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E-liquid Exposure Causes Multiple Cardiovascular Defects in Xenopus laevis Embryos

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E-LIQUID EXPOSURE CAUSES MULTIPLE

CARDIOVASCULAR DEFECTS IN XENOPUS LAEVIS

EMBRYOS

A thesis submitted in partial fulfillment of the requirement for the degree of Master of

Science at Virginia Commonwealth University.

By

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List of Abbreviations

BMP	bone morphogenetic proteins
CT3	Troponin T-C
dkk1	dickkopf WNT signaling pathway inhibitor 1
ef-1α	eukaryotic translation elongation factor 1 alpha
FGF2	fibroblast growth factor 2
flk-1	kinase insert domain receptor
Flt1	Fms Related Receptor Tyrosine Kinase 1
gata-1	GATA binding protein 1
gata-5	GATA binding protein 5
MBS	Modified Barth's Saline
myocd	myocardin
Nkx2	NK2 homeobox 1
Nkx5	H6 family homeobox 3
O-dia	o-Dianisidine
PBT	Phosphate buffered saline + Tween
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PFA	4% paraformaldehyde
Runx1	runt-related transcription factor 1
TGF-β	transforming growth factor beta
tnni3	troponin I3, cardiac type

VEGF   Vascular endothelial growth factor

Xnr1   Xenopus nodal related-1

ABSTRACT

E-LIQUID EXPOSURE CAUSES MULTIPLE CARDIOVASCULAR DEFECTS

IN XENOPUS LAEVIS EMBRYOS

By James E. Black III, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

Virginia Commonwealth University, 2020

Major Advisor: Amanda Dickinson, Ph.D. Associate Professor, Department of Biology

Over the past several years vaping has become part of the cultural zeitgeist in the United States, with ECIG usage amongst those of reproductive age rising more and more. Due to a lack of regulation and research concerning their long term effects, public opinion trends toward the idea that they are somehow safer than their traditional counterparts. As cases of lung injury and ECIG related death are beginning to be reported more and more, it is clear that the misinformation about their safety is unfounded. Congenital heart defects (CHDs) are some of the most common birth defects currently in the United States. Increased risk of these conditions is known to be associated with traditional cigarette use, but information concerning the relationship between these conditions and electronic cigarette use is not readily available, highlighting an area in need of study. *Xenopus laevis* is an ideal model to evaluate the effects of ECIG exposure on cardiac, blood, and vasculature

development due to the familiarity with the *X. laevis* genome, their transparent nature, and their ex utero development all of which make alterations to development easy to evaluate throughout multiple stages.

In this study, I show that e-liquid exposure during development reduces ventricle size and heart rate as well as altering the expression of several cardiac, blood, and vasculature related genes in *X. laevis* embryos. Additionally, I suggest that these effects are not simply due to nicotine or frequent e-liquid additives propylene glycol and vegetable glycerin, but some other chemicals used in e-liquid.

1. Introduction

The most common types of birth defects, affecting roughly 40,000 births each year in the United States, are a group of cardiovascular disorders referred to as congenital heart defects (CHDs) [22]. Roughly 2 million Americans between infancy and adulthood are currently living with CHDs. They remain the leading causes of perinatal mortality, with a current incidence rate of roughly 8% of live births globally. CHDs put a major financial and emotional strain on individuals living with them and their families, as they are synonymous with high out-of-pocket medical costs and a decrease in mental health [21]. One factor lending itself to high numbers of these congenital conditions is traditional cigarette use during pregnancy. Maternal prenatal cigarette use has a teratogenic effect on cardiovascular development, with children having a 10% relative increase in the risk of being born with a CHD, including septal and atrioventricular defects [23-24]. Over the last 12 years, a new method of smoking has continued to gain popularity in the United States as electronic cigarette (ECIG) use climbed over 78% from 2017 to 2018. This accompanied a decrease in traditional cigarette use, attributed to the way ECIGs are marketed as well as the misinformation about their safety [6]. ECIG use is particularly dangerous as there is little regulation and a major gap in information concerning their long term effects. ECIGs pose a unique threat as they tout a long list of additives in addition to the nicotine found in traditional cigarettes. Due to the relationship between traditional cigarettes and the increased risk of CHDs, it is important to examine the relationship between ECIGs and these same heart conditions. It follows logically that there is a high potential for ECIGs to result in

similar heart defects as their traditional counterparts, made even more urgent because of the misconception that they are safer.

1.1 Electronic cigarettes and their use

ECIGs are electronic devices that use an atomizer to aerosolize liquid products, often loaded as cartridges. These liquids contain nicotine, flavoring additives, and a humectant used to maintain moisture in the mixture [14]. Even though electronic cigarettes and vaping are still relatively new, the number of users is rapidly rising as it becomes normalized behavior. These devices are being hailed as a healthier, more socially acceptable method of smoking, and can be seen everywhere from designated outdoor smoking areas to school hallways, and restaurants. This prevalence is not entirely unexpected considering how these devices are marketed, and to whom they are marketed to. With a myriad of flavors made available ranging from tobacco and menthol to children's cereals and fruity cocktails, these substances seem to specifically be targeting adolescents, including women of reproductive age. This is a source of concern as studies show that roughly 15% of pregnant women are currently using ECIGs, with 43-88% of those women considered dual users –using both electronic and traditional cigarettes. Almost all women who used ECIGs reported doing so because of a sense of perceived harm reduction and as an aid in cessation from traditional cigarettes. In actuality, ECIG use tends to renormalize traditional cigarettes and perpetuate nicotine addiction [3].

While some of the chemicals that are in the e-liquids may be non-hazardous alone when aerosolized they become oxidized. This alters them in such a way that they become harmful, often converting into known carcinogens. ECIG vapor exposure has been shown to be cytotoxic to human stem cells, a fact that only further negates the wealth of public misinformation [11].

1.2 Emerging dangers of vaping

Prior to 2016, there was no FDA involvement in e-cigarette regulation. For this reason, there was no requirement for warning labels on e-liquid packaging detailing the dangers of nicotine during pregnancy [25]. This, coupled with the fact that ECIGs have been marketed as being less harmful than traditional cigarettes, has painted a very misleading picture [26]. As of February 2020, there have been 68 deaths due to vaping-related illnesses. In addition, 2,807 cases of ECIG related lung injury have been documented in the United States. These numbers continue to rise as more states are reporting [47]. In addition, there is mounting evidence from research studies in cell lines and animal models that vaping is not safe. For example, vaping or ECIG exposure has been shown to cause lung damage, increased angiogenesis, decreased heart rate and increased risk of cancer risk in rats and mice [15]. Deleterious effects on mucous membrane function and alteration of gene expression in numerous organs indicating increased cancer risk have also been observed [26]. From these studies, it is still not clear what components of e-liquids could be causing these problems. One possibility is the flavoring chemicals. For example, we know that the additive, diacetyl, causes bronchitis obliterans, commonly known as popcorn lung [1]. This chemical gives the vapor a "buttery" flavor when heated. This condition causes the bronchioles of the lungs to become irritated and can decrease their diameter causing complications and making it harder to breathe. This is the same flavoring that was once used in microwavable popcorn manufacturing, with many companies moving away from it after their employees presented large case numbers of bronchitis obliterans. Despite this, it continues to be used in some e-cigarette flavoring [2].

1.3 Dangers during development

Several studies suggest that ECIGs could have detrimental effects both to the user as well as their unborn children. Embryonic cells may be even more sensitive to ECIG solutions than adult cells. For example, some ECIG liquids were shown to be cytotoxic to human embryonic stem cells while having no effect on adult pulmonary cells [43]. ECIG exposure caused low fetal weight in rats and mice, an indicator of other complications and perinatal morbidity [17].

Electronic cigarette exposure has also been observed to have negative effects on neural development. Rats born to mothers exposed to aerosolized e-liquids had significant changes in the expression of genes in the frontal cortex. These observed alterations in the central nervous system transcriptome are associated with negative neurobiological outcomes in humans, including a decrease in cognition and memory function [19].

Developmental concerns of ECIG exposure also extend to the cardiovascular system. Some aerosolized e-liquid vapor solutions were cytotoxic to fetal rat cardiomyoblast cell lines [16], and components of aerosolized e-liquids have been shown to negatively affect cardiovascular development in Zebrafish as well. *Danio rerio* exposed to aerosolized e-liquid solution presented high frequencies of heart abnormalities not observed in embryos exposed to nicotine alone [14, 18]. Embryos exposed to particulates showed severe anatomical and physiological abnormalities including mortality, delayed development, decreased oxygen consumption, decreased heart rate and hemorrhaging. Altered angiogenesis was also observed, with some blood vessels being completely absent [18]. One of the main components of ECIG solutions called propylene glycol produced detrimental effects in Zebrafish when embryos were exposed in vivo, including reduced growth, increased fluid in the pericardial sac, and

hyperactivity in larvae. When exposed to aerosolized propylene glycol, Zebrafish embryos had a "heart-string" phenotype in which the heart failed to loop properly [31].

Previous studies have shown that e-liquid exposure resulted in a reduction of blood cells in the orofacial region of 100% of developing *X. laevis* embryos when compared to an unexposed control [1].

1.4 Transcriptome analysis of faces exposed to e-liquids

An aerosolized mixture containing only nicotine, propylene glycol, and vegetable glycerin was shown to have a dose-dependent, statistically significant effect on craniofacial development in *X. laevis*, especially the shape of the orofacial region [1]. The inclusion of flavor additives to these basic aerosolized mixtures can have varying degrees of severity on craniofacial development, some of which can result in cleft palate. To help the lab determine how e-liquids could be affecting gene expression in the face, the transcriptomic changes were evaluated in microdissected faces using next-gen sequencing (RNAseq) (manuscript in prep). The top genes in the facial tissue that were decreased were all related to blood. One such gene, *gata1*, codes for an important transcription factor necessary for the normal production of blood cells; specifically erythrocytes and platelets through the process of hematopoiesis. *gata1* knockout in mice leads to immature progenitor cells that are unable to properly differentiate and mature [4-5]. Mutations within the *gata1* gene are associated with CHDs [27]. The results of this RNA-seq analysis have led to an investigation of the effect that e-liquid exposure has on blood supply in *Xenopus laevis,* through the processes of both erythropoiesis and angiogenesis, as well as the morphological development of the heart.

1.5 Benefits of Xenopus laevis as the developmental model

The use of *Xenopus laevis* as the model in which this study will be conducted is innovative since it allows a variety of experiments to be done quickly and efficiently, ascribable to their rate of development. The current frog populations in the lab, coupled with the fact that these frogs can give up to several thousand eggs, 3-4 times a year translates to an almost "on-demand" supply of embryos so that experiments can be repeated frequently and at very large sample sizes.

The fact that they are ex utero enables me to observe and record important developmental stages in real-time, with very clear visual detail. As *X. laevis* develop, their skin is translucent enough to discern developing organs, not limited to the heart. This makes visualizing blood using both a light microscope as well as GFP-microscopy in transgenic lines easy.

One common criticism of the use of these organisms relates to the fact that they are ex utero, unlike humans. This allows for a wide variety of experiments to be performed and real-time observations to be made that would otherwise be impossible, or require more complex methodology. Another frequent misconception about the use of *X. laevis* is that observations in developmental dysfunction due to teratogens in these organisms are somehow different than those in humans, but this is not the case. There are numerous United States Government-funded studies specifically using *X. laevis* embryos as an indicator for possible toxic effects of pharmaceuticals or environmental teratogens on mammals. Several side-by-side studies have shown that certain teratogens have the same effects in both *X. laevis* and mice, strengthening our conviction that the data generated in this study will have the potential to be very relevant to public health [38-39]. Furthermore, as I am interested in prenatal effects of e-liquids wherein the

human embryo would be exposed via the placenta, *X. laevis* is once again an effective model as their embryonic dermal layer shares similar surface ion channels and transporters with the human placenta, helping to predict how e-liquid products would travel across these barriers [9, 10]. Finally, *X. laevis*, has been used in the past to model respiratory epithelium, as their skin contains cells capable of secreting mucus that act as a form of protection to the embryo. This is similar to how the ciliated epithelial cells of the human lungs act as a barrier to allow for the exchange of nutrients while protecting the body from pathogens. The similarity in structure and function between these two tissue types could give me stronger predictive capabilities about the behavior of ECIG products across the human lung. The ability to fertilize embryos in vitro allows me to generate an abundant amount of synchronized data between sibling embryos, granting a view of the differences in development between control and exposed embryos congruently. *X. laevis* are useful models for cardiac development because of their similarity in cardiogenesis in humans and might be even more useful that other model organisms for evaluating potential CHDs [27].

