

Research



Cite this article: Gauthier PT, Holloway AC, Vijayan MM. 2020 Vape flavourants dull sensory perception and cause hyperactivity in developing zebrafish embryos. *Biol. Lett.* **16**: 20200361.
<http://dx.doi.org/10.1098/rsbl.2020.0361>

Received: 19 May 2020

Accepted: 24 August 2020

Subject Areas:

behaviour, systems biology

Keywords:

fish, embryo photomotor response, neurotoxicity, fetal exposure, nicotine

Author for correspondence:

Mathilakath M. Vijayan

e-mail: matt.vijayan@ucalgary.ca

Animal behaviour

Vape flavourants dull sensory perception and cause hyperactivity in developing zebrafish embryos

Patrick T. Gauthier¹, Alison C. Holloway² and Mathilakath M. Vijayan¹

¹Department of Biological Sciences, University of Calgary, 2500 University Drive N.W., Calgary, AB T2N 1N4, Canada

²Department of Obstetrics and Gynecology, McMaster University, Hamilton, ON L8S 4K1, Canada

ACH, 0000-0002-4343-5325; MMV, 0000-0002-4300-1965

E-cigarette use (vaping) during pregnancy has been increasing, and the potential exists for the developing brain *in utero* to be exposed to chemical constituents in the vape. Vapes come in over 7000 unique flavours with and without nicotine, and while nicotine is a known neurotoxicant, the effects of vape flavouring alone, in the absence of nicotine, on brain function are not well understood. Here, we performed a screen of vape aerosol extracts (VAEs) to determine the potential for prenatal neurotoxicity using the zebrafish embryo photomotor response (PMR)—a translational biosensor of neurobehavioural effects. We screened three commonly used aerosolized vape liquids (flavoured and flavourless) either with or without nicotine. No neurobehavioural effects were detected in flavourless, nicotine-free VAEs, while the addition of nicotine to this VAE dulled sensory perception. Flavoured nicotine-free VAEs also dulled sensory perception and caused hyperactivity in zebrafish embryos. The combination of flavour and nicotine produced largely additive effects. Flavoured VAEs without nicotine had similar neuroactive potency to nicotine. Together, using zebrafish PMR as a high throughput translational behavioural model for prenatal exposure, our results demonstrate that e-cigarette flavourants that we screened elicit neurobehavioural effects worthy of further investigation for long-term neurotoxic potential and also have the potential to modulate nicotine impact on the developing brain.

1. Introduction

The use of electronic cigarettes (e-cigarette; vaping) during pregnancy has been on the rise, in part, due to the perception that they are safer than conventional tobacco cigarettes [1]. However, there is limited information regarding fetal health impacts of vaping. Exposure to chemicals during critical periods of fetal brain development potentially manifest as long-term neurodevelopmental disorders [1]. The main active ingredients in vape liquids are nicotine and flavourants. While nicotine is known to have irreversible effects on fetal brain development [2], less is understood regarding the effects of vape flavourants on fetal neurodevelopment. Flavouring compounds have the potential to be cytotoxic [3,4,5] and neurotoxic [6,7], yet the significance of these effects to the developing fetus remain unknown.

With more than 7000 vape flavours on the market [8], each having unique profiles of chemicals in the final vape aerosol [9], characterizing their potential neurotoxicity will be an onerous task. A multi-tiered approach involving initial screening of compounds for broad neurotoxicological effects has been recommended [10], and a variety of quick behavioural tests have been used to support decision-making processes for higher tier assessments of neurotoxicity

[11]. Behavioural end points have emerged as ideal biomarkers for screening neurotoxicological effects because they represent the integration of the nervous system and reflect neuronal function [12]. We were particularly interested in neurobehavioural markers during neurodevelopment in early life stages, as neurotoxicity at this stage can be teratogenic. However, because prenatal mammalian development occurs *in utero*, there have been few studies on the impact of nicotine at a system level during development.

