Produced water from North Sea offshore oil platforms causes a suite of biological effects on developing lumpfish (*Cyclopterus lumpus*) eggs.

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Environmental Toxicology
Submission date: September 2020
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Acknowedgements

This Master's thesis was written at the Department of Biology at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway. The experimental work was performed at the Center of Fisheries and Aquaculture (Sealab), NTNU, in conjunction with SITNEF Ocean.

The writing of this thesis was done under the guidance of professor Elin Kjøsrvik, at the Department of Biology at NTNU, and Bjørn Henrik Hansen, at SINTEF Ocean. I would like to thank both of my advisors for the time they invested in me throughout this process and especially for their support and understanding throughout the COVID pandemic.

I would also like to especially thank Tora Bardal for her time and invaluable knowledge and guidance throughout the analytical process.

I would finally like to thank my parents, who have supported me from afar, and my friends, without whose support my success would not have been possible.

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Abstract

This study investigated the impacts of several produced waters (PWs) from the North Sea on developing *Cyclopterus lumpus* larvae. Larvae were exposed for 96 hours early in development to 3 concentrations of 5 different PW solutions. They were then allowed to depurate until hatch. The results clearly illustrate that two of the five tested PWs are acutely toxic to embryos, even in the lowest solutions. These solutions resulted in little to no survival and hatching. Surviving larvae of the other three PWs demonstrated a myriad of sublethal effects including reduced standard lengths, reduced spinal ossification, reduced craniofacial growth. Larvae also exhibited signs of stress and extensive skeletal abnormalities. Several potentially novel phenotypes are described. The organic phase hydrocarbon components of each PW, including phenols and several groups of PAHs, were leveraged as explanatory factors. However, much of the variation in the data cannot be explained by the impact of PAHs alone. It is clear that other components, likely chemicals added to improve oil and gas production, which were not detected by the GC-MS, are equally as important in determining the toxicity of some PWs as hydrocarbon contaminants.

Keywords: produced water, *Cyclopterus lumpus*, acute toxicity, sublethal toxicity, skeletal development, skeletal abnormalities, oil production

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1. Introduction

1.1 Produced Water

Industrialization of the oil and gas industry, specifically in offshore and deep-sea marine environments, has many ways of impacting the marine environment. Acoustic surveying, oil leaks and spills, sediment cuttings, and fuel and turbine exhaust are all commonly known ways in which offshore rigs negatively impact the surrounding environment. Another such area of potential impact is produced water (PW), the complex mixture of water and dissolved chemicals and compounds that comes to the surface during oil and gas extraction.

PW, also referred to as "formation water" or "brine", likely represents the largest direct discharge of effluent into the marine environment worldwide (NOROG, 2017; Bakke et al., 2013; Neff et al., 2011). An estimated 250 million barrels of produced water are emitted worldwide daily, more than three times the approximately 80 million barrels of oil produced daily. The exact amount of PW from either a single or set of platforms is generally roughly estimated rather than precisely quantified. However, on the Norwegian continental shelf (NCS) alone, 140 million m^3 of PW has been released directly into the ocean from offshore production platforms yearly since 2003 (NOROG, 2018). This discharge occurs primarily in the epipelagic and mesopelagic zones, habitat for many commercially important fish. This discharge is also subject to offshore currents, resulting in chemical drift to areas far from the site of exposure and making risk assessment difficult.

1.1.1 Composition of PW

PW is composed of injection water, formation water (the natural water trapped in the reservoir system), naturally occurring substances within the water, including metal ions, radioactive materials, salts, dissolved solids, oil and grease, and any chemicals used in the oil extraction process.

The chemical composition of PW is largely unknown, varies over the life of the platform, and undergoes change upon release due to weathering, biodegradation, dilution, and other natural processes. However, one constant constituent, and one of the most important is oil concentration. While the average oil content of released PW on the NCS was 12.1 mg/L in 2017, below the official requirement, this still resulted in approximately 1,722 tons of oil, in various forms, being directly released to the ocean. That volume is equivalent to nearly 13,000 barrels of crude oil and thus represents the largest source of operational oil spills from the offshore oil industry (NIVA, 2019; NOROG, 2018). Moreover, the ratio of PW to oil production has shown a general increasing trend on the NCS, primarily due to the increasing average age of oilfield (NOROG, 2018; NOROG, 2012). This means that the amount of oil directly released has increased in parallel.

As a general rule of hydrocarbon production, excluding coal bed methane, the hydrocarbon-to-PW ratio is high in the beginning and decreases over the life of the platform. Number and concentration of added chemicals typically follow a similar trajectory, increasing over the life of the well as oil extraction becomes less efficient (U.S. EPA, 2019; Neff et al., 2011; Clark & Veil 2009).

Chemicals are used throughout the process of oilfield development to overcome operational inefficiencies. During production, chemical additives (also known as production chemicals, hereafter referred to as PC) are considered vital to preventing the formation of scale, which would impede the extraction and decrease not only the life of the well but also the efficiency of extraction and potentially the purity of the product (U.S. EPA, 2019; NOROG, 2018). PCs

include (1) descaling agents (2) hydrate inhibitors, (3) corrosion inhibitors, (4) oxygen scavengers, (5) biocides, (6) emulsion breakers, (7) antifoam agents, and (8) asphaltene control agents, (9) pH adjusters, among others (Exxon Mobil 2007; Henderson et al., 1999; Hudgins, 1994). These individual additives are typically found in proprietary mixtures to varying amounts, concentrations, and complexity. Moreover, the exact blend is likely to change throughout the platform and oilfield's lifetime as conditions of extraction change.

1.2 Regulation

Regulatory authorities classify known substances discharged into the marine environment according to environmental risk (xødirektoratet, 2020; U.S. EPA, 2019; OSPAR, 2017; Hart et al., 1995). Risk becomes increasingly more difficult to measure in mixtures because the number of individual chemicals, the number of potential concomitant interactions, and the number of metabolites, which have their own effects, increases. Quantifying probability of exposure, an essential component of risk, to marine toxicants is also difficult due to the rapid dilution and complex movement patterns (Johnsen et al., 1998).

Current PW-treatment techniques applied on Norwegian oil platforms prioritize the removal of free oil to 30 milligrams of oil per liter (NOROG, 2018; WEF, 2018). In some instances, PW treatment further includes the removal of specific toxic additives that are known to be especially toxic to wildlife. These techniques, unfortunately, allow many small and dissolved compounds to remain, including dispersed and dissolved oil. They also cause fragmentation of larger particles and dispersion of particles of all sizes over a large, and sometimes previously uncontaminated, volume (Hayworth and Clement, 2011; Ahmadun et al., 2009).

Dispersed and dissolved oils are notoriously difficult to eliminate from the environment and have generated a host of literature on their toxic effects in fish (Hansen et al., 2019; Beyer et al., 2016; Tissier et al., 2015; Dussauze et al., 2014; Incardona et al., 2013; Agamy 2012; Baussant et al., 2010). Dissolved PAHs, specifically, from oil exploration at concentrations as low as <1 ug/L total PAH have repeatedly demonstrated embryotoxic effects in fish (Carls et al., 2008; Carls et al., 1999; Marty et al., 1997).

1.3 Toxic effects

Petrogenic chemicals can induce toxicity both acutely and chronically (Casarett and Doull, 2013; Willows 1994; Julliard et al., 1993). While PW discharge has been considered both too low in concentration of toxic elements and too quickly diluting to have an effect, research on the endocrine system suggests that chronic toxic effects may be occurring in PW-exposed fish populations (Camus et al., 2015; Neff et al., 2011; Heintz et al., 2000; Henderson et al., 1999). Even after extreme dilution, PW-exposed fish illustrate developmental effects, including mortality (Meier et al., 2010). Moreover, acute toxicity of PW has been documented in several cases (Sørensen et al., 2019; Neff et al., 2011).

The physicochemical properties of PW may in some cases be further broken down or extracted into fractions in order to facilitate study (Sørensen et al., 2019). In this study, neither inorganics nor particles are included in the extraction procedure. Production chemicals are often excluded by this extraction procedure as well.

1.3.1 Organic phase

The two main fractions of the organic phase are composed of only DCM-extractable components in either the hydrophilic, or water-soluble, and hydrophobic, or lipid-soluble, fractions. Sørensen et al. (2019), demonstrates large variation in both the amount of

extractable compounds in various PWs as well as the ratio of each fraction within the organic phase. It is yet unclear which fraction is the more important contributor to toxicity, but it is likely that each contribute to different aspects of toxicity (Hansen et al., 2019; Sørensen et al., 2019; Farmen et al., 2010; Tollefsen and Nilsen, 2008).

Another means of quantifying the amount of soluble compounds in the PW sample is by examining the total extractable material (TEM). TEM can be more encompassing than methods identifying and measuring individual PAHs and has been predicted to be a primary explanatory factor of differences in hatching and survival (Bostick et al., 2002).

The hydrophilic fraction of PW may consist of a wide variety of compounds, including ionic metals in various forms, particulate matter, and a large portion of PCs and their degraded products. Heavy metals and metals present in substantially higher concentrations than normal seawater can be dangerous to wildlife. Concentrations of metals above a threshold concentration is documented as altering cell signaling and movement during gastrulation causing large phenotypic consequences including skeletal deformities, delayed development, and altered timing regimes (Jaishankar et al., 2014; Kobayashi and Okamura, 2004; Hardin et al., 1992). These components are not efficiently removed from PW during treatment and thus may enter the environment in relatively high concentrations (Bakke et al., 2013; Neff et al., 2011). Despite their potential impact, these compounds are not part of the extracts used as treatment conditions in this study.

Hydrophobic fractions of PWs have been investigated in previous studies and illustrate a wide range of detected concentrations both across the world and within the North Sea (Blondes et al., 2018; Lourenço et al., 2018; Neff et al., 2011). Hydrophobicity of a compound strongly correlates with the dose found in the target tissue and ability to bioaccumulate and biomagnify (Casarett and Doull, 2013; Boelsterli, 2007). For many PAHs, it is also strongly related to traditional toxicity parameters, like LC50 and EC50, and to embryotoxicity (Hodson et al., 2017; Lin et al., 2015). The dissolved portion of the hydrophobic fraction notably contains aromatic hydrocarbons like BTEX (benzene, toluene, ethylbenzene, and xylenes), phenols, and saturated alkanes. It also contains PAHs, both low-molecular weight, like naphthalenes, phenanthrenes, and fluorenes, as well as high-molecular weight PAHs, like pyrenes, chrysenes, anthracenes and fluoranthenes.

Many BTEX compounds have a high rate of volatilization, so toxic effects are likely to be limited to organisms in close contact with discharged PW. Phenols, however, are a diverse group found in PW effluent. Generally, short-chain phenols are the most abundant and have few toxic effects. Phenol toxicity, generally, is related to its rate of substitution and, in parallel, its increase in hydrophobicity. Both geological processes in oil formations and technological processes can result in the formation of long-chain phenols like nonylphenol. Nonylphenol is a potent endocrine disruptor that can disrupt hormonal homeostasis, causing changes in reproductive function and even on the immune system, and even have neurotoxic effects to developing embryonic brains (Jie et al., 2013; Schwaiger et al., 2000; Arukwe et al., 2000).

Many low-molecular weight PAHs are dissolved within the PW. High-molecular-weight PAHs, primarily 4-6 rings, are often found bound to particles and oil droplets due to their high hydrophobicity. Such compounds may be released and become dissolved in the PW when they are found in high enough concentrations or when they are dispersed by physical or chemical processes. In the environment, both particulate and dissolved forms of these PAHs

can pose a threat to developing organisms. Carls et al. (2008) found that, while dissolved PAHs, of both low and high molecular weight, make up only approximately ~15% of the PAHs embryos may be exposed to in oil-water solutions, they still result in toxic effects. Exposure to dissolved PAHs can lead to both acute and chronic effects, including histopathological changes, morphological changes, carcinogenicity, and changes in enzyme expression (Arukwe et al., 2008; Meier et al., 2012; Hansen et al., 2009). Moreover, Heintz (2000) found that incubation of eggs in a PAH-contaminated environment decreases survival rate in salmon embryos.

1.4 Production Chemicals

PCs encompass an expansive number of compounds and mixtures with divergent physiochemical properties. A previous study by Henderson et al. (1999) found that most of the chemicals tested (antifoaming agents, biocides, corrosion inhibitors, and demulsifiers) quickly induced toxic effects in exposed bacteria. Corrosion inhibitors are often lipophilic and have been shown to possess an acute toxicity hazard and bioaccumulative risk as environmental loads grow (DiNica et al., 2017; Hudgens 1991). Similarly, Pillard (1995) found that hydrate inhibitors demonstrate severe acute toxicity to aquatic life at some environmentally relevant concentrations, causing oxalate crystal formation in fish kidney, fish kills, and reduced biodiversity. Biocidal oxidants and electrophiles are well-established to cause toxic effects in higher order organisms, like fish, and especially during sensitive developmental periods (de Olmo et al., 2017; Turkiewiezc et al., 2013). Common surfactants, widely used for the pumping of viscous cude oil, include octylphenols and nonylphenols. Several of their degraded products illustrate endocrine disrupting effects in wildlife when dissolved (Sørensen et al., 2019; Neff et al., 2011).

For many of the added PCs, the primary or parent group of chemicals used has had some risk assessment performed. Generally, PCs are thought to either be removed before release or be heavily diluted beyond a no effect concentration. However, given that some of these chemicals are more soluble in oil than in water, many chemicals may remain in the dissolved portion of the hydrophobic PW and not be removed during treatment. Sørensen et al. (2019), for example, found that metabolites or degradation products of various PCs could still be found in PW and contribute to toxic effects of PW. Despite these documented effects, they are considered to pose little bioaccumulative risk and the proprietary mixtures are not well regulated (WHO 2000; Pillard, 1995). Another concern of these PCs is their potential to increase the partitioning of oil components (aromatic hydrocarbons) to the dissolved phase, thus increasing the concentration and potential toxic effects of PAHs (Henderson et al., 1999).

1.5 Combination effects

While individually the major constituents of these groups are subject to risk assessment and greater research, there are three major ways in which toxic effects may be unwittingly mediated. The first avenue of risk is via bioaccumulation, which is primarily seen in lipophilic compounds. Secondly, toxic effects may not be mediated by the chemical itself, but rather by a metabolite formed during the processes of degradation and/or biotransformation. A variety of well-known environmental toxicants fall into this category including B(a)P, a common petrogenic byproduct, which forms a genotoxic diol-epoxide metabolite. Thirdly, and potentially the greatest toxic threat posed by these additives, is the potential for interactions between chemicals and between chemicals and naturally occurring compounds.

Hypothesized interactions may be as simple as additive or even subtractive effects, however they may also result in synergism or potentiation of the individual chemical’s toxic effect

(Giannapas et al., 2011; Hannam et al., 2009; Bellas et al., 2008). For example, tests on toxicity of ethylene glycol-containing solutions illustrated higher toxicity (Jonker et al., 2016; Pillard, 1995). Co-exposure to naturally occurring nitrite and PAHs has been shown to cause DNA damage in fish, potentially due to the formation of mutagenic nitrated PAHs and nitrated derivates (Wahidulla and Rajamanickam, 2009). Some cases of antagonism or subtractive effects have also been noted, where co-exposure to metals actually reduced tissue PAH levels (Benedetti et al., 2007). Unfortunately, not only are interaction effects nearly impossible due to replicate due to number and environmental conditions, but they are also incredibly difficult to predict.

Combining toxic effects, unexpected environmental changes, and stress during the embryonic period can result in energy imbalances when they exceed maternal protective mechanisms (Hamdoun and Epel, 2006). Energy typically used for such processes as growth and skeletal development may instead be diverted to mechanisms for repair and protection, and to otherwise deal with chemical insults and environmental changes (Desforges et al., 2017; Kooijman, 1993).

1.6 Endpoints

The endpoints studied in toxicity are typically phenotypic manifestations of an adverse reaction to a chemical or chemicals (Boelsterli, 2007). Toxic responses begin at the molecular level, but are categorized into 8 main categories: functional effects, degeneration of tissues/organs, inflammation, immune-mediated toxicity, mutagenesis and carcinogenesis, transplacental toxicity and embryotoxicity, metabolic disturbance, endocrine disruption (Boelsterli, 2007). Due to inherent complexity, a range of responses, and idiosyncratic responses that may depend on concentrations, co-exposure and order of exposure among others, it can be difficult to identify the mechanisms governing the toxic endpoints studied (Casarett and Doull, 2013). Thus, for the majority of endpoints studied, they are more widely used as biomarkers of effect of toxicant exposure (Le Bihanic et al., 2014; Boglione et al., 2013).

A previous study by King-Heiden et al. (2009) showed, even when cardiovascular toxicity was expected to co-occur with craniofacial malformations, one may present itself in the absence of the other. Therefore, the use of a variety of endpoints and biomarkers is important in facilitating a clearer understanding of the overall effects of exposure on developing organisms.

Survival rate is a traditional toxicity endpoint crucial in establishing comparisons other species and forming dose-concentration curves. Hatching rate and delay are increasingly popular endpoints as both signify exposure to toxicants. PAHs like alkyl phenanthrenes and B(a)P as well as other chemicals like phthalates reduce hatching success and hatching rate with little effect on survival (Hodson, 2017; Chikae et al., 2004). Chikae et al. (2004) found that hatching effects were not clearly dose-dependent, but did correlate with other effects, including those visible into adulthood.

1.6.1 Morphometric and biometric endpoints

Despite the wealth of scientific research on chemical effects on development in vertebrates, the complexities of development have kept many exact mechanisms of toxicant influence hidden. Traditional risk assessment has focused on no-effect concentrations and lethal dosages, which are effective in the context of regulation. However, these endpoints are less effective measures of toxicity for substances that induce sub-lethal effects or chronic toxicity,

as PAHs often do (Casarett and Doull, 2013). Therefore, relevant impacts like standard length, height, width, yolk size, and lipid number of exposed embryos are a key method of illuminating specific effects as well as their magnitude. Differences in growth, which can be understood in terms of standard length, can be seen as early in development as the embryonic period and are strongly associated with high mortality both at and soon after hatching (Cowan and Shaw, 2002).

1.6.2 Skeletal endpoints

Skeletal development is a simple and reliable means of assessing development. Although skeletal development of the lumpfish, specifically of newly hatched larvae, is not well described in literature, newly hatched larvae consistently demonstrate extensive ossification across the body, with even some fin rays visible at hatch. These characteristics facilitate comparisons between treatments. Imsland et al. (2019) and Voskoboinikova and Kudryavtseva (2014) provide a guiding timeline on skeletal development and ossification of the lumpfish. Following a typical telelost pattern, ossification begins in organs vital to early life including (1) those dedicated to protection, the sucking disk as it allows larvae to adhere to surfaces that provide protection and camouflage against predators, and (2) those dedicated to feeding, including the maxilla, premaxilla, dentary (Osse and Van den Boogaart, 1995).

Monitoring and staining skeletal development also allows for the scoring of anomalies. PAHs, biocides, and nutritional deficiencies reduce skeletal development across the spine and cranium (Wu et al., 2020; He et al., 2011; Incardona et al., 2004). Craniofacial bones and their associated developmental gene pathways are sensitive to petrogenic chemicals (Chang et al., 2016; Carls et al., 2010, Colavecchia et al., 2004; Incardona et al., 2004; Mo et al., 1997). Le Bihanic et al. (2014) and King-Heiden et al. (2012) found that, of all major abnormalities studied across the skeleton, craniofacial were the most frequent to appear as a result of PAH-based chemical mixtures. By staining the skeleton, anomalies can be clearly identified, scored, and compared between individuals and treatments. Skeletal assessment results, of both development and anomalies, can be mobilized in conjunction with skeletal development and other endpoints to assess overall toxic effect.

1.6.3 Cardiac endpoints

Cardiac abnormalities beyond edema, including malformed hearts, pericardial edema, yolk-sac edema, and peritoneal edema, poor vasculature of the yolk sac, and slowed heart rate, are documented as a result of exposure to petrogenic chemicals and PCs (Sun and Liu, 2017; Brette et al., 2014; King-Heiden et al., 2012; Carls et al., 1999). Incardona et al. (2004) found that 3-ringed PAHs directly negatively impacted the cardiovascular system. Moreover, Incardona et al. (2004) found that cardiac abnormalities and poor cardiac function as a result of PAH exposure actually precede skeletal anomalies. Cardiac organ dysfunction can be lethal and thus demonstrates a close connection with morality (Brette et al., 2014; Incardona et al., 2013; King-Heiden et al. 2012).

1.7 Study specifics

1.7.1 Study species

In this study, the experimental species was *Cyclopterus lumpus*, the lumpfish. This species is increasingly commercially important in Norway as it is not only utilized for roe, but also is a suitable cold-water option for the delousing of Atlantic salmon in large-scale farming (Imsland et al., 2019; Dagbjartarson et al., 2018; Powell et al., 2018; Sunnanå and Albert, 2004). It is presently the third most important cultivated fish in Norway. However, recent

population estimates indicate a declining stock, which may be the result of habitat disturbances including oil pollution (Sunnanå, 2007)

Lumpfish is a semi-pelagic species, with adults migrating toward coastal, shallow water masses to spawn (Davenport and Kjørsvik, 1986). As a species, its range spans the majority of the North Atlantic, with large populations along the Norwegian coast. The exact spawning locations of the Norwegian coast populations are unknown, but the main populations are thought to spawn in Nordland, Troms, and Finnmark (Durif, 2020). Eggs are demersal, laid in a rocky-substrate nest, and both aerated and guarded throughout the embryonic period by males (Davenport, 1985). After hatching, larval lumpfish mature in kelp forests and in epipelagic zones until approximately one year old, when they swim out to open sea (Davenport, 1985; Blacker, 1983; Durif, 2020).

Species with that are either economically important (salmon, cod) or demonstrate a myriad of specific characteristics, including a high sensitivity to toxicants (typically due to size), high fecundity, quick development and transparency (cod, haddock, zebrafish), are typically chosen as model species (Incardona, 2017; Hall et al., 2004; Heintz et al., 1999). However, lumpfish is an ideal study species for several reasons (Davenport, 1985). The eggs have a high rate of successful fertilization in the laboratory and form a strong adhesive coating, making them easily grouped and manipulated for exposure. The eggs are lipid-rich, which may make them more susceptible to the uptake of hydrophobic compounds, like PAHs, than other species (Meador et al., 1995). The eggs are also demersal, a contrast to the majority of previous model species used to assess the impacts of oil and petrogenic chemicals (Davenport, 1985). Moreover, lumpfish also hatched highly developed, due to their long incubation period, and may then be more likely to present visible anomalies at hatch. Finally, eggs have been seen to attch to platform legs during spawning, though the effect, in terms of exposure, is unclear (Hansen, pers comm).

1.7.2 Embryonic development and study period

The study was conducted over the period of embryogenesis, from fertilization until hatching. Early life stages, encompassing up to the larval period, are more vulnerable to toxic exposure than other life stages (Le Bihanic et al., 2014; Mohammed, 2013; Foekema et al., 2012; Schmeider et al., 2000). Embryogenesis was chosen as the study period because it is a critical period in which the embryo develops an organized body plan and sets the stage for proper body functioning throughout life (Gilbert and Barresi, 2013). The development of a proper body plan and is conditioned on proper endocrine control and cell signaling (Gilbert and Barresi, 2013; Bozinovic et al., 2011). Chemical perturbations that alter gene activation or interfere with gene products or cell-signaling can induce extensive mis-regulation cascades that can then cause malformations and even death (Bozinovic et al., 2011; Boelsterli, 2007). Moreover, eggs and larvae eliminate PAHs and other lipophilic contaminants more slowly than adults; increasing the toxicant's time within the system also may increase the likelihood of toxicity occurring at the target site (Casarett and Doull, 2013; Solbakken et al., 1984; Davenport et al., 1979).

Embryonic development of the lumpfish follows a typical teleost pattern, and egg development lasting approximately 280 day degrees (d˚), between that of cod (~90 d˚) and salmon (~510 d˚) (Imsland et al., 2019; Kjørsvik et al., 2007; Gjedrem, 1993; Kjesbu, 1989). The earliest stages of development are generally considered sensitive more than later stages, with previous studies suggesting that gastrulation is one of the most vulnerable, evidencing acute, chronic, and concentration-dependent toxic effects (Kjørsvik, 1986). Hansen et al.