1.6 Heart development in X. laevis

X. laevis has a three-chambered heart with two atria and a single ventricle. This single ventricle resembles the mammalian left ventricle and is responsible for pumping oxygenated blood into the systemic circulation. Although the amphibian heart is structurally different from mammalians, the basic developmental mechanisms that underlie the formation of this structure are similar. The development of the heart starts at Stage 10, between 9 and 11 hours post-fertilization (hpf), during gastrulation. By stage 12 (~13 hpf) cardiac progenitor cells within the lateral plate mesoderm, located on both the left and right sides of the embryo, will begin to move toward the ventral midline at the anterior of the embryo. Once these cells meet at the midline by

stage 13 (~15 hpf) they fuse and soon after separate to give rise to the two heart fields. The first heart field forms the two atria and the ventricle of the heart. The second heart field gives rise to the outflow tract that is responsible for pumping blood into the systemic circulation. Between Stages 31 and 33 (~37-34 hrs pf) these cardiac progenitor cells join to form the primary heart tube [27].

For these cardiac progenitor cells to become functional cardiomyocytes, they must undergo the process of differentiation. This begins when endoderm tissue starts secreting bone morphogenetic proteins (BMP). BMP receptors activate Smad proteins initiating the transcription of several heart-specific transcription factors such as GATA5, Nkx2, and Nkx5. These transcription factors then regulate the expression of cardiac-specific genes that enable cardiac cells to contract. Myocd is one such transcription factor that specifically regulates the differentiation of cardiac and smooth muscle, and cardiac gene expression [53]. Additionally, heart development relies on the inhibition of certain signaling pathways. For example, Dkk1 inhibits Wnt/β-catenin signaling during specific early heart development stages. This inhibition ensures that proper differentiation takes place, as well as the controlled formation of important anatomical structures such as the outflow tract [52].

At stage 33 the primary heart tube is a linear structure that begins extending posteriorly. The ventricular portion of the heart tube lies anteriorly to what will become the atria at this point in *X. laevis* development. Cardiac muscle contraction takes place at stage 35 (~44-50 hpf) [27]. This contraction encourages the linear structure to loop, forming a spiral structure with the presumptive atrium moving dorsally to the ventricular portion [45]. This movement continues until the atrial region is located medially while the ventricular portion moves to the right and is displaced laterally eventually descending, residing in the pericardial cavity. This looping

continues until stage 40 when the atrial region of the heart tube comes to rest directly dorsal to the ventricular region. This process is regulated by the Xnr1 gene that controls the expression of BMP, which is expressed during the looping process solely on the left side of the linear heart tube. This gives the entire process conserved directionality and ensures proper looping to maintain function and form [46]. Finally, between stages 40-46 (~66-106 hrs pf) the looped heart tube begins to form individual chambers and the atria are finally separated into two independent structures [27] **(Table 1 A.)**

1.7 Vasculature development in X. laevis

Vasculogenesis begins with the differentiation of ventral and lateral plate mesoderm into hemangioblasts regulated by FGF signals. This occurs when FGF2 binds its receptor causing mesodermal cells to begin transcribing VEGFR2/Flk1 receptors during neurulation around stage 15. When VEGF in the cell environment binds these receptors, it promotes the formation of hemangioblasts, which become subdivided into the primitive blood islands. These blood islands go on to form the blood as well as endothelial cells, which will become the vasculature. During stages 27-32 of *X. laevis* development (31 - 40 hpf), VEGF binds its receptor (Flk1) on presumptive endothelial cells and this signaling pathway regulates their migration and differentiation into strands of cells. Next, VEGF stimulates the hemangioblasts to begin transcribing Flt1 receptors which in turn will bind VEGF leading to the promotion of tube formation. Tubes are ultimately formed from these strands of endothelial cells by the coalescence of many vacuoles into a single lumen. These newly formed tubes assemble into a primitive network or plexus and by stage 35 in *X. laevis*, circulation begins.

Next, this primary network is remodeled through angiogenesis where vessels use "filopodia" on Tip cells to reach out and connect with one another, originating and extending from previously established vessels [61]. This process is regulated by PDGF and TGF-β signaling, ensuring that the Tip cells can properly navigate their environment and ensure that vessels fuse with their complement. This is also regulated by Delta notch signaling, with higher Notch stimulation apparent in arteries compared to lower levels in veins.

By stage 46 much of the remodeling is complete and the principal blood vessels are formed [64-65] **(Table 1 C).**

1.8 Blood development in X. laevis

The early embryonic blood ultimately forms from the blood islands arising from the ventral mesoderm, before transitioning to the dorsal lateral plate (DLP) and dorsal aorta as development continues. Unlike the blood islands, the DLP contributes solely to the adult hematopoietic lineages [68].

Precursors to both immature and adult blood cells can first be seen at stage 6, at the 32-cell embryo stage. Cells destined to become the ventral blood island (VBI) begin collecting together and migrating ventrally during gastrulation between stages 10-14 (9 - 16 hpf). After being exposed to BMP the blood island precursors begin to express hematopoietic genes causing these tissues to begin differentiating into endothelial cells as well as the stem cells that will eventually give rise to blood cells between stages 14-18 (16 - 20 hpf) [69]. These hematopoietic stem cells will continue to differentiate in response to a great number of erythroid transcription factors like Runx1, which works to convert endothelial cells into blood stem cells. Runx1 expression is regulated by an intricate network of erythroid transcription factors such as SCL,

that works to ensure primitive blood cells are not converted to cardiac muscle, as well as GATA2 and Fli1. Runx1 is also important in the formation of megakaryocytes that will eventually mature to become platelets through its coexpression and interaction with GATA1 [66-68]. GATA1 is a transcription factor that is necessary for the normal production and maintenance of both RBCs and platelets. GATA1 expression, like GATA2 and SCL, is first triggered by BMP signaling. GATA1 does not work alone, as it must first complex with its cofactor FOG1 to function properly. Knockdown experiments of either GATA1 or FOG1 were shown to have lethal effects due to the lack of formation of megakaryocytes and platelets [66]. GATA1 also works to ensure the survival of red blood cells by regulating the expression of genes that alter the cell cycle to avoid apoptosis. One example is the stimulation of Bcl-Xl, an antiapoptotic protein [67].

The VBI finishes forming by stage 30 and is the primary site of hematopoiesis between stages 26-40. By stage 35 though the blood is still considered to be "primitive" circulation begins. Meanwhile, cells from the DLP have begun to migrate toward the midline, and after expressing the Flk1 receptor will form the dorsal aorta (DA) by stage 38 (~53 hpf) [70]. After stage 40 the VBI no longer exists, and the hematopoietic clusters within the DA become the site of blood development and the establishment of the adult lineages [69].

Blood cells mature over a period of 26 days, and by stage 52, adult RBCs begin to appear in circulation. Immature erythrocytes continue to be replaced by mature erythrocytes throughout metamorphosis. At stage 60, half of the primitive blood cells will have been replaced by mature RBCs, but the conversion process will continue well past metamorphosis. Sixth months after *X. laevis* adult's complete metamorphosis, the primitive erythrocytes have all been replaced by mature, functioning RBCs as the source for production changes [44]. As *X. laevis*

mature through metamorphosis the liver becomes the primary location for hematopoiesis, and then through adulthood that site becomes bone marrow [29-30] **(Table 1 B).**

Based on preliminary evidence in *X. laevis* and zebrafish, I propose that e-liquid exposure could affect cardiovascular development. Here in this thesis, I show that exposure to a popular flavored e-liquid causes abnormality in heart morphology and function, as well as blood distribution, and vascular arrangements. The effects on heart development may not be due merely to nicotine, or inert carriers since these alone had little effect on heart development. Further, both neat and aerosolized e-liquid preparations can result in similar effects on heart size suggesting that heating the e-liquids may not alter them to create any dangerous chemicals. Finally, e-liquid exposure can alter the expression of some cardiovascular regulatory genes. Thus, I have begun to uncover mechanisms to explain why these defects occur.

2. Methods and Materials

2.1 Animals

Using protocols described in Sive's laboratory manual, an adult female frog was injected with human chorionic gonadotropin (HCG) to induce egg laying. Eggs were then collected, fertilized and cultured using standard protocols [83]. Tadpoles were staged according to Niewekoop and Faber [84]. All experiments were performed following the approved IACUC protocol AD20261.

2.2 E-liquid exposures in Xenopus laevis embryos

Beginning at stage 4, embryos were cleaned and sorted to remove any unfertilized eggs as well as any embryos failing to divide. When embryos reached staged 20-24, they were pipetted using a large-bore plastic transfer pipette into sterile Petri dishes or culture plates containing 0.1X Modified Barth's Saline (MBS). Experimental solutions were added to the solution and the mixtures containing embryos were gently swirled. Chemical exposures consisted of neat Revel (1:1000, Cloud Company), aerosolized Revel (1:100, [1]), nicotine (10mg/ml), and aerosolized nicotine +PG+VG (1:100, [1]). Embryos were then incubated at 23°C. In some experiments, embryos were removed from exposure at stage 30 and processed for PCR (see section 2.8). Alternatively, embryos were exposed until stage 40. After this, they were removed and placed into 0.1xMBS until stages 45 - 46. At this time *X. laevis* embryos were processed for heart morphology (section 2.6 - 2.7), heart rate (section 2.5), blood and vascular assessments as described below (section 2.3 - 2.4) **(Fig. 1.)**

2.3 Histological blood label (o-Dianisidine staining)

The staining solution consisted of 120mL dH2O, 80mL EtOH (DeconLabs: V1016), 1.3mL 30% Hydrogen Peroxide (MilliporeSigma: HX0635-3), 0.12g Sodium Acetate (Sigma Aldrich: S2889-250G), and 0.16 o-Dianisidine (Sigma Aldrich: D9143), referred to as "O-dianisidine solution." The solution works as a marker for blood development as the hemoglobin present in fully mature red blood cells catalyzes the Hydrogen Peroxide to begin oxidizing o-Dianisidine to produce a dark red stain in any hemoglobin positive cells. After the standardized Revel exposure **(Fig 1)** embryos were fixed for 20 minutes in 4% paraformaldehyde (PFA). They were then placed in 1.5mL Eppendorf tubes and washed in phosphate-buffered saline solution with Tween (PBT) 3 times for five minutes each. All of the PBT was removed and replaced by 1.5mL of the O-Dianisidine solution to ensure the embryos were covered. The solution needs to be mixed at room temperature for at least 20 minutes before adding. The effect of the solution warming in the Eppendorf tube causes them to burst, leading to possible damage to the samples and inconsistent staining. The tubes were wrapped in foil and left mixing for 40 minutes. Embryos were washed in PBT twice for two minutes each, and then all PBT should be removed. The embryos were incubated in 4% PFA for 24 hours at 4°C while still covered in foil. Embryos were washed again in PBT, 3 times for 5 minutes each, and then 70% glycerol (Biotech: GB0232) was added and embryos were imaged on white clay (Zeiss Discovery V8 Axiocam 503 Color using Zen software.)

2.4 Transgenic X. laevis experiments

Transgenic *X. laevis* used to visualize vascular (XB-LINE-916, Flk1-GFP, FLK-1; Xla.Tg(flk1:GFP)1Mead) and blood (XB-LINE-1216, Xla.Tg(gata1:GFP)[Mead]) development

were ordered from Marine Biological Laboratory. The anterior of the embryos were imaged using Zen software from a ventral and lateral view using 1% agarose as a base (Excelitas Technologies: X-Cite Series 120 Q, Zeiss: Discovery V8 SteREO, Carl Zeiss Microimaging GmbH.)

2.5 Heart rate

Light microscopy was used to image and record heart rates of both Revel exposed and control embryos (Zeiss Discovery V8 Axiocam 503 Color using Zen software.) First, the standardized Revel exposure protocol was used to treat embryos **(Fig 1.)** The embryos were washed in 0.1xMBS and allowed to progress to stage 45. 500 µM Tricaine (Western Chemical: 051819) in 0.1xMBS was set out for twenty minutes and allowed to reach room temperature. Working in groups of five (five exposed and five control) embryos were then to be placed into the 0.1xMBS Tricaine solution for twenty minutes to acclimate to the dish temperature. Heart rates, measured in beats per minute, were assessed by counting the number of beats for 30 seconds and then multiplying by two. The mean heart rate for each of the experimental conditions is assessed. To control for any possible differences in stage, time, or temperature the experimental mean was divided by the control mean to establish a relative change in heart rate.