Zebrafish (*Danio rerio*) have emerged as an excellent vertebrate model for neurobehavioral assessments during embryogenesis, including studies involving nicotine [13]. This is because zebrafish are oviparous and embryogenesis occurs *ex utero*. Moreover, zebrafish embryos are transparent, allowing for direct behavioural observation during a critical window of early development. Using this embryo model, studies have shown that their photomotor response (PMR) is an excellent behavioural model to screen drugs for human neurological disorders [14]. The PMR is a sensorimotor response to pulses of light initiated by photosensitive opsins in the hindbrain and is distinct from basal twitching and coiling movements [15]. Disruption of sensorimotor responses can be attributed to either alterations in sensory perception or motor impairment [16]. Thus, comparing effects of basal activity and the sensorimotor response of the PMR allows researchers to infer general differences in sensory perception and motor impairment following exposure to neurotoxic chemicals. The PMR has been used in translational models for drug discovery [14], as it is easy to measure, occurs promptly after fertilization, and is sensitive to chemical exposure affecting neuronal function [15,17,18]. All these attributes make the PMR an excellent model for screening chemicals, including vape flavourants, for potential neurobehavioural effects.

Very little is known about the impacts associated with early life exposure to flavouring compounds in vape on neurobehavioural outcomes [19]. Also, there is a lack of information regarding the effect of flavourants on sensorimotor responses in early life stages of vertebrates. To address this, we investigated the potential neurotoxicity [11] of vape aerosol extracts (VAEs) of six commercial vape e-liquids, including unflavoured, cinnamon and blue raspberry, each with and without nicotine, during fetal development using the PMR. To further test whether pure flavouring compounds impact the PMR, we exposed embryos to cinnamaldehyde, a common cinnamon flavourant used in vapes. The known neurobehavioral effects of nicotine on zebrafish embryos allowed us to use this as a positive control to compare the neurotoxic effects of nicotine-free VAEs either with or without flavourants. The flavourless nicotine-free VAE was a negative control for the e-liquid carrier.

2. Materials and methods

VAEs were generated using a third-generation e-cigarette (EVOD KangerTech®, Shenzhen, China) to vapourize 3 ml of commercially available unflavoured and flavoured e-liquids (50% propylene glycol/50% vegetable glycerol) containing 0 or 12 mg ml⁻¹ nicotine [20]. The absence of nicotine in the 0 mg ml⁻¹ liquid and the concentration of nicotine in the 100% stock of e-cigarette conditioned media were confirmed by capillary electrophoresis/mass spectrometry [21]. The nicotine concentration

in the undiluted VAE was 130 µg ml⁻¹ (801 µM) [21]. The embryos were bathed in dilutions of VAE collected in 0.8% NaCl and monitored for effects on basal activity and PMRs [17].

Adult Tupfel Long-Fin (TL) strain zebrafish were maintained in 10 l polypropylene tanks at 28.5°C, pH 7.6 and 740 µS conductivity on recirculating systems (Pentair Aquatic Habitats, Apopka, FL). Zebrafish were housed in an animal care facility at the University of Calgary with a 14- to 10-h light : dark daily light cycle. Animals were fed with GEMMA Micro 300 adult zebrafish diet (Skretting, Westbrook, ME) and live *Artemia* (San Francisco Bay Brand, Newark, CA) in the morning and evening, respectively. Zebrafish were bred and the embryos were transferred to Petri dishes filled with E3 embryo medium and reared as previously described [18]. The animal protocols were approved by the Animal Care Committee at the University of Calgary and were in accordance with the Canadian Council for Animal Care Guidelines.

