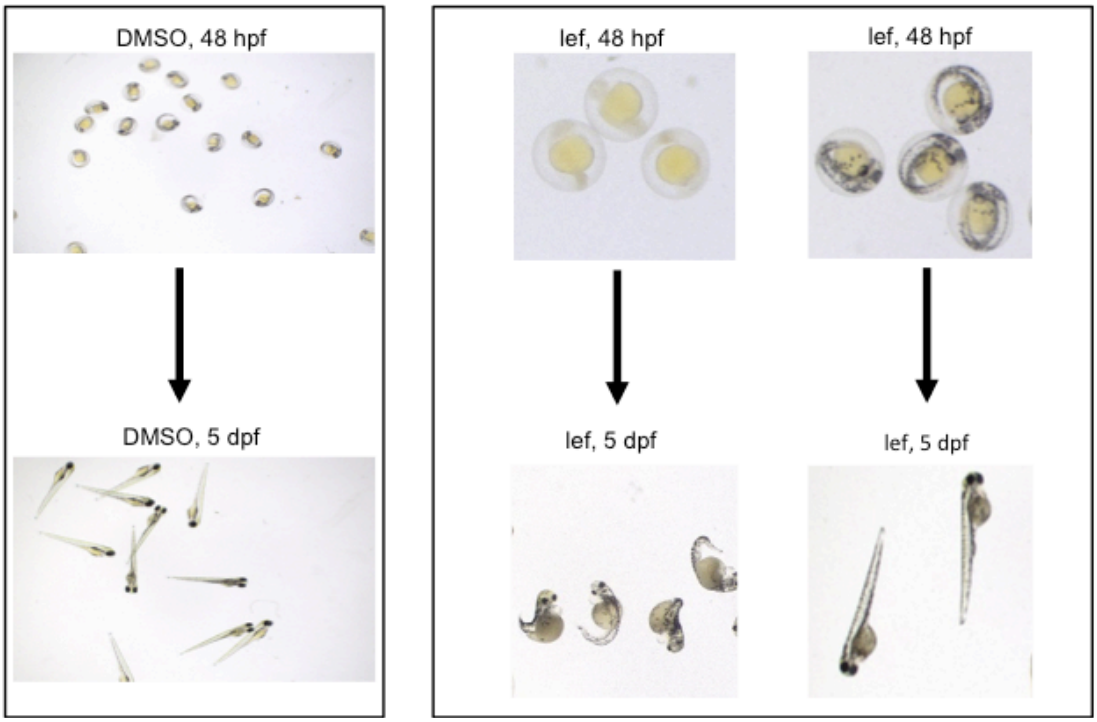


Figure 8. Lef exposure causes a delay in the onset of melanin in some embryos and this delay in melanin is correlated with increased toxicity at 5 dpf. Embryo were exposed to DMSO or 1 μM lef and separated based on presence of melanin at 48 hpf. At 5 dpf, the morphological toxicity induce by lef was consistent with the absence of melanin at 48 hpf.

