TRANSGENERATIONAL RESPONSES TO ENVIRONMENTAL STRESSORS IN VERTEBRATES: FROM ORGANISMS TO MOLECULES

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Genomic modifications occur slowly across generations, whereas short-term epigenetic transgenerational inheritance of adaptive phenotypes may be immediately beneficial to large numbers of individuals, acting as a bridge for survival when adverse environments occur. In this study we used dietary exposure to crude oil as an example of an environmental stressor to assess its effects from the molecular to the organismal levels in piscine and avian animal models. In addition, we assessed the role of the parental exposures on their offspring F1 generation. The research developed in this dissertation has contributed to several areas of investigation including molecular biology, animal physiology, and evolutionary biology. The quantitative information from these studies may be utilized to supplement information regarding the proximate and ultimate effects of environmental stressors on fish and bird populations. Furthermore, this information may be used as additional support for understanding the conservation of the responses from the molecular to the whole organismal levels across the vertebrate taxa, as well as their implications for population survival and maintenance. Additionally, the zebrafish (*Danio rerio*), the Siamese fighting fish (*Betta splendens*) and the king quail (*Coturnix chinensis*) have proven to be excellent models to start building a strong basis for understanding the effects of environmental stressors and transgenerational epigenetic phenomena using a multi-level approach. Furthermore, as more raw data and information is discovered, the concatenation of development, organismal variation, epigenetics inheritance, natural selection, speciation and evolution is being slowly decrypted.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 General Approach

Experimental approaches for understanding evolution and heritability have been expanding rapidly. The prominent technological and experimental advances in studying the role of non-genetic phenomena (epigenetics) in evolutionary biology and, in general, life sciences (Burggren, 2016; Frías-Lasserre and Villagra, 2017; Ho and Burggren, 2010; Jablonka and Lamb, 2015), has opened a new conceptual framework for understanding how animals and plants adapt to new environments (Laland et al., 2015). In this regard, experiencing new environments or stressors during the lifetime of a parental population is potentially important for the inheritance of phenotypes by subsequent generations. Furthermore, the inheritance of these traits occurs via changes in the genome or modifications in epigenomic components associated with DNA (Jablonka and Lamb, 2007) in addition to changes in the nucleotide sequence (i.e. classic genetic inheritance). The transgenerational epigenetic inheritance of phenotypes does not induce any change in the DNA sequence, and the information is transmitted from an organism to its offspring and potentially to subsequent generations of the offspring (Burggren, 2014; Ho and Burggren, 2010; Inbar-Feigenberg et al., 2013; Jablonka and Raz, 2009; Laland et al., 2015; Tollefsbol, 2014).

Epigenetic inheritance has different outcomes compared to genetic inheritance. Genetic inheritance is typically random, affects just a few individuals at a time (if not just one), is permanent until selection eliminates it, and the mutation does not have connection with its future biological significance (Burggren, 2016; Jablonka and Lamb,

2017; Jablonka and Raz, 2009). On the other hand, transgenerational epigenetic inheritance potentially affects a large proportion (if not all) of the subsequent generation (Burggren, 2016; Burggren, 2017). Additionally, since particular stimuli or stressors may modify gene expression on specific genes, the epigenetic modification may not be random and could influence specific developmental processes and stages (Jablonka and Lamb, 2015; Laland et al., 2015). These modifications potentially sunset once the stressor conditions have passed (Burggren, 2016; Burggren, 2014). Furthermore, transgenerational inheritance of phenotypic traits by epigenetic mechanisms stands out as more likely to occur when environmental conditions are unstable, offering a way for a large proportion of a population to overcome or “bridge” stressful conditions (Burggren, 2016). However, just like genetically inherited phenotypes, epigenetically induced phenotypes vary within a population, becoming a substrate for natural selection and, as a consequence, they are largely (if not completely), related to their evolutionary value (Klironomos et al., 2013). Those modifications that increase fitness of certain individuals could be pro-selected, driving genotypic frequencies across populations (Burggren, 2016; Burggren, 2017; Jablonka and Lamb, 2017; Klironomos et al., 2013).

Our current understanding about the role of adaptive transgenerational epigenetic effects and their implications within an evolutionary approach is limited and had been mostly linked with maladaptive implications in human-focused disciplines such as medicine (Baccarelli et al., 2010). This approach has marginalized the role of epigenetic inheritance as an aid for organisms (and populations) for coping with stressors and prevailing under changing conditions in short term. Although it has not been deeply studied, epigenetic transference of signals from parents to their offspring could have

adaptive implications, such as the improvement of resistance against stressors similar to those experienced by the parents, or enhancing the niche width of the offspring (Burggren, 2014; Herrera et al., 2012; Schrey and Richards, 2012). Among the common stressors that fish face every day in the environment are variation of oxygen levels (Pan et al., 2018; Pelster et al., 2018), temperature fluctuations (Cheng et al., 2017; Schulte, 2014) and exposure to chemicals compounds from anthropocentric and/or natural sources (Bhandari et al., 2015; Incardona, 2017), to list just a few such stressors.

1.2 Environmental Stressors

In natural environments, organisms are exposed to periods where oxygen availability naturally drops or raises, sometimes drastically (Welker et al., 2013). To increase the probabilities of survival, organisms cope with hypoxic or hyperoxic conditions via phenotypic acclimatory responses to increase or decrease systemic oxygen circulation (Ho and Burggren, 2012; Pan et al., 2018; Wang et al., 2018). Among other responses, these include gill remodeling, increases or decreases in surface or aerial respiration frequency, changes in the partial pressure of oxygen in swim bladder, enhancement of antioxidant capacities, changes in ventilatory responses, and behavioral modifications (Dhillon et al., 2013; Nilsson et al., 2012; Pelster et al., 2018).

In addition to variation in oxygen levels, fluctuations in temperature also exert selection pressure on fish by modifying developmental rates and metabolic responses (Arevalo et al., 2018; Gagliano et al., 2007). For example, comparison between northern and southern populations of killifish (*Fundulus heteroclitus)* acclimated to different temperatures showed differences in aerobic swimming performance and metabolic rates (Schulte, 2014). In addition, blood cell count and viability, changes in mRNA

transcriptomic levels and DNA damage also are affected by temperature stress (Cheng et al., 2017). In other animals like birds, changes in basal metabolic rates are associated with the temperature and the precipitation of the environment that they inhabit (Thompson et al., 2015; White et al., 2007).

Besides natural factors affecting individual fitness, organisms are exposed to stressors from anthropogenic sources. Chemical compounds such as endocrine disruptors (EDCs) and persistent organic compounds (POCs), enter into aquatic and terrestrial environments as a waste product of human care products and urban runoffs (Ingre-Khans et al., 2017; Mabansag et al., 2019; Piazza et al., 2019). In fish, these compounds affect fitness and reproductive success by inducing masculinization of females or feminization in males (Cai et al., 2019; Matthiessen, 2013). However, in birds and other vertebrates, the effects expand to the modification of behavioral patterns of parental care and amount of laid eggs (Kidawa et al., 2017; Weiser et al., 2018)..

In additional to the above-mentioned compounds, polycyclic aromatic hydrocarbons (PAHs) have become a concern for humans and other vertebrates in the past decade, triggering a remarkable increase on studies aimed on understanding their effects on development and physiology (Abdel-Shafy and Mansour, 2016; Greer et al., 2019; Harr et al., 2017a; Incardona and Scholz, 2018a; Pasparakis et al., 2019). PAHs are among the most important components of crude oil. These compounds have been described by the World Health Organization (2000) as large group of organic compounds composed of Hydrogen and Carbon atoms arranged in the form of two or more fused benzene rings, organized in linear, angular, or cluster arrangements. This structure grants them relatively low solubility in water but also makes them highly liposoluble (Incardona,

2017; Sims and Overcash, 1983). Physical and chemical characteristics lead to a differentiation of their environmental fate and transport, hence the route of exposure is variable. In fish, for example, exposure occurs through the gills, dietary intake or skin diffusion (Tierney et al., 2013), while in bird species exposure could occur dermally, as well as via diet and respiration (Dubansky et al., 2018). Furthermore, when PAHs enter into the vertebrate system, the effects range from the molecular, such as downregulation of genes (Edmunds et al., 2015), to the organismal level, including morphological deformities, compromised physiological performance, decrease in survivorship and behavioral alterations (Incardona and Scholz, 2016; Johansen et al., 2017; Leighton, 1993; Munilla et al., 2011; Nelson et al., 2016; O'Hara and Morandin, 2010; Sørhus et al., 2017).

Overall, environmental conditions are comprised of the interaction of natural and anthropocentric factors that may concur, thus exerting combined and emerging selective pressures on vertebrates (Schulte, 2014). However, study of the effects of *interacting stressors* on fish are rarely considered in experimental designs, and even less studied across generations.

1.3 Transgenerational Epigenetics

If a parental generation experiences acute or chronic exposure to a stressor (like crude oil or hypoxic conditions), it is probable that those adult organisms abandon the contaminated areas and, due to their detoxifying physiological capacities, they could be able to survive and reproduce after exposures. After the parental generation experiences the exposure, it is possible that their subsequent generations inherit phenotypic modifications that could be either disadvantageous or beneficial when they face similar

conditions. These transgenerational effects are presumably inherited without induction of any change in the DNA sequence and have received a considerable attention during the past two decades (Burggren, 2014; Ho, 2014; Ho and Burggren, 2012; Inbar-Feigenberg et al., 2013; Jablonka and Raz, 2009; Laland et al., 2015). Definitions for this phenomena have been proposed in more than 300 publications. For example Tollefsbol (2014), defined transgenerational epigenetics as *the transmittance of information other than that which is encoded in DNA sequence from an organism to its offspring and potentially to subsequent generations of the offspring*.

Transmission of epigenetic effects could be the result of *molecular epigenetic inheritance*, which could affect the *Molar epigenetic inheritance* in subsequent generations (Burggren and Crews, 2014). Among others and involving some that probably have not been uncovered, the mechanisms by which epigenetic inheritance occurs involve DNA methylation patterns, histone modification, non-coding RNA activity, self-sustaining loops and structural inheritance (Abdel-Shafy and Mansour, 2016; Bautista and Burggren, 2019; Burggren, 2016; Burggren, 2014; Ho, 2014; Ho and Burggren, 2012; Seemann et al., 2017). These mechanisms could result in epigenetically transmitted effects reflected as the exhibition of varied phenotypes by the offspring, which could be subject to selection. If beneficial, the frequency of these phenotypes could increase at the population level. Furthermore, since the presence of epigenetic markers varies within a population, it is likely that the genotype frequencies within a population could be also subject to change and become a substrate for natural selection (Burggren, 2016; Skinner, 2015). When the phenotypes are favorable, they could be followed by

genetic assimilation (Waddington, 1953), or, in the case of adverse phenotypes, they could be fixed via genetic drift (Burggren, 2016; Pál and Miklós, 1999).

Evidence supporting transgenerational epigenetics has been increasing in the past two decades (Bautista and Burggren, 2019; Burggren, 2016; Burggren, 2015; Ho and Burggren, 2012; Jablonka and Lamb, 2017; Klironomos et al., 2013). Notwithstanding, most of the studies intended to understand transgenerational epigenetics had been focused on probing the molecular aspect of this phenomena. Such approach has left nearly unexplored a more holistic and inclusive multi-scale (organismal to molecular) approach to determine how variation among individuals arise and function (Danchin and Pocheville, 2014; Peterson, 2017).

1.4 Research Rationale, Approach and Goals

The framework known as the Extended Evolutionary Synthesis (Laland et al., 2015) has brought back ideas and answers from early philosophers and scientists. These ideas are being recently factored into, among other fields, comparative integrative studies aimed at understanding the role of epigenetics on short-term intragenerational and intergenerational organismal responses to variable environments. Whether adaptive or maladaptive, those responses are trans-generationally transferred by means of epigenetic molecular signals that influence the expression of genes, leading to a discernible variation of responses exhibited from cellular to the behavioral phenotypic traits.

The present proposal was designed with a multi-scale approach for understanding transgenerational epigenetics. For this purpose, this dissertation presents a series of holistic experimental designs that allowed assessment from the molecular to the

behavioral levels of organization, the impacts of environmental stressors, such as dietary exposure to crude oil. The approach used in this dissertation embraces the evaluation of these effects in the populations directly exposed to the stressor (P_0), and also involves the assessment of the same (or similar) variables in their immediate offspring generations. This approach allowed us to determine if the "parental phenotype" (if any) resulting from the direct exposure to the stressor was inherited by their F_1 offspring generation The general goal, explained in more detail below in Section 1.6, is to provide more comprehensive answers of how environmental conditions shape phenotypes during development, leading to the transference of molecular signals to subsequent generations, and their implications on organismal success and fitness.

1.5 Animal Models for Studying Transgenerational Epigenetic Inheritance

This dissertation employed three different animal models: the zebrafish, the king quail, and the Siamese fighting fish.

1.5.1 The Zebrafish (*Danio rerio*)

The zebrafish was introduced into the research field by George Streisinger (considered the founding father of the zebrafish research) when, in 1981, he and his colleges published an article focused on the use of this fish as a genetic model for the study of mutagenesis screens (Spence et al., 2008; Streisinger et al., 1986; Westerfield, 2007). In addition, the importance of the zebrafish in all research areas could be taken from the fact that it has been the subject of study in more than 37,000 documents (listed in PubMed) in a number of different disciplines as Genetics, Behavioral Sciences, Ecotoxicology and Physiology (Di Paolo et al., 2015; Spence et al., 2008; Westerfield,

2007). Currently, the zebrafish is the 2nd most popular animal model after the mouse, with more than 5 million zebrafish used worldwide in biological experimentation.

Danio rerio is a tropical-freshwater species native from the Indian subcontinent. Its natural range extends from the Cauvery river basin (Southwest), Indus river (North) and Brahmaputra river (Northeastern), being the latest location where this species is naturally present in higher concentrations (Spence et al., 2008). The zebrafish, belonging to the Cyprinidae family, has a laterally compressed fusiform robust and rarely exceeds four centimeters as standard length as adults. The zebrafish has many advantageous characteristics as an animal model; its short generation time (three to four months), quick development (hatching of the larvae takes place between 48 and 72 hpf), external fertilization, large clutches and its maintenance and culture are relatively easy in comparison to other models. Notably, the zebrafish exhibits large and transparent eggs which are readily observed under a compound microscope, allowing its study with a high-resolution visualization. Taking advantage of this characteristic, the heart of the zebrafish can be studied at a single-cell level during its early development. Sophisticated analysis of physiological and morphological defects such as changes in heart rate, cardiac output, stroke volume, contractility, beat rhythm, heart chamber and body malformations, cellular composition, and edema development can be made (Di Giulio and Clark, 2015; Gagliano et al., 2007; Glickman and Yelon, 2001; Perrichon et al., 2017a; Stainier et al., 1993; Stainier and Fishman, 1994). These characteristics make *D. rerio* a highly attractive experimental model when looking for transgenerational effects induced by environmental factors. Zebrafish were the subjects of the studies described in Chapters 2 and 3.

1.5.2 The King Quail (*Coturnix chinensis*)

The king quail (*Coturnix chinensis*) offers several advantages as an avian model for developmental and transgenerational research. It is one of the smallest precocial bird species, making it suitable for the variety of experimental protocols (Chapter 5). Its monogamous behavior offers two main advantages, the first is that tracking of the offspring is more systematic since identification can be egg-specific and recollection can be performed every day, allowing for more powerful analysis of the data; secondly, a better condition status of the king quail is easier to maintain during husbandry, assuring quality and reducing aggressive behaviors in comparison with other species (Adkins-Regan, 2016; Mcdonald, 2010; Puddephatt, 2014). In addition, being a species used in aviculture, most of the good practices for husbandry are well known. Consequently, experimental designs, set ups, management and costs are easier/cheaper in comparison with wild species, allowing for more systematized and controlled exposure conditions. Finally, the king quail is considered to be a prolific avian species. able to lay 1 or 2 eggs a day reducing the probability of experimental failure (Mcdonald, 2010).

C. chinensis is a well-suited species to use as a proxy for understanding the effects of crude oil exposures on parental and subsequent generations. In addition, little is known about the morphological and physiological effects of oil on any bird species exposed to oil (Dean et al., 2017; Harr et al., 2017a; Harr et al., 2017b; Murphy et al., 2016), and even less is known about their effect on reproduction and the early life stages of the offspring. Because of this, research aimed to determine the impact of environmental stressors on birds has a significant benefit to the scientific community and the general public, since those species are ecologically important and for some of them the Gulf of

Mexico represents a migratory step over and natural habitat (Dean et al., 2017; Mazet et al., 2002).

1.5.3 The Siamese Fighting Fish (*Beta splendens*)

B. splendens is a freshwater anabantid fish native from South Asia (Dzieweczynski et al., 2018). It has been widely used in behavioral and medical research on sedatives and other compounds due to its well-known stereotypical aggressive displays (Clotfelter et al., 2006; Dzieweczynski and Kane, 2017; Jaroensutasinee and Jaroensutasinee, 2003; Lane and Briffa, 2017; Monvises et al., 2009). The Siamese fighting fish (*Betta splendens*) relies heavily on stereotypical aggressive behaviors for territory defense and reproductive purposes. To acquire territory and mating opportunities, male betta fish compete fiercely with each other by employing well documented behavioral displays that include gill flaring, darting, finning and lateral displays during swimming (Brown and Clotfelter, 2012; Gozlan et al., 2003). Once males have successfully defended a territory, they must build a bubble nest and then defend it from egg and larva predation for a few days following egg laying by the female (HedayatiRad et al., 2017). Nest building behavior in betta fish is thus a key component needed for successful reproduction (Jaroensutasinee and Jaroensutasinee 2003). These characteristics make the *Betta* fish a suitable fish model to address behavioral changes, and potential underlying morphological changes, induced by dietary exposure to crude oil.

1.6 General Aims and Hypothesis

This dissertation contributes quantitative information to the field of transgenerational epigenetics by establishing the following general goals:

a) Determine the proximate effects of dietary exposure to crude oil on a piscine and an avian species.

b) Determine if dietary exposure crude oil in a parental population (P_0) of a piscine and an avian model lead to transgenerational epigenetic inheritance in their subsequent immediate generation (F_1).

c) Determine the maternal and paternal P_0 contribution on epigenetic inheritance on F_1 offspring populations.

d) Provide quantitative data correlating molecular, tissue, physiological, morphological and behavioral responses to environmental stressors in a transgenerational epigenetic framework.

e) Improve our understanding about the role of transgenerational effects and epigenetics in an evolutionary context.

The general hypothesis tested in this dissertation was:

Hypothesis: Epigenetic transgenerational inheritance will arise from the dietary exposure to crude oil in fish and birds, resulting in effects from the molecular to the reproductive levels of organization.

To meet these goals and test the general hypothesis and its sub-hypotheses, a series of experiments were performed, each comprising a separate chapter of this dissertation. Additionally, introduction and background material specifically supporting the rationale behind each proposed experiment is given at the beginning of each chapter, as are the specific aims and hypothesis for each experiment. Finally, each chapter in this dissertation has been constructed as an individual unit with the purpose to stand as publishable manuscript*, for this reason the findings of each study are extensively discussed at the end of each chapter.

* Chapters 2 and 4 have already been subjected to peer review and have been published. Details are provided at the beginning of each chapter.

CHAPTER 2

PARENTAL STRESSOR EXPOSURE SIMULTANEOUSLY CONVEYS BOTH ADAPTIVE AND MALADAPTIVE LARVAL PHENOTYPES THROUGH EPIGENETIC INHERITANCE IN THE ZEBRAFISH (*Danio rerio*)*

2.1 Abstract

Genomic modifications occur slowly across generations, whereas short-term epigenetic inheritance of adaptive phenotypes may be immediately beneficial to large numbers of individuals acting as a bridge for survival when adverse environments occur. Crude oil was used as an example of an environmental stressor. Adult zebrafish (P_0) were dietarily-exposed for three weeks to no, low, medium or high concentrations of crude oil. The F_1 offspring obtained from the P_0 groups were then assessed for transgenerational epigenetic transfer of oil-induced phenotypes. The exposure did not alter body length, body and organ mass or condition factor in the P_0. However, when the P_0 were bred, the fecundity in both sexes decreased in proportion to the amount of oil fed. Then the F_1 larvae from each P_0 were exposed from hatch to 5dpf to oil in their ambient water. Remarkably, F_1 larvae derived from oil-exposed parents, when reared in oiled water, showed a 30% enhanced survival compared to controls ($P<0.001$). Unexpectedly, from day 3 to 5 of exposure, the F_1 larvae from oil-exposed parents showed poorer survival in clean water (up to 55 % decreased survival). Additionally, parental oil exposure induced bradycardia (presumably maladaptive) in F_1 larvae in both clean and oiled water. We

* This entire chapter is reproduced from Bautista, N.M., Burggren, W.W., 2019. Parental stressor exposure simultaneously conveys both adaptive and maladaptive larval phenotypes through epigenetic inheritance in the zebrafish (*Danio rerio*). The Journal of Experimental Biology, jeb.208918.

conclude that epigenetic transgenerational inheritance can lead to an immediate and simultaneous inheritance of *both* beneficial and maladaptive traits in a large proportion of the F_1 larvae. The adaptive responses may help fish populations survive when facing transient environmental stressors.

2.2 Introduction

Transgenerational epigenetic inheritance enables parent-to-offspring transference of modified phenotypes without alteration in genomic sequence. In its broadest interpretation, this can include maternal/paternal effects – for an introduction into the extensive literature see (Burggren, 2016; Burggren, 2019; Burggren and Crews, 2014; Heard and Martienssen, 2014; Thorson et al., 2017). Our current understanding of the implications of epigenetic inheritance within the framework of dynamic, stressful environments is limited. Indeed, epigenetically inherited phenotypes have been mostly characterized as maladaptive, especially in human medicine (Baccarelli et al., 2010; Burggren, 2016; Lester et al., 2016; Moosavi and Ardekani, 2016) Unfortunately, this 'maladaptive perspective' of epigenetic inheritance has largely overshadowed the potential role of epigenetic inheritance as a positive mechanism enabling individual animals (and populations) to cope with stressors and survive and even thrive under short-term environmental challenges (Burggren, 2016). Yet, epigenetic inheritance can also result in acquisition of *adaptive* phenotypes that could potentially aid organismal survival (Burggren, 2016; Burggren, 2014; Laubach et al., 2018; Motta et al., 2015; Vogt, 2017). Such adaptive phenotypes could include improvement of resistance against the stressors experienced by their parents, or even result in increased niche width for the offspring (Herrera et al., 2012; Schrey and Richards, 2012). For example, in the zebrafish (*Danio*

rerio), 2-4 weeks of parental exposure to chronic hypoxia confers hypoxic resistance to the F_1 generation (Ho and Burggren, 2012). In killifish (*Fundulus heteroclitus*), F_1 and F_2 embryos from parents inhabiting creosote-polluted sites exposed to creosote contamination showed a lower incidence of cardiac deformities compared to embryos from parents inhabiting non-polluted areas (Clark et al., 2014). Clearly, resistance inherited by the offspring is related to the parental experiences, though the specific mechanisms of epigenetic inheritance have yet to be fully determined. However, experimental protocols exploring epigenetic inheritance are scarce (in part because of their complexity and required time and other resources). Consequently, we still have only a poor understanding of the role of transgenerational epigenetic inheritance during exposure to environmental stressors (Seemann et al., 2017; Seemann et al., 2015).

Epigenetic adaptive responses can be generated in response to either natural environmental stressors (e.g. temperature, hypoxia) or anthropogenic stressors (e.g. crude oil and the PAHs it contains). In fact, the line between natural stressors and anthropogenic stressors is increasingly blurring – consider ambient temperature, for example. Such stressors can have serious consequences for aquatic organisms, and especially fish populations. The actions of these stressors may be through some common pathways, such as the aryl hydrocarbon receptor originally implicated in hydrocarbon exposure effects (Incardona, 2017), but now also implicated in hypoxia responses (Button et al., 2017; Nie et al., 2001). Exposure to crude oil and the basic cellular and molecular responses it evokes thus represents a contemporary and important environmental challenge. As importantly, the study of the effects of crude oil exposure go beyond toxicology, in fact potentially providing important insights into basic principles behind how

individual and population-level survival is affected by numerous environmental stressors, and how epigenetic inheritance may alter survival.

Whether acute or chronic, exposure to crude oil and the thousands of compounds it contains can be a potent environmental stressor. In particular for fish, crude oil exposure may occur via the gills, via diet or by skin contact (Tierney et al., 2013), deeply affecting all developmental stages of fish, from molecular to behavioral levels of organization (Bautista et al., 2019; Brette et al., 2014; Carls et al., 2008; Dubansky et al., 2013; Edmunds et al., 2015; Esbaugh et al., 2016; Frantzen et al., 2012; González-Doncel et al., 2008; Incardona et al., 2004; Incardona et al., 2012; Khursighara et al., 2016; Mager et al., 2014a; Nelson et al., 2016; Perrichon et al., 2016; Sørhus et al., 2017; Xu et al., 2017a; Xu et al., 2017b). For example, some studies have reported the existence of a link between embryonic exposures to oil and modified phenotypes exhibited during later developmental stages, such as reduced swimming performance and interference with normal heart development (Hicken et al., 2011; Huang et al., 2014; Incardona et al., 2015; Mager et al., 2014a). Parental dietary exposure to benzo[a]pyrene, an extensively studied polycyclic aromatic hydrocarbon (PAH), increased mortality and the presence of body deformities in its F_1 generation of zebrafish, lasting up to the F_3 generation (Corrales et al., 2014). However, the F_1 generation from a parental zebrafish population dietarily exposed to pyrolytic PAHs failed to show significant differences in hatching success or morphological abnormalities, but did exhibit reduced heart rate and differences in yolk sac surface and the ratio of yolk-sac/whole-larval surface (Perrichon et al., 2015). Thus, while the findings of individual studies vary, crude oil and its components can, along with the natural stressors of hypoxia or elevated temperature, serve as a useful ‘probe’ for

exploring transgenerational phenomena and their mechanisms.

Experimentation testing the role on subsequent generations of acute and chronic exposures to natural or anthropogenic stressors is rarely practical with parental wild fish populations. Consequently, the zebrafish has been widely used as a model to perform acute and chronic effect-directed analysis of stressors in several disciplines such as genetics, behavioral sciences, ecotoxicology and physiology (Burggren and Dubansky, 2018; Di Paolo et al., 2015; Jaspers et al., 2014; Milash et al., 2016; Pitt et al., 2018; Spence et al., 2008; Zhou et al., 2019).

2.2.1 Goals

Dietary exposure to crude oil in fish has not been widely study. Consequently, little is now about its proximate effects on the exposed population. Even less is known if dietary exposure to crude oil in a parental population elicits transgenerational epigenetic effects on their offspring population. Thus, the goals of this chapter were

(i) To establish what are the proximate phenotypic and reproductive effects of dietary exposure to crude oil within environmentally relevant concentrations in a parental population of zebrafish, and

(ii) To determine if the parental exposure to crude oil elicited any transgenerational epigenetic effect on their offspring generation

2.2.2 Hypotheses

The current study uses the zebrafish to test the following hypotheses:

1) Dietary exposure to crude oil, within environmentally relevant levels, will affect primary indicators of fish health such as body mass and length, organ mass, condition factor (K) and Specific Growth Rate (SGR) (Barnham and Baxter, 1998; Cook et al., 2000; Williams, 2000).

2) Dietary exposure to crude oil, will affect variables directly related to reproductive success such as fecundity, fertilization and egg, and sperm quality in the parental population.

3) Dietary exposure to crude oil, will induce tissue disruption of the gonads and cardiac collagen deposition, which have been associated with oil exposure (Chablais et al., 2011; Gemberling et al., 2013; Grivas et al., 2014; Horn and Trafford, 2016; Kikuchi, 2014; Marro et al., 2016).

4) Dietary crude oil exposure of a parental population will enhance resistance of their larvae (i.e. enhance their survival and heart rate) by means of non-genomic inheritance.

2.3 Materials and Methods

Two separate but complementary experiments were completed during this study. The first, hereafter called the “fecundity experiment” was performed with 280 zebrafish. Its aim was to determine if dietary exposure to crude oil affects variables directly related to their reproductive success. The second, the “inheritance experiment”, was performed with 120 adult zebrafish, and was focused on determining if parental dietary exposure to crude oil elicits enhanced survival in their F_1 generation during exposure to crude oil via water. For both experiments, similar protocols, fish care and maintenance, preparation of dietary treatments, parental exposures, and F_1 larval exposures were used, unless otherwise specified.

All experiments were approved and performed in compliance with the Institutional Animal Care and Use Committee (IACUC-Protocol #15003) at the University of North Texas.

2.3.1 Fish Care and Maintenance: Parental Generation (P_0)

Adult AB strain zebrafish were obtained from a local supplier and maintained individually in 1 L tanks at the University of North Texas. Prior to experimentation, the fish were acclimated for two weeks under recommended husbandry conditions for this species (~27±0.5 ºC, pH ~7.8, 14:10h light:dark cycle, ~ 7.8 DO mg/L) (Spence et al.,

2008; Westerfield, 2007). Fish were fed ~3% of body weight per day with commercial flake food (TetraMin Tropical Food).

2.3.2 Experimental Design

2.3.2.1 Preparation of Dietary Treatments for P_0 adults

Dietary exposure to crude oil was used as the stressor in experiments with adult zebrafish. To prepare oiled diets, solutions of High Energy Water Accommodated Fractions of crude oil (HEWAF) were prepared following standard protocols (Bautista et al., 2019; Forth et al., 2017; Mager et al., 2014a; Reddam et al., 2017). Source oil “B” (SOB) sampled from the Gulf of Mexico MC252 well on May 22–23, 2010 was used for this experiment (British Petroleum acknowledges the use of a defoamer (Nalco EC9323A), oxygen scavenger (Nalco VX9831) and methanol during the collection of this type of crude oil. Although their presence in SOB cannot be dismissed, the direct sampling from the riser insertion tube may reduce the possibility of incorporation of these compounds into the oil (de Soysa et al., 2012)). In brief, 2000mg of crude oil were added into 1L of conditioned aquarium water and blended for 30 s in a commercial blender (WaringTM CB15). After blending, the mixture was placed into a separation funnel for 1 h, after which 100 ml of the solution was taken out through a bottom port of the funnel and discarded. 600 ml of the remaining mixture (considered as 100%HEWAF) and two diluted solutions (10% and 50% HEWAF in conditioned aquarium water) were used for diet preparation.

Four dietary treatments were used for parental exposures for adult fish: a) Control, b) 10%HEWAF, c) 50%HEWAF, and d) 100%HEWAF. To make these dietary treatments, two g of commercial flake food (Tetramin®) were evenly distributed across the bottom of

plastic weighing boats (135L ×135W ×20mmH). The food was sprayed 5 times (5ml total solution volume) with conditioned water (Control) or one of the three HEWAF solution concentrations described above. The spraying process was performed under a fume hood, after which the treated food was allowed to dry for ~12 hours. The dried food was then collected from the weighing boats, and stored at 4º C in amber glass bottles covered with aluminum foil.

Representative samples of the treatment diet were analyzed by ALS Environmental (ALS Environmental, Kelso, WA, USA) to obtain total polycyclic aromatic hydrocarbons (PAH) concentrations (ΣtotPAH). PAHs are petroleum components well-known to affect the cardiac system, swimming capacity, performance, and morphology throughout developmental stages in fish (Incardona et al., 2014; Incardona and Scholz, 2018a; Mager and Grosell, 2011; Stieglitz et al., 2016). Thus, determination of [PAH] in the diets offers a valid indication of the toxicity level of each treatment used during this study (Bautista et al., 2019; Mager et al., 2014a). Also measured for each food treatment was the sum total of 50 commonly selected PAHs (Σ50PAH) used for the Deepwater Horizon Natural Resource Damage Assessment toxicity testing program (Dubansky et al., 2018; Esbaugh et al., 2016; Johansen et al., 2017) . Total PAH levels as assayed for each specific dietary composition were proportional to the percentage of HEWAF used to spike the food, indicating the validity of stressor (oil) PAH delivery via this pathway. The Control group had a Total PAH concentration of less than 0.14 mg/kg of food. Total PAH concentrations of 10%HEWAF, 50%HEWAF and 100%HEWAF diets were 2.3, 12.8 and 24.2 mg/kg, respectively (Fig. A2-1), and 65-70% of total PAHs for all three diets comprised the 50 selected PAH analytes (Table. A2-1). Unfortunately, estimating the

[PAHs] in specific organs was not feasible because of the small size of zebrafish. Thus, ~12g of fish (pools of whole animals) per treatment were also sent for analysis. Total [PAH] concentrations for each experimental treatment were 15.5, 36.86, 28.3, and 62.82 ug/Kg for female and 10.89, 9.73, 26.2 and 18.29 for male µg/kg respectively for Control, 10%HEWAF, 50%HEWAF and 100%HEWAF (Fig. A2-1B and Table A2-2).

2.3.2.2 P_0 Crude Oil Exposure

Adult male and female zebrafish were randomly divided into four groups, each receiving a control diet. After two weeks of acclimation to the holding conditions, exposure to petroleum was initiated by feeding the experimental groups the specific diet (control, low, medium or high HEWAF concentration) twice daily during three week period. To prevent possible non-dietary oil exposure through the gills via water, or by coprophagia, during each feeding event fish were allowed to eat for 10 min, after which non-eaten food and feces were removed. In addition, since the fish were maintained in a closed system, 30% of water volume of each 1-liter tanks was also changed after each feeding event (60% per day).

For the fecundity experiment, after the second daily feeding event on day 21 of crude oil exposure, 21 breeding tanks (3L) per group were established, each containing one female and one male fish from the same parental exposure treatment. The fish were maintained separated by sex overnight. The following morning (day 22) at the start of the light period, in 15 of the tanks the two sexes were placed together for courtship, mating and breeding. Adults in the remaining 6 tanks were used for histologically assessing the testis and gonadal morphology, and to test sperm motility in the males (see below).

2.3.2.3 F_1 Larvae and Crude Oil Exposure

In the inheritance experiment, after the exposure period, female and male fish from the same parental treatment were placed into 10L tanks. Fish were also kept separated by sex overnight. The following morning, the fish were allowed to breed for 2h. The eggs were then collected and rinsed with deionized water and placed in clean conditioned water at 27±0.5ºC. Stereoscopic microscopy was employed to confirm fertilization and cell division of the embryos, and any non-viable embryos were discarded.

Crude oil exposures were made on early F_1 larvae 5 days post fertilization (dpf), which are among the most sensitive developmental stages (Mager et al., 2017a; McKim, 1977; Mohammed, 2013; Réalis-Doyelle et al., 2016; Woltering, 1984). Zebrafish embryos and early larvae subsist on yolk absorbance during the first 5-6 days post fertilization (Anderson et al., 2011; Kimmel et al., 1995), which prevented using dietary crude oil exposure as for the P_0 parents. Consequently, oil exposure for the offspring was performed via branchial and cutaneous exposure in ambient water, an exposure equally relevant as through diet.

F_1 larvae obtained from each parental treatment were subsequently separated into four groups and each group was grown to 5 dpf in one of the following environmental conditions: a) clean water (control), b) 10%HEWAF, c) 50%HEWAF or, d) 100%HEWAF. The exposure conditions were renewed each other day. All larval populations were maintained at 27±0.5ºC.

2.3.3 Phenotype Measurement

2.3.3.1 Parental (P_0) Generation

Adult mortality was assessed daily for each parental treatment. Body mass of

individual adults was recorded with a Symmetry EC-Series Portable Top-loading Balance (100g X 0.001g, 120V). Individual fish were carefully netted and then immediately placed into a previously tared 100 ml water-filled container to obtain body mass to the nearest mg. The measurement was completed within <30 sec. To estimate body length, a lateral photograph of each fish was acquired (Nikon Coolpix AW130, 16Mpx) during body mass determination, and the measurement was estimated by digital analysis with ImageJ Software (https://imagej.nih.gov/ij/). Both body mass and length were measured every second day during the exposure period for experiment one, and at the end of acclimation, first, second and third week of exposure for the fecundity experiment.

Body length and mass were used to calculate the specific growth rate (SGR) of each group (Cook et al., 2000). Also determined was the condition factor (K) (Barnham and Baxter, 1998; Williams, 2000), using the formula $K = ((10^5 * W) / L^3)$ where; K= condition factor of the fish/quantitative index of fish wellness, W= weight of the fish in grams (g), and L= length of the fish in (mm).

After breeding, the P0 adult fish were euthanized by exposure to a solution of ~300mg MS-222 /L buffered with sodium bicarbonate to pH = 7.4. Fish were maintained in the solution for 10 min after opercular movements had ceased, following institutional guidelines. Immediately after euthanasia, fish were fixed in Z-Fix (Anatech LTD) for two days. The ventricle, liver, gonads and the gut were extracted from each fish, weighed and stored in 70% ethanol. Ventricles of the fish were processed histologically, first embedding them in paraffin, and then sectioning them at 4µm for staining with Masson's trichrome. This staining technique allowed pixel density assessment by digital analysis to determine whether dietary exposure to crude oil could cause damaging collagen

deposition among the extracellular matrix in heart tissue, potentially leading to compromised cardiac activity (Carson, 1990; Huang et al., 2014; Sheehan and Hrapchak, 1973). In brief, photographic images from the heart slices were acquired using a Zeiss Axio Imager.M2 and then analyzed with ImageJ to quantify the area containing collagen. All images were acquired using the same microscopy parameters (scale, zoom, opening of diaphragm). We used gill tissue and bulbus arteriosus slices as positive control for the stain (Fig. A2-4). Based on the staining of these tissues, we determined the color threshold values for blue coloration of collagen (zoom 40X, brightness ratio 150:255, saturation 10:255, and HUE ratio 140:190), and used them to standardize the analysis. After setting these parameters in each image, we used the function “analyze particles” in the ImageJ software to obtain the number of pixels that meet the assumptions for collagen coloration. Six to seven ventricles were analyzed per exposure population, with three different sections per individual analyzed and averaged. Gonadal tissues of both male and female fish were also histologically processed and stained with Hematoxylin and Eosin (H&E). The tissue sections were analyzed under optic microscopy and photographed using the equipment mentioned above. To test if fecundity was impaired due to crude oil exposure, after allowing the breeding pairs to court and mate for 1 h, the number of parental pairs that spawned was recorded for each experimental group. All the eggs from each breeding pair were carefully collected by using a disposable pipette to siphon them from the bottom of the tank. Stereoscopic microscopy was used to determine the total egg number, the number of fertilized eggs, the number of non-fertilized eggs and the number of fertilized but non-viable eggs for each breeding pair.

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To assess sperm quality in the fecundity experiment, the remaining 6 breeding pairs from each experimental group were set and maintained in overnight conditions as if for breeding, as described above. However, instead of allowing the fish to breed, the next morning, the male fish from each tank were transferred to a specific three-litter container per group. Sperm characteristics were assessed on these adult males following the protocol described elsewhere (Wilson-Leedy and Ingermann, 2007). In brief, males were anesthetized using 100 mg/L MS-222 solution buffered to pH 7.4. After anesthesia, each fish was carefully netted and dried using a Kimwipe®. Fish sperm become activated when making contact with water, so special attention was paid to drying the area surrounding the male's vent before sampling. After drying, the fish was rinsed in sperm immobilizing medium (ZSI – 140mM NaCl, 10mM KCL, 2mM $CaCl_2$, 20mM HEPES, buffered to pH of 8.5 with 1.0 M NaOH), and then transferred to a sponge previously set for stereoscopic microscopy (Wilson-Leedy and Ingermann, 2007). Fresh seminal fluid was obtained by carefully squeezing the ventral area of the fish and collected by placing a capillary tube in the opening of the vent (Westerfield, 2007). The fish was then placed into a container with aquarium water maintained in recommended conditions (Spence et al., 2008; Westerfield, 2007) and allowed to recover. No mortalities were recorded as a result of this

procedure. An average of 1.8 µl of seminal fluid was obtained per fish, from which 1.5 µl were diluted in 10 µl of ZSI. Activation of the sperm was attained by diluting 2 µl of the diluted sperm into 10 µl of conditioned aquarium water. 5 µl of this dilution was placed in a depression slide, covered with a coverslip and immediately placed under the microscope for video recording (Zeiss Axio Imager.M2). Three-second videos at 30 frames per second were recorded for each fish using 100X magnification. The videos were recorded at 20°C and within 20 to 45 sec after sperm activation.

Video analysis was performed using the ImageJ software plugin Computed Assisted Sperm Analysis (CASA - availability and documentation: http://rsb.info.nih.gov/ij/plugins/casa.html). Analyzed variables were: percent of motile sperm; curvilinear velocity (VCL µm/s), velocity of the head of the sperm on its curvilinear path; average velocity on a path (VAP, µm/s), which refers to the velocity of the head of the sperm along its trajectory; velocity in straight line (VSL, µm/s), velocity of sperm between its initial and final position on a linear path; linearity (LIN), and sperm count (Wilson-Leedy and Ingermann, 2007).

