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Since 2007, electronic cigarette (e-cigarette) sales in the U.S. have surpassed those of tobacco cigarettes. This is due, in part, to manufacturer's claims that they are a safer alternative to tobacco cigarettes. However, formaldehyde, acrolein, and diacetyl have been detected in e-cigarettes and public knowledge of e-cigarette composition and potential bioactivity is conspicuously lacking. We evaluated the toxicity of nine e-cigarette flavor mixtures and their constituents in the developmental zebrafish, an excellent whole animal biosensor of chemical hazard. Seven of the nine flavors (78%) elicited adverse developmental responses at 1% by volume. The number of toxic endpoints varied greatly between flavors. Two flavors, Grape and Bubble Gum, had very similar chemical compositions, but different toxicity profiles. We hypothesized that the toxicity was driven by a constituent present only in the Bubble Gum flavor, cinnamaldehyde. To replicate this toxicity, we built our own defined mixture, and added varying concentrations of cinnamaldehyde. Cinnamaldehyde drove the bioactivity of these mixtures and demonstrated that e-cigarette toxicity is flavor dependent, largely driven by a few key ingredients in a flavor mixture.

Profiling Zebrafish Developmental Responses to E-Cigarette Flavor Mixtures

by
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Laura Louise Holden, Author

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CONTRIBUTION OF AUTHORS

In all chapters, Dr. Robert Tanguay contributed to intellectual formulation, writing, and study design.

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CHAPTER ONE – INTRODUCTION

Electronic Cigarette Devices

Electronic cigarettes, e-cigarettes, or "vape" have become a popular alternative to tobacco cigarettes. These products are touted as "safer" alternatives based upon the belief that they neither produce emissions nor contain toxins such as carbon monoxide, phenol, or arsenic (Al-Delaimy et al., 2015; Grana et al., 2014; Kennedy et al., 2017a; Muthumalage et al., 2017; Palpant et al., 2015; Spindel and McEvoy, 2016). This is a gross misconception because their sole operating principal remains the low temperature combustion of organic compounds, a process fraught with undesirable products (Goniewicz et al., 2014; Jensen et al., 2017; Kosmider et al., 2018). Despite this, usage of e-cigarettes among children, young adults and pregnant women is high (Arrazola et al., 2015; Suter et al., 2015). In 2012, the CDC determined that at least 1.78 million American students between grades 6 - 12 had used e-cigarettes once (Arrazola et al., 2015). As of July 2015, the FDA banned sales of e-cigarettes to anyone under the age of 18 (FDA, 2018a; HHS, 2016; Smith et al., 2016; Tierney et al., 2016). Roughly ten percent of female smokers in the US continue to smoke while pregnant and they may also turn to e-cigarettes as an ostensibly safer tobacco alternative (Cnattingius, 2004; Colman, 2003; Kennedy et al., 2017a; Kennedy et al., 2017b; Spindel and McEvoy, 2016; Suter et al., 2015; Wickstrom, 2007). While the effects of tobacco cigarette exposure on the fetus are well documented, few studies have evaluated the hazards of e-cigarette exposure (Al-Delaimy et al., 2015; Bahl et al., 2012; Bruin et al., 2010; Grana et al., 2014; Longo et al., 2013; Muthumalage et al., 2017; Slotkin, 2004; Smith et al., 2016; Spindel and McEvoy, 2016; Suter et al., 2015; Wickstrom, 2007).

E-cigarette devices contain a battery and a heating element to vaporize the contents of a cartridge mixture. These cartridges contain propylene glycol (PG), vegetable glycerin (VG), nicotine (from 0 – 36 mg/mL), and various chemical flavorings (Bahl et al., 2012; Grana et al.,

2014; Massarsky et al., 2017; Muthumalage et al., 2017; Spindel and McEvoy, 2016; Suter et al., 2015; Tierney et al., 2016). Zebrafish exposure to PG concentrations as low as 1.25% by volume during development was associated with reduced body size, hyperactivity and edemas (Massarsky et al., 2017). Furthermore, the average concentration of PG in an e-cigarette mixture was estimated to be 500 – 600 mg/mL, while the concentration of VG was estimated to be 400 – 500 mg/mL (Schober et al., 2014). Together, PG and VG make up more than 90% of a given e-cigarette cartridge, suggesting that the long-term potential hazards of these compounds be investigated (Jensen et al., 2017; Massarsky et al., 2017; Suter et al., 2015).

Electronic Cigarette Flavorings

There is also scant information regarding the safety of e-cigarette flavoring agents (Grana et al., 2014; Kennedy et al., 2017a; Kennedy et al., 2017b; Muthumalage et al., 2017; Smith et al., 2016; Suter et al., 2015; Tierney et al., 2016). A study from Tierney et. al. (2016) determined that flavoring chemicals make up roughly 1 – 4% of cartridges by weight, and consist of known or probable respiratory irritants such as benzaldehyde, vanillin, and cinnamaldehyde (Bahl et al., 2012; Behar et al., 2014; Tierney et al., 2016). Many adult users have reported throat, mouth and lung irritation after using various cinnamon-flavored cartridges, of which cinnamaldehyde and cinnamic acid are major constituents (Bahl et al., 2012; Behar et al., 2014; Czégény et al., 2016; Tierney et al., 2016). Thermal decomposition of cinnamic acid at 300°C in the presence of oxygen leads to the formation of toluene, phenol, and phenanthrene (Czégény et al., 2016). When oxygen is not present, no decomposition products are formed (Czégény et al., 2016). Combustion aside, the base toxicity of the flavoring agents remains an important knowledge gap. Filling this gap will help assess the risk these compounds may present to human health.

As mixtures, the toxicity of e-cigarette chemicals becomes more complicated to characterize and little is known about what compounds are in the mixture and at what

concentrations (FDA, 2018a, b; Kennedy et al., 2017a; Sears et al., 2017; Smith et al., 2016; Tierney et al., 2016). Under the FDA, e-cigarette chemicals are required to be generally recognized as safe (GRAS). It is important to note that this certification only applies to oral ingestion of the chemical (Borgerding et al., 2012; Carmines and Gaworski, 2005; Kennedy et al., 2017b; Sears et al., 2017; Tierney et al., 2016). An *in vitro* mixture study by Bahl et. al. (2012) exposed human pulmonary fibroblasts, human embryonic stem cells, and mouse neural cells to 35 different e-cigarette flavors and measured cytotoxicity. The human stem and mouse neural cells were more sensitive than the adult human lung cells, and cytotoxicity was related to the concentrations of the individual flavors rather than nicotine content (Bahl et al., 2012; Behar et al., 2014). *Xenopus laevis* craniofacial defects were more severe in embryos exposed to e-cigarette flavors with a fruit, candy, or vanilla flavor profile than exposures to PG, VG or nicotine alone. This suggests flavor-specific, and thus chemical specific effects within a given mixture (Kennedy et al., 2017a).

Animal Model

To overcome the limited ability to translate *in vitro* data into human hazard potential, we leveraged the advantages of the developmental zebrafish (Bugel et al., 2014; Garcia et al., 2016; Massarsky et al., 2017; Palpant et al., 2015; Truong et al., 2014). With a short generation time and significant physiological and genetic homology to humans, the zebrafish has proven a useful model to study phenotypic and genotypic outcomes in response to chemical insults (Garcia et al., 2016; Howe et al., 2013; Truong et al., 2016; Truong et al., 2014). By 120 hours post fertilization (hpf), all of the major organ systems of the larval zebrafish have developed, resulting in a dynamic, whole organism model in which numerous omic level responses can be anchored to complex phenotypic outcomes (Garcia et al., 2016; Truong et al., 2014; Zoupa and Machera, 2017). By assessing biological activity, chemicals that are a “hit” in morphological and behavioral endpoints can be prioritized for further studies (Geier et al., 2018a; Truong et

al., 2014). Furthermore, the outcomes of early life exposure can be studied and precise "windows of sensitivity" can be determined (Bugel et al., 2014; Garcia et al., 2016; Truong et al., 2014; Zoupa and Machera, 2017). Since the underlying molecular processes driving development are highly conserved between humans and zebrafish, this sensitive tool can assess the hazard various mixtures may pose to biological systems (Geier et al., 2018a; Geier et al., 2018b; Hill et al., 2005; Monosson, 2004; Rennekamp and Peterson, 2015; Truong et al., 2014; Wiley et al., 2017; Zoupa and Machera, 2017).

Zebrafish and E-cigarette Devices

Few studies have utilized the zebrafish model to evaluate the effects of e-cigarette exposure. Palpant et. al. (2015) used the zebrafish to examine the cardiac effects of developmental exposure to purified nicotine, conventional cigarette smoke or e-cigarette vapor during the first three days of development (Palpant et al., 2015). Overall, tobacco and e-cigarette smoke-exposed animals showed significantly more heart defects compared to the purified nicotine group. The heart defects in animals exposed to tobacco and e-cigarette smoke were also more severe than in animals exposed to nicotine alone (Palpant et al., 2015). Additionally, the morphological and behavioral effects of propylene glycol, a primary e-cigarette vehicle, are well established in the developing zebrafish (Lahnsteiner, 2008; Maes et al., 2012; Massarsky et al., 2017). However, the effects of native e-cigarette flavorings, alone or in mixtures, on zebrafish development have not been examined (Palpant et al., 2015). A significant challenge is that the exact ingredients of e-cigarette cartridges and their concentrations are mostly unknown (Kennedy et al., 2017a; Muthumalage et al., 2017; Smith et al., 2016; Suter et al., 2015; Tierney et al., 2016).

Rationale

In the present study, we observed highly toxic flavors (Bubble Gum, Cotton Candy), as well as relatively non-toxic flavors (Grape, Coffee) in the same dilution range. Two mixtures, Grape and Bubble Gum had similar chemical compositions, but different morphological outcomes. We hypothesized that the toxicity was driven by cinnamaldehyde, present only in the Bubble Gum flavor. Using the developmental zebrafish model to examine the bioactivity of these mixtures and their components, we have begun a whole animal-response data collection that will ultimately inform better regulatory and consumer product choices.

CHAPTER TWO – MATERIALS AND METHODS

Chemicals and Reagents

Eight e-cigarette flavors used in the Tierney et. al. (2016) study: Bubble Gum, Coffee, Cotton Candy, French Vanilla, Grape, Nicotine (24 mg/mL), Unflavored, and 555 were ordered online from Mt. Baker Vapor (Lynden, WA; https://www.mtbakervapor.com/) (Tierney et al., 2016). Each cartridge had a total volume of 15 mL, and each flavor contained 12 mg/mL of nicotine, except for the Nicotine (24 mg/mL) and Unflavored (0 mg/mL) cartridges. We received an additional flavor as a gift with our order: Banana Crème Pie; which was not included in the Tierney study (Tierney et al., 2016). Analytical grade glycerin (≥99%), propylene glycol (≥99%), nicotine (≥99%), DMSO (≥99.5%), cinnamaldehyde (≥95%), ethyl butyrate (≥99%), ethyl vanillin (≥98%), maltol (≥98.5%), and vanillin (99%) were ordered from Sigma-Aldrich (St. Louis, MO). Benzyl alcohol (99%) and ethyl acetate (≥99%) were ordered from Alfa Aesar (Haverhill, MA). For exposures that used DMSO, the maximum DMSO concentration used was 0.64%.

Zebrafish Maintenance and Embryo Collection

Adult zebrafish were housed following the approved Institutional Animal Care and Use Committee (IACUC) protocols at the Oregon State University Sinnhuber Aquatic Research Laboratory (SARL) (Corvallis, OR) and maintained on a 28°C recirculating water system with a 14:10 h light/dark cycle. The fish were fed twice daily with the appropriate Gemma Micro (Skretting Inc. Tooele, France) without supplementation of any live feed. All experiments were conducted using the wild-type (WT) 5D Tropical line. Spawning funnels were placed in tanks the night prior to spawning. The following morning embryos were collected, staged, and maintained in an incubator at 28°C (Kimmel et al., 1995; Westerfield, 2007). To increase bioavailability, the chorion was enzymatically removed using 83 µL of 25.3 U/µL pronase

(Roche, Indianapolis, IN, USA)) at 4 hours post fertilization (hpf) using a custom automated dechorionator (Mandrell et al., 2012).

Morphological and Behavioral Endpoints Measured in Larval Zebrafish

For all exposures, animals were evaluated for 22 morphological endpoints and 2 behavioral assays at 24 and 120 hpf. All behavioral assays were conducted before the morphological screening took place.

24 hpf Developmental Toxicity Endpoints and Behavioral Assay

At 24 hpf, mortality, developmental progression, spontaneous movement, and notochord distortion were evaluated by viewing animals under a microscope in 96 well plates (Hagstrom et al., 2018; Truong et al., 2016). For behavior, the embryonic photomotor response (EPR) was assessed in all animals, using the Photomotor Response Assessment Tool (PRAT) (Reif et al., 2016). The PRAT test consisted of Background, Excitation and Refractory periods. The Background period consisted of 30 seconds of darkness (IR light). Next, a one second pulse of intense visible light, and 9 seconds of darkness made up the Excitation period. Finally, another 1 second pulse of visible light was emitted followed by 10 seconds of darkness, which made up the Refractory period (Hagstrom et al., 2018; Truong et al., 2014). Statistical significance was calculated for each interval using a Kolmogorov-Smirnov test (K-S) with a threshold of $p < 0.01$ (Hagstrom et al., 2018; Reif et al., 2016).