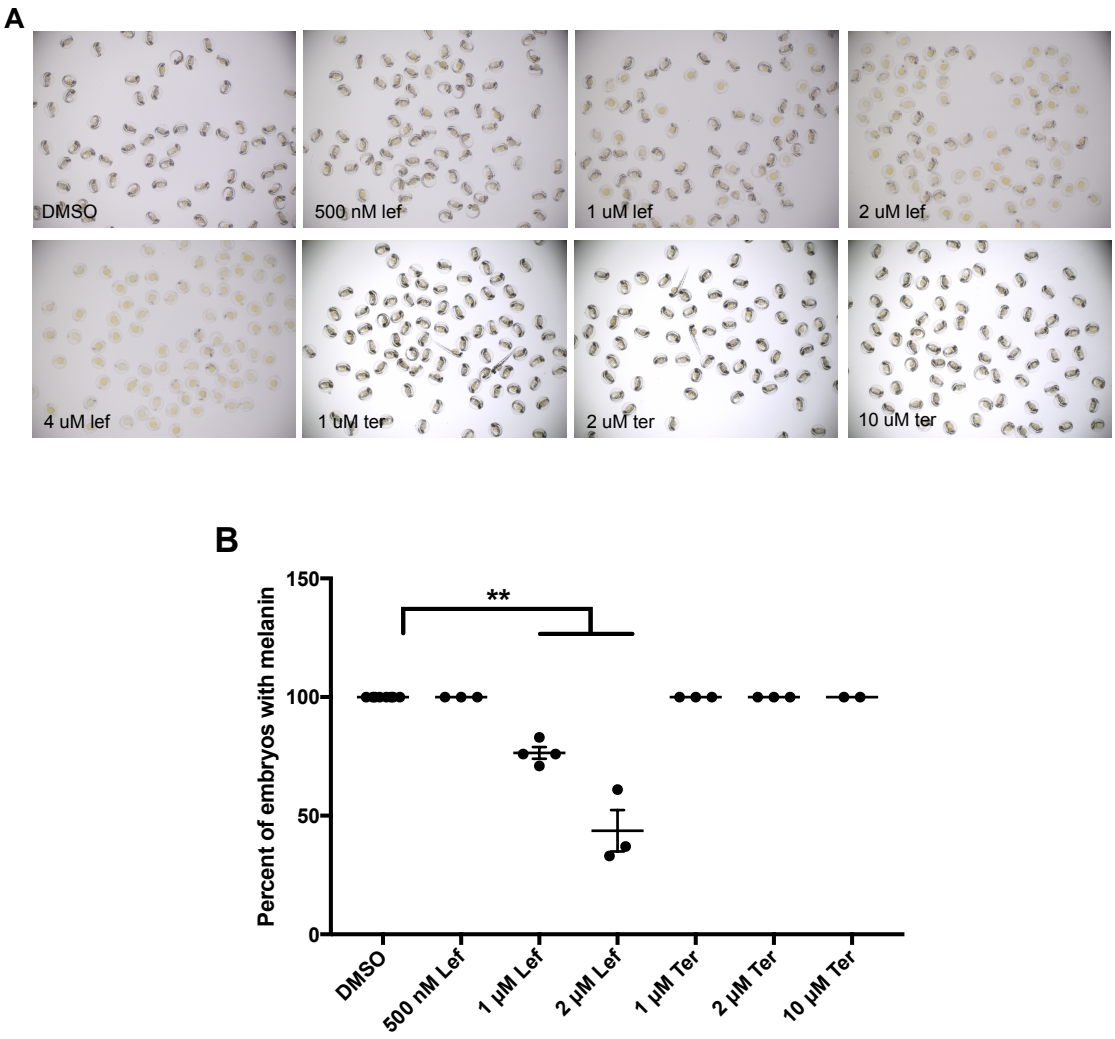


Figure 9. Lef, but not ter, exposure causes a delay in the onset of melanin in a dose dependent manner. (A) Images are representative of 48 hpf embryos continuously exposed to DMSO, lef, or ter. (B) Percent of embryos with melanin at 32 hpf under varying experimental conditions. n=3 experiments with 100 embryos/experiment, **p-value <0.05, One-way ANOVA with Tukey post hoc.