2.3.3.2 Larval F_1 Generation: Inheritance Experiment

Larval mortality experiments were conducted in two phases. In the first phase, the effects of parental HEWAF exposure as a stressor was determined on the survival of F_1 larvae in clean water. The second phase determined the effects of parental oil exposure on the subsequent survival of F_1 larvae when they, themselves, were exposed to varying concentrations of HEWAF. In both phases of this experiment, mortality (evidenced by absence of heart beat) of fish embryos and larvae was assessed on a daily basis from fertilization through 5 dpf.

Heart beat cycles were recorded daily in resting embryos and larvae from each treatment during the 5 days of HEWAF exposure. Heart cycles were videoed over a ~20 sec period using a stereomicroscope (Nikon SMZ1000) adapted with a camera (iPhone 5S). Heart rate (f_H), in beats per minute, was determined in embryos from the video recordings using ImageJ and Adobe PhotoShop CS6 Extended.

This experiment was replicated three times using different adult fish for each replication. With the exception of the subgroups obtained from the parental control group, which were divided into 26 individuals per subgroup for the first replicate, the remainder of the subgroups and subsequent replicates had a density 50 embryos per 50ml beaker. Consequently, 16 groups per replicate were obtained in total (Fig. 2.1).

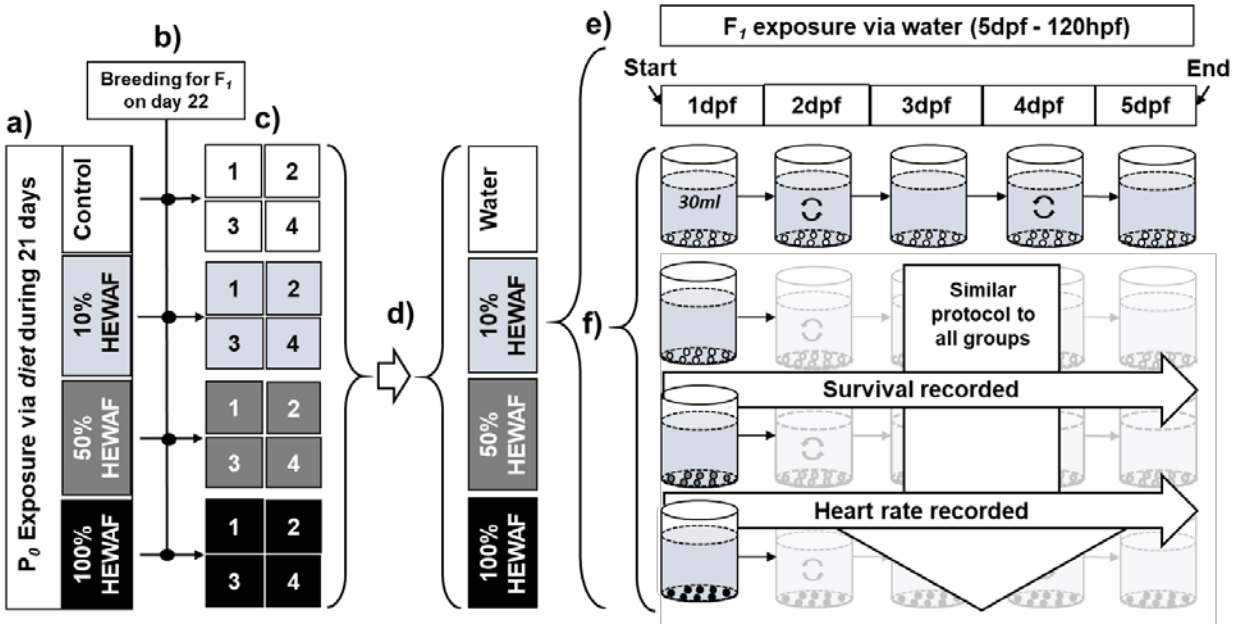

Figure 2.1: Experimental protocol. a) A parental population of adult zebrafish was divided into 4 exposure groups and exposed via diet to water or any of the HEWAF diets for 21 days. b) Offspring (F_1) were obtained from breeding within each one of the parental groups on day 22. c) The F_1 larvae from each group was subsequently divided into 4 subgroups, 1 to 4. d) One subgroup from each F_1 was exposed to water or any of the three HEWAF concentrations for 5 days. For simplification, the F_1 exposure protocol is exemplified by illustrating just the F_1 exposure protocol to "10% HEWAF" via water. e) Exposure to 10% HEWAF began at ~ 3 hours post fertilization and ended at 5dpf. f) The F_1 from the four different parental groups were placed into 50ml beakers filled with 30ml of water or one of the three HEWAF solutions. To maintain levels of exposure, the solution was changed on

day 2 and 4. Survival and heart rate recordings on all populations were performed throughout the 5 days of HEWAF exposure.

2.3.3.3 Larval F_1 Generation: Fecundity Experiment

After determination of fecundity variables mentioned above, the eggs from all parental pairs from the same dietary treatment were mixed. Twenty-five eggs per parental group were placed into a petri dish containing clean egg water and photographed using stereoscopic microscopy. The area of the chorion and the yolk (mm^2) were estimated for each egg by processing the pictures using ImageJ. Using the area and the radius, the volume of the chorion and the yolk were calculated from the formula of the sphere ($V= ¾ \pi r^3$) and, in turn, used to calculate yolk to chorion ratio.

From the remainder of the mixed eggs, samples of 50 eggs were taken to recreate F_1 offspring exposures conditions mentioned above. A total of four beakers per F_1 exposure condition were set for this experiment. Fifteen larvae from two beakers per condition were used to estimate body length at 2 and 5 dpf by image analysis in ImageJ. These measurements were used to estimate SGR. The third and fourth beakers were used to determine the presence or absence of cardiac and yolk edema and/or head and tail deformities at 5dpf under exposure conditions. Determination of these parameters was performed using stereoscopic microscopy.

2.3.4 Statistical Analysis

2.3.4.1 Parental (P0) Generation

For both fecundity and inheritance experiments, a three-way ANOVA was conducted for the parental P_0 generation to test if level and time of stressor exposure and the sex of the fish induced effects on body mass, body length and condition factor. Holm-

Sidak method was employed to determine pairwise comparisons as post hoc tests. Specific growth rate in the inheritance experiment was analyzed with one-way ANOVA. Similarly, the mass of the organs and extent of collagen deposition in the heart was compared between dietary treatment-groups with one-way ANOVAs.

For the fecundity experiment, a chi-square test was used to compare the proportion of mating pairs that spawned. To assess if the number of eggs spawned per female was different among the parental groups, analysis of covariance (ANCOVA) was performed using female mass as covariate. One Way Analysis of Variance was used to compare for fertilization rate, the number of fertilized eggs, the number of non-viable eggs and the number of non-viable but fertilized eggs among parental groups. Similarly, sperm quality variables were compared among treatments using One Way Analysis of Variance.

2.3.4.2 Larval F_1 Generation

For the inheritance experiment, the survival slopes of the offspring in the different replicates was compared with Log-Rank survival tests. No differences were found between slope rates ($P>0.05$) within exposure conditions. Thus, we pooled the data of the three replicates and analyzed and plotted them together. Thus we considered *n* to be 3, where each replicate had 26 – 50 embryos per condition, as above explained.

To assess the significance of differences in survival rate of F_1 offspring in the inheritance experiment, a Cox Stratified Model of Survival was employed. The F_1 exposure conditions (clean water, 10% HEWAF, 50% HEWAF or 100% HEWAF) were selected as “strata” in this analysis while the parental exposure-background (Control, 10% HEWAF, 50% HEWAF or 100% HEWAF) was designated as a covariate. Subsequently, to determine differences between groups within each stratum, Survival Log-Rank tests

were employed. Because statistical assumptions of survival analysis do not allow determination of differences between groups at specific points in time, chi-square tests were performed at each developmental day. Finally, using time (1, 2, 3, 4 and 5 days post fertilization), parental exposure-experience and F_1 exposure condition as factors, heart rate of F_1 larvae was compared with a three-way Analysis of Variance.

Assessment of differences in specific growth rate of F_1 larvae from the fecundity experiment was tested by Two Way Analysis of Variance, in which parental treatment group and the F_1 exposure conditions were used as factors.

Finally, to assess, differences in presence of edemas and body deformities among F_1 exposures within parental groups, chi-square tests were performed.

Statistical significant level was set at $P<0.05$ for all analyses. Data are expressed as means ± standard error of the mean (SEM), unless other indicated.

2.4 Results

2.4.1 Parental P_0 Population

2.4.1.1 Survival

None of the dietary treatments caused any mortality in the P_O generation during the 21-day time course of the experimental PAH exposures in either the fecundity or inheritance experiments.

2.4.1.2 Body Morphology

From the beginning to the end of the dietary exposure to oil, adult female mean mass increased from 399±21 mg to 466±21 mg (fecundity experiment) and from 369±39 mg to 417±42 mg (inheritance experiment). For the fecundity experiment, male body mass was 314.4±16 mg and 347.6±13 mg at the beginning and end of the exposure,

respectively. Male mean mass was 300±14 mg and 336±12 mg, at the beginning and at the end of the exposure period, respectively for the inheritance experiment. Sex was the only factor associated with a significant difference in adult mass in either experiments ($P<0.001$). Either level of dietary stressor nor day of measurement (or any of their interactions) had any significant effect on adult body mass ($P>0.05$).

In both fecundity and inheritance experiments, experimental time, but not sex nor dietary treatment, had a significant effect on adult total body length ($P=0.027$ and $P=0.001$, respectively). No significant interactions between factors were found in either of the experiments ($P>0.05$). For the fecundity experiment, body length increased from 26.3±0.4 mm up to 28.9±0.3 mm in females, and from 27.6±0.5 mm to 28.307±03 mm in males and fecundity experiments. For the inheritance experiment, female and male mean body length during the oil exposure period increased from 25.8±0.4 mm up to 28.8±0.4 mm and 27.8±0.5 mm to 29.2±0.4 mm, respectively. The condition factor for fish in the fecundity experiments (1.69±0.06) and the inheritance experiment (–1.56±0.16) was constant and did not differ among any population throughout the experiments. Similarly, growth rate did not differ between treatment groups (~0.3±0.2% of body mass/day).

In the inheritance experiment, no significant differences were found in the mass of the ventricle, liver, and gut (0.21, 0.39 and 3.42% of body mass, respectively) between sexes or between treatments (Fig. 2.1A). However, gonadal mass was significantly larger in female compared to male adults (10.2±1.1% and 1.5±0.3% of body mass, respectively), so data were analyzed separately by sex. In contrast to female gonad mass, which was unaffected by treatment, male gonads differed significantly between treatments ($P=0.035$) (Fig. 2.2B), although the effects were complex. The gonads were significantly smaller

than control (~1.0±0.1% of body mass) with 10%HEWAF treatment, but were significantly larger (2.6±0.7% of body mass) with the 100%HEWAF treatment.

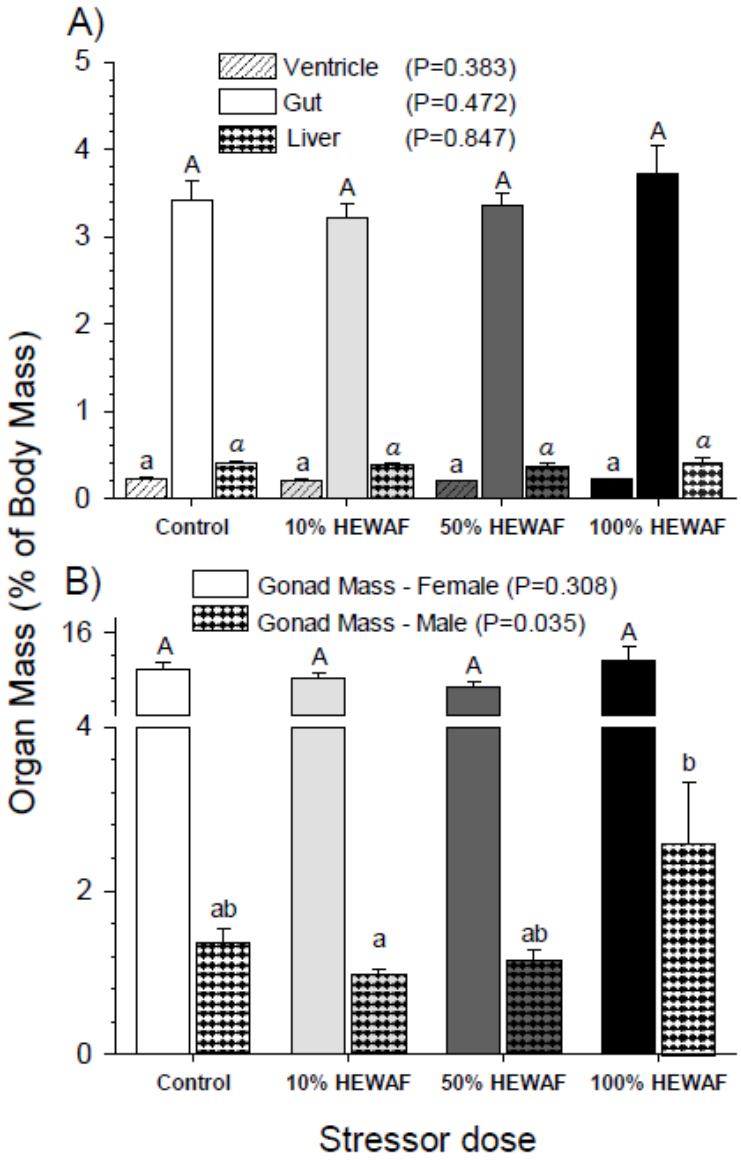

Figure 2.2: Adult organ mass as percentage of whole body mass, as influenced by crude oil exposure. A) Comparison of: ventricle, gut and liver mass between treatments (n=27-30/bar). B) Female (n=15–18/bar) and male (n=11-12/bar) gonadal mass. Data are presented as mean ± SEM. Significance level was considered with P value < 0.05. Different letters indicate statistical significant differences between groups.

Upon histological examination, gross morphology of the ventricular tissues appeared to be visually similar in the four groups of P_0 adults (Fig. A2-4). This observation was confirmed by digital quantification of collagen density in the images, and no

significant difference between oil-treated populations was observed (Fig. 2.3). Similarly, no apparent disruptions of gonadal tissue integrity was found in relation to crude oil exposure, as evident from the normal conformation of the lumina, spermatocysts and spermatogonia for male fish (Fig. A2-2), and the normal conformation of previtellogenic oocytes and vitellogenic oocytes in female gonadal tissue (Fig. A2-3).

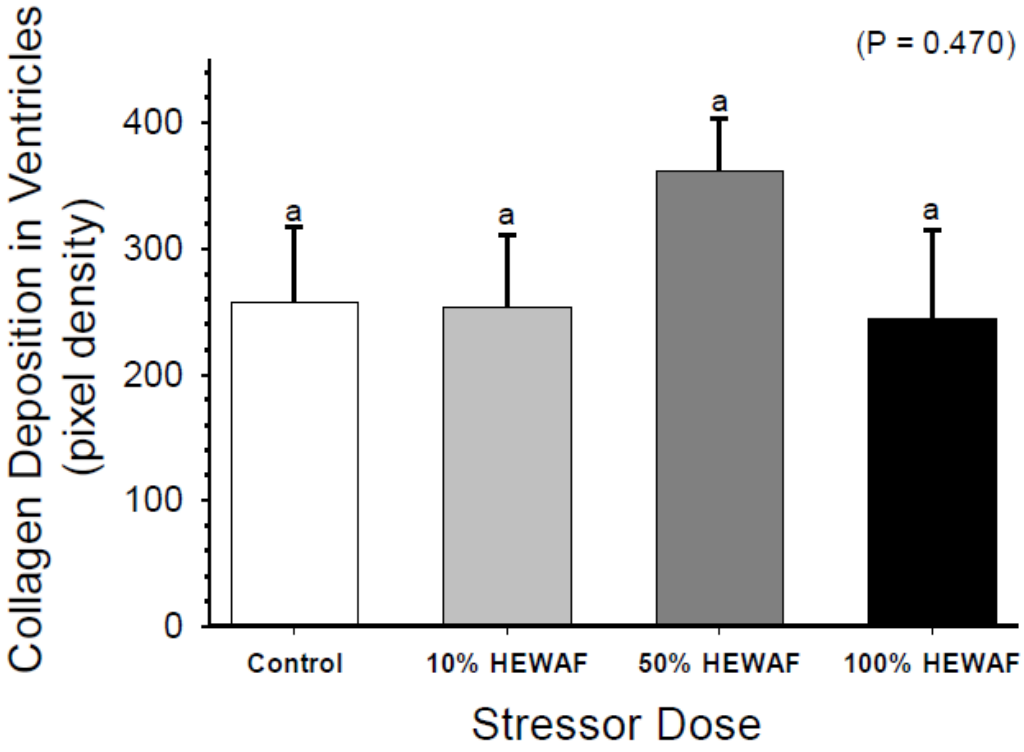

Figure 2.3: Collagen in adult ventricles, as influenced by crude oil exposure. Collagen area is expressed as pixel density, in the images of the ventricles from adult zebrafish exposed to different levels of HEWAF. There were no statistical significant differences between treatments (mean ± SEM, P=0.470). n=6-7 per group.

Comparison of egg laying variables among treatments are reported in Table 2.1. In brief, the total number of eggs laid per female in the higher HEWAF% groups were statically lower than the number of the laid eggs in the control groups. Similarly, fertilization rates were also lower in the HEWAF groups, as were the percentages of fertilized and viable eggs.

Volume of the chorion and the yolk and the yolk to chorion volume ratio were not

significantly different among treatments in the fecundity experiments (Table 2.2A). From the six sperm quality variables (Table 2.2B) estimated from each parental group, only the sperm count per area differed among treatments. In general all levels of oil exposure reduced sperm count, and in particular sperm count was 50% lower in the highest concentration of crude oil exposure compared to the control group.

Table 2.1: Fecundity variables resulting from HEWAF exposure in adult male and female zebrafish. Different superscript letters indicate differences among dietary treatment groups.

A) *Fecundity variables*	Treatment				p value
	Control	10%HEWAF	50%HEWAF	100%HEWAF	
"n" number	15	15	15	15	α = 0.05
Spawned (#Yes/#No)	15/0 ^{A}	15/0 ^{A}	14/1 ^{A}	7/8 ^{B}	0.0001
Total egg #	5352	4130	2366	2090	NS
Average egg # / female	356.8 ± 51.7 ^{A}	275.3 ± 28.8 ^{AB}	157.7 ± 23.9 ^{BC}	139.3 ± 40.7 ^{C}	0.002
% of fertilized eggs	75.8 ± 5.4 ^{A}	38 ± 4.6 ^{B}	61 ± 5.9 ^{B}	58.9. ± 8.1 ^{B}	0.001
% of non-fertilized eggs	24.2 ± 5.4 ^{A}	62 ± 4.6 ^{B}	43.3 ± 7 ^{AB}	41.1 ± 8.1 ^{AB}	0.001
% of non-viable fertilized eggs	5.9 ± 2.7 ^{A}	38.1 ± 5 ^{B}	26.1 ± 7.1 ^{AB}	14.6 ± 6.3 ^{AB}	0.001

Table 2.2: Egg variables A), and sperm quality variables B) in female and male zebrafish exposed to varying HEWAF concentrations. Different superscript letters indicate differences among dietary treatment groups.

A) *Egg variables*	Treatment				p value
	Control	10%HEWAF	50%HEWAF	100%HEWAF	
"n" number	25	25	25	25	α = 0.05
Chorion volume (mm^2)	1.02 ± 0.01	0.95 ± 0.03	0.97 ± 0.02	1.34 ± 0.06	NS
Yolk volume (mm^2)	0.24 ± 0.004	0.24 ± 0.01	0.24 ± 0.01	0.3 ± 0.03	NS
Yolk/chorion volume ratio	0.23 ± 0.004	0.26 ± 0.01	0.25 ± 0.01	0.22 ± 0.01	0.073

(table continues)

B) *Sperm quality variables*	Treatment				p value
	Control	10%HEWAF	50%HEWAF	100%HEWAF	
"n" number	6	5	5	6	α = 0.05
% Motility	55 ± 4.9	70.8 ± 6.6	74.9 ± 6.5	67 ± 12.3	0.391
Curvilinear velocity (VCL)	55.7 ± 5.7	57.3 ± 6.7	58.8. ± 9	78 ± 10.4	0.202
Velocity average path (VAP)	41.7 ± 2.3	37.1 ± 4.6	46.3 ± 3.8	48.3 ± 3.6	0.165
Velocity straigth line (VSL)	27.1 ± 2.1	23.9 ± 5.8	35.7 ± 1.3	25.8 ± 2.9	0.104
Linearity (LIN)	66 ± 6.4	60.8 ± 13.5	74.9 ± 6.5	67 ± 12.3	0.145
Count / 0.006 mm^2	18 ± 1.4 [a]	10.8 ± 1.7 [b]	9.4 ± 2.5 [b]	9.3 ± 3 [b]	0.039

2.4.2 Larval (F_1) Population

2.4.2.1 Effects of P_0 Exposure on F_1 Survival

Survival rates of all four of the F_1 larval populations reared in clean water are indicated in Fig. 2.4A. Parental exposure had a significant effect on the survival rates of their larval offspring when developing in clean water (Cox Stratified Model, P = 0.001). Essentially, F_1 larvae from parents lacking any oil exposure, or exposed to just 10% HEWAF through diet, showed little to no survival differences compared to the F_1 control offspring when developing in clean water. However, parental exposure to 50% or 100% HEWAF resulted in greatly reduced survival rates of the F_1 larvae when developing in clean water, especially from 3 dpf to 5 dpf (Log Rank survival test, P<0.001)

Parental exposure to 10%HEWAF had no significant effect on mortality of F_1 larvae also exposed to 10% HEWAF, at any monitored point in the development (Fig. 2.5A). In part, this lack of significant change resulted from higher variation within the population, with some larvae surviving through the developmental period and others succumbing early on.

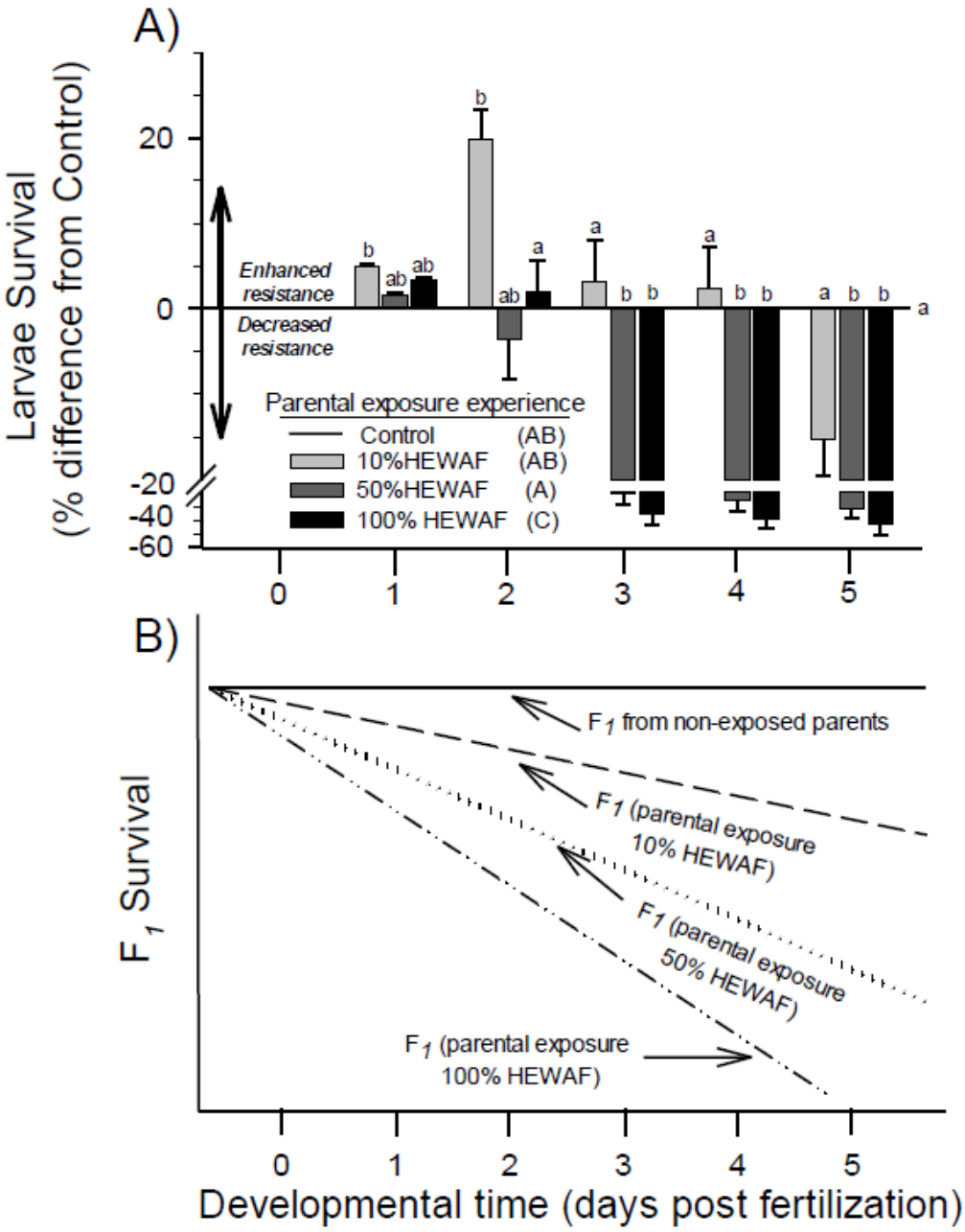

Figure 2.4: Effect of parental HEWAF exposure on larval survival. A) Survival of F1 larvae raised in clean water. Presented are the differences in survival % between F_1 obtained from control parents (zero-line), and F_1 obtained from treated parental groups (bars), at specific developmental time (dpf). Bars above or below the zero-line are interpreted as enhanced or decreased resistance, respectively. Different upper case letters by each parental treatment in the legend indicate significant (P<0.001) differences occurred between populations. Different lower case letters above the bars indicate difference between groups at specific days. "a" was assigned to the control group for all days, and is showed at the end of the zero-line. B) Schematic representation of survival patterns of F_1 from exposed parents raised in clean water, derived from Panel A. Each bar in panel A represents mean and SEM of the three replicates. The backwards arrows are just indicating which label belongs to each treatment one of the conceptual trends.

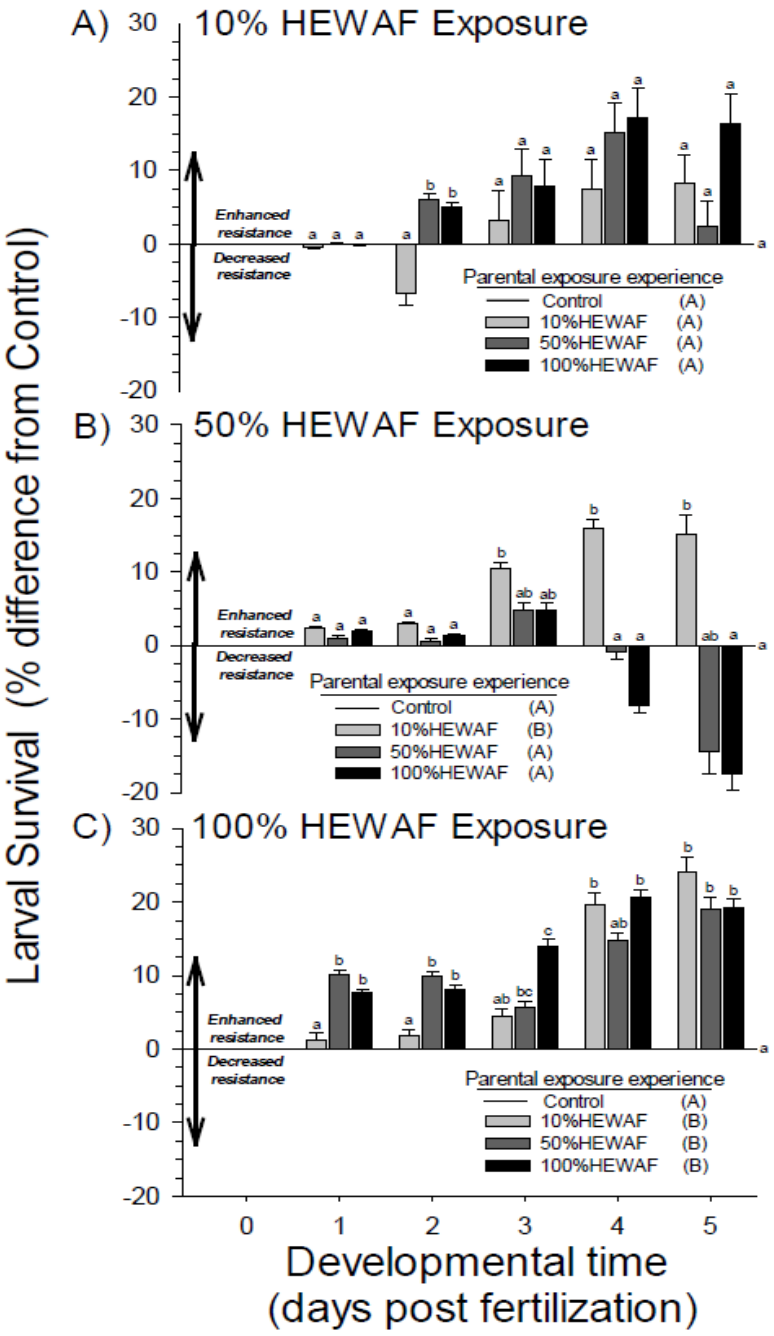

Figure 2.5: Synergistic and antagonistic effects of parental and larval HEWAF exposure on larval survivorship. A) F_1 larval exposure to 10% HEWAF. B) F_1 larval exposure to 50% HEWAF. C) F_1 larval exposure to 100% HEWAF. Data are presented as the difference in survival % between F_1 obtained from control parents (zero-line), and F_1 obtained from treated parental groups (bars), at specific developmental time (dpf). Bars above or below the zero-line are interpreted as enhanced or decreased resistance respectively. Different upper case letters by each parental treatment in the legend indicate significant (P<0.001) differences occurred between populations. Different lower case letters above the bars indicate difference between groups at specific days, "a" was assigned to the control group for all days, and is showed at the end of the zero-line. See Results for additional explanation. Each bar represents mean and SEM of the three replicates.

Reflecting a dose response to crude oil exposure, parental exposure to 50%HEWAF induced significant changes in survival when the F_1 larvae were exposed to the three HEWAF concentrations, especially later in day 5 of the developmental period (Fig. 2.5). There was an interesting dichotomy created by parental exposure levels. F_1 larvae from parents exposed to 10%HEWAF showed improved survival when they, themselves, were exposed to 50%HEWAF. However, this parentally-induced protective effect for larvae in 50%HEWAF was reversed by parental exposures of 50% or 100% HEWAF. Interestingly, during exposure to 100% HEWAF solution, F_1 larvae survival rate from parents exposed to oil was significantly enhanced in comparison to that of the offspring from the control parental group (Fig. 2.5C). Essentially, all F_1 larval groups obtained from parents exposed to any level of oil exhibited enhanced resistance to 100%HEWAF throughout the measured developmental period.

2.4.2.2 Effects of P_0 Exposure on F_1 Specific Growth Rate, Edemas and Body Deformities

Total body length in larvae increased from 3.1±0.02 mm at 2dpf to 3.9±0.02 mm at 5 dpf. Larval specific growth rate was 3.1±0.01% of body length/day, and did not differ among treatment groups ($P>0.05$). Neither the parental exposure condition ($P>0.05$), nor the F_1 exposure condition ($P=0.424$), nor their interaction ($P=0.9$), had an effect on larvae growth rate.

Presence of edemas and body abnormalities were assessed at 5 dpf in all larval populations. Comparisons between offspring HEWAF exposure conditions within the same parental group are reported in Table 2.3. When F_1 offspring from Control parents were exposed to control conditions, no cardiac nor yolk edema, or head or tail deformities were observed. However, there was a proportional dose-response increase in the

percentage of larvae exhibiting those phenotypes when the Control larvae were raised in oil conditions. In particular, exposure to 100%HEWAF induced both edema types in 100% of the larvae and more than 80% of them exhibited deformities in their heads or tails. When F1 offspring from 10%HEWAF exposed parents were exposed to control and 10%HEWAF, none of the modified phenotypes emerged in the larvae. However, when larvae were raised in 50% and 100%HEWAF conditions, the percentage of larvae exhibiting edemas or deformities increased proportionally. Importantly, however, the proportion of deformities was smaller than the percent exhibited by offspring from control parents.

Table 2.3: Fecundity variables resulting from HEWAF exposure in adult male and female zebrafish. Different superscript letters indicate differences among dietary treatment groups. * and ** indicate that the value of the adjusted residuals from the chi-square analysis were equal to (or beyond) 2 or -2 , which deviate them from the H_0 (equal proportions) respectively (Agresti and Kateri, 2011).

Exposure condition		% of individuals exhibiting malformations				SGR
Parental	F_1 Offspring	Cardiac edema	Yolk edema	Head deformities	Tail deformities	% body length/day
		n = 15	n = 15	n = 15	n =15	n= 12-15
Control	Control	0 .00 *	0.00 *	0.00 *	0.00 *	3.3 ± 0.1
	10%HEWAF	25.00	8.34 *	16.67	16.67	3.2 ± 0.1
	50%HEWAF	46.60	40.00	33.34	26.67	3.3 ± 0.1
	100%HEWAF	100 **	100 **	83.34 **	100 **	3.1 ± 0.2
Pearson Chi square / P value		25.712 / 0.0001	34.195 / 0.0001	23.094 / 0.0001	33.3 / 0.0001	
10%HEWAF	Control	0.00 *	0.00	0.00	0.00	3.1 ± 0.1
	10%HEWAF	0.00 *	0.00	0.00	0.00	3.2 ± 0.1
	50%HEWAF	26.67	26.67	26.67	33.34	3.2 ± 0.1
	100%HEWAF	46.67 **	33.34	40 **	60 **	3.2 ± 0.2
Pearson Chi square / P value		15.473 / 0.001	10.850 / 0.013	12.960 / 0.005	21.242 / 0.0001	
50%HEWAF	Control	13.34	13.34	0.00	0.00	2.9 ± 0.1
	10%HEWAF	20.00	20.00	13.34	20.00	3 ± 0.1
	50%HEWAF	26.67	26.67	26.67	26.67	3 ± 0.1
	100%HEWAF	46.67	40.00	40.00	46.67	3.1 ± 0.1
Pearson Chi square / P value		4.773 / 0.189	3.111 / 0.375	8.333 / 0.04	9.317 / 0.025	
100%HEWAF	Control	26.67	26.67	0.00	40.00	3 ± 0.1
	10%HEWAF	6.67	6.67	6.67	6.67 *	2.8 ± 0.1
	50%HEWAF	40.00	20.00	0.00	53.34	3 ± 0.1
	100%HEWAF	60.00	40.00	46.67 **	66.67	3.1 ± 0.1
Pearson Chi square / P value		10.2 / 0.017	4.845 / 0.184	19.615 / 0.0001	12.274 / 0.007	

Offspring obtained from parents exposed to 50%HEWAF and 100%HEWAF exhibited cardiac and yolk edemas when raised in clean water conditions. Neither tail nor head abnormalities were found in offspring from 50%HEWAF parents in clean water, but 40% of the offspring from 100%HEWAF parents exhibited tail abnormalities in this condition. Similarly, when the offspring of these parental groups were exposed to any of the three oil conditions, the F_1 population percentage exhibiting edemas or body deformities was also proportionally increased.

To summarize, exposure to crude oil conditions in F_1 larvae from oil-exposed parents also led to the presence of edemas and body deformities. However, the percentage of the population exhibiting these phenotypes was smaller in F_1 larvae from oil-exposed parents in comparison to the larvae from control parents. These results suggest that parental exposure attenuate adverse effects in their offspring during stressor conditions.

2.4.2.3 Effects of P_0 Exposure on F_1 Heart Rate

Resting heart rate in control larvae from control parents was ~160 bpm on day 1 and 2, increasing significantly ($P<0.001$) to 190-200 bpm on day 3 and 4, before declining slightly on day 5 to 180 bpm (Fig. 2.6).

There was a significant interaction between time, parental crude oil-dietary exposure and acute oil F_1 exposure via water affecting resting f_H in the F_1 offspring (three-way ANOVA, $P=0.001$). The patterns of change were complex, however. Parental oil exposure had a marked effect on f_H of F_1 larvae developing in clean water. Essentially, at 3 dpf a depressed f_H (bradycardia) occurred when in clean water in those F_1 larvae from parents who had been exposed to as little as 10% HEWAF (Fig. 2.6). Bradycardias were

induced by parental exposure to higher HEWAF levels at this stage of development. Thus, at days 1 and 2, parental exposure to 100%HEWAF led to a larval f_H depression of 50 bpm, even when these larvae were raised in clean water. This larval group continued exhibiting bradycardia through 5 days of development in comparison with control-derived offspring.

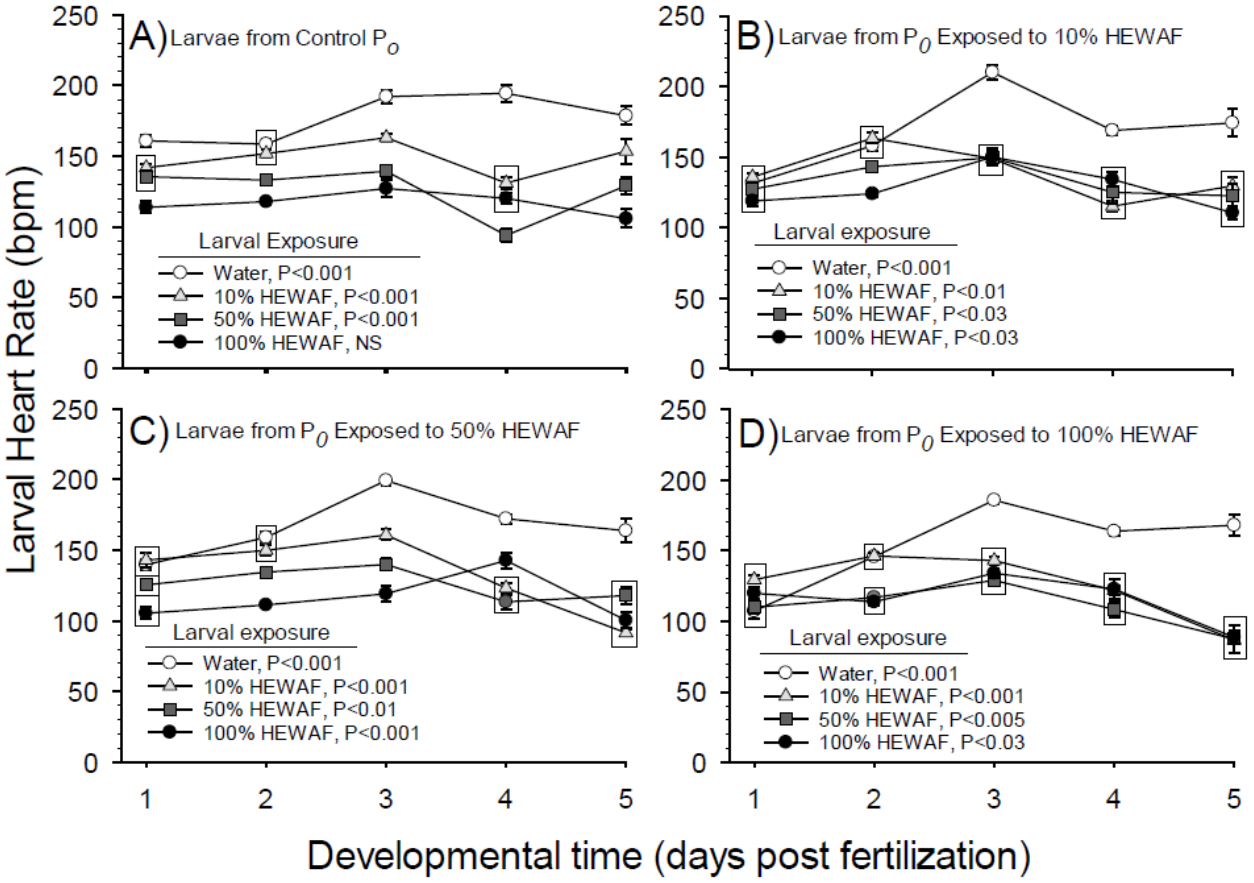

Figure 2.6: Heart rate in 1 to 5 dpf F_1 zebrafish larvae as a function of P_0 parental crude oil exposure. A) Larvae from control parents, B) Larvae from 10%HEWAF exposed parents, C) Larvae from 50%HEWAF exposed parents and D) Larvae from 100%HEWAF exposed parents. Larvae raised in Clean water, 10%HEWAF, 50%HEWAF or 100%HEWAF are indicated with white circles, gray triangles, dark-gray squares, and black circles, respectively. Data are expressed as means ± 1 SEM. Means for any given developmental day that are grouped within boxes are not significantly different (P>0.05). P-values beside the legend refer to differences across developmental time for each treatment. n= 8-74 per data point.