(2019) and Kazuyuk et al. (1988) showed that the effect of PW is most pronounced during the gastrulation subperiod. This may be explained by specific processes within gastrulation, including the formation of tissue layers and the embryo axis, early organogenesis, and the transition of gene regulation from maternal to embryo control. Thus, for this study, exposure occurred over a 96-hour period, similar to a previous study by Hansen et al. (2019), covering the earliest stages of development including blastulation, gastrulation, and early organogenesis.

1.8 Aim

The aim of this study was to i) investigate whether PW causes embryotoxicity in lumpfish, ii) describe the s potential symptoms of embryotoxicity, iii) investigate if there are differences between PWs from different oilfields, and iii) describe the potential differences and suggest reasons for these.

Embryos were exposed to three concentrations of five different PWs during a 96-hour window, beginning approximately 18 hours post fertilization, and then allowed to develop normally until hatch in filtered seawater. The study environment was standardized and equivalent across all individuals and treatments to minimize any potential effects of other factors that have been demonstrated to cause skeletal anomalies including pH, water temperature, and water currents (Boglione et al., 2013; Vadstein et al., 2004). Several endpoints were then investigated 2-3 days post hatch, including survival, hatching, biometric specifics, skeletal development and anomalies, heart rate measurements, and craniofacial development. A host of endpoints were examined as toxication is likely to present in a variety of way and in a variety of organs. Moreover, complex mixtures are often characterized by a lack of predictability in effects when compared to exposure to a single component of the mixture, a similar combination, or even a previous trial.

2. Methods

2.1 Produced Water

PW samples were supplied by oil production companies operating on the NCS. PW was collected from platforms on five different mature North Sea oil fields that represent a range of crude oil types. Samples from each field were then prepared by Lisbet Sørensen (SINTEF Ocean, Trondheim, Norway) in three concentrations, 10%, 50%, and 100% (undiluted). The solutions were bubbled with air at 10˚C and pH adjusted. Samples were taken for separate chemical analysis.

Exposure treatments will henceforth be referred to by location, or type, as PW followed by the identifying letter, A, B, C, D, or E. When the concentration of treatment is relevent, an addition -L, for low, -M, for medium, or -H, for high, will be attached to the treatment type. This results in a specific identification for each exposure solution, for example, PWA-L. "Ctrl" refers to the seawater control treatment and "SV" refers to the solvent control treatment. Control and solvent control treatment concentrations were designated "N", or neutral.

2.2 Fertilization

Lumpfish eggs were obtained from a 4.0376 kg female from Skjerneset Fisk AS (Averøy, Norway). Eggs were fertilized via batch method within two hours of arrival using previously cryopreserved milt from a single male supplied by Cryogenetics AS (Hamar, Norway) (supplementary methods 2.2). Eggs were allowed to sit in filtered seawater for between two and three minutes and then spread into a monolayer circle approximately 2-cm in diameter, approximately 80 eggs per circle. Circles were then allowed to set in filtered seawater until hardened (approximately 10 to 12 minutes) before being transferred into a flow-through filtered seawater incubator at 10˚C. They were kept in a 16:8 (light: dark) light regime for the remainder of the experiment.

2.3 Exposure and recovery

After 20 hours, three egg circles per treatment were then transferred, in duplicate, to glass containers filled with approximately 100 mL of exposure solution. Exposure solutions were refilled and dissolved O_2 concentration checked every 24 hours for 96 hours.

At 96 hours, one egg circle per container was sampled for a separate study. The remaining egg circle was transferred into mesh-lined plastic bottles in a 10˚C filtered seawater incubator, with water exchange at 12 L/hour, and allowed to develop normally until hatching. Individual eggs were imaged every 48-72 hours, until 25 days post fertilization (dpf), to track development.

2.4 Survival and hatching analysis

Hatching began at 30 dpf and lasted for 96 hours, but the majority of hatching occurred over the course of 24 hours, at approximately 31-32 dpf or 310-320 d˚. Hatching was checked twice daily during the period and hatched larvae were counted, removed, and replicates pooled. At the completion of hatching, up to 96 hours after the first hatch observations, the remaining eggs in each container were removed, determined to be either unhatched living larvae, dead embryos, or unfertilized, and counted.

Hatching, survival, and fertilization rate were determined for each individual container and later averaged across treatment condition for statistical analysis. Survival rate was calculated as the total number of alive specimens divided by total number of fertilized larvae. Hatching rate was calculated as the total number of hatched larvae out of total number of surviving

organisms. Some embryos were alive at the completion of hatching, but did not hatch; it could not be assumed that these larvae would hatch, even with more time. Fertilization rate was calculated as the number of eggs successfully fertilized divide by the total number of eggs.

Eggs hatching delayed were determined as those hatching after 1) complete hatching of both the control and solvent control solutions, 2) more than ~90% hatching in the low concentration of a specific PW and more than ~50% in the medium concentration, and 3) a 12-hour period in which less than 5% of larvae hatched followed by a group hatching event.

2.5 Biometry

Of hatched larvae, approximately 15 were sampled from each pooled treatment for biometric analysis (n = 15 per treatment condition). Larvae were embedded alive in methylcellulose and immediately photographed in a straight plane two positions, ventral and dorsal, using a dissecting microscope (Leica M205, Leica Microsystems, Germany) equipped with a CCD camera with video function (Nikon, DS-5M/l2, Nikon Corporation, Japan). Video was also taken for heart rate analysis. Previous tests illustrated no change in stress response, specifically heart rate, during this procedure. Larvae were sacrificed by blunt force immediately afterward. Pictures were used for analyses of biometric endpoints. Biometry was completed by computer automated analysis standardized for lumpfish.

Biometric analysis was performed to quantify fourteen parameters: side body area, standard length, myotome length, myotome height, side yolk area, eye area, eye max diameter, eye min diameter, ventral body area, ventral body length, ventral body width, ventral yolk area, number of lipids, and total lipid area. After several measures were determined to be unreliable and thus excluded, a correlation analysis was then performed on the remaining measurements (appendix figure 2.5). In the reduced dataset, analyses were checked for accuracy and remeasured if necessary. From these measurements, 6 were chosen: standard length, myotome height, side yolk area, eye min diameter, ventral body width, and number of lipids. Heart rate measurement was done by counting the number of heart beats in 30 seconds and scaling accordingly to get beats per minute (bpm).

2.6 Skeletal Analysis

Skeletal analysis was performed on approximately 15 randomly sampled individuals pooled from each treatment condition. Excessively curled positioning of some individuals, often a result of impaired development or early death within the egg, made determinants of axis deviations difficult to properly assess. Larvae were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) and stored at 10˚C until analysis, when they were stored at 4˚C. Larvae were stained with alizarin red according to Kjørsvik et al. (2009).

Bone staining took place in transparent plastic well-trays, with each well containing approximately 5 larvae or eggs. Before staining, eggs were dechorionated to facilitate uptake of the solutions as well as a clear view of the unhatched embryo.

The bone staining procedure (supplementary methods 2.5) took place in five distinct steps: rehydration, bleaching, clearing, staining, and preservation. Rehydration occurred in solutions of decreasing ethanol concentration. Larvae were then bleached in a 1:9 3% hydrogen peroxide in 1% potassium hydroxide solution under strong light until all pigmentation was removed, approximately 3 hours. Subsequently, larvae were cleared in a trypsin buffer to transparent, approximately 22 hours. In some cases, the yolk remained slightly opaque. Then,

larvae were immersed in an alizarin red solution for ~22 hours to stain bones. Finally, larvae were washed and preserved in 40% glycerol in 1% KOH for 2 days, during which time they were photographed under a stereomicroscope (Leica MZ75, Germany) equipped with a camera (Nikon Digital Sigth DS-5M L1, Japan), and after which preserved in increasing concentrations of glycerol.

Photographed larvae were analyzed according to modified versions of Hansen et al. (2019), Cheng et al. (2018), Voskoboinikova and Kudryavtseva (2014), Herbing et al. (1996) for standard length, ossification of vertebrae, dorsal and anal ray number, cranial bones, and anomalies using ImageJ (version 1.8.0_172); Figure 2.5 illustrates the craniofacial morphological endpoints measured using abbreviations and guidelines of Table 2.5. Further examples are provided in appendix figure 2.5. The level of development in the craniofacial area was not assessed as differences in size and degree of staining because such factors could not definitively be tied to development rather than abnormal growth. Therefore, they are described under craniofacial abnormalities.

Table 2.6. Craniofacial endpoint measurement definitions.

Measurement	Abbrev.	Definitions
Head length	HL	From base of spine, typically at or near the intersection of preoperculum and first visible neural arches, through the middle of the eye, to the edge of the face
Jaw length	JL	From the base of the tooth, following along the hyomandibular (hm) to the beginning of the opercle
Jaw-to-eye length	JtE	From the edge of the jaw to the center of the eye along the HL line
Jaw point angle	Q	The angle created where the JtE measurement line intersects the HL line measurement

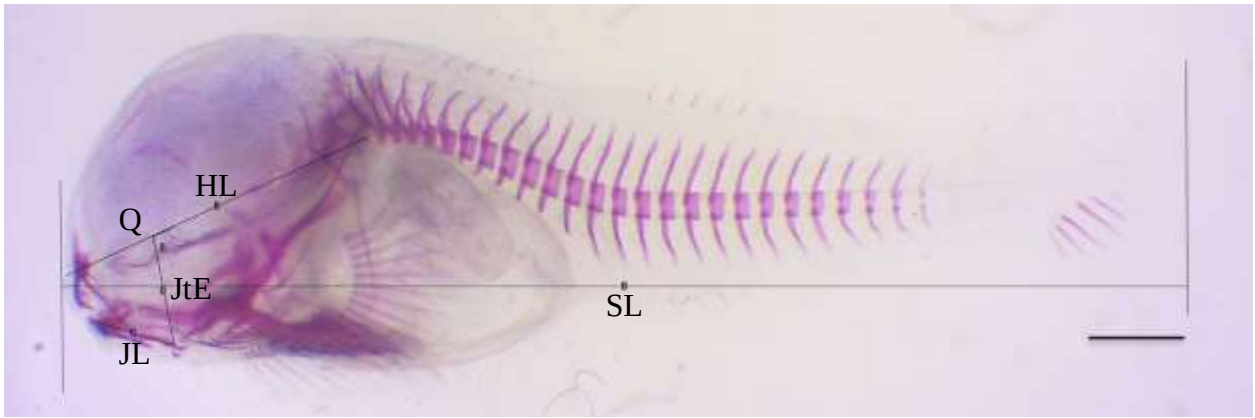

Figure 2.6. Craniofacial and standard length endpoint measurements of bone-stained lumpfish larvae. Black bar is 500 µm.

2.6.1. Ossification of vertebrae

Because all larvae are in the same newly-hatched developmental stage, with a standard length of less than 6.5mm, they are expected not to illustrate full ossification or compactness within their vertebrae segments. Therefore, vertebrae segments were classified as near-compact (NC), thoroughly ossified (TO), partially ossified (PO), and transparent (T) in order to provide greater detail (Figure 2.5.1)

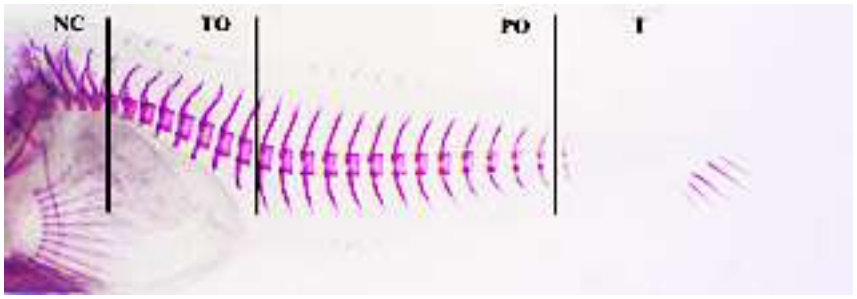

Figure 2.6.1. NC vertebrae demonstrate consistent staining and touching of the vertebrae to the previous or to the base of the spine. TO vertebrae are stained throughout the arches. PO vertebrae illustrate staining to various degrees. T vertebrae show little to no staining, with ossification not reaching the middle of the vertebrae.

2.6.2 Ossification of fins

As general ossification of fin rays is low in young larvae, stained dorsal and caudal fin rays were counted rather than an ossification level scored. Any visible ossification of a fin rays was counted.

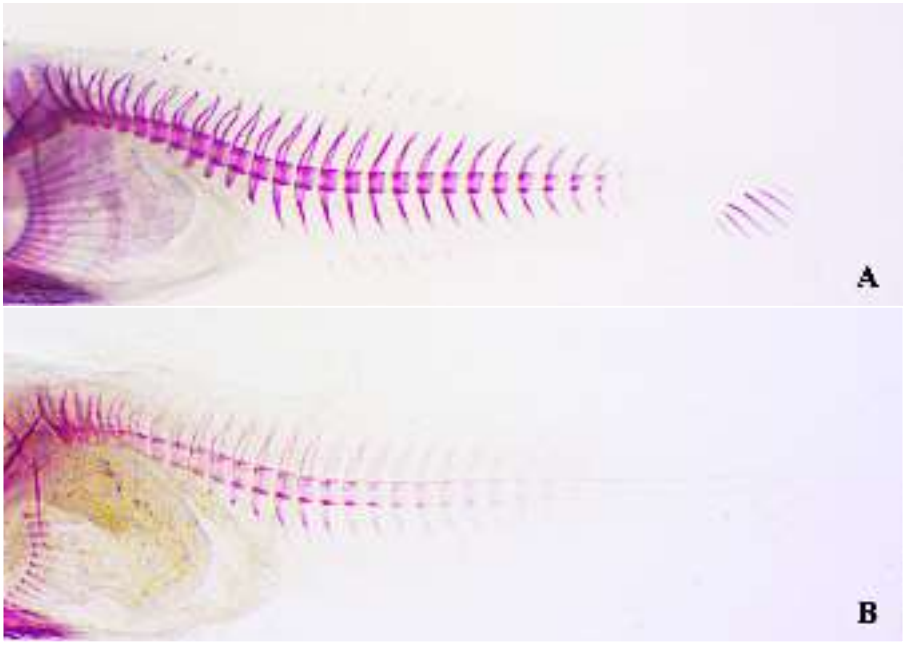

Figure 2.6.2. Fin ray ossification count. Picture A shows ossification in 14 dorsal and 6 caudal fin rays. Picture B shows no fin ray ossification.

2.6.3 Skeletal anomalies

Anomalies, or deviations from the normal shape of an organ, across the larval body were categorized and counted. Due to the overall low level of ossification across all stained larvae, anomalies were not scored for severity. Anomalies were primarily grouped into two main categories, spinal and craniofacial, and further delineated as seen in Table 2.5.3. Within these groups, anomalies were further delineated with according to type. Skeletal anomalies were not reported according to region due to general lack of development and lack of literature on regionality within lumpfish. Provided definitions are partially derived from Incardona et al. (2004), Boglione et al. (2001), and Carls et al. (2010).

Deviations, even within the same bones, are expected to co-occur. Other anomalies, for

ample, the hunchback phenotype, may occur, but those in table 2.5.3 were considered the most important and relevant to consideration in current literature.

Table 2.6.3. List of considered abnormalities. Each abnormality was found in several (>5) individuals.

Abnormality	Definition
Spinal	
1. Axis deviations	
a. Break	A break in the spinal column not due to specimen handling
b. Curvature	Severe sideways deviation of the spine not returning to a straight spinal axis
c. Kyphosis	Upward pointing dorsal-ventral curvature
e. Scoliosis	Lateral curvature of the spine, from and returning to a straight spinal axis, in either a "S" or "C" shape
2. Vertebral malformation	
a. Stunted spine	A short, non-existent, or stub of one or both arches of a vertebrae
b. Wavy arches	Spines illustrate excessive curvature, often several
c. Twisted arches	Spines form a spiral at the apex
d. Vertebral expansion	A drastic increase in or stretching of the vertebral area in comparison to nearby vertebrae; phenotypically similar to a vertebral fusion, but with no sharing of arches
e. Vertebral compression	A notable shrinking of vertebral area in comparison to nearby
Craniofacial	
1. Flat face	Cranial formation, from the premaxilla past the top of the eye and to the dorsal side of the skull, is upright (covers atop the majority of the eye) rather than proceeding at an angle (bone begins approximately halfway through the eye)
2. Underbite	Lower jaw at or beyond the upper jaw
3. Extremely open mouth	Distance between the premaxilla and dental is large and/or the maxilla bone is at an open angle in relation to the premaxilla
5. Lack of chin	Lower jaw bones undeveloped, specifically including but not limited to the lower dentary, retroarticular, anguloarticular, quadrate, and symplectic bones
6. Elongated face	Best seen when looking dorsally; there is a noticeably greater distance between the parasphenoid, prootic, and basioccipital bone/channel for dorsal aorta, as well as the parasphenoid and the premaxilla

2.7 PW components

The control solution was filtered seawater, equivalent to the filtered seawater used for incubation. The solvent control was prepared in the same manner as the reconstituted PW solutions, using only dichloromethane (no extract).

Gas chromatography and mass spectrometry (GC-MS) quantification identifies a host of PAH compounds in each sample (Figure 2.7, appendix table 2.7). Individual components of each PW were investigated by GC-MS chemical analysis.

The GC analysis included a separate determination of the total extractable material (TEM) in the DCM-extractable fraction (appendix table 2.7). The resulting data was then analyzed in conjunction with the various endpoints of this study in order to provide explanation to the effects seen under the different PW types.

PAHs were divided into subgroups. Naphthalenes, though 2-ring PAHs, were not considered within the 2-3 ring group because they are generally too large and mask other PAHs within this groups. Phenanthrenes, dibenzothiophenes and fluorenes are considered within the 2-3 ring group, however they are also separate to clarify their contribution to the larger group. 4-6 ring PAHs are grouped together for better quantification.

Table 2.7. Concentration (µg/L, TEM in total area recovered) obtained by GC-MS of various major compound groups found in each PW, at all doses, as well as the controls. PDF refers to the group of phenanthrenes, dibenzothiophenes, and fluorenes.

PW	Dose	TEM	Phenols	Naphth-alenes	2-3 ring PAHs	PDF	4-6 ring PAHs	Total PAH
Ctrl	N	3147	1.00	0.39	0.24	0.23	0.01	0.64
PWA	L	3650	15.62	3.75	1.63	1.61	0.31	5.69
	M	12664	284.98	22.93	8.83	8.72	1.81	33.57
	H	21367	218.98	46.59	13.08	12.91	2.54	62.21
PWB	L	18059	148.02	11.10	13.01	12.36	1.31	25.41
	M	78855	607.48	50.05	69.18	62.37	6.39	125.62
	H	149114	1390.44	106.33	156.47	142.67	12.40	275.20
PWC	L	5760	19.33	1.66	4.51	4.39	0.43	6.60
	M	14360	112.91	6.54	22.67	22.12	1.59	30.79
	H	24419	215.77	14.37	46.57	45.375	2.94	63.88
PWD	L	3995	17.70	0.74	1.78	1.775	0.30	2.82
	M	8181	92.21	1.61	6.95	6.92	1.12	9.68
	H	12957	185.32	3.37	14.12	14.055	2.21	19.70
PWE	L	6367	41.26	8.59	24.53	24.12	2.62	35.74
	M	27137	292.75	50.08	135.89	133.47	14.45	200.42
	H	48027	587.04	111.19	268.76	263.37	29.02	408.98
SV	N	1912	1.62	0.48	0.19	0.185	0.02	0.70

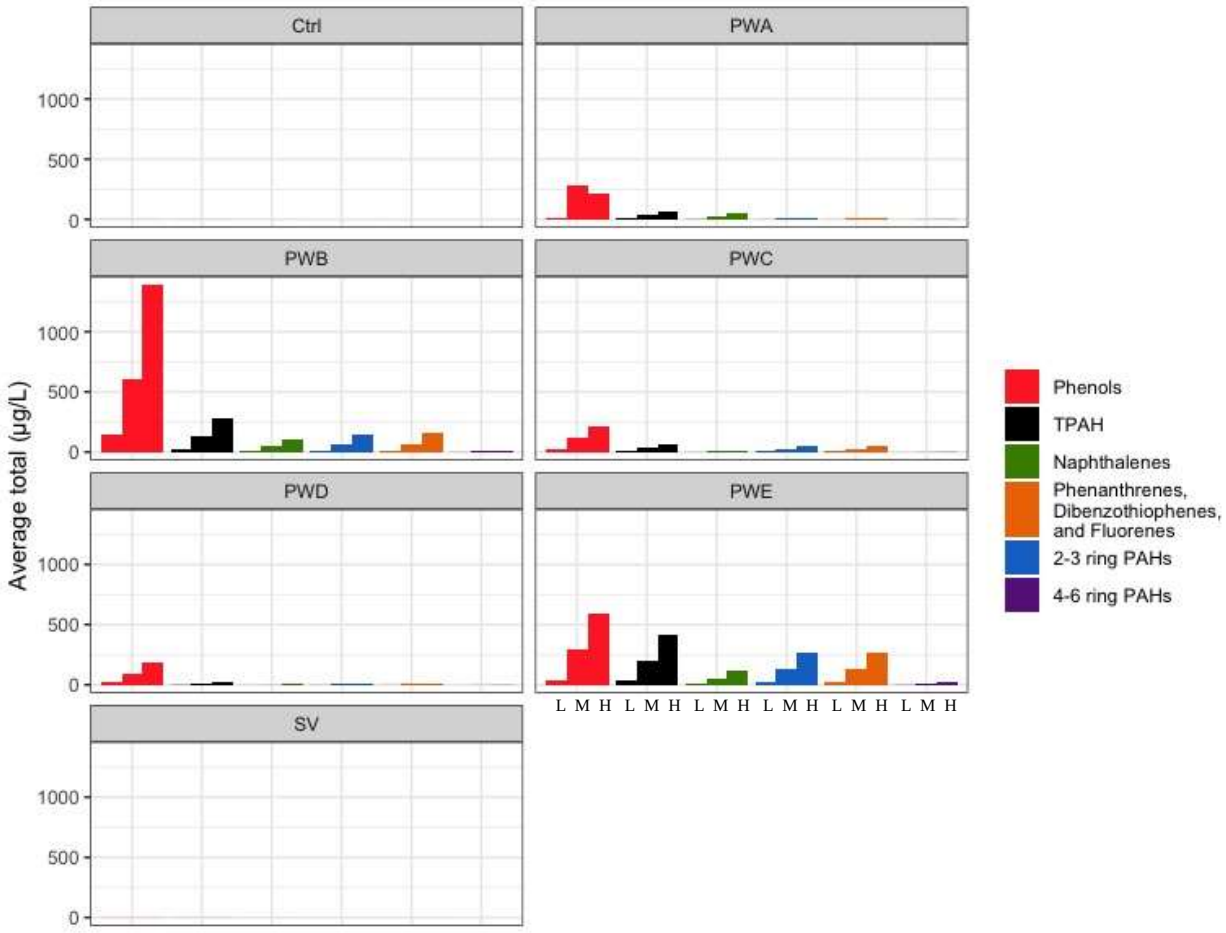

Figure 2.7. Average total amount (from two separate GC-MS analyses) of various major compound groups in µg/L found in each PW tested, at all doses, as well as the seawater and solvent controls. The different levels of each compound represent the concentration at doses low, medium and high.

2.8 Oilfield data

Oilfield data was gathered from online publications of the corresponding platforms to study the effect of oilfield age and major oil-type on toxicity measures.

Table 2.8. Most recent available information (2019). Volumes in millions of standard cubic meters of oil equivalents.

PW	Production start	Total current product	Oil product	Gas product	LNG product	Oil % of total product
A	22.12.1986	2.74	2.74	0	0	100
B	18.10.1995	4.64	3.03	1.47	0.14	65.3
C	06.11.1997	1.27	0.43	0.71	0.13	33.9
D	24.11.1979	2.59	0.83	1.09	0.67	32.0
E	06.10.1988	0.32	0.3	0	0.02	93.8

2.9 Statistical Analysis

Data exploration were performed to investigate the effect of various factors on chosen endpoints. RStudio (version 1.2.5001) was used for all statistical analysis. Plots reflect mean ± standard error of the mean (SEM) unless otherwise noted. Plots reflect hatching rate rather than survival rate unless otherwise noted.