120 hpf Developmental Toxicity Endpoints and Behavioral Assay

At 120 hpf, animals were evaluated under a microscope for the following 18 morphological endpoints: mortality, yolk sac edema, curved or bent body axis, missing or smaller/larger eye(s), shortened or malformed snout, malformed jaw, malformed or missing otic vesicle, pericardial edema, malformation of the brain, malformed, missing, or disorganized somites, malformed or missing pectoral and/or caudal fins, hypo or hyperpigmentation, lack of circulation, truncated body, failure of swim bladder to inflate, bent notochord and/or tail, and

response to touch (Hagstrom et al., 2018; Truong et al., 2016). At this timepoint, zebrafish behavior was assessed using the Larval Photomotor Response (LPR) Assay in Viewpoint LifeScience Zebraboxes (Truong et al., 2014; Zhang et al., 2017). The LPR assay assesses individual zebrafish larvae motor response to a series of light-dark changes at 120 hpf. The assay consists of four 3-minute light and dark alternating periods (Truong et al., 2014; Zhang et al., 2017). By measuring motor response to a light stimulus in exposed organisms, the phenotypic effect of a chemical can be monitored at an early point during development. Wells with mortality or malformed animals were excluded from the subsequent analysis (Truong et al., 2016). An entropy score was calculated for each interval and compared to the control to compute a relative ratio, as described in (Zhang et al., 2017). Statistical significance was determined using a K-S test ($p < 0.01$) and a relative ratio of > 10% or < 10%. All analyses were conducted using custom R scripts previously described (Team, 2016).

Developmental Exposures

Propylene Glycol and Vegetable Glycerin Screening

Prior to working with the e-cigarette flavors, zebrafish embryos were exposed to their major vehicle components: propylene glycol (PG) and vegetable glycerin (VG). All exposures took place in 96 well plates in a total volume of 100 µL; 90 µL of which was Embryo Medium (EM) (Westerfield, 2007). Animals were exposed to 10 µL of a 1:100, 1:1000, 1:10,000, 1:100,000, or 1:1,000,000 dilution of PG, VG or a 50:50 mix (by volume) of PG and VG in EM. To start, 1:10 dilutions were prepared by adding 5 mL of propylene glycol or glycerin to 45 mL of EM in 50 mL conical tubes. Tubes were vortexed for 5 minutes. The 1:100 – 1:1,000,000 dilutions were made by removing 5 mL of the previous dilution and mixing thoroughly with 45 mL of EM. The 1:10 PG and VG mixture dilution was made by adding 2.5 mL of PG and 2.5 mL of VG to 45 mL of EM in a 50 mL conical tube in the same manner as

described. Plates were preloaded with 90 µL of EM, and then loaded with 10 µL of propylene glycol, glycerin, PG/VG mixture dilution, or EM (control). After all chemicals were added, plates were covered in parafilm and mixed for 5 minutes using a plate shaker at 400 RPM. Plates were then handloaded with 6 hpf dechorionated zebrafish embryos. After all embryos were added, plates were sealed with silicone plate covers (to prevent volatization) and shaken overnight at 235 RPM in an incubator room at 28ºC (Truong et al., 2016). After chemical exposure, embryos were not exposed to visible light until the EPR test at 24 hpf (Reif et al., 2016). Two replicate plates were used for a total of 32 animals for each dilution. As described in Truong et. al. (2014), embryos were statically exposed until 120 hpf. At 24 and 120 hpf embryos were assessed for morphology and behavior as described above (Truong et al., 2014).

Nicotine Screening

Zebrafish embryos were exposed to a nicotine standard in order to determine the effects of nicotine alone for later comparison with e-cigarette mixtures. Plates were preloaded with 100 µL of EM and automatically loaded with dechorionated 6 hpf embryos using an automated embryo placement system (Mandrell et al., 2012). A Hewlett Packard D300e chemical dispenser was used to dispense nicotine at 0, 1, 5, 15, 20, and 30 µM from a 10 mM stock in 100% DMSO. All exposure wells were normalized to 0.64% DMSO. After all chemicals were added, plates were sealed with silicone plate covers and shaken overnight at 235 RPM in an incubator room at 28ºC. Replicate plates totaled an n of 64 animals for each concentration. Morphological endpoints and behavioral assays were assessed as described above.

E-Cigarette Flavor Screening

Zebrafish were exposed to 10 µL of a 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, or 1:1,000,000 serial dilution of flavor in a 1:1,000 PG:EM mixture. The 1:10 dilutions were prepared as described in the PG and VG screening section. The 1:10 dilutions led to 100% mortality in all animals across all flavors (data not shown). As a negative control, a 1:1,000

propylene glycol vehicle exposure was added to each plate. As discussed previously, plates were preloaded with 90 µL of EM, and loaded with 10 µL of flavor dilution, PG dilution, or EM. After all embryos were added, plates were sealed with silicone plate covers and shaken overnight at 235 RPM in an incubator room at 28ºC. Replicate plates totaled an n of 32 animals for each dilution. Plates were set up with the same dilution series for each flavor. Morphological endpoints and behavioral assays were assessed as described above.

Chemical Constituent Flavor Screening

The seven most common chemical constituents in the flavors examined in our study appear in Table 1 (Tierney et al., 2016). Stock solutions of 100 mg/mL were made in 100% DMSO for benzyl alcohol, cinnamaldehyde, ethyl acetate, ethyl vanillin, and maltol. A stock solution of 200 mg/mL in 100% DMSO was made for ethyl butyrate, and a stock solution of 1g/mL was made for vanillin. As described in the nicotine exposure section, plates were preloaded with 100 µL of EM and automatically loaded with dechorionated 6 hpf embryos. A Hewlett Packard D300e chemical dispenser was used to dispense the target concentration (Table 1) of each constituent. Replicate plates totaled an n of 32 animals for each concentration. All plates were normalized to 0.64% DMSO; other procedures were as described above.

Recreating a Mixture

To determine which compound(s) was driving the toxicity of an e-cigarette flavor, we compared two flavors with similar compositions but different morphological outcomes, Bubble Gum and Grape. The major tested constituents of each were ethyl acetate and ethyl butyrate, while Bubble Gum also contained cinnamaldehyde. The morphological endpoint associated with exposure to the Bubble Gum flavor was mortality between 24 and 120 hpf. We chose a binary endpoint, since incidence can be counted as yes or no, 0 or 1, enabling the proportion of affected animals to be less subjective (Truong et al., 2016; Truong et al., 2014). EC_{50} values were calculated using the drm function within the drc package in R, as described (Truong et

al., 2016; Truong et al., 2014). First, we generated a mixture of ethyl acetate and butyrate where we varied the concentration of ethyl butyrate but kept the ethyl acetate constant.

A 100 mg/mL working stock solution of ethyl acetate in a 1:1,000 propylene glycol:EM mixture was made, as propylene glycol was a major component of the e-cigarette flavors. From this working stock, five different vials were created. Each vial had a final volume of 20 mL and a final ethyl acetate concentration of 10 µg/mL, the highest concentration that produced no morphological effects. Next, a 100 mg/mL stock of ethyl butyrate in a 1:1,000 PG:EM mixture was made. Varying volumes of ethyl butyrate were added to each vial for final concentrations of: 1,000, 100, 10, 1, and 0.1 µg/mL. To ensure all vials were the same volume, 1:1,000 PG:EM was added as needed to total 20 mL. Animals were exposed to 10 µL of a given vial. Replicate plates were used for a total of 32 animals per concentration. Exposures took place without the Bioprinter, as in the e-cigarette flavor section, and 24 and 120 hpf analyses were carried out as described previously. To decrease the likelihood of carryover effects (due to ethyl acetate's high volatility) controls were carried out on a separate plate (n=48), where all animals were exposed to the 1:1,000 PG:EM mixture.

Next, we generated a mixture of 10 µg/mL ethyl acetate and 10 µg/mL ethyl butyrate. These concentrations were selected as they were the highest concentrations that produced no morphological effects (Figure A1). To this mixture, we added varying amounts of cinnamaldehyde to determine whether we could recapitulate Bubble Gum's toxic response. Using the same ethyl acetate and butyrate working stocks, five different vials were created, each with a final volume of 20 mL, a final ethyl acetate concentration of 10 µg/mL, and a final ethyl butyrate concentration of 10 µg/mL. A 100 mg/mL stock of cinnamaldehyde was made up in a 1:1,000 PG:EM mixture. Cinnamaldehyde was added to each vial for final concentrations of: 1,000, 100, 10, 1, and 0.1 µg/mL. Replicate plates were used for a total of

32 animals per concentration. Exposures took place as described above, and control animals were on a separate plate.

To determine whether cinnamaldehyde was driving toxicity in a real mixture, we added the same concentrations of cinnamaldehyde to the Grape flavor (which contained ethyl acetate and ethyl butyrate). Nine mLs of a 1:100 dilution of Grape:propylene glycol solution (from the E-cigarette screen above) was pipetted into five different vials. Cinnamaldehyde was added to each vial for the same final concentration range as listed above. To ensure all vials were the same volume, 1:1,000 PG:EM was added as needed to total 20mL. Replicate plates were used for a total of 32 animals per concentration. Exposures took place as described above, and control animals were on a separate plate.

Table 1: E-cigarette Chemical Constituents Screened in Zebrafish

The seven most common chemical constituents detected in six different e-cigarette mixtures from Mount Baker Vapor in Lynden, WA (Tierney et al., 2016) and concentration range tested in zebrafish. Neither the nicotine nor the unflavored cartridges appear in this table as neither contained any additional chemicals aside from PG, VG, and Nicotine. Additionally, the Banana Crème Pie flavor is not included in the table as its chemical constituents could not be confirmed.

Chemical Constituent Name	**CAS Number**	**Present in E-Cigarette Flavor(s)**	**Concentration Range (µg/mL)**
Benzyl alcohol	100-51-6	Coffee	1,000; 100; 10; 1; 0.1
Cinnamaldehyde	104-55-2	Bubble Gum	1,000; 100; 10; 1; 0.1
Ethyl acetate	141-78-6	Bubble Gum, Grape	1,000; 100; 10; 1; 0.1
Ethyl butyrate	105-54-4	Bubble Gum, Grape	1,000; 100; 10; 1; 0.1
Ethyl vanillin	121-32-4	555, Cotton Candy, French Vanilla	1,000; 100; 10; 1; 0.1
Maltol	118-71-8	555, French Vanilla	1,000; 100; 10; 1; 0.1
Vanillin	148-53-8	555, Coffee, Cotton Candy, French Vanilla	10,000; 1,000; 100; 10; 1

CHAPTER THREE – RESULTS AND DISCUSSION

E-Cigarette Flavor Developmental Toxicity

Public information on the safety, biological activity and the health risks of e-cigarette flavorings lags behind their surging popularity (Al-Delaimy et al., 2015; Arrazola et al., 2015; Bahl et al., 2012; Behar et al., 2014; FDA, 2018a, b; Grana et al., 2014; Kennedy et al., 2017a; Massarsky et al., 2017; Palpant et al., 2015; Sears et al., 2017; Smith et al., 2016; Suter et al., 2015). We used the developmental zebrafish to profile the biological responses to nine different e-cigarette mixtures and 10 of their most common constituents. At six hours post fertilization (hpf) dechorionated zebrafish embryos were exposed to a dilution range of e-cigarette flavors in propylene glycol (PG) and assessed for morphological and behavioral outcomes at 24 and 120 hpf (Figures 1A, 1B, A1 and Tables A1 and A2).

Overall, at 24 hpf, embryonic zebrafish behavior in animals exposed to e-cigarettes did not differ significantly from controls (Table A1). At 120 hpf, larval zebrafish exposed to the majority of e-cigarette flavors, except for nicotine, exhibited hyperactive behavior (Table A2). This is consistent with previous studies demonstrating PG exposure resulted in hyperactive larval zebrafish behavior (Maes et al., 2012; Massarsky et al., 2017). However, our propylene glycol exposures alone did not recapitulate this hyperactivity (Table A2). Our dilutions started at 1% by volume, while zebrafish propylene glycol hyperactivity has been reported at 1.25% by volume (Massarsky et al., 2017). While the concentration of PG in our e-cigarettes could not be verified it was likely much more concentrated as e-cigarettes generally contain at least 90% propylene glycol by weight (Jensen et al., 2017; Tierney et al., 2016). Additionally, the concentration of PG in e-cigarettes has been determined at 500 – 600 mg/mL, suggesting that PG in the e-cigarette flavor mixtures was much more concentrated than our PG exposures (Schober et al., 2014).

Zebrafish exposed to the nicotine flavor or a nicotine standard (0 – 75 μM) exhibited hypoactivity, an effect also previously associated with nicotine in zebrafish (Table A2) (Klee et al., 2011; Svoboda et al., 2002; Thomas et al., 2009). As shown in Klee et. al. (2011), embryonic nicotine exposure results in swimming paralysis for prolonged periods of time (Klee et al., 2011). Thus in the larval photomotor response assay, animals exposed to nicotine are unable to move, and are unable to respond to the light changing stimulus. Furthermore, as discussed in Thomas et. al. (2009), this paralysis was determined to be due to exogenous overstimulation of the nicotinic acetylcholine receptor (nAChR) following nicotine exposure (Thomas et al., 2009). Furthermore, zebrafish possess nACh receptors homologous to those in humans (Papke et al., 2012; Svoboda et al., 2002; Thomas et al., 2009). This further demonstrates the use of the zebrafish as a tool to study e-cigarettes and their biological effects. It is also important to note that the nicotine flavor contained 24 mg/mL of nicotine versus 12 mg/mL in all of the other flavors (except unflavored), thus the strength of the nicotine content likely outweighed any hyperactive effects of propylene glycol.