Larval offspring obtained from parents exposed to 10%HEWAF showed significant differences in f_H during their early development compared to control-derived offspring

(Fig. 2.6A). At 1 dpf, no differences in f_H occurred between exposure condition groups (128±3 bpm). At 2 dpf fish exposed to clean water or 10%HEWAF showed a similar f_H of 161±3 bmp. However, larval groups exposed to 50% and 100%HEWAF exhibited significantly lower f_H values (143±1 and 124±2bpm, $P<0.05$) in similar conditions. From 3 dpf to 5 dpf, regardless of the exposure concentration of HEWAF, oil-exposed larvae exhibited significant bradycardia (decrease of 50bpm, ~30%) in comparison to larvae raised in clean water.

Offspring obtained from the 50%HEWAF-exposed parents exhibited similar f_H patterns to those obtained from 10%HEWAF-exposed parents on at 1 and 2 dpf (Fig. 2.6B). At 3 dpf the four larval groups differed between each other ($P<0.001$), with f_H ranging from 199±3 down to ~119±6 bpm. Although the larvae exposed to 100% HEWAF differed from all treatments at lower concentrations, over the last two days all three oil-exposed larval groups showed bradycardia (103±5 bpm) in comparison to larvae raised in clean water (164±8 bpm).

Finally, exposure to clean water or any of the three HEWAF concentrations had no effect on f_H at 1dpf (~117 bpm) in offspring obtained from parents exposed to 100%HEWAF (Fig. 2.6C). At 2 dpf, the offspring exposed to clean water and 10%HEWAF showed similar levels of f_H (146±2bpm), which were significantly higher than the 50% and 100%HEWAF-treated groups (~ 115). From 3 dpf to 5 dpf the pattern of f_H was similar to that of the larval offspring obtained from 10%HEWAF exposed parents. All three larval groups exposed to oil exhibited a bradycardia ranging from 135±3 down to 88±6 bpm in comparison with those raised in clean water, which ranged from 186±2 down to 168±7bpm ($P<0.05$).

Heart rate effects are summarized in Fig. 2.7, which shows that a bradycardia resulted from 100% HEWAF exposure at all developmental times and all parental HEWAF exposures.

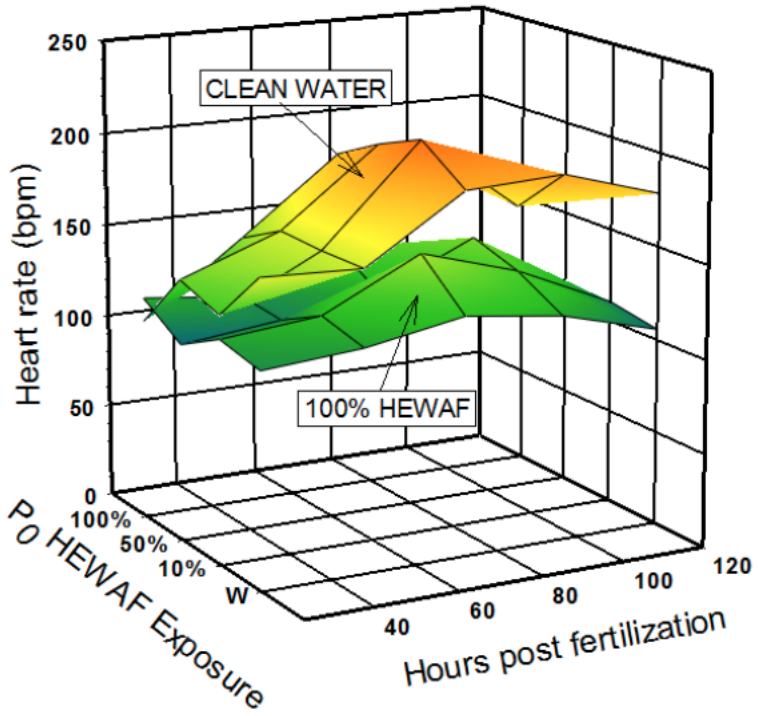

Figure 2.7: Comparison of heart rate between offspring exposed to clean water (top plane) and offspring exposed to 100% HEWAF (bottom plane). Surfaces for 10% and 50% HEWAF exposures are intermediate and have been omitted for clarity.

2.5 Discussion

Interest in epigenetic inheritance has burgeoned in the last two decades, and has been largely dominated by the demonstration of the transgenerational transfer of *maladapative* phenotypes. In contrast, studies focused on demonstrating and interpreting *adaptive* transgenerational epigenetic inheritance are still relatively scarce (Burggren, 2016; Manjrekar, 2017). Yet, such inheritance could be highly influential in individual- and population-level survival. Consequently, the current study has tested if exposure to a stressor, in the form of dietary parental crude oil, could actually enhance resistance to

that stressor in their offspring, through non-genomic inheritance.

2.5.1 Parental Responses to Crude Oil Exposure

The effects that crude oil and other similar toxicants have on fish have mainly been studied in early developmental stages. However, some studies have evaluated juvenile and adult fish in this context (Pasparakis et al., 2019). For example, in comparison with controls, 24 h exposure to 8.4 µg L^{-1} of 50 selected PAHs from crude oil induced a 14% decrease in maximum sustained swimming speed (U_{crit}) in young adult mahi-mahi (*Coryphaena hippurus*) (Stieglitz et al., 2016). Similar exposures to 20% HEWAF solution in cobia (*Rachycentron canadum*) induced an 18% increase in heart rate, but an offsetting 36% decrease in stroke volume, in oil-exposed fish relative to control (Nelson et al., 2017)

At the tissue level, exposure during early development to oil compounds can induce collagen deposition in the heart of zebrafish at later developmental stages (Huang et al., 2014). Similarly, excessive oil exposure induces collagen build-up in the heart of juvenile salmon (Alderman et al., 2017). However, in the present study on zebrafish there was no difference in collagen content between the ventricles of the various exposure groups of adults (Fig. 2.3). Similarly, we did not find any indications of gonadal morphological abnormalities for either female or male tissue (Figs. A2-3 and A2-4). This finding coincides with the reported literature for the polar cod (Bender et al., 2016), where seven months of dietary exposure still did not induce morphological differences in gonadal tissue. However, in the same study, indicators of sperm viability (curvilinear path velocity, percentage of motile sperm and velocity in straight line) were affected by exposure. This differs from the findings of the current study, in which only sperm count per area was reduced in fish exposed to any of the three HEWAF conditions (Table 2.2B). This

difference may be a function of the different exposure periods.

2.5.2 Parental History and Inheritance of Adaptive Phenotypes in F_1 Larvae

2.5.2.1 Survival of F_1 Population

During early development, fish are highly sensitive to multiple stressors – both natural and anthropogenic. Their survival depends on several factors such as length of exposure, rates of exposure, and emergent stressors from the interaction of several factors and even parental experiences (Blaxter, 1991; Burggren and Dubansky, 2018; Ehrlich and Muszynski, 1982; Siefert et al., 1973). Hence, offspring phenotypic traits are determined by both genotype and non-genetic contribution of their own or their ancestors' environmental experiences (Auge et al., 2017). However, the ability for offspring to inherit resistance to stressors experienced by the parental population, while potentially adaptive, may also carry trade-offs if these offspring then experience different environmental conditions for which the adaptations leading to resistance may be ill-suited.

Compared with F_1 offspring from control parents, offspring from oil-treated parental groups showed higher survival rates when they, themselves, were raised in HEWAF conditions (Fig. 2.5). This is clearly an important adaptation to help survive an adverse environment. At first glance, these results resemble those reported for killifish (Meyer and Di Giulio, 2002; Meyer and Di Giulio, 2003; Ownby David et al., 2009). In those studies, F_1 and F_2 larvae from killifish parents residing in PAH-contaminated areas of the Elizabeth River in VA, USA exhibited increased survival and normal development when exposed to contaminated sediments, when compared with offspring from a reference, non-polluted site. Importantly, however, their experimental design provided only correlations, and was unable to differentiate between genetic effects, in which the resistance had been selected

for in the adult populations, and epigenetic inheritance, in which acute exposure of adults led to transfer of modified phenotype through an epigenetic marker or another similar mechanism. Indeed, until the present study the most parsimonious explanation was that the adult killifish had evolved resistance through natural selection, and “simply” passed this resistance on to their offspring through genetic inheritance. Our studies on zebrafish suggest that there may have been transgenerational epigenetic inheritance in these killifish populations.

Although is yet to be determined if the levels of sensitivity correlates with the manifestation of effects, environmental and anthropogenic stressors appear to affect larval stages to a greater extent than they affect embryonic stages (Hutchinson et al., 1998; Mohammed, 2013; Stieglitz et al., 2016). In the present study, differences in the survival rate of the F_1 from control parents, when exposed to clean water or any of the three HEWAF concentrations, were more pronounced from 3 to 5 dpf than earlier developmental stages (Fig. 2.4-2.5). These results are similar to other studies (Perrichon et al., 2016) where, compared with controls, larval zebrafish exposed to water accommodated fractions of heavy fuel oil exhibited decreased survival at 6 dpf compared to earlier developmental stages.

The experimental design of the current study tested the influence of parental experiences on offspring survival. Differences in survival rates in the present study were evident only after hatching had occurred (Fig. 2.4). This could be explained by the fact that the chorion of the embryos may act as an impermeable, or at least partially selective, barrier to crude oil compounds, as it does for the drug amiloride in medaka fish, for example (Cameron and Hunter, 1984). However, there are documented examples of oil-

induced changes in embryonic function prior to rupturing of the chorion (Greer et al., 2019; Pasparakis et al., 2016; Pasparakis et al., 2017). An alternative explanation could be that even if dissolved oil components reach the embryo by passing through the chorion, the effects of oil do not become apparent until larval stages in zebrafish, for example by increasing metabolic demands (Pasparakis et al., 2017). Additionally, once hatched, larval fishes also face direct exposure to the environment, becoming readily susceptible to phenotypic modification from environmental stressors.

2.5.2.2 F_1 Developmental Abnormalities

Exposure to crude oil via water during early development in fish induces cardiac and yolk edema and body abnormalities in a dose-response fashion (Incardona et al., 2014; Incardona and Scholz, 2018b). However, we only poorly understand the effects of parental exposures on larval structure and performance. Our results suggest that 21 days of dietary exposure to crude oil with any of the dietary treatments used in this experiment may attenuate the development of cardiac and yolk edemas and body abnormalities in F_1 offspring during exposure to oil via water.

2.5.2.3 Heart Rate in the F_1 Population

Heart rate in the zebrafish through all developmental stages is affected by temperature, oxygen availability and anthropogenic toxicants (Barrionuevo et al., 2010; Barrionuevo and Burggren, 1999; Burggren, 2017; Cypher et al., 2017; Horri et al., 2018). In the present study, exposure to crude oil induced bradycardia in control larvae derived from non-exposed parents. These results are similar to those reported for yellow and blue fin tuna and amberjack, where oil exposure created a decrease in heart rate of ~30%, ~55% and ~40% in comparison to control fish, respectively (Incardona et al., 2014).

Similarly, oil exposure produced a pronounced bradycardia in embryos of the pacific herring (Incardona and Scholz, 2018b; Incardona et al., 2012), and also decreased heart rate, stroke volume, and cardiac output in the red drum in a dose-dependent fashion (Khursighara et al., 2016). The general assumption in the literature on fishes is that this persistent bradycardia, opposite to the tachycardia that often occurs in mammals, is maladaptive – or at least not adaptive - especially when accompanied by reduced cardiac output (Perry and Desforges, 2006). However, further experiments are warranted in this regard, as theoretical arguments for an adaptive role for bradycardia have been posited for adult fishes (Farrell, 2007). Moreover, whether bradycardia conveys the same physiological effects in larval and adult fishes is unresolved

Notably, in the present experiment, major heart rate differences between larval groups only developed at 3 dpf. One explanation for this could be that, during the initial development period (<3 dpf), the timing for significant differences in cardiac traits between treatments align well with the change from intrinsic to extrinsic factors controlling cardiac function in the zebrafish (Lema et al., 2007; Pelster et al., 2005; Schwerte et al., 2006). Similarly to our results, exposure to three-ring PAHs compounds (e.g. phenanthrene and dibenzothiophene) did not disrupt the time of onset of heartbeat in zebrafish embryos at 1dpf, and bradycardia and arrhythmias were present until 3dpf (Incardona et al., 2004).

Those differences in survivorship and heart rate in F_1 offspring were larger after the hatching period, raising the question about the function of the chorion as a protective physical barrier against chemical stressors (see above). Additionally, it is possible that transgenerational maternal provisioning and programming effects could be protecting the embryos until they rely on their own means of protection against stressors (Meyer and Di

Giulio, 2003).

2.5.3 Transgenerational Epigenetics of F_1 Phenotypes

The present study demonstrates that dietary exposure to crude oil extracts, within environmentally relevant concentrations (Vignet et al., 2014), did not affect major indicators of fish health such as condition factor or organ mass of the P_0 adult zebrafish (Fig. 2.2, 2.3). However, 21 days of dietary exposure to crude oil did affect male testes mass and sperm count and female egg laying variables. Remarkably the parental toxicant experience clearly improved the performance of their offspring experiencing a similar stressor, as measured by larval survival. One of the most remarkable findings of this study is that when offspring obtained from HEWAF-exposed parents were raised in clean water, their survival actually strongly decreased and they also developed cardiac and yolk edemas (Fig. 2.4A). In contrast, when offspring obtained from oil-exposed parents were challenged to survive in HEWAF, their survival was significantly higher than those offspring from parents that were not exposed (Fig. 2.5), and the percentage of them exhibiting edemas was also smaller in comparison with offspring from control parents exposed to highest HEWAF concentration. A major finding of our study is thus an *adaptive* phenotype can be conferred upon offspring through parental exposure to an environmental stressor. Moreover, when combined with the epigenetic inheritance of a bradycardia, we believe this to be the first demonstration of simultaneous inheritance of adaptive as well as maladaptive traits, making for an increasingly complex landscape for epigenetic inheritance.

2.5.4 Potential Mechanisms for Epigenetic Inheritance of Larval Phenotype

Epigenetically transferred signals from parents to their offspring could induce

altered larval gene expression, allowing the larvae with temporally low fitness to survive and even exhibit improved resistance against stressors (Burggren, 2016; Ho and Burggren, 2012; Jablonka and Lamb, 2015). Some studies have shown that resistance to PAHs in subsequent generations did not show differences in methylation patterns in CpG sites of the CYP1A promoter (Timme-Laragy et al., 2005), a gene highly involved in detoxification of PAHs (Dubansky et al., 2013; Meyer et al., 2002). Their results do not exclude the potential role of other epigenetic mechanisms as complementary means to genetic factors (Nacci et al., 2010) for achieving this end.

Furthermore, since the presence of epigenetic markers varies within a population, it is likely that the genotype frequencies within a population could be also subject to change and indirectly become a substrate for natural selection (Burggren, 2015; Skinner, 2015). Since epigenetic inheritance could increase organismal fitness (Klironomos et al., 2013), it has adaptive implications by providing a mechanism for populations to prevail during exposure to anthropogenic stressors (i.e. oil spills, temperature increases) and non-stable natural environments (i.e. seasonal changes in oxygen availability and stochastic temperature fluctuations) (Burggren, 2017; Burggren, 2019; Burggren and Crews, 2014).

Transgenerational effects inherited without induction of any change in DNA sequence, have received considerable attention during the past two decades (Burggren, 2016; Hu et al., 2018; Inbar-Feigenberg et al., 2013; Jablonka and Raz, 2009). The study of transgenerational epigenetic effects had been linked mostly with maladaptive implications in human-focused disciplines such as medicine (Baccarelli et al., 2010). Consequently, our understanding of the adaptive role of epigenetic inheritance is limited.

Studying how epigenetic markers could aid organisms and populations to cope with stressors and prevail under adverse conditions requires implementation of more detailed experiments in which the studied phenotypic variables must embrace a continuum among different levels of organismal organization. In addition, some studies have demonstrated that transgenerational effects that influence offspring phenotypes could arise from both maternal (Nye et al., 2007) and paternal (Lombó et al., 2015) lines.

2.6 Conclusions and Future Studies

Our study demonstrates that parental experiences in the form of transient exposure to an environmental stressor prompts a signal transfer to the F_1 generation through non-genomic (i.e. epigenetic) inheritance. The inherited phenotype imbues the the F_1 larvae with enhanced survival and attenuation of maladaptive effects when facing similar stressors to those experienced by the P_0 generation. However, our finding that exposure to crude oil during early development induced bradycardia even in offspring obtained from oil-exposed parents indicates that potentially both adaptive and maladaptive traits may be simultaneously inherited through none genomic means, opening a window for further studies aimed at understanding how populations overcome challenges imposed by changing environments and their stressors. In this sense, experimental designs should be directed to test and reveal epigenetic mechanisms involved in gene expression, and the relative contributions of parental experiences on offspring performance. Finally, while crude oil has been used as the stressor in this study, we emphasize that these findings may have broad applicability to other stressors, both natural and anthropogenic. Consequently, this type of experiment will provide information for building new foundations and improving our understanding of the transgenerational

effects that environmental stressors (e.g. algal blooms causing hypoxia, weather events creating hypo-or hyperthermia, anthropogenic events such as oil spills) can have on natural animal populations, as well as the repercussions for the survival and prevalence of the species.

2.7 Appendix: Supplemental Tables and Figures

Table A2-1: List of components and nominal concentrations (ug/Kg and mg/kg) for each diet treatment. The sum of all the components listed below was considered the "Total PAH concentration". The components highlighted with gray color were considered for the 50 PAHs most frequently measured PAHs (Dubansky et al., 2018; Johansen et al., 2017). ND= not determined.

COMPONENT	CONTROL FOOD (ug/Kg)	10% HEWAF (ug/Kg)	50% HEWAF (ug/Kg)	100% HEWAF (ug/Kg)	BLANK (ug/Kg)
cis/trans-Decalin	117	63.2	72.6	113	ND
C1-Decalins	ND	116	115	212	ND
C2-Decalins	ND	175	269	422	ND
C3-Decalins	ND	ND	412	745	ND
C4-Decalins	ND	ND	518	940	ND
Benzo(b)thiophene	ND	ND	ND	ND	ND
C1-Benzothiophenes	ND	ND	ND	29.3	ND
C2-Benzothiophenes	ND	ND	ND	33.1	ND
C3-Benzothiophenes	ND	ND	ND	41.6	ND
C4-Benzothiophenes	ND	ND	ND	ND	ND
Naphthalene	7.78	36.7	164	312	0.493
C1-Naphthalenes	ND	133	666	1330	ND
C2-Naphthalenes	ND	274	1340	2470	ND
C3-Naphthalenes	ND	245	1270	2140	ND
C4-Naphthalenes	ND	218	781	1310	ND
Biphenyl	ND	19.5	99.6	174	ND
Dibenzofuran	ND	ND	17.0	33.6	ND
Acenaphthylene	ND	ND	D	ND	ND
Acenaphthene	ND	ND	9.28	18.9	ND
Fluorene	ND	18.9	102	206	ND

(table continues)

COMPONENT	CONTROL FOOD (ug/Kg)	10% HEWAF (ug/Kg)	50% HEWAF (ug/Kg)	100% HEWAF (ug/Kg)	BLANK (ug/Kg)
C1-Fluorenes	ND	53.9	294	563	ND
C2-Fluorenes	ND	126	466	858	ND
C3-Fluorenes	ND	ND	465	810	ND
Anthracene	ND	ND	ND	ND	ND
Phenanthrene	7.16	46.0	232	456	ND
C1-Phenanthrenes/Anthracenes	ND	114	580	1130	ND
C2-Phenanthrenes/Anthracenes	ND	114	686	1260	ND
C3-Phenanthrenes/Anthracenes	ND	85.5	466	908	ND
C4-Phenanthrenes/Anthracenes	ND	ND	249	639	ND
Retene	ND	ND	13.7	22.3	ND
Dibenzothiophene	ND	ND	30.2	55.3	ND
C1-Dibenzothiophenes	ND	ND	112	207	ND
C2-Dibenzothiophenes	ND	ND	163	327	ND
C3-Dibenzothiophenes	ND	ND	109	256	ND
C4-Dibenzothiophenes	ND	ND	ND	ND	ND
Benzo(b)fluorene	ND	ND	9.14	20.0	ND
Fluoranthene	ND	ND	ND	11.0	ND
Pyrene	ND	ND	20.6	39.2	ND
C1-Fluoranthenes/Pyrenes	ND	ND	67.1	122	ND
C2-Fluoranthenes/Pyrenes	ND	ND	141	255	ND
C3-Fluoranthenes/Pyrenes	ND	ND	136	274	ND
C4-Fluoranthenes/Pyrenes	ND	ND	ND	227	ND
Naphthobenzothiophene	ND	ND	ND	16.4	ND
C1-Naphthobenzothiophenes	ND	ND	ND	85.8	ND
C2-Naphthobenzothiophenes	ND	ND	ND	ND	ND
C3-Naphthobenzothiophenes	ND	ND	ND	ND	ND
C4-Naphthobenzothiophenes	ND	ND	ND	ND	ND
Benz(a)anthracene	6.31	ND	ND	11.6	ND
Chrysene	ND	ND	53.4	77.2	ND
C1-Chrysenes	ND	ND	103	191	ND
C2-Chrysenes	ND	ND	163	294	ND
C3-Chrysenes	ND	ND	ND	ND	ND
C4-Chrysenes	ND	ND	ND	ND	ND

(table continues)

COMPONENT	CONTROL FOOD (ug/Kg)	10% HEWAF (ug/Kg)	50% HEWAF (ug/Kg)	100% HEWAF (ug/Kg)	BLANK (ug/Kg)
Benzo(b)fluoranthene	ND	ND	ND	ND	ND
Benzo(k)fluoranthene	ND	ND	ND	ND	ND
Benzo(a)fluoranthene	ND	ND	ND	ND	ND
Benzo(e)pyrene	ND	ND	ND	14.7	ND
C30-Hopane	ND	ND	55.5	98.9	ND
Benzo(a)pyrene	ND	ND	ND	ND	ND
Perylene	ND	ND	ND	ND	ND
Indeno(1,2,3-cd)pyrene	ND	ND	ND	ND	ND
Dibenz(a,h)anthracene	ND	ND	ND	ND	ND
Benzo(g,h,i)perylene	ND	ND	ND	ND	ND
4-Methyldibenzothiophene	ND	8.04	48.6	101	ND
2-Methyldibenzothiophene	ND	ND	20.2	39.9	ND
1-Methyldibenzothiophene	ND	ND	16.2	28.8	ND
3-Methylphenanthrene	ND	18.5	101	211	ND
2-Methylphenanthrene	ND	22.4	120	249	ND
2-Methylanthracene	ND	ND	ND	ND	ND
9-Methylphenanthrene	ND	23.8	135	275	ND
1-Methylphenanthrene	ND	21.3	106	207	ND
2-Methylnaphthalene	ND	95.5	506	1010	ND
1-Methylnaphthalene	ND	92.4	491	858	ND
2,6-Dimethylnaphthalene	ND	72.6	467	913	ND
2,3,5-Trimethylnaphthalene	ND	71.3	313	575	ND
Carbazole	ND	ND	ND	ND	ND
Fluorene-d10	82	78	80	73	87
Fluoranthene-d10	94	84	92	83	90
Terphenyl-d14	88	87	95	85	90
SUM TOTAL PAH	138.25	2264.54	12774.12	24227.60	0.493
SUM TPAH50	21.25	1484.50	8994.32	17102.70	0.493
SUM TOTAL PAH	0.14	2.27	12.77	24.23	0.00
SUM TPAH50	0.02	1.485	8.994	17.10	0.00

Table A2-2: List of components and nominal concentrations (ug/Kg and mg/kg) of PAHs in whole body fish per experimental group. The sum of all the components is listed at the bottom of each column. ND= not determined.

Component	Control Female	Control Male	10%HEWAF Female	10%HEWAF Male	50%HEWAF Female	50%HEWAF Male	100%HEWAF Female	100%HEWAF Male	Blank method
Naphthalene	ND	1.4	1.8	0.95	1.1	ND	1.0	1.2	ND
2-Methylnaphthalene	1.6	ND	2.0	ND	1.4	1.9	2.0	2.3	ND
1-Methylnaphthalene	1.4	ND	2.1	ND	1.3	1.4	1.5	1.8	ND
C2-Naphthalenes	ND	ND	ND	ND	8.1	6.8	15	ND	ND
C3-Naphthalenes	ND	ND	6.7	ND	7.3	8.5	17	ND	ND
C4-Naphthalenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Biphenyl	ND	ND	ND	ND	ND	ND	ND	2.3	ND
Acenaphthylene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dibenzofuran	1.6	0.79	0.96	ND	ND	ND	ND	0.99	ND
Acenaphthene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluorene	1.1	1.3	1.9	0.98	1.6	1.1	1.6	1.6	ND
C1-Fluorenes	ND	ND	ND	ND	ND	ND	6.1	ND	ND
C2-Fluorenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C3-Fluorenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dibenzothiophene	ND	ND	1.1	ND	ND	ND	0.92	ND	ND
C1-Dibenzothiophenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C2-Dibenzothiophenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C3-Dibenzothiophenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phenanthrene	7.5	6.3	12	7.8	7.5	6.5	9.1	8.1	ND
Anthracene	ND	ND	ND	ND	ND	ND	ND	ND	ND
C1-Phenanthrenes/Anthracenes	ND	ND	7.0	ND	ND	ND	8.6	ND	ND
C2-Phenanthrenes/Anthracenes	ND	ND	ND	ND	ND	ND	ND	ND	ND

(table continues)

Component	Control Female	Control Male	10%HEWAF Female	10%HEWAF Male	50%HEWAF Female	50%HEWAF Male	100%HEWAF Female	100%HEWAF Male	Blank method
C3-Phenanthrenes/Anthracenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C4-Phenanthrenes/Anthracenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluoranthene	1.2	1.1	1.3	ND	ND	ND	ND	ND	ND
Pyrene	0.62	ND	ND	ND	ND	ND	ND	ND	ND
C1-Fluoranthenes/Pyrenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benz(a)anthracene	0.48	ND	ND	ND	ND	ND	ND	ND	ND
Chrysene	ND	ND	ND	ND	ND	ND	ND	ND	ND
C1-Chrysenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C2-Chrysenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C3-Chrysenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
C4-Chrysenes	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(b)fluoranthene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(k)fluoranthene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(e)pyrene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(a)pyrene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Perylene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Indeno(1,2,3-cd)pyrene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dibenz(a,h)anthracene	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(g,h,i)perylene	ND	ND	ND	ND	ND	ND	ND	ND	ND
SUM TOTAL	15.5	10.89	36.86	9.73	28.3	26.2	62.82	18.29	0

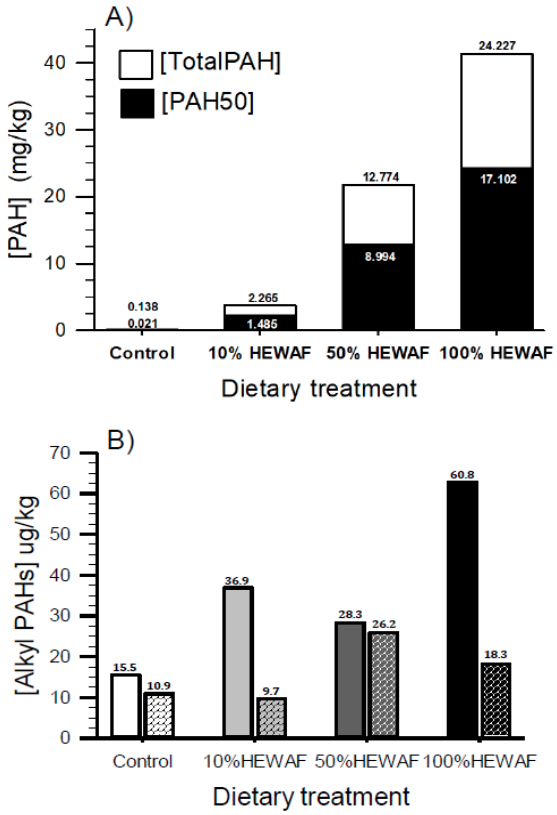

Figure A2-1: A) Concentration of PAHs in dietary treatments. ΣTotPAH is the sum of all the different PAHs compounds found in the diet. ΣPAH50 represents the fifty most common PAHs in the toxicology literature. B) Concentration of PAHs estimated from pooled whole body fish per treatment group. Empty bars and patterned bars refere to female and male fish respectively.

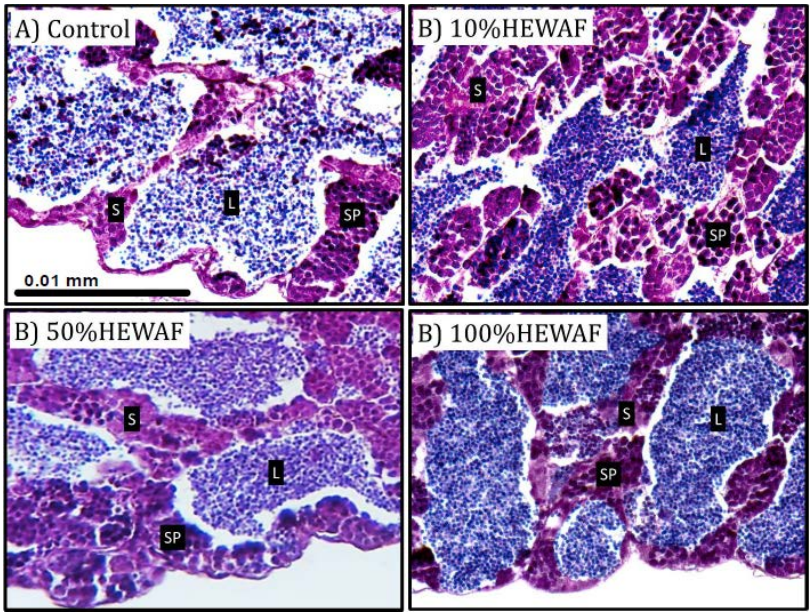

Figure A2-2: Male gonadal sections. A) Control, B) 10%HEWAF, C) 50%HEWAF and D) 100%HEWAF. L= lumina, S= spermatogonia, SP= spermatocysts

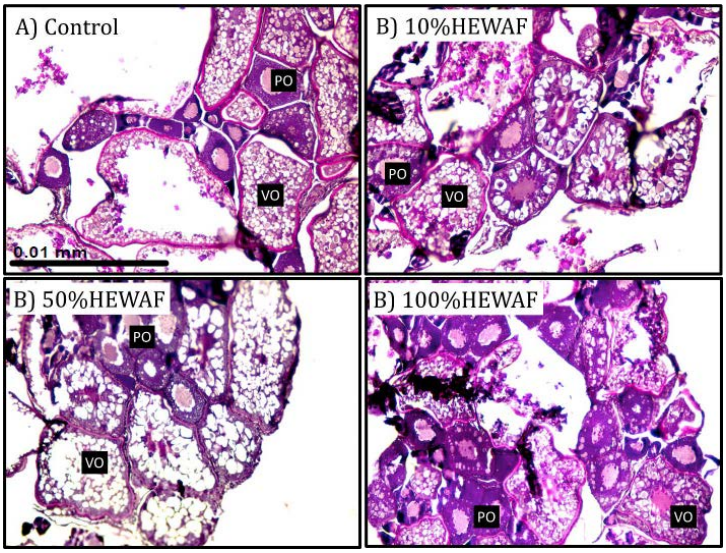

Figure A2-3: Female gonadal sections. A) Control, B) 10%HEWAF, C) 50%HEWAF and D) 100%HEWAF. PO = Previtellogenic oocytes, VO = vitellogenic oocytes.

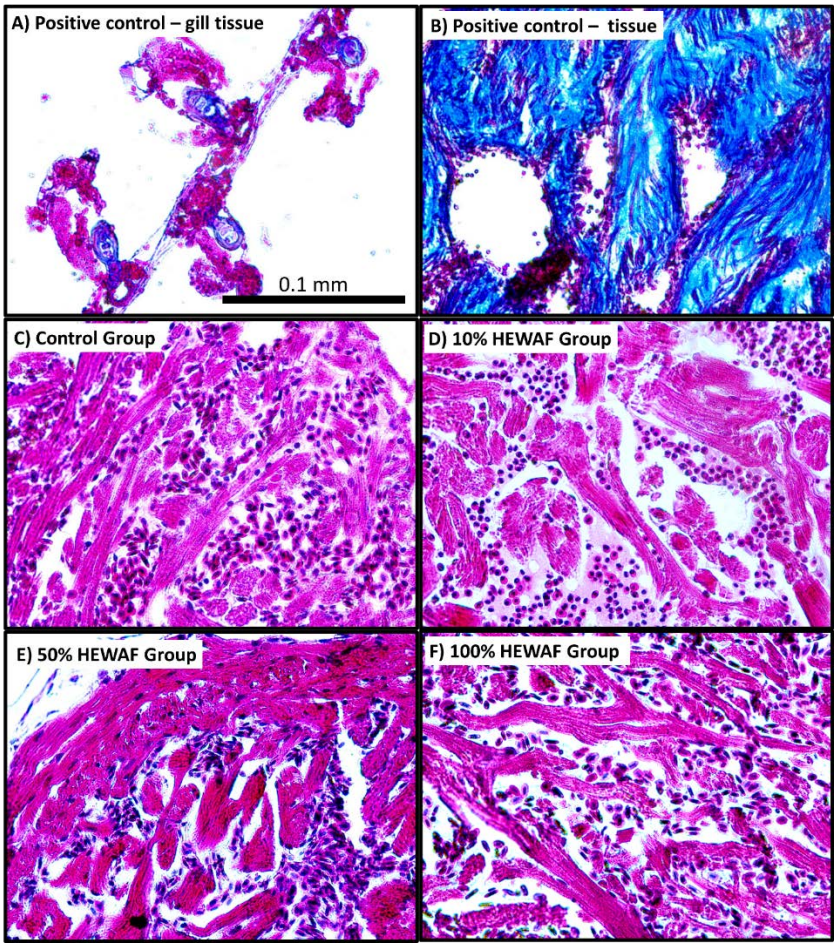

Figure A2-4: Masson's trichrome staining technique in ventricular tissue. Positive control stains, A) gill tissue and B) bulbus arteriousus tissue. C) Control, D) 10%HEWAF, E) 50%HEWAF and F) 100%HEWAF groups, respectively.

CHAPTER 3

MATERNALLY AND PATERNALLY DERIVED TRANSGENERATIONAL EPIGENETIC INHERITANCE OF DIETARY CRUDE OIL RESISTANCE IN THE ZEBRAFISH

3.1 Abstract

Transgenerational inheritance from both parental lines can be transmitted not only by classic genetic inheritance, but also via the influence on the genome of epigenetic markers whose state results from environmental stressors, resulting in various offspring phenotypes. It is widely recognized that the maternal line has more opportunities to influence offspring survival and fitness by provisioning its offspring. Somewhat neglected has been the paternal contribution to offspring fitness. Consequently, adult zebrafish were separated into four experimental groups (two female and two male groups): a male and female group served as control groups, while the other two groups were exposed to water accommodated fractions (HEWAF) of petroleum via diet for 21-days. Subsequently, four offspring groups were obtained: 1) control F_1 (non-exposed parents – C♀C♂), 2) paternally-exposed (C♀E♂) F_1, 3) maternally-exposed (E♀C♂) F_1, and 4) both parents exposed (E♀E♂). Phenotypic responses were then evaluated in the parental (P_0) and offspring (F_1) generations. Growth rate, hypoxia resistance, and heart rate did not differ among parental groups. However, global DNA-methylation in heart tissue was decreased in oil-exposed compared to non-exposed parents P_0 fish. Compared with offspring from control parents, global DNA-methylation was decreased in the three F_1 offspring groups obtained from all groups of oil-exposed parents. Regardless of male or female parental exposure history, F_1 larvae exposed to oil exhibited bradycardia (~125 bpm). Our results show that patterns of global methylation in offspring fish can be inherited from both

parental lines. Hence, phenotypic responses during exposure to environmental stressors in F_1 fish could be influenced by either or both paternal and maternal experiences.

This chapter has been completed in collaboration with the following researchers:

- Amelie Crespel: Conception of experimental design; data acquisition, analysis and interpretation; manuscript drafting, writing and editing.
- Janna Crosley: Assistance with molecular techniques
- Pamela Padilla: Assistance and supervision with immunohistochemical techniques
- Warren W. Burggren: Assistance with conception of experimental design; manuscript editing.

3.2 Introduction

Organismal survival and performance depends upon the capacity to cope with stressors based on their own experiences and also the transgenerationally inherited contribution from their parents (Stein et al., 2018). Variation in the parent-to-offspring inherited information in the offspring population may result in various phenotypes that, in some instances, can be potentially beneficial when coping with persistent stressors (Bautista and Burggren, 2019; Dey et al., 2016). These phenotypes can then become subject to selection (Burggren and Dubansky, 2018; Corrales et al., 2014). For example, at the physiological-organismal level, when F_1 and F_2 embryos of the killifish (*Fundulus heteroclitus)* were exposed to creosote contamination, the offspring descendent from parents that previously inhabited creosote-polluted areas exhibited lower incidence of cardio toxic phenotypes compared to embryos from non-polluted areas (Clark et al., 2014). Additionally, Chapter 2 reported that exposure to crude oil via diet in the parental population enhanced the survival of their offspring challenged to grow in similar conditions (Bautista and Burggren, 2019). In contrast, the presence of detrimental phenotypes in F_1

offspring as a result of parental exposure to pyrolytic polycyclic aromatic hydrocarbons (PAHs) have also been described (Perrichon et al., 2015). In some occasions, the mal-adaptive effects of the parental exposure last up to three generations (Corrales et al., 2014).

Offspring phenotypes are clearly related to their parental experiences and regardless of whether they are adaptive or non-adaptive, parent-to-offspring transgenerational inheritance may occur through genetic mechanisms (Clark et al., 2014), and/or non-genetic mechanisms (Jablonka, 2017; Knecht et al., 2017). Although the transmission of information occurs from both maternal and paternal lines (Emborski and Mikheyev, 2019; Jablonka and Lamb, 2017; Knecht et al., 2017), it is generally assumed that the maternal line has more opportunities to contribute to the survival and fitness of their offspring by provisioning the eggs with nutritive resources (Dey et al., 2016; Herman and Sultan, 2016; Ho et al., 2014; Siddique et al., 2017). Thus, experimental designs have been mainly focused in understanding the influence of the maternal line in offspring survival and performance (Pick et al., 2016). For example, larger eggs in the brown trout (*Salmo trutta*) have lower metabolic costs and higher yolk conversion efficiencies in comparison to smaller eggs (Régnier et al., 2012). Also, fatty acid composition in the yolk of Atlantic silverside is directly correlated with survival during exposure to high CO_2 (Snyder et al., 2018). However, this experimental approach focusing on the maternal line has overshadowed the potential role of paternal inheritance, which similarly to the maternal line, may result in variated phenotypes that are substrate for selection (Jiang et al., 2013; Siddique et al., 2017). As an example, F_1 and F_2 offspring obtained from fathers exposed to Bisphenol A experienced dose-dependent increases in embryo mortality and

body malformations (Lombó et al., 2015). Consequently, inheritance from the paternal line may be the unique way to transmit information to the offspring. For instance, the DNA methylome in zebrafish embryos was inherited just from the spermatozoids and not from oocytes (Jiang et al., 2013), highlighting the importance of the paternal inheritance for offspring development and performance.

Although the maternal and the paternal lines are both directly involved in offspring survival and fitness, the current understanding of the separate contributions of each line of inheritance for offspring performance and the molecular mechanisms that allow them to cope with persistent environmental stressors is limited. Hence, understanding processes that are potentially involved in the regulatory mechanisms of phenotypes and their inheritance deserves much additional attention.

Epigenetic regulation and its phenotypic consequences involve various different molecular processes including modification of histones and/or DNA methylation (Jablonka and Lamb, 2017). For instance, DNA methylation and post-translational modification in the N-terminal of histones are major modifications in the epigenome associated with repression or activation of genes that are crucial for embryogenesis, cell differentiation and organismal development (Jiang et al., 2013; Toni and Padilla, 2016). Indeed, it has been observed that both of these modifications are involved in the inheritance of reproductive defects (Yang et al., 2018) and organismal transgenerational responses to environmental and anthropogenic stressors (Falisse et al., 2018; Nilsson et al., 2018). Furthermore, any environmental or anthropogenic stressor that modifies these epigenetic markers will potentially induce broad ecological impacts for population maintenance in wild populations (Bhandari et al., 2015).