Because the effect of treatment on hatching and survival rates were significant, further statistical analysis was completed to better understand how the components of treatment effected hatching rate. A mixed effect model could not be used because the data is categorical and thus not linear.

Standard length (SL) was measured in both the automated biometric analysis as well as the non-automated skeletal analysis. Results of a Student's T-test illustrate that the two methods result in significant differences in the SL measurement in each treatment (Appendix Figure 2.9a). Therefore, throughout the results and discussion paper, the non-automated SL was used for analyses.

Correlation analyses were first performed. ANOVAs were run to investigate the effect of causative agents on chosen endpoints, including hatching and survival rate, hatching delay rate, biometric measurements, and craniofacial measurements. Comparison between groups was done by Student's T-test. When ANOVAs could not be performed due to high correlation between independent variables, which prevented each compound groups' analysis as an individual explanatory factor (Appendix Table 2.9), a PCA was performed to summarize variables' influence, understand their correlation, and reduce dimensionality. The PCA was created using data of: TEM, phenols, naphthalenes, 2-3 ring PAHs, phenanthrenes, dibenzothiophenes, and fluorenes, 4-6 ring PAHs, and TPAH.

Table 2.9. Compilation of samples sizes for endpoints and explanatory factors. In PWE, 22 individuals were used for standard length analysis, but only 17 of those were used for the remaining skeletal analysis because excessive curvature prevented proper analysis.

Sample size							
Treatment	Dose	Hatching and survival	Biometric analysis	Heart rate	Skeletal analysis	Component analysis measurement	Oilfield statistics
Ctrl	N	6	16	16	15	2	NA
PWA	L	3	0	0	0	2	1
	M	3	0	0	0	2	
	H	3	0	0	0	2	
PWB	L	3	17	18	15	2	1
	M	3	16	14	15	2	
	H	3	14	15	15	2	
PWC	L	3	15	15	17	2	1
	M	3	15	15	16	2	
	H	3	16	15	16	2	
PWD	L	3	15	15	16	2	1
	M	3	15	15	15	2	
	H	3	15	16	15	2	
PWE	L	3	17	15	17*	2	1
	M	3	0	0	0	2	
	H	3	0	0	0	2	
SV	N	3	15	16	15	2	NA

3. Results

3.1 Fertilization

As demonstrated in Figure 3.1, there was no difference in fertilization rate between the exposure treatments and the controls. The mean fertilization rate was 94.5%. The lowest fertilization rate was 88.6%, seen in a single replicate of PWB-H; the other replicates subjected to this treatment condition had fertilization rates of 91.0% and 91.7%. Differences in fertilization rate were random and could not be caused by treatment because exposure occurred approximately 20 hours after fertilization.

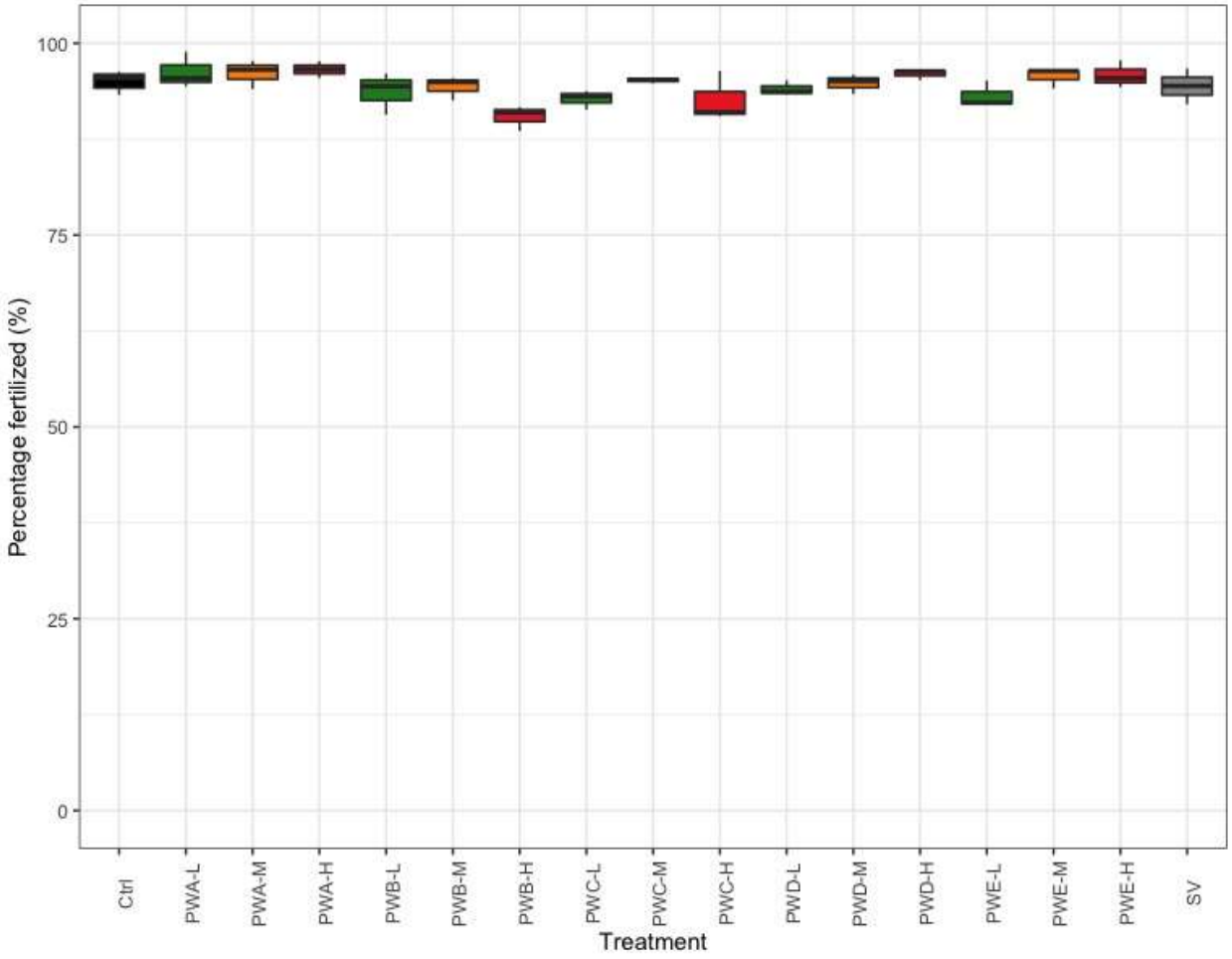

Figure 3.1. Box and whisker plot of fertilization rate per treatment. There were no differences in fertilization rates between individual treatments and control treatments.

3.2 Hatching and survival

Lumpfish larvae hatching lasted for approximately four days, from 30 dpf to 34 dpf (~300 d˚ - 340 d˚). The majority of hatching occurred in the first 48 hours. Larval hatching and survival information was compiled at 34 dpf. The hatching rate demonstrated clear differences depending on both PW type (A, B, C, D, or E) and concentration (L, M, or H).

No hatching was observed in any replicate of PWA at any concentration or in PWE at the medium and high concentrations. The hatching rate was significantly lower than the control (Figure 3.2, $p < 0.001$). The hatching rate of individuals in PWB in the high concentration and PWE in the low concentration was significantly lower in comparison to the control (Figure 3.2, $p < 0.001$).

There was a significantly higher rate of hatching delay in the high concentrations of PWB and PWC (Figure 3.2.2, $p < 0.001$). Hatching delay and the ratio of unhatched to hatched embryos generally followed an increasing trend paralleling the increasing concentration of PW (Figure 3.2.2).

Survival rate closely followed the hatching rate with significantly fewer larvae surviving in all concentrations of PWA and PWE as well as in the PWB-H treatment (Figure 3.3).

An interaction model of PW type and concentration revealed both factors individually as well as in interaction to be significant predictors of variation in the dataset, explaining 96.9% of the variations in the hatching dataset and 98.1% in the survival dataset. The PW type predicted more of the variation observed in both datasets (Appendix tables 3.2.1, 3.2.2).

3.2.1 Hatching

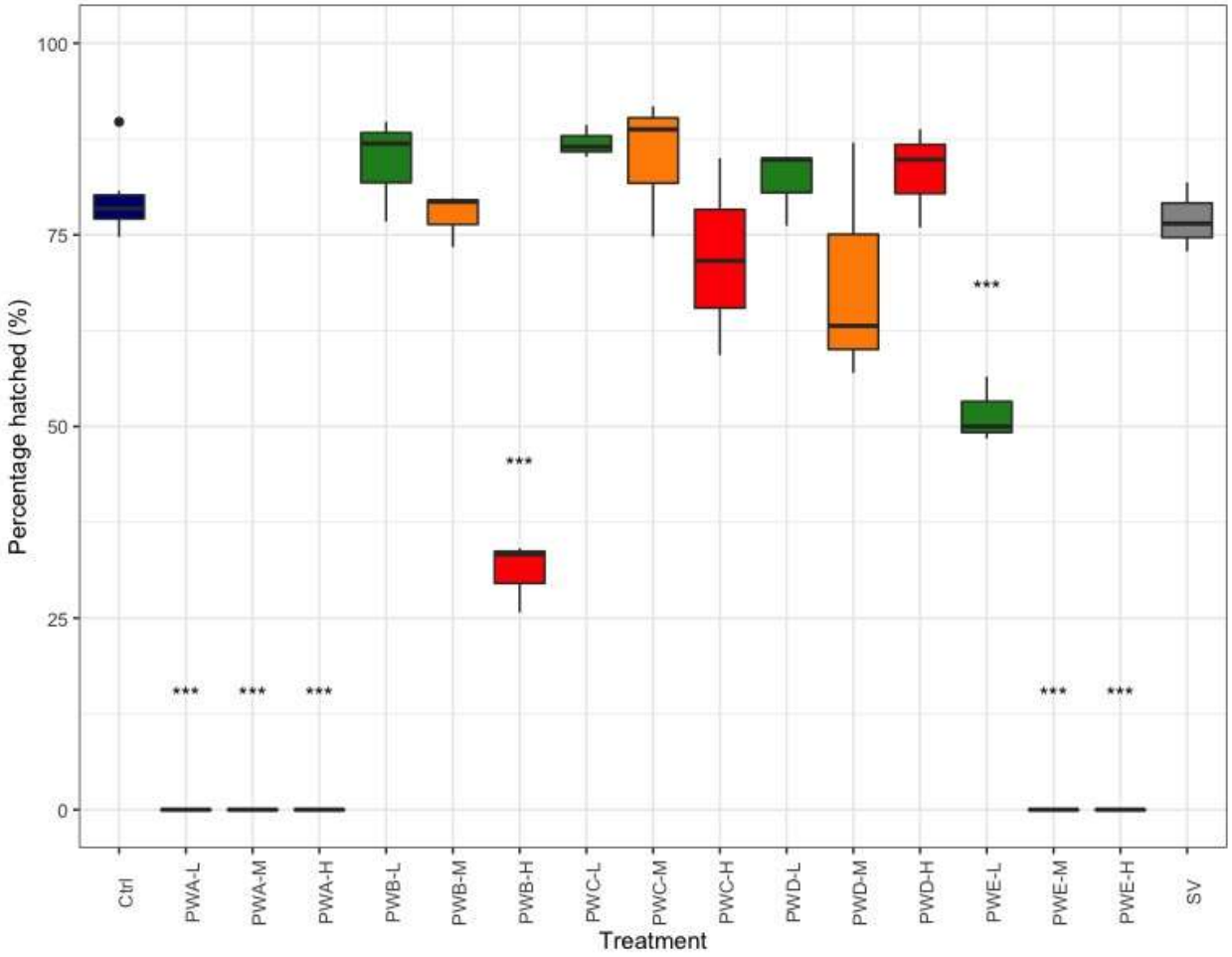

Figure 3.2.1. Box and whisker plot of hatching rate per treatment. No larvae hatched in all levels of PWA. Significantly fewer larvae hatched in all concentrations of PWE as well as PWB-H in comparison to the control ($p < 0.001$). *** denotes $p < 0.001$

3.2.2 Hatching delay

Figure 3.2.2. Hatching outcome (in terms of mean percentage that hatched on time, delayed, or did not hatch) of living embryos for each treatment and condition. Error bars represent SEM. Significance represents deviation from the control in terms of percentage of embryos that hatched on time. Produced waters A and E at all concentrations led to little to no hatching, which was significantly less than the control. Treatments PWB-H and PWC-H led to significantly more embryos either hatching with a delay or not at all in comparison to the control. *** denotes $p < 0.001$

3.2.3 Survival

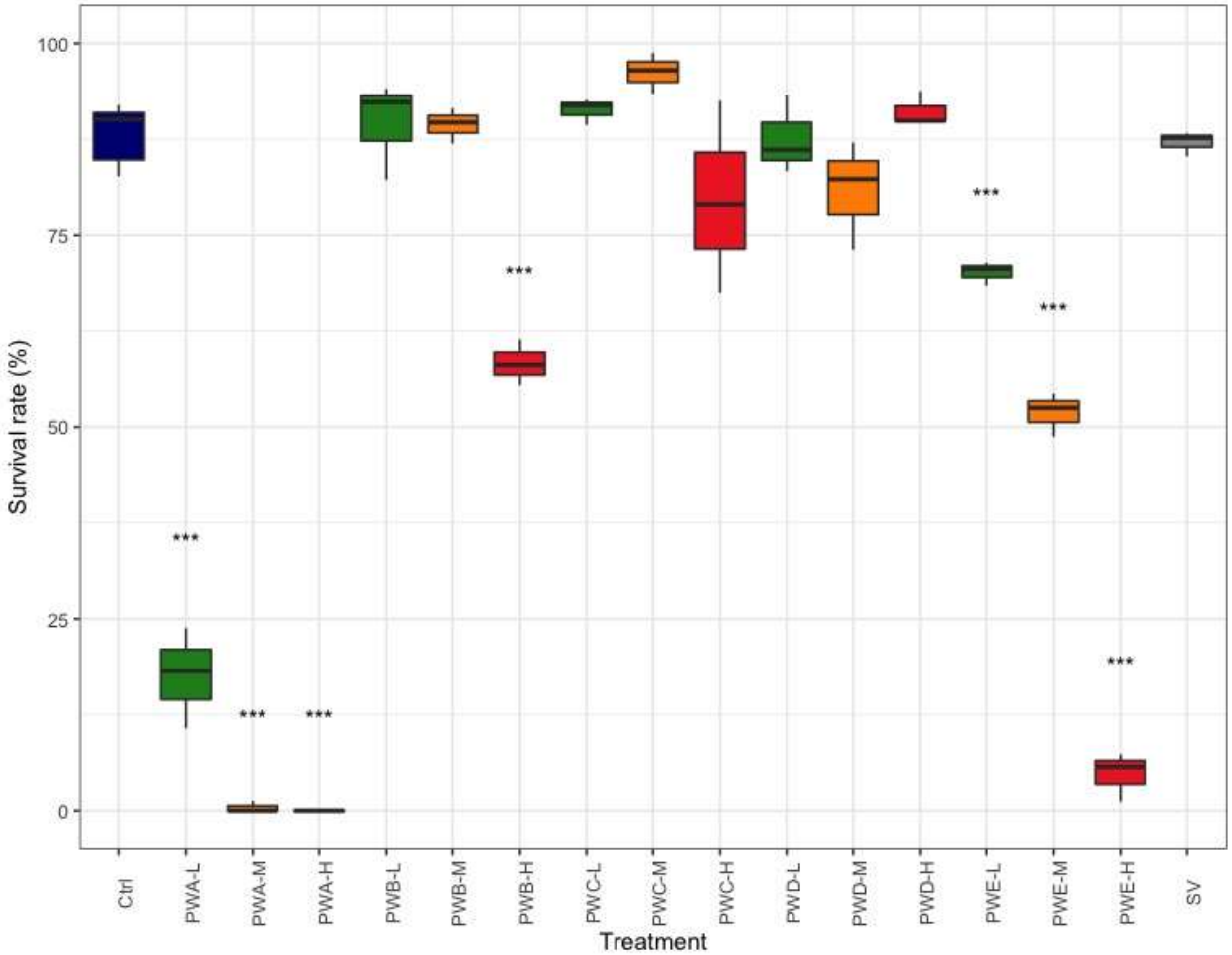

Figure 3.2.3 Box and whisker plot of survival rate per treatment. Some individuals that did not hatch were still alive. Significantly fewer larvae survived in all levels of PWA, PWE, and PWB-H in comparison to the control ($p < 0.001$). The results for PWC and PWD indicated that increasing concentration did not always correspond with a decrease in survival rate. *** denotes $p < 0.001$

3.3 Hydrocarbons and petrogenic chemicals

TEM was predicted to explain some portion of variation seen in regard to treatment. TEM did not reliably predict hatching rate (Figure 3.3.1). An overlay of TEM onto a boxplot of hatching rate (as well as a corresponding regression in Appendix Figure 3.3.1) illustrates that, while various PWs showed negative relationships between both hatching rate and TEM, the overall model predicted only 2.78% of variation in the data (Figure 3.3.1).

Hydrocarbon content was also predicted to explain some portion of variation seen in regard to treatment. Together, PC1 and PC2 captured >99% of the information in the input component parameters (Figure 3.3.2). PC1 was positively and strongly correlated to all of the input parameters; PC2 was positively and most strongly correlated with TEM and phenols as well as with 4–6 ring PAHs to a lesser and negative degree. Results of the component PCA illustrated a connection between the two primary principal components and hatching rate. However, PC1 explained only 27% of the variation in the hatching data and demonstrated a negative linear relationship. PC2 explained only 5% and showed a positive linear relationship (Figure 3.3.3). A significant portion of variation was not explained by components analyzed under the testing method used in this study.

3.3.1 Total extractable matter (TEM)

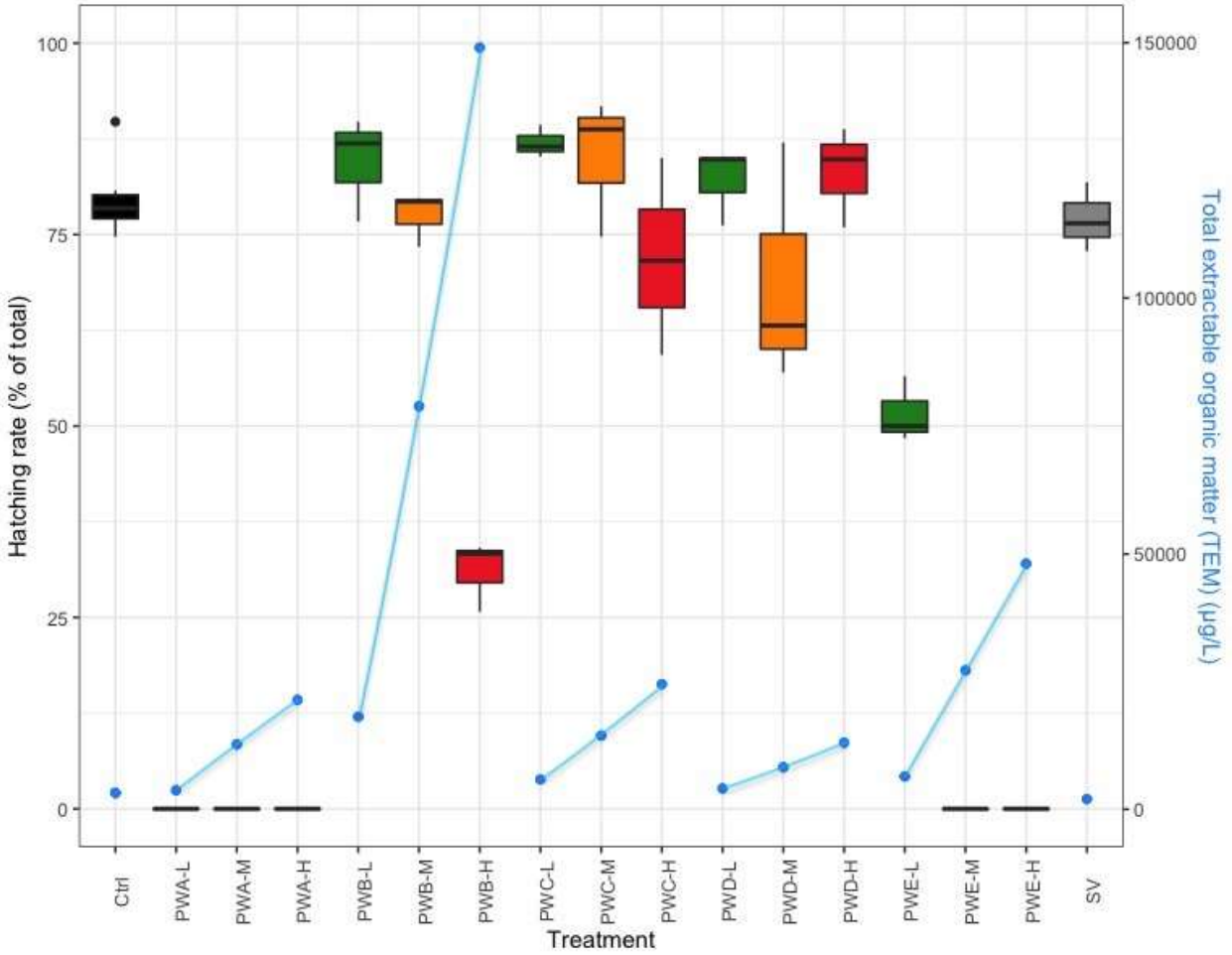

Figure 3.3.1. Comparison of TEM levels with boxplot of hatching rate. TEM showed a clear increasing pattern with increasing concentration. However, it explained only 2.78% of the variation in the data.

3.3.2 Hydrocarbon components PCA

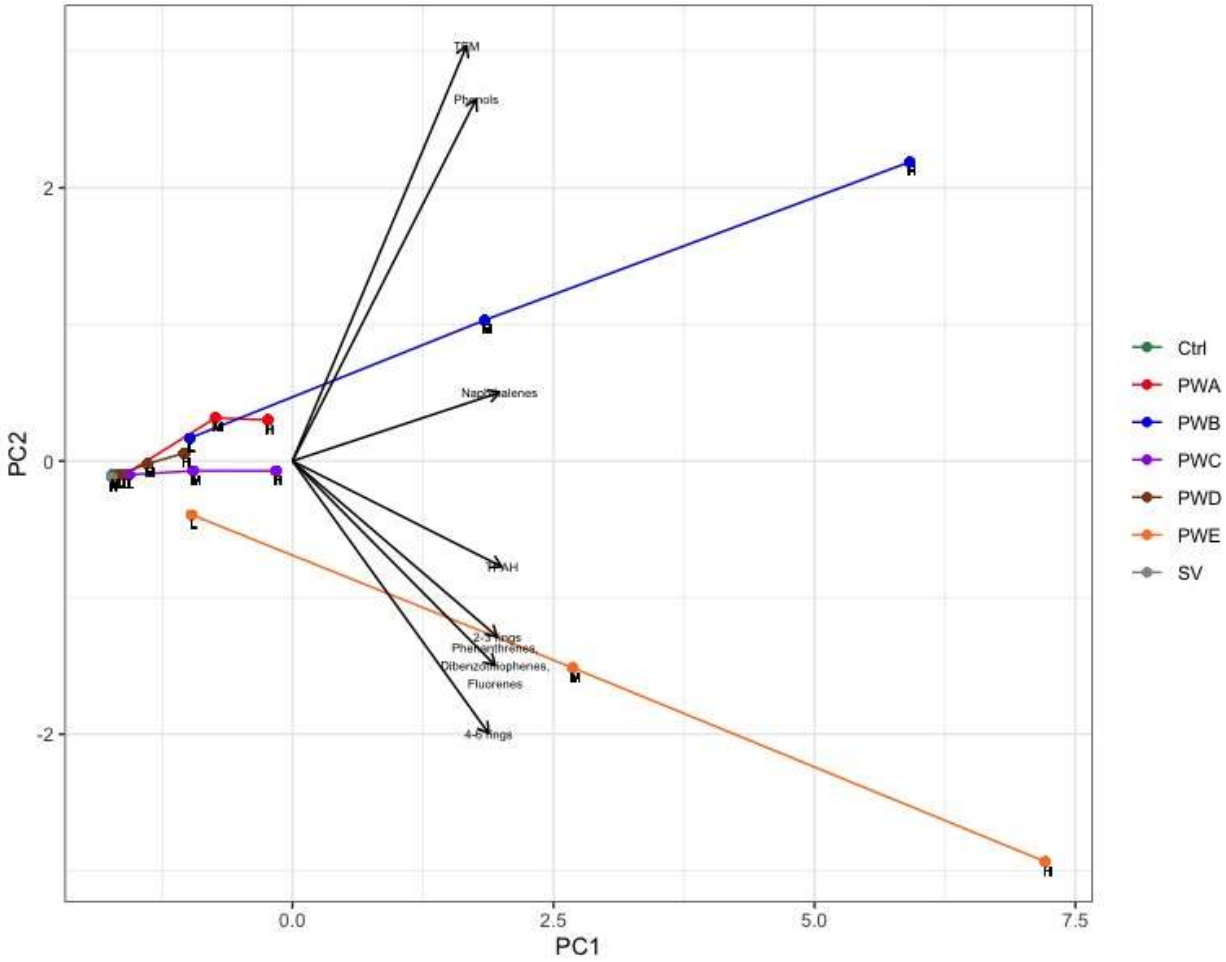

Figure 3.3.2. PC1 (87.03%) and PC2 (12.08%) with component loadings to visualize influence and correlation. All loadings positively correlated with PC1; PC2 was strongly positively correlated with TEM and phenols and was negatively correlated with 4–6 ring PAHs. Concentration showed a clear effect on PC1.