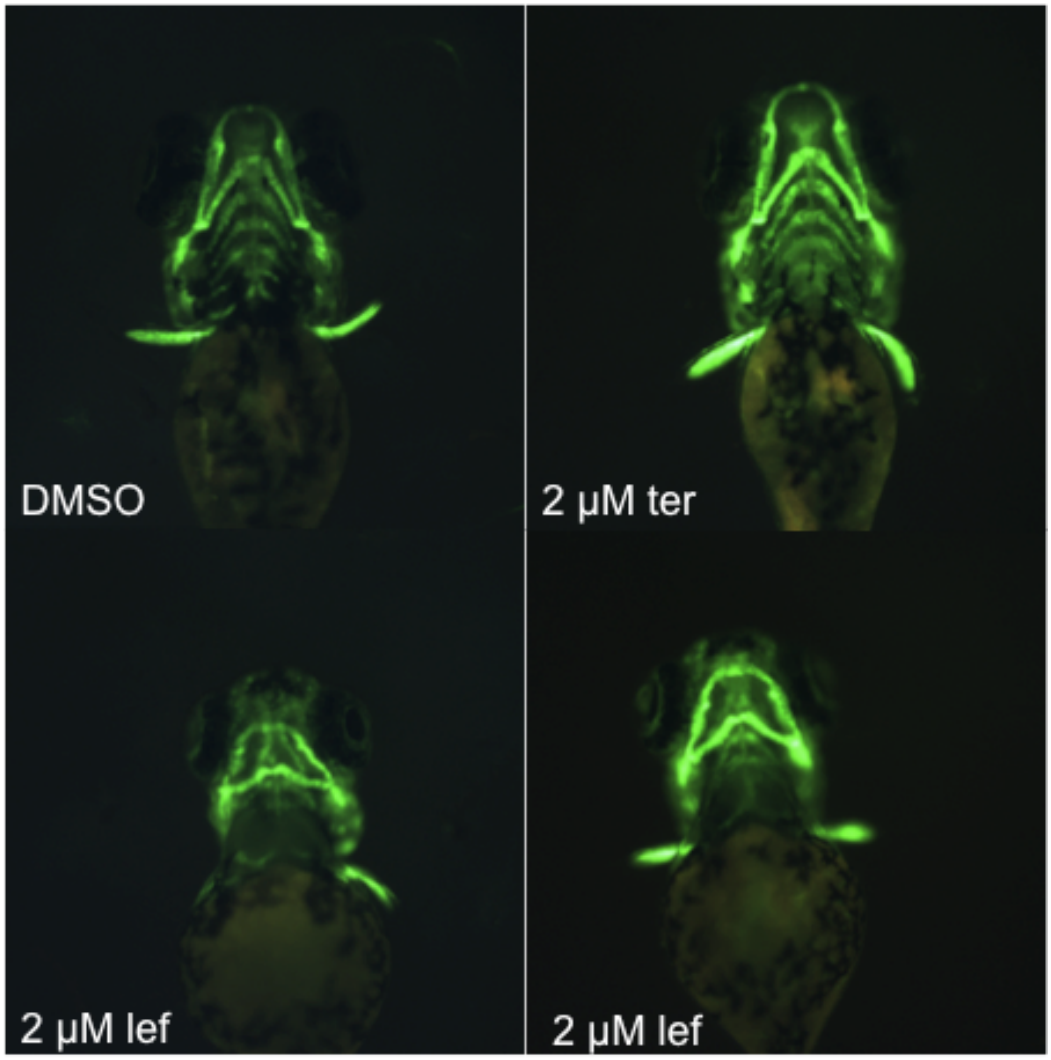


Figure 10. Exposure of Tg(*sox10*:eGFP) embryos to lef causes craniofacial malformations in zebrafish larvae. Exposure of sox10:egpf embryos exposed to DMSO 2 μM lef, or 2 μM reveals alterations in patterning of neural crest cells at jaw at 96 hpf.

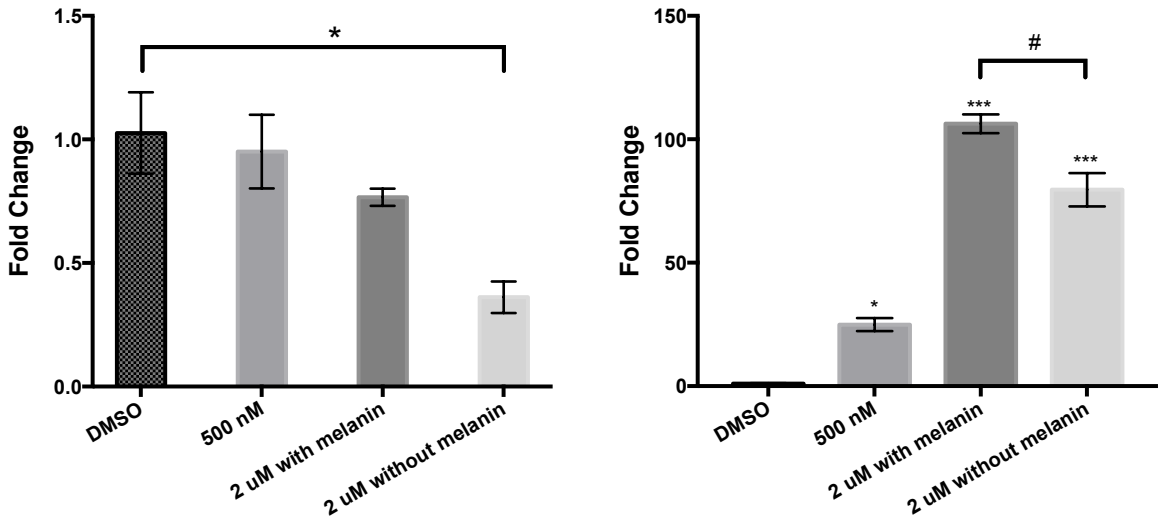


Figure 11. Differential melanin expression is coupled with differential upregulation of *cyp1a*. Graphs represent fold change for *tyr* (left) and *cyp1a* (right) at 48 hpf in response to lef-exposure. The 2 μM lef-exposed group was split into groups based on presence of melanin. *Cyp1* is a gene classically known to be upregulated in response to AhR activation and the difference in upregulation (2 μM) indicates a difference in some part of the AhR pathway. n=3, *p-value <0.05 compared with DMSO, ***p-value <0.0001 compared with DMSO, # p-value <0.01, One-way ANOVA with Tukey post hoc.