At 24 h post-fertilization (hpf), embryos were dechorionated in 1 g l⁻¹ pronase (Sigma) and transferred (six embryos per well) to the centre 48 wells of a 96-well plate (Greiner, Sigma) with 225 µl of embryo medium per well as described previously [18]. The plates were returned to the incubator until ready for exposures. Beginning at 31.5 hpf [17,18], embryos were exposed to 0, 1, 5 and 10% dilutions of VAEs for 30 min prior to behavioural screening. We also tested 0.01, 0.1, 1 and 10% volume/volume (v/v) of the flavour compound, cinnamaldehyde (Sigma), diluted in E3. This concentration range was chosen as the initial rangefinder tests showed that cinnamaldehyde concentrations greater than 10% v/v were acutely lethal. Final well volumes were 300 µl following 75 µl addition of VAE and cinnamaldehyde stocks. Treatment groups were randomly assigned by column on the 96-well plates (i.e. each 96-well plate had eight columns) for behavioural screens as described previously [18]. After 10 min of exposure (i.e. 20 min prior to behavioural screening), well plates were transferred to a Zebrafish behavioural acquisition system (Viewpoint Life Sciences, Montreal, QC, Canada) held at 28.5°C and allowed to equilibrate in total darkness. Following 30 min of exposure, embryo PMRs were recorded. The PMR trials were 30 s in duration and were carried out in total darkness except for one 57 295 lux light pulse at 10 s as described previously [18]. In total, 5616 and 564 embryos were used for VAE and cinnamaldehyde testing, respectively.

Embryo activity was quantified as Δ pixel intensity from each frame with ZebraLab software (Viewpoint). Basal activity was considered as embryo movement prior to light stimulation. Photosensitivity was calculated by subtracting basal activities from maximum predicted activities during the PMR to give relative changes in activity following light stimulation. Maximum predicted activities were obtained by fitting a nonlinear mixed-model to the raw PMR data between 10 and 20 s [17]. An asymmetric Lorentzian mixed model was chosen to match the response profile of the PMR, and to account for within-subject repeated measurements over time [17]. For VAE PMRs, mean activities over time within columns (i.e. treatment groupings) on each well plate were considered as replicates ($n=1$ per plate), and column by plate was included as the within-subject random effect. For cinnamaldehyde PMRs, individual wells on the well plate were considered replicates in order to obtain sufficient replication ($n=8$ per plate), and well by column by plate was included as the within-subject random effect. For VAE's, effects on mean basal activity and photosensitivity were assessed with linear mixed-models, with flavour (flavourless, blue raspberry, cinnamon), nicotine (absent, present) and concentrations included as fixed terms in the model (i.e. full factorial design). Well plate was included as a random effect to account for temporal variability in responses among well plates. For cinnamaldehyde, temporal variability among well plates could not be included as a random effect as there was insufficient crossover