Interest in understanding the transgenerational effects of environmental stressors (e.g. crude oil) in fish has increased in the past decade (Jasperse et al., 2019; Simning et al., 2019). Crude oil is a persistent environmental stressor that impacts the development, cardiac performance, swimming activity and behavior of fish (Bautista et al., 2019; Incardona and Scholz, 2018a; Johansen et al., 2017; Mager et al., 2018). Although exposure to crude oil in fish may happen through their skin, gills and diet (Tierney et al., 2013), they are also able to detect the presence of oil in the water and escape (Bøhle, 1986; Schlenker et al., 2019). Furthermore, exposure will still occur through ingestion of contaminated prey (Olsvik et al., 2011) without reducing their appetite (Christiansen and George, 1995), highlighting the importance of the exposure through this route.

Because of its nature (time, resources and space, principally), experimentation with wild fish populations aimed to understand the transgenerational implications of the parental exposure to oil is rarely practical and consequently little is presently known about its molecular epigenetic transgenerational effects. Therefore, to test the effects of parental acute and chronic exposures to environmental stressors on their offspring, the use of animal models has increased in several disciplines such as genetics, physiology, behavior and ecotoxicology (Burggren and Dubansky, 2018; Pitt et al., 2018; Zhou et al., 2019)

3.2.1 Goals

Studies aimed to establish if dietary exposure to crude oil, within environmentally relevant concentrations, elicits changes in gene expression and epigenetic markers (i.e DNA methylation, histone modifications) in a parental population are scarce.

Consequently, little is known if those changes are transmitted to their offspring from the maternal or the paternal line, and their specific contributions to offspring survival and performance. Thus, the goals of this chapter are to:

(i) Establish if dietary exposure to crude oil elicits changes in global DNA methylation levels and in the patterns of expression of three histone-related antibodies (associated with gene expression and silencing) in the gonads and the hearts of the parental population;

(ii) Establish the maternal and paternal contributions for offspring survival and performance under transgenerational epigenetic framework, and

(iii) Establish weather larval tissue exhibits changes in global DNA methylation levels and determine the patterns of larval expression of three histonee-related antibodies due to the parental exposure to crude oil.

3.2.2 Hypotheses

The current study uses the zebrafish to test the following hypothesis:

1) Regardless of the sex of the P_0 fish, dietary exposure to crude oil will affect:

    a. phenotypic traits directly related with fish health such as growth and heart rate in the parent;

    b. the levels of global DNA methylation in the gonadal and heart tissues of the parental population;

    c. the patterns of presence of histone-related antibodies (associated with gene expression and silencing) in the gonadal and heart tissues of the parental population.

    d. the upregulation of genes related with cardiac development, stress and DNA methylation maintenance.

2) The maternal line of inheritance will better enhance the survival and performance of its offspring in comparison with the paternal line.

3) Global DNA methylation and the patterns of expression of histone-related antibodies will change in the offspring populations regardless of the parental line exposed.

4) Regardless of the parental line exposed to petroleum, the transcript levels of genes involved in heart development, stress and maintenance of DNA methylation in offspring will increase in comparison with offspring from non-exposed parents.

Additionally, immunohistochemical staining of the gonadal tissue of the parental groups was performed and compared with the expression patterns of histones related to gene activation or silencing differed among groups in the parental and also in the offspring generation. Furthermore, global DNA methylation levels in the gonadal and heart tissue and the expression of genes related with cardiac function in the parental population were quantified to see the effects of exposure. Finally, to determine if the maternal or the paternal experience had influence in offspring performance, the F_1 offspring were challenged with crude oil exposure via their ambient water and heart rate recorded during the first five days of development. In addition, F_1 larvae were challenged to loss of equilibrium tests under hypoxia and Global DNA methylation and gene expression levels quantified in pools of whole larvae obtained from the different P_0 parental lines. Finally, immunohistochemical analysis for histone-related antibodies were also conducted in 30 dpf larvae.

3.3 Materials and Methods

3.3.1 Maintenance of Parental Population (P_O)

One hundred twenty adult AB strain zebrafish (60 female, 60 male), were obtained from a local supplier and maintained individually in 1L tanks at the University of North Texas facilities. Before experimentation, the fish were divided into 6 groups (20 fish/each) as follows: 1) Non-treated females (female control - two groups), 2) Non-treated males (male control – two groups), 3) Treated female and 4) Treated male. Because of experimental design (see below), the female and male control groups were duplicated. Prior to exposure, the fish were acclimated during two weeks under recommended husbandry conditions for this species (~27±0.5 ºC, pH ~7.6, 14:10h light:dark cycle, ~ 7.8

DO mg/L) (Spence et al., 2008; Westerfield, 2007). The fish were fed 3% of body mass twice per day with commercial flake food (TetraMin Tropical Food). All experiments described in this study were performed in the strict compliance with the Institutional Animal Care and Use Committee (IACUC-Protocol #15003) at the University of North Texas.

3.3.2 Experimental Design

3.3.2.1 Preparation of Dietary Treatments

To prepare the oiled diet used in this experiment, High Energy Water Accommodated Fractions of crude oil (HEWAF) were prepared following standard protocols (Bautista et al., 2019; Forth et al., 2017; Incardona et al., 2013; Mager et al., 2014b; Reddam et al., 2017). Briefly, 2000 mg of Source Oil B were added into 1L of conditioned aquarium water (60mg/L of instant ocean salts buffered at 7.6 pH with HCO_3) and blended for 30 s in a commercial blender (WaringTM CB15). The mixture was allowed to set during one hour into a separation funnel, after which 100 ml were taken through the bottom port of the funnel and discarded. 600ml of the remaining solution was considered 100% HEWAF solution and was used to prepare the oiled-dietary treatment used during this experiment.

Two g of flake food were evenly spread across the bottom of plastic weighing boats (135mmL X 135mmW X 20mmH) and sprayed with 5ml of conditioned water (control food), or with 5ml of 100%HEWAF (treatment food). The spraying process was performed under the fume hood, where the food was allowed to dry for 12 h. After drying, the food was collected and stored at 4º C in amber glass containers covered with aluminum foil. In Chapter 2 (Bautista and Burggren, 2019), we have reported that the concentrations of

Polyaromatic hydrocarbons (PAHs) in these diets are 0 and 24.2 mg/kg, for the control and 100% HEWAF diets respectively.

3.3.2.2 Dietary Crude Oil Exposure in the P_0 Parental Population

Female and male groups were fed twice a day (3% of body mass per event) with their respective dietary treatment for 21 days. To avoid coprophagia as well as the possibility of exposure via the gills, the fish were allowed to eat for 10 min in each feeding event, after which the non-eaten food and feces were siphoned out and 300ml of water in their containers was removed and replaced with fresh water. .

3.3.2.3 F_1 Acquisition

Immediately after the exposure period, the parental groups were divided and selectively mated to produce the following groups of offspring crosses: 1) Control offspring, obtained from non-exposed parents (C♀C♂), 2) Paternally-exposed offspring, obtained from mating pairs were the males were the only exposed fish (C♀E♂), 3) Maternally-exposed offspring, obtained from mating pairs where the female were the only exposed individuals (E♀C♂), and 4) Both-parents exposed offspring, obtained from mating pairs were both parents were exposed (E♀E♂) (Fig.3.1.). Breeding was carried out by placing three female and three male fish from the respective parental exposure condition into 3L tanks. The fish were maintained separated by sex overnight. The following morning, at the start of the photoperiod, both sexes were allowed to begin courtship and mate for two hours. After this period, the resulting eggs were collected from each tank and rinsed with clean aquarium water at 27±0.5ºC. Eggs obtained from similar parental crosses were mixed and inspected under stereoscopic microscopy to confirm fertilization and cell division of the embryos. Any non-viable embryos were discarded.

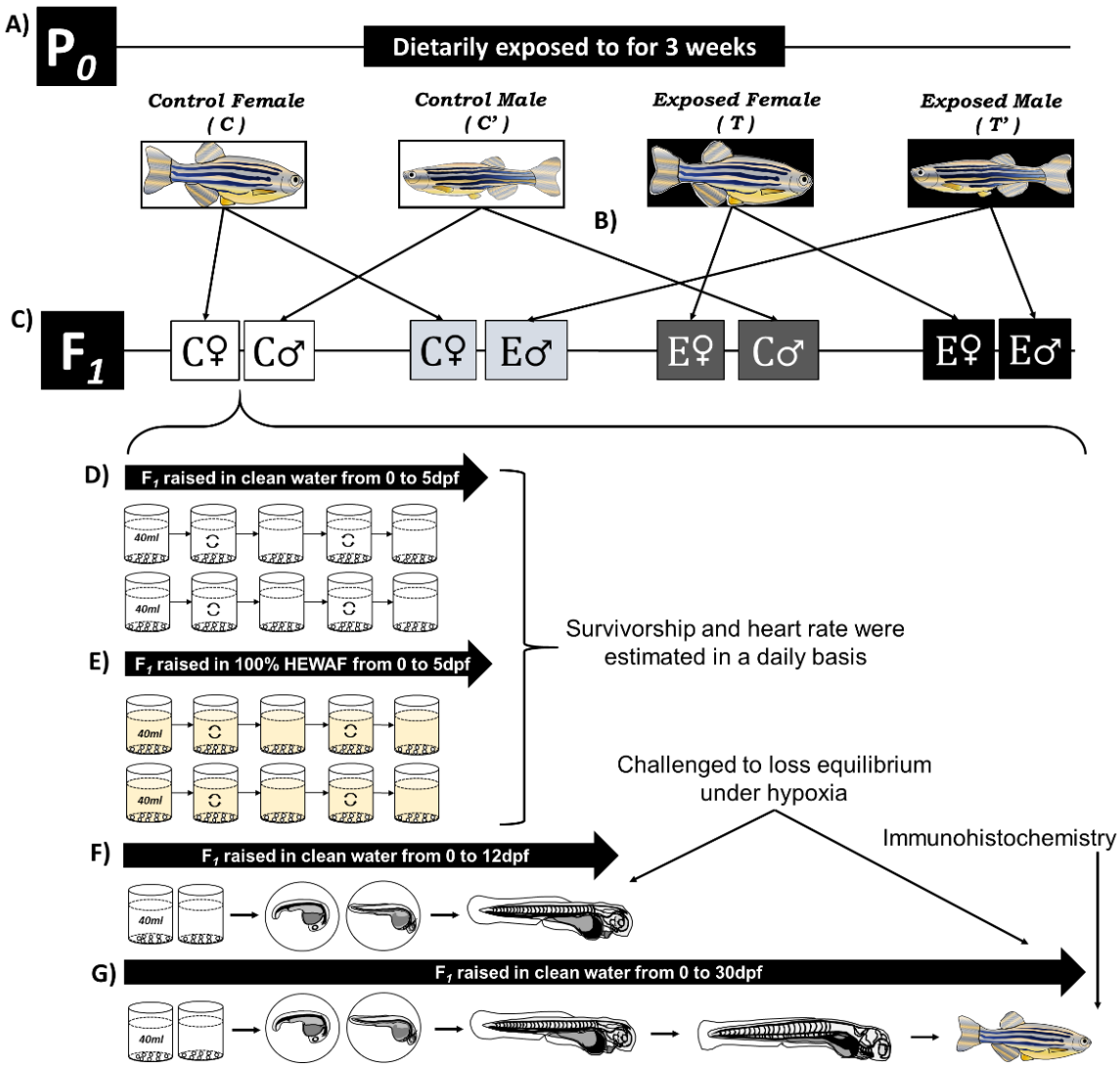

Figure 3.1: Experimental design. A) A parental population of adult zebrafish was divided into four groups (two female and two male) and exposed via diet to water or HEWAF diet for 21 days. B) The following morning after the last day of exposure the parental pairs were bred to obtain the offspring generation. C) The offspring from each parental group (C♀C♂ - C♀E♂ – E♀C♂ and E♀E♂) was divided into eight F1 subgroups (25 individuals / 40ml beaker) obtained from the parental crosses. For clarity purposes we limited the point, the explanation for the offspring obtained just from the C♀C♂ parental cross. The same protocol was applied to the offspring from the rest of the parental crosses. D) Two of the subgroups from each parental cross were raised in clean water from fertilization to 5dpf. E) Two of the subgroups from each parental cross were raised in 100% HEWAF (yellow color) from fertilization to 5dpf. For both conditions the survivorship and the heart rate were estimated in a daily basis during the 5 days of exposure. F) Two of the subgroups from each parental cross were raised in clean water from fertilization to 12 dpf.larvae were challenged to loss of equilibrium under hypoxia (10%DO). In addition, pools of larvae were flash-frozen for molecular analysis. G) The last two subgroups from each parental cross were raised in clean water from fertilization to 30dpf. At this point, the larvae were challenged to loss of equilibrium under hypoxia (10%DO), additionally at this point we took larvae samples for immunohistochemistry staining.

3.3.3 Phenotype Assessment of Parental Population (P_0)

3.3.3.1 Body Length in P_0

Adult fish body length was recorded at the end of the two week acclimation period, at the first, second and third week of exposure. To estimate body length, individual adults were photographed (Nikon Coolpix AW130, 16Mpx) and each photo was digitally analyzed with ImageJ software (https://imagej.nih.gov/ij/).

3.3.3.2 Estimation of Heart Rate in P_0

During the third week of exposure of P_0 fish (average mass 0.46 ± 0.01 g, n= 20 per parental group), measurements of heart rate were performed in the parental population using electrical impedance. An experimental setup of four channels (10 cm L X 8cm W receiving aerated and temperature-controlled (28±0.5°C) fresh conditioned water (60mg/L Instant ocean Salts, 90mg/L of MS222 and buffered at pH 7.8 with HCO_3). The water was delivered to the fish through a needle previously sanded to avoid injuring the fish. The fish (n = 20 per condition) were anesthetized in 100 mg/L MS222 solution, weighed, and placed with the ventral side facing up into a v-shaped sponge. Two monopolar needle electrodes (XLTEK) held in place using manual micromanipulators (World precision instruments) were carefully positioned on the skin of the fish cardiac area for the measurements. The electrodes were then connected to an impedance converter (Model 2991 UFI Morro Bay, California), connected to an amplifier (Model PL3504, PowerLab 4/35) and the signals were recorded using Labchart software V7.

The adult fish were allowed to stabilize in the experimental setup for 30 min prior to intraperitoneal injections of cardio-active drugs. To elicit maximum heart rate by blocking vagal input and maximize sympathetic influence in the hearts, atropine sulfate

(1.2 µg/g body mass) was first injected to increase the heart rate by blocking the activity of cholinergic fibers. This was followed, after 15 min, by an injection of the Beta-adrenergic agonist isoproterenol (7.8 ng/g of fish) to maximize the sympathetic influence of the nervous system on cardiac contractility, and finally after another 15 min propanolol (1.2 µg/g of fish), a non-selective beta-adrenergic receptor antagonist that decreases heart rate and contractility, was injected. All drugs were dissolved in physiological saline solution (0.9% NaCl). Heart rate signals were recorded, for each fish, at one minute intervals for ten time points (during the 5 min prior to atropine injection, and at 5, 10 and 15 min after each drug injection). At the end of the procedure, the fish were carefully removed from their channel and returned to their rearing tanks. A recovery rate of 75% was observed after this procedure.

3.3.3.3 Loss of Equilibrium (LOE) during Hypoxia Challenge in P_0

Loss of equilibrium (LOE) is an indication of resistance against stressors in fish (Ho and Burggren, 2012) and the representation of systemic impairments, and thus could be considered as ecological death (Beitinger et al., 2000). After breeding, adults were placed individually in separated chambers (9 × 8 × 7 cm) within an air-saturated and temperature-controlled 120L water tank, and left undisturbed overnight. The following morning, all the chambers were transferred into a 40L tank of hypoxic water (10% DO) and covered with a grid (to prevent any possible aquatic surface respiration). The oxygen level of the water was controlled by bubbling nitrogen gas into the tank and was monitored by an oxygen meter (ProODO Optical Dissolved Oxygen Instrument, YSI, USA).

Time to loss of equilibrium (LOE) of each fish was recorded starting at the moment when the chambers were fully submerged in the hypoxic water. Once the fish were unable

to maintain equilibrium, they were quickly removed from the challenge tank, identified and transferred into a fully aerated tank for recovery. The corresponding recovery time was recorded for each fish and the experiment ended when the last fish was recovered.

3.3.3.4 Dissection and Tissue Sampling in P_0

One day after the recovery from the hypoxia challenge, the adult fish were anesthetized with MS222 (300mg/L), and their body mass and standard length were measured. Three-quarters of the fish were dissected, their ventricle and gonads were excised and their wet mass was recorded and expressed as percentage of body mass to determine the ventriculo- and gonado-somatic indexes. After mass determination, the tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until processed for molecular analysis (see below). The remaining 25% of the fish were fixed with Zfix solution (formaldehyde based fixation with Zinc buffer - Anatech Ltd) for 24h, and then stored in PBS for five days. Gill, liver, guts, ventricle, gonads were then excised. With exception of the gills, the wet mass of the organs was recorded and expressed as percentage of body mass to determine their specific mass index. The tissues were then stored in 70% Ethanol for subsequent histological processing.

3.3.3.5 Immunohistochemistry in P_0

Female and male gonadal tissue were processed for histological analysis following standard procedures (Carson, 1990). In brief, the tissues were dehydrated in a series of ethanol baths after which they were washed three times in Histochoice® clearing agent. After clearing, tissues were embedded in paraffin and subsequently sliced into 5µm sections with a manual microtome (Leica RM2245).

To determine if exposure to crude oil leads to changes in the localization of

histones associated with gene expression or silencing, gonads from three adult female and three male were immunohistologically stained. Antibodies were localized using the VECTASTAIN® Elite® ABC Universal Plus kit, Peroxidase following the instructions of the manufacturer. In brief, after acquiring slides of organs following the protocol mentioned above, the sections were deparaffinized and hydrated through a series of Histochoice® and graded alcohol series. After unmasking and quenching, the sections were incubated with mouse or rabbit primary antibodies:

a) rabbit polyclonal (mAbcam 8580) anti-histone H3 (tri methyl K4 - epigenetic marker for promoters of active genes and initiated forms of RNA polyhmerase II – H3K4me3), and

b) mouse monoclonal (mAbcam 6002) anti-histone H3 (tri methyl K27-epigenetic marker for inactive genes – H3K27me3) (Toni and Padilla, 2016). After staining, the slides were photographed with light microscopy (Zeiss Axio Imager.M2) adapted with an AxioCam camera MRc.

Indirect determination of antibodies expression in the tissues was carried out by digital analysis (ImageJ). All photographs were acquired using the same microscopy parameters (scale, zoom, opening of diaphragm, lighting). Control reagent stains were performed for each antibody and each slide, and used to determine the level of background staining in an area of 5000 μm^2. Based on the control-reagent stains, we determined the color threshold values for positive signals for each antibody at 40X (HUE ratio 0:255; saturation 0:255; and brightness ratio 40:113), and used these to standardize the analysis. After setting these parameters for each image, we used the function “Analyze Particles” in the ImageJ software to obtain the number of pixels that meet the assumptions for positive signals of each antibody.

3.3.3.6 Molecular Assessment in P_0

Six pools of three hearts and three gonads per treatment and sex in the parental

population were homogenized and split into two equal parts. DNA and total RNA were then extracted using DNAzol and RNAzol (Sigma Aldrich) solutions according to the manufacturer's protocols. The integrity of the extraction was assessed by electrophoresis on 1% agarose gel and the purity and concentrations using the Nanodrop (Nanodrop 2000 Spectrophotometer, Thermo Scientific, USA). The samples were stored at -80°C until further processed.

3.3.3.7 Global DNA Methylation in P_0

The relative levels of global DNA methylation (5 mC) percentage in the DNA samples was quantified using a MethylFlashTM Methylated DNA Quantification kit (Epigentek Group, USA), according to the manufacturer's instructions.

3.3.3.8 Quantitative PCR in P_0

Total RNA obtained from the different pools was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) and stored at -20°C until analysis. Quantitative real-time polymerase chain reaction (qPCR) was used to analyze: one candidate gene involved in response to PAHs (CYP1a), four genes involved in cardiac development (GATA4, MyH7, MyL7, and NKX2.5) and three genes involved in cardiac stress (NPPA, TNNT2a, and ATP2a2a). Additionally, ten genes were analyzed that were specifically involved in: DNA methylation maintenance (DNMT1), establishment of new methylation (DNMT3, DNMT4, DNMT5, DNMT6, DNMT7), methylation transfer (GNMT), and demethylation (TET1, TET2, TET3). The design of the primers for each gene was performed using Primer3Plus software based on cDNA sequences available in the public database NCBI (https://www.ncbi.nlm.nih.gov) (Table 3.1). qPCR was performed using Evagreen Supermix (Bio-Rad Laboratories, USA).

Table 3.1: Oligonucleotide primers used for quantitative PCR

Gene name	Abbreviation	Accession number	Species	Forward (F) and reverse (R) primers sequences (5'-3')
Cythochrome P450 1a	CYP1a	NM_131879.1	*D. rerio*	F: CTTCCCTTCACCATTCCTCA R: GGTTGACTTGCCACTGGTTT
GATA binding protein 4	GATA4	NM_131236.2	*D. rerio*	F: CCGGTGTCCAGATTCACTTT R: TCCCGGCTTGTATAAGTTGC
Myosin heavy chain 7	MyH7	NM_001112733.1	*D. rerio o*	F: CGACATAATCGCAAAGCTGA R: CCTTGGTGAGGGTGTTGACT
Myosin light chain 7	MyL7	NM_131329.3	*D. rerio*	F: ACCCAGAGGAAACCATCCTT R: GGGTCATTAGCAGCCTCTTG
NK2 homeobox 5	NKX2.5	NM_131421.1	*D. rerio*	F: GCATCAGAGCTTGGTGAACA R: TCCCAGCCAAACCATATCTC
Natriuretic peptide A	NPPA	NM_198800.3	*D. rerio*	F: ACACAGAGAAATCGCCTGCT R: AGGGTGCTGGAAGACCCTAT
Troponin T 2a	TNNT2a	NM_152893.1	*D. rerio*	F: GACGCAAACCTCTGGACATT R: CATCCAGCTCCACAATTCC
ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2a	ATP2a2a	NM_200965.1	*D. rerio*	F: CTGTGCCTTGTGCAATGACT R: GCAGGTTAGAGCCGTTTCTG
DNA methyltransferase 1	DNMT1	NM_131189.2	*D. rerio*	F: GGTGTGGTGTCTGTGAGGTG R: AGGTTGGGACACCTCCTCTT
de novo DNA methyltransferase 3	DNMT3	AF135438.1	*D. rerio*	F: GTGGACCCTTGGACCTGTTA R: GGCAAAGAACTCCTGCACTC
DNA methyltransferase 4	DNMT4	AB196915.1	*D. rerio*	F: GAGATGCTGCTGTGTGGAAA R: GTCCAGATTACGGGCACTGT

(table continues)

Gene name	Abbreviation	Accession number	Species	Forward (F) and reverse (R) primers sequences (5'-3')
DNA methyltransferase 5	DNMT5	AB196916.1	*D. rerio*	F: GCTGACCAGTCAATGAAGCA R: AGGGCCAACAAGCTTAGGAT
DNA methyltransferase 6	DNMT6	AB196917.1	*D. rerio*	F: TCGGTCGAATTCCATAAAGC R: CTCTCCATCTCAGTGCACCA
DNA methyltransferase 7	DNMT7	AB196918.1	*D. rerio*	F: TGACAGAGCCAGTTCCTGTG R: CTTGACCATCACGAGCTTCA
Glycine N-methyltransferase	GNMT	NM_212816.1	*D. rerio*	F: GCAGCTGTACATCGGAGACA R: ACATCCAGCACCCTCTGAAC
Ten-eleven-translocation 1	TET1	KC689999.1	*D. rerio*	F: GCTTTCTCTCCTCTCGCTGA R: TGACTCGCTGTCAAATGAGG
Ten-eleven-translocation 2	TET2	KC690000.1	*D. rerio*	F: CATGCTCACAGAGACCTCCA R: TTCCAATCTCGCGATTATCC
Ten-eleven-translocation 3	TET3	KC690001.1	*D. rerio*	F: TTGCTTCAGACTGTCGGTTG R: GCCATTGTGGAGGTTATGCT
Actin beta 2	ACTB2	NM_181601.4	*D. rerio*	F: GTGCCCATCTACGAGGGTTA R: TCTCAGCTGTGGTGGTGAAG
WD and tetratricopeptide repeats 1	WDTC1	NM_001130606.1	*D. rerio*	F: AGCAGTGGCTCTGGTGATCT R: GAGCTGAATGGCTTGAGTCC

WDTC1 and ACTB2 genes were chosen as the housekeeping genes for the gonads and the heart, respectively, as they showed minimal variance in their transcript levels, both within and among conditions. No amplification was observed in negative controls. In brief, we used 150 µg for the heart tissue and 500 µg for the gonadal tissue that were converted into cDNA. From the obtained cDNA, 20 µl were used to prepare a 1:25 and a 1:50 dilution for the heart and the gonads, respectively; from this dilution, 2µl were used in a total dilution of 10 µl per reaction. The cycling characteristics were: 30 s at 95 ºC followed by 5 s at 95 ºC, then 5 s at 60 ºC and retuned to 95 ºC. The cycling process alternated from 60 to 95 ºC 50 times. The CFX Manager program (Bio-Rad Laboratories, USA) was used to determine the threshold cycle (C_t) values. The qPCR efficiency (*E*) was calculated for each gene according to the equation $E=10^{(-1/slope}$. We also used the formula $E^{CT(gene\ of\ interest)}$ / $E^{CT\ (housekeeping\ gene)}$ to calculate the expression.

3.3.4 F_1 Generation

3.3.4.1 F_1 Rearing Conditions and Exposure

Early developmental stages are considered to be highly sensitive to environmental stressors (Burggren and Dubansky, 2018; Mager et al., 2017b; Reddam et al., 2017), thus they represent a good model to assess organismal performance and the influence of their parental inheritance during exposure to environmental stressors. To test if the parental exposure experience had any influence on offspring survival or heart rate, 200 eggs per parental cross (C♀C♂, C♀E♂, E♀C♂ or E♀E) where sampled and divided into 8 subgroups of 25 larvae each. Each larval subgroup was placed into a 40ml beaker and grown up to 5 dpf in either clean water or 100% HEWAF. From the 8 beakers obtained from each parental cross, 4 beakers were exposed to each experimental condition. Two

of those groups were used to measure survival and the other two beakers were used to measure heart rate (Fig.3.1.).

The remaining F_1 offspring from each parental cross were raised to 12 or 30dpf in control conditions until further physiological or molecular analysis was performed (see below). During this period, the larvae were fed twice a day with Otohime™A1 and A2 commercial food (www.otohime.us). To maintain water quality, F_1 larvae were allowed to eat for 30 min during each feeding event, and then the remaining food was removed. All larval exposures and maintenance in this experiment were performed at 28±0.5ºC (Fig.3.1F-G.)

3.3.4.2 Survival in F_1

Survival of the F1 larvae obtained from each parental cross was estimated for 5 days during exposure to clean water or 100% HEWAF. Survival was assessed daily from fertilization through 5dpf, the length of the exposure. Larvae were considered dead when absence of heartbeat was observed under a stereoscopic microscopy.

3.3.4.3 Heart Rate

Heart rate of the offspring population was measured under stereoscopic microscopy on a daily basis during the 5 days of exposure. Twenty sec video recordings were acquired from larvae of each P_0 parental group and exposure condition. The videos were subsequently processed with Photoshop CS6 extended version and analyzed using ImageJ software.

3.3.4.4 Loss of Equilibrium (LOE) in Hypoxia Challenge in F_1

At 12 and 30 dpf, zebrafish larvae that had been raised in clean water but were

from the different parental exposures were placed individually in small cylindrical glass chambers (4 × 0.6 cm – 1 ml) covered at both ends with a fine netting, which prevented the fish from moving but allow water to flow through the chamber. Groups of four chambers were then transferred into a tank containing 10L hypoxic water (13% DO). The % of O_2 level of the water was controlled by bubbling nitrogen gas into the tank and monitored with an O_2 meter (ProODO Optical Dissolved Oxygen Instrument, YSI, USA). The time to loss of equilibrium (LOE) of each larva was recorded, starting at the moment when the chambers fully entered into the hypoxic water. After the larvae were unable to recover from their loss of equilibrium for 3 s, they were quickly removed from the hypoxic water and transferred into a tank containing fully aerated water for recovery. The corresponding time for recovery of each larva was recorded and the experiment ended when the last larva had recovered.

3.3.4.5 F_1 Sampling and Tissue Processing in F_1

Zebrafish larvae (13 dpf) from the different parental background raised under clean water were anesthetized with MS222 (100 mg/L) and 10 pools of 15 larvae per cross were sampled. The pools were directly frozen in liquid nitrogen and stored at -80°C for subsequent molecular analysis. Additionally, 4 to 6 larvae (30 dpf) per condition were fixed in Zfix solution for 24 h, rinsed and stored in PBS for 5 days and then transferred to 70% ethanol until histology analysis.

Tissues and larvae stored in 70% ethanol for histology analysis were processed as mentioned above. Briefly, the samples were dehydrated through a series of ethanol washes (80%, 95%, 100% ethanol), cleared using three washes of Histochoice® clearing agent, and then embedded using three baths of paraffin. The samples were stored at

room temperature for subsequent staining.

3.3.4.6 Immunohistochemistry in F_1

To determine if parental exposure leads to changes in the patterns of localization and presence of histones related to gene activation or silencing, three 30dpf embedded larvae from each parental cross were sectioned at 5µm and stained with the same antibodies as the parental population (see above). After staining, photograph of the heart and skeletal muscle of the larvae were acquired with light microscopy (Zeiss Axio Imager.M2). Indirect determination of the expression levels of the antibodies were estimated following the same procedures as for the parental population.

3.3.4.7 Molecular Analysis

Nine pools of 15 larvae per parental cross were homogenized and split into 2 equal parts. DNA and total RNA were then extracted with a similar protocol to the parental population. After the integrity of the extractions and the purity and concentrations were assessed, the samples were stored at -80°C until further processing.

3.3.4.8 Global DNA Methylation

The relative levels of global DNA methylation (5 mC) percentage in the F_1 DNA samples were quantified similarly to the samples from the parental population.

3.3.4.9 Polymerase Chain Reaction in F_1

Total RNA obtained from the different pools was reverse transcribed into cDNA as described above and stored at -20°C until use. The qPCR protocol was similar to the parental samples, but only the ACTB2 gene was chosen as the housekeeping gene for the larvae. Similarly, no amplification was observed in negative controls, the threshold

cycle (C_t) values and the efficiency (E) of qPCR were estimated as for the parental population.

3.3.5 Statistical Analysis

Statistical significance was assumed with an α value of 0.05. All data was tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Brown-Forsy tests, respectively. If the data did not meet these assumption, appropriate non-parametric analyses were performed.

3.3.5.1 Statistics for the Parental (P_0) Generation

Body length of the adults was compared among parental groups with three-way ANOVA, using treatment, sex and time as factors. Similarly, heart rate measurements in the parental generation were analyzed with three-way ANOVA using sex, time and treatment as factors. The hepato-somatic, gut-somatic, ventriculo-somatic and gonado-somatic indexes were analyzed using two-way ANOVAs with diet and sex as factors. Gonado-somatic index was rank-transformed and statistical procedures were applied on ranks (Quinn and Keough, 2002). Time to loss of equilibrium under hypoxia was analyzed using two-way ANCOVA with diet exposure and sex as factors and body mass as a covariate. Analysis of antibody expression in the tissues was estimated by three-way-ANOVA using fish sex, group, and the slide (control reagent and stained) as factors for each antibody. Global DNA methylation level and gene expression profiles in the heart and gonads of the parental generation were analyzed using two-way ANOVAs with diet and sex as factors.

3.3.5.2 Larval F_1 Generation

Larval survival was analyzed with the Cox Stratified model using the F_1 exposure

condition as strata and the maternal and paternal exposures as covariates. Because differences were found among groups, Survival Log-Rank tests were employed to analyze each exposure condition by separate and Holm-Sidak method employed for multiple comparisons.

Heart rate of larvae was rank transformed and analyzed with General Linear Model (GLM) - multifactor ANOVA, using the time, treatment, and the maternal and paternal exposure experience as categorical factors. Because the interaction of the factors induced significant differences (P=0.011), each F_1 exposure condition (clean water and 100%HEWAF) subsequently analyzed separately with a GLM – three-way ANOVA, where time and the parental exposure conditions were employed as factors. Similarly to the parental population, time to loss of equilibrium under hypoxia was rank transformed for the larvae, and three-way ANOVAs was performed on ranks. Analysis of antibody presence in the F_1 larvae tissues was performed by two-way ANOVA using maternal and paternal exposure as factors for each antibody. Finally, the global DNA methylation levels at 13 dpf and the gene expression profiles were analyzed using two-way ANOVAs with maternal exposure and paternal exposure as factor.

Statistica version 7.0 (StatSoft, USA), Sigmaplot V.14, and Statgraphics centurion XVI software were used for analyzing the data. All data are expressed as mean ± s.e. mean unless other indicated.

3.4 Results

3.4.1 Parental Population P_0

3.4.1.1 Survival and Body Length in P_0

No mortality was observed due to any dietary treatment in the parental populations.

Total body length ranged from 28.9±0.4 mm and 28.4±0.4 mm for female and male fish at the end of the acclimation period, to 31.6 ± 0.5 mm and 31.2 ± 0.5 mm, respectively, but these differences were not significant (P>0.05).

3.4.1.2 Heart Rate and Loss of Equilibrium in P_0

Diet and sex had no significant effect on heart rate frequency in the parental generation ($F_{1,689} = 0.05$, $P = 0.82$; $F_{1,689} = 7.30$, $P = 0.007$) with no difference revealed in the post hoc test, respectively (Fig. 3.2). However, the measuring time points had a significant effect ($F_{9,689} = 5.14$, $P < 0.001$). Additionally, heart rate measured under isoproterenol was significantly higher than heart rate measured both before injection and after propanolol injection. However, no statistically significant effects were induced by atropine.

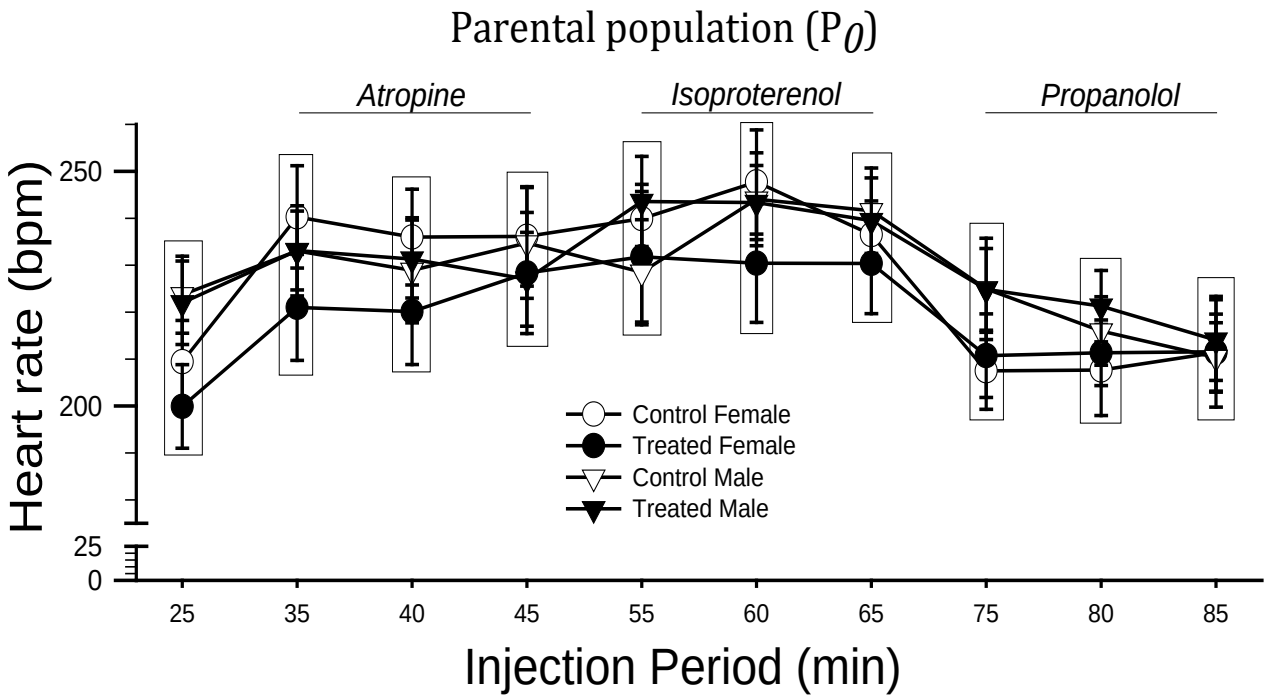

Figure 3.2: Heart rate (bpm) in the parental (P_0) population. Heart rate in the parental population during the injection protocol. n = 20 per group.

Similarly diet and sex did not have a significant effect on the time to loss of

equilibrium in the parental generation (F1,88 = 0.003, P = 0.95; F1,88 = 0.009, P =0.92, respectively). The average time to loss of equilibrium was 2.0 ± 0.1 h (Fig.3.3).

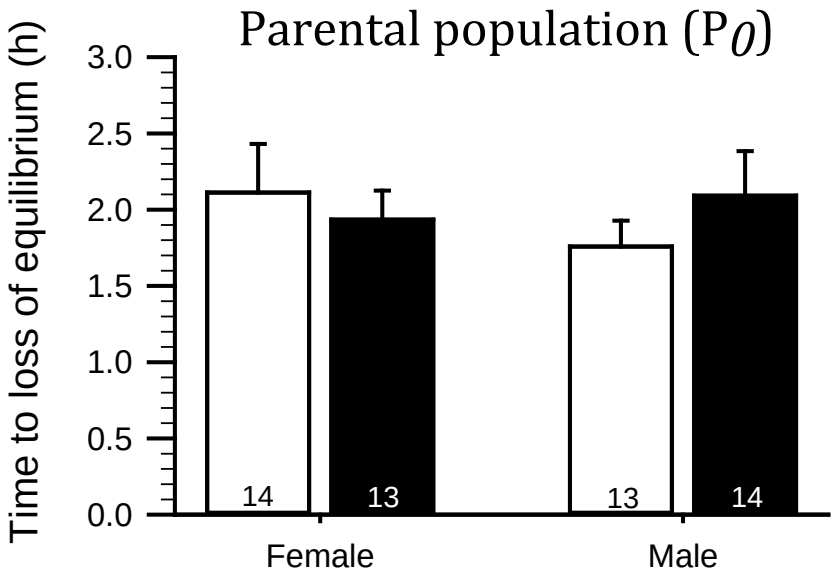

Figure 3.3: Time to loss of equilibrium under hypoxia challenge in the parental (P_0). Non-exposed control group are demarked with white bars; dietarily exposed fish are indicated with black bars.

3.4.1.3 Organo-Somatic Index, and Immunohistochemistry in P_0

Neither diet nor sex significantly affected hepato-somatic index (F1,22 = 0.22, P = 0.64; F1,22 = 3.19, P = 0.09, respectively) (Table 3.2). Similar results were observed for the gut-somatic index for which no difference was observed according to diet or sex (F1,24 = 0.13, P = 0.73; F1,24 = 0.02, P = 0.89, respectively). However, sex had a significant effect on the ventriculo-somatic index and the Gonado-somatic index (F1,122 = 9.60, P = 0.002; F1,111 = 226.57, P < 0.001, respectively). Females had a smaller ventriculo-somatic index but a higher gonado-somatic index compared to males (Table 3.2). The diet did not have an effect on the ventriculo-somatic index and the Gonado-somatic index (F1,122 = 0.03, P = 0.85; F1,111 = 0.04, P = 0.85, respectively).

Table 3.2: Organo-somatic index (%) of the control and dietary exposed fish from the parental generation. Values are expressed as mean ± s.e.m. Different superscript letters indicate significant differences among groups (P<0.05).