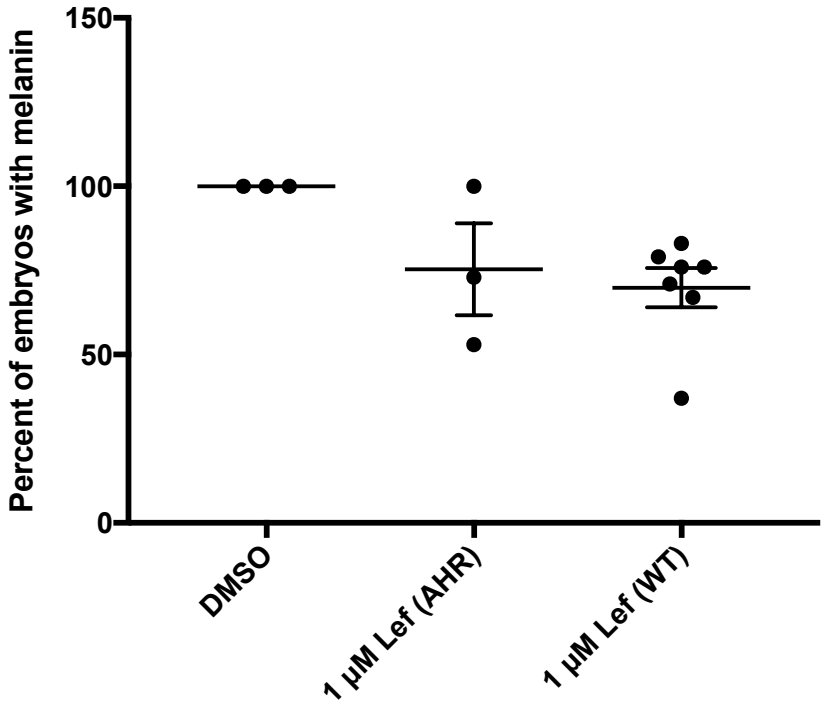


Figure 12. Lef-exposed AhR2-null fish are not more susceptible to a delay in the onset of melanin than WT. Graph reports percent of embryos with melanin at 32 hpf. Embryos were homozygous for *ahr2* mutation. n=3 experiments with 100 embryos/experiment, NS, One-way ANOVA with Tukey post hoc.