At the 1:1000 dilution, several morphological endpoints were affected by a majority of the e-cigarette flavors (Figure 1A). The common endpoints affected were yolk sac and pericardial edema (YSE, PE, respectively), as well as Eye malformations. YSE was present in six of the nine flavors – 555, Banana Crème Pie, Coffee, French Vanilla, Nicotine and Unflavored. Four of these six flavors, with the exception of Coffee and Unflavored also exhibited eye malformations and PE. There was no significant bioactivity associated with exposure to the Grape mixture in our study (Figure 1A, 1B). From Figure 1A, we suggest that the flavor, not the nicotine content, drove the toxic response of a given e-cigarette mixture. This was consistent with previous work in both *in vivo* and *in vitro* models (Bahl et al., 2012; Behar et al., 2014; Kennedy et al., 2017a). All zebrafish morphological concentration response plots are available in the Appendix (Figure A1).

The most severe malformations in the head and cardiac regions were present in animals exposed to 555, Banana Crème Pie, and French Vanilla (Figure 1A, 1B). The main chemical constituents in 555 and French Vanilla were ethyl vanillin, maltol, and vanillin (Table 1; (Tierney et al., 2016)). The chemical constituents of Banana Crème Pie could not be determined (Table 1), but it is likely that the Banana Crème Pie flavor contained some of the same or related flavoring agents as 555 and French Vanilla. Overall, this suggests that embryonic exposure to these flavor profiles is associated with severe craniofacial defects in the developmental zebrafish in agreement with previous findings in *X. laevis* (Kennedy et al., 2017a).

Bubble Gum and Cotton Candy flavor exposures were associated exclusively with mortality, though sufficient animals survived to detect some malformations at the 1:1000 and 1:10000 dilutions (Figure A1). Bubble Gum was the only flavor that lead to a high incidence of 24 hpf mortality (MO24), at the 1:100 dilution, while Cotton Candy and French Vanilla were associated with a high incidence of mortality only at 120 hpf at the 1:100 dilution (Figure A1). These three flavoring mixtures or the sum of their parts may have been the most biologically active in the zebrafish (Table 1; (Tierney et al., 2016)).

Chemical Constituent Developmental Toxicity

To profile the developmental toxicity to each of these constituents, it was essential to expose the animals to a concentration range that included a no observable adverse effect level (NOAEL). We used a wide concentration range to determine the sensitivity of the model to each native constituent. The actual concentration of each flavor component was unavailable, though each flavor made up no more than 4% of the final mixture by weight, and often less than 1% (Tierney et al., 2016). Thus, a one percent measure by weight would roughly correspond to 10 mg/mL (Tierney et al., 2016). This dosage was ten times greater than the maximal concentration zebrafish were exposed to in our study, 1 mg/mL or 1000 µg/mL, aside

from the vanillin exposure (Table 1). Overall, this demonstrates the sensitivity of the zebrafish model. Similar to Figure 1A, Figure 2 shows the results of the morphology and behavioral screen for zebrafish exposed to seven common chemical flavorings contained in our e-cigarette flavors. Six hpf embryos were exposed to 0.1, 1, 10, 100 and 1,000 µg/mL of each constituent, except for vanillin. Due to limited stock material, animals were exposed to 1, 10, 100, 1,000 and 10,000 µg/mL of vanillin. All stocks were made in 100% DMSO to enhance solubility.

In Figure 2, several morphological endpoints were common: YSE, PE, and eye malformation. Four chemicals: benzyl alcohol, cinnamaldehyde, ethyl vanillin and vanillin were associated with YSE, PE, and trunk defects in the 100 to 10,000 µg/mL concentration ranges. Cinnamaldehyde, ethyl vanillin and vanillin led to axial, eye, and jaw malformations by 120 hpf in the same concentration range. The craniofacial endpoints affected in this screen were similar to those affected by French Vanilla, 555, and Banana Crème Pie flavors (Figure 1A, Table 3). These chemicals may affect a common toxic mechanism. Additionally, Table 3 compares the flavors and chemical constituents side-by-side revealing common morphological endpoints (YSE, PE) associated with ethyl vanillin, vanillin and their respective flavors (Table 3). Anchoring the similar phenotype to their underlying transcriptomic and metabolomic changes would provide a clearer indication of whether the toxic mechanism is shared (Bugel et al., 2014; Garcia et al., 2016; Haggard et al., 2017; Truong et al., 2014; Zoupa and Machera, 2017). Transcriptomic profiling could reveal new markers to study human disease etiology and outcomes, since more than 80% of potential human disease-related genes have at least one zebrafish ortholog, further demonstrating the utility of the zebrafish model (Garcia et al., 2016; Howe et al., 2013). Additionally, chemical exposure during the first five days of development covers the expression of the entire transcriptome increasing the number of significant biological changes at the molecular level, including potentially novel targets (Garcia et al., 2016; Haggard et al., 2017; Zoupa and Machera, 2017). Thus, the use of the zebrafish in forward genetic

screens may uncover the toxicity mechanisms for a given chemical or class of chemicals (Garcia et al., 2016; Zoupa and Machera, 2017).

For instance, in our screen cinnamaldehyde was the most bioactive chemical, with a lowest effect level (LEL) of 0.1 µg/mL (Figure 2). Cinnamon-flavored cartridges have been ostensibly associated with throat, mouth, and lung irritations in e-cigarette user forums (Behar et al., 2014). Furthermore, cinnamaldehyde is recognized as an irritant by the North American Skin Contact Dermatitis Group (Nguyen et al., 2008). Future studies using the developmental zebrafish model could employ forward genetics techniques to uncover the underlying mechanism(s) (Garcia et al., 2016; Zoupa and Machera, 2017).

Bahl et. al. (2012) found that the most cytotoxic refill fluids to human stem cells, human pulmonary fibroblasts, and mouse neural stem cells had cinnamon, caramel, butterscotch, and vanilla flavor profiles. These flavors had lower IC_{50} values than propylene glycol, vegetable glycerin and menthol flavorings, again suggesting their greater bioactivity (Bahl et al., 2012). Cells in this study were exposed to a percent by volume solution of refill cartridges, similar to our study, although the exposure concentrations did not exceed 1% (Bahl et al., 2012). A follow-up study using GCMS and HPLC identified cinnamaldehyde (from 0.005 – 5M) and vanillin (from 0.0025 – 0.075M) in the cinnamon-flavored cartridges (Behar et al., 2014).

Mixture Recreation

In Table 1, the Bubble Gum and Grape mixtures had similar components, yet from Figure 1A they were associated with different outcome profiles (Figure 1A, Table 3). While ethyl acetate and ethyl butyrate were associated with few morphological effects, cinnamaldehyde was associated with a high percent mortality as well as morphological effects (Figure 2). We hypothesized that cinnamaldehyde was driving the toxicity of the Bubble Gum mixture (Figure 1A). Using a component-based mixture approach, we built a series of mixtures

and varied the final concentration of a given component (Geier et al., 2018b; Monosson, 2004; Simmons et al., 2004). Since we have established that the flavoring agents, not nicotine, drove the adverse outcomes in Figure 2, nicotine was omitted from the mixture (Figure 2). Additionally, vegetable glycerin was omitted as it was previously associated with relatively little bioactivity (Bahl et al., 2012; Carmines and Gaworski, 2005; Kennedy et al., 2017a). Finally, since e-cigarettes typically contain 90% (or more) propylene glycol by weight, PG and embryo medium served as the base for each mixture (Kimmel et al., 1995; Massarsky et al., 2017; Tierney et al., 2016).

Three mixtures: ethyl acetate + ethyl butyrate (EA+EB), ethyl acetate + ethyl butyrate + cinnamaldehyde (EA+EB+C), and the Grape flavor + cinnamaldehyde (G+C) were tested and 120 hpf mortality incidence was used to calculate EC_{50} values (Table 2, 3). Figure 3 shows the concentration-response curves associated with the mortality incidence in each mixture. The estimated EC_{50} values for each chemical or mixture are listed in Table 2. A range of cinnamaldehyde concentrations were added to the 10 µg/mL ethyl acetate + 10 µg/mL ethyl butyrate vials. We were not able to account for all of the possible sub-components in the mixtures, thus we added different concentrations of cinnamaldehyde to a 1% solution of the Grape flavor, as this concentration did not lead to any adverse effects at a statistically significant level (Figure 1A and 1B).

The percent mortality associated with the Bubble Gum flavor was at least four-fold higher than the Grape (Figure 3A). At the highest dilution tested, 1:100 or 1%, there was less than 20% mortality by 120 hpf in the embryos exposed to Grape. Bubble Gum was associated with more than 80% mortality at the 1:100 concentration (Figure 3A). The concentration-response curves of ethyl acetate and ethyl butyrate differed (Figure 3B). Ethyl acetate was associated with less than 10% mortality at any concentration, thus an EC_{50} value could not be estimated due to lack of bioactivity (Table 2, Figure 3B). Ethyl butyrate was associated with a

near five-fold mortality increase between 10 and 100 µg/mL (Figure 3B). The estimated EC_{50} for ethyl butyrate was 117 ug/mL (Table 2). The incidence of mortality is much lower in the EA + EB mixture than in ethyl butyrate alone (Figure 3B) with an estimated EC_{50} for the EA+EB mixture that is approximately four-fold greater than that of ethyl butyrate alone. This could suggest a synergistic effect of the ethyl butyrate on the ethyl acetate when in the mixture, enhancing ethyl acetate's toxicity that is otherwise not observed (Cedergreen, 2014).

The concentration-response curves for the cinnamaldehyde mixtures appear in Figure 3C. The EA+EB+C and G+C mixtures had much steeper concentration-response curves than their components without cinnamaldehyde (Figure 3A, 3B). Overall, the G+C mixture was more potent than the EA+EB+C mixture, and lead to 100% mortality at both the 100 and 1000 µg/mL cinnamaldehyde concentrations (Figure 3C). Alone, cinnamaldehyde was associated with an EC_{50} of 11.4 µg/mL while the EA+EB+C mixture was associated with an EC_{50} of 45.1 µg/mL. At the same time, the G+C mixture had an EC_{50} of 28.6 µg/mL, and, while less potent than cinnamaldehyde alone; this suggests an additive effect that makes the G+C mixture more potent than the EA+EB+C mixture. Thus, cinnamaldehyde drove the mortality of a given mixture – mixtures of both known composition (EA+EB+C) and unknown composition (Bubble Gum and G+C) (Figure 3C).

The present study demonstrated flavor-specific e-cigarette toxicity in the developmental zebrafish, not associated with nicotine content (Figure 1). By evaluating mixtures, i.e. the commercial e-cigarette flavors, we captured morphological effects that might otherwise have been missed (Figure 1). For example, the Grape flavor was associated with few phenotypic effects and we concluded that it was non-toxic up to 1% (Figure 1). If we had only tested the Grape components ethyl acetate and ethyl butyrate individually, we would have missed the moderating effect of ethyl acetate on ethyl butyrate bioactivity (Figure 2, Figure 3B). Our study was not without limitations. We did not know the concentration of each

component in each flavor (Tierney et al., 2016). Due to the lack of regulation of e-cigarette flavors, batch-to-batch effects are likely, and concentration values may only offer rough estimates of actual exposures (Bahl et al., 2012; Behar et al., 2014; FDA, 2018a, b; Grana et al., 2014; Sears et al., 2017; Smith et al., 2016; Suter et al., 2015; Tierney et al., 2016). There are currently thousands of different e-cigarette flavors on the market, making safety testing a significant challenge. However, using the developmental zebrafish as a means to assess bioactivity, we can prioritize flavors (and constituents) that warrant further investigation. In this study, flavor-specific, morphological effects suggested that chemical composition of the different flavors targeted different aspects of vertebrate development (Figure 2). This alone should serve as incentive for more comprehensive hazard evaluations of e-cigarettes flavoring in whole animal models.