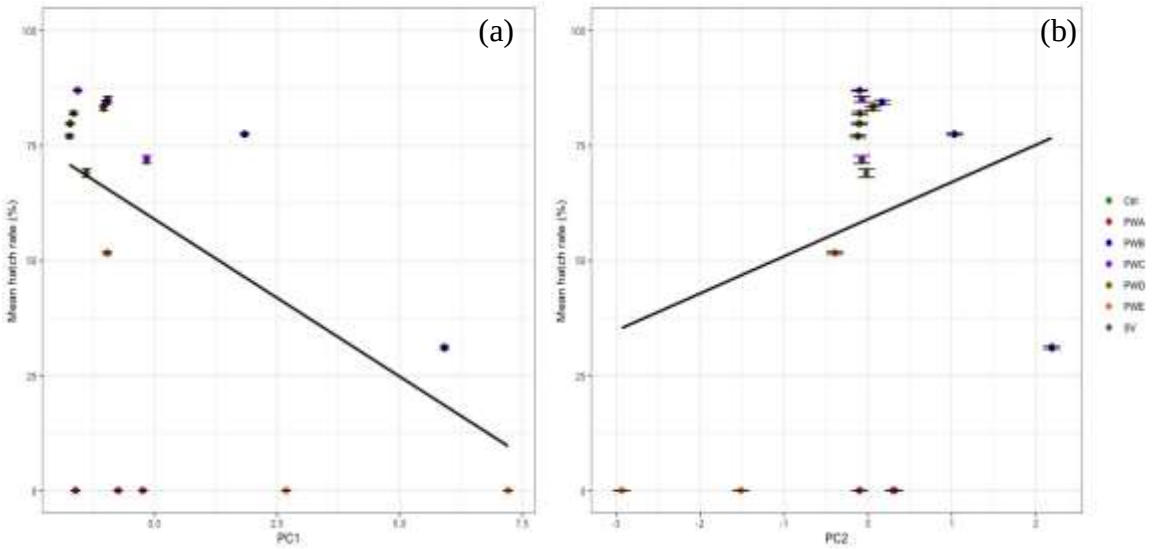

Figure 3.3.3. Impact of PC1 (a) and PC2 (b) on hatching rate. PC1 had a negative linear relationship with hatching rate ($r^2 = 0.2696$, $p < 0.001$). PC2 had a positive linear relationship with hatching rate ($r^2 = 0.05031$, $p < 0.001$).

3.4 Oilfield metrics

Oilfield statistics (oilfield age and percent oil product) were compiled and plotted against hatching rate to assess effect. Percent oil product refers to the percentage of total product that is oil (out of a total that includes also natural gas and liquified natural gas; Table 2.8). Oilfield age demonstrated no trend in relation to hatching rate and explains only 3.8% of the variations in hatching data (Figure 3.4a).

Percent oil product, however, was a significant factor influencing hatching rate ($p < 0.001$) (Figure 3.4b). It demonstrated a clear negative linear relationship with hatching rate and explained 65.6% of the variations in the data. Oil product did not explain the variation in either PC1 ($r^2 = 0.07437$, $p = 0.1514$) or PC2 ($r^2 = -0.03014$, $p = 0.4771$; Appendix Figure 3.4b).

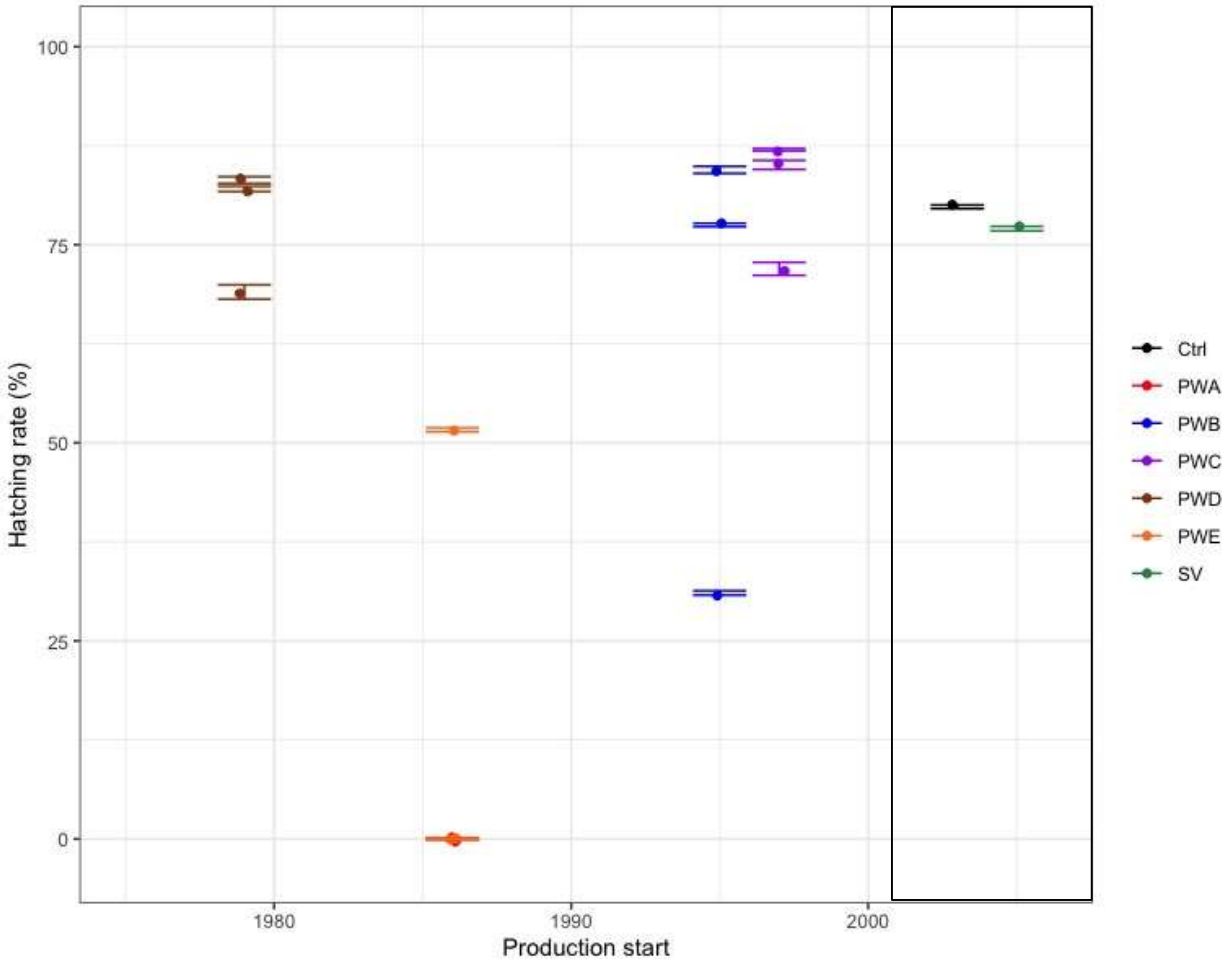

Figure 3.4a. Impact of oilfield age on mean hatching rate ± SEM. For reference, Ctrl and SV have been added in a separate box because they are not subject to oilfield age. For the tested fields, oilfield age explained little of the variation in the data (adjusted $R^2 = 0.03825$, $p = 0.1045$).

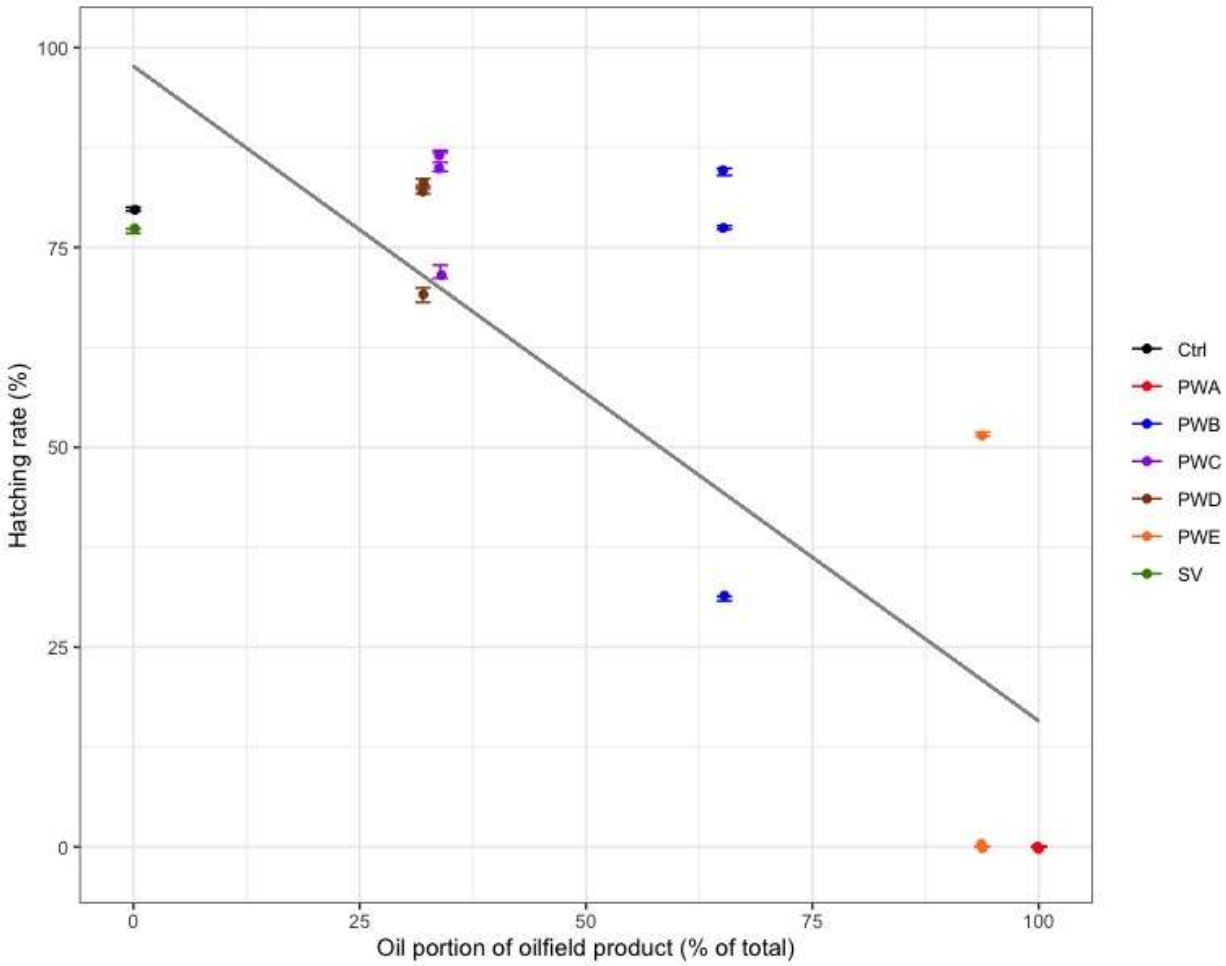

Figure 3.4b. Impact of the percentage of oil in the total oilfield product on mean hatching rate ± SEM. There was a moderately strong negative linear relationship between the variables; oil portion explained a significant portion of the variation in hatching rate (adjusted $R^2 = 0.6563$, $p = 7.132 \times 10^{-14}$).

3.5 Biometry

The biometric endpoints assessed included side yolk area, eye minimum diameter, ventral body width, number of lipids, and heart rate. The effect of PW treatment and of increasing concentration was not consistent across biometry endpoints (Figure 3.5.1, 3.5.2, 3.5.3; Appendix Figure 3.5).

Side yolk area was only significantly larger than the control (0.944 ± 0.024; p = 0.038) in the PWE-L treatment (1.013 ± 0.021; Appendix Figure 3.5). Other treatments indicated a possible decreasing trend in side yolk area with increasing treatment concentration. Eye minimum diameter was significantly larger than the control in PWC-H, all PWD concentrations, and PWE-L treatments, but was significantly smaller than the control in PWB-H (Figure 3.5.1). Average ventral body width was significantly wider in PWB-M and H, PWC-L and M, and PWD-L and M, as well as in the solvent control (SV). PWD-H and PWE-L were also (non-significantly) wider on average and displayed a greater amount of variation in the data. The number of lipids was significantly greater in the PWD-L treatment (1.533 ± 0.165) than the control (1.063 + 0.063) (Appendix Figure 3.5.1). All treatments showed a higher average number, but also showed more variation. While both lipid numbers in the PWD-M and PWB-H treatments were not significantly different from the control (p = 0.061, p = 0.051, respectively), they require more data.

Heart rate was highly variable both within and across treatments. Concentration had a varied effect in each PW condition (Figure 3.5.3). In PWB, increasing concentration lowered the average heart rate. In PWC, increasing treatment concentration correlated with increasing heart rate. Individuals exposed to PWB-H, PWC-L, PWD-L, and PWE-L treatments had significantly reduced heart rates in comparison to the control. Larvae exposed to the SV exhibited significantly lower heart rates as well as more variation in comparison to the control. Larvae exposed to PWC-H had significantly higher heart rates than the control.

3.5.1 Eye minimum diameter

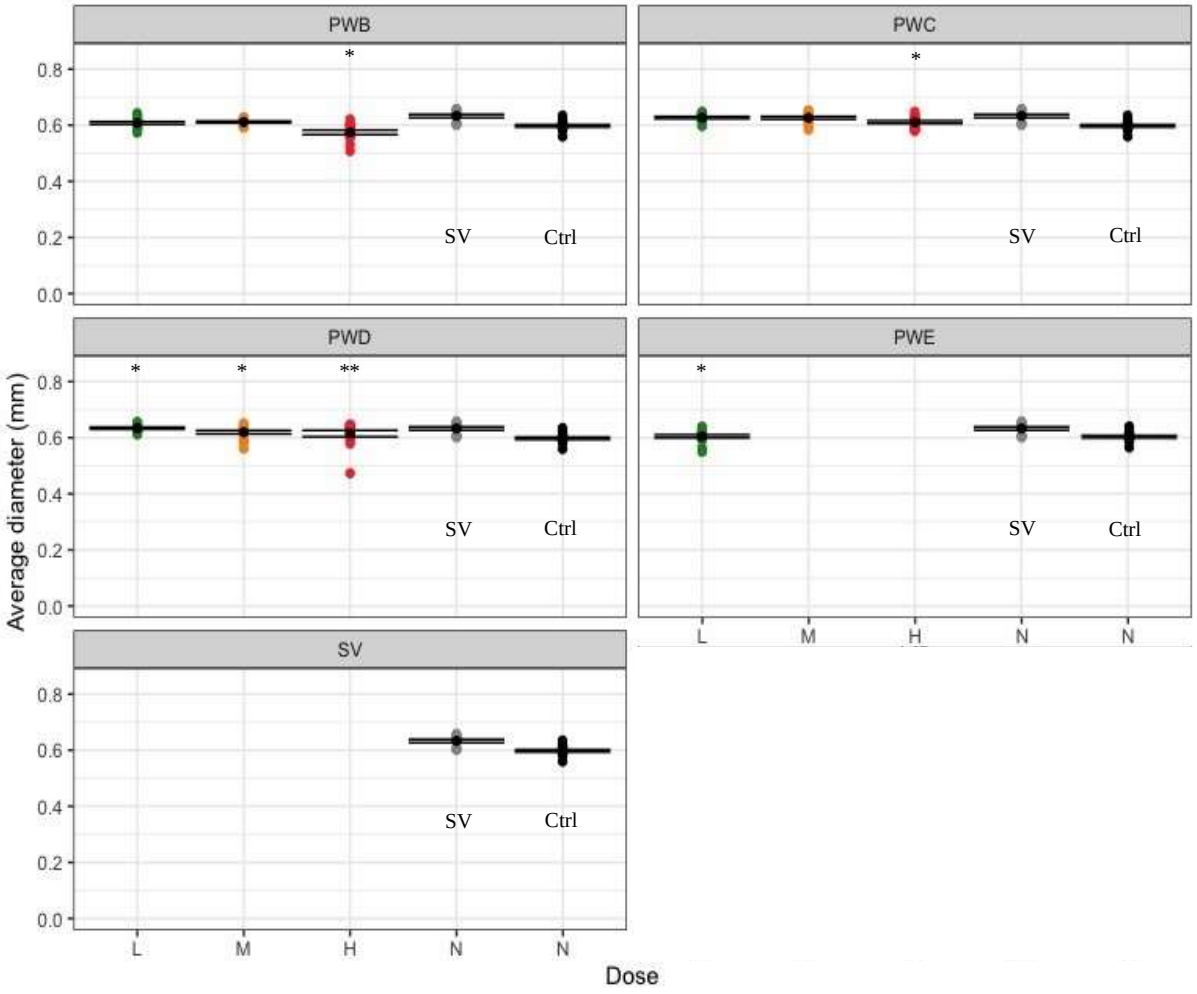

Figure 3.5.1. A comparison of minimum eye diameters for each treatment. The black point and error bars represent the mean (mm) ± SEM. PWB-H, PWC-H, PWD (all concentrations), and PWE-L demonstrated significantly larger minimum eye diameters than the control. There was a trend of decreasing diameter with increasing dose.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

3.5.2 Ventral body width

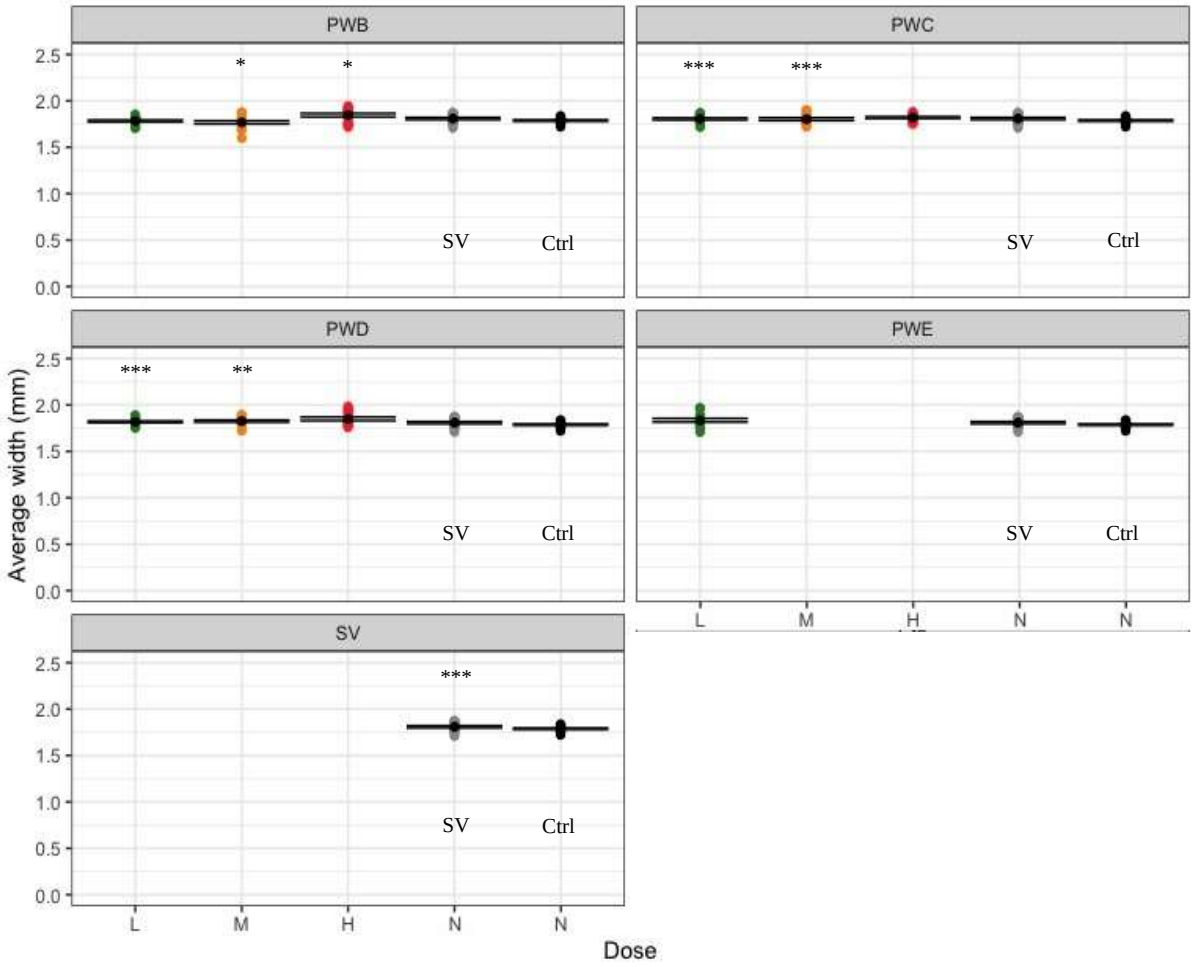

Figure 3.5.2. A comparison of average widths of individuals in each treatment. The black point and error bars represent the mean (mm) ± SEM. PWB-M and -H, PWC-L and -M, and PWD-L and -M, were all significantly wider than the control solution. SV was also significantly wider than the control. PWD-H and PWE-L were, on average, wider, but displayed a greater amount of variation in the data. * $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

3.5.3 Heart rate

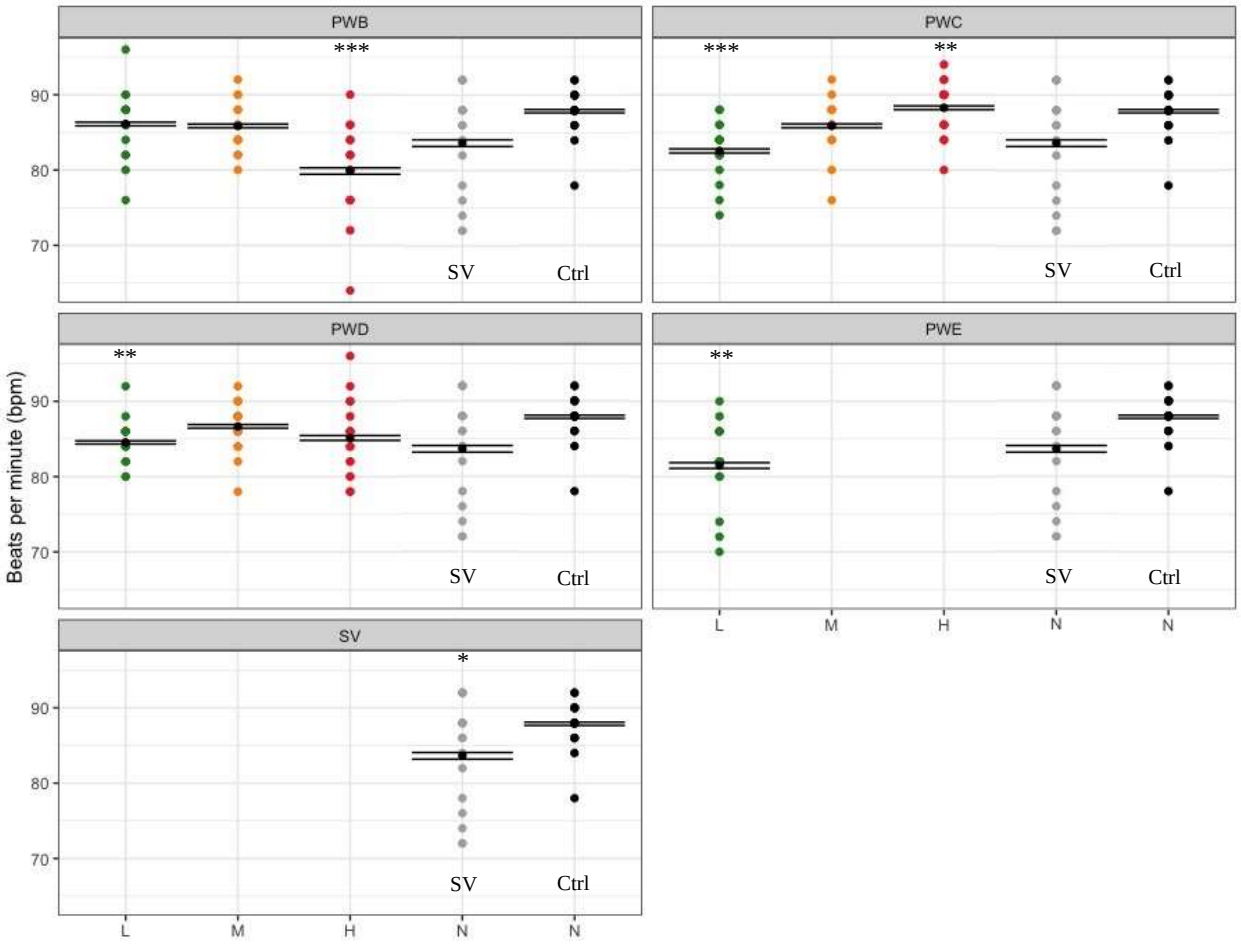

Figure 3.5.3. Comparison of heart rate across each treatment to the control. The black point and error bars represent the mean (bpm) ± SEM. The effect on heart rate was not consistent across treatments. Produced water B-H, PWC-L, PWD-L, and PWE-L as well as the SV had significantly lower heart rates than the control. PWB-H and PWC-H had significantly higher heart rates than the control.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

3.6 Skeletal development

Newly hatched larvae in all exposure solutions exhibited ossification in the lower craniofacial area, sucking disk, and spine (Figure 2.6). Unhatched embryos from the PWE-L group also exhibited ossification across the majority of the skeleton to a lesser extent.