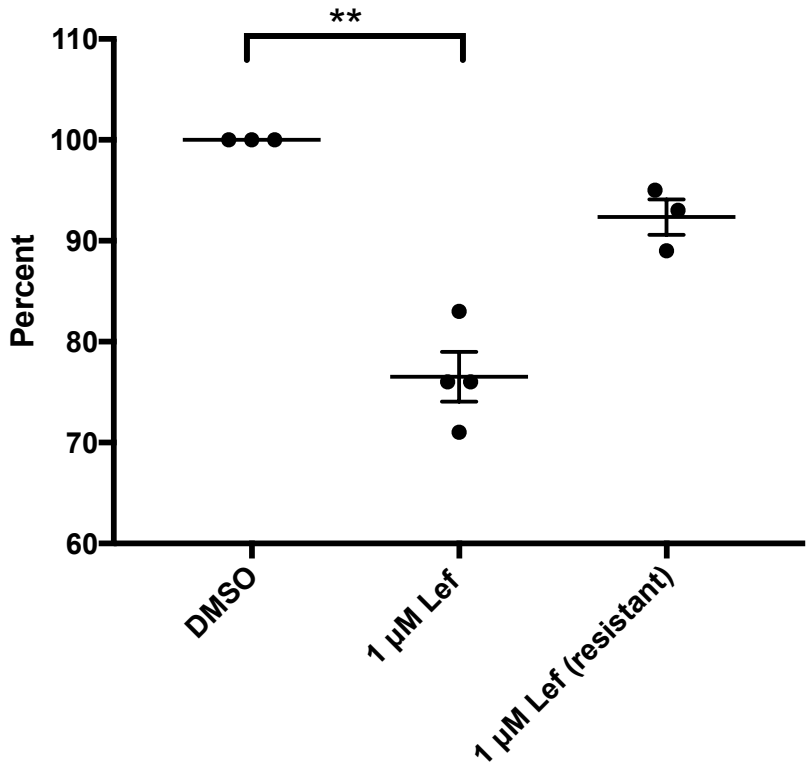


Figure 13. The resistance to lef-induced lack of melanin is heritable. Graph reports percent of embryos with melanin at 32 hpf. 1 µM lef group is naïve exposed WT and 1 µM lef (resistant) group is the offspring of fish that were selected for their resistance to lef-induced delay in melanin onset when exposed developmentally. They were reared to determine if the resistance could be passed to the next generation. n=3 experiments with 100 embryos/experiment, p-value <0.001, one way ANOVA with Tukey post hoc.

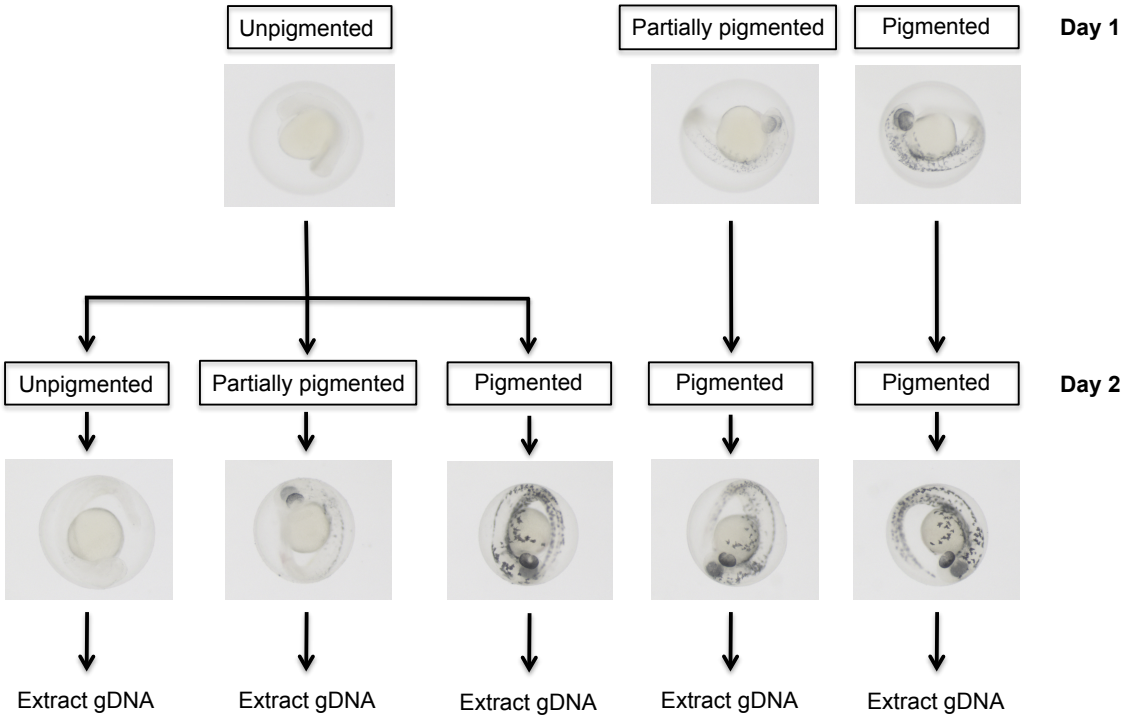


Figure 14. Lef-exposed embryos are separated for genomic sequencing based on amount of melanin. The schematic above represents how the embryos are divided into groups based on the presence of pigment. On day 2, genomic DNA was extracted and will be sequenced to look for genetic variations that could affect the susceptibility to lef-induced delay in the onset of melanin.