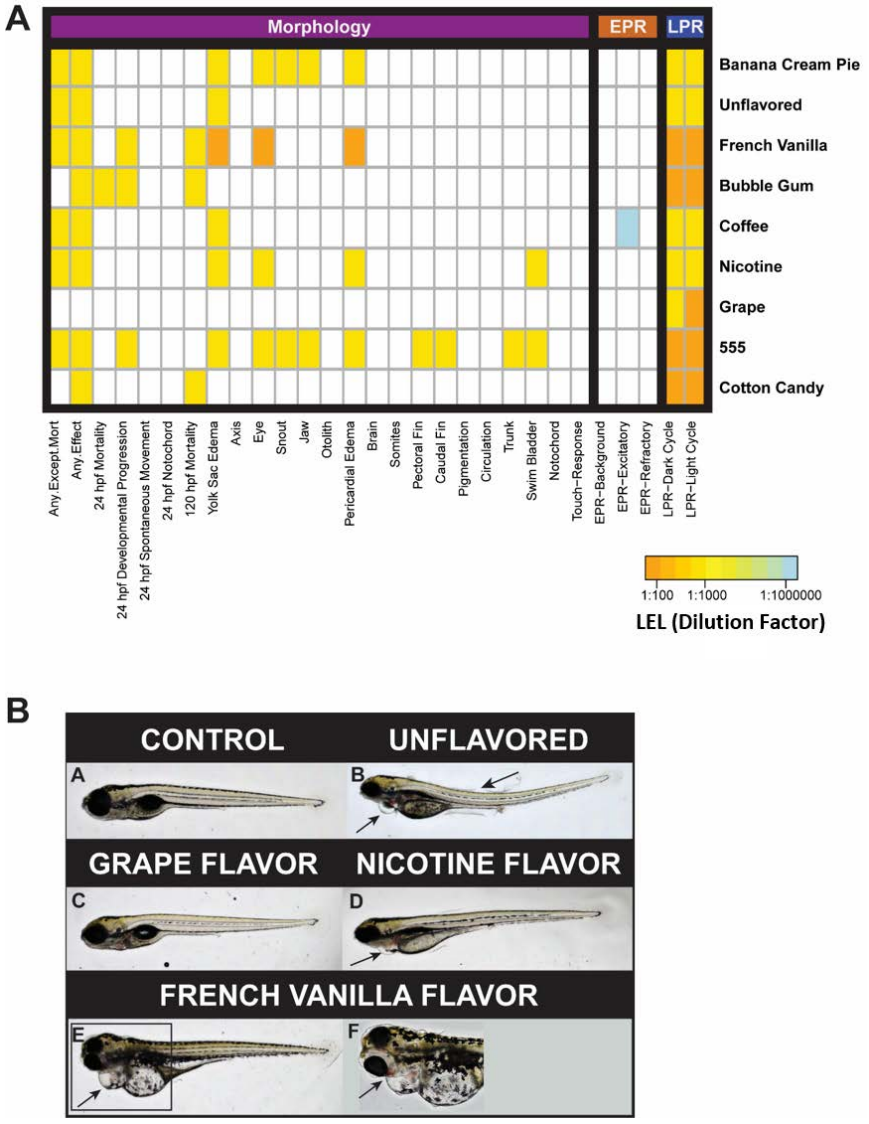

Figure 1A. Developmental toxicity heatmap displaying morphological and behavioral endpoints assessed in embryos exposed to nine e-cigarette mixtures. Color scale indicates relative potency, based upon the Lowest Effect Level (LEL) determined for a given mixture and endpoint. E-Cigarette flavors appear in the top right column. All morphological and behavioral endpoints are listed across the bottom of the figure. Orange color denotes greater potency (1:100 dilution factor), while green-blue colors indicate less potency (1:1000000 dilution factor). White indicates that there was no significant observable LEL for a particular mixture and endpoint. "Any Effect" and "Any Except Mortality" (left two columns) are aggregates of all morphological end points. The Unflavored mixture contains only PG and VG while the Nicotine flavor contains PG, VG and 24 mg/mL of Nicotine. **Figure 1B.** Representative images of 120 hpf zebrafish exposed to four different e-cigarette flavors. Images **A-E** were taken at 2X magnification using a Keyence BZ100 microscope under the bright field setting. Image **F** was taken at 10X magnification under the bright field setting. **A**: Control animal, exposed to 1:1000 PG:EM. **B:** 1:100 dilution of Unflavored e-cigarette flavor, presence of some edemas and a slightly curved body axis. **C:** 1:100 dilution of Grape flavor, note the lack of malformations. **D:** 1:100 dilution of Nicotine e-cigarette flavor, presence of some minor edemas. **E and F:** Zebrafish exposed to a 1:1000 dilution of French Vanilla flavor. Note the blood pooling in the eye and midbrain regions.

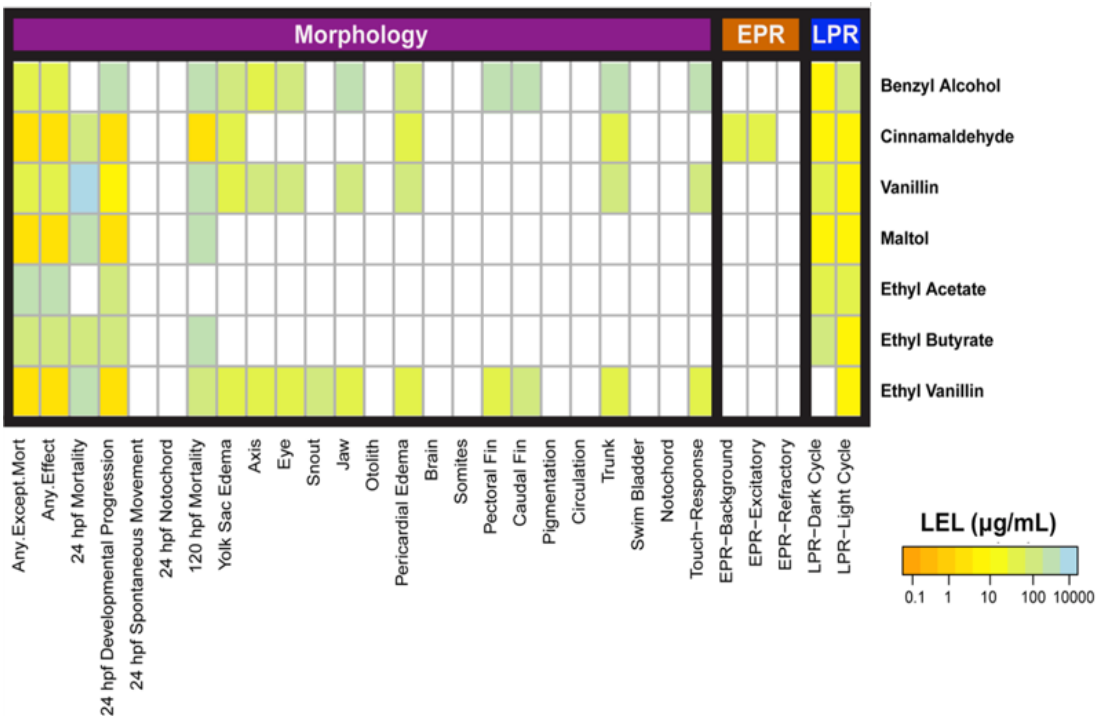

Figure 2. Developmental toxicity heatmap displaying morphological and behavioral endpoints screened in embryos exposed to the seven most common chemical flavorings (Table 1) of the e-cigarette mixtures tested (Figure 1A). Color scale indicates relative potency, based upon the Lowest Effect Level (LEL) determined for a given chemical and morphological or behavioral endpoint. Chemical names appear in the top right column. All morphological and behavioral endpoints are listed across the bottom of the figure. Orange color denotes smaller LEL (µg/mL) and thus greater potency, while green-blue colors indicate a higher LEL and less potency. White indicates that there was no significant observable LEL for a particular endpoint and mixture. “Any Effect” and “Any Except Mortality” (left two columns) are aggregates of all morphological end points.

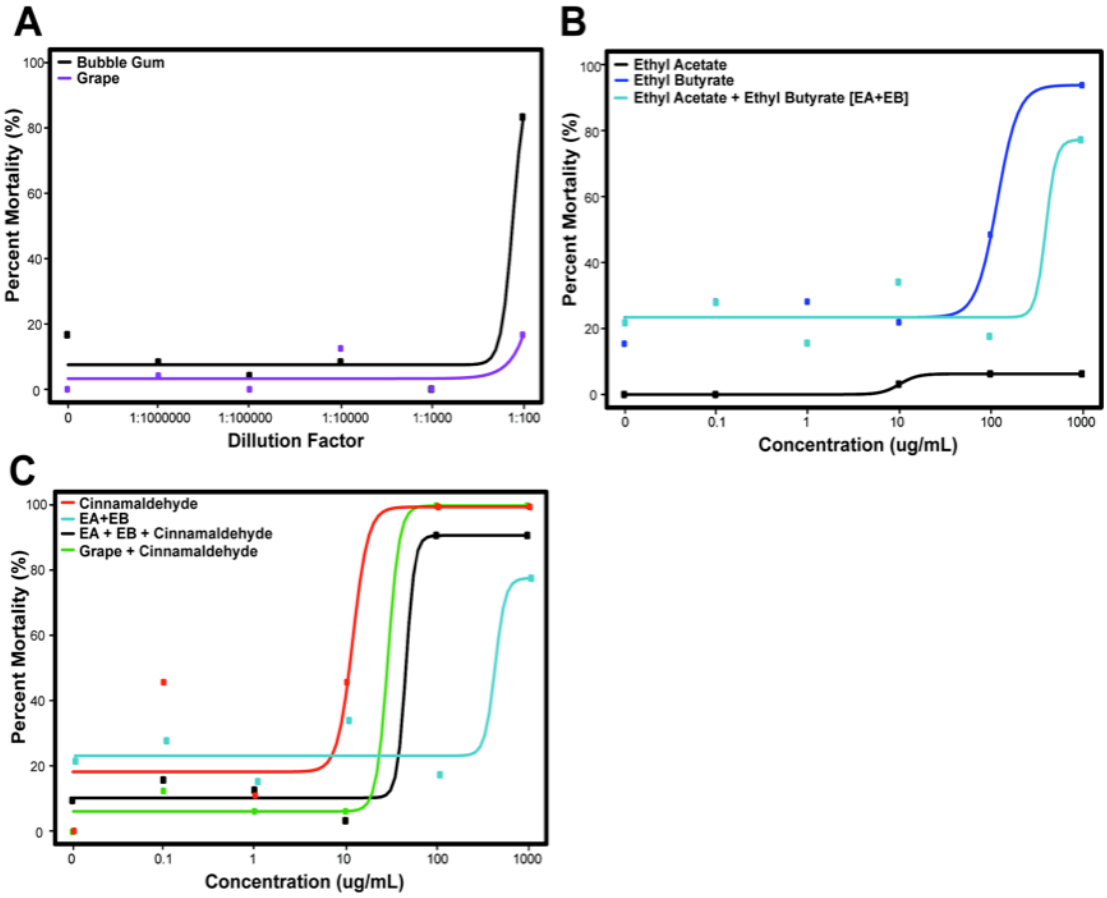

Figure 3. Concentration-response curves for the 120 hpf mortality (MORT) endpoint in two flavor mixtures with similar compositions – Bubble Gum and Grape. **3A**: Concentration-response curve for Bubble Gum is much steeper than Grape, suggesting an additional component(s) driving toxicity. Concentration values are based on serial dilutions, denoted as dilution factor across the x-axis. **3B:** Concentration-response curve of ethyl acetate, ethyl butyrate, and ethyl acetate + ethyl butyrate. Concentration is in units of ug/mL. Ethyl butyrate does not appear to be driving toxicity up to 1000 ug/mL. **3C**: Concentration-response of the ethyl acetate + ethyl butyrate mixture (for comparison), cinnamaldehyde, the ethyl acetate + ethyl butyrate + cinnamaldehyde mixture and the Grape mixture + cinnamaldehyde. The high mortality rate is recapitulated at the 100 and 1000 ug/mL cinnamaldehyde concentrations for both mixtures, suggesting the large role that cinnamaldehyde is likely playing in driving toxicity.

Table 2: Estimated EC_{50} Values for MORT Endpoint for Mixture Study

Estimated EC_{50} values for e-cigarette mixture flavors Bubble Gum and Grape as well as their mixture components: ethyl acetate, ethyl buytrate and cinnamaldehyde, in addition to the component-based mixtures created in the study. EC_{50} values are calculated from the percent incidence of 120 hpf mortality, MORT. For the Grape mixture and ethyl acetate, we were unable to calculate estimated EC_{50} values due to the lack of mortality by 120 hpf (Figures 1, 2, 3). Ethyl acetate + ethyl butyrate = EA+EB; ethyl acetate + ethyl butyrate + cinnamaldehyde = EA+EB+C; Grape mixture + cinnamaldehyde = G+C.

Chemical or Mixture	Estimated EC_{50} Value
Bubble Gum Flavor	1:100 dilution factor
Grape Flavor	NA (dilution factor)
Ethyl Acetate	NA µg/mL
Ethyl Butyrate	117 µg/mL
Cinnamaldehyde	11.4 µg/mL
EA+EB	406 µg/mL
EA+EB+C	45.1 µg/mL
G+C	28.6 µg/mL

Table 3: Summary Table

Morphological endpoints affected and LELs from our e-cigarette flavor screen (Figure 1A) and our chemical constituent screen (Figure 2). The left hand column lists each e-cigarette flavor or created mixture and its chemical constituents. The three right-hand columns list the most common endpoints affected, LEL values, and, where applicable, calculated EC_{50} values. The top row of the table lists each chemical constituent, as well as which flavor it was in, represented by an ✕. The bottom three rows in the left-hand column list the most common endpoints affected, LELs, and, where applicable, calculated EC_{50} values. All EC_{50} values were calculated using the mortality endpoint (MORT) and ND stands for Not Determined. As seen in Figures 1 and 2, endpoints common to 555 and French Vanilla were also common to their chemical constituents (Ethyl Vanillin, Vanillin, and Maltol), suggesting a similar mechanism of toxicity.

Mixture/Chemical	**Benzyl Alcohol**	**Ethyl Vanillin**	**Vanillin**	**Cinnamaldehyde**	**Maltol**	**Ethyl Butyrate**	**Ethyl Acetate**	**Endpoints**	**LEL (Dilution Factor)**	**EC50**
555		✕	✕		✕			YSE, EYE, SNOUT, JAW, PE	1:1000	ND
Banana Crème Pie								YSE, Eye, SNOUT, JAW, PE	1:1000	ND
Coffee	✕							YSE	1:1000	ND
French Vanilla		✕	✕		✕			MORT, YSE, EYE, PE	1:100	ND
Cotton Candy		✕	✕					MORT	1:1000	ND
Bubble Gum				✕		✕	✕	MO24, MORT	1:1000	1 to 100
Grape						✕	✕	NA	NA	NA
Nicotine								YSE, EYE, PE	1:1000	ND
Unflavored								YSE	1:1000	ND
EA+EB						✕	✕	MO24, MORT	100 ug/mL EB	405 ug/mL
EA+EB+C				✕		✕	✕	MO24, MORT	10 ug/mL C	45.1 ug/mL
G+C				✕		✕	✕	MO24, MORT	10 ug/mL C	28.6 ug/mL
Endpoints	MORT, YSE, EYE, JAW	YSE, EYE, JAW, PE	YSE, EYE, SNOUT, JAW, PE	MORT	MO24, MORT	MO24, MORT	DP24			
LEL (µg/mL)	100	10	100	0.1	1000	100	100			
EC50	ND	ND	ND	11.4 ug/mL	ND	117 ug/mL	NA			

CHAPTER 4 – CONCLUSION

In the United States, the sale of e-cigarettes has surpassed that of tobacco cigarettes. This is due in part to the wider appeal of numerous flavor choices and the common misconception that they are a safe smoking alternative. At the same time, e-cigarettes have largely been unregulated despite containing vehicle and flavoring agents that are known respiratory hazards. The developmental zebrafish was instrumental in associating e-cigarette hazard potential with distinct flavor bioactivity profiles and chemical constituents. Flavors with a candy, dessert, or vanilla flavor profile were the most developmentally toxic. This effect was ostensibly driven by components such as cinnamaldehyde, ethyl vanillin, and vanillin, and indeed, in isolation cinnamaldehyde recapitulated the toxicity of these flavors in an otherwise non-toxic mixture. Our study supports the continued exploration of e-cigarette hazard potential in the developmental zebrafish. The logical next steps will examine zebrafish developmental responses to low temperature combustion products captured from e-cigarette smoke.