Skeletal development in terms of length is shown in Figure 3.6.1; individuals from PWB-H, PWC-H, PWD-H and PWE-L treatments had significantly shorter skeletal lengths. Higher variation in standard length (SL) was found in individuals from PWB-H, PWC-M, and PWE-L groups (Figure 3.6.1). Individuals from the PWB-M treatment were significantly longer than the control. The average standard length of individuals from the PWD-L was also longer, although not significantly so. Variations in SL was significantly explained by both hatching rate ($r^2 = 0.888$, $p < 0.001$) (Appendix Figure 3.6.1) and PC1 ($r^2 = 0.1312$, $p < 0.001$). Oil product ratio did not significantly explain the variations in the data, but demonstrated a negative linear trend.

Table 3.6.2 and Figure 2.6.2 show skeletal development in terms of the extent of ossification of the spine. Individuals from the control treatment had an average of 28.67 vertebrae, 4.8 of which showed the deepest level of ossification seen in newly hatched lumpfish. There was also extensive ossification of the dorsal and fin rays in the control group (Table 3.6.2). PC1 and ossification level were negatively, linearly correlated and PC1 explained a significant portion of the variations in the tested endpoints of ossification levels, deeply ossified, transparent, and dorsal rays ($p < 0.001$). There were significantly fewer visible vertebrae in all treatment groups. There were also fewer individuals with vertebrae showing the deepest level of ossification. Dorsal and fin rays could be seen in some fish exposed to the PW treatments (Figure 2.6), but some treatments resulted in significantly lower average numbers of ossified dorsal and fin rays (Table 3.6.2; corresponding Appendix Figure 3.6.2). Treatments that resulted in fewer dorsal rays than the control did not necessarily also result in fewer fin rays.

3.6.1 Standard length

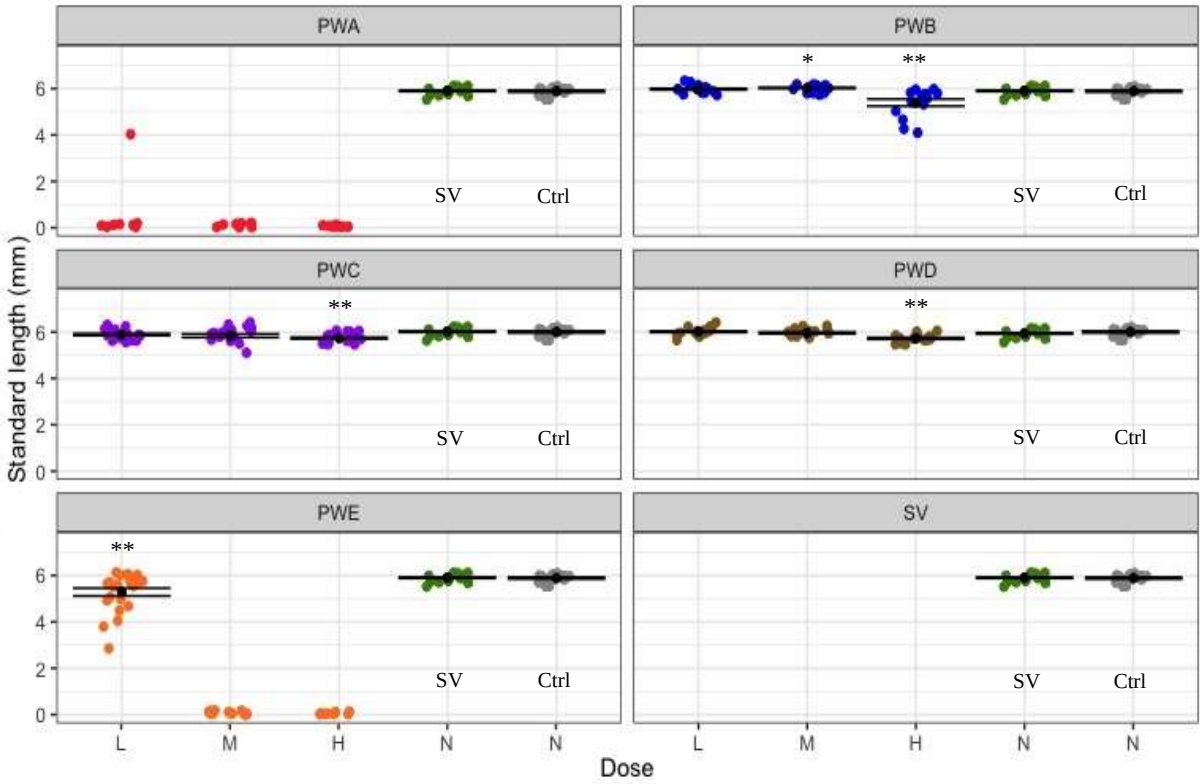

Figure 3.6.1. Comparison of non-automated calculation of SL across treatments to the control. The black point and error bars represent the mean (mm) ± SEM. Samples unable to be measured due to death before adequate development are presented visually at 0, but are not included in the analysis. PWB, PWC, and PWD in the high concentrations as well as PWE-L are significantly shorter than the control. PWB-M is significantly higher than the control.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

3.6.2 Spinal ossification

Table 3.6.2. Average number of specified vertebral measurements for each treatment. All treatment conditions significantly reduced the average number of partially ossified vertebrae; all treatments except SV significantly reduced the total number of visible vertebrae. Some treatment conditions caused significant decreases in the number of visible dorsal and fin rays.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$.

PW	Dose	n	Deeply ossified	Totally ossified	Partially ossified	Transparent	Total	Dorsal rays	Fin rays
Ctrl	N	15	4.80 ± 0.15	6.13 ± 0.34	14.00 ± 0.47	3.73 ± 0.32	28.67 ± 0.25	11.00 ± 0.43	4.87 ± 0.26
PWA	L	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	M	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	H	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
PWB	L	15	4.53 ± 0.22	7.13 ± 0.45	14.07 ± 0.37	1.33 ± 0.21 ***	27.07 ± 0.18 ***	11.73 ± 0.25	4.93 ± 0.25
	M	15	3.87 ± 0.19 ***	7.93 ± 0.23 ***	13.12 ± 0.31	1.67 ± 0.27 ***	26.60 ± 0.34 ***	8.27 ± 0.89	3.67 ± 0.49 *
	H	15	2.20 ± 0.44 ***	4.60 ± 0.48 *	14.13 ± 0.74	2.80 ± 0.24 *	23.73 ± 0.37 ***	5.47 ± 0.95	1.73 ± 0.47 ***
PWC	L	17	4.18 ± 0.15 **	8.00 ± .27 ***	13.18 ± 0.27	1.47 ± 0.21 ***	26.82 ± 0.18 ***	7.82 ± 0.87 **	3.76 ± 0.36 *
	M	16	3.00 ± 0.35 ***	8.44 ± 0.41 ***	13.25 ± 0.35	1.69 ± 0.20 ***	26.38 ± 0.22 ***	5.69 ± 1.04 ***	2.88 ± 0.46 ***
	H	16	2.88 ± 0.46 ***	4.81 ± 0.63	16.25 ± 0.74 *	1.81 ± 0.29 ***	25.75 ± 0.30 ***	4.81 ± 0.90 ***	2.88 ± 0.42 ***
PWD	L	16	4.69 ± 0.22	7.25 ± 0.19 **	13.81 ± 0.19	1.12 ± 0.15 ***	26.88 ± 0.09 ***	11.13 ± 0.50	5.06 ± 0.23
	M	15	3.67 ± 0.13 ***	8.33 ± 0.16 ***	13.53 ± 0.22	0.93 ± 0.15 ***	26.47 ± 0.19 ***	9.00 ± 0.41 **	4.07 ± 0.45
	H	15	3.47 ± 0.40 **	6.33 ± 0.70	14.73 ± 0.73	1.87 ± 0.19 ***	26.40 ± 0.19 ***	6.93 ± 0.97 **	3.80 ± 0.35 *
PWE	L		3.41 ± 0.45 **	7.35 ± 0.79	12.41 ± 0.59 *	2.52 ± 0.21 **	25.71 ± 0.59 ***	7.94 ± 1.01 *	4.00 ± 0.68
	M	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	H	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
SV	N	15	5.20 ± 0.20	7.07 ± 0.18 *	13.20 ± 0.58	2.53 ± 0.19 **	28.00 ± 0.46	11.00 ± 0.68	4.53 ± 0.54

3.7 Spinal abnormalities

The spinal abnormalities found in lumpfish larvae and embryos include spinal break, kyphosis, lordosis, scoliosis, compression or expansion, stunted arches, and wavy or twisted arches (Figure 3.7, Table 3.7). These abnormalities were combined under the heading “axis deviations” in order to provide an overall account. Several axis deviations illustrated an extreme and a subtle phenotype. Figure 3.6 presents kyphosis in both extreme (3.7; b.1) and subtle (3.7; f) phenotypes. The majority of larvae exposed to a PW treatment showed one or more deviations.

The average number of axis deviations was significantly higher in individuals exposed to PW treatment. Larvae from the control group had almost no spinal abnormalities, with only one individual exhibiting wavy or twisted arches. The number of individuals with wavy and twisted arches was significantly higher in all treatment conditions. Scoliosis was significantly higher in all treatment conditions except PWD-L and -H.

Several phenotypic effects were unique to a single PW type or treatment group. The stunted arches shown in Figure 3.7 (d.2) only occurred in PWB at the high concentration (Table 3.7), with 40% of individuals presenting this type of abnormality. The presence of vertebral expansion or compression was significantly higher in PWC groups and was also seen to a low

degree in both PWB-L and PWD-L groups (Table 3.7). Similarly, kyphosis was seen in various treatments and occurred at a significantly higher rate in PWB-H and PWC-H.

While significant axis deformations were explained by PW type and showed increases with concentration, no other explanatory variable (hatching rate, oilfield type, or hydrocarbon components) identified in this study demonstrated a significant explanation or correlation.

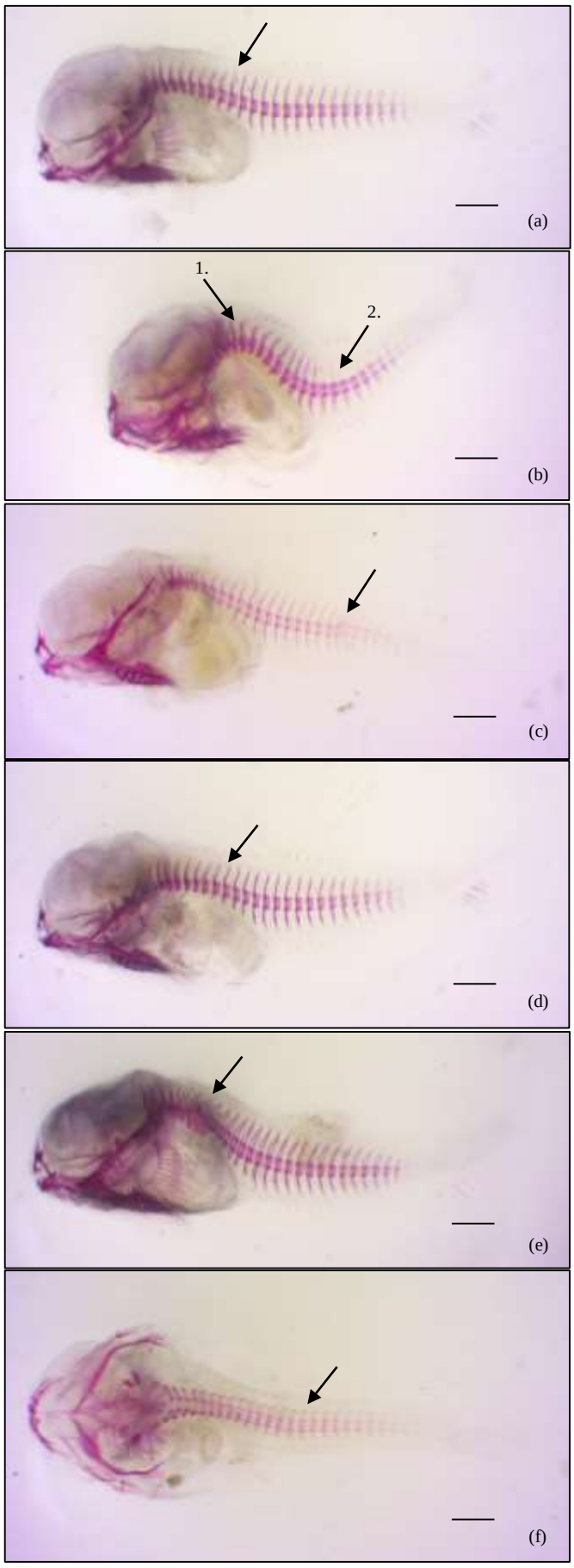

Figure 3.7. Example of axis deviations: (a) twisted or wavy arches; (b) 1. kyphosis, 2. stunted arches; (e) break; (f) minor kyphosis; (g) expansion or compression; (h) scoliosis. Bar represents 500µm.

Table 3.7. Percent of total individuals per condition with specified deviation. Axis deviation represents a percent of the total with one or more deviations. Some fish co-possessed multiple types of spinal deformations. All solutions induced a significantly higher rate of total deformities ($p < 0.001$) in comparison to the control.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$.

PW	Dose	n	Axis deviation	Break	Kyphosis	Scoliosis	Compression or Expansion	Stunted arches	Wavy or twisted arches
Ctrl	N	15	0	0	0	0	0	0	6.67
PWA	L	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	M	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	H	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
PWB	L	15	53.33 ***	13.33	0	26.67 *	6.67	0	86.67 ***
	M	15	46.67 **	6.67	6.67	20.00 *	0	0	66.67 ***
	H	15	60.00 ***	0	40.00 **	33.33 **	0	40.00 **	100 ***
PWC	L	17	58.82 ***	11.76	11.76	17.65 *	11.76 *	0	58.82 **
	M	16	56.25 ***	12.5	6.25	43.75 **	6.25 *	6.25	81.25 ***
	H	16	81.25 ***	0	18.75 *	43.75 **	6.25 *	6.25	87.50 ***
PWD	L	16	31.25 **	0	0	12.50	6.25 *	0	68.75 ***
	M	15	40.00 **	0	6.67	33.33 **	0	0	100.00 ***
	H	15	46.67 **	0	13.33	0	0	0	66.67 ***
PWE	L	17	29.41 *	0	0	23.53 *	0	0	88.24 ***
	M	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	H	0	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
SV	N	15	0	0	0	0	0	0	13.33

3.8 Craniofacial endpoints

Craniofacial development was measured as head length, jaw length, jaw-to-eye length, and jaw point angle (Figures 3.8.1a to 3.8.1d). The relationship between all craniofacial development endpoints except head length were significant; hatching rate predicted >85% of the variations in jaw length, jaw-to-eye length, and jaw point angle ($p < 0.001$). PC1 predicted approximately 12% of the variations in all craniofacial developmental endpoints, including head length ($p < 0.001$).

The results shown in Figure 3.8.1a indicated that head length was significantly longer than the control in the PWB-L, PWD-L, and PWD-M treatments. In both the highest concentration treatments and PWE-L, head length was shorter on average. However, this latter result requires more data before significance can be proven.

Jaw length was less than or equal to that of the control in all treatment groups (Figure 3.8.1b). The highest concentrations all showed significant decreases in length. The SV also demonstrated a significantly shorter jaw length than the control ($p = 0.044$).

The average jaw-to-eye length of all individuals treated with PW was lower than that of the control (Figure 3.8.1c). This was significant in PWB-M and H, PWC-M and H, PWD-L and M, and PWE-L. Jaw-to-eye length also increased between the medium and high concentrations of each treatment. However, more data is required to prove significance.

Jaw point angle was larger in all PW treatments and generally decreased with increasing concentration (Figure 3.8.1d). The average angle was significantly larger in PWB-L and M and in all concentrations of PWC and PWD.

The craniofacial abnormalities observed included flat face or underbite, open mouth, lack of jaw, and elongated face (Table 3.8.2). They also included jaw abnormalities (i.e. the number of jaw-specific abnormalities that did not include anomalies of the upper cranial or facial region unless they co-occurred with or resulted in jaw abnormalities). 20.1% of the variations in jaw abnormalities was explained by hatching rate ($p < 0.001$) and 19.9% was explained by PC1. Approximately half of all PW-exposed embryos developed craniofacial abnormalities.

The incidence of flat face or underbite phenotype was significantly higher in PWB-H, PWD-H, and PWE-L treatment conditions (Table 3.8.2). The percentage of larvae or embryos exhibiting the open mouth phenotype was significantly higher in all PW treatment conditions (except PWC-L). It was also associated with increased jaw angle (Table 3.8.2; Figure 3.8.1c). Lack of jaw was a novel phenotype (Figure 3.8.2) observed only in PWE-L (and thus to a significantly higher degree than the control). Incidence of elongated faces was significantly higher in PWC-H; only one other larvae, treated with PWE-L, illustrated this phenotype.

3.8.1 Craniofacial development

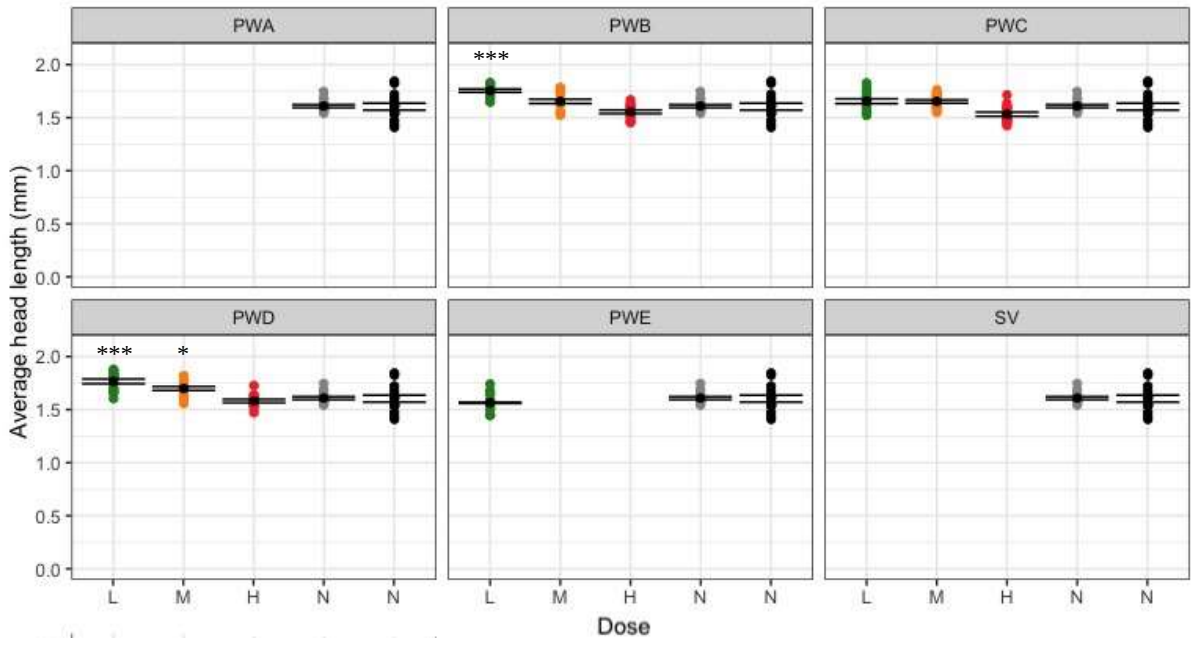

Figure 3.8.1a. A comparison of average head lengths of individuals in each treatment in comparison to the control solution. The black point and error bars represent the mean (mm) ± SEM. PWB-L, PWD-L and M all had significantly longer heads than the control solution.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

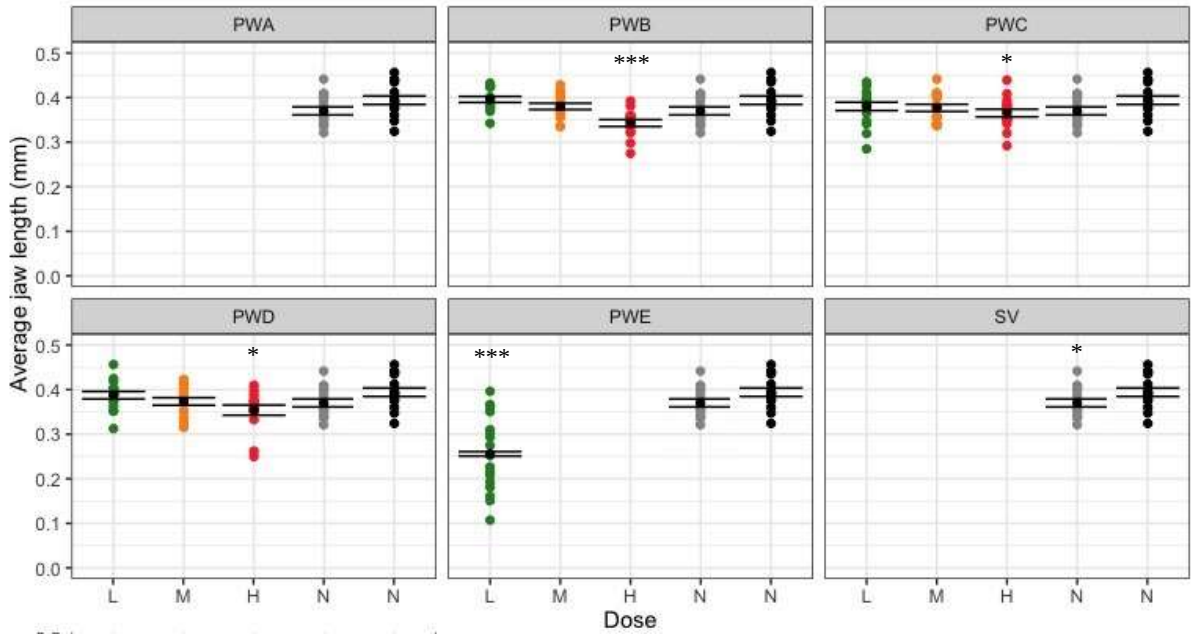

Figure 3.8.1b. A comparison of average jaw lengths of individuals in each treatment to the control. The black point and error bars represent the mean (mm) ± SEM. PWB-H, PWC-H, PWD-H, and PWE-L all had significantly shorter jaws than the control. A large amount of variation was seen in PWE-L. * 0.05 < p < 0.01, ** 0.01 < p < 0.01, *** p < 0.001