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APPENDIX

APPENDIX A

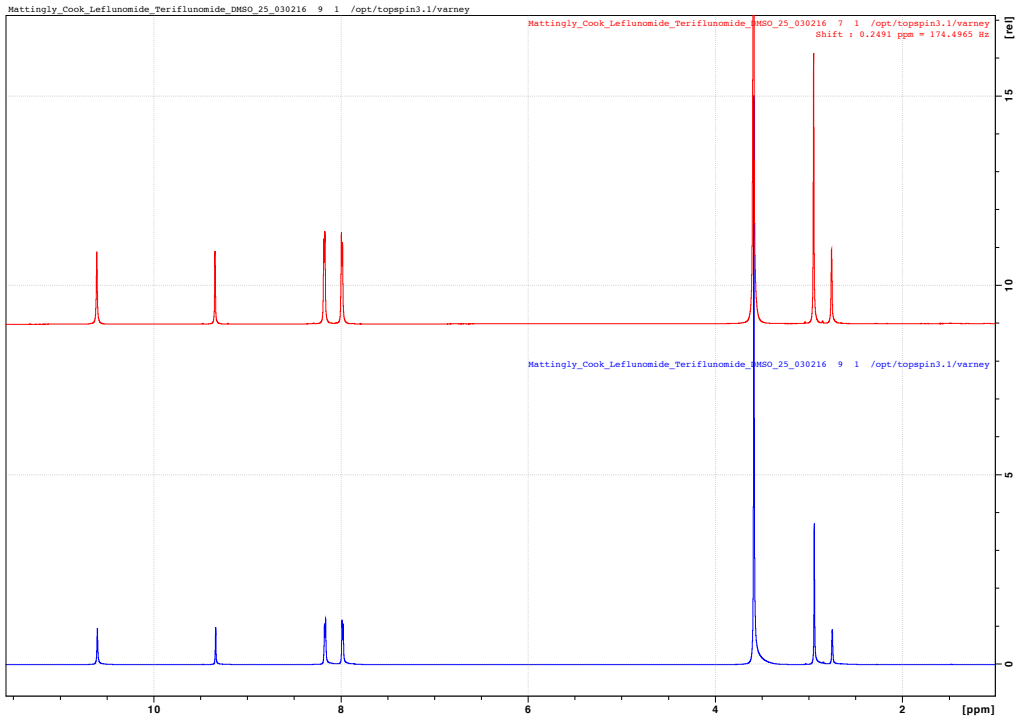

Figure 15. Lef proton nuclear magnetic resonance (NMR ^{1}H) spectrum. The NMR spectrum for lef was initially recorded after suspension in DMSO (top) and 1 month later after storing at 4°C (bottom). There was no change between spectra, indicating a lack of conversion of lef to ter in these storage conditions.