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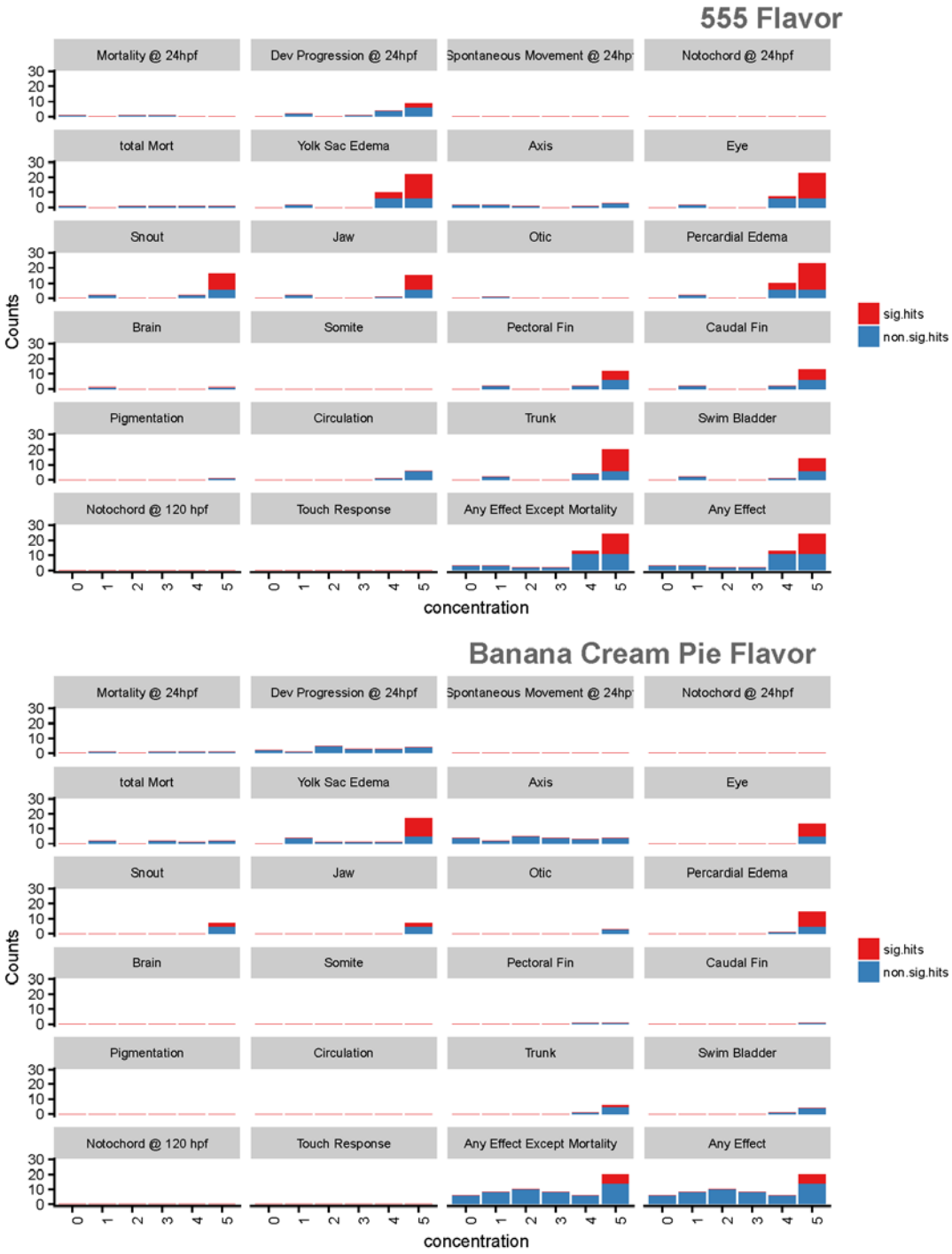
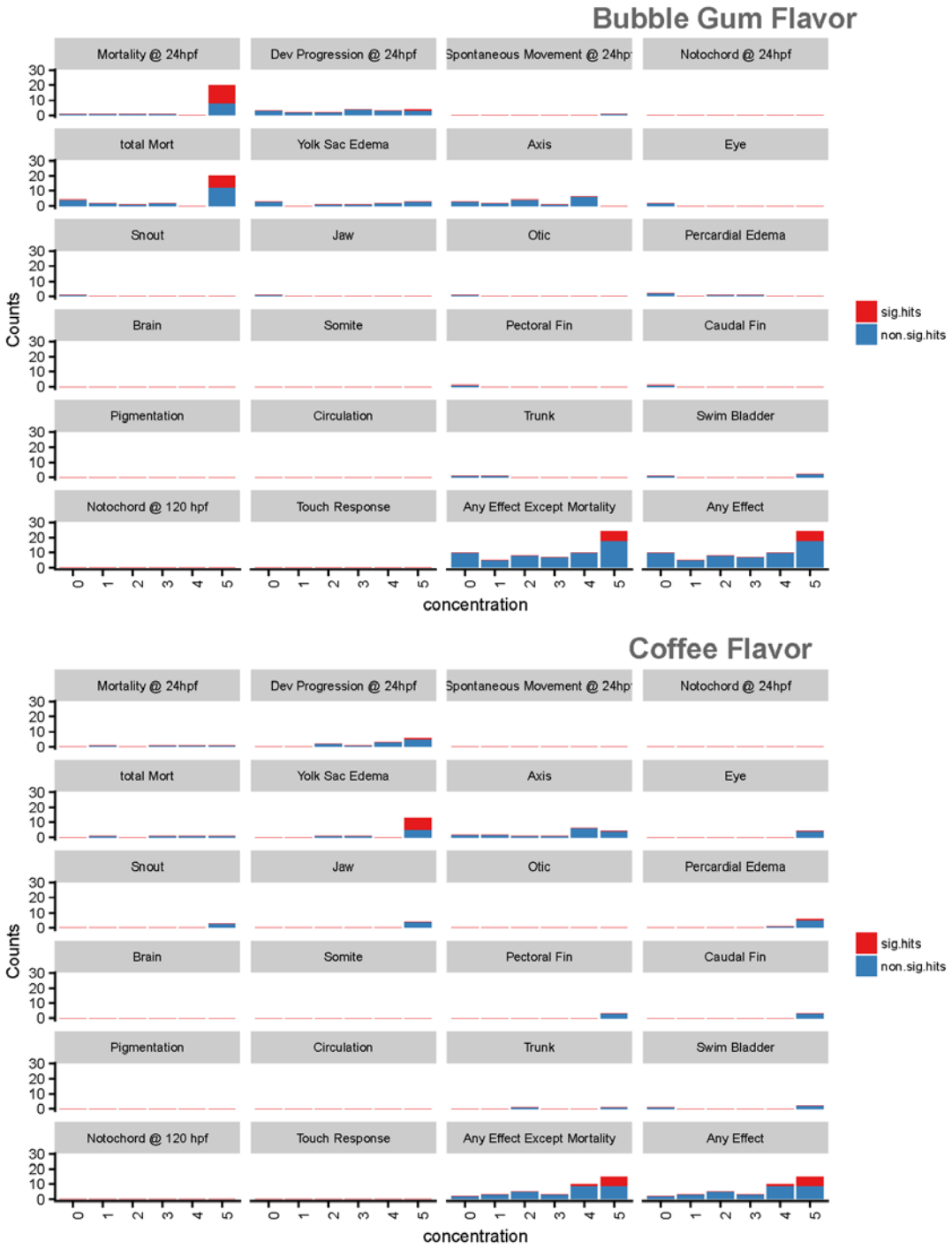
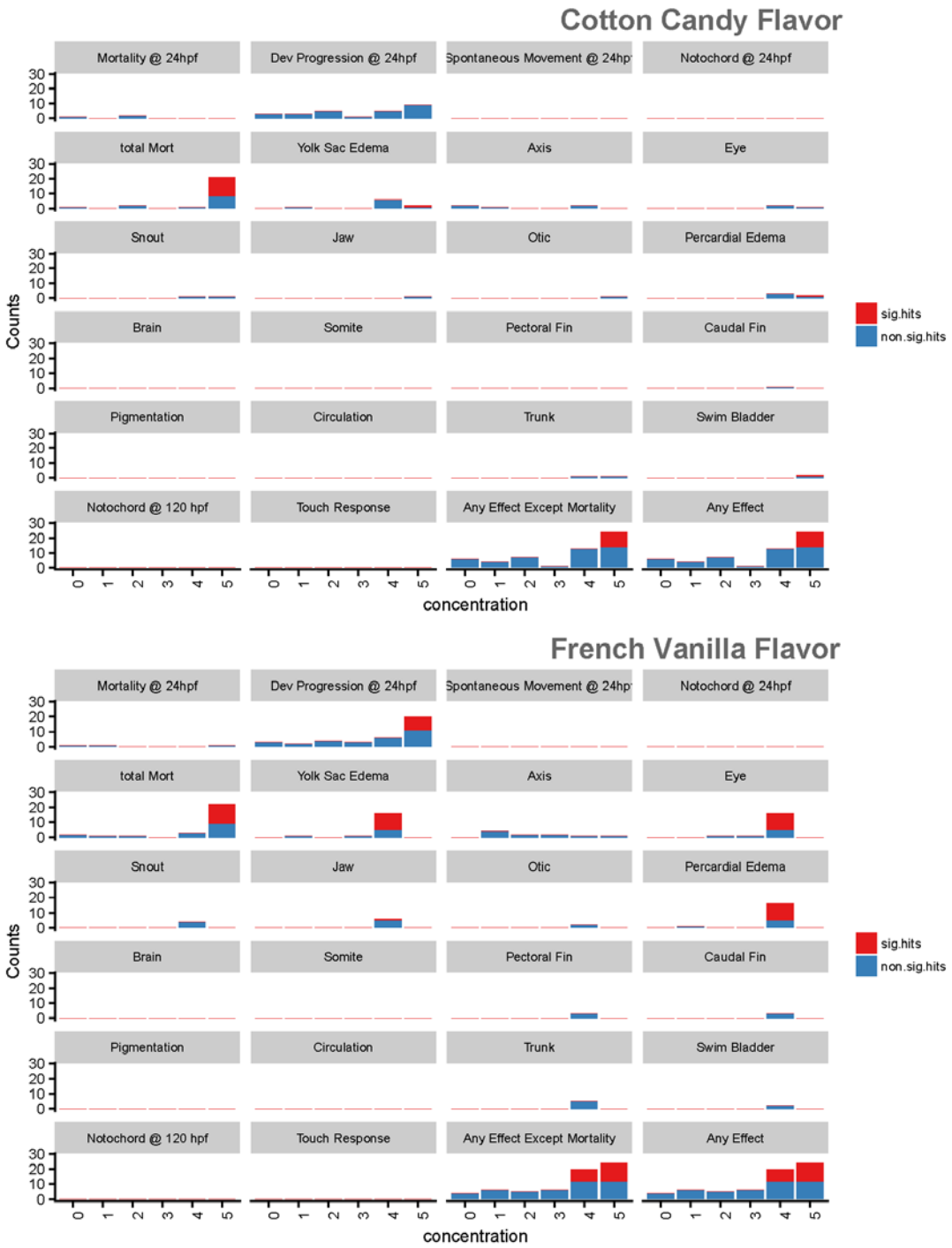
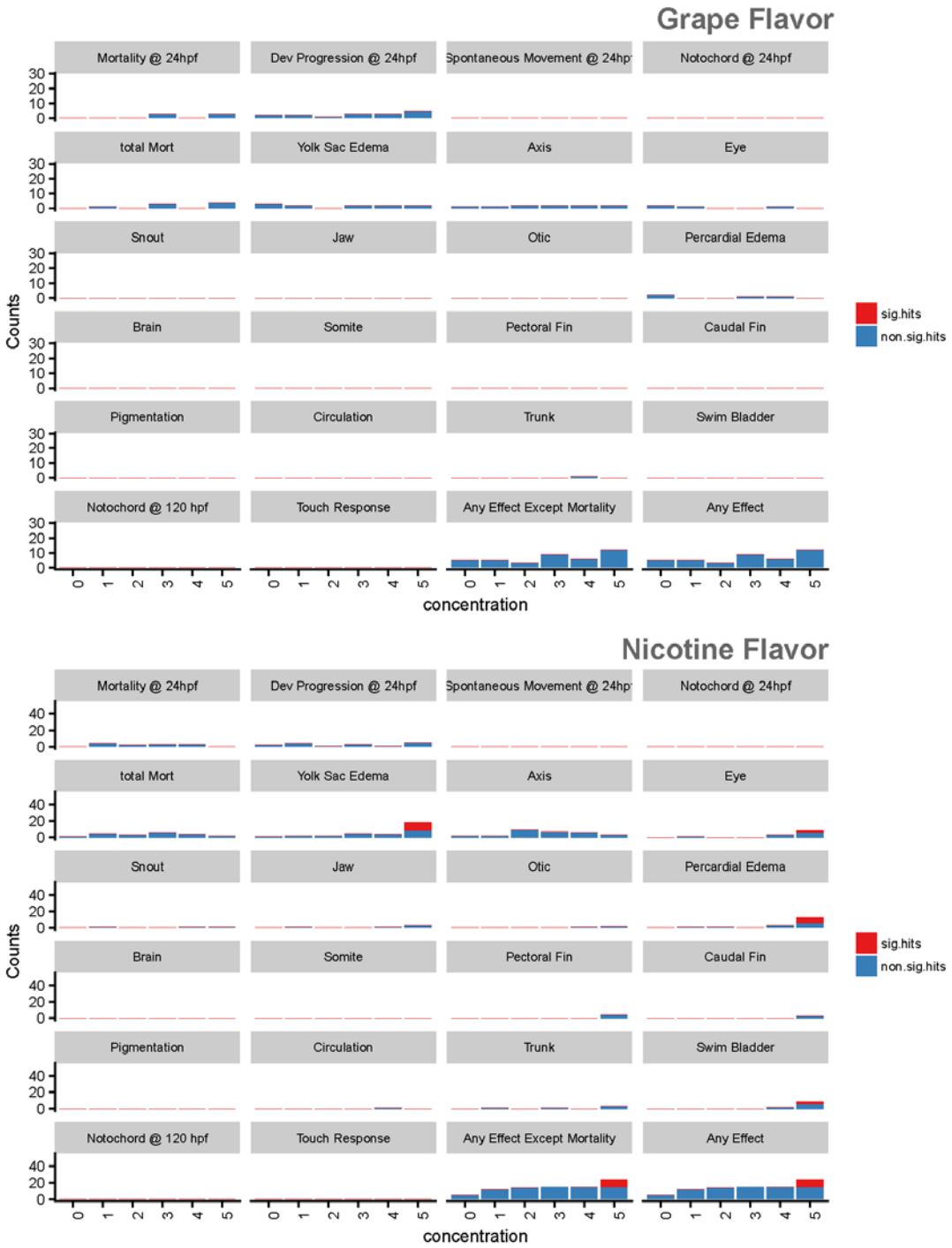
APPENDICES