2.6 Light microscopy

After experimental embryos are exposed to the Revel solution using the standardized protocol **(Fig 1),** the embryos were washed in 0.1xMBS and allowed to mature to stage 45. At stage 45 embryos were fixed in 4% PFA and imaged using a stereoscope (Zeiss Discovery V8 Axiocam 503 Color using Zen software.)

2.7 Anti-Troponin T-C (CT3) staining

After experimental embryos are exposed to the Revel solution using the standardized

protocol **(Fig 1),** embryos were washed in 0.1xMBS and allowed to reach stage 45, at which

point they were fixed in 4% PFA overnight at 4°C. Embryos were then placed into 1.5mL

Eppendorf tubes, limiting each tube to 10 embryos. The fixative was removed and replaced with

PBT. Embryos were left in fix for 24 hours, no more and no less, as any variance, limits the

efficacy of the stain or causes them to deteriorate respectively. The embryos were then washed in

PBT three times for one hour. During the final wash, a primary antibody solution consisting of

20µl of CT3 antibody (Developmental Studies Hybridoma Bank: AB_528495) in 500µl of PBT

was prepared for each tube containing embryos. After the final wash, all of the PBT was

removed from the tubes and then 0.5ml of the primary antibody solution was added. The

embryos were left in the primary antibody solution at 4°C for seven days. After seven days, the

primary antibody solution was removed and replaced with PBT. Three washes of one hour were

performed. During the final wash, a 1:500 secondary antibody solution was prepared by adding

2µl of green fluorescent secondary antibody Alexa 488 goat anti-mouse (ThermoFisher: A-

11001) in 1mL of PBT. After the final wash, all of the PBT was removed and replaced with

0.5ml of the secondary antibody solution. At this point, the reaction is highly light-sensitive, so

tubes were promptly covered with foil. The embryos were kept in the secondary antibody

solution at 4°C for six days. Next, the secondary antibody solution was removed, and the

embryos were washed three times in PBT for at least one hour each. The embryos were

equilibrated in series in 50% glycerol until the embryos sank to the bottom of the Eppendorf

tube. Next, the 50% glycerol was removed, and 75% glycerol was added. Once again time was

taken to allow the embryos to sink. Once the embryos sank, they were imaged in 75% glycerol

using 1% agarose as a base (Excelitas Technologies: X-Cite Series 120 Q, Zeiss: Discovery V8

SteREO, Carl Zeiss Microimaging GmbH.)

2.8 RT-PCR experiments

At stage 20-24, *X. laevis* embryos were randomly split into two groups and placed into

25mL of 0.1xMBS. The experimental groups were exposed by adding 25µl of neat Revel

solution and then incubated at 23°C until stage 30 for early heart markers and early *gata1*

experiments, or stage 40 for later stage *gata1* experiments. They were then washed in 0.1xMBS

and placed into 800µl of Trizol (Ambion Fisher: 15596026) that was resting in ice. The embryos

were incubated for 10 minutes in the lab at room temperature (24°C) and then stored in a -80°C

freezer overnight for roughly 24 hours. The next day the tissue was thawed on ice and then

"homogenized" using both pestles and a Fisherbrand™ Mini Vortex Mixer (Fisher Scientific:

14-955-151) to begin the RNA extraction process. Next, the resulting mixture was centrifuged at

max speed for 10 minutes, and roughly 800µl of the supernatant was placed into a new 1.5mL

Eppendorf tube, while the remaining insoluble material was discarded. Using proper PPE

(goggles, gloves, and a lab coat), 200µl of Chloroform (Fisher Scientific: C606SK-1) was added

in the fume hood to the tube, vortexed for 15 seconds, and then left to incubate at room

temperature for 3 minutes. Tubes were then placed into a Sorvall™ Legend™ Micro 21R

Microcentrifuge (Thermo Fisher Scientific: 75002447) and centrifuged for 30 minutes at 12000g

speed and 4°C. Next tubes were removed carefully and 200µl of the aqueous phase containing

RNA was removed and placed into a new tube, while the rest was discarded. 500µl of isopropyl

alcohol was added to the solution, incubated for 10 minutes, and then centrifuged again for 20

minutes at 12000g speed and 4°C. The resulting supernatant was then discarded, 1 ml of 75% Ethanol was added, and the mixture was centrifuged at 7500g speed and 4°C. Again, the supernatant was discarded, and the remaining material was centrifuged for 10 seconds. Any additional liquid was removed, and the resulting pellet was left to dry. 50µl of ultrapure distilled water was added to the pellet, followed by 25µl of a LiCl Precipitation Solution (Thermo Fisher Scientific: AM9480) and then left to incubate overnight at -20°C. The next morning samples were defrosted on ice and then centrifuged at max speed for 30 minutes at 4°C. The supernatant was discarded and then 1ml of 70% ethanol was added before centrifuging again for 30 minutes at max speed and 4°C. The supernatant was discarded, and the tube was respun for 10 seconds. The resulting pellet was set to dry for 5 minutes before being resuspended in 30µl of Nuclease-Free Water (Thermo Fisher: AM9938). Next, resulting RNA was tested for concentration and purity before being used in RT-PCR.

In order to quantify expression levels of early cardiac genes and *gata1* with *ef1-α* as a housekeeping gene (BioMolecular Systems micPCR, using micPCR v2.6.5 software) a Luna® Universal One-Step RT-qPCR Kit (New England BioLabs: E3005S) was used in which the generated RNA can be used directly for RT-qPCR experiments. Materials were thawed at room temperature and then placed on ice. Master mixes were created using 10µl of Luna Universal One-Step Reaction Mix (2X), 1µl of Luna WarmStart® RT Enzyme Mix (20X), 0.8µl of 10µM forward primer and 0.8µl of 10µM reverse primer, and 6.4 µl of Nuclease-Free water. After 19µl of this master mix was placed into the appropriate qPCR tube 1µl of 10ng/µl Template RNA was added. Samples were placed into the micPCR machine and the following protocol was used. A single reverse transcriptase phase of 10 minutes at 55°C, a single initial denaturation cycle at 95°C, followed by 45 cycles of a Denaturation stage of 10 seconds at 95°C, an extension phase

of 30 seconds at 60°C, followed by a single melt curve cycle between 60°C and 95°C. Results were gathered using the micPCR v2.6.5 software. Primers used were as follows: *myocd*, 5′-GCC CAA AGC AAA TTA CAA GAA-3′ (forward) and 5′-GGA AGT CGG TGT TGA AGA TAC-3' (reverse) [53]; *dkk1*, 5′-TCC CAG AAG AAC CAC ACT GAC-3′ (forward) and 5′-GGT GCA CAC CTG ACC TTC TT-3′ (reverse); *tnni3* 5′-CTG CCG ACG CCA TGA TG-3′ (forward) and 5′-GTT TGA GAC TGG CCC GTA GGT-3' (reverse) [52].

Stage 40 *gata1* PCR experimental embryos were exposed to Revel solution according to the standard exposure protocol **(Fig 1),** removed at stage 40**,** and were then placed into 0.1xMBS and a few drops of Tricaine (Western Chemical: 051819) was added. 30 heads from both the control and exposed groups were dissected and collected in 800µl of Trizol (Ambion Fisher: 15596026), taking care to avoid including the heart and anything posterior to it. RNA was generated using the same protocol described above. cDNA was then synthesized using a Sensifast™ cDNA Synthesis Kit (Bioline Meridian Bioscience: Bio-65054). A master mix was made for control and Revel treated RNA separately consisting of 4µl of a SensiFast 5x TransAmp Buffer, 1µl of Reverse Transcriptase, 1µg of RNA, and enough Nuclease-Free water to bring the total volume of each tube to 20µl. Tubes were placed into a thermocycler set to the following protocol: an annealing step at 25 °C for 10 minutes, a reverse transcription step at 42 °C for 15 minutes, an inactivation step at 85 °C for 5 minutes, and finally a hold step at 4 °C. The resulting cDNA was then used in RT-PCR. RT-PCR was performed using a SensiFAST™ SYBR® No-ROX Kit (Bioline Meridian Bioscience: BIO-98005) to quantify expression levels of *gata1* using *ef1-α* as a housekeeping gene (BioMolecular Systems micPCR, using micPCR v2.6.5 software.) Primers were suspended in Nuclease-Free water from 100µM stock solutions to 10µM aliquots of 25µl. Master mixes were made using 10µl of 2x SensiFAST™ SYBR® No-

ROX Mix, 1µl of the template cDNA, and 8µl of dH_2O. Primer mixes were made using 0.5µl of

10µM forward primer and 0.5µl of 10µM reverse primer. Once these were created, 19µl of the

Master mix was added to a qPCR tube, followed by 1µl of the appropriate primer mix. Results

were gathered using the micPCR v2.6.5 software.

3. Results

3.1 Revel exposure causes alterations in heart morphology

To assess heart morphology the heart was imaged at stage 45 when the looping process is complete and septation has occurred in the atria, resulting in the three individual chambers of the heart being formed. Basic light microscopy, as well as immunofluorescence using an antibody that binds to a heart-specific muscle protein, cardiac troponin (Tnni3), was used.

In Revel exposed embryos, altered heart morphology was observed using both techniques. Specifically, the ventricles were smaller, and the position of the ventricle and outflow tract appeared different than in controls. **(Fig 2 A-H.)** Outflow tracts in the Revel exposed embryos lacked the distinct curvature observed in all controls often making the hearts appear longer **(Fig 2 B, D)**

To quantify differences in heart size, measurements of ventricle diameter, from anterior to posterior in a ventral view were recorded**(Fig 2 A, C.)** A statistically significant difference was found between the embryo ventricle diameters of exposed (N=30) compared to control (N=41) embryos (student t-test, p-value = 0.009) **(Fig 2 J.)** To further compare the changes in heart size between the two groups a categorical analysis where the ventricle diameter of each embryo was equally categorized into large, medium, small, and extra small was created. Each category was then visualized on a stacked bar graph in an attempt to better see trends in the data. Results indicate that 37% of the Revel exposed embryo ventricle measurements fell into the "small" range (230.36 µm to 285.54 µm), compared to only 11% of the control. None of the control embryos fell into the "extra small" range (175.19 µm to 230.35 µm), while 16% of Revel exposed embryos did **(Fig 2 I.)** Additional differences in morphology were apparent as the Revel

exposed embryo hearts tended to appear "longer," and more random in orientation, perhaps due to the disrupted looping patterns.

Together, the results indicate that Revel exposure causes defects in the morphology of the heart, specifically in the size of the ventricle.

3.2 Revel exposure reduces heart rate

To assess the function of the heart, I used light microscopy to measure heart rates of both Revel exposed and control embryos. A statistically significant reduction in heart rate was observed in Revel exposed embryos (N=60) when compared to the unexposed controls (N=60), with a relative decrease of 14% (3 biological replicates, Mann-Whitney Rank Sum, P = <0.001) **(Fig 2 K.)** A categorized analysis and stacked bar graph revealed that of the Revel exposed embryos, 42% were shown to have a decreased heart rate in a range (110.4-131.6 bpm) that fell below any of the control specimens **(Fig 2 L.)** Additionally, ventricle contraction in Revel exposed embryos at times appeared sporadic and less uniform than the controls.

These results indicate that in addition to morphological malformations, Revel exposure also causes a reduction in heart rate.

3.3 Exposure to neat and aerosolized e-liquid solution preparations have similar effects

Next, to determine whether the process of aerosolization alters e-liquids in such a way that could affect the heart differently, sibling embryos were exposed to both aerosolized and neat Revel. Preliminary experiments in the lab were performed to test various concentrations of each preparation and it was determined that 1:100 dilution of aerosolized had almost identical effects

as a 1:1000 dilution of neat Revel [1]. This is consistent with calculations of nicotine concentrations in the aerosolized Revel preps.