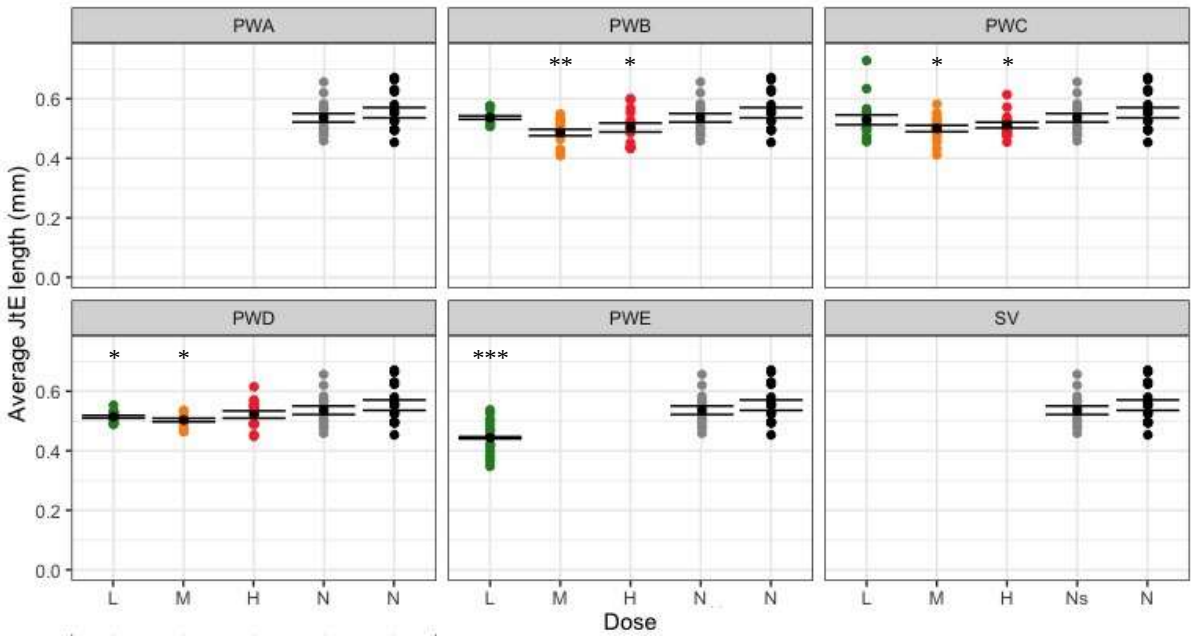

Figure 3.8.1c. A comparison of mean jaw-to-eye lengths of individuals in each treatment with the control. The black point and error bars represent the mean (mm) ± SEM. PWB-M and H, PWC-M and H, PWD-L and M, and PWE-L all had significantly shorter jaw-to-eye lengths than the control solution. * 0.05 < p < 0.01, ** 0.01 < p < 0.01, *** p < 0.001

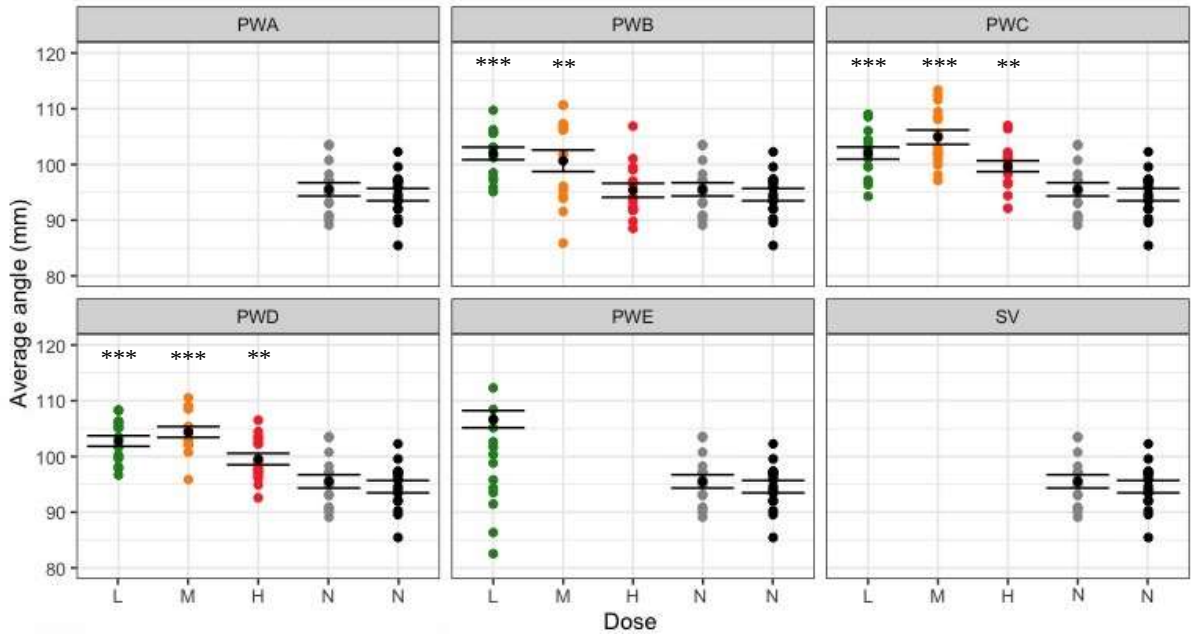

Figure 3.8.1d. A comparison of average jaw point angles of individuals in each treatment. The black point and error bars represent the mean (mm) ± SEM. PWB-M and -H, PWC-L and -M, and PWD-L and -M, L all had significantly wider jaw angles than the control solution. Produced water E-L displayed a greater amount of variation in the data.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

3.8.2 Craniofacial abnormalities

Figure 3.8.2. Examples of observed craniofacial anomalies: (a) 1. flat face, 2. underbite; (b) open mouth; (c) lack of jaw; (d) elongated face. Bar represents 500µm.

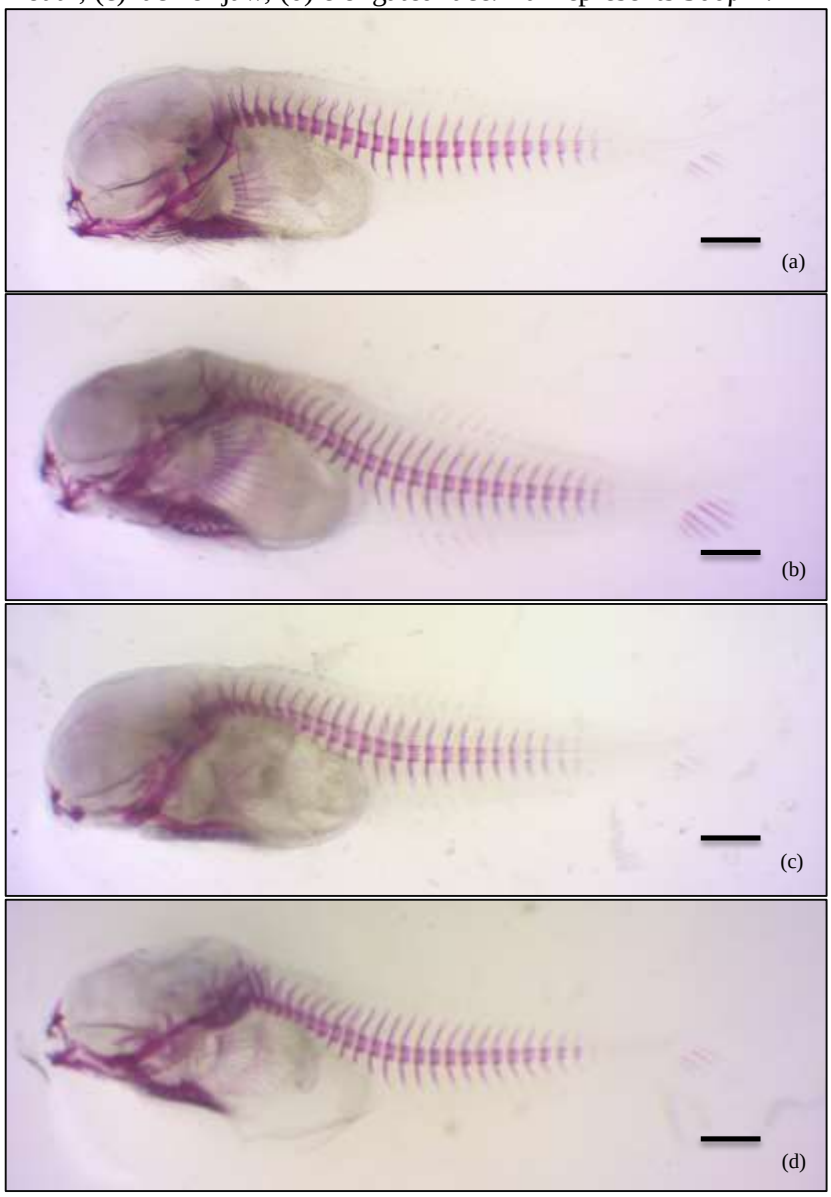

Table 3.8.2 Percent of total larva or embryos with anomalies per treatment (%). Jaw abnormalities represented a percent of the total with one or more of deviations; some fish co-possessed multiple types of craniofacial deformations. Individuals in all PW groups (except PWC-L) demonstrated significantly higher numbers of jaw abnormalities (specifically the open mouth phenotype) than the control. Several treatments produced unique phenotypes (e.g. lack of jaw, elongated face) to a significantly higher degree than the control. * $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$.

PW	Dose	n	Jaw abnormalities	Flat face or underbite	Open mouth	Lack of jaw	Elongated face
Ctrl	N	15	0	0	0	0	0
PWA	L	0	*NA*	*NA*	*NA*	*NA*	*NA*
	M	0	*NA*	*NA*	*NA*	*NA*	*NA*
	H	0	*NA*	*NA*	*NA*	*NA*	*NA*
PWB	L	15	0	0	0	0	0
	M	15	40.00 **	6.67	33.33 **	0	0
	H	15	46.67 **	26.67 *	26.67 *	0	0
PWC	L	17	6.25	0	6.25	0	0
	M	16	18.75 *	6.25	18.75 *	0	0
	H	16	56.25 ***	11.76	25.00 *	0	38 **
PWD	L	16	25.00 *	0	25.00 *	0	0
	M	15	20.00 *	0	20.00 *	0	0
	H	15	46.67 **	20.00 *	41.18 **	0	0
PWE	L	17	76.45 ***	53.85 **	29.41 *	47.06 ***	5.88
	M	0	*NA*	*NA*	*NA*	*NA*	*NA*
	H	0	*NA*	*NA*	*NA*	*NA*	*NA*
SV	N	15	0	0	0	0	0

4. Discussion

4.1. The effects of PW on hatching and survival

The hatching and survival of lumpfish larvae were impaired by treatment with several PWs compared to the seawater control. The results agree with previous studies reporting acute toxicity to PW and PW fractions (Le Bihanic et al., 2014; Carls et al., 1999; Heintz et al., 1999). Exposure to PWs A and E demonstrated high acute toxicity at all concentrations. Acute toxicity is an important endpoint for regulatory use and has been repeatedly seen in studies of waterborne exposure of fish to PAHs (Carls et al., 1999; Heintz et al., 1999). However, treatment conditions that result in decreases in hatch rate (rather than in survival rate) may allow for a larger dataset in analysis going forward. Moreover, differences in hatching rates were more extreme than differences in survival rates (Figure 3.2.2; Figure 3.2.3) suggesting that hatching rate may be a more sensitive and overall more useful indicator of effect.

PW type (A, B, C, D, or E) was a strong predictor of differences in hatching rate. Therefore, determination of the individual components of each PW and exploration of oilfield specifics was hypothesized to further explain the variation in hatching rate and other endpoints. However, TEM, a commonly used metric for regulatory and other analyses, was not a determining factor in any acute toxicity or depreciation in hatching rate (Figure 3.4.1b). Interpretation of the PCA suggested that PC1 was primarily correlated with organic character and PC2 to represent the effect of dissolvability of a compound. As expected, hatching rate demonstrated a negative linear relationship with PC1 and thus with all petrogenic compound groups tested (Hansen et al., 2019; Cherr et al., 2017; Beyer et al., 2016; Le Bihanic et al., 2014; Figure 3.3.3a). Together, these groups explained a significant portion of the toxicity (approximately 32%).

The explanatory power of TEM or PC1 would likely be significantly greater if not for PWA. Treatment of embryos with PWA caused complete mortality at all concentrations, despite having the lowest rates of petroleum-based compounds (Figure 2.7). The toxicity of PW cannot therefore be completely and accurately estimated by current testing measures and reflected in the corresponding regulatory guidelines. These results confirm the need for a greater study of the specific components of PW. This may require a new methodological approach as suggested by Sørensen et al. (2019) and Bostick et al. (2002).

The amount of petrogenic compounds in a PW is often assumed to be influenced by the age of the oilfield and the proportion of oil in the total product of the field (Neff et al., 2011). Older oilfields may be expected to have lower amount of petrogenic compounds because they have been repeatedly filled with reinjected seawater to facilitate product removal (Ngene and Tota-Maharaj, 2020). They may also be expected to have higher amounts of production chemicals to further facilitate product extraction as this becomes less efficient over time (Ngene and Tota-Maharaj, 2020). These expectations were not supported by the results of this study (Figure 3.4a). While the exact type of oil product could not be determined from the available information, the proportion of oil in the product was strongly correlated with both hatching rate and survival rate. It is likely that the proportion of oil in the product is associated both with petrogenic compounds and with certain toxic production chemicals.

The interaction of PAHs with cellular enzymes, DNA, and cellular proliferative signals causes cellular damage by hindering proper function, inducing reactive oxygen species, and otherwise shunting resources from skeletal growth and development (Casarett and Doull, 2013; Shi et al., 2012; He et al., 2011). Larvae are thus less developed at hatching. PAHs can

delay hatching time without dose dependence and may result in non-dose dependent influences lasting to adulthood, including sex ratio and fecundity indices (Chikae et al., 2004).

4.2 The effects of PW on development

Growth was represented by several endpoints including body measurements (standard length, ventral width, head length, eye minimum diameter, jaw length, jaw-to-eye length, and jaw point angle), spinal ossification scoring, and yolk parameters (side yolk area and lipid number). Samples exposed to all concentrations of PWA or to PWE in the M and H concentrations died before reaching a developmental stage where growth endpoints could be assessed. For several endpoints, it was impossible to separate and fully rule out the possibility of toxicity as a result of SV. Despite this, growth endpoints showed alterations after treatment with all PWs, illustrating sublethal toxicity.

While all parameters were affected, there was no strong pattern in terms of which treatments affected developmental parameters or how they did so. Generally, high concentrations of PWB and PWC and low concentrations of PWE were most reliable in inducing aberrations in growth endpoints. These responses included decreased standard length, increased ventral width, altered heart rate, decreased spinal ossification, and decreased jaw and head lengths. PWE also resulted in the most variation in individual responses.

Standard length is often affected by exposure to toxic solutions (Hansen et al., 2019; Vignet et al., 2014). A negative linear relationship between both PC1 and PC2 and standard length was found. The relationship of PC1 to standard length was much stronger than that of PC2. This relationship has not been well-established for lumpfish, but it is weaker than that typically seen in cod (Frantzen et al., 2015; Bellas et al., 2005). There are likely several causes for this weak relationship, which may include 1) literature primarily concerned with cr1) limited exposure and long depuration times; 2) the robustness of lumpfish in comparison to other study species; 3) combination of sexes despite dimorphism in size and toxicant uptake rates (Madenjian et al., 2016; Vignet et al., 2014; Casarett and Doull, 2013); 4) differences in the components and component ratios of PW (Sørensen et al., 2019; Bostick et al., 2002; Røe Utvik, 1999); and 5) few data points.

While exposure ended before the likely beginning of cardiogenesis (heartbeat becomes evident at around 120 d° in lumpfish), cardiac cells may be designated as early as gastrulation, which coincided with the treatment period of this study (Imsland et al., 2019; Gilbert and Barresi, 2016; Langeland and Kimmel, 1997). Brown et al. (2017), Cherr et al. (2017) and Incardona et al. (2013) found heart development to be extremely sensitive to low levels of PAHs. The impact of sublethal toxicity on the heart can manifest in several ways, making heart rate a reliable (though not perfect) predictor (Brown et al., 2017).

Cardiac toxicity, primarily in the form of edema, has been considered by some to be the primary and most sensitive phenotype of toxicity (Incardona et al., 2013). However, no incidence of edema was seen in any individuals in this study. In accordance with the literature, cardiac dysfunction was also expected to present as a narcosis-like effect (Incardona et al. 2003). Incardona et al. (2003) further suggest that cardiac disfunction is primarily related to exposure to 3-ring PAHs. While solutions with high levels of 3-ring PAHs did exhibit lower heart rates, other solutions exhibited similarly low heart rates. Altered cardiac performance, (both increased and decreased rhythms) can be reliably related to impaired swimming performance, increasing the chances of predation (Brown et al., 2017). Moreover, early differences in cardiac function and size have been shown to persist into adulthood, which can

further decrease survival rate (Tang et al., 2018; Brown et al., 2017; Incardona et al., 2015). The surprising findings in the current study may be a result of the oil type, which could not be determined for the PWs used in this study. Edema is generally found in embryos exposed to crude oil with high rates of 3-ring PAHs. It may also suggest that cardiotoxicity may present itself less strongly with PW or may not always be a sensitive endpoint (Jung et al., 2013; Jung et al., 2015).

Bone ossification is a known target of endocrine-disrupting chemicals including alkylphenols and dioxin-like hydrocarbon compounds (Agas et al., 2019; Holz et al., 2007). Disruption may occur in a variety of ways, including disruption of the hormone balance of the skeletal system, alteration of collagen turnover rates, decreasing bone mineralization activity, and other disruption the energy balance of the larvae away from growth and toward mitigation of toxicants (Agas et al., 2019; Desforges et al., 2017; Boelsterli, 2007; Holz et al., 2007; Singh et al., 2000; Koojiman, 1993). Decreased bone development was evident across all treatment solutions.

While increasing PC1 explained some variation in the decreased spinal development, increased PC2 actually resulted in an increase in some spinal development measures, including the total number of ossified vertebrae. The reasons for such a response are unclear, but they suggest that more data is necessary to confirm these results and support research findings that phenols play an important role in altering ossification, through a plurality of effects.

The large yolk reserves of lumpfish (in comparison to other common study species) may play an important role in allowing the fish to continue growth even when under stress. However, developmental metrics like ventral width and, specifically, yolk parameters are often used as measures of larval stress and body condition (Hansen et al., 2019; Olsvik et al., 2012; Incardona et al., 2004). Ventral width correlated strongly with other indicators of toxicity and may be a sensitive indicator. Increases in yolk number typically signals inteferences in early stages of development and cell signaling. While yolk size across treatment conditions showed no differences, yolk number was (non-significantly) both higher and more variable across all solutions. Decreases in yolk size and larval standard length in comparison to control populations typically signifies icnreased nutritional needs due to energy being diverted toward various forms of stress maintenance, detoxification, and repair processes (Olsvik et al., 2012; Incardona et al., 2004). Differences in yolk number likely suggest and result in a hindered ability to use yolk nutrients normally and an inability to attain proper nutrition, especially between hatching and first feeding.

Incardona et al. (2004), documented reduced head growth resulting from exposure to solutions high in phenanthrenes, dibenzothiophenes, and fluorenes. While this group was too highly correlated with other components to allow individual comparison, the results of this study suggest that head length is not always significantly impacted by exposure to PW, with only PWB-L showing a significantly reduced average head length.

Lumpfish larvae demonstrate allometric growth; the development of bones supporting important life processes such as feeding, locomotion, and respiration are prioritized (Osse and Van de Boogaart, 1995). As lumpfish typically begin feeding within several days of hatching, their mouths often are developed and open at the point of hatching (Imsland et al., 2019; Voskoboinikova and Kudryavtseva, 2014; Kjørsvik et al., 2007). As expected, jaw development was strongly associated with hatching rate (and, to a lesser extent, standard

length). It may therefore be used as a reliable indicator of delayed development in juvenile fish. Jaw point angle may be the most sensitive indicator of abnormal development and is likely to correlate with jaw abnormalities. However, as exemplified by the lack of observable effect in the PWB-H treatment, which resulted in significant decreases in hatching rate and standard length, jaw point angle may be hidden by delayed hatching and the resulting reduced jaw development.

Generally, the increases in variation of growth metrics demonstrated in the current study can be problematic for a species when extrapolated to the population level even in the absence of a change in the average. It can also be problematic if the parameter scales with other important life history traits such as fecundity or predation risk, both of which can have large-scale consequences (Meekan and Forteir, 1996).

4.3 The effects of PW on abnormalities

A host of abnormalities as a result of exposure to PAHs and crude oil have been documented (Hansen et al., 2019b; Sørensen et al., 2019; Hodson, 2017; Sørhus et al., 2015; Vignet et al., 2014; Incardona et al., 2013; Jung et al., 2013; Li et al., 2011; Incardona, 2004). Typically, these toxicity tests impart a heavy pollutant load. The resulting abnormalities can occur throughout the body and organs. However, in the current study, the endpoints assessed for abnormality included only the early ossified bone structures: the spine and the craniofacial area.

It is well-established in the literature that abnormalities in fish embryonic development correlate strongly with pollution load (Adeogun et al., 2019; Gilbert and Barresi, 2016; Casarett and Doull, 2013; Incardona et al., 2012). Pollution load refers both to the amount of a pollutant that is discharged (typically measured as TEM or total PAH concentration) as well as the amount of stress placed on a system by that pollution. In this case, the system was an individual embryo and the pollution load was the amount of matter dissolved within the treatment PW and the stress it induced on the embryo.

The principal components, PC1 and PC2, may best account for pollution load in this experiment, but they only explain a moderate portion of the spinal abnormalities in PW treatment conditions. All treatment conditions resulted in a myriad of spinal abnormalities including wavy or twisted arches and axis deviations (Table 3.7). PWE, which had the highest overall PAH pollutant load, resulted in both acute and sublethal toxicity. Of the sublethal toxicity outcomes, the spinal abnormalities are both fewer in number and fewer in type than in other treatment conditions.

The specific toxicants encapsulated within the PC1 and PC2 groups have the ability to interact with cellular systems, enzymes, and even nucleic acids, all of which can alter important regulation cascades and cause a multitude of abnormalities (Gilbert and Barresi, 2016; Casarett and Doull, 2013). For example, B(a)P, pyrene, and phenanthrene inhibit Na^+/K^+-ATPase activity in a dose-dependent manner. This results in increased abnormal spinal curvature, specifically scoliosis, lordosis, and kyphosis (Brette et al., 2014; Li et al., 2011). However, the results of the current study suggest that the amount of 3- and 4-ring PAHs do not scale in a linear fashion with abnormal spinal curvature. Lordosis was not seen in any individual and treatment conditions that were lower in PAHs demonstrated higher rates of scoliosis and kyphosis than their high 3- and 4-ring PAH counterparts. Moreover, these abnormalities did not scale in a dose-dependent manner.