Group	Hepato-somatic index	Gut-somatic index	Ventriculo-somatic index	Gonado-somatic index
Control Female	1.32 ± 0.35	5.89 ± 0.69	0.13 ± 0.01[a]	10.72 ± 1.35[b]
Control Male	0.84 ± 0.21	4.83 ± 0.37	0.18 ± 0.01[b]	1.58 ± 0.41[a]
Exposed Female	1.52 ± 0.37	4.70 ± 0.54	0.14 ± 0.01[a]	11.71 ± 1.46[b]
Exposed Male	0.92 ± 0.29	5.60 ± 0.78	0.17 ± 0.01[b]	1.23 ± 0.28[a]

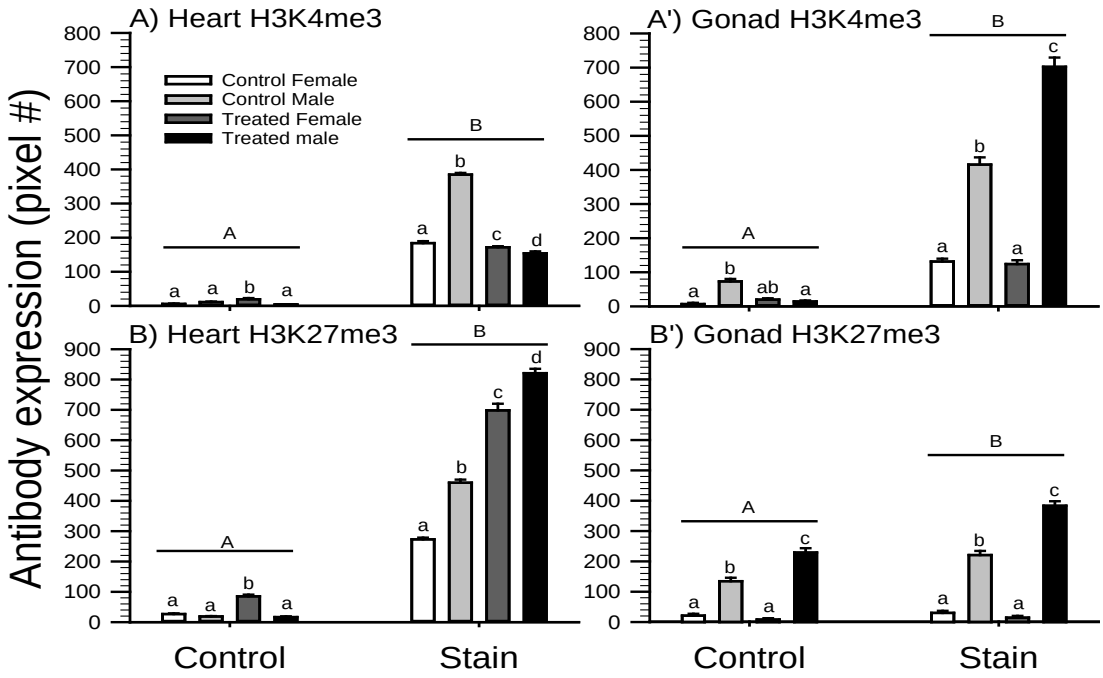

Figure 3.4: Antibody presence (pixel density) in the adult population. In the heart: A) H3K4me3 and B) H3K27me3; In the gonads: A') H3K4me3 and B') H3K27me3. Statistical significance was considered with a P value lower than 0.05. Each bar represents the average of three animals.

The levels of antibodies presence quantified by indirect digital analysis in the heart tissue of the adults revealed that control female and male fish exhibited higher pixel density in comparison with exposed fish (Fig. 3.4). In heart tissue, the pixel density of H3K4me3 (active genes) was significantly reduced by half in the exposed fish compared

to control fish (Fig. 3.4A). However, the expression of K3K27me3, associated with gene silencing, was significantly increased by in exposed fish (Fig. 3.4). In contrast, the levels of exposure of the two antibodies in the gonads of the exposed adult males were significantly higher in comparison to the control male adult fish gonads (Fig. 3.4A-B). In the gonadal tissue, exposed female exhibited a similar pixel density.

3.4.1.4 Global DNA Methylation in P_0

Dietary exposure to crude oil had a significant effect on the global DNA methylation in the heart of the parental generation ($F_{1,20} = 5.00$, $P = 0.04$). The exposed adult fish exhibited lower percentage of global DNA methylation than the control adults (Fig. 3.5). In contrast, the sex of the fish did not significantly impact the global DNA methylation level in the heart ($F_{1,20} = 2.06$, $P = 0.17$). Additionally, neither diet nor sex had an effect on the global DNA methylation level in the gonadal tissue ($F_{1,20} = 0.07$, $P = 0.79$, $F_{1,20} = 0.61$, $P = 0.44$, respectively, Fig.3.5).

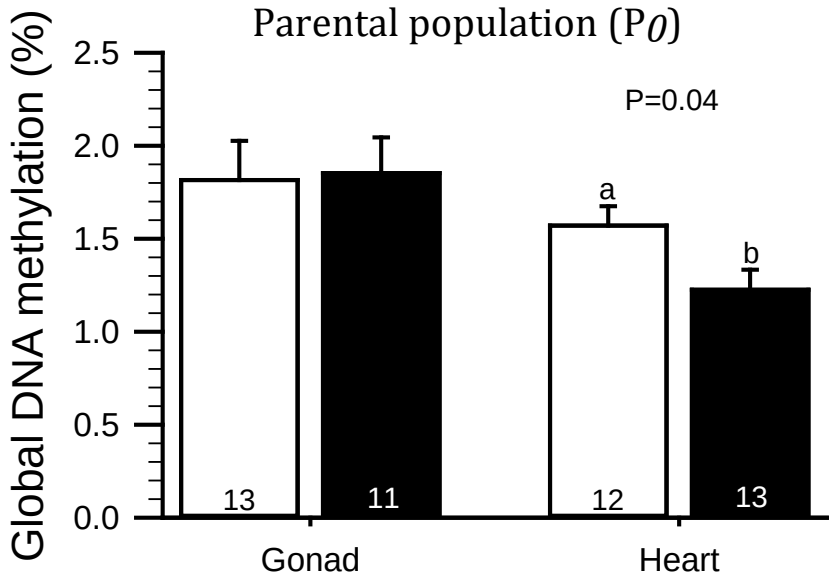

Figure 3.5: Global DNA methylation level (%) of Gonadal and heart tissue of the control (white bars) and dietarily exposed fish (black bars) from the parental generation.

3.4.1.5 PCR and Epigenes in P_0

The transcripts levels of the gene involved in the response to PAHs (CYP1a, $F_{1,15} = 0.14$, $P = 0.71$), cardiac development (GATA4, $F_{1,16} = 0.11$, $P = 0.75$; MyH7, $F_{1,16} = 1.81$, $P = 0.20$; MyL7, $F_{1,16} = 0.74$, $P = 0.40$; NKX2.5, $F_{1,16} = 0.11$, $P = 0.75$) and cardiac stress (NPPA, $F_{1,15} = 0.0002$, $P = 0.99$; TNNT2a, $F_{1,14} = 0.60$, $P = 0.45$; ATP2a2a, $F_{1,16} = 0.20$, $P = 0.66$) in the heart of the parental generation were not significantly influenced by the diet. However, the sex had significant impact on the transcript levels of CYP1a ($F_{1,15} = 4.99$, $P = 0.04$) and ATP2a2a ($F_{1,16} = 1.06$, $P = 0.02$), in which the male exhibited higher levels of transcript than the female (Table 3.3).

Table 3.3: Standarized transcript levels of genes involved in the response to PAHs (CYP1a), cardiac development (GATA4, MyH7, MyL7, NKX2.5) and cardiac stress (NPPA, TNNT2a, ATP2a2a) from heart and gonads of the control and dietary exposed adults fish from the parental generation and from whole 13 dpf zebrafish larvae from the offspring generation obtained from both parents control (C♀C♂), only father exposed (C♀E♂), only mother exposed (E♀C♂), and both parents exposed (E♀E♂) to HEWAF. Values are expressed as mean ± s.e.m. Different superscript letters indicate significant differences among groups (P<0.05).

	CYP1a	GATA4	MyH7	MyL7	NKX2.5	NPPA	TNNT2a	ATP2a2a
Heart tissue								
Control Female	0.74 ± 0.05[a]	0.89 ± 0.08	1.07 ± 0.22	0.95 ± 0.12	0.89 ± 0.09	0.89 ± 0.11	0.87 ± 0.06	0.83 ± 0.09[a]
Control Male	1.03 ± 0.19[b]	1.11 ± 0.11	0.96 ± 0.07	1.02 ± 0.03	1.02 ± 0.03	0.85 ± 0.03	1.11 ± 0.20	0.96 ± 0.11[b]
Exposed Female	0.85 ± 0.08[a]	0.93 ± 0.09	0.82 ± 0.07	0.87 ± 0.10	0.92 ± 0.11	0.85 ± 0.10	0.83 ± 0.09	0.78 ± 0.07[a]
Exposed Male	0.99 ± 0.03[b]	1.02 ± 0.03	0.87 ± 0.03	0.94 ± 0.04	1.05 ± 0.09	0.89 ± 0.04	0.99 ± 0.06	1.09 ± 0.10[b]
Gonadal tissue								
Control Female	1.03 ± 0.07	1.04 ± 0.05	0.72 ± 0.06[a]	0.79 ± 0.04[a]	0.74 ± 0.04[a]	0.77 ± 0.04[a]	1.01 ± 0.22	1.05 ± 0.06[b]
Control Male	0.93 ± 0.01	1.31 ± 0.04	1.29 ± 0.19[b]	1.41 ± 0.18[a*]	1.23 ± 0.21[b]	1.25 ± 0.08[b]	1.10 ± 0.10	0.90 ± 0.01[a]

(table continues)

	CYP1a	GATA4	MyH7	MyL7	NKX2.5	NPPA	TNNT2a	ATP2a2a
Exposed Female	0.93 ± 0.02	1.14 ± 0.12	0.76 ± 0.07[a]	0.75 ± 0.07[b]	0.77 ± 0.05[a]	0.78 ± 0.06[a]	0.90 ± 0.08	1.16 ± 0.06[b]
Exposed Male	0.89 ± 0.04	1.24 ± 0.12	1.43 ± 0.26[b]	1.01 ± 0.06[b*]	0.96 ± 0.02[b]	1.41 ± 0.15[b]	0.93 ± 0.02	0.89 ± 0.04[a]
Larvae								
C♀C♂	0.97 ± 0.07	0.92 ± 0.04[a]	0.93 ± 0.05	0.90 ± 0.05	1.04 ± 0.11[b]	0.84 ± 0.04[a]	1.02 ± 0.07	1.05 ± 0.08
C♀E♂	1.04 ± 0.07	1.16 ± 0.08[b]	1.10 ± 0.08	1.02 ± 0.03	1.25 ± 0.08[b]	1.09 ± 0.06[b]	1.16 ± 0.02	1.07 ± 0.05
E♀C♂	1.02 ± 0.06	1.01 ± 0.04[ab]	0.94 ± 0.03	0.97 ± 0.03	1.02 ± 0.06[a]	0.97 ± 0.06[ab]	1.23 ± 0.08	1.05 ± 0.04
E♀E♂	0.99 ± 0.09	0.91 ± 0.03[a]	0.90 ± 0.07	0.87 ± 0.06	0.92 ± 0.07[a]	0.87 ± 0.06[a]	0.94 ± 0.10	0.96 ± 0.05

In the gonads, only the expression level of MyL7 (involved in cardiac development) was significantly down-regulated in the exposed fish compared with controls ($F_{1,14}$ =4.94, P = 0.04) (Table 3.3). The transcripts levels of all the other genes (GATA4, $F_{1,16}$ =0.01, P = 0.91; MyH7, $F_{1,12}$ = 0.36, P = 0.56; NKX2.5, $F_{1,16}$ = 1.48, P = 0.24; NPPA, $F_{1,14}$ = 1.18, P = 0.30; TNNT2a, $F_{1,15}$ = 1.00, P = 0.33; ATP2a2a, $F_{1,16}$ = 0.59, P = 0.45), including the one involved in the response to PAHs (CYP1a, $F_{1,16}$ = 1.93, P = 0.18), were not influenced by dietary exposure to oil. The sex of the fish had significant effect on ATP2a2a ($F_{1,16}$ = 10.79, P = 0.005), the transcript level of the female was up-regulated compared to the transcript level of the male. In contrast the transcript levels of the genes MyH7, MyL7, NKX2.5 and NPPA ($F_{1,12}$ = 18.21, P = 0.001; $F_{1,14}$ = 19.67, P = 0.001; $F_{1,16}$ = 12.49, P = 0.003; $F_{1,14}$ = 52.17, P < 0.0001; respectively), in the males were up-regulated compared to the transcript levels of the female fish.

The expression level of GNMT (involved in the transfer of DNA methylation) was significantly up-regulated in the heart of the exposed fish compared to the hearts of the control fish ($F_{1,16}$ = 8.05, P = 0.01) (Table 3.4). In contrast, the expression level of TET3

(involved in DNA demethylation) was down-regulated in the hearts of the exposed fish compared to the control ($F_{1,16} = 4.78$, $P = 0.04$). However, the expression levels of the all the others genes, involved in the methylation maintenance (DNMT1, $F_{1,16} = 2.34$, $P = 0.15$), the *de novo* methylation (DNMT3, $F_{1,15} = 2.12$, $P = 0.17$; DNMT4, $F_{1,16} = 1.84$, $P = 0.19$; DNMT5, $F_{1,16} = 1.66$, $P = 0.22$; DNMT6, $F_{1,15} = 0.11$, $P = 0.75$; DNMT7, $F_{1,16} = 1.81$, $P = 0.20$) and the demethylation (TET1, $F_{1,16} = 0.24$, $P = 0.63$; TET2, $F_{1,16} = 0.25$, $P = 0.62$) in the heart of the parental generation, were not significantly influenced by the dietary exposure. Nonetheless, the heart of the female exhibited higher expression level of DNMT5 than the heart of the male ($F_{1,16} = 12.23$, $P = 0.003$).

In the gonadal tissue, the expression level of DNMT1 (involved in the methylation maintenance) was significantly influenced by the interaction between the dietary treatment and the sex of the fish ($F_{1,16} = 5.24$, $P = 0.04$). Exposed female had a higher level of transcript than the control female and male (Table 3.4). The transcripts levels of all the other genes (DNMT3, $F_{1,16} = 3.90$, $P = 0.07$; DNMT4, $F_{1,15} = 0.25$, $P = 0.62$; DNMT5, $F_{1,15} = 0.63$, $P = 0.44$; DNMT6, $F_{1,15} = 0.87$, $P = 0.36$; DNMT7, $F_{1,16} = 1.54$, $P = 0.23$; GNMT, $F_{1,16} = 0.02$, $P = 0.89$; TET1, $F_{1,16} = 1.38$, $P = 0.25$; TET2, $F_{1,16} = 1.70$, $P = 0.21$; TET3, $F_{1,16} = 0.07$, $P = 0.79$) were not influenced by the diet of the fish. In contrast, the sex of the fish induced a significant effect on DNMT3 ($F_{1,16} = 6.06$, $P = 0.03$), for which the transcript level of the female was higher compared to the transcript level of the male, and also on DNMT6, TET1, TET2 and TET3 ($F_{1,15} = 21.88$, $P = 0.0003$; $F_{1,16} = 27.39$, $P < 0.0001$; $F_{1,16} = 33.50$, $P < 0.0001$; $F_{1,16} = 11.91$, $P = 0.003$; respectively), for which the transcript levels of the male were higher than the transcript levels of the female.

Table 3.4: Relative transcript levels of genes involved in maintenance of DNA methylation (DNMT1), establishment of new methylation (DNMT3, DNMT4, DNMT5, DNMT6, DNMT7), transfer of methylation (GNMT) and demethylation (TET1, TET2, TET3) from heart and gonads of the control and dietary exposed adults fish from the parental generation and from whole 13 dpf zebrafish larvae from the offspring generation obtained from both parents control (C♀C♂), only father exposed (C♀E♂), only mother exposed (E♀C♂), and both parents exposed (E♀E♂) to HEWAF. Values are expressed as mean ± s.e.m. Different superscript letters indicate significant differences among groups (P<0.05).

	DNMT1	DNMT3	DNMT4	DNMT5	DNMT6	DNMT7	GNMT	TET1	TET2	TET3
Heart tissue										
Control F	0.78 ± 0.04	0.79 ± 0.05	0.82 ± 0.04	0.84 ± 0.02^{b}	1.05 ± 0.15	0.75 ± 0.06	0.82 ± 0.06^{a}	0.92 ± 0.05	0.99 ± 0.07	1.09 ± 0.05^{b}
Control M	0.71 ± 0.08	0.70 ± 0.08	0.83 ± 0.04	0.65 ± 0.04^{a}	1.08 ± 0.08	0.69 ± 0.07	0.89 ± 0.06^{a}	0.94 ± 0.08	0.99 ± 0.05	1.17 ± 0.03^{b}
Exposed F	0.94 ± 0.11	0.90 ± 0.09	0.92 ± 0.06	0.89 ± 0.05^{b}	1.08 ± 0.07	0.88 ± 0.12	1.06 ± 0.05^{b}	0.99 ± 0.11	0.93 ± 0.09	0.94 ± 0.09^{a}
Exposed M	0.80 ± 0.04	0.80 ± 0.06	0.85 ± 0.03	073 ± 0.06^{a}	1.13 ± 0.09	0.79 ± 0.04	0.95 ± 0.04^{b}	0.94 ± 0.05	0.98 ± 0.09	0.99 ± 0.09^{a}
Gonadal tissue										
Control F	0.93 ± 0.03^{a}	1.13 ± 0.05^{b}	0.88 ± 0.04	1.05 ± 0.11	0.87 ± 0.05^{a}	1.08 ± 0.04	0.89 ± 0.05	0.72 ± 0.04^{a}	0.75 ± 0.05^{a}	0.81 ± 0.04^{a}
Control M	0.90 ± 0.01^{a}	0.98 ± 0.03^{a}	1.02 ± 0.05	0.79 ± 0.09	1.49 ± 0.17^{b}	1.03 ± 0.05	1.08 ± 0.08	1.01 ± 0.07^{b}	1.04 ± 0.05^{b}	1.02 ± 0.03^{b}
Exposed F	1.14 ± 0.05^{b}	1.00 ± 0.04^{b}	0.99 ± 0.03	0.82 ± 0.05	0.91 ± 0.07^{a}	1.02 ± 0.02	1.06 ± 0.04	0.65 ± 0.05^{a}	0.71 ± 0.04^{a}	0.84 ± 0.05^{a}
Exposed M	0.89 ± 0.04^{a}	0.93 ± 0.03^{a}	0.95 ± 0.02	0.88 ± 0.08	1.26 ± 0.10^{b}	0.99 ± 0.01	0.93 ± 0.03	0.94 ± 0.05^{b}	0.96 ± 0.01^{b}	0.97 ± 0.05^{b}
Larvae										
C♀C♂	0.93 ± 0.05	1.00 ± 0.05	0.84 ± 0.02	1.18 ± 0.14	0.85 ± 0.04	0.95 ± 0.06	0.85 ± 0.04	0.98 ± 0.09	1.02 ± 0.05	1.05 ± 0.03
C♀E♂	0.94 ± 0.04	1.01 ± 0.04	0.84 ± 0.03	1.17 ± 0.09	0.93 ± 0.03	1.03 ± 0.09	1.03 ± 0.03	1.06 ± 0.07	1.03 ± 0.02	1.10 ± 0.04
E♀C♂	0.88 ± 0.03	0.98 ± 0.04	0.81 ± 0.02	1.23 ± 0.08	0.82 ± 0.02	0.94 ± 0.03	0.94 ± 0.03	1.00 ± 0.06	1.08 ± 0.08	1.19 ± 0.06
E♀E♂	0.85 ± 0.06	0.87 ± 0.06	0.79 ± 0.07	0.99 ± 0.05	0.84 ± 0.05	0.87 ± 0.07	0.93 ± 0.07	0.89 ± 0.08	0.98 ± 0.06	1.11 ± 0.05

3.4.2 F_1 Generation

3.4.2.1 Survival under Exposure Conditions in F_1

Survival rates of all four of the F_1 larvae populations were significantly but

differently influenced by the parental exposure experience. All larval populations also exhibited differences in both experimental conditions (Fig. 3.6). During exposure to clean water, the survival of the larvae obtained from the C♀C♂ (control) parental condition decreased from 100% to 94% from the first to the second day of exposure and maintained this value throughout the experimental period (Fig.3.6A). The survival rates from the paternal-exposed (C♀E♂) and both parents exposed (E♀E♂) offspring groups were different from the control group (C♀C♂) ($P<0.05$), varying from 100% to 92% and 100% to 84% at the beginning and end of the exposure, respectively. In contrast, the maternally-exposed (E♀C♂) offspring group did not significantly differ ($P<0.05$) from the control (C♀C♂) group and maintained a survival rate of 94% throughout the experimental time.

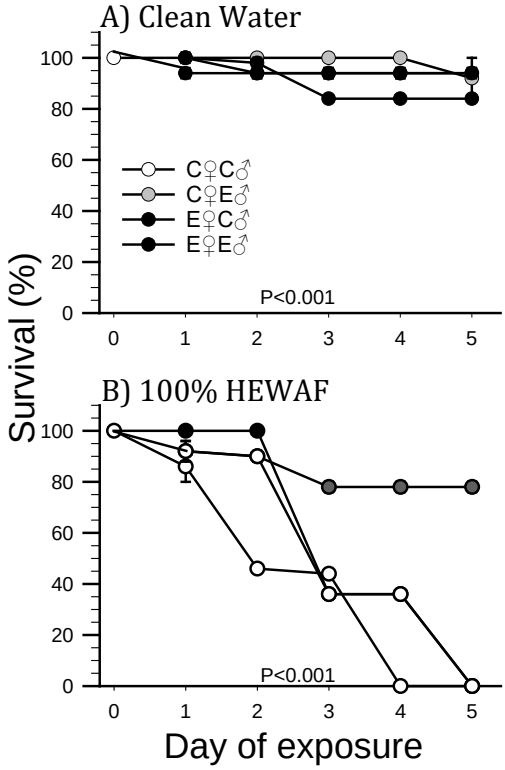

Figure 3.6: Larval survival under exposure to clean water (A) and 100%HEWAF (B) during 5dpf. White circles, Clear-gray circles, Dark-gray circles and Black circles demark offspring obtained from control (non-exposed parents – C♀C♂), paternally-exposed parents (C♀E♂), maternally-exposed parents (E♀C♂), and both-parents exposed (E♀E♂) groups. For survival graphs n = 2 replicates with 25 individuals per replicate.

Exposure to crude oil via the ambient water induced a remarkable and highly significant decrease in survivorship from 100% to 44% from the first to the third day of exposure and reaching 0% at the fourth day in the control offspring group (Fig.3.6B.). In general, the offspring obtained from the parental crosses were the maternally (E♀C♂), paternally (C♀E♂), or with both parents exposed (E♀E♂) lines exhibited enhanced survival rates in comparison with the C♀C♂ derived offspring (Fig.3.6B).

3.4.2.2 Heart Rate under Exposure Conditions in F_1

Larval heart rate exhibited differential responses depending on exposure conditions (Fig.3.7). Under exposure to clean water larval heart rate exhibited a complex pattern (Fig.3.7A). Offspring obtained from the maternally-exposed group (E♀C♂) exhibited bradycardia as soon as 1 dpf compared with the other three groups. However, this difference was lost at the second day, in which the paternally-exposed offspring exhibited higher heart rates compared with the both-parents exposed (E♀E♂) group. At the third day of exposure the E♀E♂- offspring exhibited bradycardia compared with the other three groups, which was maintained through the fourth and fifth days. However, larval heart rate was not significantly different from the C♀C♂ and E♀C♂ derived offspring at the fourth day. Finally, at the fifth day, the F_1 offspring obtained from the control parental group exhibited increased heart rate in comparison with all the offspring obtained from all the treated parental groups. Exposure to 100% HEWAF induced bradycardia in all offspring groups compared with offspring raised in clean water throughout the exposure period (Fig.3.7A). No significant differences were found among groups within days of exposure. However, the four groups exhibited higher heart rates at 2 days post fertilization ($P<0.05$) compared with the other days.

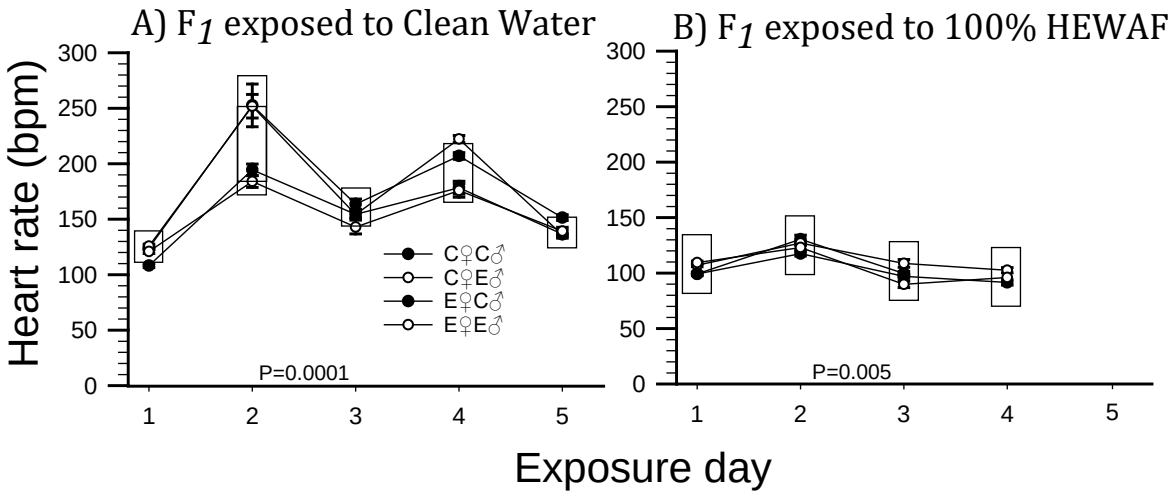

Figure 3.7: Heart rate frequencies (bpm) in the offspring F_1 generation. A) Offspring heart rate under exposure to clean water and (B) Offspring heart rate under exposure to 100%HEWAF. White circles, clear-gray circles, dark-gray circles and black circles demark offspring obtained from control parents – C♀C♂; paternally-exposed parents (C♀E♂), maternally-exposed parents (E♀C♂), and both-parents exposed (E♀E♂) groups. n = 17-50 individuals per data point. In A) data points within boxes are not statistically different.

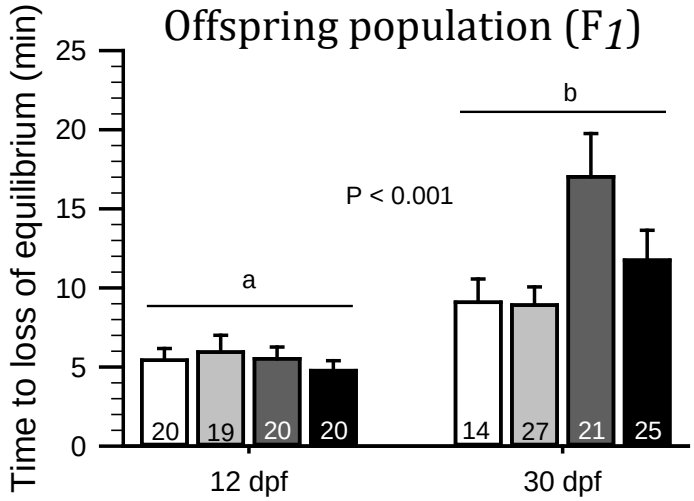

Figure 3.8: Time to loss of equilibrium under hypoxia challenge in the offspring (F_1) generation. Offspring obtained from non-exposed parents (C♀C♂ – white bars), paternally-exposed (C♀E♂ – light gray bars), maternally-exposed (E♀C♂ – dark grey bars), and both parents exposed (E♀E♂ – black bars) to HEWAF. Values are expressed as mean ± s.e. mean. Different letters indicate significant difference (P<0.05). n is indicated within bars.

3.4.2.3 Loss of Equilibrium in Hypoxia in F_1

Developmental time induced a significant effect on time to loss of equilibrium ($F_{1,113} = 58.62$, $P<0.001$, Fig. 3.8), with larvae at 30 dpf being more resistant to oil

exposure than the 12 dpf larvae (16.2 ± 1.6 and 5.4 ± 0.4 minutes, respectively). No significant difference was observed among parental exposure combination ($F_{3,113}$ = 1.81, P = 0.15).

3.4.2.4 Immunohistochemistry in F_1

The pattern of expression levels of the antibodies in larval hearts was complex (Fig. 3.9). The pixel density levels of H3k4me3 were significantly (P>0.05) higher in the offspring obtained from the groups where just one or both of the parents were exposed to crude oil. Nonetheless, the levels of expression among offspring obtained from the mother or the father exposed groups were not significantly different (P>0.05) (Fig. 3.9A). Regarding the expression levels of H3K27me3, the E♀E♂ offspring group exhibited lower levels in comparison to the control and the C♀E♂ groups (Fig. 3.9B).

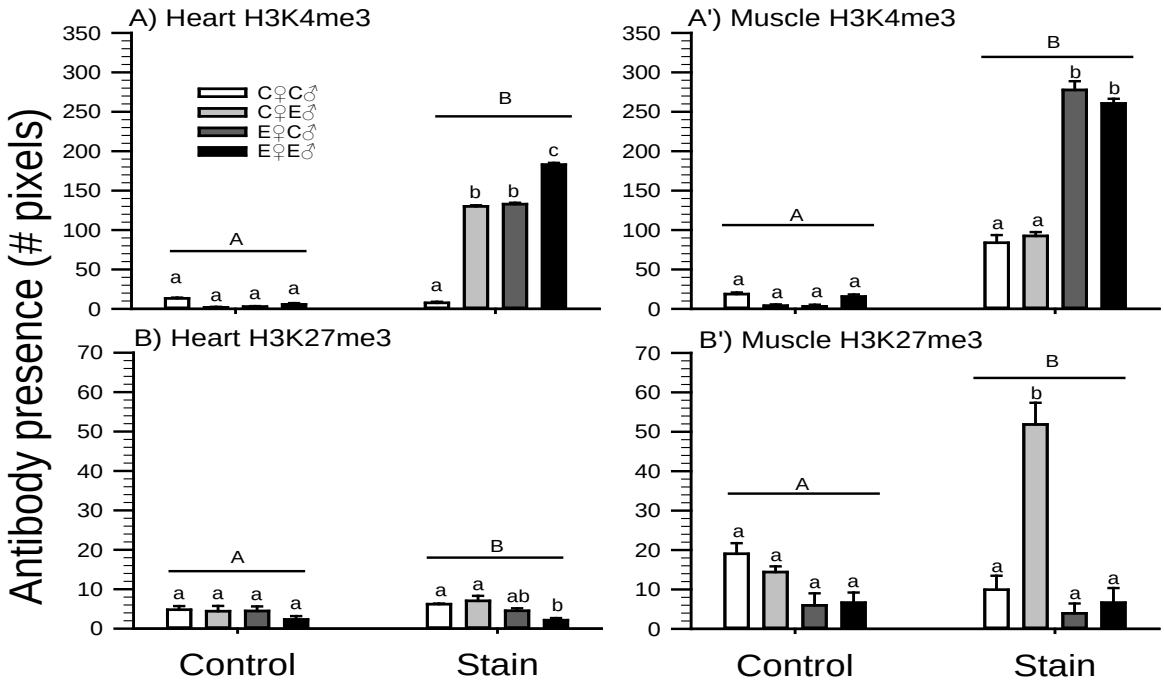

Figure 3.9: Antibody expression (pixel density) in the offspring F_1 population. In the heart: A) H3K4me3 and B) H3K27me3; In the gonads: A') H3K4me3 and B') H3K27me3. Statistical significance was considered with a P value lower than 0.05. Each bar represents the average of three animals.

In the muscle tissue, H3K4me3 expression levels were higher in E♀C♂ and E♀E♂ larvae groups in comparison with control or the C♀E♂ offspring groups (Fig. 3.9.A). H3K27me3 density levels were significantly (P<0.001) higher in the C♀E♂ group in comparison with the rest of the offspring. (Fig. 3.9B').

3.4.2.5 Global DNA Methylation in F_1

The interaction between maternal and paternal crude oil exposure had a significant effect on the global DNA methylation level of the 13 dpf larvae ($F_{1,29} = 6.20$, $P = 0.02$). Remarkably, the larvae obtained from either the maternally-, paternally- or both parents-exposed groups, exhibited lower percentage of global DNA methylation than the larvae obtained from the control parents (Fig. 3.10).

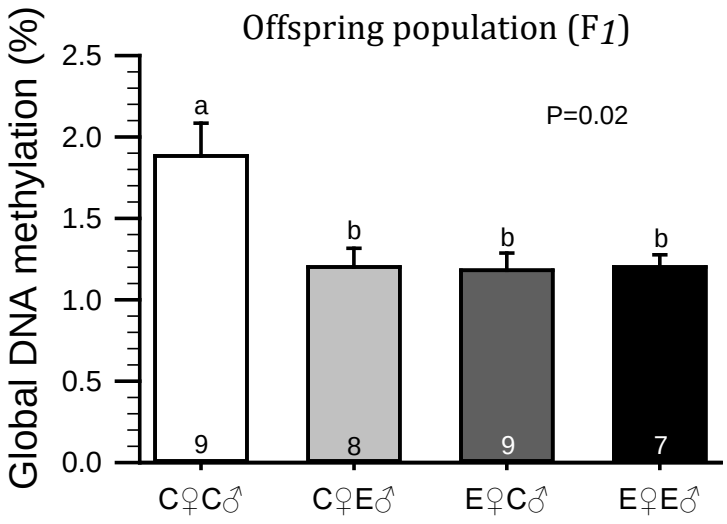

Figure 3.10: Global DNA methylation % in the whole 13 dpf larvae. Offspring generation obtained from non-exposed parents (C♀C♂), paternally-exposed (C♀E♂), maternally-exposed (E♀C♂), and both parents exposed (E♀E♂) to HEWAF. Values are expressed as mean ± s.e.m. Different letters indicate significant difference (P<0.05). n is indicated within bars.

3.4.2.6 PCR and Epigenes in F_1

The interaction between the maternal and paternal exposure significant affected

expression levels of GATA4 (involved in cardiac development) and NPPA (involved in cardiac stress) in the F_1 offspring (F1,20 = 9.51, P = 0.006; F1,20 = 10.65, P = 0.004; respectively). The transcript levels of the 13 dpf larvae obtained from the paternally-exposed (C♀E♂) group were significantly up-regulated compared to the transcript levels of the larvae obtained from either, the control parent (C♀C♂) or the group in which both parents were exposed (E♀E♂') group (Table 3.3). Additionally, exposure in the maternal line affected transcript levels of NKX2.5 (involved in cardiac development) (F1,20 = 4.67, P = 0.04). Thus, F_1 larvae obtained from the maternally-exposed group decreased in the abundance of their transcript compared to larvae obtained from control non- exposed females. The transcripts levels of all the other genes (MyH7, F1,20 = 3.26, P = 0.09; MyL7, F1,19 = 0.91, P = 0.35; TNNT2a, F1,14 = 0.65, P = 0.43; ATP2a2a, F1,20= 0.98, P = 0.34), including the one involved in the response to PAHs (CYP1a, F1,19 = 0.51, P = 0.48), were not significantly influenced by the parental exposure background. Finally, the parental exposure did not significantly influence the expression level of any of the genes involved in the methylation modification in the offspring generation (DNMT1, F1,19 = 0.16, P = 0.69; DNMT3, F1,20 = 1.31, P = 0.27; DNMT4, F1,19 = 0.06, P = 0.82; DNMT5, F1,20 = 1.46, P = 0.24; DNMT6, F1,20 = 0.58, P = 0.44; DNMT7, F1,20 = 1.20, P = 0.29; GNMT, F1,19 = 3.49, P = 0.08; TET1, F1,20 = 1.58, P = 0.22; TET2, F1,20 = 0.94, P = 0.34; TET3, F1,20 = 1.83, P = 0.19) (Table 3.4).

3.5 Discussion

3.5.1 General View

Population success and maintenance rely on the quality of the subsequent generations in their capacity to cope with persistent stressors, using both genetic and

non-genetic mechanisms (Siddique et al., 2017). Environmental stressors, such as anthropogenic chemicals, may induce broad ecological consequences by reducing performance and survivability of the exposed populations (Burggren and Dubansky, 2018). The effects that crude oil and other pollutants have on fish may have effects from the whole organismal to the molecular levels (Pasparakis et al., 2019). In some scenarios, those effects may be transgenerationally inherited by their subsequent generations (Bhandari et al., 2015; Nilsson et al., 2018).

Although transgenerational inheritance occurs from both parental lines, demonstration of the influence of the P_0 generation in F_1 offspring fitness and survival has been dominated by experimental designs testing only one parental line at a time (Lombó et al., 2015; Nye et al., 2007). In this study we exposed female and male fish to crude oil extracts via diet, within environmentally relevant concentrations (Vignet et al., 2014), and obtained F_1 larvae crosses that allowed testing of the separate contribution of the maternal and paternal inheritance lines on their offspring.

3.5.2 Crude Oil Effects on the P_0 Males and Females

Studies aimed at understanding the effects of crude oil exposure on adult fish are scarce and have been surpassed by those focusing on early-life stages (Pasparakis et al., 2019). However, some studies on juvenile and young adult mahi mahi (*Coryphanea hippurus)* and red drum (*Sciaenops ocellatus*) indicated that at the whole organismal level, exposure to crude oil via water decreases swimming performance and metabolic rate (Johansen and Esbaugh, 2017; Mager et al., 2014b; Stieglitz et al., 2016). Additionally, at the tissue level, cardiac function is compromised as are action potentials and ion fluxes at cellular levels (Brette et al., 2017; Nelson et al., 2016).

In this chapter, dietary exposure to a sublethal concentration of crude oil for three weeks did not compromise survivorship or adult growth. These results suggest that the adult fish were in a healthy state during experimentation. This notion is further supported by the lack of difference in heart rate capacities (~240 bpm), as well as no differences in time to loss of equilibrium under hypoxia challenge (Fig, 3.3), or tissue-specific somatic indexes among groups (Table 3.2). These results are consistent with the results of Chapter 2 in which fish dietarily exposed to the same concentrations of crude oil experienced, decreased fecundity of both female and male fish (decrease in the number of eggs spawned and reduction in sperm count, respectively) (Bautista and Burggren, 2019).

In general, the antibody presence levels (measured as pixel density) in the heart tissue were variable among the groups, however, the responses were tissue-specific for each antibody (Fig. 3.4). H3K4me3, associated with promoters of activated genes (Toni and Padilla, 2016), was less expressed in both groups of exposed adults, suggesting a state of downregulation of genes due to the crude oil exposure. Accordingly, indirect measurement of the expressionof H3K27me3, an epigenetic marker for inactive genes, indicates higher levels of expression in the exposed fish adults in comparison with non-exposed adults (Fig. 3.4B).

The levels of expression of H3k4m23 and H3k27me3 in the gonadal tissue of the female adults did not differ among control and treated individuals (Fig. 3.4A-B), nonetheless, both antibodies exhibited lower levels in comparison with exposed male fish. These results suggest that male gonads may be more plastic than female gonads in remodeling DNA methylation levels as a response to environmental stressors. This notion

deserves appropriate future investigation.

At the molecular level, global DNA methylation in heart tissue was reduced in the oil-treated adults (Fig. 3.5), suggesting higher levels of gene expression in the tissue. Although DNA methylation patterns are established early during embryogenesis (Fang et al., 2013), these results suggest highly plastic responses of this epigenetic modification to environmental exposures also at later life stages. Such epigenetic response may then be adaptive and can allow individuals to rapidly cope with environmental stressors (Burggren, 2016; Heckwolf et al., 2019). Additionally, the global DNA methylation decrease and the up-regulation of GNMT1, involved in the transfer of methyl groups from S-adenyl methionine (SAM) to glycine S-adenosylhomocysteine (Table 3.4), align with a previous report in zebrafish in which exposure to 2.4 µg/L of the model PAH Benzo(a)pyrene, increased GNMT activity, in turn decreasing SAM supply to the embryo leading to demethylation (Fang et al., 2013). In contrast to the cardiac tissue, the global DNA methylation level of the gonads was not affected by exposure to oil. This suggests that DNA methylation patterns as a response to environmental stressors may be tissue specific. However, changes in DNA methylation at specific sites (i.e. promoters) may still not happen (Falisse et al., 2018; Fang et al., 2013). In addition, the transcription level of the gene TET3, involved in demethylation and previously reported to affect DNA methylation levels in fish (Xiong et al., 2018), was downregulated (Table. 3.4), suggesting a role of this protein in modulating methylation levels in gonadal tissue as a response to oil exposure.

3.5.3 Parental Experience and Transgenerational Inheritance on F_1 Offspring

Early developmental stages in fish are highly sensitive to environmental stressors

and their survivability depends upon their own capacities to cope with the environment as on their inherited phenotypes from their parents (Auge et al., 2017; Burggren and Dubansky, 2018; Mager et al., 2014b). In fact, the role of maternal and paternal effects on F_1 offspring fish populations to cope with stressors has been reported elsewhere (Green, 2008; Lombó et al., 2015; Nye et al., 2007; Ownby et al., 2009). Although the effects of parental exposure experiences are mostly related to detrimental outcomes for the offspring populations (Corrales et al., 2014), depending upon the conditions, the inheritance of characters may also be adaptive by enhancing the resistance against stressors or even increasing the niche width of the offspring (Bautista and Burggren, 2019; Herrera et al., 2012).