Whole embryos exposed to neat and aerosolized Revel each showed similar reductions in overall length and a curved body axis when compared to controls. Also, similar levels of edema in the anterior portion of the embryo, as well as gut abnormalities, were observed in embryos exposed to both preparations, and absent in controls. In both preparations, e-liquid exposure led to a phenotype in which the eyes were located closer together when viewing dorsally. Similar lens defects in the eye were also observed in both groups, but absent in controls **(Fig 3 A-C.)**

To determine if the heart ventricle size was affected in the same way by both preparations, ventricle sizes were measured from a ventral view as mentioned above. There was an apparent reduction in the ventricle size when visualized using light microscopy **(Fig 3 D-F.)** To visualize the relationship between ventricle size between the two groups I performed a categorial analysis and created a stacked bar graph with a small sample of embryos (n=36, 1 replicate). 75% of control embryos fell into a range of 294.67μm and 346.00μm, compared to only 23% of aerosolized, and 14% of neat e-liquid exposed embryos. Roughly 70% of both the aerosolized and neat exposed embryos fell into a range of 242.33μm and 293.67μm, compared to only 25% of the control. Finally, 8% of the aerosolized Revel and 14% of the neat Revel exposed embryos fell into the smallest range of 190μm and 241.33μm, while none of the controls were found to be that small **(Fig 3 G.)**

Together, these results suggest that the effects of e-liquid exposure are similar regardless of the preparation. Most importantly, concerning the observed decreases in heart size and heart rate in this study, the effects of the teratogenic chemicals observed do not seem to be different when the e-liquid is aerosolized.

3.4 Exposure to neither neat nicotine nor a solution of aerosolized nicotine, propylene glycol, and vegetable glycerin reduced ventricle size or heart rate

To determine whether the observed changes in heart morphology and function recorded were due to additives in the Revel flavorings or due solely to nicotine and other common carrier fluids in e-liquids. To assess this, sibling embryos were exposed to a neat preparation of nicotine and an aerosolized mixture of nicotine, propylene glycol, and vegetable glycerin (referred to as nicotine+PG+VG.) Light microscopy and immunofluorescence using an antibody that binds to Troponin, a cardiac-specific muscle protein, were used to evaluate heart rate and ventricle size in these embryos compared to controls.

No significant difference was found in ventricle diameters between the embryos exposed to neat nicotine, nicotine+PG+VG, or control embryos (Kruskal-Wallis One Way Analysis of Variance on Ranks, p-value = 0.418) **(Fig 4 B.)** Ventral images of control, nicotine, and nicotine+PG+VG exposed embryos showed no observable difference in ventricle size, shape, or orientation **(Fig 4 C-E.)**

Similarly, no significant difference was observed in the heart rate between neat nicotine, nicotine+PG+VG, and control embryos (Kruskal-Wallis One Way Analysis of Variance on Ranks, p-value = 0.533) **(Fig 4 A.)**

These results indicate that neither neat nicotine nor nicotine plus the common e-liquid additives Propylene Glycol and Vegetable Glycerin had significant effects on heart morphology or heart rate.

3.5 Revel exposure alters vascular organization

To assess any alteration in developing vasculature in embryos exposed to Revel I utilized a transgenic line in which GFP is driven by the *flk1* promoter. *flk1* is expressed in the developing vasculature and therefore allows visualization of GFP in the blood vessels.

Unexposed control embryos showed a consistent and mostly symmetrical vascular network in the orofacial region across 100% of specimen. While there was some observable vasculature supplying the face in experimental embryos, what resulted was a spectrum of phenotypes. Only 20% of embryos appeared to have similar vascular networks and branching patterns when compared to controls. The remaining 80% had varying numbers of vessels present, and the patterns of vascular branching were less organized in mild phenotypes. In some more extreme phenotypes, large parts of the network seemed to be absent altogether. Edema was noted in the anterior portion of 80% of exposed embryos. On the whole, experimental embryos were shown to be of a reduced size when compared to controls **(Fig 5 I-L.)**

These results suggest that e-liquid exposure does, in fact, alter vasculature development.

3.6 Revel exposure alters the localization of blood in embryos

3.6a) O-Dianisidine staining

To assess the effect that e-liquid had on the location of blood in the orofacial region of exposed embryos, I used light microscopy and O-Dianisidine staining to detect hemoglobin [42].

100% of control embryos had vivid networks of O-Dianisidine labeling in the face, and from most ventral and dorsal views there was extensive vascular branching to other areas around the craniofacial region **(Fig 5 A.)** By contrast, 87% of Revel exposed embryos had little or no O-

Dianisidine labeling in the head accompanied by excess labeling near the heart and digestive system (n=80, 4 biological replicates) **(Fig 5 B-C.)** The remaining 13% of Revel exposed embryos that had staining were still distinct from the control group. In these embryos, a random scattering of blood cells with no real consistent pattern in location was observed **(Fig 5 D)**. These embryos showed similar signs of blood pooling around the gut or tail.

These results suggest that e-liquid exposure alters the amount of hemoglobin containing red blood cells present in the orofacial region of developing embryos.

3.6b) GATA1 Transgenic blood marker

To confirm that there was indeed a difference in blood localization in embryos exposed to Revel I next used another technique to visualize blood cells. Here I utilized a GATA1 transgenic line that expresses GFP in circulating blood cells. Individual blood cells were imaged as they moved throughout the embryo in real-time. Conserved networks of moving fluorescing cells marking the presence of Gata1 expression was observed in 100% of control embryos **(Fig 5 E.)** However, while exposed embryos did show varying amounts of blood cells anterior to the gut, only 61% of them had similar levels to those of the control. Further, of that 61% of exposed embryos, the observed branching patterns lit by Gata1 in blood cells were not as conserved and appeared more random when compared to controls **(Fig 5 H.)**

The remaining 39% appeared to have far lower numbers of blood cells in the orofacial region, with many of the conserved branching patterns of the controls completely absent **(Fig 5 F, G.)** Using RT-qPCR, the micro-dissected faces of Revel exposed embryos at stage 40 showed a reduction in the level of *gata1* expression in the orofacial region, with a fold change of 0.58 (student t-test, p-value = 9.91×10^{-6}) **(Fig 5 M.)**

3.7 Revel exposure alters the expression of regulatory genes prior to the start of circulation

3.7a) Blood regulator GATA1

One could hypothesize that the changes in blood distribution could simply be due to a reduction in heart function and/or vascular malformation. On the other hand, the observed changes in blood localization could be due to a reduction in the expression of genes that regulate hematopoiesis and erythropoiesis. Therefore, to test this, I measured the level of an important blood regulator GATA1 using RT-qPCR. Relative levels of GATA1 were measured at stage 30 (35 hpf), a time when the blood islands are forming and GATA1 is vital for proper blood development as red blood cell differentiation has begun. Alterations during this time could negatively affect both the proliferation and viability of RBCs.

GATA1 levels in Revel exposed embryos were reduced at stage 30 by a fold change of 0.33 relative to housekeeping (student t-test, p-value = 0.000715) **(Fig 6 A)**

The results from this experiment indicate that Revel exposure reduces GATA1 expression, most importantly concerning development, at a time when it is critical for initial erythropoiesis.

3.7b) Heart development regulators: myocd, dkk1, and tnni3

I next asked if the reductions in ventricle size and heart rate were due to the effects of Revel on the early development of the heart. To assess this, expression levels of genes integral to heart development were measured. RT-qPCR was used to quantify relative expression levels of a gene necessary for early development (*dkk1*), a transcription factor required for

cardiomyocyte differentiation (Myocd), and a gene that when transcribed is a protein necessary for heart cell contraction (*tnni3*) in Revel exposed embryos at stage 30 (35 hpf). Stage 30 was chosen because at this stage the primary heart tube is beginning to form and soon the looping process will begin. Assessing any alterations in the expression of important regulatory genes and transcription factors at this state would help to clarify the effect that e-liquid exposure has during critical developmental stages.

Revel exposed embryos showed a reduction in the level of expression of all three genes in whole embryos: *tnni3* with a fold change of 0.59 (student t-test, p-value = 0.0193), *dkk1* had a fold change of 0.02 (student t-test, p-value = 2.64 x 10^{-8}), and *myocd* had a fold change of 0.27 (student t-test, p-value = 0.0456) **(Fig 6 B.)**

The results of these RT-qPCR experiments indicate that Revel e-liquid exposure reduced the expression levels of genes important for heart development.

4. Discussion

The present work details the effects of an e-liquid containing popular flavors on cardiovascular morphology, heart function, and gene expression. Further, I begin to examine the effects this e-liquid has on the development of vasculature and blood. Additionally, it was assessed whether the observed defects were due solely to nicotine in combination with common inert carriers or instead caused some other substances in the e-liquid solution, perhaps flavoring additives. Finally, whether there is something in the aerosolized form that results in different phenotypes from the neat preparation was investigated.

This work demonstrates the potential dangers of ECIG use during development, specifically the risks associated with heart defects. Due to the previously established link between traditional cigarettes and their negative effect on development and increased risk of CHDs, the importance of examining the effects of ECIG exposure on cardiovascular development is paramount.

4.1 The effects of e-liquid exposure on cardiovascular development

4.1a) Examining the effects of e-liquid exposure on heart morphology

Revel exposure resulted in abnormal heart morphology in *X. laevis* embryos. The heart ventricles were smaller, and an aberrant arrangement of the heart was seen. These observations are consistent with previous reports of e-liquid and cigarette exposure on zebrafish [14]. *X. laevis* embryos were exposed over a period of heart development so this effect is not likely due to non-specific effects at gastrulation. I also determined that genes required in the early induction

of the heart and later differentiation of cardiomyocytes were reduced. This is consistent with previous studies that have established that e-liquid exposure reduced the expression of transcription factors in immature cardiac cells leading to a disruption in the timing of cardiac cell differentiation [14]. Therefore, the reduction in ventricle size and abnormal heart morphology could be due to a decrease in the number of viable heart cells that differentiate. The proper morphology might be dependent on having sufficient tissue. Alternatively, other genes required for proper heart looping may be disrupted. While the initial contractions of the cardiac muscle play an important role in triggering the looping process, the continued contractions may play a supporting role in other factors affecting morphogenesis [51]. Research suggests that the actual flow of blood, powered by the developing heart, has a substantial effect on the process of heart development, specifically in embryos. While the overall mechanism has not yet been determined, certain processes and genes that specifically respond to blood flow continue to be discovered [50]. This helps to illustrate the importance of proper conservation of these key processes, which seem to be disrupted in those embryos exposed to e-liquid solution in this study.

Further, the alterations in ventricle morphology may be due to the disruption of early cardiac gene expression around the time of heart tube formation. Proper development of the heart relies on the synergistic cooperation of several important genes and transcription factors [56]. *X. laevis* embryos exposed to an e-liquid solution had a reduction in the expression of three genes that regulate cardiac development.

The expression of one gene reduced by e-liquid exposure was *dkk1*. *dkk1* expression levels in embryos exposed to e-liquid were 98% lower. *dkk1* is an important gene that codes for a protein that acts to inhibit Wnt signaling during cardiogenesis to ensure proper cardiac cell differentiation. Wnt/β-catenin signaling plays a crucial regulatory role in several phases of heart

development due to its ability to bind to its receptor, influence gene expression, and in doing so activate a multitude of signaling pathways [52]. Throughout development, levels of canonical Wnt/β-catenin signaling are tightly regulated and will change depending on the stage. For example, while canonical Wnt/β-catenin signaling is first necessary to ensure the formation of mesoderm and the proliferation of cardiomyocytes in the heart, it must be lowered to ensure that cardiac specification and differentiation are carried out correctly. *dkk1* knockdown experiments resulted in a reduction in outflow tract, atria, and ventricle size as well as a significant reduction in the heart rate [52]. Similarly, embryos that were exposed to Revel e-liquid solution had a smaller ventricle size and slower heart rate. Thus, these defects could be attributed to e-liquid chemical(s) modulating *dkk1* expression.

Levels of *myocd* expression were reduced by 73% in embryos exposed to e-liquid. *myocd* is expressed exclusively in cardiac and smooth muscle throughout development. Myocd plays a role in early heart development, cardiac cell differentiation, as well as smooth muscle vasculature differentiation. It does this by being a transcriptional co-activator of serum response factor (SRF) that regulates the expression of heart and smooth muscle-specific SRF-target genes, making it important for the differentiation of smooth muscle and heart development [89]. Myocd is an important transcription factor for the initiation of cardiac gene expression as overexpression of this gene was shown to cause ectopic expression of multiple heart-related genes [55]. Knockdown of *myocd* in *X. laevis* resulted in the complete absence of cardiac differentiation as well as disruptions in the normal morphogenic movements that take place during the formation of the heart tube [53]. This reduction suggests that the e-liquid effects on heart morphology could be due to the lack of differentiation of the cardiac muscle. The reduction

in *myocd* expression in embryos exposed to e-liquids could be one reason why the heart is smaller and malformed, due to the lack of differentiation of the cardiac muscle.