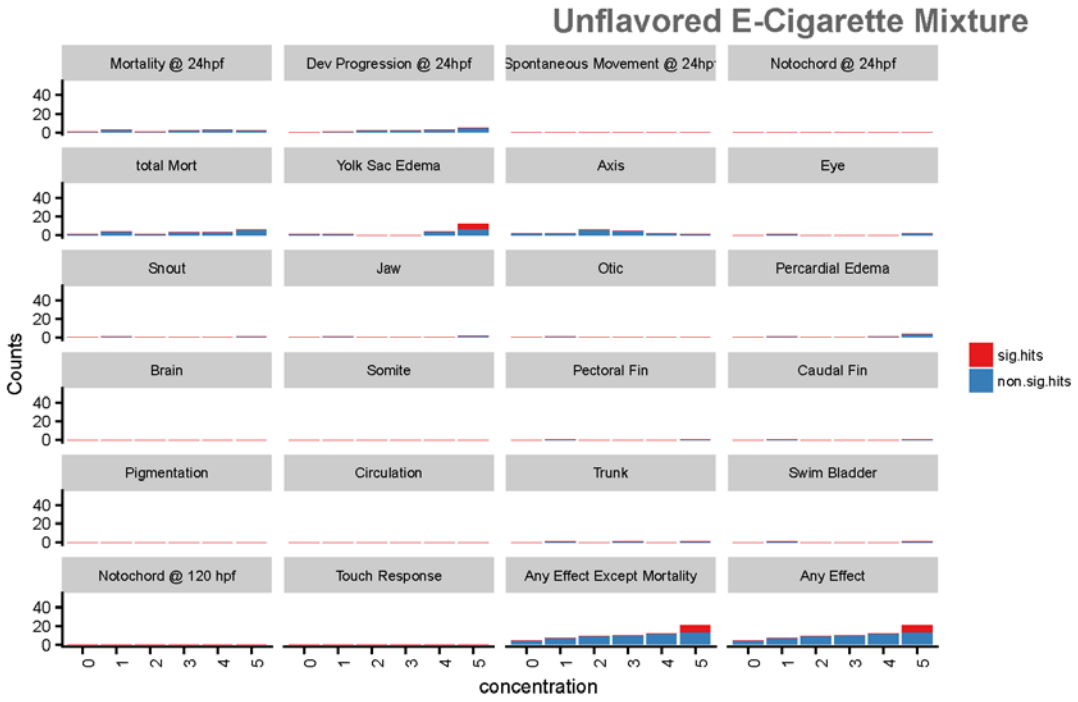
Figure A1. Morphology Barplots for all tested Mixtures and Chemicals

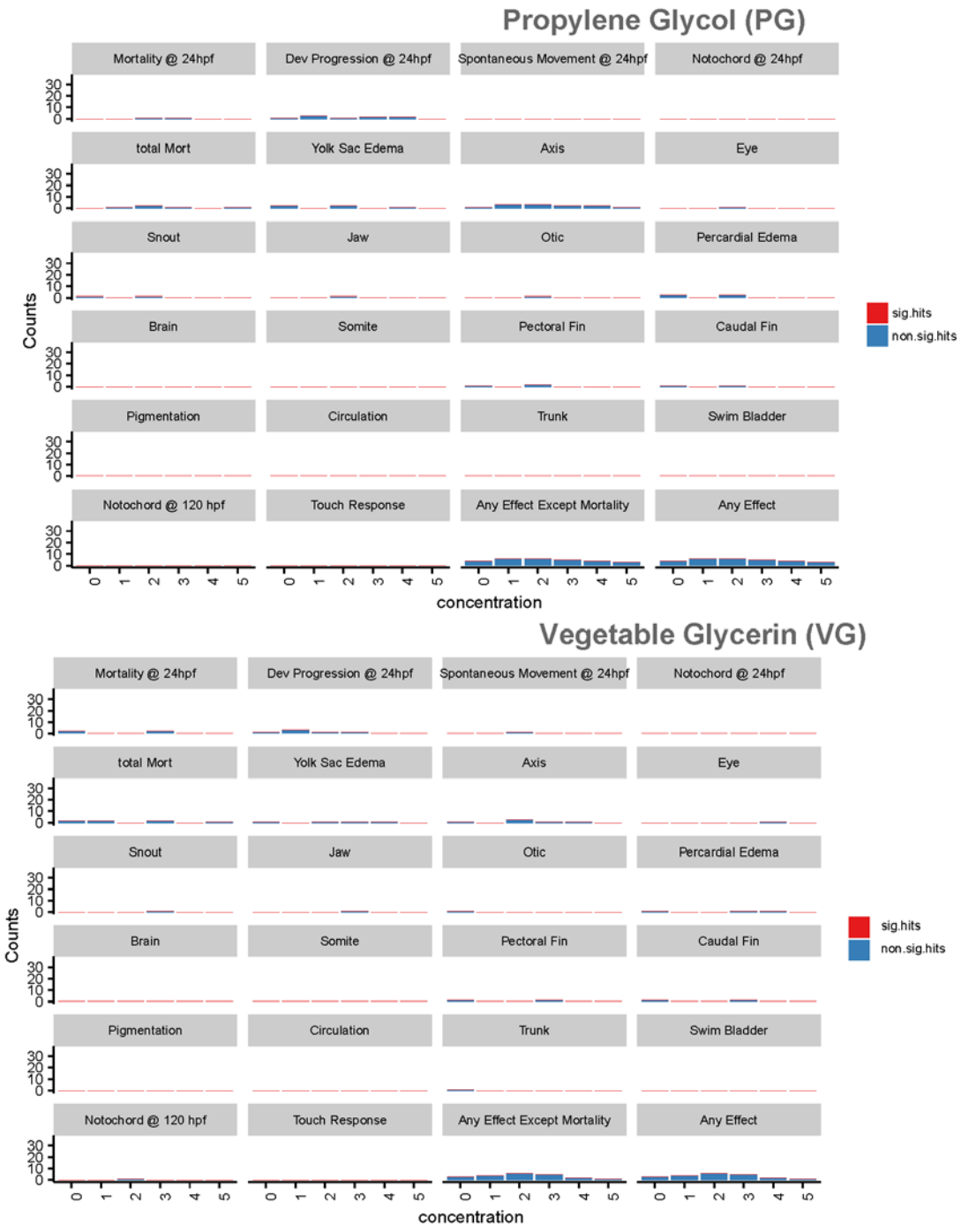


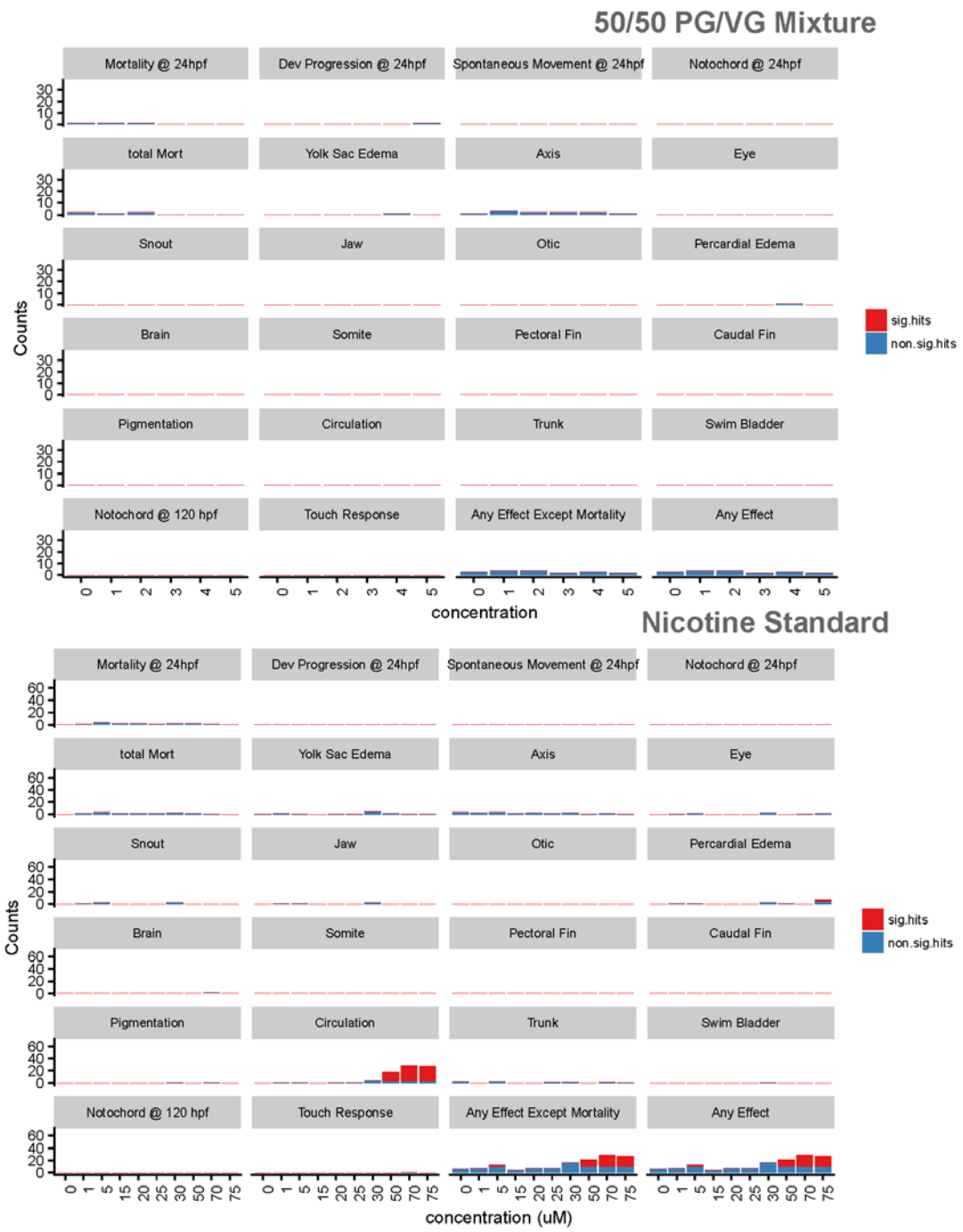

Bubble Gum Flavor
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits
Coffee Flavor
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits

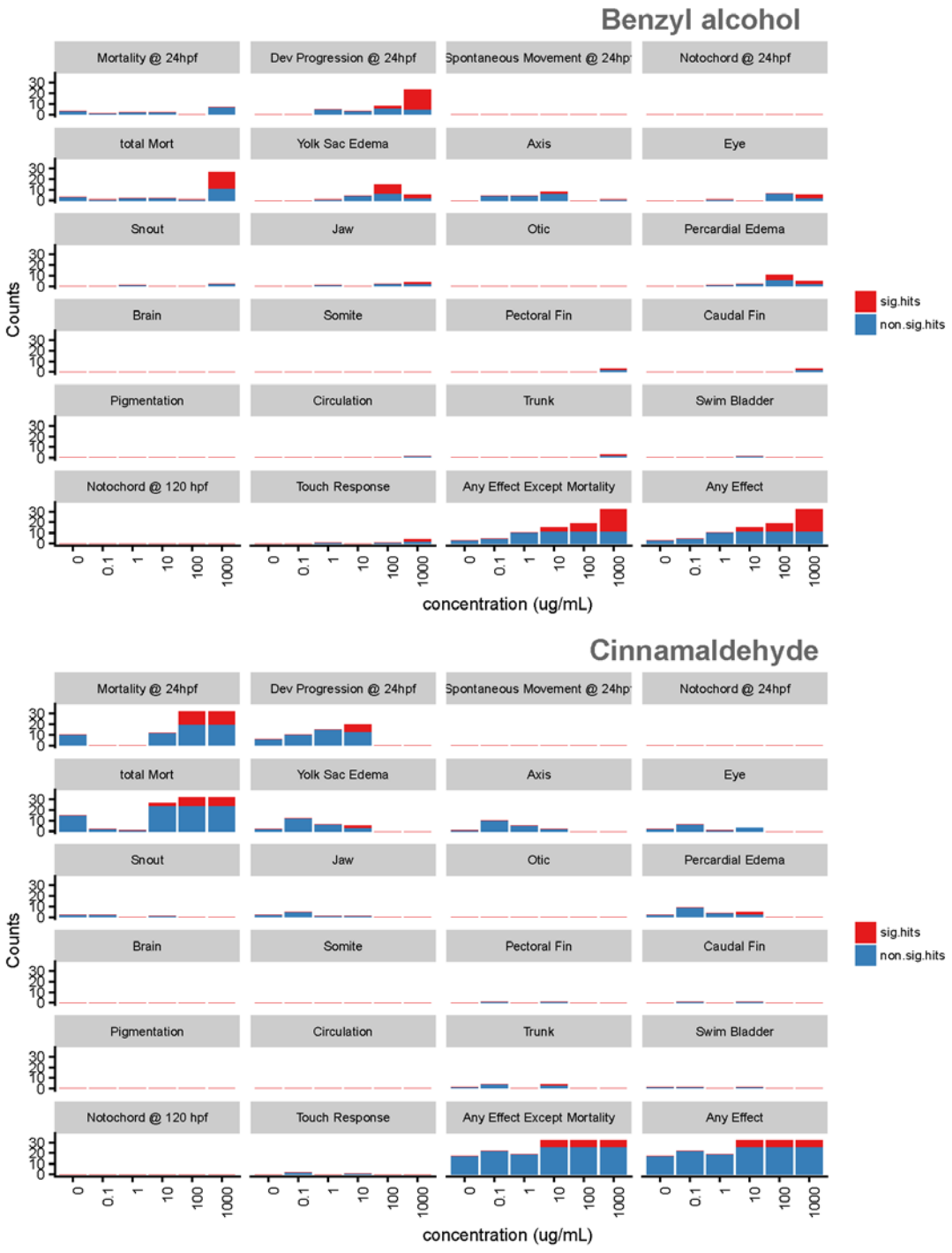

Cotton Candy Flavor
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits
French Vanilla Flavor
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits

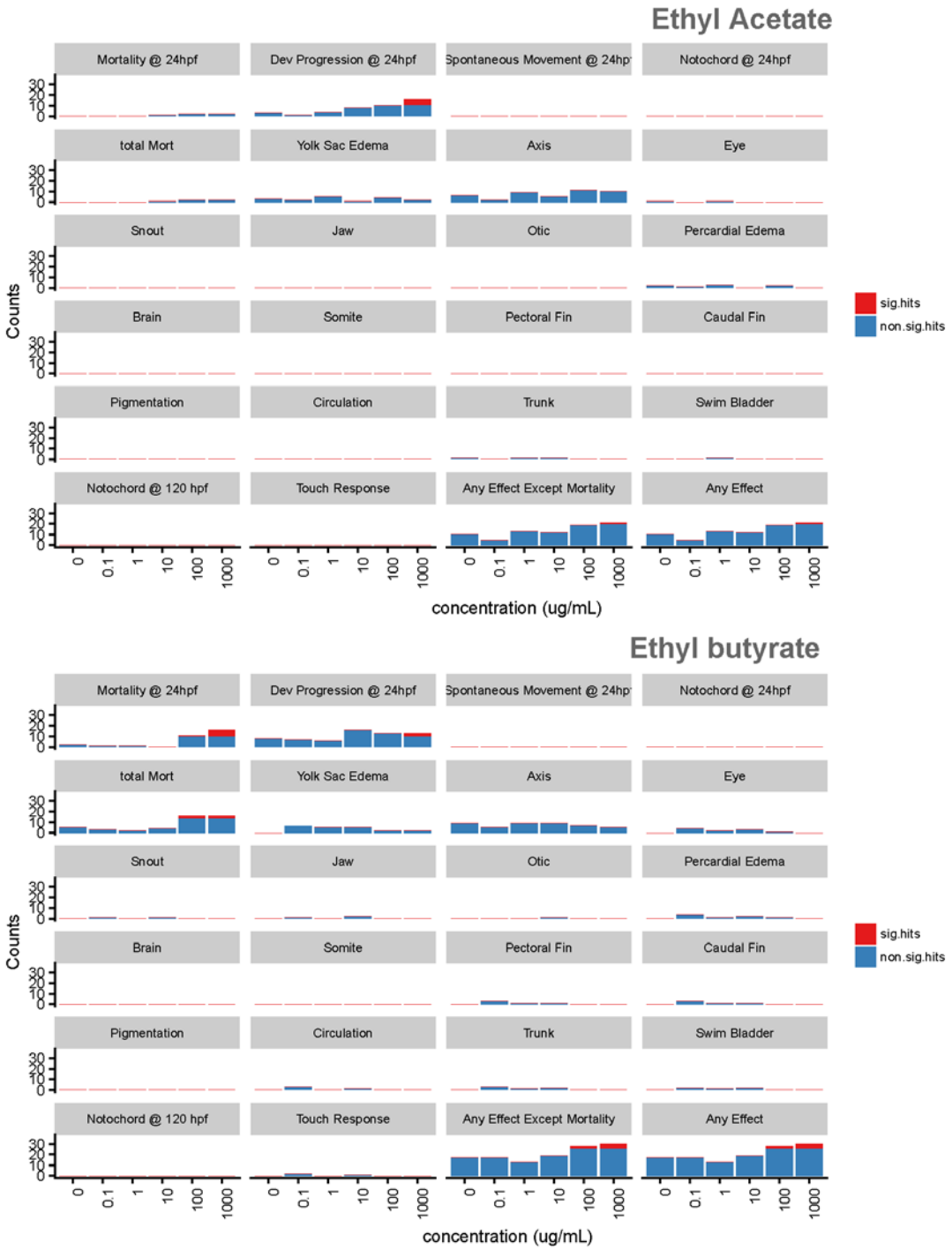

Grape Flavor
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits
Nicotine Flavor
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits

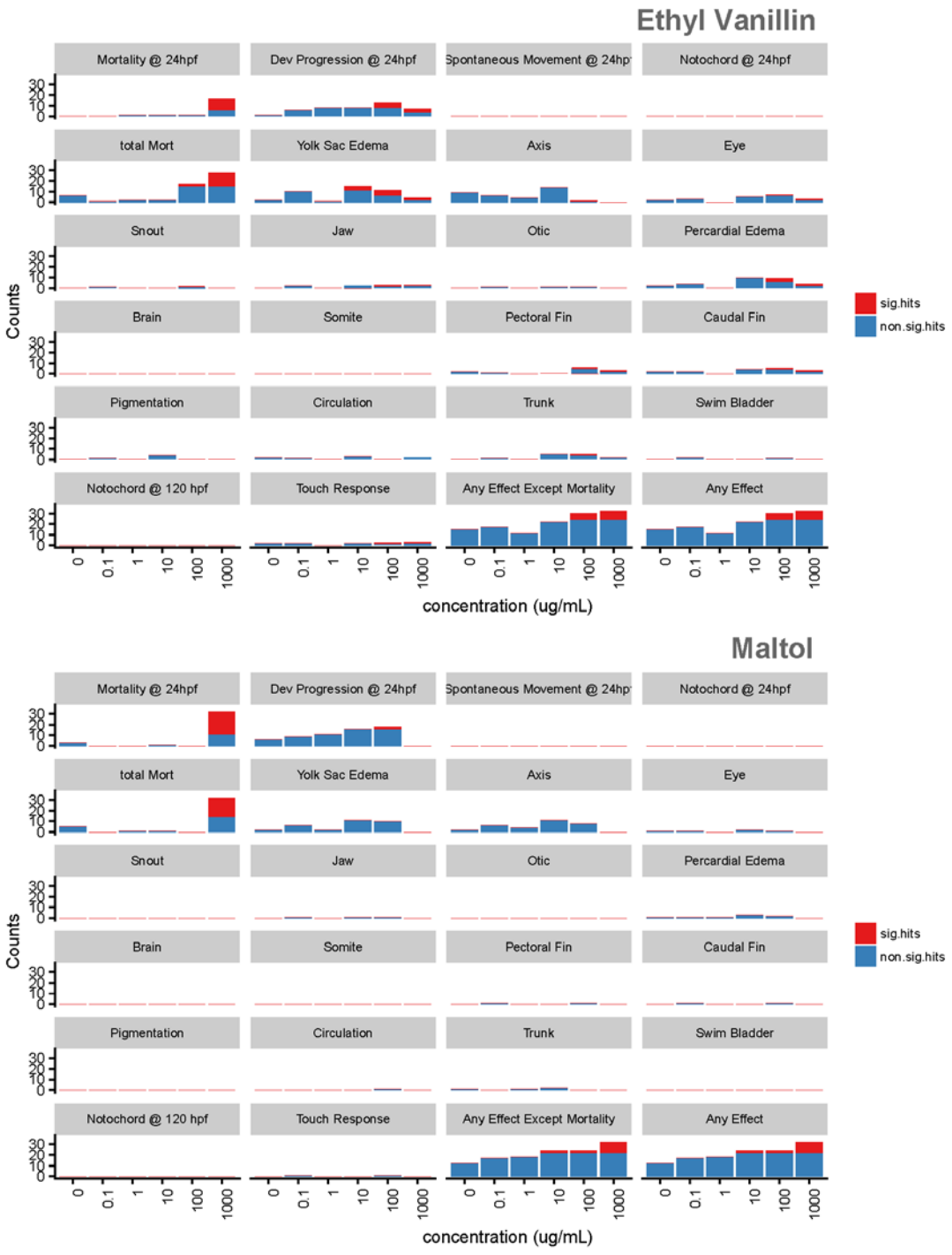

Unflavored E-Cigarette Mixture
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
0
20
40
0 1 2 3 4 5
concentration
sig.hits
non.sig.hits


Propylene Glycol (PG)
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits
Vegetable Glycerin (VG)
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits


50/50 PG/VG Mixture
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration
sig.hits
non.sig.hits
Nicotine Standard
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration (uM)
sig.hits
non.sig.hits


Benzyl alcohol
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
0
10
20
30
0
0.1
1
10
100
1000
concentration (ug/mL)
sig.hits
non.sig.hits
Cinnamaldehyde
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration (ug/mL)
sig.hits
non.sig.hits


Ethyl Acetate
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
0
10
20
30
0
0.1
1
10
100
1000
concentration (ug/mL)
sig.hits
non.sig.hits
Ethyl butyrate
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration (ug/mL)
sig.hits
non.sig.hits