In this study, survival rates of the in F_1 offspring depended upon which parental line was exposed. For example, while the E♀C♂ offspring did not exhibit survival rate differences compared with the C♀C♂ (control) group the C♀E♂ and E♀E♂ offspring had lower survival rates when raised in clean water conditions. These results are similar to previous studies where killifish embryos obtained from parental groups where both of the parents have been exposed to creosote-contaminated environment exhibited lower survival when raised in clean non-polluted water (Meyer and Di Giulio, 2003). Interestingly, in the present study larvae with lower survivability in clean water were obtained from both of the groups where the paternal line was exposed to crude oil, suggesting a strong role of the male fish in affecting offspring survival. Nonetheless, when the in F_1 larval groups were exposed to crude oil via their ambient water, the three groups obtained from exposed parents exhibited enhanced survivability compared to the control groups (Fig.3.6). These results align with studies in killifish and zebrafish were offspring

from exposed parents exhibited enhanced resistance to the stressor to which the parents were exposed (Bautista and Burggren, 2019; Clark et al., 2014). The present finding highlights that *both* parental lines are involved in the regulation of survival capacities of their offspring. However, the male adult fish may be more involved in the survivability of their offspring during early embryogenesis. This speculation warrants further investigation.

At the physiological level, parental exposure to crude oil did not have any effect on offspring performance when the F_1 larvae were challenged to loss of equilibrium under hypoxia (Fig. 3.8). However, when the larvae were raised in clean water conditions, heart rate of the offspring obtained from the parental exposed groups (C♀E♂, E♀C♂ and E♀E♂) exhibited bradycardia in comparison with the control group (C♀C♂ - Fig. 3.7). However, major differences were seen after the hatching period (2 dpf), and align with the change from intrinsic to extrinsic control of cardiac function in this species (Lema et al., 2007; Schwerte et al., 2006). In addition, regardless of the parental exposure experience, exposure to crude oil via their ambient water induced bradycardia in all experimental groups. Nonetheless, it still a topic for discussion of whether the decrease in heart rate in larvae fish is adaptive or maladaptive under stressful conditions (Farrell, 2007; Perry and Desforges, 2006).

In addition to organismal-physiological assessment, at the cellular-molecular scale, the present study analyzed two epigenetic mechanisms in larvae to determine if parental exposure to crude oil could influence offspring phenotypes. Firstly, we assessed chromatin changes by analyzing histone H3 associated with gene activation (H3K4) or silencing (H3K27) (Toni and Padilla, 2016). The fact that the level of expression of

H3K4me3 in the heart of the C♀E♂, E♀C♂ and E♀E♂ larvae were higher in comparison to the control larvae (Fig. 3.9A) suggests that, regardless of the parental line that was exposed to crude oil, exposure in a parental population led to the potential activation of genes in their offspring even when they were raised in clean water conditions. Remarkably, when both parents were exposed, the larval F_1 expression was still higher, suggesting that there was actually an additive effect. This is further supported by the fact that H3K27me3 did not differ among groups (with exception in the E♀E♂ group where the level was lower). Accordingly, the responses in the muscular tissue were somewhat similar (Fig. 3.9B'). With the exception of the C♀E♂ group, where H3K27me3 exhibited higher levels of expression in comparison with the rest of the groups, an increase in H3k4me3 levels was seen in parent-exposed derived offspring groups. Overall these results suggest gene expression in the offspring groups was tissue specific and potentially related to the specific gene function.

The second epigenetic mechanism analyzed was global DNA methylation, which was assessed in the F_1 larvae. DNA methylation, the most studied epigenetic mechanism, is also associated with modifications in gene expression, and can be inherited by subsequent generations when parental populations have been exposed to environmental stressors (Cavalieri and Spinelli, 2017; Olsvik et al., 2014). In fish, the maternal DNA methylation levels are reset during early embryogenesis, but the paternal inherited methylome is maintained constant (Ci and Liu, 2015; Jiang et al., 2013). This potentially lead to a differential effect on offspring performance, but the functional implications to evolution are currently unknown.

In the present study we found that the global DNA methylation levels in the F_1

larval groups derived from one or both crude oil-exposed parents were decreased in comparison with the control larvae (Fig. 3.4B). Interestingly, high levels of methylation (presence of 5-methylcytosine-5Mc) represses DNA transcription (Nilsson et al., 2018). Furthermore, the finding that the parental exposure leads to activation of genes in their offspring is confirmed by the decrease in larval global methylation levels.

Previous studies have suggested that the use of pooled larvae may complicate global DNA methylation responses due to the heterogeneity of tissues (Cavalieri and Spinelli, 2017; Falisse et al., 2018). The findings of this chapter suggest that this may be specific to the experimental design, exposure conditions and questions of interest. Although more specific studies of methylation patterns at specific sites (such as genes promoter sites) are needed to elucidate the specific role of DNA methylation on gene regulation, the determination of global DNA methylation levels may enable general deciphering of transgenerational epigenetic effects.

The differential upregulation of GATA4 and NPPA (genes involved in cardiac development) and the downregulation of NKX2.5 (involved in cardiac stress) induced by paternal and maternal crude oil exposure, respectively, in the parent-exposed derived offspring, suggest that the parental exposure experience can indeed be translated into an alteration of the regulation of the cardiac function in the offspring. Notwithstanding, none of the transcript levels of the genes involved in modifications of DNA methylation were affected by the parental exposure experience (Fig. 3.10) in any of the F_1 larval groups. The previous findings, and the fact that CYP1A was not upregulated, suggest that the change in DNA methylation in the offspring population is an inherited character derived from the parental exposure to crude oil.

3.6 Conclusion and Future Studies

Interest in understanding the role of the maternal and paternal influence in offspring performance has burgeoned during the past two decades. Although, it has been speculated that inheritance of phenotypic traits can be attributed to epigenetic mechanisms, empirical evidence addressing this phenomenon is scarce, partially due to the impracticality of experimentation (Akhter et al., 2018) . In addition, studies aimed to disentangle the separate role of both; the maternal and paternal lines of inheritance on offspring survival and performance are also lacking. This study we tested weather epigenetic inheritance in offspring may be derived from both parental lines. The results demonstrate that crude oil exposure is an epigenetic modifier in which both parental lines are differentially involved in offspring inheritance and response. Finally, more studies should focus on understanding the particular role of methylation patterns at the CpG islands of promoters or in the gene body *per se* which may cause stable transcriptional gene silencing or enhance expression, respectively (Cavalieri and Spinelli, 2017). However, epigenetic factors may be as important to sense and respond to the variable interaction of environmental conditions. However, studies considering more than one concurrent stressor should be performed.

3.7 Appendix: Supplemental Table A3-1

Heart rate frequency (BPM min^{-1}) of the control and dietary exposed fish from the parental generation before any injection (Acclimation - 25 min), 5, 10 and 15 after the injection of atropine (Atropine 35, 40 and 455 min respectively), 5, 10 and 15 after the injection of isoproterenol (Isoproterenol - 55, 60 and 65, min respectively), and 5, 10 and 15 after the injection of propanolol (Propanolol - 75, 80 and 85 min respectively). Values are expressed as mean ± s.e.m. n= 20 / group. Different letters indicate significant differences among injection periods (P<0.05).

Groups	25 min(ab)	35 min (bcd)	40 min (abcd)	45 min (abcd)	55 min (bcd)	60 min (d)	65 min (cd)	75 min (abc)	80 min (ab)	85 min (a)
Control Female	209.4 ± 8.8	240.3 ± 10.9	236 ± 10.2	236.1 ± 10.6	239.9 ± 7.3	247.8 ± 11.1	236.5 ± 7.2	207.5 ± 8.2	207.7 ± 9.7	211.4 ± 8.2
Control Male	223.7 ± 8.9	233.1 ± 11.3	228.9 ± 11.3	229.0 ± 12.9	228.5 ± 13.9	244.1 ± 12.6	241.6 ± 10.7	225.0 ± 8.9	216 ± 7	210.4 ± 11.8
Exposed Female	200 ± 8.2	221 ± 9.6	220.1 ± 11.2	228.3 ± 11.8	231.8 ± 11.2	230.4 ± 9.9	230.4 ± 9.1	210.7 ± 10.8	211.4 ± 7.3	211.6 ± 7.4
Exposed Male	222 ± 8.9	233.1 ± 8.4	231.3 ± 8.3	227 ± 10	243.6 ± 9.6	243.4 ± 7.9	239.5 ± 9.1	224.9 ± 8.7	221.23 ± 7.6	214.1 ± 8.6

CHAPTER 4

BEHAVIORAL CONSEQUENCES OF DIETARY EXPOSURE TO CRUDE OIL EXTRACTS IN THE SIAMESE FIGHTING FISH (*Betta splendens*)*

4.1 Abstract

Uptake by fishes of crude oil and its polycyclic aromatic hydrocarbons (PAHs) components occurs via gills, dietary intake, or diffusion through the skin. Dietary exposure to crude oil and its components is environmentally relevant and induces physiological and morphological disruptions in fish. However, the impacts of crude oil on fish social and reproductive behaviors and thus the possible influences on reproductive success are poorly understood. As a part of their intraspecific interactions, male Siamese fighting fish (*Betta splendens*) exhibit highly stereotypic behavioral and territorial displays. This make this species a tractable model for testing crude oil effects on behavior. After 2 weeks of acclimation at 29°C, male adult betta fish were divided into three groups and fed for 4 weeks with food spiked with water (control), low oil concentrations or high oil concentrations (∑Total PAH concentrations 340, 3960 or 8820 ng/g dw, respectively) to determine subsequent alterations in behavioral displays. Compared with control fish, the aggressive display of “opercular flaring” was significantly increased ($P<0.03$, n= 14-16) in oil-exposed fish. Bubble nest building, as well as testes and brain mass, were significantly reduced in treated fish ($P<0.05$). Hematocrit of treated groups was increased significantly ($P < 0.02$) from 21% in control fish to ~27% in both oil exposure groups. Dietary exposure over a 4-week period to low, relevant levels of crude oil thus leads to an increase in

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aggressive behavioral displays, a decrease in reproductive activity and additional morphological changes.

4.2 Introduction

Reproductive success and fitness in fishes are highly dependent upon successful resource acquisition. In many species this is associated with aggressive behavioral displays for acquiring mates and fending off competitors, which collectively lead to increased fitness (Chen et al., 2016b; Tubert et al., 2012). For example, in the sunbleak (*Leucaspius delineatus*), aggressive males gained territory and were preferred by females for mating in comparison with shier male counterparts (Gozlan et al., 2003). In the zebrafish (*Danio rerio*) more aggressive male individuals attained up to 33% higher rates of fertilization in comparison with shier and less bold fish (Ariyomo and Watt, 2012). This enhanced aggressive behavior apparently has heritability value and is subject to evolution under selective pressures (Ariyomo and Watt, 2012). However, high levels of aggression in fishes may also involve disadvantages because individuals can get engaged in potentially fatal fights, experience elevated metabolic costs, and consume time that individuals might otherwise be investing in foraging or mating(Alton et al., 2013; Arnott and Elwood, 2009; Forsatkar et al., 2016; Lane and Briffa, 2017; Regan et al., 2015). These disadvantages are particularly important in fish species where paternal care is the prevailing reproductive strategy, since females often choose their mates based upon behavioral performance during contests and courtship (Clotfelter et al., 2006; Dzieweczynski and Kane, 2017; Forsatkar et al., 2016).

The Siamese fighting fish (*Betta splendens*) relies heavily on stereotypical aggressive behaviors for territory defense and reproductive purposes. To acquire territory

and mating opportunities, male betta fish compete fiercely with each other by employing well documented behavioral displays that include gill flaring, darting, finning and lateral displays during swimming (Brown and Clotfelter, 2012; Gozlan et al., 2003). Once males have successfully defended a territory, they must build a bubble nest and then defend it from egg and larva predation for a few days following egg laying by the female (HedayatiRad et al., 2017). Nest building behavior in betta fish is thus a key component needed for successful reproduction (Jaroensutasinee and Jaroensutasinee, 2003)..

Any anthropogenic material that disrupts these complex territorial and reproductive behaviors in betta fishes will potentially reduce their fitness. Crude oil, with its complex chemical composition, is an example of such a material. Crude oil and especially the polycyclic aromatic hydrocarbons it contains has been increasingly recognized as a concern for humans and animal populations (Abdel-Shafy and Mansour, 2016; CDC, 2013). Crude oil components enter aquatic environments during accidental spills, governmentally-authorized disposal or other primarily anthropogenic sources. Crude oil is present in complex mixtures that can be fresh or “weathered”, factors that affect their persistence and bioavailability (Booth, 2004; Burgess et al., 2003; Peterson et al., 2003).

Compounds in crude oil may impose a challenge on individuals and populations to survive and reproduce. Fishes, in particular, may be exposed to oil via gills, skin contact, and diet (Tierney et al., 2013), potentially negatively affecting much of the aquatic or marine food web (Hylland, 2006; Peterson et al., 2003). Exposure to crude oil as a mixture, or to individual or groups of components (e.g. PAHs) in fish has been related to increased mortality and morphological and physiological disruptions in all developmental stages. Consequently, the study of oil exposure to fish has received considerable

attention in the past two decades (Burggren and Dubansky, 2018; Cherr et al., 2017; Cox et al., 2017; Hedgpeth and Griffitt, 2016; Perrichon et al., 2016; Perrichon et al., 2017b).

Perhaps relating to the relative difficulty of behavioral measurements, studies aimed to understand oil's impacts on behavioral traits in fishes are still scarce. As one example, habitat choice of two species of marine damselfish (*Pomacentrus amboinensis* and *P. moluccensis)* was severely affected after acute exposure to oil, resulting in a nearly 3-fold increased mortality due to predation as a consequence of adopting riskier behavior (Johansen et al., 2017). Those results suggest that exposure to oil affects cognitive processes in fish and, hence, it is possible that other behavioral traits directly related with fitness and reproductive success (i.e. aggressive displays and nest building) could also be affected.

In the present study we used the Siamese fighting fish (*Betta splendens*) to assess the impact of dietary exposure to crude oil on behavioral parameters related with reproductive success in this species. *B. splendens* has been widely used in behavioral and medical research on sedatives and other compounds due to its well-known stereotypical aggressive displays (Clotfelter et al., 2006; Dzieweczynski and Kane, 2017; Jaroensutasinee and Jaroensutasinee, 2003; Lane and Briffa, 2017; Monvises et al., 2009). This makes the Siamese fighting fish a suitable fish model to address behavioral changes, and potential underlying morphological changes, induced by dietary exposure to crude oil. We hypothesize that chronic dietary exposure to low levels of crude oil in male betta fighting fish could lead to alterations in levels of aggressiveness and bubble nest building behavior.

4.2.1 Goals

Studies aimed to establish if dietary exposure to crude oil, within environmentally relevant concentrations, elicits changes in behavioral traits associated with fish fitness and reproductive success of exposed animals are scarce. Thus, the goals of this chapter were to:

(i) Establish if dietary exposure to crude oil elicits changes in primary indicators of fish health in the Siamese fighting fish.

(ii) Establish if dietary exposure to crude oil elicits changes in the stereotypic aggressive behaviors of the male Siamese fighting fish.

(iii) Establish if dietary exposure to crude oil elicits changes in behaviors associated with reproductive success in the Siamese fighting fish.

4.2.2 Hypotheses

The current study uses the Siamese fighting fish to test the following hypotheses:

1) Dietary exposure to crude oil will not impact primary indicators of fish health in the betta fish.

2) Dietary exposure to crude oil will increase the aggression levels in the betta fish.

3) Dietary exposure to crude oil will affect the performance of behaviors related with reproductive success in the betta fish.

4.3 Materials and Methods

4.3.1 Fish Care and Maintenance

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC-Protocol #16002) at the University of North Texas. Male Siamese fighting fish (*Betta splendens*) were purchased from a local supplier. Holding conditions were~29°C, pH ~7.6, 14:10h light:dark cycle, and a dissolved oxygen concentration of ~7.2 mg O_2/l, conditions representing their optimal environment (Carey et al., 2015; Curtis

and Maclean, 2014). Since male betta fish are highly territorial and tend to attack, if not kill, intruders (Monvises et al., 2009), we maintained them individually in 1L tanks. This extremely aggressive male behavior extends even towards females if they are kept into the male tank prior to courtship or after spawning and fertilization, due to paternal care until some days after hatching (Carey et al., 2015; Tate et al., 2017). Thus, to avoid stress due to constant behavioral responses to with fish visible in neighboring tanks, three sides of each holding tank were covered in black plastic sheeting to prevent its occupant from seeing any other fish. In addition, the tanks were pitched at a 25^{o} angle with respect to the incubator shelf to prevent eye contact with the researcher and to aid container cleaning. Each tank was cleaned daily (aggression behavior experiment) or weekly (nest building experiment, to prevent nest disturbance) by carefully siphoning feces and changing ≃500 ml of water.

4.3.2 HEWAF and Diet Preparation

Treatments with either control diets or diets supplemented with different concentrations of crude oil were very similar in both the aggression behavior and nest building experiments. High Energy Water Accommodated Fractions (HEWAF) of oil were prepared according to standard protocols that examine HEWAF effects on organisms (Incardona et al., 2014; Mager et al., 2014a; Perrichon et al., 2016; Singer et al., 2000). Source oil “B” (SOB) sampled directly from the MC252 well on May 22-23, 2010 was used for this experiment. British Petroleum acknowledges the use of a defoamer (Nalco EC9323A), oxygen scavenger (Nalco VX9831) and methanol during the collection of this oil. Although their presence in SOB cannot be dismissed, the direct sampling from the riser insertion tube may reduce the possibility of incorporation of these compounds into

the oil (de Soysa et al., 2012).

To briefly describe the HEWAF preparation process, 2000 mg of were added into 1L of conditioned aquarium water (60mg Instant Ocean Salts/L dH_2O, used to generate a typical freshwater level of common anions and cations). This mixture was then blended for 30s in a commercial blender (WaringTM CB15). The resulting HEWAF mixture was placed into a separation funnel for 1, after which 150 ml of the solution were discarded from the bottom of the funnel. The remaining ~600 ml of solution (designated 100% HEWAF) were used for experimental purposes.

Dietary treatments were prepared by spraying commercially purchased pellet fish food (AquaExcel Cargill Inc.) with conditioned water (comprising the control), 50% HEWAF (mixture of 100%HEWAF and conditioned water in a 1:1 ratio) or 100% HEWAF. The diet preparation process started by spreading two g of commercial food out into a thin layer in a plastic weighing boat (135 mm long, 135 mm wide, 20 mm high). Using a plastic bottle atomizer, the food pellet layer was sprayed 5 times with either conditioned water (Control) or one of the two HEWAF solution concentrations indicated above. The spraying process was performed under a fume hood, and the treated food was allowed to dry for ~12 hours. The dry treated food pellets (mean pellet eight was 0.916mg ±0.03mg) were then collected from the weighing boats, and stored at 4º C in amber glass bottles (20ml) covered with aluminum foil until subsequent use.

Crude oil is a complex mixture composed of thousands of compounds, a high percentage of them are yet to be categorized). Their bioavailability depends on their particular and physicochemical characteristics (Booth, 2004). Some of these compounds fall in the category known as Unresolved Complex Mixtures (UCMs) of hydrocarbons that

conforms the majority of the hydrocarbons present in petroleum (Scarlett et al., 2007). Those compounds have shown to be toxic to sediment-dwelling organisms (Booth, 2004; Booth et al., 2008; Booth et al., 2007; Melbye et al., 2010; Scarlett et al., 2007) that often are part of the diet of fishes (Vignet et al., 2014).

Polycyclic aromatic hydrocarbons (PAHs) are a specific component of crude oil well-known to impact cardiac performance, swimming activity and performance and morphological characteristics in developing and adult fish (Brette et al., 2014; Incardona et al., 2015; Incardona and Scholz, 2016; Incardona and Scholz, 2018a; Mager and Grosell, 2011; Stieglitz et al., 2016). Thus, determination of [PAH] in the experimental diets of this study offers a valid indication of the diet's toxicity. In the present study we determine the total concentration of PAH compounds as a proxy of toxicity levels of exposure of each dietary treatment used in this experiment. Representative samples of each pellet type (control, 50% and 100% HEWAF-treated) were collected, stored in amber glass bottles at 4 ºC and shipped to ALS Environmental (Kelso, WA, USA) for analysis. PAH-analytes quantification was performed as described in EPA method 8270D using a gas chromatograph coupled with a mass spectrometer (Forth et al., 2016). The compound list of PAH analytes is provided as appendix information (Table A4-1).

4.3.3 Experimental Protocols

Two separate experiments were completed during this study. The first, performed on 45 adult male bettas, was focused on determining if dietary exposure to crude oil altered aggressive behavior. The second experiment, performed on 28 adult male bettas, examined oil effects on reproductive behavior and morphology. In both experiments the acclimation and exposure protocols were similar. The fish were allowed to acclimate for

three weeks under the conditions mentioned above.

The feeding protocol for exposure consisted of feeding each individual fish with 15 pellets (~14 mg of food) of its respective dietary treatment (control, 50% HEWAF-or 100% HEWAF treated diets) once a day at 13:00 hr. Regardless of HEWAF level, all fish consumed all the 15 pellets offered to them daily during the four weeks of exposure.

4.3.3.1 Aggressive Behavior Assessment

Sixteen fish were randomly assigned to each of the Control, 50% HEWAF and 100% HEWAF treatments at the beginning of the 3 week acclimation period, during which they received 15 pellets daily of control food. After this acclimation period, fish then received one of the three dietary treatments of 15 pellets daily, which were then provided for a four week treatment period. Mortality was during the nearly two month long course of the experiment was very low – two in the control group (final n=14), one in the 50% HEWAF group (final n=15), and zero in the 100% HEWAF group (final n=16).

Fish aggressiveness during the four week treatment period was determined daily on weekdays, at the same time for each day. A 10 cm X 16 cm mirror was fastened to the transparent side of the tank for a 3 min test period while assessing the male betta's behavioral responses to its own image. The use of mirrors to investigate aggressive behavioral displays in fish is well established in studies of fighting behavior (Ariyomo and Watt, 2012; Arnott et al., 2016b; Desjardins and Fernald, 2010; Elwood et al., 2014). The use of mirror is validated in lab conditions since fish display almost identically to mirror images as to live opponents (Arnott et al., 2016b; Balzarini et al., 2014). However, such displays may not reflect real agonistic encounters in the wild, which would involve physical contact (Elwood et al., 2014).

In the present study, four different behavioral displays were assessed: A) “flaring” - the protrusion of the operculum for aggressive-defensive purposes; B) “circling” - swimming back and forward in a circular motion along the perimeter of the tank where the reflection took place; C) “darting” - burst swimming in an agitated fashion; and D) “finning” - the fanning of pectoral fins at a visible increased rate without resulting in body movement. The four different displays during each exposure session were scored on a scale of 1-3: absent (0), meek (1), relatively aggressive (2), or highly aggressive (3). Scores for each behavior were averaged for all fish to produce an end-of-week average score for each one week time period.

4.3.3.2 Nest Building Assessment

For the second experiment, 28 male bettas were randomly assigned to one of the control group (n=8), 50% HEWAF group (n=10) or 100% HEWAF group (n=10). After three weeks of acclimation to holding conditions and a control diet, males were exposed to control food, 50% or 100% HEWAF treated for 4 weeks

Male *B. splendens* typically created surface bubble nest that clung to the perimeter of the tank’s water surface. Sometimes the nest was a discrete, oval-shaped structure, but often it comprised a series of bubbles spread along the periphery of the tank (Fig. 4.1). Occasionally, two discrete nests were created by a single fish within a single tank. To quantify the various forms of nest construction, photographs of each fish in its container, including any bubbles in small groups or in structured nests, were acquired (Nikon Coolpix AW130, 16 Mpx camera). Photographs were taken horizontally from each of the four sides of the container and one taken vertically looking down on the container surface. The container where the fish were kept had a rectangular form, so nest presence

was scored by counting the number of quadrants containing a part of or an entire bubble nest (Fig. 4.1). Thus, if a portion of the nest was present in each of the four sides of the container, quadrant presence was scored as 100%. This value was calculated for each fish's nest building activity at the end of each week, and averaged for each fish for each dietary treatment for each week. In addition, total bubble nest surface area, and maximum width and depth of the bubble nest were calculated from the photos by digital image analysis using ImageJ Software (Schneider et al., 2012).

4.3.3.2.1 Blood Collection and Hematocrit

High levels of aggression in fish are related to elevated energetic demands to which they adjust by physiological modification (Harter and Brauner, 2017; Regan et al., 2015). For example, hematocrit (Hct), % packed red cell volume in blood, has been used as proxy for estimation of oxygen carrying capacity and as an indicator of organismal health in fish (Cyriac et al., 1989; Ghaffar et al., 2018; Houston, 2011). In the present study hematocrit was determined at the end of four weeks of either crude oil exposure following the nest building experiment. Siamese fighting fish, were deeply anesthetized with MS222 solution (~300mg Methanesulfonate/L) buffered to pH 7.0. Immediately after anesthesia, evident by cessation of gill ventilation and other body movements, the heart was surgically exposed and blood samples for hematocrit determination were acquired directly from the ventricle using heparinzed capillary glass tubes (World Precision Instruments Inc. #1B100-3). The blood-filled capillary tubes were then centrifuged for 4 min at 8000 rpm (Clay Adams Readacrit Microhematocrit Centrifuge 426591). After centrifugation, hematocrit was determined.

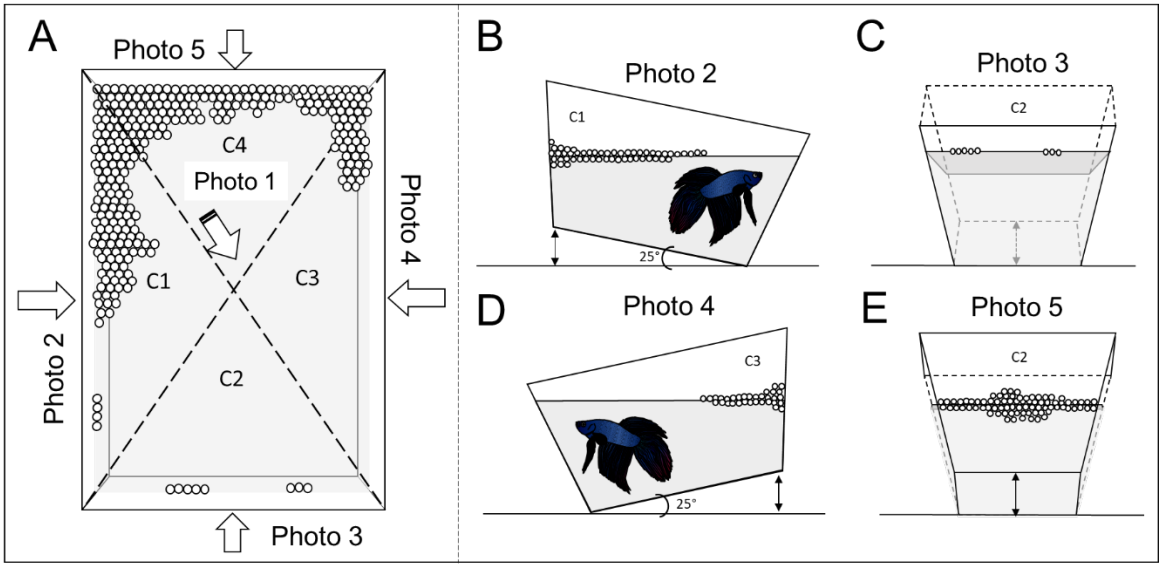

Figure 4.1: Methodology for bubble nest building assessment. A) Schematic diagram of Photo Angle 1 of the tank taken from above. Dashed lines represent division of tank into quadrants (C1 – C4) subsequently superimposed onto the images. Panels B) to E) shows aspects for Photos 2-5, acquired from all four sides of the tank.

4.3.3.2.2 Body and Organ Masses

At the end of each treatment week, each fish was carefully netted from its tank and then placed into a 100 ml water-filled, tared container placed on a top-loading balance (Symmetry EC-Series, Cole Parmer) to determine mass (nearest mg) of the living fish. After weighting, fish were immediately returned to their tank.

Organ weights were determined immediately after blood collection at the end of the experiment. The ventricle (blotted free of blood within), gills, brain and testes were surgically extracted and weighed with a Microbalance (Metter Toledo XA105DR).

4.3.4 Statistical Analysis

Differences in aggressive behavioral displays, nest building variables and body masses were assessed with two-way ANOVA. All comparisons were subjected to normality tests if this assumption was not met, data were rank transformed and the

ANOVA analysis performed on ranks. Hematocrit after arcsine transformation from percentage and organ mass were compared among treatments by one-way ANOVA. With the exception of one-way ANOVA tests, where dietary treatment was the only factor considered, dietary treatment and exposure time period were considered as factors for all two-way ANOVA tests. Assessment of nest characteristics was performed with ANCOVA analysis where body mass was used as covariate.

Statistical significance for all tests was considered below an α of 0.05. Data are presented as mean ± standard error of the mean (SEM) unless otherwise indicated.

4.4 Results

4.4.1 Oil-Concentration in Food

As described above, total PAH concentrations ([PAH]) were used as a proxy for different concentrations of crude oil in diets. The validity of crude oil delivery via diet is indicated by the proportional dose-related increases of [PAH] contained in the various groups of sprayed food pellets. The Control group had a [Total PAH] concentration less than 340 ng/g of food. Total PAH concentrations of 50% HEWAF and 100% HEWAF diets were 11.5X and 25.4X higher, respectively (Fig. 4.2). Also indicated in Fig. 4.2 are the concentrations of the 50 analytes used for the Deepwater Horizon Natural Resource Damage Assessment toxicity testing program (Forth et al., 2016) and most frequently measured PAHs (Dubansky et al., 2018; Johansen et al., 2017), which comprised 80-85% of total PAHs for all three diets. These levels of exposure are above concentrations considered environmentally relevant, contained in natural foods of some fish (Perrichon et al., 2015; Vieweg et al., 2018; Vignet et al., 2014).

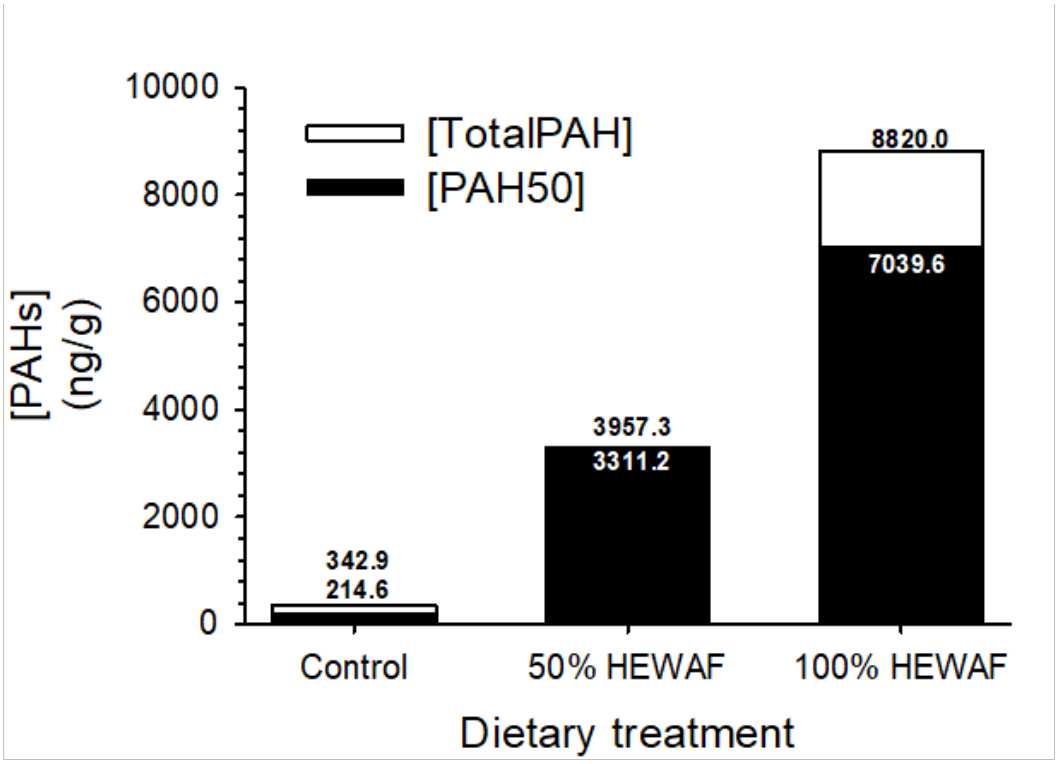

Figure 4.2: Concentration of total PAHs and the sum of 50 select PAHs in each of the three dietary treatments. Concentrations are expressed in ng/g. See appendix for the list of compounds analyzed.

4.4.2 Mortality

Dietary exposure to HEWAF treated diets did not appear to cause mortality, *per se*, in any of the treatments during the time course of the experiments. Three of 48 males died during the course of the behavioral display experiment, but these occurred in the control (2 deaths) and 50% HEWAF (1 death) groups, with no mortality occurring in the 100% HEWAF group in either the behavioral display or nest building experiments.

4.4.3 Morphological Parameters

4.4.3.1 Body Mass

Males in all three dietary groups of the nest building experiment increased in body mass during the four week treatment period, indicating that they were thriving on their

respective diets. Average body mass of control and oil-treated fish ranged from 0.90±0.06 g at the start of treatment to 1.14±0.04 g after four weeks of dietary treatment. Body mass was from 0.92±0.04 g to 1.21±0.02 g for the 50% HEWAF treatment group and 0.99±0.04 g to 1.26±0.05 g for the 100%HEWAF group at the beginning and end of the treatment period, respectively. Body mass did not significantly differ among treatments at any of the four weekly measurement periods (P>0.05, Fig. 4.3), and there was no statistical interaction between length of exposure time and treatment group (two way ANOVA, P=1.00).

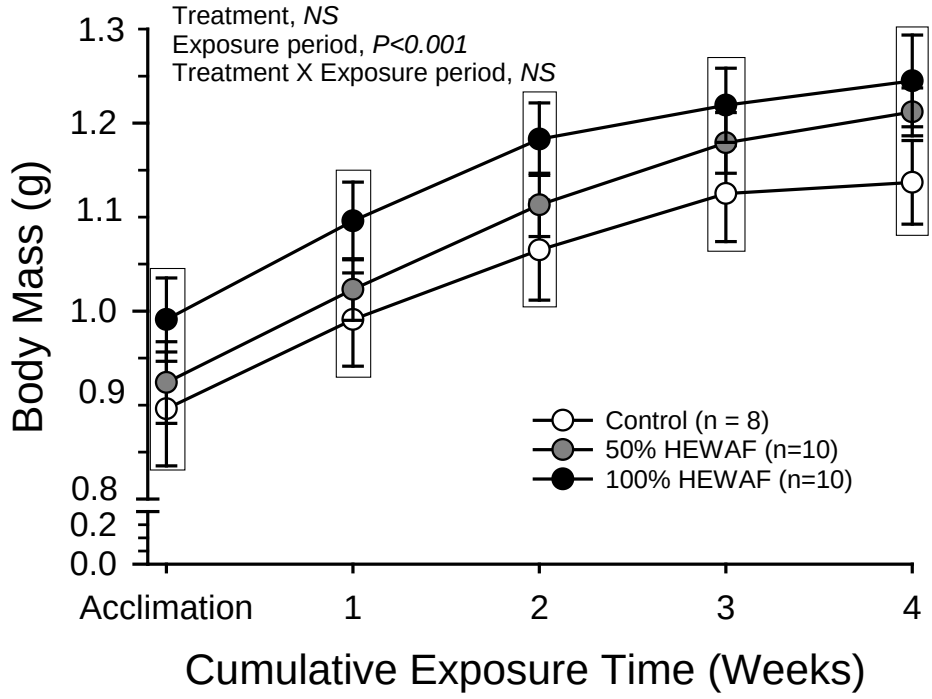

Figure 4.3: Body mass changes of betta fish at the end of the three week acclimation period and at the end of each week of the four week crude oil treatment period for the nest building experiment.

4.4.3.2 Organ Mass

Ventricular mass (1.63±0.09 mg) did not significantly differ (P>0.05) among oiled dietary treatments (Fig. 4.4). Similarly, gill mass was unaffected by HEWAF exposure

(33.8±1.7 mg, P>0.05). In contrast, testes mass (4.22±0.3 mg) was significantly smaller (P<0.002) in bettas exposed to 4 weeks of a 100% HEWAF diet in comparison with the 50% HEWAF and the control groups (5.62±0.4 mg and 5.8±0.44 mg, respectively), which were not significantly different from each other.

Both oil-treated groups had significantly (P<0.05) smaller brain mass (8.33±0.5 mg and 8.28±0.6 mg for 50% HEWAF and 100% HEWAF, respectively) compared to control fish (10.5±1.0 mg) after 4 weeks of dietary HEWAF exposure.

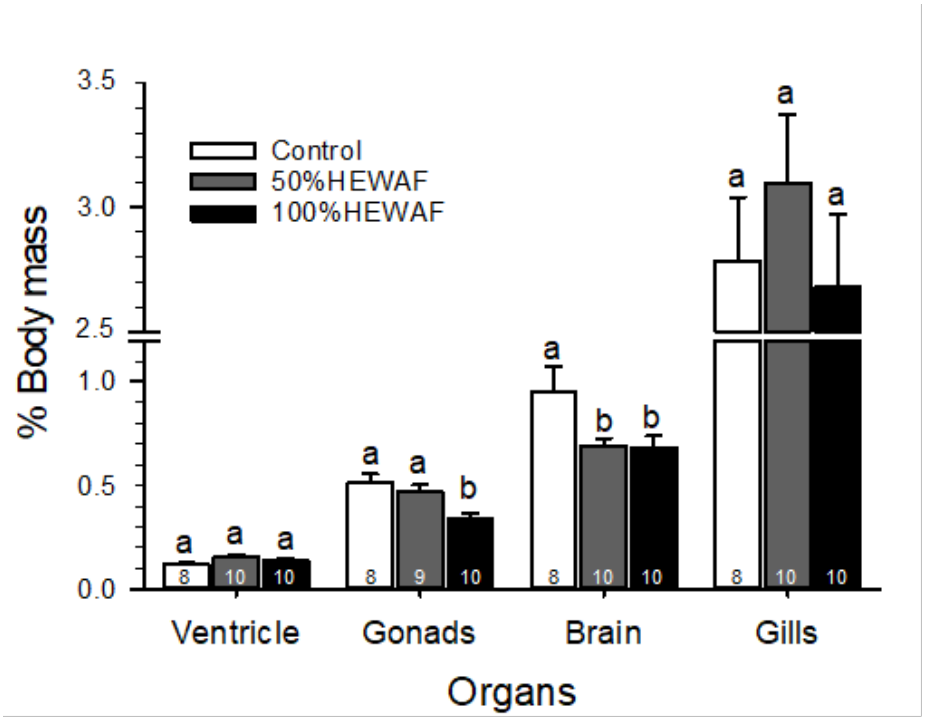

Figure 4.4: Organ mass as a function of dietary exposure to crude oil and exposure time in fish from the nest building experiment. Statistical differences among groups are indicated with different letters above the bars. n values are shown within bars.

4.4.3.3 Hematocrit

Hematocrit was 20.9±0.8% in control adult male measured after the nest building experiment. Males exposed to both doses of HEWAF exhibited significantly higher (P<0.02) hematocrit percentages than control fish, 26.6±1.8% for 50% HEWAF and

27.4±1.6% for 100% HEWAF. There was no significant difference (P>0.05) in Hct between the two HEWAF-treated groups (Fig. 4.5).

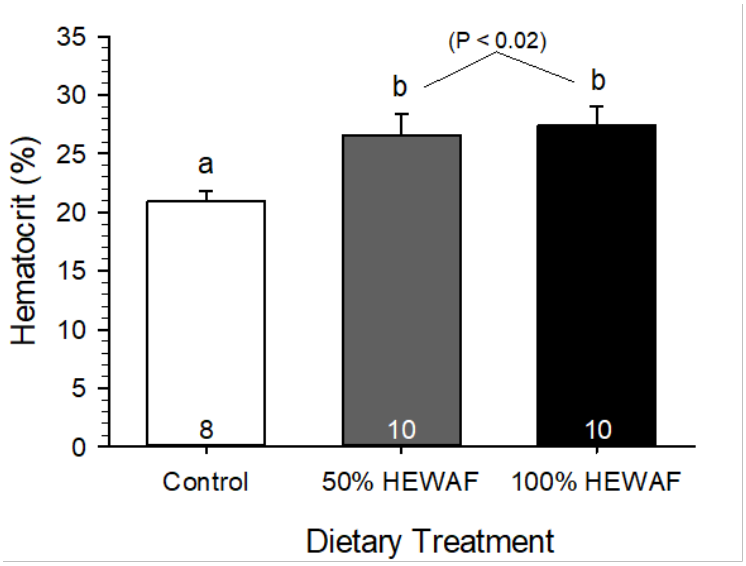

Figure 4.5: Hematocrit at the end of the four week treatment period for the three dietary groups in fish from the nest building experiment. Statistical differences between groups are indicated with different letters above the bars. n values are shown within bars.