APPENDIX B

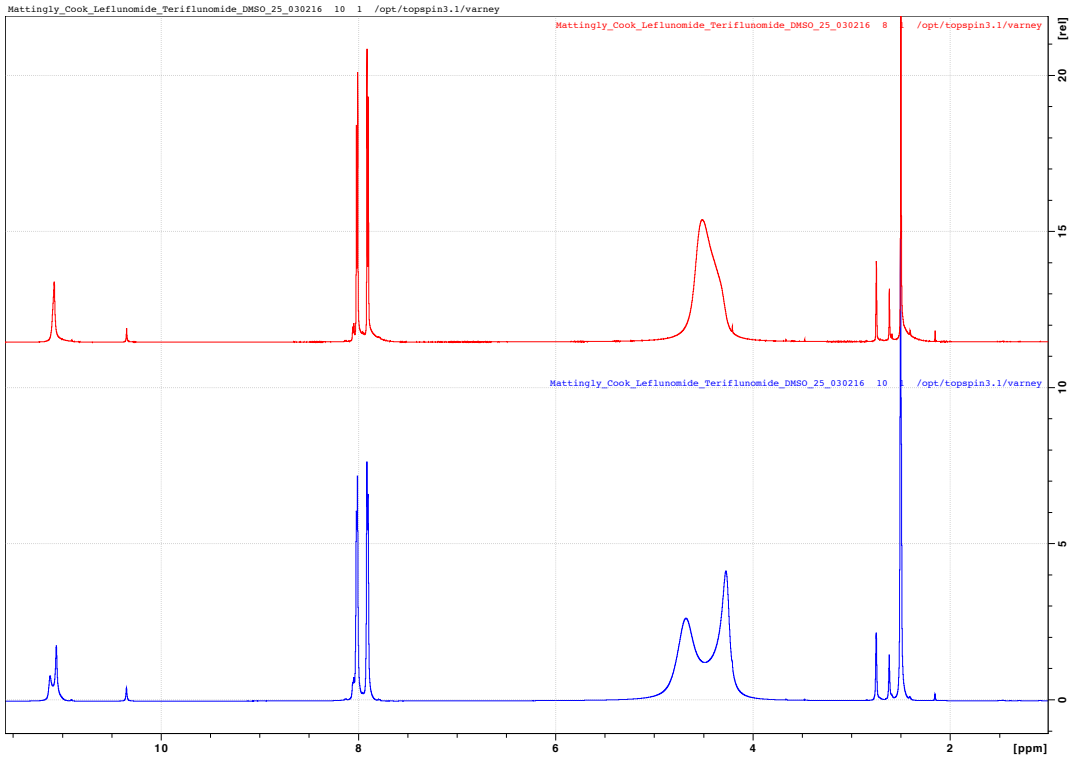

Figure 16. Ter NMR ^{1}H spectrum. The NMR ^{1}H spectrum for ter was initially recorded after suspension in DMSO (top) and 1 month later after storing at 4°C (bottom). There was no change between spectra.

APPENDIX C

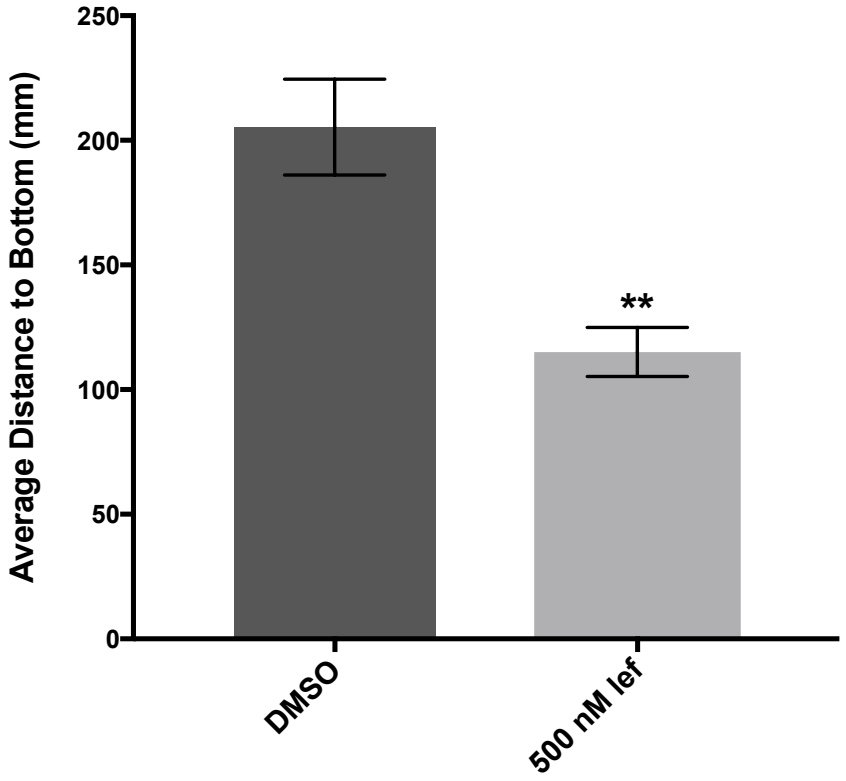

Figure 17. Lef induces an anxiety-like phenotype in adult zebrafish. After an eight-day exposure to DMSO or 500 nM lef, fish were individually placed in a novel tank and distance to bottom of the tank was recorded for 5 minutes. Zebrafish exposed to lef spent more time at the bottom of a novel tank when compared with DMSO controls. ** P-value <0.01, t-test, n=2 experiments.

APPENDIX D

Table 1. Lef exposure causes a decrease in dopamine (DA), but not 3,4-dihydroxyphenylacetic acid (DOPAC) or homovanillic acid (HVA). Embryos were exposed to DMSO, 250 nM lef, or 500 nM lef for 48 hours and then homogenized for analysis with high performance liquid chromatography. DA was down in the 500 nM lef exposed group; however, the metabolite of DA, DOPAC, was unaltered. Another metabolite of DA, HVA, was down in the 250 nM lef exposed group and not detected (ND) in the 500 nM lef exposed group. Measurements represent ng of metabolite per mg of total protein.

	DA (ng/mg)	DOPAC (ng/mg)	HVA (ng/mg)
DMSO	1.582486907	0.42709084	0.213712427
250 nM lef	1.684402189	0.32015056	0.032704689
500 nM lef	0.777047991	0.4486652	ND