Interaction of several toxicants at the receptor level may also offer some level of mitigation. Receptors can interact and be activated through a mechanism known as "cross-talk." This, in some cases, might allow for the activation of other detoxifying enzymes before a typical agonist has been received (Holth et al., 2008; Mortensen and Arukwe, 2007; Safe, 2001). Similarly, higher concentrations of certain pollutants may increase the cellular presence of detoxifying enzymes (Boelsterli, 2007). In effect, more PAHs and toxicants might then be mitigated in a stronger solution as compared to a weaker one. This effect may explain the relatively equivalent (and occasionally higher) rate of toxicity seen in PWD in the medium concentration compared to the corresponding high concentration (Figures 3.2.1, 3.2.2, 3.3.3, 3.5.2, 3.8.1a-d, and Table 3.7).

Another significant finding was the presence of a novel phenotype (stunted arches) in individuals treated with PWB-H. This phenotype is not explained by any parameter explored in this study. A more complete account of the PW contents is likely to account for a significantly greater portion of the both the abnormalities, including stunted arches, as well as other explored endpoints.

The impact of skeletal abnormalities outside of the laboratory can be severe. Wittenrich et al. (2009) and Isaac et al. (2017) have shown that abnormalities correspond with significantly impaired survival due to low feeding rates, impaired swimming performance, and increased disease susceptibility. While this results in economic loss in aquaculture, in the wild it results in recruitment failure and reduced breeding stock.

PAHs are also shown to reduce the expression of various cellular proliferative signals (and thus cell proliferation) as well as collagen enzymes (Shi et al., 2012; He et al., 2011). Both of these reductions lead to reduced craniofacial skeletal cell development and deformed cartilage, which may result in associated craniofacial deformities (Shi et al., 2012; He et al., 2011). Disruptions in jaw growth in larvae typically become malformations in adults. While the patterns of abnormal phenotypic effect are complex and intertwined, all PW treatments illustrated jaw abnormalities and many also exhibited cranial-specific abnormalities (e.g. flat and elongated face).

However, in some cases, a specific PW may feasibly be linked to the occurrence of specific abnormalities. For example, PWE-L alone resulted in the "lack of jaw" phenotype. Similarly, PWC-H was the only treatment to result in the "elongated face" phenotype. The other abnormalities were induced by various PWs at several concentrations. The reason for the exceptional nature of these phenotypes can only be speculated at, but they suggest an impact of production chemicals or other explored elements.

4.4 Considerations

The results of this study are both revealing and complicating. It is obvious that the effect of PW in the environment is complex and cannot be fully elucidated even in a comprehensive review such as this. However, the mortality rate and hatching failure in PWE is likely a result of the additive effects of various PAHs. While PWE may illustrate synergistic or potentiative effects, evidence of chemical interactions is scant. It is promising that PW generally undergoes rapid and extensive dilution. A model by Niu et al. (2016) suggested that the mixture may disperse to 1% within the first 12 hours. The effect of 1% of a PW solution is likely minimal for many PWs, but may still result in acute toxicity for other PWs similar to PWA and PWE (which demonstrate acute toxicity at 10%).

Even if PW only increases local background levels, the effects can still be problematic in an environmental context. Organic contaminants may decrease an organism's ability to cope with other threats, toxic or otherwise. If the contaminant is a lipophilic compound or a metal, it may accumulate in the tissue. This may result in further toxicity during tissue mobilization or via mutagenesis and corresponding carcinogenesis (Gilbert and Barresi, 2016; Foekema et al., 2012). In fact, markers of PW have been found up to 10km from the discharge point (Johnsen et al., 1998). Moreover, various tissue several resident fish populations have shown elevated levels of toxicants (Brooks et al., 2013; Lourenço et al., 2018; Neff, 2002).

Increasing evidence suggests that modified PAHs (such as metabolites, photo-degraded products, or other derivatives) are widely distributed in the environment and often exhibit greater toxicity than their parent compounds (Hansen et al., 2018; Xie et al., 2006). Geier et al. (2018) found that a focus on well-known and "priority" PAHs that are detectable by GC-MS under-represents the range of toxicity in PAH mixtures. This is specifically problematic for regulatory purposes and may be relevant to this study. The developmental toxicity of substituted PAHs cannot be predicted by their definitive chemical characteristics and is undoubtedly affected by the individual and the environment (Casarett and Doull, 2013; Gilbert and Barresi, 2016; Boelsterli, 2007; Singh, 1996).

Oil and gas platforms can act as artificial reefs and thus carry large resident fish populations (Brooks et al., 2013 Jørgensen et al., 2002; Løkkeborg et al., 2002; Franks, 2000; Farrell, 1974). Models suggest that migratory or planktonic species primarily located within a dense area of oil platforms may be at the highest risk of toxic exposure and bioaccumulation (Niu et al., 2016; Rye et al., 1996). Behavior studies in fish suggest a general avoidance of contaminated areas, but it is unclear whether such populations are more or less contaminated due to proximity to the discharge plume and potential background exposure (Claireaux et al., 2018; Niu et al., 2016). Moreover, depending on the complexity of outfall at oil and gas platforms, the surrounding area may exhibit higher rates of primary production (Oviatt et al., 2007; Fucik and El-Sayed, 1979; Farrell, 1974). Plentiful food could potentially increase fish residence despite contamination. Oil and gas platforms are also sites of small-scale oil and chemical spills, with 100-150 documented spills occuring yearly in the NCS (NIVA, 2019). Direct exposure to crude oil and dispersed oil droplets due to oil spills is highly toxic and the effects may be compounded by the presence of toxicants already present in the body.

Lumpfish larvae are unlikely to be exposed to pollution solely via waterborne sources (Pichtel, 2015). The Nui et al. (2016) model suggest that PW plumes sink to the ocean floor and spread (Johnsen et al., 1998). For sediment-associated eggs, such as those of lumpfish, larger toxic compounds can be adsorbed to the sediment and transferred to the egg at a higher rate (Dale et al., 2019; Cherr et al., 2017; Le Bihanic et al., 2014; Neff et al., 2011).

PW is also often emitted at a different pH and salinity to the surrounding water (Ahmadun et al., 2019; Neff et al., 2011). Differences in pH can cause altered chemical states and bioavailability of dissolved metals and ions. In fact, altered pH and ion levels can alter the permeability of the embryonic chorion, induce cardiac and craniofacial anomalies, and impair gill functioning in adults (Pichtel, 2015; Marco et al., 1999; Fletcher, 1978). Increasing salinity also contributes to toxicity by raising sorption levels of toxic mixtures, specifically of PAHs (Ahmandun et al., 2017; Faskness et al., 2008; Barron et al., 2003; Turner and Rawling, 2001).

Metals and PAHs often coexist in PW, interacting in ways that are both positive and negative for the organism (Casarett and Doull, 2013). For example, the common PAH phenanthrene is rapidly photodegraded and becomes a more toxic metabolite (9,10-phenanthrenequinone). While this product alone results in the production of reactive oxygen species, it shows synergistic toxicity during co-exposure with copper, a widely available essential metal (Xie et al., 2006). It is thus important to consider the effect of all elements of PW and the environment as they interact, in conjunction, with an organism.

The results of this study, through the extensive analysis using explanatory variables, suggest that toxicants not explored in PW analysis strongly contribute to toxicity, inducing acute toxicity (PWA) and likely to specific effects such as skeletal deformities and cardiotoxicity (Bellas et al., 2005). Focusing on the components, oil product, and hatch rate ultimately improves the general understanding of differences between PWs and of what factor exerts more control over phenotypic manifestations (e.g. is the change in jaw length a direct effect, caused by a interaction at the site of phenotypic response, or an indirect effect, caused by the effect of a chemical on hatch rate and energy usable that can cause downstream effects like jaw shortening).

Many of the dissolved production chemicals in PW possess known toxic potential. These include hydrate inhibitors, which have demonstrated severe acute toxicity to aquatic life at environmentally relevant concentrations. Results include kidney failure, fish kills, and reduced biodiversity (Pillard, 1995). Corrosion inhibitors, which are primarily lipophilic compounds, pose an acute aquatic toxicity hazard (Singh, 1996). Their widespread and continuous use and release is hypothesized to cause harmful effects as residuals environmental loads grow (DiNica et al., 2017). Remnants of H_2S scavenging chemicals are found in PW that induces toxic effects; it may be a component contributing to toxicity (Sørensen et al., 2019; Boyd, 2014; Casarett and Doull, 2013). Biocidal oxidants and electrophiles are well-established causes of toxic effects in higher order organisms such as fish, especially during sensitive developmental periods (del Olmo et al., 2015; Turkiewizc et al., 2013). Finally, surfactants like octylphenols and nonylphenols are known toxicants and endocrine disruptors that are widely used in the pumping of viscous crude oil (Sørensen et al., 2019; Neff et al., 2011; Maddin and Schlumberger, 1981).

Research on PCs and PC-PW interaction is valuable for increasing the efficiency of platforms and decreasing oil release content because PCs can increase the oil content of PW by chemically breaking down and thus increasing the dissolved fraction. Remnants and degradation products of PCs such as butoxyethyoxy ethanol, carbazoles, and amines are found in PW, as are a host of unreported or rarely reported compounds from both oil and PCs (Sørensen et al, 2019). Only after identifying the constituents of a specific PW can appropriate regulatory guidelines and treatment techniques be selected and applied.

The effects of skeletal impacts and reduced growth may be amplified in natural populations by low feeding rate due to mouth abnormalities and impaired swimming performance (Meier et al., 2010; Tilseth et al, 1984). This could lead to nutritional deficiencies that cause further effects and increase the likelihood of early mortality Valente et al., 2013; Cahu et al., 2009). However, other studies suggest that, of fish that survive treatment with dispersed oil, early reductions in performance and growth disappear within several weeks after exposure (Frantzen et al., 2015).

4.5 Limitations and future directions

This study is limited in several respects: firstly, with respect to sex, secondly, with respect to enzyme and metabolic activities, and thirdly, with respect to geographic context.

Studies by Vignet et al. (2018) and Foekema et al. (2012) found that growth was inhibited to varying degrees in fish exposed to different fractions of PAHs. Results varied in relation to the combination of species, age, and sex. Future studies should incorporate a delineation of sex as well as age. The uptake and excretion of pollutants in a fertilized egg have been shown by Vignet et al. (2014) to be low in comparison to that of newly hatched larvae, likely due to the protective role of the chorion (Villalobos et al., 2000). Foekema et al. (2012) also demonstrated that tissue concentration of highly lipophilic compounds peaks at the moment of lipid reserve depletion, which typically occurs around hatching. In light of this information and the results of this study, species that hatch early in development (such as cod), may be planktonic near PW outflows (in a susceptible developmental stage and unable to avoid highly contaminated areas), and which demonstrate higher general PAH absorption rates (due to high surface area to volume ratios) may be especially susceptible to the acute effects of PW exposure (Heintz et al., 1999). Populations of lumpfish that attach to the platform may also be especially susceptible. Future research should also be performed on populations of resident fish at offshore platforms in order to better understand the local effect of PW on various species and ages of fish. Moreover, research should investigate the effects of both dietary and waterborne exposure, as several important food sources have been shown to accumulate PAHs (Hansen et al., 2020; Hansen et al., 2017). These sources of exposure may cause bioaccumulation and biomagnification. It is also important for future studies to continue research later into species lives as delayed effects, like biomagnificaiton, have been shown to have population level consquences (Heint et al., 2000).

Future study of enzyme and metabolic activities is important due to the presence of known toxicants such as PAHs as well as endocrine disruptors like B(a)P, nonylphenol, and TCDD-like compounds. An analysis of enzymes and metabolism pathways related to detoxification, the endocrine system, and stress are important to better understand the mechanisms toxic effects (Qian et al., 2018; Blanchfield et al., 2015; Oziolor et al., 2016; Arukwe et al., 2008). It is likely that molecular analysis may further explain the phenotypic manifestations seen here and reveal some of the potential for chronic effects. Further samples were taken in this study for future analysis of the lipid content and gene alterations. Both of these are important areas of study for a more complete understanding of toxicity and how it contributes to phenotypic manifestations and immunity (Qian et al., 2018; Cherr et al., 2017; Neff et al., 2011; Gündel et al., 2007;). This information may increase understanding of PWA toxicity, contribute to understanding of sublethal effects (including endocrine disruption), and illuminate the potential impacts in adulthood (including premature death and reduced fecundity).

Finally, future research should include a similar study with a set of geologically distinct PWs from a variety of oilfield product types, including various crude oils as well as natural gas. It is to be expected that some of these effects will be consistent across regions; the literature documents common toxicity syndromes across distinct regions (U.S. EPA, 2019; Lourenço et al., 2018; Incardona et al., 2013; Jung et al., 2013; Hansen et al., 2019). The predictive use of principal components in this study was limited by the relatively large amount of endpoint data corresponding to a narrow set of PW information. The effect of this limitation in power is evident in Figure 3.3.3. In this case, one PW type had a strong effect on the overall calculation, causing hatching rate to have a small, but positive linear association with PC2 (TEM and phenols).

5. Conclusions

A comprehensive assessment of the studied endpoints suggests that the manifestations of toxicity straddle response categories including mutagenesis (abnormalities), embryotoxicity, metabolic disruption (reduced growth and hatch rate), cell signal interference (skeletal impacts), endocrine disruption (skeletal impacts), and likely a multitude of others. Differences in outcomes from previous research support the unpredictable nature of PAH-toxicity manifestation (Geier et al., 2018; Gilbert and Barresi, 2016; Casarett and Doull, 2013). Moreover, the results prove that while PW toxicity shows some similarities to that of crude-oil and PAHs, it can be very different. Therefore, the effects and subsequent regulation predictions of these groups cannot be reliably mapped to each other. It is highly likely that, even without a clear determination of the corresponding permanent changes in adult fish, adverse life history outcomes may be expected for both individuals and populations exposed to many PWs (DeSesso and Scialli, 2018; Regnault et al., 2018). In the context of mutagenetic, metabolic, and endocrine effects, the likelihood of multigenerational effects is high (King-Heiden et al., 2012; Regnault et al., 2018; Xin et al., 2015; Corrales et al., 2014; Casarett and Doull, 2013).

These results also highlight the need for continued site-based testing coupled with a more extensive investigation of PW components and their impact on resident fish populations (Sørensen et al., 2019; Jørgensen et al., 2002; Løkkeborg et al., 2002). PW is the largest waste stream in the oil and gas industry and is produced at the majority of drilling sites to some extent. Moreover, the results of this and similar species are important for informing regulations regarding economically and socially important species such as salmon and cod. As seen in the Great Lakes (USA) trout populations, the combined damage of overfishing (which may be happening with North Sea fish populations) and organic compounds has resulted in successive recruitment failure despite decades of re-stocking efforts (Sunnanå, 2007; King-Heiden et al., 2012). A lack of regulatory focus on PW exposes not only fish but also developing larvae and eggs to a wide variety of potential toxicants that demonstrate teratogenicity, persistence, and bio-accumulative potential. Risk assessment and the understanding of the chemical fate of produced water is important because it has been established that “the solution to pollution is [not] dilution” (Hayworth and Clement, 2011; Hull, 2002).

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Appendix

Appendix I: Supplementary methods

I.I Fertilization

200 mL of fresh eggs was fertilized with 4 mL milt from a single male. Milt was cryopreserved (Cryogenetics AS, Hamar, Norway) and stored at -80˚C until use. Frozen milt was activated by mixing well in a 25˚C water bath for 30 seconds before mixing with eggs and diluting the mixture to 50% in filtered seawater. Fertilized eggs hardened within 10 to 12 minutes.

I.II Dechorionation

Unhatched larvae and dead embryos were washed in PBS buffer and then water. Under the dissecting microscope, embryos were freed from the eggshell using tweezer and a small (acupuncture) needle. Caution was taken to avoid causing mechanical damage.

I.III Bone staining procedure

Modified bone staining procedure after Balon, 1985 (modified from Taylor, 1967, Dingerkus and Uhler, 1977, and Gavaia et al., 2000).

Larvae and embryos	<10mm in length
1. Fixation	
Fix in 10% neutral formalin Rinse in distilled water 1b. Neutralize if necessary	 2 x 5’
Absolute ethanol bath with freshly added 1% KOH	A few minutes
Store in sodium borate buffer if necessary	
2. Rehydration	
96% ethanol 50% ethanol 15% ethanol Distilled water	2 x 25-30’ (depending on size) 30’ 30’ 30’
3. Bleaching	
Bleach in 1:9 3% H_2O_2: 1% KOH under strong light	1-2.5 h
4. Clearing	
Clear in trypsin buffer until all color in eyes is gone and larvae is nearly transparent	16-20 h
5. Staining	
Stain bones in Alizarin working solution	20 h (maximum)
6. Preservation	
Rinse in distilled water Rinse in 1% KOH 40% glycerol in 1% KOH Take pictures 70% glycerol in 1% KOH Store in 100% glycerol	5’ 2x 48 h 2-24 h

Appendix II: Supplementary figures

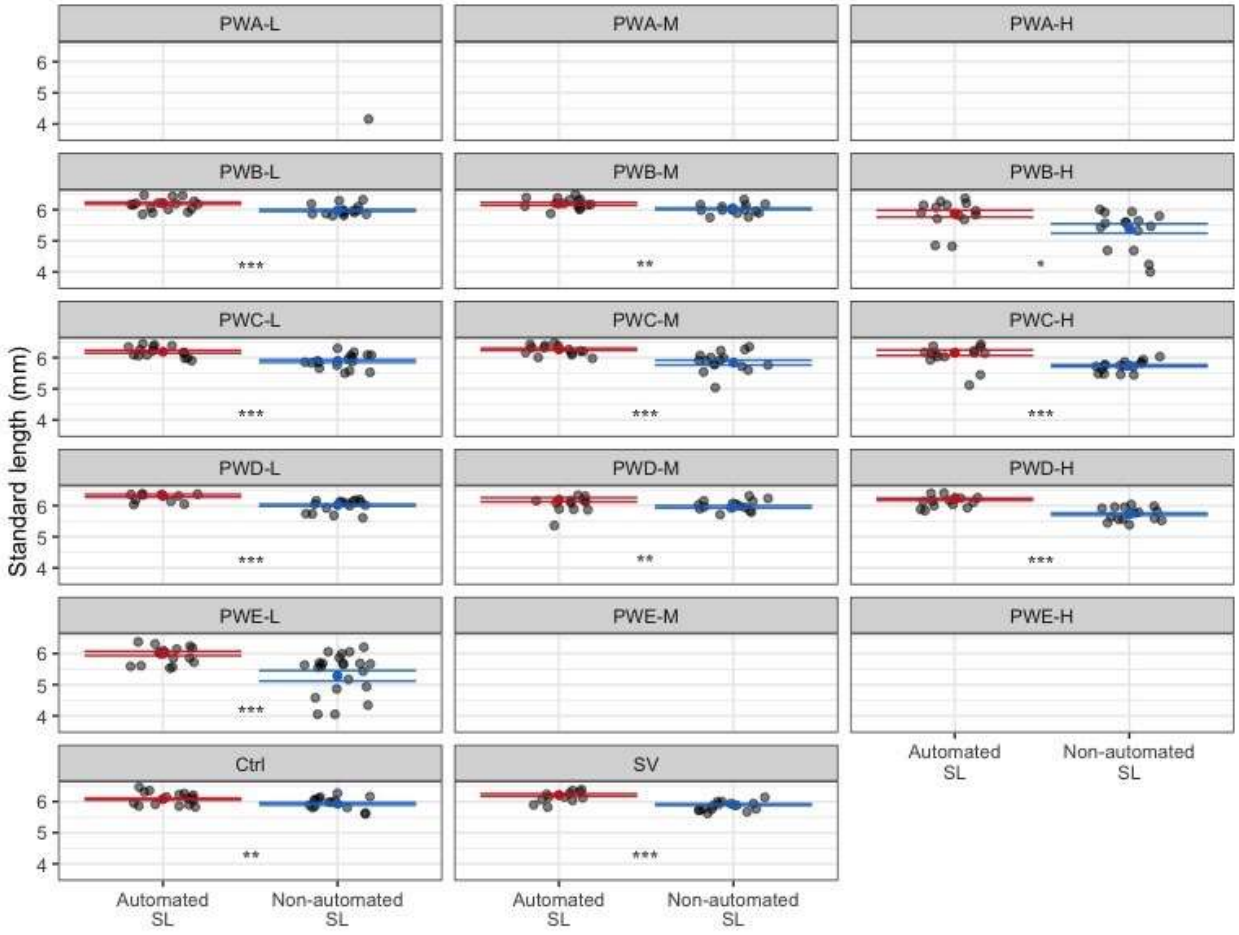

Figure 2.9a. Comparison of two SL measurement methods, automated (biometric) and non-automated (ImageJ from skeletal analysis), by Student's T-test. SLs were significantly different across all treatments. Automated measurements were significantly larger across all treatment conditions.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

Table. 2.9. Corresponding table to Figure 2.9 of comparison of two SL measurement methods, automated (biometric) and non-automated (ImageJ from skeletal analysis), by Student's T-test. p-value is comparison of automated and non-automated SLs.

		(a) Non-automated			(b) Automated			
		Mean	SEM	n	Mean	SEM	n	p
Control	N	5.93	0.040	15	6.08	0.032	16	0.0062
PWA	L	*NA*	*NA*	1	*NA*	*NA*	0	*NA*
	M	*NA*	*NA*	0	*NA*	*NA*	0	*NA*
	M	*NA*	*NA*	0	*NA*	*NA*	0	*NA*
PWB	L	5.98	0.032	15	6.21	0.036	17	4.46×10^{-5}
	M	6.03	0.025	15	6.19	0.047	16	0.0055
	H	5.39	0.15	15	5.87	0.111	14	0.0177
PWC	L	5.89	0.044	17	6.19	0.045	15	4.35×10^{-5}
	M	5.84	0.077	16	6.27	0.036	15	5.71×10^{-5}
	H	5.74	0.034	16	6.16	0.088	16	2.67×10^{-4}
PWD	L	6.01	0.031	16	6.31	0.043	15	6.51×10^{-6}
	M	5.96	0.038	15	6.19	0.067	15	0.0073
	H	5.73	0.038	15	6.20	0.036	15	7.32×10^{-10}
PWE	L	5.29	0.168	23	6.00	0.068	17	4.71×10^{-4}
	M	*NA*	*NA*	0	*NA*	*NA*	0	*NA*
	H	*NA*	*NA*	0	*NA*	*NA*	0	*NA*
SV	N	5.90	0.025	15	6.21	0.041	13	3.23×10^{-6}

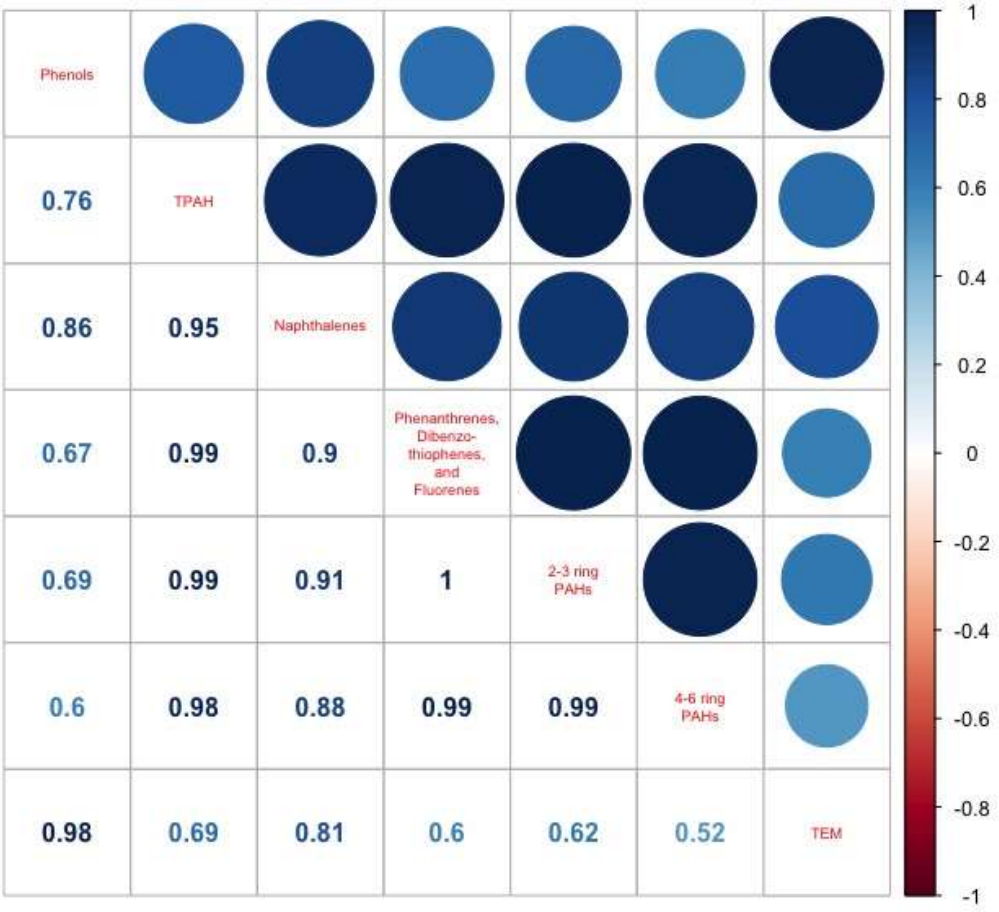

Figure 2.9b. Correlogram via Pearson method demonstrating the strength and direction of correlation between different analyzed chemical groups.