4.4.4 Behavioral Parameters

4.4.4.1 Aggressive Behavior

The four behaviors that were assessed as indicators of aggressive behavior for *Betta splendens* were gill flaring, darting, circling (Fig. 4.6). Opercular flaring was significantly elevated by HEWAF exposure (Fig. 4.6A.). In fact, significant differences in behavior were recorded as early as the end of the first week of exposure, when the 50% HEWAF group exhibited a higher behavioral score for opercular flaring (1.7±0.2) in comparison with the control or 100% HEWAF treatments (~1.4±0.1 and ~1.3±0.2, respectively). Gill flaring reached its peak during Week 2 of the exposure period in all three populations, after which it slowly declined to week 4. By the second week of treatment, both the 50% HEWAF and the 100% HEWAF treatment groups exhibited

higher flaring scores in comparison with the control group, an elevation of score that continued throughout the remainder of the trials.

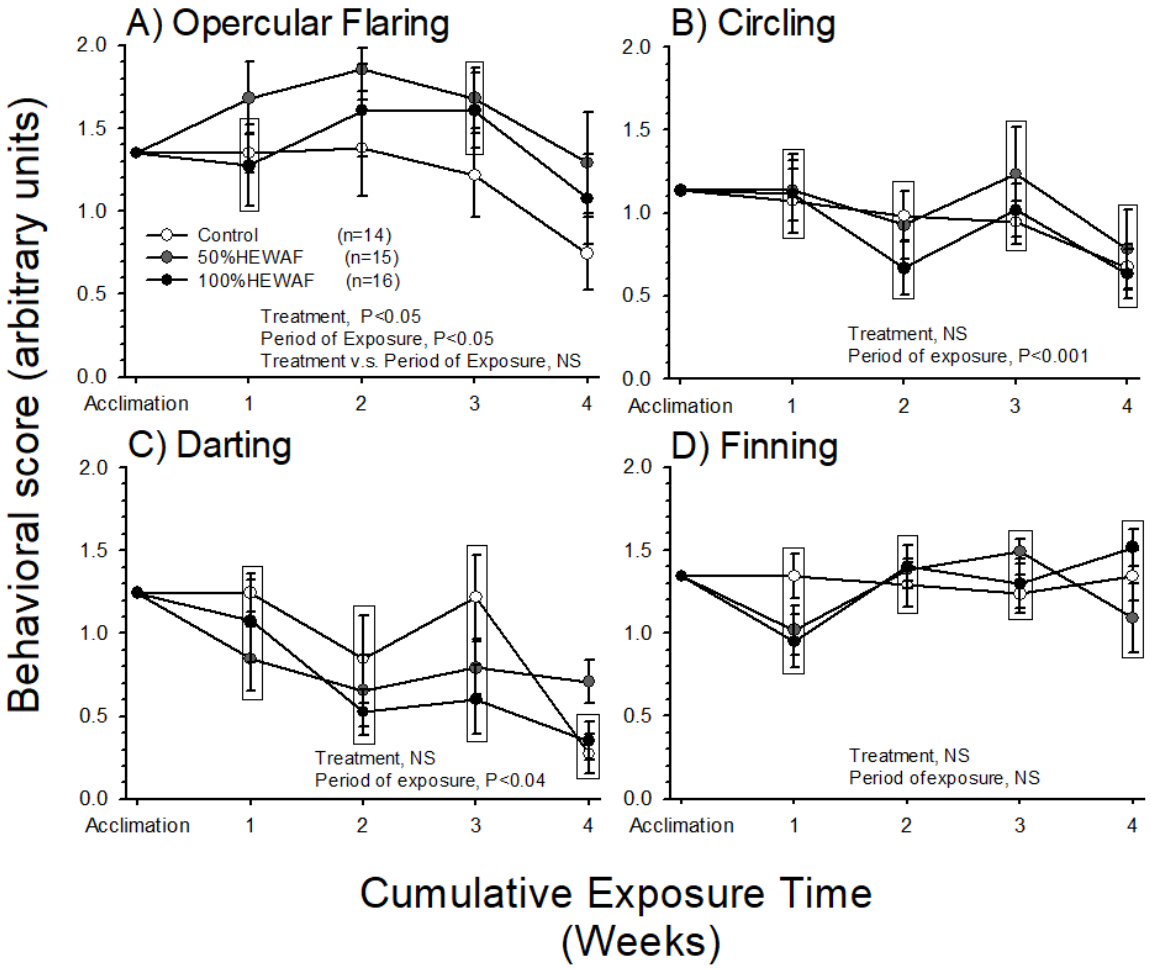

Figure 4.6: Analysis of changes in aggressive behavioral displays in male bettas as a function of dietary exposure to crude oil and exposure time. A) Opercular Flaring, B) Circling, C) Darting and D) Finning. n values in panel A apply to all other panels. No measurements were recorded for the acclimation period for any of the behaviors, instead, a theoretical starting point where all treatments exhibited the control score measured on week 1 was added and extrapolated to the actual data for Week 1 (dashed lines) for visualization purposes, but acclimation numbers were not factored into the statistical analysis. Mean values enclosed within a box are not significantly different.

Circling and darting displays showed no significant variation (P>0.05) between the three dietary treatments. Similarly, analysis of finning behavior showed no significant effect of HEWAF dosage and no significant signs of change throughout the trial period.

4.4.4.2 Nest Building

Nest presence declined significantly during the course of the trial in all treatments (Fig. 4.7A). However, by the third week of exposure the 100% HEWAF group clearly showed a significant, sharp decline compared to the control (<40%). By the fourth week of dietary treatment, both 50% and 100% HEWAF-treated groups also exhibited lower bubble nest presence in comparison to the control group ($P<0.05$). Although both cumulative length of exposure and dietary exposure to oil induced significant effects on nest presence, there were no significant interactions between factors (Fig. 4.7A.). In general, from the acclimation period to the end of the experiment, surface area of the nest decreased 6.22 cm^2, 12.5 cm^2, and 11.2 cm^2 for the control, 50% HEWAF and 100% HEWAF groups, respectively (Fig. 4.7B). However, these changes were unrelated to oil exposure levels, which did not significantly alter bubble nest area. There was no interaction between exposure treatment and exposure time ($P>0.05$).

In addition to nest surface area, maximum nest width and depth were also compared among experimental groups (Figs. 4.8C and 4.8D, respectively). Dietary exposure to oil did not affect nest width or depth ($P>0.05$). In contrast, cumulative length of exposure was a factor inducing significant ($P<0.05$) differences within groups. Additionally, the interaction between factors was significant for nest depth. At the beginning of the trial both treated groups exhibited wider and deeper nests (width 7.8±1.8 cm and 8.1±1.4 cm and, depth 5.96±1.5 mm and 6.1±1.4 mm for 50% HEWAF and 100% HEWAF, respectively) in comparison with the control (3.9±0.5 cm and 4.2±1.4 mm). A sharp decrease in nest width and depth in both groups is seen at the second week of exposure. These decreases put the values of both groups at the same level as the Control

for the rest of the experimental trial.

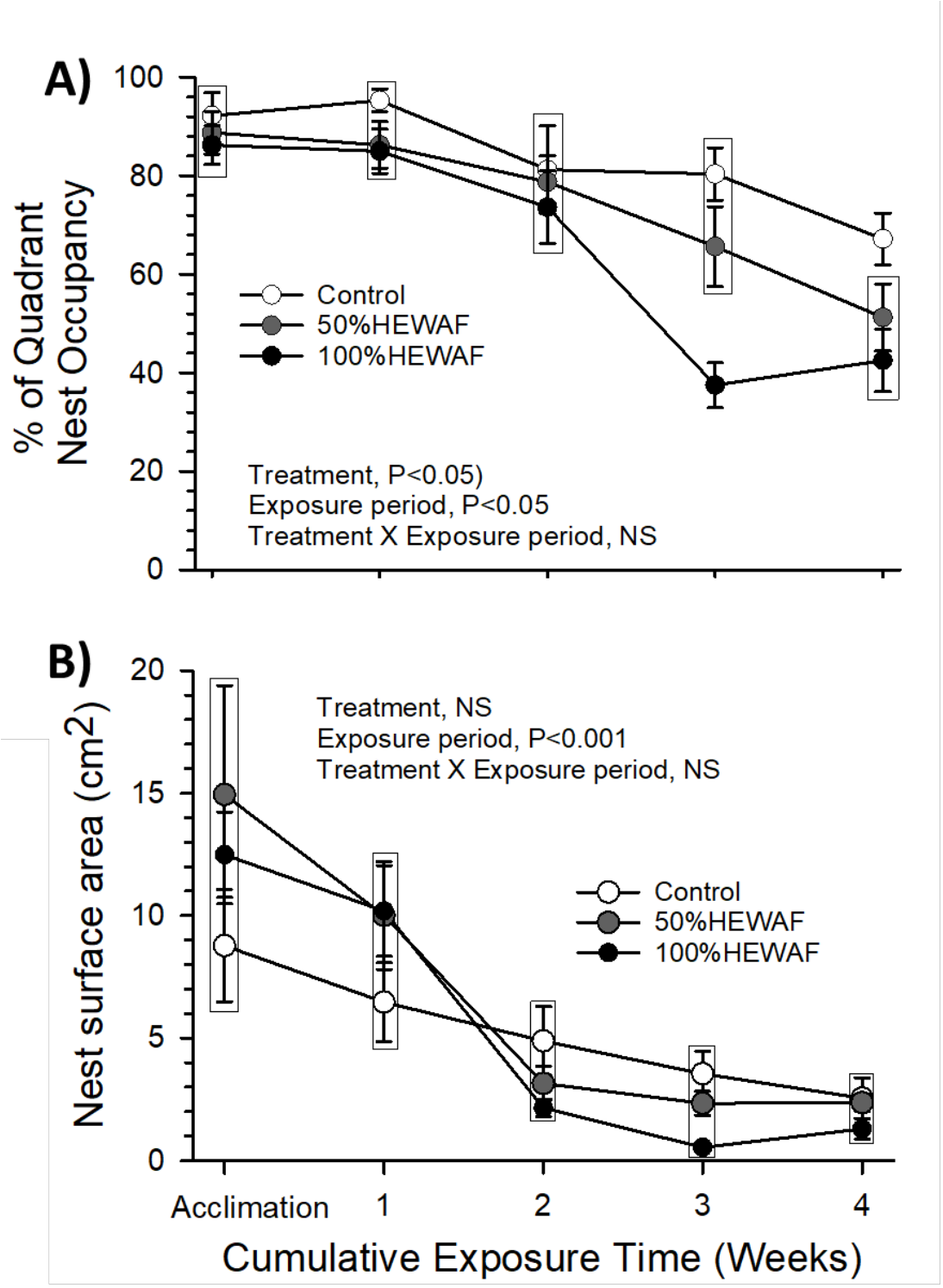

Figure 4.7: Changes in nest presence (A) and nest surface area B) in response to dietary exposure to crude oil in *B. splendens*. Mean values enclosed within boxes are not statistically different.

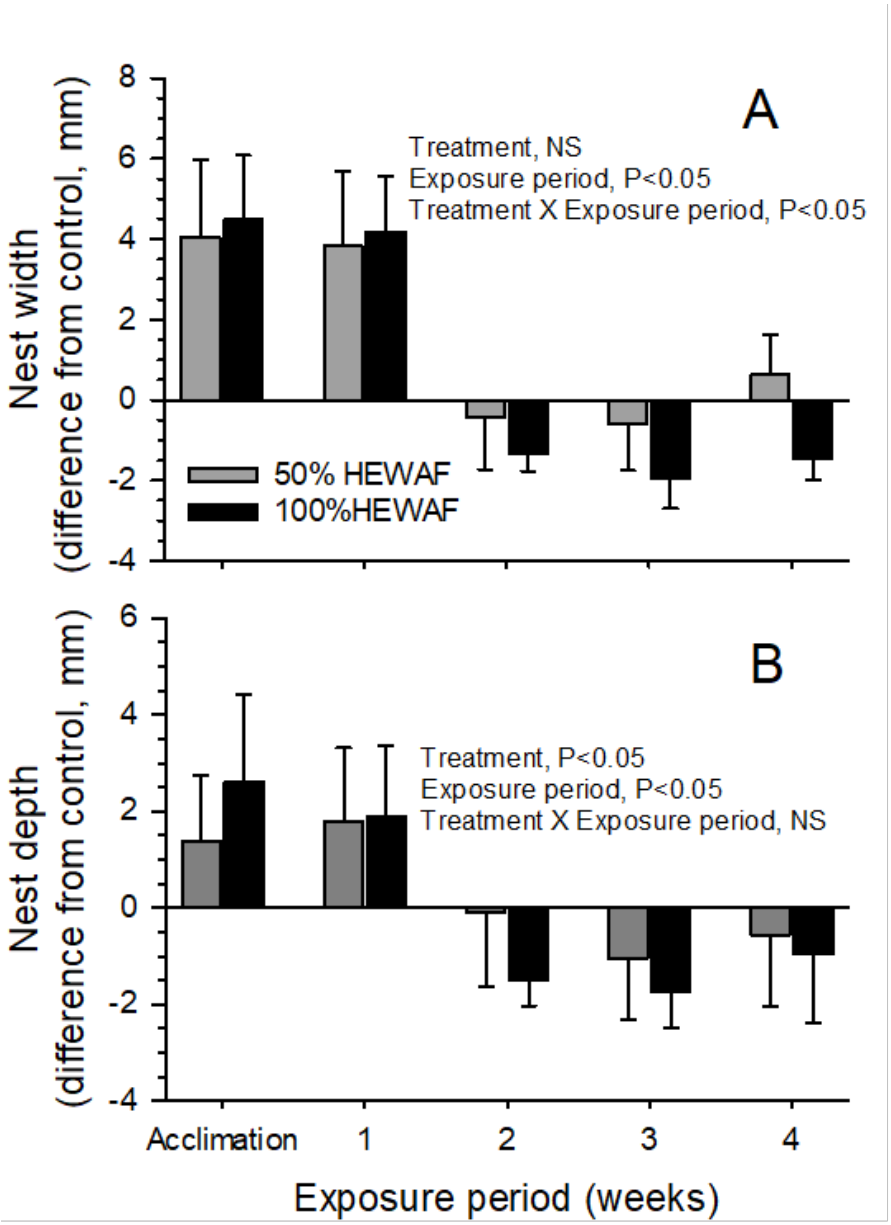

Figure 4.8: Percentage changes in maximum nest width A) and nest depth B). The "0" line represents the value of the control group at each particular time period. n= 7-10 per data point.

4.5 Discussion

Interest in understanding the behavioral and fitness consequences of exposure to environmental toxicants in fish has increased in the last decade (Burggren and Dubansky, 2018; Dickins and Dickins, 2018; Dzieweczynski et al., 2018; Mager and Grosell, 2011).

We know that exposure to crude oil affects fish fitness and survival by disrupting swimming activity and performance (Khursigara et al., 2017; Mager et al., 2014a; Mager et al., 2018; Stieglitz et al., 2016; Wang et al., 2018). However, determination of cause and effect consequences of crude oil exposure on behavioral aggressive and reproductive displays in wild fish populations is complicated by, several confounding factors (Elliott et al., 2007; Nash et al., 2004). Fish are exposed to crude oil via skin, gills and diet (Tierney et al., 2013), potentially leading to differential responses. Adult codfish are able to detect oil presence in the water and then avoid those polluted areas (Bøhle, 1986). However, in some scenarios exposure to oil will still result from feeding on contaminated prey that is not able to avoid oil exposure via water (Olsvik et al., 2011). Additionally, fish appetite seems to not decrease in fish fed with oil-contaminated food (Christiansen and George, 1995), highlighting the importance of dietary oil exposure. The current study has tested the consequences of dietary exposure to crude oil on aggressiveness and nest building behavior in the Siamese fighting fish, a venerable animal model for addressing this inquiry (Arnott et al., 2016a; Dzieweczynski et al., 2018).

The oil exposure levels used in the present study did not affect mortality nor body mass, even though they were above concentrations found in the natural diets of fish after oil spills occurred, and considered environmentally relevant concentrations (Perrichon et al., 2015; Vieweg et al., 2018; Vignet et al., 2014). In fact, fish mass continued increasing during the experiments in HEWAF as well as control groups (Fig. 4.3), suggesting that a primary indicator of fish health was not affected by dietary exposure to petroleum during experimentation (Anderson and Gutreuter, 1983; Curtis and Maclean, 2014). Although this may suggests that our fish had not reached fully grown state at the start of the

experiments, it is unlikely that our results of nest building behavior would be different in older fish, since this reproductive behavior has been reported in fish as young as 53 days old (Braddock and Braddock, 1959).

To our knowledge, no study has reported on the ontogeny of aggressiveness in *B. splendens* fish. Inter-individual aggressiveness due to competition for food and cannibalistic behaviors starts at early developmental stages in fish species such as *Epinephelus septemfasciatus* and *Seriola quinqueradiata* (Sabate et al., 2009; Sakakura and Tsukamoto, 1996) suggesting that similar results to those obtained in this study might be observed even in fully grown betta fish.

Employing mirrors to elicit aggressive responses in betta fish has its limitations. Although mirrors might evoke responses by visual clues, sensorial clues through the lateral-line are not received, and mirror responses fail to elicit intrinsic organismal responses, such as hormonal release (Arnott et al., 2016a; Villars, 1983) and brain gene expression (Desjardins and Fernald, 2010). Bias to lateralization in responses (left-side, left-eye), have been reported during combat when using mirrors but not when fish faced real opponents (Arnott et al., 2016a; Elwood et al., 2014). Although those studies failed in showing differences in aggressive displays between the routes of stimulation, the question of whether behavioral effects evoked by a mirror following oil exposure would be even more severe with a live opponent is yet to be answered.

Habituation of responses and active avoidance of opponents have been reported in aggressive displays by *B. splendens* after repeated exposures to an stimulus in as little as 6 days of experimentation (Peeke and Peeke, 1970; Rhoad et al., 1975) . No such habituation of aggressive displays was observed in the present study (Fig. 4.6), perhaps

because mirror exposures were limited to 3 min daily. Indeed, the most common aggressive behavior (gill flaring) was increased in oil-exposed fish throughout the four weeks of exposure to oil (Fig. 4.6A). Although, aggressiveness and boldness have been correlated with higher reproductive success, the negative effects on nest building and gonadal mass found in this study strongly suggest reduced reproductive success. Additionally, performance of aggressive displays is metabolically costly (Regan et al., 2015). Fish may adjust physiological traits to overcome O_2 delivery to the tissues under challenging situations (Harter and Brauner, 2017). Moreover, it is possible that the increase in aggressiveness elicited by dietary exposure to oil also imposes higher demand of O_2, and thus it is probable the observed increase in hematocrit (Fig. 4.4) may be an attempt to facilitate O_2 uptake and transport. Besides any metabolic costs, the more time spent performing aggressive behaviors, the less time is available to that fish to spend foraging or in courtship/mating behaviors (Arnott and Elwood, 2009; Lane and Briffa, 2017).

Additionally to altered aggressive behavior, sexual characteristics and courtship behavior in the Siamese fighting fish and other fish species are highly sensitive to anthropogenic chemical compounds such as endocrine disruptors (Chen et al., 2016b; Dzieweczynski and Hebert, 2013; Stevenson et al., 2011), and to pharmaceuticals entering the aquatic environment (Eisenreich et al., 2017). Such compounds are correlated with decreased reproductive success and individual fitness (Dzieweczynski and Kane, 2017). For example, life-term exposure to 5ng/L of ethynylestradiol (EE_2) in a F_1 population of zebrafish resulted in complete reproductive failure (Nash et al., 2004). This failure was accompanied by the absence of secondary sexual phenotypic

characteristics in males, the lack of expressible sperm and normal morphology of their testes (Nash et al., 2004). In the present study, the mass of the testes of fish exposed to 100% HEWAF concentration was significantly smaller in comparison to control and 50% HEWAF diet fish (Fig. 4.5).It is yet to be determined if crude oil exposure leads to sterility in these fish, blocking successful reproduction. Some PAHs induce endocrine disruption partly by activation of aryl-hydrocarbon receptor (Hawliczek et al., 2012) or by, estrogenic (van Lipzig et al., 2005), anti-estrogenic (Santodonato, 1997) and anti-androgenic activity (Kizu et al., 2003). It will be important to determine if dietary exposure to crude oil induces endocrine disruption in adult fish.

It is probable that exposure to oil could impact mating success through behavioral alterations. For example after exposing male betta fish to EE_2, female bettas spent less time near, tracking and orientating towards exposed males in comparison with unexposed individuals (Dzieweczynski and Kane, 2017). Furthermore, behavioral displays such as female responsiveness to exposed males could be also playing an important role in population maintenance.

Exposure to crude oil in the present study led to the significant reduction of bubble nest presence in fish exposed to the highest dose in comparison with control fish and those exposed to medium oil concentration (Fig. 4.7A). Although nest surface area, width and depth were not affected by the oil treatment, crude oil exposure appears to have an “all or nothing” effect on presence or absence of a bubble nest.

4.6 Future Studies

Research on betta fish has focused on male aggression and boldness behavior (Ariyomo and Watt, 2012; Tubert et al., 2012) . However, further investigation is needed

to determine how female fish could be affected by exposure to contaminants. For example, along with reduced female responsiveness towards males exposed to EE_2, exposure to the same compound in females lead to the reduction in mate-tracking behavior (Dzieweczynski and Kane, 2017). The fact that female mate choice and mate behavior are affected, suggests that target traits needed for successful reproduction may be affected in both sexes. Thus, future studies should be expanded to both sexes to provide a comprehensive understanding of the effects of pollutants like crude oil on fish fitness. Finally, effects of oil exposure affect both the perception of the external environment and also intrinsic characteristics of the internal environment of individuals. Since some behaviors were affected in this study and others were not, future experiments should include multiple behavioral factors and levels of interaction.

4.7 Conclusion

Crude oil exposure in fish induces organismal responses that range from molecular to whole organismal responses (Burggren and Dubansky, 2018; Khursigara et al., 2018; Mager et al., 2014a; Xu et al., 2017a). The present study demonstrates that in the Siamese fighting fish dietary exposure to crude oil induces alterations in aggressive behaviors necessary for reproductive success. Changes in fish behavior related to crude oil exposure have also been identified in marine fishes (Johansen et al., 2017; Khursigara et al., 2018; Pan et al., 2018). Collectively, these results suggest that exposure to crude oil may be inducing impairments of cognitive processes that may induce fish to perform behaviors that may affect fitness, survival and population maintenance. Although the cellular and molecular mechanisms underpinning these behavioral modifications are yet to be described, our findings open new windows for further studies of oil-induced effects

on behavior, and highlights the importance of considering a multi-scale approach aimed at understanding how fish populations, and especially their collective and individual behavior, are affected by oil exposure. Considering the effects of crude oil exposure on aggressiveness and reproductive behaviors, offers a more holistic approach to assessing impacts of toxicants on fish fitness and population maintenance in fish populations. Finally, these type of experiments will provide information for building up new foundations and improving our understanding of the effects that accidents (i.e. oil spills) induce on wildlife populations, as well as the repercussions on the survival and prevalence of species.

4.8 Appendix: Supplemental Table A4-1

List of components and nominal concentrations (mg/Kg) for each diet treatment. The sum of all the components listed below was considered the "Total PAH concentration". The components highlighted with gray color were considered for the 50 PAHs most frequently measured PAHs (Dubansky et al., 2018; Johansen et al., 2017). ND= not determined.

Component	Dietary treatments			
	Control_ (mg/Kg)	50% HEWAF (mg/Kg)	100% HEWAF (mg/Kg)	BLANK (mg/Kg)
cis/trans-Decalin	ND	ND	ND	ND
C1-Decalins	ND	ND	0.0225	ND
C2-Decalins	ND	ND	ND	ND
C3-Decalins	ND	ND	ND	ND
C4-Decalins	ND	ND	0.273	ND
Benzo(b)thiophene	ND	ND	ND	ND
C1-Benzothiophenes	ND	ND	ND	ND
C2-Benzothiophenes	ND	ND	ND	ND
C3-Benzothiophenes	ND	ND	ND	ND
C4-Benzothiophenes	ND	ND	ND	ND
Naphthalene	0.0150	0.0277	0.0546	0.00672
C1-Naphthalenes	0.0267	0.137	0.292	0.0205
C2-Naphthalenes	0.0248	0.339	0.725	ND

(table continues)

Component	Dietary treatments			
	Control_ (mg/Kg)	50% HEWAF (mg/Kg)	100% HEWAF (mg/Kg)	BLANK (mg/Kg)
C3-Naphthalenes	0.0359	0.331	0.732	ND
C4-Naphthalenes	ND	0.450	0.773	ND
Biphenyl	0.0123	0.0376	0.0674	0.0100
Dibenzofuran	0.00761	0.0115	0.0200	ND
Acenaphthylene	0.00418	0.00600	ND	ND
Acenaphthene	0.00583	0.00635	0.00984	ND
Fluorene	0.00875	0.0408	0.0884	0.00373
C1-Fluorenes	ND	0.112	0.260	ND
C2-Fluorenes	ND	0.165	0.375	ND
C3-Fluorenes	ND	0.183	0.407	ND
Anthracene	0.00286	0.00586	0.0148	ND
Phenanthrene	0.0191	0.0826	0.175	0.00512
C1-Phenanthrenes/Anthracenes	ND	0.196	0.415	ND
C2-Phenanthrenes/Anthracenes	ND	0.282	0.581	ND
C3-Phenanthrenes/Anthracenes	ND	0.279	0.501	ND
C4-Phenanthrenes/Anthracenes	ND	ND	0.247	ND
Retene	ND	ND	0.0110	ND
Dibenzothiophene	ND	0.0104	0.0232	ND
C1-Dibenzothiophenes	ND	0.0390	0.0949	ND
C2-Dibenzothiophenes	ND	0.0667	0.156	ND
C3-Dibenzothiophenes	ND	0.0597	0.151	ND
C4-Dibenzothiophenes	ND	0.0165	0.0305	ND
Benzo(b)fluorene	ND	0.00661	0.0131	ND
Fluoranthene	0.00605	0.00565	0.0110	0.00287
Pyrene	0.00462	0.00821	0.0170	0.00262
C1-Fluoranthenes/Pyrenes	ND	0.0314	0.0706	ND
C2-Fluoranthenes/Pyrenes	ND	0.0500	0.106	ND
C3-Fluoranthenes/Pyrenes	ND	0.0558	0.132	ND
C4-Fluoranthenes/Pyrenes	ND	0.0469	0.112	ND
Naphthobenzothiophene	ND	0.00446	0.00898	ND
C1-Naphthobenzothiophenes	ND	ND	0.0399	ND
C2-Naphthobenzothiophenes	ND	ND	ND	ND

(table continues)

Component	Dietary treatments			
	Control_ (mg/Kg)	50% HEWAF (mg/Kg)	100% HEWAF (mg/Kg)	BLANK (mg/Kg)
C3-Naphthobenzothiophenes	ND	ND	ND	ND
C4-Naphthobenzothiophenes	ND	ND	ND	ND
Benz(a)anthracene	0.00800	0.00858	0.0144	0.00822
Chrysene	0.00323	0.0168	0.0444	0.00441
C1-Chrysenes	ND	0.0384	0.0840	ND
C2-Chrysenes	ND	0.0924	0.147	ND
C3-Chrysenes	ND	ND	ND	ND
C4-Chrysenes	ND	ND	ND	ND
Benzo(b)fluoranthene	0.00645	0.00824	0.0102	0.0183
Benzo(k)fluoranthene	ND	ND	ND	ND
Benzo(a)fluoranthene	ND	ND	ND	ND
Benzo(e)pyrene	ND	0.00423	0.00911	ND
C30-Hopane	0.0277	0.0447	0.0698	ND
Benzo(a)pyrene	0.00227	ND	0.00383	ND
Perylene	ND	ND	ND	ND
Indeno(1,2,3-cd)pyrene	0.0139	0.00412	0.00990	ND
Dibenz(a,h)anthracene	0.00311	ND	0.00568	ND
Benzo(g,h,i)perylene	0.00394	ND	0.00686	ND
4-Methyldibenzothiophene	ND	0.0177	0.0441	ND
2-Methyldibenzothiophene	ND	0.00842	0.0231	ND
1-Methyldibenzothiophene	ND	0.00543	0.0130	ND
3-Methylphenanthrene	ND	0.0387	0.0880	ND
2-Methylphenanthrene	0.00451	0.0457	0.101	ND
2-Methylanthracene	ND	0.00192	0.00525	ND
9-Methylphenanthrene	ND	0.0486	0.114	ND
1-Methylphenanthrene	ND	0.0390	0.0859	ND
2-Methylnaphthalene	0.0195	0.102	0.219	0.0109
1-Methylnaphthalene	0.0169	0.0833	0.173	0.0185
2,6-Dimethylnaphthalene	0.00900	0.120	0.276	ND
2,3,5-Trimethylnaphthalene	0.0309	0.117	0.234	ND
Carbazole	0.0198	0.0183	0.0280	ND
Fluorene-d10	79	83	70	67

(table continues)

Component	Dietary treatments			
	Control_ (mg/Kg)	50% HEWAF (mg/Kg)	100% HEWAF (mg/Kg)	BLANK (mg/Kg)
Fluoranthene-d10	90	94	82	80
Terphenyl-d14	83	87	76	81
SUM TOTAL PAH	0.34	3.96	8.82	0.11
SUM TPAH50	0.21	3.31	7.04	0.08

CHAPTER 5

TRANSGENERATIONAL EFFECTS ON RESPIRATORY PHYSIOLOGY IN THE KING QUAIL (*Coturnix chinensis*)

5.1 Abstract

Environmental contaminants, such as crude oil, represent a constant threat for marine and marsh birds species that could be exposed dermally, dietarily or via respiration, leading to detrimental biological effects. Unfortunately, oil spills may coincide with the breeding season for these birds populations, potentially affecting their reproductive success and fitness. Exposure to crude oil, and the thousands of compounds that it contains, elicit transgenerational effects in fish and mammals, affecting the developmental trajectories of their offspring. This study used the king quail as animal model and involved exposure to two different concentrations of high energy water accommodated fractions of crude oil (1%HEWAF or 10%HEWAF) via their diet. Indicators of bird health (body mass, temperature, oxygen consumption) and fitness (laying, fertilization, hatching success) were analyzed in the adult quails. Then we assessed, from the cellular to the systemic levels, if the respiratory physiology of their offspring was also impaired. Body mass did not vary among adult groups (~69.5g female, ~46.4g male) however, oxygen consumption was increased in the group exposed to the higher dose compared with control animals (5.95ml O_2*g*h and 5.10ml O_2*g*h, $P<0.05$, respectively). Although egg mass and size did not differ among groups, fertilization percentage was reduced in oil-exposed groups by up to 20%. Additionally, egg water loss from the oil exposed parents was increased (~0.11g/day oil-exposed vs 0.05g/day control, respectively). Our results suggest that crude oil exposure in adult quails may compromise

organismal metabolic costs and directly affects reproductive success. Additionally, offspring respiratory physiology may be compromised. This experiment highlights the relevance of performing experiments considering more than one generation to better understand the ecological impact of environmental stressors, such as oil-spills, on bird physiology and population maintenance.

This chapter was completed in collaboration with the following researchers:

- Lara do Amaral-Silva: Data acquisition; performance of experiments; manuscript editing.
- Edward Dzialowski: Conceptualization of experimental design; equipment, data interpretation; manuscript editing.
- Warren Burggren: Conceptualization of experimental design; data analysis and interpretation; performance of experiments; drafting of manuscript and editing.

5.2 Introduction

The impacts of the Deepwater Horizon oil spill extended from the pelagic zone in the ocean to estuaries along the northern coast of the Gulf of Mexico (Michel et al., 2013; Pasparakis et al., 2019). The weathering process separates the oil into different phases, depending on its physicochemical characteristics and its interaction with the environment (Tierney et al. 2013). Some of the oil was mixed in the water column, exposing aquatic species (Mager et al. 2014; Dubansky et al. 2013). However, another part of the oil and its constituents came in contact with the shorelines, where sediments are still laden with it (Dubansky et al. 2018; Pennings et al. 2014). Consequently, oil represents a constant threat for marine and especially marsh species of birds that are exposed dermally, dietarily and via respiration (Dubansky et al. 2018). Thus, the biology of numerous bird species was also highly perturbed by the Deepwater Horizon oil spill (Harr et al., 2017a; Harr et al., 2017b; Horak et al., 2017; Perez et al., 2017).

Crude oil exposure in avian species has acute lethal effects on heavily exposed individuals (Perez et al., 2017). External oil-contamination of the feathers is considered to have the most harmful effects by reducing their capacity to repel water, their insulating properties, buoyancy and flight performance (Leighton, 1993; Munilla et al., 2011; O'Hara and Morandin, 2010). Beyond that, after an oil spill occurs many birds may also be exposed via diet to varying concentrations of petroleum, and this exposure may significantly affect their biology (Alexander et al., 2017). For example, increased oxidative levels due to increased activity of total glutathione, glutathione reduced and glutathione disulfide in hepatic tissue were reported in double-crested cormorants after dietary exposure to oil-injected prey (Pritsos et al., 2017). Additionally, potential impairment of blood oxygen carrying capacity was reported in sandpipers, cormorants, homing pigeons, and laughing gulls (Bursian et al., 2017; Dean et al., 2017). It was suggested these changes resulted from damage to the tertiary structure of hemoglobin by means of epoxide-formation in the liver through the cytochrome P450 complex. However, few studies have been aimed at directly assessing if dietary exposure to crude oil affects whole organismal respiratory physiological variables in the exposed population.

Not only were there severe effects of crude oil exposure directly on exposed adult bird populations produced by the Deepwater Horizon spill, but this spill coincided with the breeding season of numerous bird species (Bernardo, 1996; Harr et al., 2017a; Harr et al., 2017b; Horak et al., 2017; Perez et al., 2017). Consequently, it is possible that petroleum exposure on parental populations potentially lead to effects on subsequent generations through epigenetic transgenerational inheritance. However, to our knowledge, no study has aimed to determine if dietary exposure to crude oil in birds could

lead to transgenerational effects on their immediate F_1 generation. Performing this type of study on wild avian species is possible, but it is expensive, time consuming and potentially requires large spaces for husbandry in laboratory conditions. Not surprisingly, the use of animal models or domesticated species to address these types of questions have burgeoned in the past decade (Guerrero-Bosagna et al., 2018).

In the present study we used the king quail (also known as Chinese painted quail) *Coturnix chinensis* as a proxy for understanding the effects of crude oil on a parental P_0 generation and the immediate subsequent F_1 generation of birds exposed to crude oil during a spill.

5.2.1 Goals

The overall goal of this chapter is to provide a detailed description of the effects of crude oil exposure (from the Deepwater Horizon oil spill) on adult bird phenotype (parental population) and determine its effects on offspring phenotypic characteristics and respiratory physiology. Classic, state-of-the-art and proof of concept methodologies will be used to evaluate dietary exposure to crude oil in the king quail with the following goals:

(i) To determine the proximate morphological and physiological effects of dietary exposure to crude oil in the parental (P_0) generation.

(ii) To determine if the parental exposure leads to transgenerational effects on F_1 offspring phenotypic characteristics.

5.2.2 Hypotheses

1. Dietary exposure to crude oil at the levels of exposure used will not affect primary indicators of adult P_0 bird health such as body mass.
2. Dietary exposure to crude oil will affect organ mass and hematological variables of the parental population.

3. Dietary exposure to crude oil will increase oxygen consumption of the parental population.

4. Parental exposure to crude oil will affect morphometric and physiological characteristics such as mass, length, width and water loss of the eggs of their F_1 offspring.

5. Parental exposure to crude oil will affect F_1 hatchling mass and their capacity to cope with low temperatures.

5.3 Materials and Methods

5.3.1 *Coturnix chinensis* as an Animal Model

The king quail (*Coturnix chinensis*), is the smallest “old world” true quail. This species has been bred extensively in captivity for human commercial and recreational interests because they are considered game birds and are endangered. *C. chinensis* offers several advantages as an avian model for research. It is one of the smallest precocial species, ranging from 40 to 70 g as adults. This characteristic makes it suitable for the variety of experimental protocols described below. In addition, being a species extensively used in aviculture, most of the optimal practices for husbandry are well known. Also, experimental designs, set ups, management and costs are easier and less expensive in comparison with wild species, allowing for more systematized and controlled experimental exposure conditions (MacDonald, 2010). The king quail’s monogamous behavior also offers two main advantages. The first is that tracking of the offspring’s lineage is more systematic, allowing for more powerful analysis of the data. Second, it is easier to maintain a healthier condition status of king quails during husbandry, assuring quality and health of the animals, and reducing aggressive behaviors in comparison with other species (Adkins-Regan, 2016; MacDonald, 2010; Puddephatt, 2014). Finally, the king quail is considered to be a prolific avian species capable of laying 1 or 2 eggs a day,

thus allowing to test for transgenerational effects on the offspring in relatively short periods of time (MacDonald, 2010).

5.3.2 Animal Husbandry

Fifteen pairs of adult king quail were obtained from a local supplier (Elm Ridge Enterprises) and kept at the avian storage and incubation facilities at the University of North Texas. The birds were maintained at ~24ºC ~60% humidity in a 14:10 hours light:dark cycle, and provided with food and water ad libitum. As mentioned above, *C. chinesis* are monogamous. Thus, one male and one female were kept together in a 18 gallon container (60cm L X 47.3cm W X 34.6cm H). The bottom of the tanks were covered with wood shavings to prevent injuries in the feet of the birds and to absorb moisture (MacDonald, 2010; Puddephatt, 2014). Cleaning of the tanks was performed on a weekly basis, but all tanks were checked, stocked and cleaned (if necessary) during every morning and afternoon.

All experiments in this study were approved and performed in strict follow up with the Institutional Animal Care and Use Committee (IACUC-Protocol #18022) at the University of North Texas.

5.3.3 Experimental Design

5.3.3.1 Diet Preparation

Dietary exposure to crude oil was used as the stressor in experiments with adult quail. To prepare oiled diets, solutions of High Energy Water Accommodated Fractions of crude oil (HEWAF) were prepared following standard protocols (Bautista et al., 2019; Forth et al., 2017; Mager et al., 2014a; Reddam et al., 2017). Source oil “B” (SOB) sampled from the Gulf of Mexico MC252 well on May 22–23, 2010 was used for this

experiment. British Petroleum acknowledges the use of a defoamer (Nalco EC9323A), oxygen scavenger (Nalco VX9831) and methanol during the collection of this type of crude oil. Although their presence in SOB cannot be dismissed, the direct sampling from the riser insertion tube may reduce the possibility of incorporation of these compounds into the oil (de Soysa et al., 2012). In brief, 1000mg of crude oil were added into 1L of conditioned aquarium water and blended for 30 s in a commercial blender (WaringTM CB15). After blending, the mixture was placed into a separation funnel for 1 h, after which 100 ml of the solution was taken out through a bottom port of the funnel and discarded. 600 ml of the remaining mixture (considered as 100%HEWAF) and two diluted solutions (1% and 10% HEWAF in conditioned aquarium water) were used for diet preparation.

Three dietary treatments were used for parental exposures for adult quail: a) Control, b) 1%HEWAF, and c) 10%HEWAF. To make these dietary treatments, 100 g of quail food was prepared from equal parts of 24% chick starter (Dumor poultry #5078197), Reptile diet (Mazuri 006267) and wild bird food mixed seeds (Blain's farms and fleet). This mixture was then evenly distributed across the bottom of aluminum trays (100cm L X 30cm W). The food mixture was sprayed with 60 ml of water (Control) or one of the two HEWAF solution concentrations described above. The treated food was allowed to dry for ~12 hours. The dried food was then collected from the trays and stored at room temperature in glass bottles.

Representative samples of each treatment diet were analyzed by ALS Environmental (ALS Environmental, Kelso, WA, USA) to obtain total polycyclic aromatic hydrocarbons (PAH) concentrations (ΣtotPAH). PAHs are petroleum components well-known to affect the cardiac system, swimming capacity, performance, and morphology

throughout developmental stages in fish and birds (Fallon et al., 2018; Harr et al., 2017b; Harr et al., 2017c; Incardona et al., 2014; Incardona and Scholz, 2018a; Mager and Grosell, 2011; Stieglitz et al., 2016). Thus, it is widely accepted that determination of [PAH] in the diets offers a valid indication of the toxicity level of each treatment used during this study (Bautista et al., 2019; Mager et al., 2014a).

In addition to dietary samples, eggs obtained from each parental group were also sent for PAH determination analysis to ALS Environmental.