Finally, the third gene reduced in my expression analysis of embryos exposed to an e-liquid was *tnni3* whose expression was reduced by 41%. The Tnni3 protein is exclusively expressed in cardiac muscle tissues and is a definitive cardiac marker that is commonly used to test for altered morphogenesis [54]. *tnni3* knockdown studies in mice resulted in death within two weeks, due to a decrease in both the size and function of the left ventricle [75]. Considered together, the significant decrease in ventricle diameter in embryos exposed to e-liquid could be due to the disruption of *tnni3* expression.

Multiple studies have detailed the detrimental effect that both over and underexpression of key cardiac genes can have on successful cardiogenesis [52-56]. Revel exposed embryos showed lower expression levels of all three of the genes examined and displayed similar phenotypes to those described in knockdown experiments. Therefore, the reduction in ventricle size and altered heart morphology in embryos exposed to e-liquid may be, at least in part, due to the misexpression of such key regulatory genes.

4.1b) Examining the effects of e-liquid exposure on heart function

In addition to reduced ventricle size heart function, as assessed by ventricular contraction, was decreased. Heart rates of Revel exposed embryos showed a significant decrease of 14% when compared to that of the control. This is consistent with previous studies that have shown that exposure to e-liquid results in significant decreases in zebrafish heart rate [18]. This reduction is further demonstrated when analyzing the distribution of the heart rates between the

two groups. Measurements showed that 42% of the Revel exposed embryos had heart rates that were below 131.6 bpm, lower than any of the control heart rates in this study.

One explanation for the reduction in heart rate could be due to the observed decrease in ventricle size as well as the malformed heart structure. As structural morphology is altered, it stands to reason that the function would undoubtedly be affected. The altered heart rate may be due to some insurmountable morphological change. Alternatively, I have shown that Revel exposure leads to a reduction in the expression of Tnni3, a cardiac-specific protein that is associated with the regulation of heart contraction due to its interaction with the sarcomere [90]. Therefore, the irregularities in heart rate may stem from a lack of this critical protein, reducing the literal number of viable contractile units within the developing myocardium. Studies in mice have shown that as cardiac troponin levels were reduced sarcomeres began to shorten altering function [75]. Additionally, Tnni3 must respond directly with varying calcium levels to maintain a coordinated rhythm [90]. It is possible that due to the reduced expression levels there is not an adequate amount of Tnni3 to interact with the cellular calcium leading to irregular contraction patterns and overall rhythm. This is in line with previous knockdown studies that found that there was a decreased response to calcium in cardiac troponin depleted in mice [78]. This could perhaps also explain why Revel exposed embryo heartbeats would appear to be sporadic compared to the even, rolling contractions of the controls. Experiments in this work also show a reduction in genes that regulate the differentiation and proliferation of cardiomyocytes, such as *dkk1*. As cardiomyocytes are the cells responsible for generating contractile force, the reduction in heart rate may be due to a lack of these cells. It is also possible that e-liquids cause disruptions in the physiological environment such as increases in intracellular calcium levels leading to irregular rhythms and slowed heart rate. E-liquid, as well as nicotine exposure, has

been shown to lead to increases in mitochondrial calcium levels resulting in altered function in neural and stem cells [79]. Additionally, neat and aerosolized e-liquid exposure was shown to alter intracellular calcium levels independent of nicotine in epithelial cells [88]. E-liquid exposure could have similar effects in other tissues as well. Further, this suggests that calcium fluctuations are due to chemicals within the e-liquid and not in products formed by combustion. Further experiments are needed to examine e-liquid exposure and calcium levels in *X. laevis* cardiac tissues. Another possibility is that Revel exposure disrupts the natural *X. laevis* heart conduction system. Previous studies have shown that *X. laevis* heart conduction responds similarly to that of humans in response to multiple cardiac drugs [71]. Further, in humans, it has been noted that malfunctions in the electrical conduction system often present themselves alongside malformations in anatomy [71]. It is possible therefore that the disruption to heart rate is due to malfunctioning pacemaker cells responsible for the electrical conduction in the cardiovascular system, presenting alongside the noted heart malformations.

More experiments are needed to truly understand why Revel causes defects in heart morphology and function.

4.2 Clarifying causative agents for the observed alterations in the X. laevis heart

4.2a) Assessing the effects of nicotine on heart morphology and function

I first wanted to assess whether the observed reductions in ventricle size and heart rate were simply due to nicotine since this chemical is known to alter heart rate in humans [87]. Similarly, my goal was to rule out the possibility that the observed cardiac changes might be due to a combination of common carrier additives in e-liquid solutions, that might be making

nicotine more potent. To test this, embryos were exposed to identical concentrations of a neat nicotine solution and an aerosolized mixture of nicotine, propylene glycol (PG), and vegetable glycerin (VG), hereafter referred to as "nicotine+PG+VG," at levels that were identical to those in the concentrations of Revel solution used to expose embryos in this study; ventricle diameters and heart rates were then recorded. No significant difference was found between any of the ventricle diameters between the groups. Similarly, there was no significant difference found in the heart rates between any of the groups. There appeared to be no difference in ventricle orientation, or shape between any of the groups either. These results suggest that other chemicals in e-liquids are responsible for the changes described in this study. This theory is in line with the results of previous experiments that have found that popular flavoring additives in e-liquids were more toxic than PG and VG alone [77]. Further, studies have shown that the cytotoxic effects of ECIGs were not due to nicotine but instead caused by various flavoring chemicals [43]. These experiments suggest that at concentrations used in this study, the heart defects in form and function in embryos exposed to Revel e-liquid solution are not simply due to nicotine, by itself, or complexed with propylene glycol (PG) and vegetable glycerin (VG). Further experiments are needed to conclude what specific chemicals are causing these changes, but it is likely due to chemicals such as those that are used to add flavoring.

Chemicals that provide flavor are numerous and include benzyl alcohol, benzaldehyde, vanillin [76]. Some flavor additives, such as diacetyl, which lends a creamy flavor to vapor are currently on the FDA's Generally Recognized As Safe list [77]. For this reason, diacetyl may seem safe as it may not cause any detriment when digested, however, when inhaled it causes bronchitis obliterans [1]. This is partially due to the method of administration, where inhaled substances avoid the first-pass metabolism, but also because when aerosolized, these substances

undergo chemical changes that convert them into hazardous compounds [58]. Many of these

chemicals are converted to carcinogens some of which, like benzene, present major danger to

users as well as those exposed to vapor second hand [60]. Chemicals that lend vapor cinnamon

(cinnamaldehyde) flavoring have now been shown to be highly toxic and poisonous to *D. rerio*

and human stem cells [14].

4.2b) Neat and aerosolized e-liquid exposure results in similar phenotypes

After concluding that nicotine was not the cause for the altered morphology, I wanted to

assess whether there was something different in aerosolized e-liquids that was capable of causing

heart defects. Embryos exposed to either aerosolized or neat Revel appeared very similar

phenotypically, with a shorter axis, curved bodies, and a reduction in ventricle size. The results

of these experiments strongly suggest that neat and aerosolized Revel exposure has similar

effects, at least on heart size and general embryonic morphology. However additional

experiments and quantification would be necessary. The observed similarities in phenotype

between preparations lead me to the conclusion that there is something in the actual e-liquid

itself that goes unchanged when heated, which ultimately results in the alterations. This

conclusion is in line with previous studies that found no difference in relative toxicity in cells

between aerosolized and neat e-liquid exposure [77, 82]. This suggests that despite studies

indicating that propylene glycol (PG) and vegetable glycerin (VG) become harmful substances

like formaldehyde and acetone when heated [85], these chemicals are not responsible for the

reduced ventricle size and heart rates in this study. Interestingly, formaldehyde has been

identified in inhalers used medicinally at similar levels to those found in certain aerosolized e-

liquids [85]. These results suggest that focusing specifically on chemicals that are altered when

heated may not hold the key to identifying the compounds that cause these defects in morphology.

One of the difficulties in identifying the chemicals responsible for the adverse effects of electronic cigarettes is the high level of variability of their ingredients. In a study that submitted 122 e-liquids to chemical analysis, 171 unique compounds were detected. 41 of the most common compounds detected carried GHS Warning codes, some indicating reproductive and organ toxicity [86]. This indicates that the defects observed from e-liquid exposure could be due to chemicals known to have damaging health effects that are unaltered during the combustion process during vaping.

4.3 Alterations in blood development and distribution as a result of e-liquid exposure

In this study, I focus on the distribution of blood and vasculature in the head region of *X. laevis* embryos, as this is a region of interest in our lab. Additional experiments are required to fully investigate the effect of e-liquid exposure on developing blood and vasculature in other areas. Further, previous studies in our lab found that the presence of blood in the trunk and tail of embryos exposed to e-liquid appeared similar to controls [1]. Thus, I did not examine these areas deciding to concentrate instead on the head.

My data and previous studies in our lab, found a change in the localization of blood present in the developing *X. laevis* embryo face using both a histological stain for hemoglobin and transgenic embryos carrying a transgene for red blood marker Gata1 at the larval stage. To further characterize this, I also measured levels of a *gata1* expressed in the head using PCR, noting a 42% reduction in expression. Additionally, while blood was visible in the contracting ventricle without any staining in almost all of the control embryos, this was not as common in

exposed groups. Instead, while contractions were evident, the organ remained translucent and without color. Occasionally this would be accompanied by blood that was visible without stain pooling around the gut or gills. Gata1 is a transcription factor that codes for two proteins that bind DNA and regulate the expression of genes that play an important role in the formation and maturation of red blood cells (RBCs) [30]. Therefore, the reduction in blood cells in the head might be because the development of the blood itself is altered. Reductions in *gata1* expression were noted during a critical time when blood islands are forming, and red blood cell differentiation has begun. Any scenario that would lower *gata1* expression or inactivate all together will likely result in one of several deleterious outcomes [4]. First, there may be a lower overall amount of red blood cells and platelets present. It is also possible that those that are present will have a reduction in function as the Gata1 transcription factors help to regulate the maturation of RBCs. Another observed outcome has been a complete lack of these cell types; the absence of blood. In a previous study, *gata1* knockdown mice all died around the 11-day mark as erythrocyte progenitor cells began undergoing apoptosis and large amounts of immature megakaryocytes were not able to differentiate into functioning platelets [4].

Alternatively, the number of red blood cells could be reduced in the head because of the noted alterations in heart size, shape, and function. It is possible that due to this disrupted morphology, the blood simply is not able to reach the anterior portion of the embryo due to some loss of contractile strength. Studies in human cardiomyocytes have found that electronic cigarette exposure resulted in decreases in expression of sarcomeric genes [14]. The sarcomere is the contractile unit in muscle tissue. Perhaps it goes beyond the just strength of contractions and instead stems from some insurmountable change to the overall structure that lowers cardiac output. Previous studies in zebrafish have noted that cigarette smoke exposure resulted in

reduced ventricle size and heart rate accompanied by a decrease in stroke volume (the volume of blood pumped systemically by the ventricle) [80]. Chemicals in e-liquid vapor might have similar effects on cardiac output due to the similarities in ventricle size and heart rate reductions.

It is also possible that though the heart still beats, it does so in a disorganized way that never achieves full recruitment of cardiomyocytes limiting ejection fraction. Further, it could be a combination of these two things: a weak contraction that is lacking coordination and regularity.

Finally, the lack of blood in the face may be due in some part to a lack of blood vessel formation. Many of the Flk1 Transgenic embryos exposed to Revel solution had alterations in the patterns of the vasculature, with some embryos missing large portions of the observed networks in controls.

The reduction in blood in the head of embryos exposed to e-liquid is likely due in part to a reduction in the expression of important transcription factors that regulate blood cell development. While further investigation is necessary, it might also be caused by a reduction in the amount of blood the malformed heart can pump systemically.