Table 3.2.1. Analysis of variance table of interaction effect model of PW type and concentration predicting hatching.

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
PW type	5	4.984	0.9967	241.98	$< 2x10^{-16}$
Concentration	3	1.138	0.3794	92.11	$< 2x10^{-16}$
Interaction	8	0.684	0.0855	20.75	$1.7x10^{-11}$
Residuals	37	0.152	0.0041	*NA*	*NA*

Table 3.2.2. Analysis of variance table predicting delay of hatching.

Source	Degrees of freedom	Sum of squares	Mean square	F-value	P-value
PW type	5	959.56	159.93	7.7048	$2.052x10^{-5}$
Concentration	3	750.71	375.36	18.0835	$3.326x10^{-16}$
Interaction	8	1477.73	184.72	8.8991	$1.059x10^{-06}$
Residuals	37	768.00	20.76	*NA*	*NA*

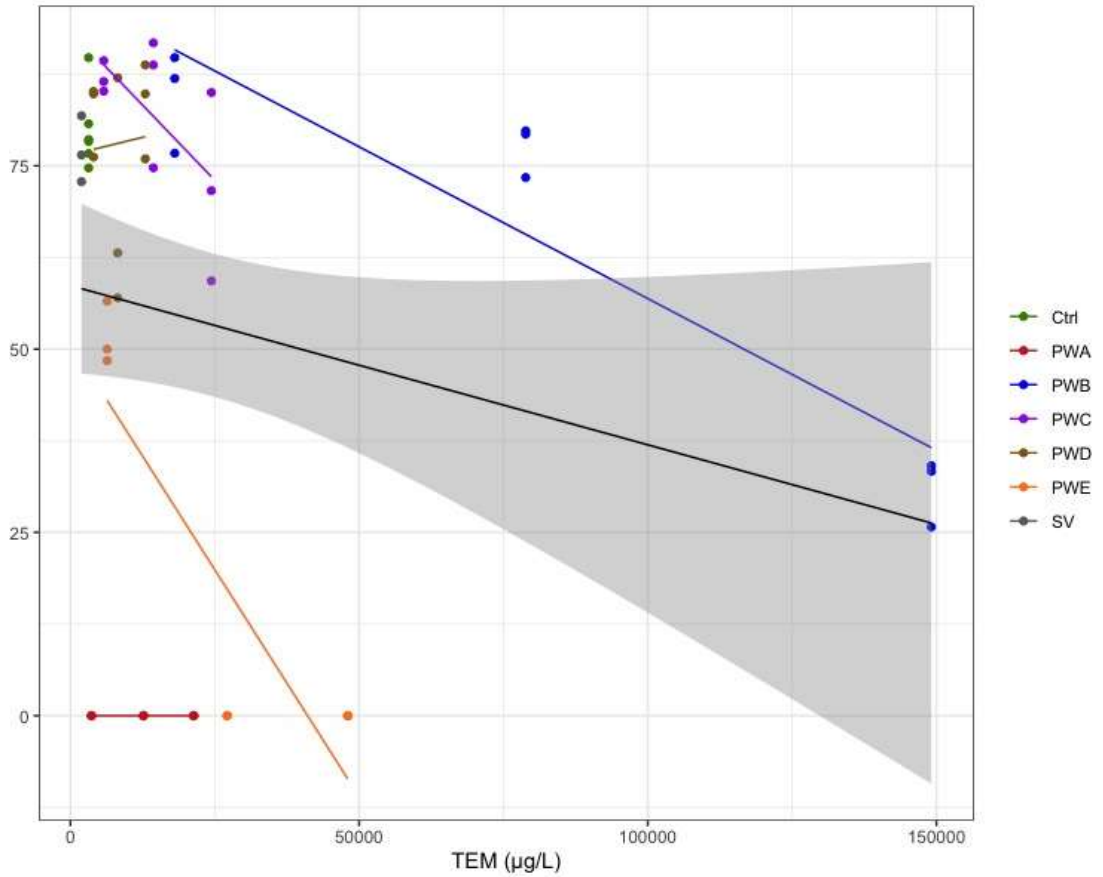

Figure 3.3.1. Impact of TEM on hatching percentage with colors indicating PW type. TEM alone explains only explains 2.78% of the variance in the data (adjusted R^2 = 0.02778). The black line represents the linear model of TEM predicting hatching percentage with 95% confidence intervals shaded in grey. Colored lines in demonstrate the regression of each PW type with TEM.

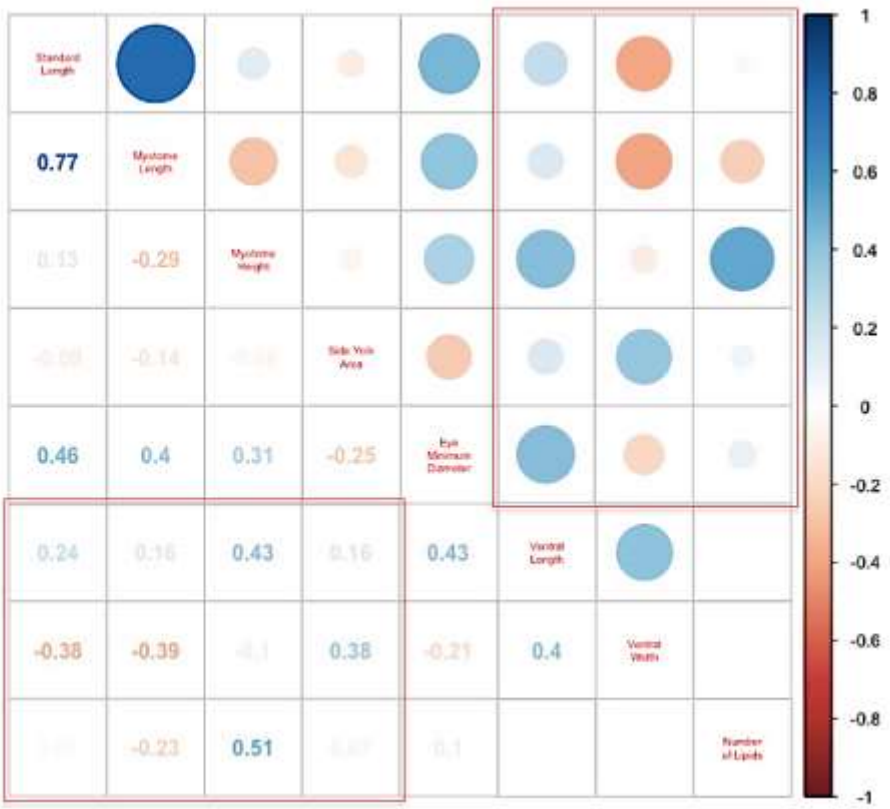

Figure 2.5. Correlogram of biometric measurements showing correlations and the strength of the correlation between different endpoint. Correlations boxed in red were calculated from a summary dataset, allowing full pairwise comparisons of measures between photograph positions (ventral and dorsal).

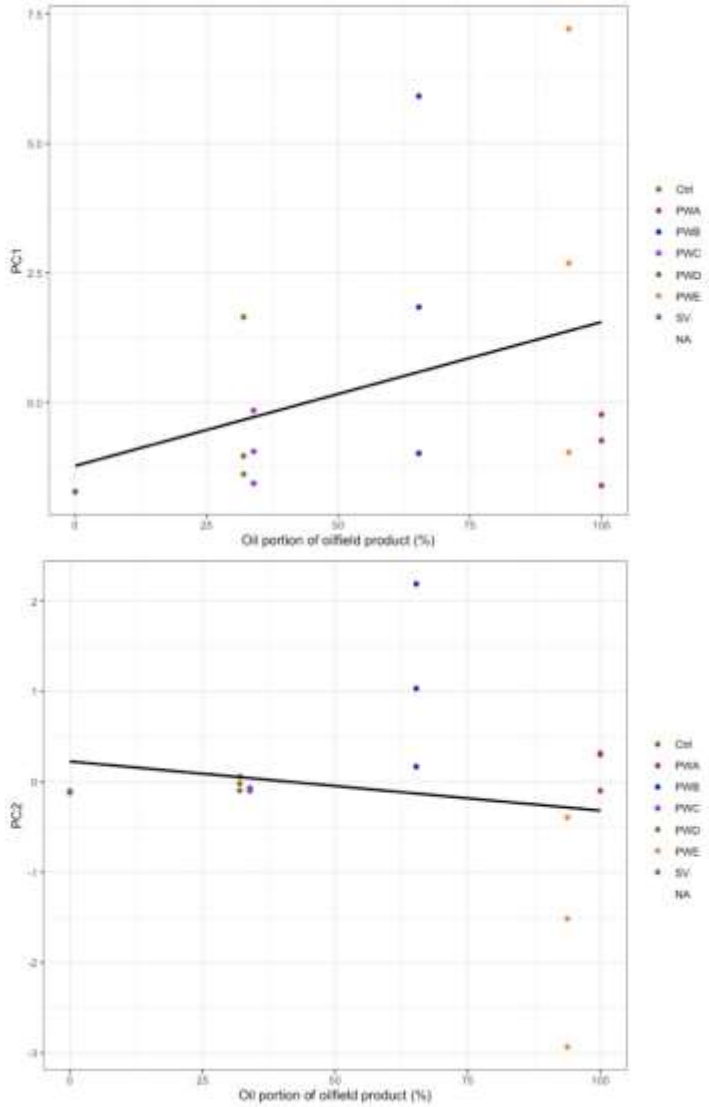

Figure 3.4b. Impact of oil portion of product on the principal components. Oil portion did not explain the variation in either principal component. (a) Regression with PC1 demonstrated a positive, but weak relationship with increasing oil portion; (r^2 = 0.07437, p = 0.1514) (b) a regression with PC2 demonstrated a weak, negative relationship with increasing oil portion (r^2 = -0.03014, p = 0.4771).

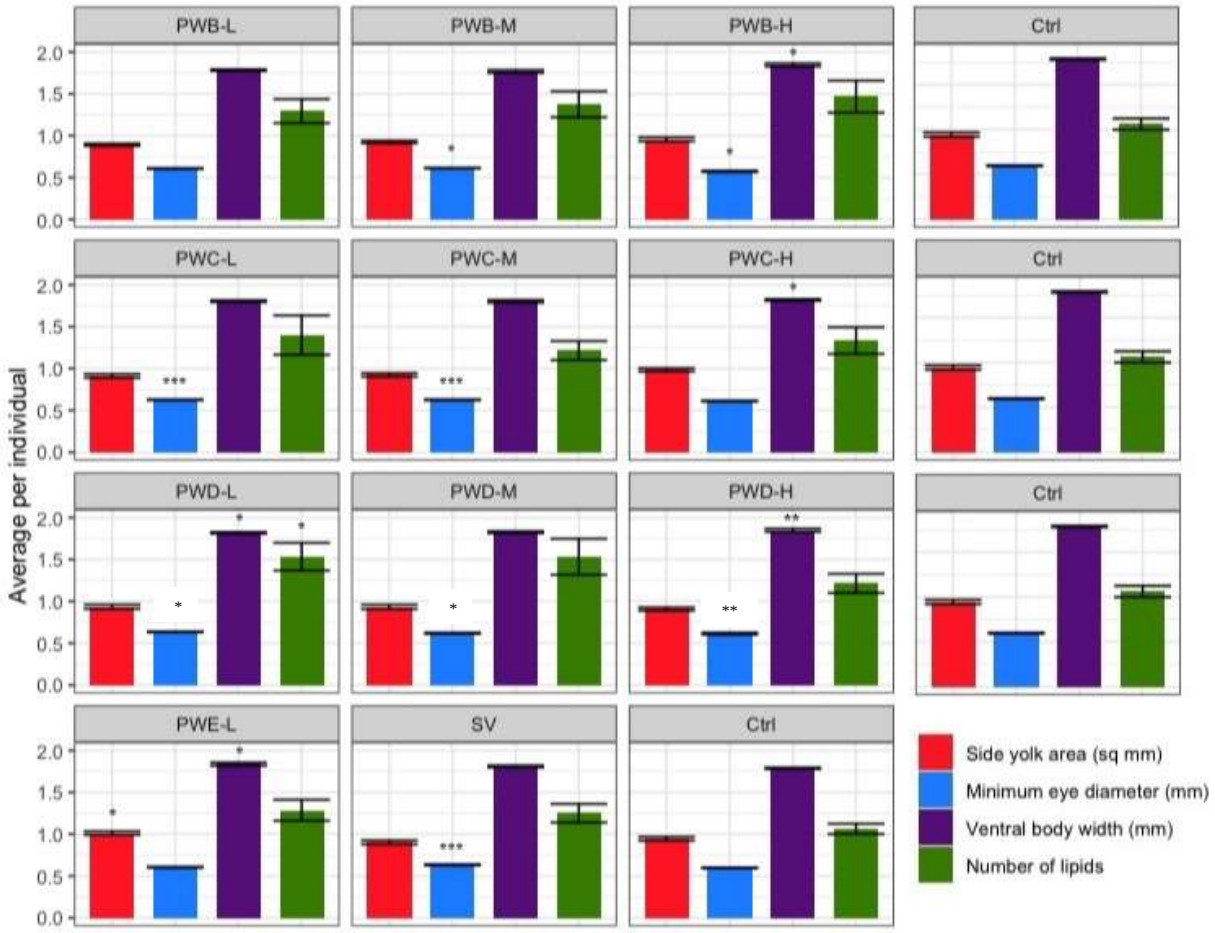

Figure 3.5. Barplot of the effect of treatment on chosen biometric parameters. Bars represent ± SEM. The effect was not explained by hatching rate. Side yolk area was significantly larger than the control in PWE-L. Eye minimum area showed the most difference across treatments in comparison to the control. PWD-M, PWC-L and M, PWD-L and M, and the SV all were significantly larger in eye diameter than the control. PWB-H however had a significantly smaller eye diameter. All tested high treatments as well as PWD-L and PWE-L were significantly wider than the control. Number of lipids is only significantly more in PWD-L, but all treatments show an increased amount of variation.
* $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

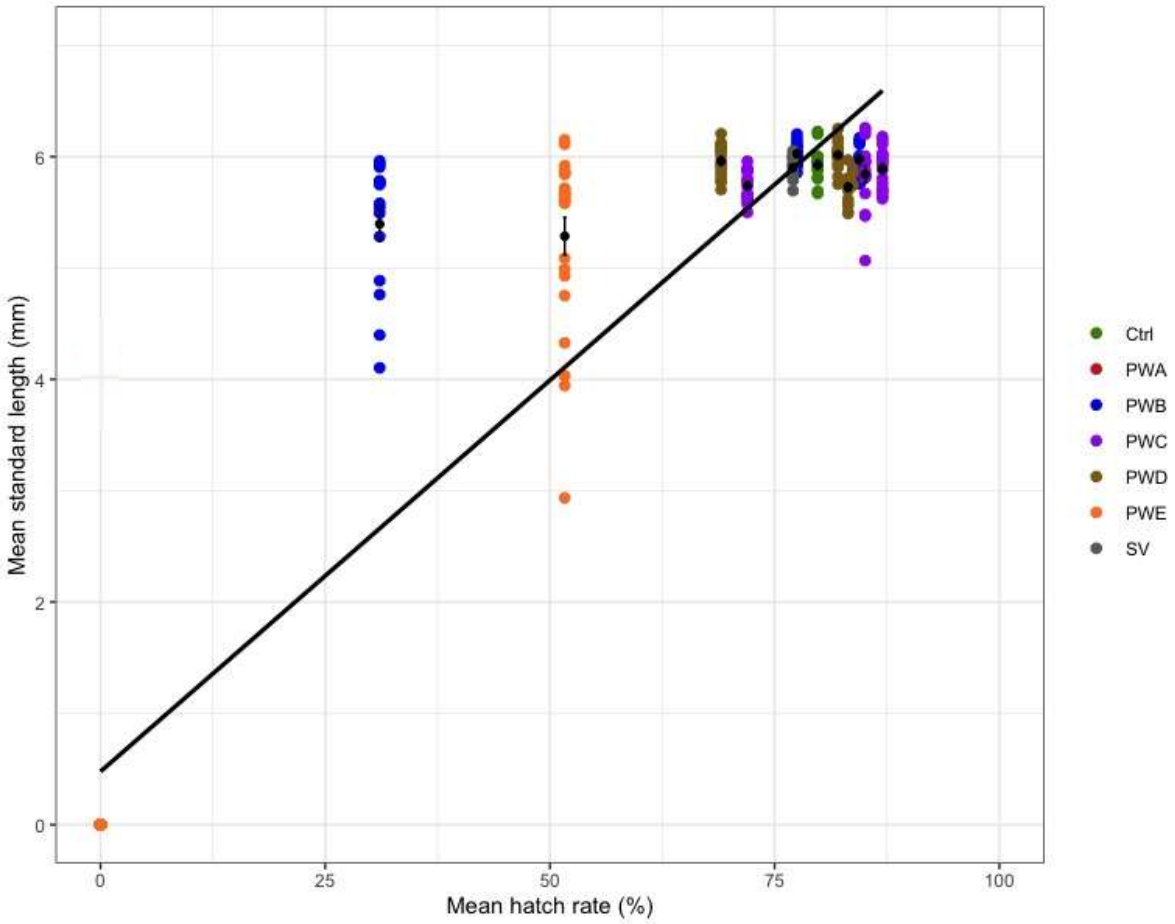

Figure 3.6.1. A plot of standard length vs hatch rate. Variation in SL was significantly explained by hatching rate ($r^2 = 0.888$, $p < 0.001$). Black point and error bars represent the mean of that treatment ± SEM.

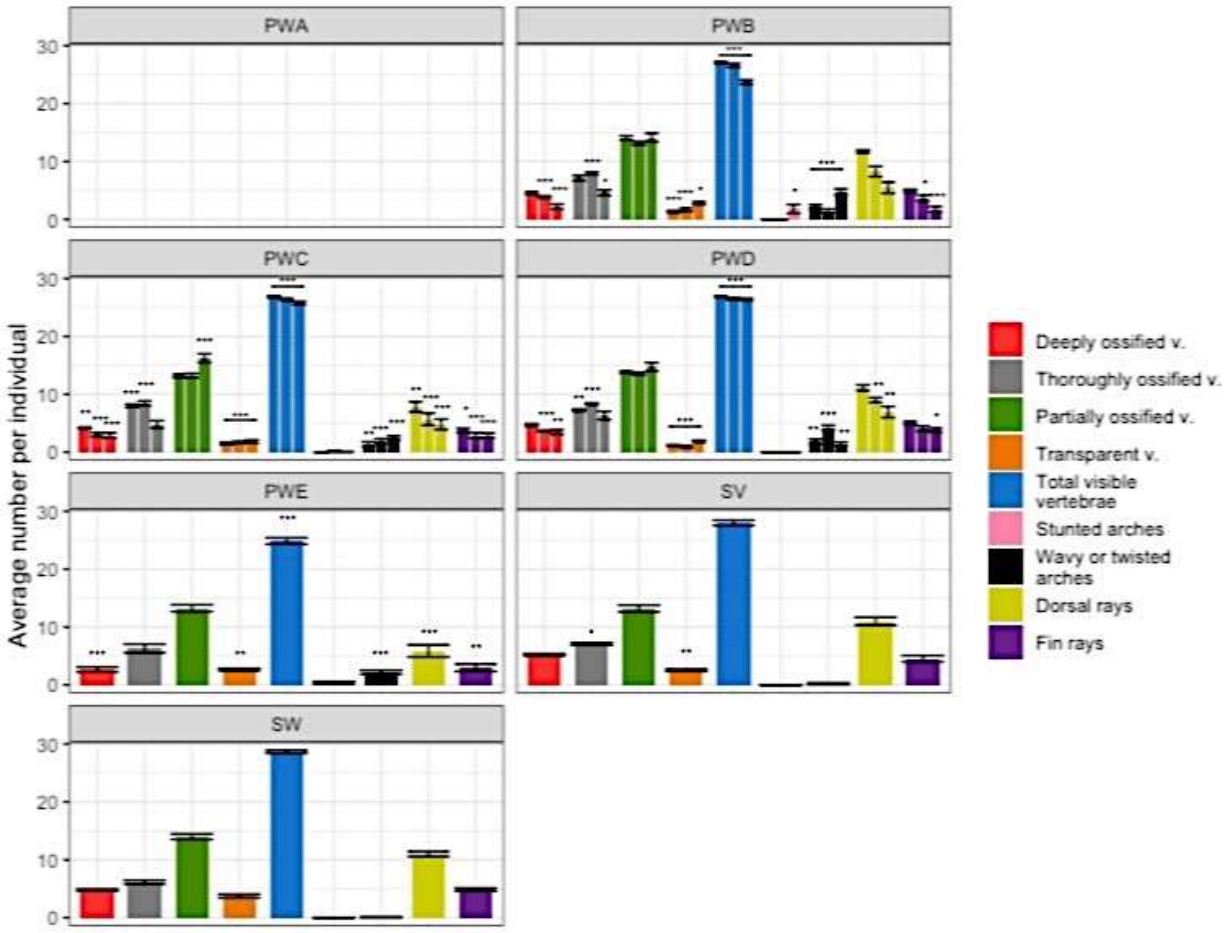

Figure 3.6.2. A barplot of spinal ossification and ray ossification. Bars represent mean ± SEM. Each set of three bars represents the measurements of the three concentrations in order L, M, H, when applicable. Statistical analysis via student's t-test in comparison to control. The majority of treatments (except PWB-L and PWD-L) had significantly fewer deeply ossified vertebrae than the control. Total visible vertebrae was significantly lower in all treatments and all concentrations in comparison to the control. The phenotype wavy or twisted arches was seen significantly more often in all treatment conditions than in the control. A novel phenotype "stunted arches" was seen in PWB-H and no other solution. * $0.05 < p < 0.01$, ** $0.01 < p < 0.01$, *** $p < 0.001$

Table 3.7. Skeletal analysis also included a separate measure, 'twist or curvature', seen in the associated pictures (a) twist and (b) curvature. It is unlikely that such an effect was caused by a larva being euthanized in a specific position, however the possibility could not be ruled out.

Treatment	Dose	n	Curve or twist
Ctrl	N	15	0
PWA	L	0	*NA*
	M	0	*NA*
	H	0	*NA*
PWB	L	15	20.00 *
	M	15	20.00 *
	H	15	33.33 **
PWC	L	17	41.18 **
	M	16	25.00 *
	H	16	37.50 **
PWD	L	16	25.00 *
	M	15	13.33
	H	15	33.33 **
PWE	L	16	5.88
	M	0	*NA*
	H	0	*NA*
SV	N	15	0