5.3.4 Dietary Exposure Protocol

Adult birds were allowed to acclimate for 14 days prior exposure. Before the beginning of exposure, all birds were screened for condition. Dietary exposures began at day 15 of the experiment. Food and water were provided ad libitum. Then, adult quail were dietarily exposed to crude oil during 21 consecutive days before any laid eggs were considered for analysis. The exposure to crude oil continued until the desired number of F_1 offspring was obtained. None of the dietary treatments induced any mortality in the experimental P_0 groups.

5.3.5 Phenotypic Parameters

5.3.5.1 Parental Population (P_0)

Body mass (g) of all adult quail was recorded with 7-day periodicity and every time that oxygen consumption was estimated for each particular animal. Body mass in grams (g) was recorded by placing the bird on a top balance (Mettler Toledo, XA105 DualRange).

Oxygen consumption was estimated by flow-through respirometry. A gas mixture of 79% N_2 and 21% O_2 (Model 0154 Brooks Instruments) was delivered to a 500ml

aluminum respirometer chamber and maintained at a flow rate of ~370 ml*min^{-1} at 23 ºC in darkness, inside of an incubator. The gas mixture was controlled with a mass-flow control Model R-1(Applied electrochemistry Inc.) (Fildes et al., 2009). The gas from the outlet of the respirometer was passed through Drierite to absorb any humidity before analysis in a FC-1B O_2 Analyzer (Sable Systems International). Cloacal temperature of the animals was measured after measurements. After oxygen consumption measurement, all birds were checked for signs of injury and returned to their respective husbandry-container.

At the end of the crude oil exposure period, adult quail were anesthetized by placing them into a sealed container with a cotton humidified with isoflurane. Anesthetized birds were placed in a dissection tray, and the head of the bird was inserted into a tube connected to a vaporizer to maintain constant flow of isoflurane vapors to maintain anesthesia. An incision was made at the top of the breast area to obtain an unobstructed view of the heart under the sternum. Blood analysis was performed as described elsewhere (Flores-Santin et al., 2018; Tazawa et al., 2002). In brief, 400 µl blood samples were obtained directly from the right brachiocephalic artery using a 1 ml syringe previously heparinized, and placed in sterile 1.5 ml Eppendorf tube. Blood collection was performed within 1 minute. Immediately after collection, the samples were processed and analyzed as follows; 120µl of were analyzed for pH, pO_2 and pCO_2 in mmHg, SO_2 (blood oxygen saturation as a % of 100%), and [HCO_3] (mmol/L) using a blood gas analyzer ABL5 (Diamond Diagnosis); 10µl were used to estimate MCV and Hgb using an hematology analyzer (Beckman Coutler *diff*); 10µl were used to estimate osmolality with a vapor pressure osmometer (Vapro 5520); 10µl were used to estimate lactate

concentration (mmol/l) with a lactate meter (Nova biomedical - 39654); finally 140 µl of blood were used to measure hematocrit in duplicate using 70µl capillary tubes (Fisher Scientific 22-362-566) and spun for 5 min in a micro hematocrit centrifuge (LW Scientific M24). With exception of the hematocrit, the other of the measurements were obtained within 2 min after the blood was sampled.

Immediately after blood extraction, the anesthetized birds, were decapitated to assure death. The major organs (brain, eyes, lungs, heart, liver, spleen, kidneys, stomach and intestines and gonads) were excised and weigthed. Tissues were fixed in zinc-formalin solution (Z-fix, Anatech LTD).

5.3.5.2 Offspring Population (F_1)

Egg mass was obtained immediately after collection by placing each egg on a digital balance (Mettler Toledo, XA105 DualRange). Egg length and width were obtained to the nearest mm using a caliper.

Water vapor flux through the eggshell was determined by measuring the mass of the intact egg, after which the egg was placed into a sealed container filled with Drierite and allowed to settle for 24 h. After this period, water loss throughout the eggshell was calculated as the difference in mass before and after the desiccation period (Ackerman and Rahn, 1981; Paganelli, 1980; Spotila et al., 1981).

Summit metabolic rate (maximum oxygen consumption reached by thermoregulation), the maximum rate of resting metabolic thermogenesis (Minnaar et al., 2014), was measured in the 1 week old F_1 hatchling. To achieve M_{sum}, hatchlings were deprived from food 1 h prior to start of the oxygen consumption measurement. Each bird was placed into a ~250ml metal can with an inlet and outlet port affixed in the lid of the

chamber. Temperature of the chamber was controlled by placing the chamber into an incubator previously set at 37 ºC for acclimation period. A thermocouple, adapted with a plastic base, passing through lid of the chamber was inserted into the hatchling's cloaca and glued to the feathers, was used to record body temperature (Tb) during measurements. A gas mixture of 79% N_2 and 21% O_2 was provided to the respirometer at a constant flow rate of ~360 ml*min^{-1}. As for the apparatus for the parental population, the gas from the outlet of the respirometer was passed through Drierite to absorb any humidity. $\dot{M}O_{2sum}$ was achieved by exposure to cold. The initial temperature for the test was set at 37 ºC, and each bird was allowed to acclimate at this temperature during 45 min. Then, temperature was progressively decreased by 3ºC every 30 min (Swanson and Liknes, 2006). After oxygen consumption measurements, birds were checked to ensure no injury and then returned to their respective husbandry-container. After oxygen consumption measurements were finished, the hatchlings were anesthetized with isoflurane in a sealed container and allowed to breathe the vapors for 5 min before decapitation.

Hematological variables of the F_1 offspring, were measured as for the parental population. Because of the small size of the animals (~5.42g) the maximum sampled blood volume was 300µl.

As for the adult birds, the offspring quail were immediately decapitated while still under anesthesia after blood collection, all the major organs were excised, weighed and fixed. Organ mass data was normalized to whole organismal body mass.

5.3.6 Statistical Analysis

Body mass and basal metabolic rate of the adult quail were compared among

groups with three way ANOVA, using experimental group, sex and time as factors. Hematological variables and organ mass were compared between groups with one-way ANOVA. All variables of the offspring population were analyzed with one way ANOVA. Statistical significance was considered with a p value equal or lower than 0.05 and alpha value of 0.05 and data is expressed as mean ± s.e.m.

5.4 Results

Total sum of PAH concentrations in the dietary treatments was 0, 0.8 and 2.4 µg/g for the Control, 1% and the 10% HEWAF diets (Fig. 5.1).

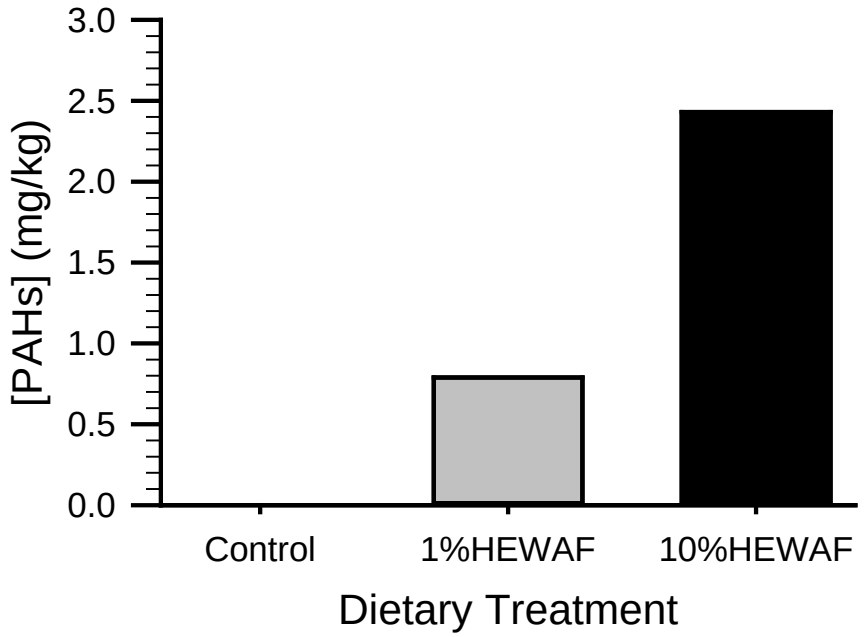

Figure 5.1: Concentration of PAHs in dietary treatments of the adult population.

5.4.1 Parental Population

None of the dietary treatment induced any mortality in the experimental groups. Indeed, we did not observed changes in appetite. However, the birds exposed to 10% HEWAF experienced diarrhea.

5.4.1.1 Body Mass

Neither experimental treatment nor the weeks of crude oil exposure, had an effect on the body mass of the adult population (P>0.05). However, female birds exhibited statistically larger body mass (58.7g to 63.8g) in comparison with male birds (44.5g to 45.8g) from the beginning to the end of the experiment (Fig. 5.2).

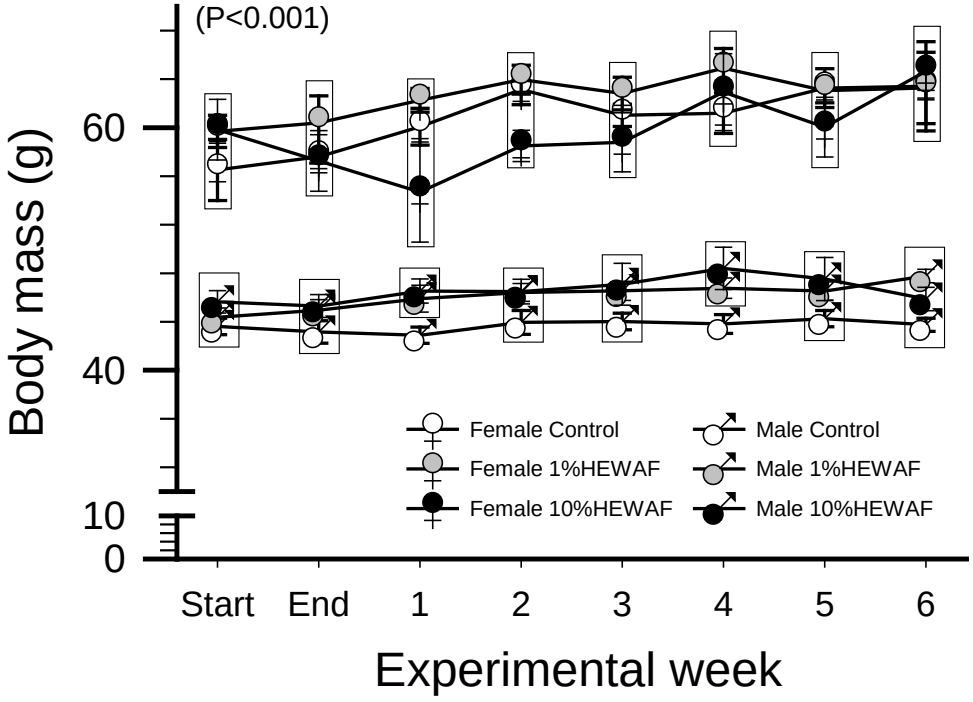

Figure 5.2: Female and male adult quail body mass through experimental period. "Start" and 'End" refer to the start and the end of the acclimation period. Data within boxes are not statistically significant. n = 9-10 per data point.

5.4.1.2 Basal Metabolic Rate (BMR)

Metabolic rate of the 10%HEWAF increased progressively during experimentation from 5.1 to 5.9 ml*g*h. In comparison with the Control and the 1% HEWAF groups, the 10%HEWAF exposed group exhibited statistically higher oxygen consumption values than the other two groups (Fig. 5.3). The difference was evident after the third week of exposure.

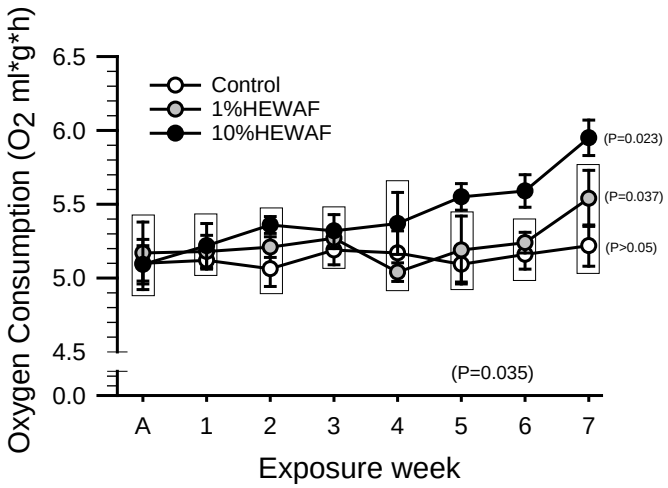

Figure 5.3: Oxygen consumption in the adult groups. "A" refer to the measurement acquired in the las 5 minutes of the acclimation period. n = 9-10 per data point. Data within boxes are not statistically significant. n=4 per data point.

5.4.1.3 Hematological Variables

Dietary exposure to crude oil induced changes in three of the measured hematological parameters. The control group exhibited the lowest P_{CO2} levels in comparison with the two HEWAF-treated groups (Table 5.1). However, the oil treated groups exhibited lower P_{O2} and S_{O2} levels in comparison with the control groups. None of the other variables were statistically different among groups.

Table 5.1: Hematological variables for the adult (P_0) and the offspring (F_1) populations. Different superscript letters indicate statistical differences among groups.

	P0			F1		
	Control	1% HEWAF	10% HEWAF	Control	1% HEWAF	10% HEWAF
N	9	10	10	17	22	20
pH	7.1 ± 0.4	7.4 ± 0.1	7.4 ± 0.1	7.4 ± 0.04	7.4 ± 0.0	7.5 ± 0.02
P_{CO2} (%)	25.8 ± 0.8^{a}	27.5 ± 1.3^{b}	31.7 ± 0.7^{b}	39.5 ± 3.7	36.2 ± 3.3	31.5 ± 0.8
PO_2 (%)	87.6 ± 0.6^{a}	88.6 ± 0.6^{a}	85.6 ± 0.6^{b}	99.5 ± 4.8	99.3 ± 5.1	98.9 ± 3.2
SO_2 (%)	97.5 ± 0.3^{a}	96.2 ± 0.5^{b}	94.8 ± 0.3^{b}	95.5 ± 1.8	94.0 ± 2.6	97.2 ± 0.5
HCO_3 (mmol/L)	22.7 ± 0.9	23.1 ± 0.6	21.8 ± 0.6	22.4 ± 1.1	21.7 ± 0.5	24.5 ± 0.7

(table continues)

	P0			F1		
	Control	1% HEWAF	10% HEWAF	Control	1% HEWAF	10% HEWAF
Hgb	12.5 ± 0.5	11.9 ± 0.3	11.8 ± 0.5	10.3 ± 0.3	10.5 ± 0.2	10.4 ± 0.3
OSM	305.8 ± 0.9	305.0 ± 1.3	305.3 ± 1.8	297 ± 2.8	298.8 ± 3.2	291.3 ± 2.1
HcT (%)	28.9 ± 4.6	27.4 ± 6.1	34.2 ± 3.6	29.2 ± 2.5	32.9 ± 0.7	32.3 ± 0.8
Lac (mmol/L)	3.3 ± 0.4	3.8 ± 0.6	4.0 ± 0.4	4.1 ± 0.6	4.7 ± 0.5	5.5 ± 0.9

5.4.1.4 Hematocrit

There were no statistical differences in the hematocrit levels (~30.2 ± 4.8 %) among the parental experimental groups (Fig.5.4).

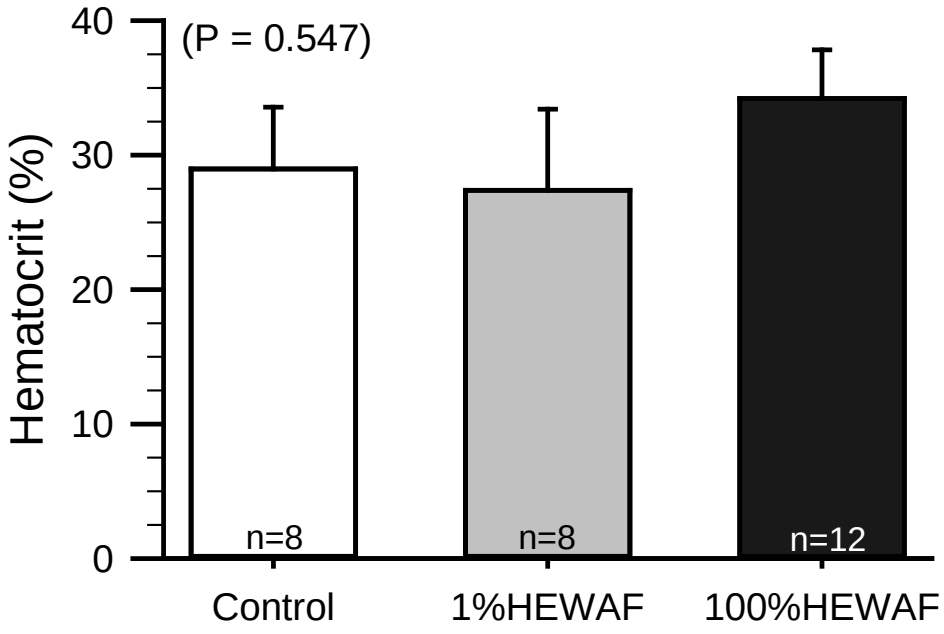

Figure 5.4: Hematocrit percentage in the parental population.

5.4.1.5 Organ Mass

The heart was the only organ exhibiting a difference in mass among experimental groups (Table 5.2). Heart mass was smaller in both crude oil-treated groups (0.7 ± 0.04

g for 1% HEWAF and 0.7 ± 0.02 g for 1% HEWAF) than the control group (0.8 ± 0.04 g %).

Table 5.2: Organ mass as percentage (%) of body mass, for the adult (P_0) and the offspring (F_1) populations. Different superscript letters indicate statistical differences among groups.

	P_0			F_1		
	Control	1%HEWAF	10%HEWAF	Control	1%HEWAF	10%HEWAF
n	8	10	10	8	11	10
Heart	0.8 ± 0.04[a]	0.7 ± 0.04[b]	0.7 ± 0.02[b]	1.2 ± 0.04	1.2 ± 0.04	1.1 ± 0.03
Liver	2.4 ± 0.3	2.4 ± 0.2	2.4 ± 0.2	4.3 ± 0.4	4.4 ± 0.2	4.7 ± 0.3
Lungs	0.8 ± 0.06	0.8 ± 0.05	0.7 ± 0.04	0.7 ± 0.04	0.8 ± 0.04	0.8 ± 0.02
Gut	2.1 ± 0.1	2.3 ± 0.2	2.2 ± 0.2	5.7 ± 0.9	6.3 ± 0.4	6.3 ± 0.5
Eyes	0.8 ± 0.1	0.7 ± 0.04	0.7 ± 0.04	2.5 ± 0.2	2.3 ± 0.12	2.2 ± 0.1
Brain	1.0 ± 0.1	0.9 ± 0.05	0.9 ± 0.05	3.3 ± 0.2	3.2 ± 0.2	2.9 ± 0.1
Kidneys	0.4 ± 0.1	0.7 ± 0.09	0.4 ± 0.04	1.0 ± 0.04	1.0 ± 0.08	1.1 ± 0.03
Ceca	0.8 ± 0.06	0.9 ± 0.07	0.8 ± 0.06	1.3 ± 0.2	1.1 ± 0.08	1.3 ± 0.2

5.4.2 Offspring Population (F_1)

None of the egg samples from the parental groups exhibited any detectable content of PAHs after analysis.

5.4.2.1 Egg Metrics

The mass of the eggs obtained from the 1% and 10% HEWAF-fed groups exhibited higher values in comparison with the eggs from the control parental group. These egg mass differences were constant from the beginning to the end of the exposure and are not attributable to the exposure treatment. Egg length and width were not different among groups (Fig.5.5)

Neither the weeks of exposure nor parental exposure experience had significant effects on egg mass in the offspring population

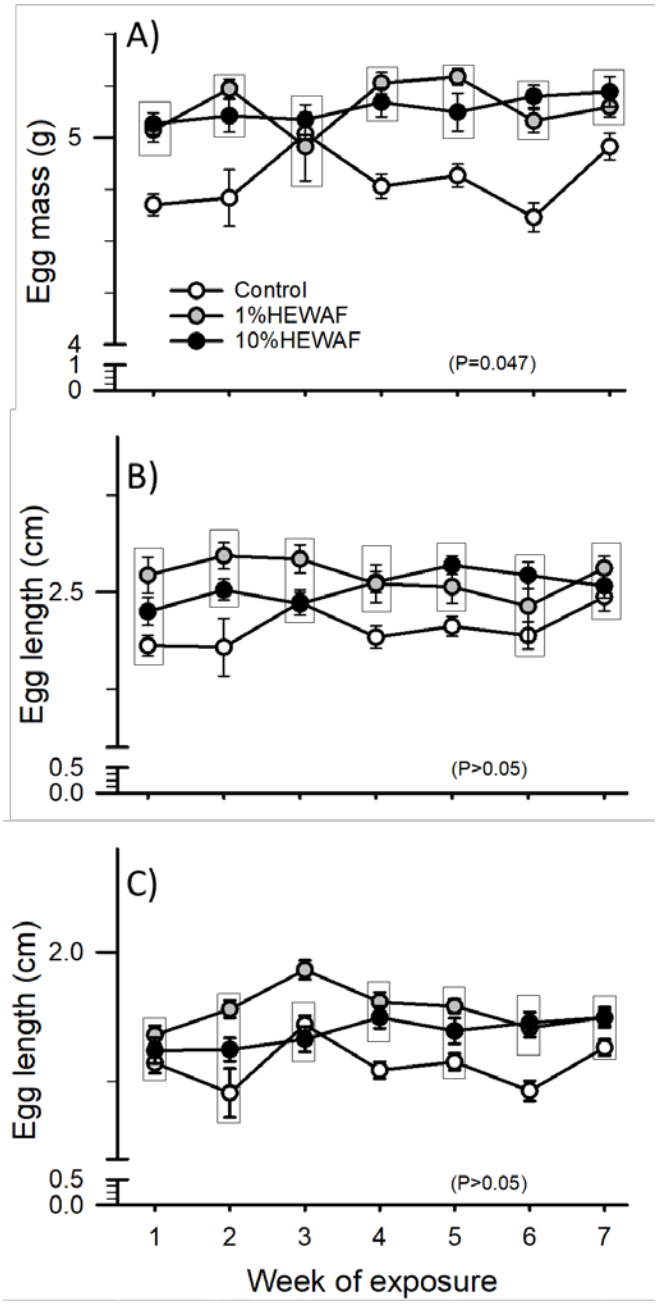

Figure 5.5: Egg metrics throughout experimental time. A) Egg mass, B) egg length, and C) egg width.

5.4.2.2 Water Loss

Water loss was significantly higher in the eggs obtained from the 10%HEWAF group in comparison with the control and the 1% HEWAF offspring, which were not significantly different from each other (Fig. 5.6).

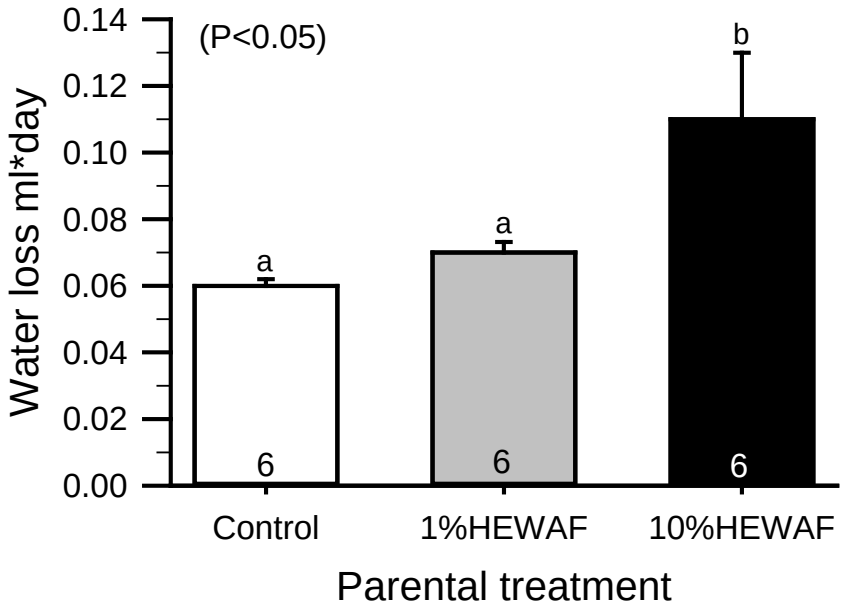

Figure 5.6: Water loss in the eggs obtained from the different parental groups.

5.4.2.3 Hatchling Body Mass

There were no statistical differences in the body mass of 7 day post-hatch F_1 quail among the experimental groups (~8.2g ± 0.3).

5.4.2.4 Summit Oxygen Consumption ($\dot{M}O2_{sum}$)

Metabolic rate in the offspring population differed among experimental groups (Fig 5.7). The offspring obtained from the 10%HEWAF exposed parents exhibited higher metabolic rates than the control and the 1%HEWAF-exposed group. The difference was maintained until 25 ºC, below which the 10%HEWAF group was not able to cope with the decreasing temperature and oxygen consumption began to drop to levels below the level at the start of the experiment. The control and 1%HEWAF group exhibited an increase in metabolism until 22 ºC, a temperature at which both groups reached their maximum O_2 consumption. Below this temperature the control and the 1% HEWAF group dropped to similar O_2 consumption levels as the 10%HEWAF group.

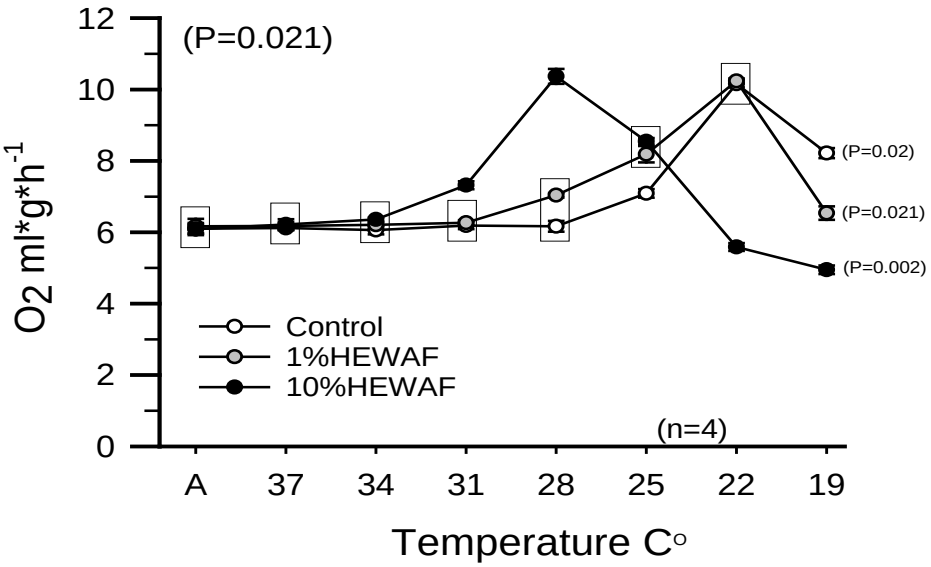

Figure 5.7: Oxygen consumption in the offspring population. Data within boxes are not statistically different.

5.4.2.5 Hematological Variables

There were no significant differences in any of the hematological variables in the F_1 offspring populations (Table 5.1). Similarly, there were no differences in hematocrit values (29.2% ± 2, 32.9% ± 0.8, 32.3% ± 0.8 for Control, 1%HEWAF and 10%HEWAF, respectively) among the offspring populations (Fig.5.8).

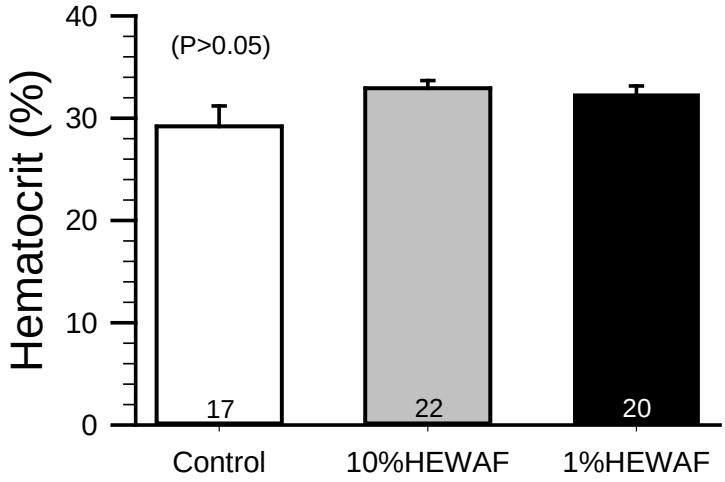

Figure 5.8: Hematocrit values from the offspring groups.

5.4.2.6 Organ Mass

Similarly to the parental population, there were no differences in the organ mass of the offspring (Table 5.2).

5.5 Discussion

5.5.1 Direct Effects of Crude Oil Exposure in the P_0 Parental Generation

Exposure to crude oil in avian species induce life-threatening effects on the exposed organisms. For example, impairment of thermoregulatory capacities, decrease in buoyancy and changes in navigational abilities have been reported in cormorants, gulls and western sandpipers after dermal exposure to crude oil (Harr et al., 2017c; Maggini et al., 2017a). In addition, dietary exposure to crude oil induced loss of appetite, loss of weight, diarrhea, and hepatic and renal diseases (Burger, 1997; Harr et al., 2017a; Harr et al., 2017b). In contrast, to these studies, in the present study we did not observed changes in appetite or loss of body mass in the exposed groups (Fig. 5.2). In addition, none of the dietary treatments induced mortality in the experimental groups. However, we did observed diarrhea in the 10%HEWAF group, suggesting a potential disruption of the hepatic function and the digestive tract integrity.

Respiratory distress (changes sin ventilation rates) due to crude oil exposure has been reported previously in birds (Mazet et al., 2002). Here we reported that exposure to 10%HEWAF elicited higher oxygen consumption rates evident after the third week of exposure (Fig. 5.3). This is consistent with the time line of an increase of flight energy cost up to 45% in western sandpipers exposed to oil (Maggini et al., 2017b). In addition, we found higher levels of P_{CO2} and lower levels of P_{O2} and S_{O2} in the blood of treated animals (Table 5.1) suggesting an impairment in blood O_2 transport function. Similar

results have previously been reported as a side effect of the hemolytic anemia caused by oil exposure in the double crested cormorant (Harr et al., 2017a). However, we found no significant differences in the hematocrit of the various groups. Thus, future studies focused on determining the molecular implications of oil exposure in hemoglobin function are needed.

5.5.2 Transgenerational Effects of Crude Oil in the F_1 Generation

Crude oil exposure and its components induce transgenerational effects in fish (Bautista and Burggren, 2019; Corrales et al., 2014). However, the study of transgenerational effects of crude oil exposure in avian species has received little attention. In the present study we found that although morphological characteristics of the eggs (Fig. 5.5) and hatchlings (Table 5.2) may not be impaired by the parental exposure, their responses at the physiological level may not be consistent. For example, egg water loss in the offspring obtained from the 10% HEWAF-exposed parental group was increased compared to the control group (Fig. 5.6). Maintenance of humidity and temperature is of critical importance for eggshell quality and the calcification process during the egg's passage through the female oviduct, thus affecting survival during early development in avian embryos (Branum et al., 2016; Yang et al., 2010). Our results showing modified water vapor flux across the egg shell in the experimental groups suggest a functional disturbance of the female oviduct due to stress that potentially arose from the oil exposure, as has been reported other environmental stressors (Mazzuco and Bertechini, 2014; Stewart and Munn, 2014). Furthermore, the survivability of the eggs while facing fluctuating environmental conditions may be impaired (Lewis and Malecki, 1984).

Similarly to the parental population, hematological variables and the mass of the organs did not significantly differ among offspring groups (Table 5.1-5.2). However, at the whole organismal level, the 10% HEWAF offspring reached their maximum summit oxygen consumption rate at higher temperatures in comparison with the control and the 1% HEWAF groups. Noteworthy is the fact that the offspring obtained from the 10% HEWAF-exposed parental group also exhibited decrease in oxygen consumption at higher temperatures, when they were no longer able to cope with declining temperature. To our knowledge this is the first study to demonstrate that crude oil exposure in a parental population affects the F_1 offspring respiratory physiology and performance. However, studies aimed to determine if these effects still present in adult F_1 offspring, are highly warranted.

Finally, it is widely accepted that the development of the subsequent generation is affected by the yolk components deposited by the mother (Guerrero-Bosagna et al., 2018). However, the fact that in the present study there were no PAHs detected in the offspring from the different parental groups. This suggests that there were no direct effects of PAHs in the offspring, however, it is possible that different components may be inherited from the mother through the yolk (Hagmayer et al., 2018). Consequently, the effects seen in the offspring population may well be due to the inheritance of epigenetic markers

5.6 Conclusions and Future Directions

Despite the current knowledge of the devastating effects of external exposure to petroleum on the mortality of the exposed populations, only a few studies have attempted to understand the effects of different routes of exposure and their consequences for avian

species (Harr et al. 2017; Dean et al. 2017; Murphy et al. 2016). Although dietary exposure to crude oil may not lead to increased mortality in the exposed birds, it may affect their physiological functions. The present study has demonstrated that exposure to a parental generation to crude oil via diet may impact the performance of the subsequent immediate generations. Thus, to better understand the impacts of environmental stressors such as crude oil on organism and populations, future studies should focus on exposure effects on more than one generation. In addition, as responses at the whole organismal level may not be complementary with molecular responses, a multi-level approach aimed to decipher the molecular basis of these effects will provide more complete answers.

CHAPTER 6

OVERALL CONCLUSIONS AND SYNTHESIS

6.1 Summary of Contributions of this Dissertation

The research developed in this dissertation has contributed to several areas of investigation including molecular biology, animal physiology, and evolutionary biology. The quantitative information from these studies may be utilized to supplement information regarding the proximate and ultimate effects of environmental stressors on fish and bird populations. Furthermore, this information may be used as additional support for understanding the conservation of the responses from the molecular to the whole organismal levels across the vertebrate taxa, as well as their implications for population survival and maintenance.

6.2 Summary of the Most Important Contributions of this Dissertation

1) Determination of the proximate effects of dietary exposure to crude oil within environmentally relevant concentrations from the molecular to the behavioral levels in piscine and avian models. (Chapters 2, 3, 4 and 5)

2) Determination of the proximate effects of dietary exposure to crude oil on the fecundity and the performance of behaviors associated with reproductive success in piscine models. (Chapter 2)

3) Providing quantitative information supporting the idea hypothesis that transgenerational epigenetic inheritance may result in both adaptive and maladaptive phenotypes in offspring generations obtained from exposed parents. (Chapters 2 and 3)

4) Determination of the separate contributions of the maternal and the paternal lines of inheritance on offspring survival and performance by means of epigenetic inheritance in a piscine model. (Chapter 3)

5) Determination of the proximate respiratory physiological effects of the dietary exposure to crude oil in an avian model. (Chapter 5)

Table 6.1: Summary of specific hypothesis of each chapter and decision

Chapter	Hypothesis	Accepted/ Rejected
Chapter 1. General Introduction	Epigenetic transgenerational inheritance will arise from the dietary exposure to crude oil regardless of the vertebrate group tested.	Accepted
Chapter 2. Parental Stressor Exposure Simultaneously Conveys Both Adaptive and Maladaptive Larval Phenotypes through Epigenetic Inheritance in the Zebrafish (*Danio rerio*)	Dietary exposure to crude oil, within environmentally relevant levels, will affect primary indicators of fish health such as body mass and length, organ mass, condition factor (K) and Specific Growth Rate (SGR) (Barnham and Baxter, 1998; Cook et al., 2000; Williams, 2000).	Rejected
	Dietary exposure to crude oil, will affect variables directly related to reproductive success such as fecundity, fertilization and egg, and sperm quality in the parental population.	Accepted
	Dietary exposure to crude oil, will induce tissue disruption of the gonads and cardiac collagen deposition, which have been associated with oil exposure (Chablais et al., 2011; Gemberling et al., 2013; Grivas et al., 2014; Horn and Trafford, 2016; Kikuchi, 2014; Marro et al., 2016).	Rejected
	Dietary crude oil exposure of a parental population will enhance resistance of their larvae (i.e. enhance their survival and heart rate) by means of non-genomic inheritance.	Accepted
Chapter 3. Maternal and Paternal Transgenerational Epigenetic Effects of Dietary Crude Oil Exposure in the Zebrafish	Regardless of the gender of the fish, dietary exposure to crude oil will not affect phenotypic traits directly related with fish health such as growth and heart rate on the pare.	Accepted
	Regardless of the gender of the fish, dietary exposure to crude oil will affect the levels of global DNA methylation in the gonadal and heart tissues of the parental population.	Accepted
	Regardless of the gender of the fish, dietary exposure to crude oil will affect the patterns of expression of histone-related antibodies (associated with gene expression and silencing) in the gonadal and heart tissues of the parental population.	Accepted
	Regardless of the gender of the fish, dietary exposure to crude oil will elicit the upregulation of genes related with cardiac development, stress and DNA methylation maintenance.	Rejected

(table continues)

Chapter	Hypothesis	Accepted/ Rejected
	The maternal line of inheritance will better enhance the survival and performance of its offspring in comparison with the paternal line.	Rejected
	Global DNA methylation and the patterns of expression of histone-related antibodies will change in the offspring populations regardless of the parental line exposed.	Accepted
	Regardless of the parental line exposed to petroleum the transcript levels of genes involved in heart development, stress and maintenance of DNA methylation in offspring will increase in comparison with offspring from non-exposed parents.	Rejected
Chapter 4. Behavioral Consequences of Dietary Exposure to Crude Oil Extracts in the Siamese Fighting Fish (*Betta splendens*)	Dietary exposure to crude oil will not impact primary indicators of fish health in the betta fish.	Accepted
	Dietary exposure to crude oil will increase the aggressivity levels in the betta fish.	Accepted
	Dietary exposure to crude oil will affect the performance of behaviors related with reproductive success in the betta fish.	Accepted
Chapter 5. Transgenerational Effects on Respiratory Physiology in the King Quail (*Coturnix chinensis*)	Dietary exposure to crude oil will not affect primary indicators of bird health such as body mass.	Accepted
	Dietary exposure to crude oil will affect the hematological variables of the parental population.	Partially accepted
	Dietary exposure to crude oil will affect the organ mass of the parental population.	Rejected
	Dietary exposure to crude oil will increase oxygen consumption of the parental population.	Accepted
	The parental exposure to crude oil will affect morphometric and physiological characteristics such as mass, length, width and water loss of the eggs of their offspring.	Partially accepted
	The parental exposure to crude oil will affect hatchling mass and their capacity to cope with low temperatures.	Accepted

6) Quantitative information for the transgenerational effects on eggshell morphology and physiology that arose from the parental exposure to crude oil in an avian model. (Chapter 5)

7) Quantitative information for the transgenerational effects on hatchlings physiology that arose from the parental exposure to crude oil in an avian model. (Chapter 5)

6.3 Summary of Hypotheses

Table 6.1 lists each specific hypothesis raised in each chapter and their status based on the findings of these studies.

6.4 Broad Context

The findings of this dissertation offer new basis for understanding the importance of transgenerational epigenetic inheritance in organismal survival, and their overall capacities to cope with environmental stressors. Although the definite implications for speciation and evolution are still to be discovered, this dissertation provide quantitative information that supports the fact that epigenetic inheritance may help organismal populations to bridge environmental stressors.

To better understand the role of epigenetics, we need to consider that the presence of epigenetic markers, and thus the modified phenotypes that they induce, may be present in species for decades without getting fixated in the genome (Cubas et al., 1999; Katz et al., 2009). However, even when genomic assimilation has not been induced, epigenetic modifications may induce variation subject to natural selection (Klironomos et al., 2013), potentially leading to epigenetically-driven evolution (Burggren, 2016; Burggren and Crews, 2014; Jablonka, 2017; Jablonka and Lamb, 2017). This information is further supported by the fact that some epigenetic mechanisms, like specific chromatin configurations, may initiate molecular loops, the products of which (i.e. methylation

patterns or protein formation) may enhance its own maintenance (Chen et al., 2016a). Thus, the preconditioned notion (central dogma) that parent to offspring transmission of information must involve replicative processes deserves rethinking. Furthermore, as more raw data and information is discovered, the concatenation of development, organismal variation, epigenetics inheritance, natural selection, speciation and evolution is being slowly decrypted.

6.5 Final Remarks

The research developed for this dissertation has shown that transgenerational epigenetic inheritance could arise from the parental exposure to environmental stressors in vertebrate populations. Additionally, the zebrafish (*Danio rerio*), the Siamese fighting fish (*Betta splendens*) and the king quail (*Coturnix chinensis*) have proven to be excellent models to start building a strong basis for understanding the effects of environmental stressors and transgenerational epigenetic phenomena using a multi-level approach. The results reported throughout this dissertation provide supportive information for guiding and interpreting new findings obtained from future research focused in deciphering the molecular mechanisms and their implications on whole organismal and even population maintenance under an ecological-focused approach. In this sense, studies aimed to determine epigenetic phenomena in the wild and its implication in this context are increasingly needed and demanded.

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