4.4 The effects of e-liquid exposure on vascular development

To further evaluate the relationship between e-liquid exposure and developing blood vessels, a vasculature marker, Flk1, that highlights areas of both vasculogenesis and angiogenesis was used. Most of the Flk1 Transgenic embryos that were exposed to Revel solution had variances in the patterns of the vasculature, notably with a reduction in the overall amount of blood vessels present. This apparent reduction in vasculature could be due to alterations in gene expression that play a role in vascular development. Previous studies noted a reduction in VEGF, a vascular differentiation marker, in embryos exposed to e-liquid [1]. When

addressing the observed lack of blood cells in the head, many embryos exposed to e-liquid were missing large portions of the observed vascular networks in controls entirely. This may be due in some part to a disruption during development that simply resulted in a lower number of blood vessels forming. Cigarette condensate was shown to disrupt vascular patterns and cause extensive hemorrhaging in chick embryos [81]. Chemicals in e-liquids could be capable of similar disruptive effects. Also, e-liquid exposed groups consistently exhibited edema proximal to the heart. The lack of blood in the face may not simply be due to a reduction in viable blood cells, but perhaps disrupted networks of vasculature that are "leaky" leading to the observed blood pooling near the gut of some embryos.

Another possible explanation for the apparent reductions in vasculature could be traced back to *gata1*. Again, Gata1 levels were reduced at early developmental stages when angioblasts are first beginning to migrate and begin to differentiate into the strands of cells that will go on to form tubes and then the primary plexus. Gata1 plays an important role in the expression of an angiogenic factor (AGGF1) that promotes endothelial cell proliferation, and when AGGF1 expression levels are altered results in vascular defects [5]. *gata1* knockdown cultures were observed to have significant decreases in this angiogenic factor that led to apoptosis of the cell layer that lines the interior of blood vessels, and the inhibition of the formation of capillaries from endothelial cells [5]. Therefore, the reduction in blood vessels could be due to the alteration of genes that play important roles in the formation and maintenance of endothelial cells that will eventually become the vasculature.

Alternatively, the observed changes in the appearance of vascular networks could be due to something other than the direct development of the vessels themselves. Revel liquid exposure may alter the environment in which the endothelial sheets begin to form tubes by disrupting the

Retinoic acid gradient at these stages. Previous studies have shown that disruptions in the retinoic pathway can lead to malformation of the major vessels as well as the outflow tract of the heart [72].

The results of this transgenic experiment suggest that there is a disruption and a reduction in the vascular network supplying the head. Further experiments need to be done to determine if levels of *flk1* expression are altered.

5. Conclusion

Based on the data collected in this work I am proposing a mechanism of how e-liquids exposure could be affecting various aspects of cardiovascular development.

E-liquid exposure alters regulatory genes and transcription factors during critical developmental stages when the earliest cardiac cells are differentiating, and structures are forming. These disruptions result in morphological changes that include a reduction in ventricle diameter. These changes are not limited to size, but include composition, with alterations in the number of functional contractile cells that result in an alteration in heart rate. This early pattern of disruption does not rule out the potential for other detrimental effects in later developmental patterning and physiology.

Further, e-liquid exposure reduces the expression of transcription factors that are critical for the establishment of the embryonic blood cell lineages and the maintenance of these immature blood cells as they mature. This reduction results in a decrease in the number of viable cells, and ultimately a lack of blood as progenitor cells are unable to differentiate and avoid apoptosis.

Further, I suggest that e-liquid exposure alters signaling pathways during vasculogenesis and angiogenesis that disrupt the formation of functioning capillary networks and instead results in a disorganized network of blood vessels that are leaky, resulting in edema and blood pooling around the gut and tail.

The result of these events culminates in defective heart morphology, a reduction in cardiac function, and reduced blood flow in the head **(Fig 7).**

These cardiovascular changes help to highlight the dangers of ECIG use during pregnancy, as the same disruptions observed in *X. laevis* could translate to similar defects in humans. Due to the use of chemical ingredients suspected to cause damage to the developing fetus, certain e-liquids and vapors have the potential to disrupt a myriad of developmental processes. As the risk of congenital heart defects increases with prenatal cigarette use, the changes in heart form and function in this study indicate that ECIGs could present similar hazards. In addition to being dangerous on their own, defects due to ECIGs could become even more severe in dual users of both traditional and electronic cigarettes. Further, as we learn more about the variety of e-liquid ingredients, there is the potential for interactions with other substances such as alcohol that are yet unseen.

6. Future Directions

6.1 To determine which components of e-liquid solution cause cardiovascular defects

I have shown that electronic cigarette exposure results in a reduction in *X. laevis* ventricle size and heart rate, as well as variability in positioning. The observed heart defects were shown not to be caused by nicotine or inert humectants alone. Further studies need to be done to try to determine what specific ingredient(s) in Revel e-liquid could be causing the alteration in form and function observed. These ingredients could be tested independently as well as in synergistic experiments.

I have done some preliminary work examining exposure to a common e-liquid flavoring component, vanillin, to get a precursory idea of its effect on development. Exposed embryos exhibited edema that was so extreme it caused them to have a spherical appearance, impairing their ability to move. Also, hearts in exposed embryos were arranged in such a way that seemed longer, and "uncoiled". Heart rates were detected, but not assessed. Due to the elongated heart phenotype, it was difficult to judge the efficacy of contractions in most embryos as blood did not seem to settle in any specific part of the anatomy before moving to the next. Further, eyes were closer together when compared to controls. Shorter axis and body curvatures similar to Revel exposed embryos were also detected.

6.2 To determine the effects of nicotine and inert carriers on blood and vasculature development

Though nicotine did not appear to cause the observed changes in heart morphology or function, I cannot rule out the possibility that it is still altering blood development and vasculature in some way. Previous studies have found that nicotine exposure resulted in a significant increase in both *VEGF* mRNA and proteins in pigs. Increases in VEGF have been shown to result in increases in angiogenesis as well as increased permeability of forming vasculature [73]. Similarly, some studies in zebrafish found that exposure to nicotine in total particulate matter from traditional cigarettes results in a decrease in the number of blood vessels supplying the brain [74]. It is possible that, despite seeing no difference in cardiac phenotype, blood and vasculature were still affected. To assess whether or not nicotine or nicotine+PG+VG altered blood development and vasculature, similar PCR and Transgenic experiments to those in this study could be conducted.

To evaluate changes in vasculature development, levels of *VEGF* expression in nicotine and nicotine+PG+VG exposed embryos could be examined through the use of RT-qPCR at multiple stages. For example, stage 22 when VEGF is responsible for the formation of hemangioblasts, stage 27 when VEGF influences migration and differentiation of angioblasts, and finally stage 46 when principal blood vessels have finished forming.
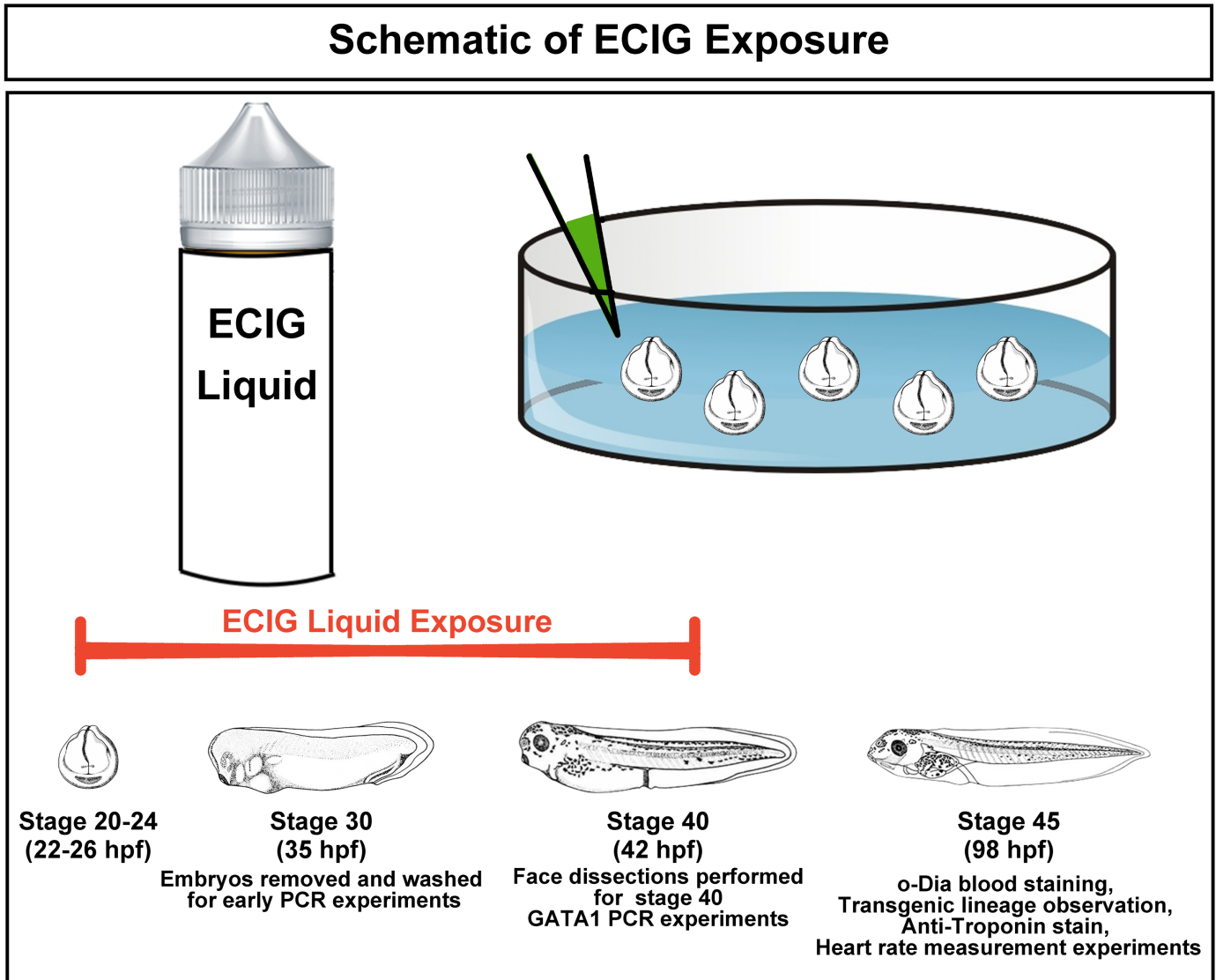
In this work, I showed a decrease in *gata1* associated with a reduction in the number of blood cells present. To evaluate the effects of nicotine and inert carriers on blood development, *gata1* levels in these embryos could be investigated as well, at stage 30 when the blood islands are forming and stage 40 when the dorsal aorta has formed. Alternatively, to visualize any

reductions in blood cells or blood vessels, transgenic lines could be used that would allow these structures to be visualized using fluorescent microscopy.