Ethyl Vanillin
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
0
10
20
30
0
0.1
1
10
100
1000
concentration (ug/mL)
sig.hits
non.sig.hits
Maltol
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
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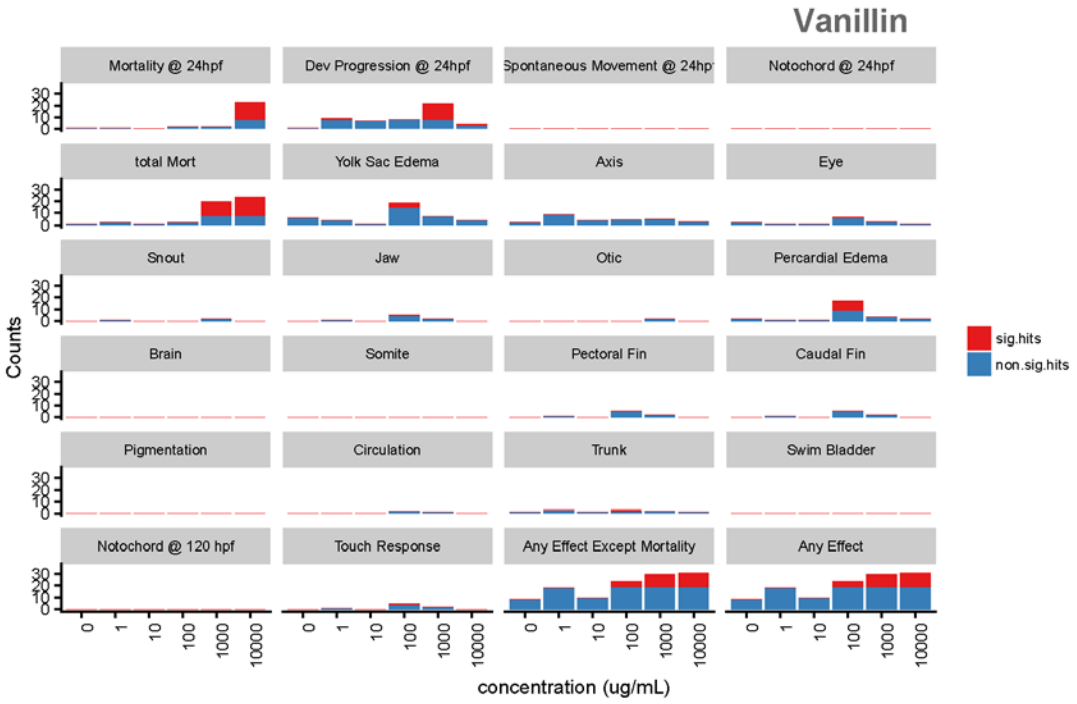
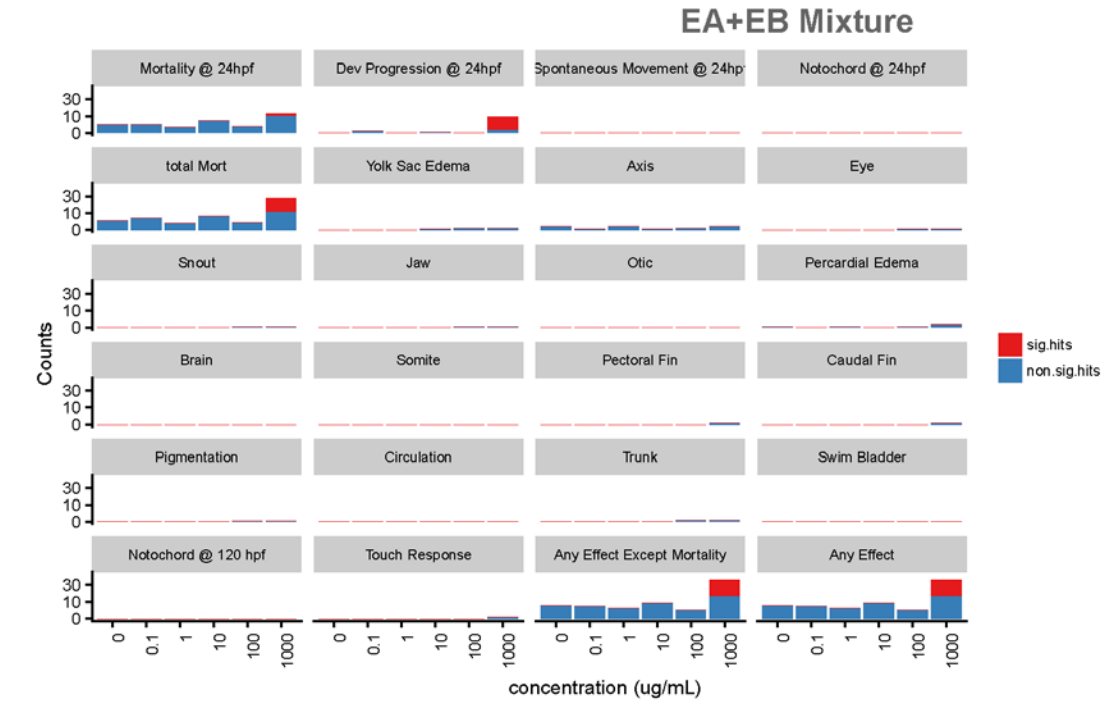
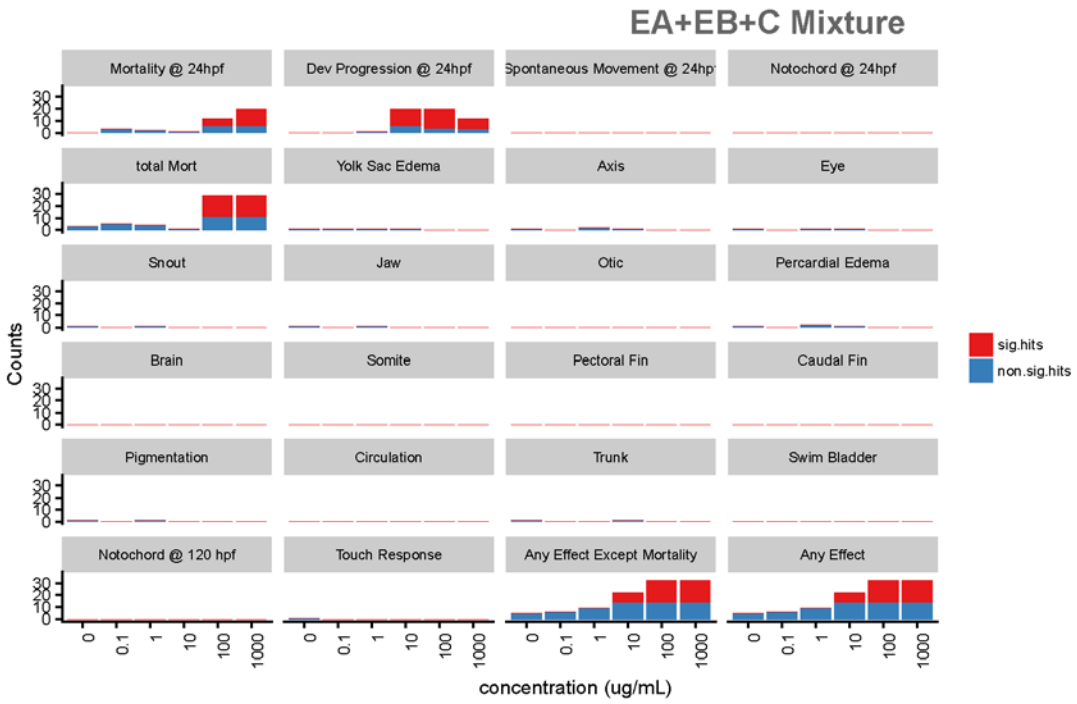
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration (ug/mL)
sig.hits
non.sig.hits


Vanillin
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hpf
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
0
10
20
30
0
1
10
100
1000
10000
concentration (ug/mL)
sig.hits
non.sig.hits


EA+EB Mixture
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hp
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration (ug/mL)
sig.hits
non.sig.hits


EA+EB+C Mixture
Mortality @ 24hpf
Dev Progression @ 24hpf
Spontaneous Movement @ 24hp
Notochord @ 24hpf
total Mort
Yolk Sac Edema
Axis
Eye
Snout
Jaw
Otic
Percardial Edema
Brain
Somite
Pectoral Fin
Caudal Fin
Pigmentation
Circulation
Trunk
Swim Bladder
Notochord @ 120 hpf
Touch Response
Any Effect Except Mortality
Any Effect
Counts
concentration (ug/mL)
sig.hits
non.sig.hits

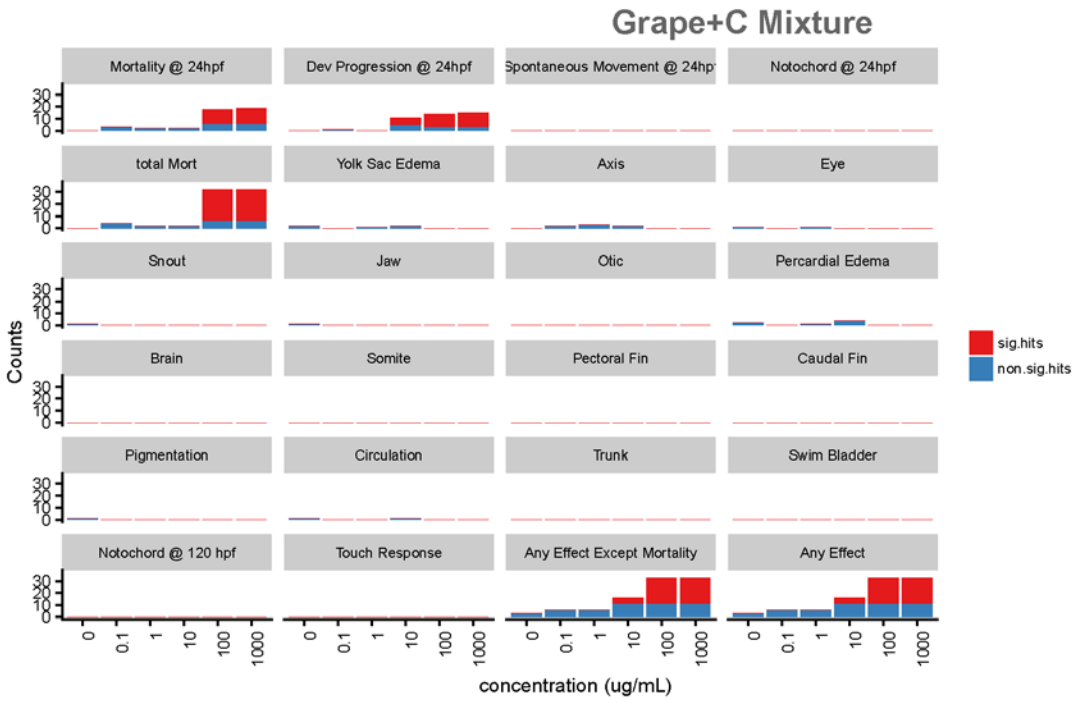


Figure A1. Barplots showing the results of the 24 and 120 hpf morphology screens for nine e-cigarette mixtures, their 10 most common chemical constituents, and the three mixtures (EA+EB, EA+EB+C, and Grape+C) generated in this study. For all graphs, the y-axis represents the number of counts for a particular endpoint in a given mixture or chemical. For e-cigarette mixtures, animals were exposed to 1:100, 1:1000, 1:10000, 1:100000, and 1:1000000 serial dilutions of the given flavor in a 1:1000 propylene glycol:embryo medium (PG:EM) background. On the x-axis, 0 refers to an EM control, while 1 refers to the 1:1000000 dilution and 5 refers to the 1:100 dilution (highest concentration). PG, VG or PG/VG mix (50:50% by volume) exposures were also carried out as serial dilutions, from 1:100 to 1:1000000. However, on the x-axis, 0 corresponds to an EM control, while 1 refers to the 1:100 (greatest) concentration and 5 refers to the 1:1000000 (lowest) dilution tested. No morphological significance was detected for any endpoint at any concentration for the PG, VG, and PG/VG Mix exposures. Animals were exposed to the chemical constituents and our generated mixtures in units of µg/mL, while the nicotine standard exposure was in units of µM. Statistical significance was calculated using a Fisher's exact test across the proportion of affected and unaffected animals for a given chemical or mixture. For concentrations where the proportion of affected animals is significant vs. the proportion of unaffected animals, this is indicated by the presence of red on the bar plot. Compounds and concentrations that were not significant are indicated by the blue bar only. Overall, these data help illustrate the strong developmental effects of vanilla and candy e-cigarette flavor profiles and their corresponding constituents.

Table A1 Embryonic Photomotor Response (EPR) Significance Table

Direction of significant changes in EPR behavior, for mixtures and chemicals with significant behavior effects in at least one phase. + indicates hyperactive, - indicates hypoactive compared to the controls, NA denotes phases that were not significantly different from controls in all tested concentrations.

Compound Name	Background	Excitatory	Refractory
555 E-Cigarette Flavor	NA	NA	NA
Banana Crème Pie E-Cigarette Flavor	NA	NA	NA
Bubble Gum E-Cigarette Flavor	NA	NA	NA
Coffee E-Cigarette Flavor	NA	+	NA
Cotton Candy E-Cigarette Flavor	NA	NA	NA
French Vanilla E-Cigarette Flavor	NA	NA	NA
Grape E-Cigarette Flavor	NA	NA	NA
Nicotine E-Cigarette Flavor	NA	NA	NA
Unflavored E-Cigarette Flavor	NA	NA	NA
Benzyl Alcohol	NA	NA	NA
Cinnamaldehyde	+	+	NA
Ethyl Acetate	NA	NA	NA
Ethyl Butyrate	NA	NA	NA
Ethyl Vanillin	NA	NA	NA
Maltol	NA	NA	NA
Vanillin	NA	NA	NA
Propylene Glycol	NA	NA	NA
Glycerin	NA	NA	NA
Propylene Glycol/Glycerin Mix (50:50% by volume)	NA	NA	NA
Nicotine Standard	NA	NA	NA
Ethyl Acetate + Ethyl Butyrate Mixture	NA	NA	NA
Ethyl Acetate + Ethyl Butyrate + Cinnamaldehyde Mixture	NA	NA	NA
Grape Flavor + Cinnamaldehyde Mixture	NA	NA	NA

Table A2 Larval Photomotor Response (LPR) Significance Table

Direction of significant changes in LPR behavior, for mixtures and chemicals with significant behavior effects in at least one phase. + indicates hyperactive, - indicates hypoactive compared to the controls, NA denotes phases that were not significantly different from controls in all tested concentrations. “All” phase a comparison activity across the entire assay.

Compound Name	All	Dark	Light
555 E-Cigarette Flavor	+	+	+
Banana Crème Pie E- Cigarette Flavor	NA	NA	NA
Bubble Gum E-Cigarette Flavor	+	+	+
Coffee E-Cigarette Flavor	+	+	+
Cotton Candy E-Cigarette Flavor	+	+	+
French Vanilla E-Cigarette Flavor	+	+	+
Grape E-Cigarette Flavor	+	+	+
Nicotine E-Cigarette Flavor	-	-	-
Unflavored E-Cigarette Flavor	+	+	+
Benzyl Alcohol	+	+	+
Cinnamaldehyde	+	+	+
Ethyl Acetate	+	+	+
Ethyl Butyrate	+	+	+
Ethyl Vanillin	NA	NA	+
Maltol	NA	NA	NA
Vanillin	+	+	+
Propylene Glycol	NA	NA	NA
Glycerin	NA	NA	NA
Propylene Glycol/Glycerin Mix (50:50% by volume)	NA	NA	NA
Nicotine Standard	-	-	-
Ethyl Acetate + Ethyl Butyrate Mixture	+	+	+
Ethyl Acetate + Ethyl Butyrate + Cinnamaldehyde Mixture	+	+	+
Grape Flavor + Cinnamaldehyde Mixture	+	+	+