6.3 To better understand the mechanisms by which e-liquid exposure causes cardiovascular malformations

I have shown that e-liquid exposure during development resulted in altered heart morphology including a reduction in ventricle size as well as decreases in heart function. Embryos exposed to Revel solution continually showed phenotypes in which the heart appeared long and "unwound." Further studies need to be done to determine the specific mechanism that leads to the observed malformations. The results of this study suggest a few possibilities. First, e-liquid exposure may alter the expression of regulatory genes during cardiogenesis that ultimately leads to the observed morphological anomalies. Second, e-liquid exposure might alter structures that then, in turn, results in some alteration in gene expression similar to those observed in this study. These changes could then lead to further morphological change. Similarly, decreases in heart function at early stages could lead to disruption in the later stages of development. To elucidate this cause and effect relationship the experiments performed in this study would need to be extended in scope to include other cardiogenic regulatory genes over many more stages.
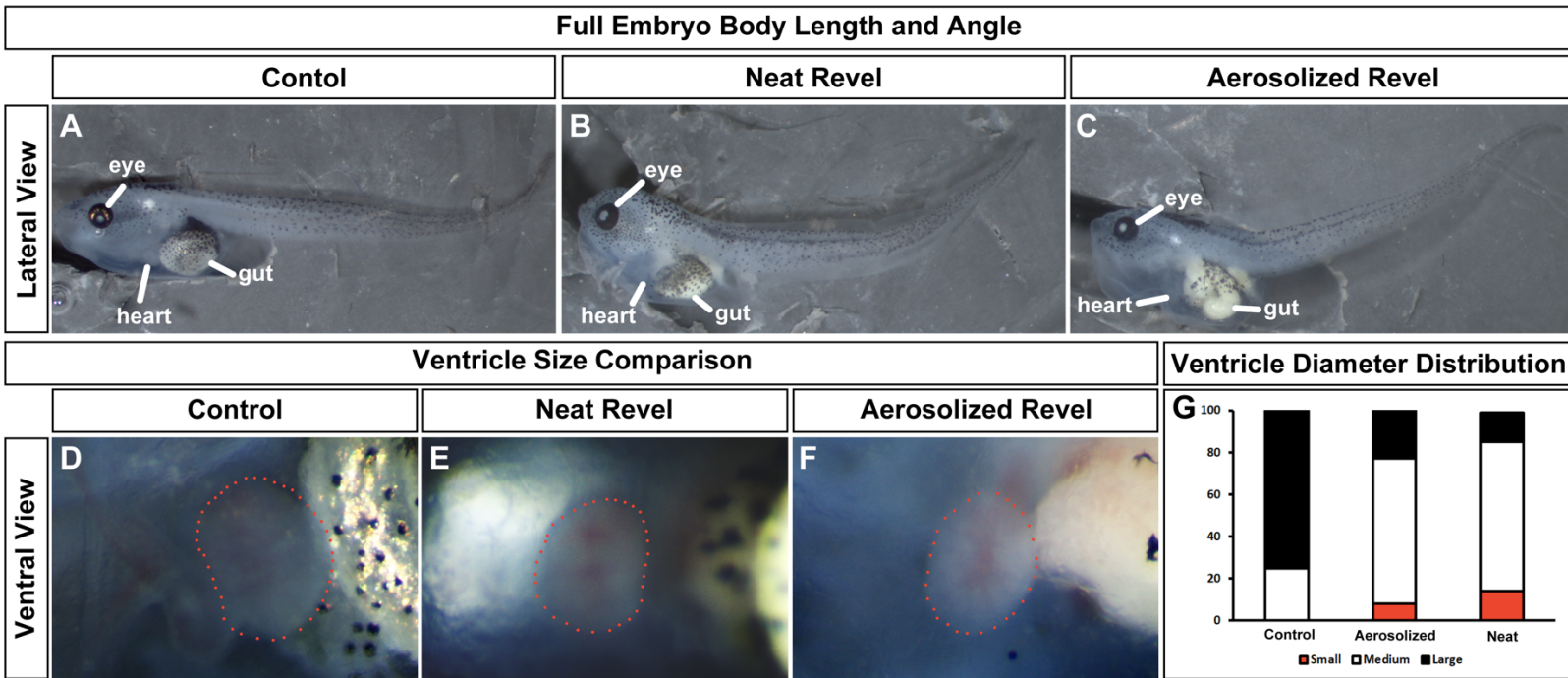
Figures

Schematic of ECIG Exposure

ECIG Liquid

ECIG Liquid Exposure

**Stage 20-24
(22-26 hpf)**

**Stage 30
(35 hpf)**
Embryos removed and washed
for early PCR experiments

**Stage 40
(42 hpf)**
Face dissections performed
for stage 40
GATA1 PCR experiments

**Stage 45
(98 hpf)**
o-Dia blood staining,
Transgenic lineage observation,
Anti-Troponin stain,
Heart rate measurement experiments

Figure 1. Schematic of e-liquid exposure Experimental solutions were added at stage 20-24. Embryos were then incubated at 23°C until stage 30 for early PCR experiments or stage 40 where they were washed in 0.1xMBS. At this point, depending on the specific aim, embryos were either left to mature to stage 45, or fixed and dissected for GATA1 RT-PCR. Embryo images: adapted from Nieuwkoop and Faber (1994) Normal Table of *Xenopus laevis* (Daudin). Garland Publishing Inc, New York ISBN 0-8153-1896-0 and Karimi K, Fortriede JD, Lotay VS, et al. Xenbase: a genomic, epigenomic and transcriptomic model organism database. *Nucleic Acids Res*. 2018;46(D1):D861–D868. doi:10.1093/nar/gkx936
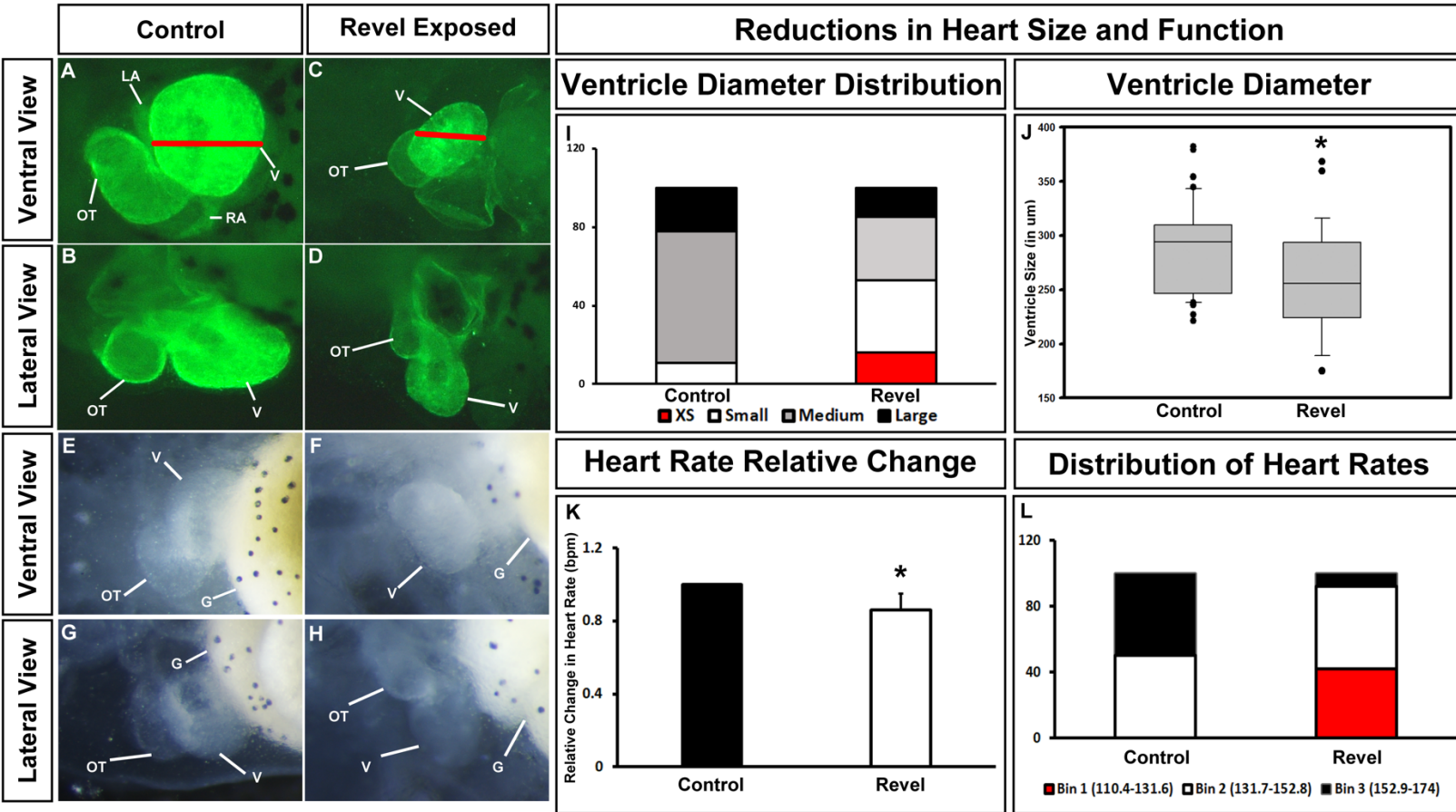
**Figure 2. Effects of Revel e-liquid exposure on Heart morphology and function**
(A-D) Representative fluorescent microscope images with Anti-troponin labeling of cardiac protein (green) in representative images showing ventral and lateral views in controls. **(A, C)** Red lines model how ventral ventricle measurements were taken. **(E-H)** Representative light microscope images showing ventral views of controls **(E, G)** and Revel exposed **(F, H)** embryos. **(I)** Distribution of ventricle diameter measurements (µm) among groups. **(J)** Ventricle diameters (µm) of experimental groups (n=71, student t-test, p-value = 0.009) **(K)** Relative changes in heart rate (bpm) in control and Revel exposed embryos (n=120, 3 biological replicates, Mann-Whitney Rank Sum, P = <0.001) **(L)** Distribution of heart rate measurements (bpm) among groups. **Abbreviations**: **OT** = outflow tract, **V** = ventricle, **G** = gut

Figure 3. Effect of neat Revel vs aerosolized Revel exposure on X. laevis heart morphology.
(A-C) Representative light microscope full embryo images displaying typical phenotypic body length and body angle of control, neat and aerosolized exposed embryos. **(D-F)** Representative light microscope images showing ventral views of controls **(D)**, neat Revel exposed **(E)**, aerosolized Revel exposed **(F)** with ventricles outlined in red. **(G)** Distribution of ventricle diameter measurements among groups.
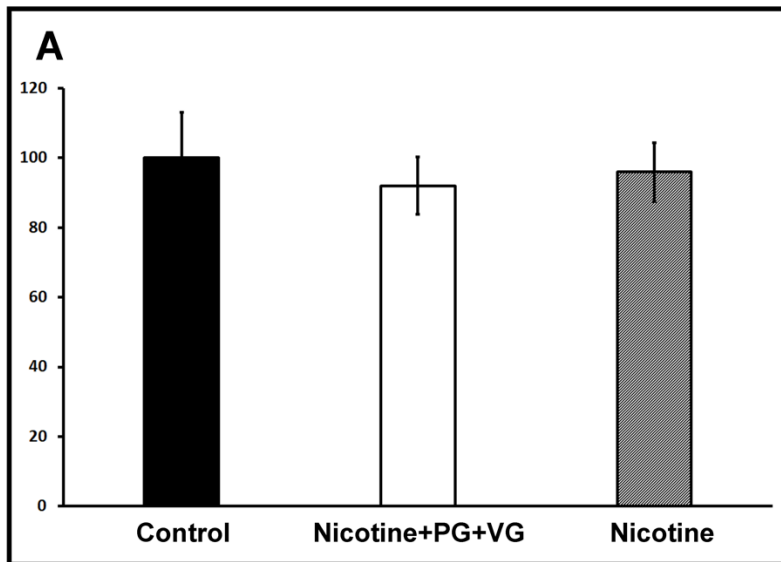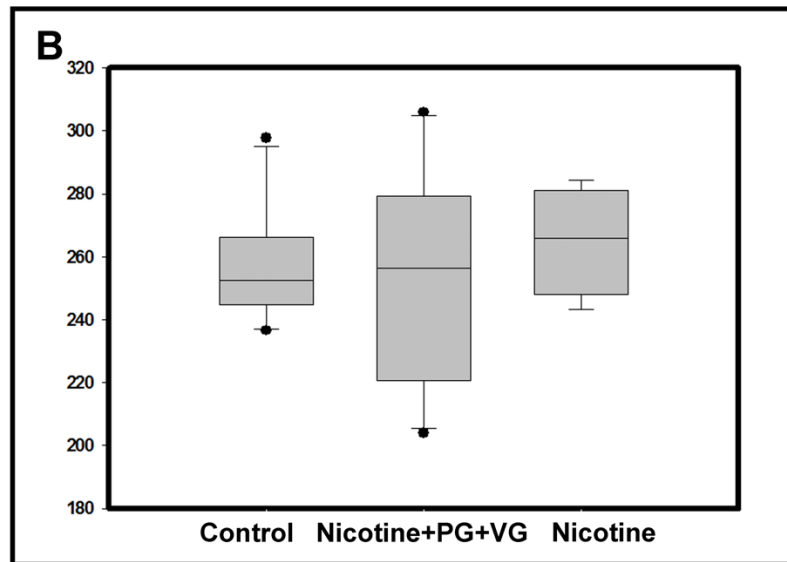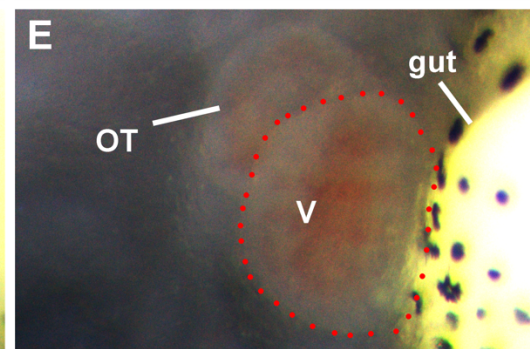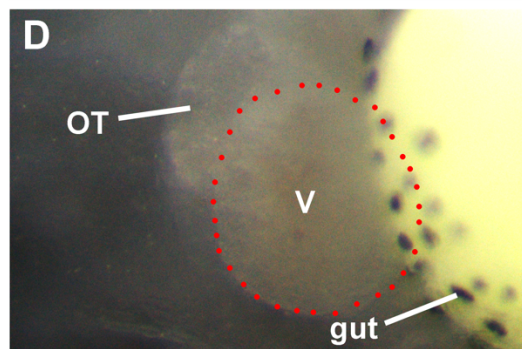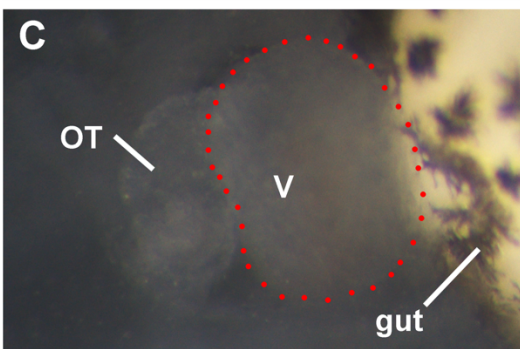
Figure 4. Neat nicotine and nicotine+PG+VG effect on heart morphology and function
(A) Relative heart rates (bpm) in nicotine+PG+VG and neat nicotine compared to control (n=60, 1 replicate, Kruskal-Wallis One Way Analysis of Variance on Ranks, p-value = 0.533) **(B)** Ventricle diameter measurements in control, nicotine+PG+VG, and neat nicotine exposed embryos (n=60, 1 replicate, Kruskal-Wallis One Way Analysis of Variance on Ranks, p-value = 0.418) **(C-E)** Representative light microscope images showing ventral views of controls **(C)**, nicotine+PG+VG exposed **(D)**, neat nicotine exposed **(E)** with ventricles outlined in red. **Abbreviations**: **OT** = outflow tract, **V** = ventricle
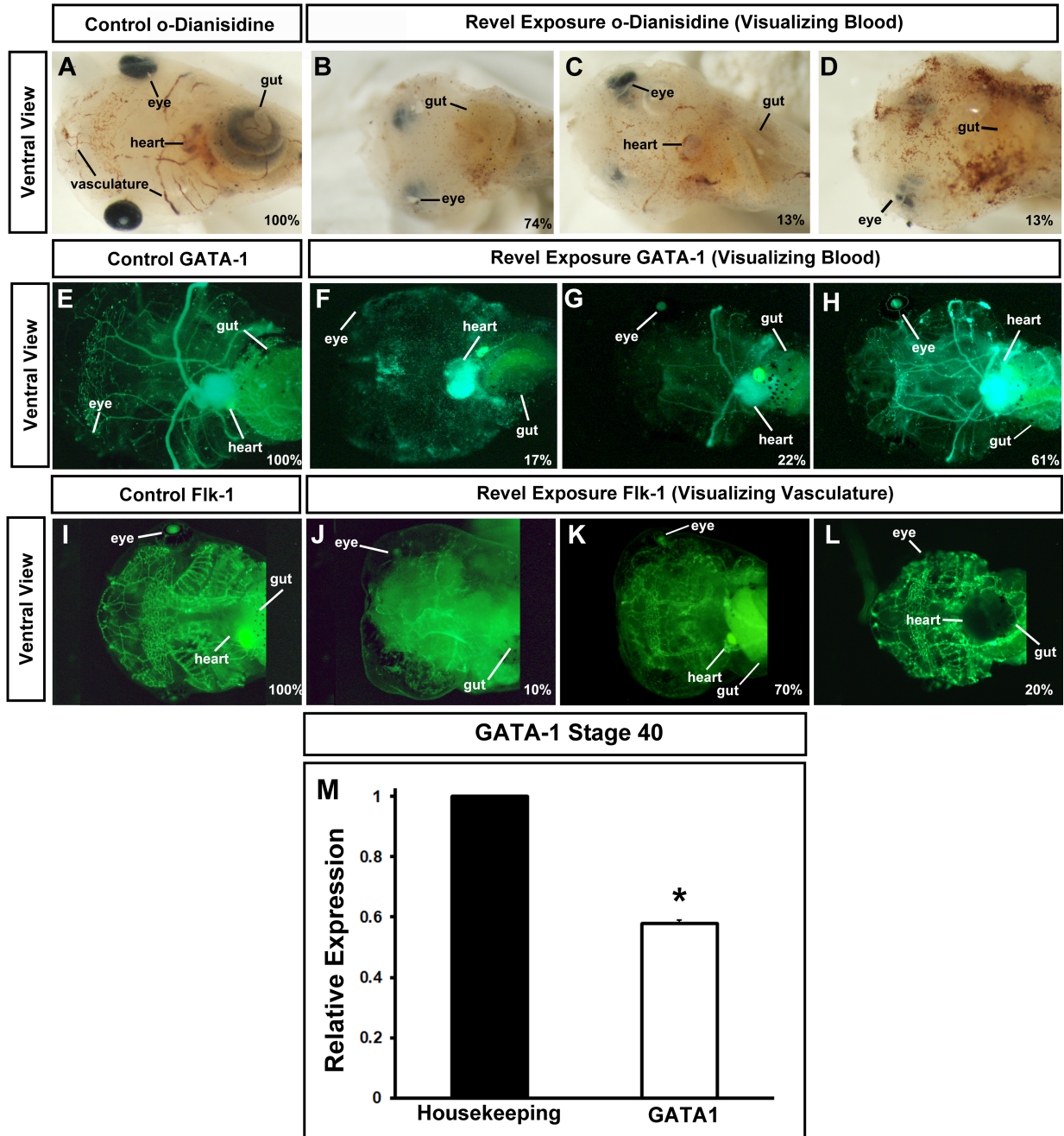
***Figure 5. o-Dianisidine and Transgenic experiments* A-D)** Light microscope images of control **(A)** and Revel Exposed **(B-D)** embryos using an o-Dianisidine stain to detect blood. **E-H)** Fluorescent microscope images of control **(E)** and Revel **(F-H)** exposed Transgenic Gata1-GFP embryos used to detect blood. **I-L)** Fluorescent microscope images of control **(I)** and Revel exposed **(J-L)** Transgenic FLK-GFP *X. laevis* embryos used to detect vasculature. **(M)** Relative fold change of *gata1* expression as determined by qPCR at stage 40 (n=30, 3 replicates, student t-test, p-value = 9.91 x 10^{-6})

RT-PCR

GATA-1 Stage 30

Cardiac Genes Pre-Heartbeat
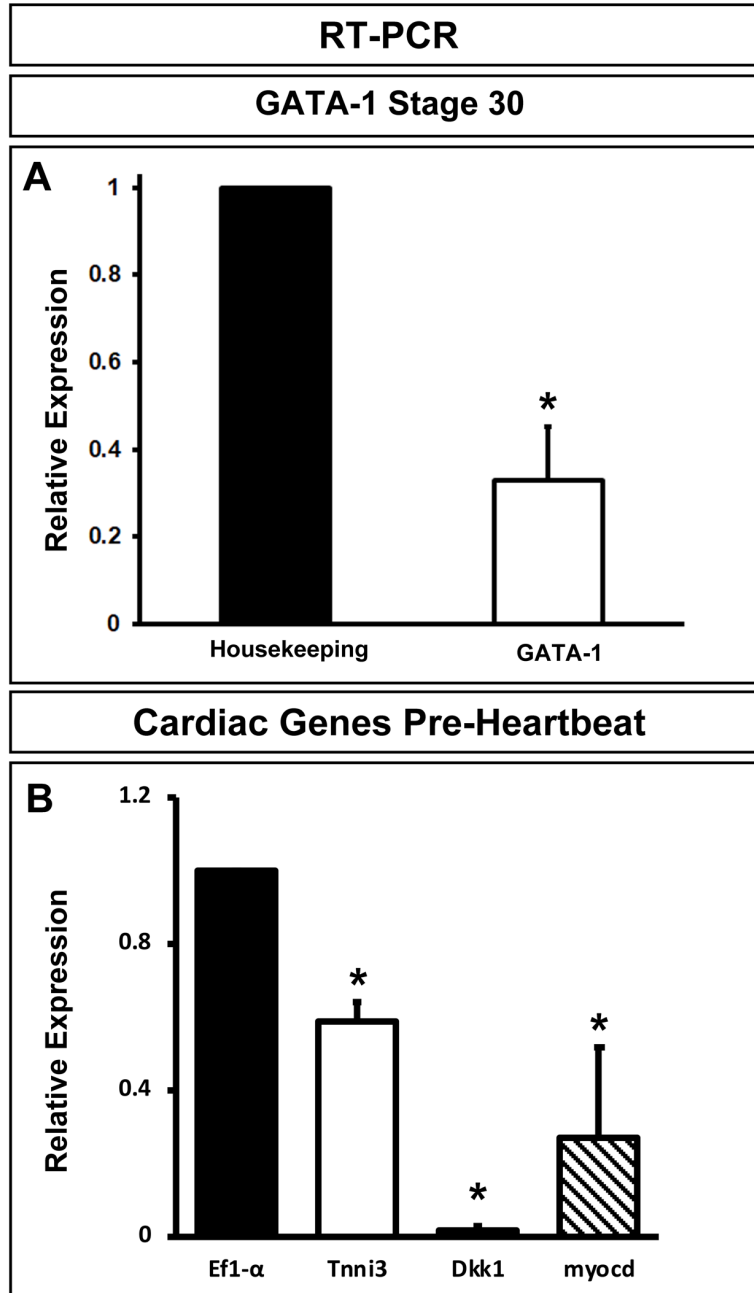
Figure 6. E-liquid exposure PCR Experiments
(A) Relative fold change of GATA1 expression as determined by qPCR at stage 30 (n=40, 1 replicate, student t-test, p-value = 0.000715) **(B)** Relative fold change of multiple heart gene expression levels at stage 30. (N=40, 1 replicate) *tnni3* fold change of 0.59 (student t-test, p-value = 0.0193), *dkk1* fold change of 0.02 (student t-test, p-value = 2.64×10^{-8}), and *myocd* fold change of 0.27 (student t-test, p-value = 0.0456)
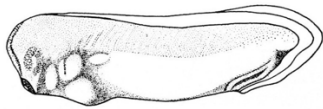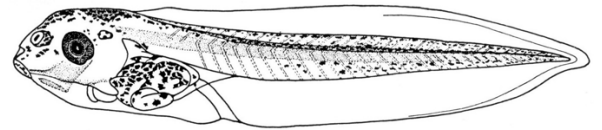
Model of e-liquid Exposure of Cardiovascular Development

Stage 30	Stage 45

Effect of ECIG Exposure

Heart Tube Forming
Blood Islands Forming

Heart Formed
Circulation Has Begun

⬇ **GATA1 Expression**
⬇ **Cardiac Cell Development & Differentiation Gene Expression**

Heart Malformation
Reduced Ventricle Size
⬇ **Blood In Head**
Blood Pooling/Hemorrhaging
⬇ **Heart Rate**

Figure 7. Model of e-liquid Exposure on Cardiovascular Development
Schematic summary of the proposed model outlining the effects of Revel exposure on cardiovascular development in *Xenopus laevis*. Embryo images adapted from Nieuwkoop and Faber (1994) Normal Table of *Xenopus laevis* (Daudin). Garland Publishing Inc, New York ISBN 0-8153-1896-0 and Karimi K, Fortriede JD, Lotay VS, et al. Xenbase: a genomic, epigenomic and transcriptomic model organism database. *Nucleic Acids Res*. 2018;46(D1):D861–D868. doi:10.1093/nar/gkx936

Table 1. Important Developmental Events in Heart, Vasculature, and Blood Development

A	Stage 12-13	Stage 31-33	Stage 33-36	Stage 35	Stage 39-40	Stage 45
Major events in heart development	Migration to ventral midline	Heart tube forms	Heart looping occurs	Heartbeat begins	Chambers form	Septation concludes and the heart is formed
B	Stage 10-14	Stage 26-30	Stage 35	Stage 35-38	Stage 52	Adult
Major events in blood development	Mesoderm begin expressing hematopoietic genes	Ventral Blood Island forms	Circulation begins	Dorsal Aorta forms	Adult RBCs first appear in circulation	Blood is mature
C	Stage 15	Stage 20-22	Stage 27-32	Stage 35	Stage 35-36	Stage 36-46
Major events in vasculature development	Mesoderm transcribe Flk1 receptors	Hemangioblasts form	Angioblasts migrate and differentiate	Circulation begins	VEGF promotes tube formation. Tubes form a primary network	Angiogenesis remodels the primary network

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