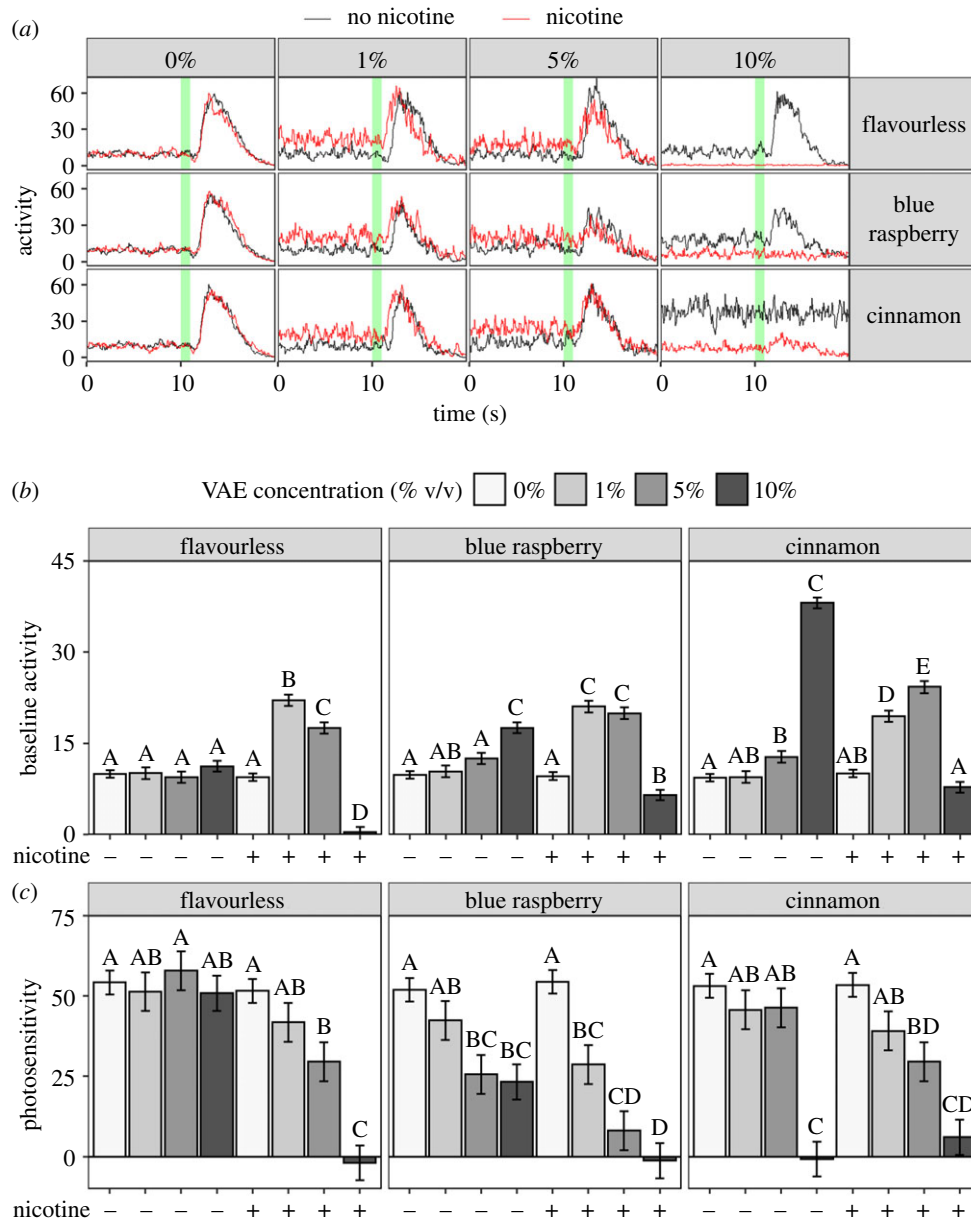


Figure 1. Neurobehavioural testing of vape aerosolized extracts (VAEs) with the zebrafish embryo. Video recordings of basal activity and photomotor response (PMR) are simultaneously acquired and represented as activity profiles. (a) Representative images of real-time basal activity measurements and the zebrafish embryo PMR in response to 1 s light pulse (green bar) at 10 s. The effects of compounds on (b) basal activity $\pm$ standard errors (s.e.) and (c) photosensitivity $\pm$ s.e. (i.e. intensity of the PMR response above basal activity) are shown as bar graphs. Bars with different letters are significantly different within each VAE flavour (b,c, interactive effect of nicotine and concentration; $F_{3,120} = 89.5$; $p < 0.0001$).

in treatment concentrations among well plates. Thus, the effects of cinnamaldehyde concentration on mean basal activity and photosensitivity were assessed with linear models. Linear analyses were followed by ANOVAs as omnibus tests, and then by Tukey-style post-hoc tests to compare flavour, nicotine, VAE concentration, cinnamaldehyde concentration and interactions when appropriate. All data are reported as means $\pm$ standard errors and significance was assessed with $\alpha = 0.05$. Main effects were not reported when interactive effects were detected. All statistical analyses were carried out in R [22] using the nlme [23], emmeans [24] and car [25] packages. All data as well as R scripts of the statistical analyses are provided in the Dryad Digital Repository [26].

3. Results and discussion

There was an interactive effect of VAE flavour (flavourless, blue raspberry, cinnamon), nicotine and their concentrations

for both basal activity ($F_{6,120} = 28.1$, $p < 0.0001$) and photosensitivity ($F_{6,120} = 4.5$, $p = 0.0004$) following 30 min of exposure. Zebrafish embryos exposed to dilutions of flavourless nicotine VAE became hyperactive (figure 1a–c). At 1% ($t_{120} = 10.9$; $p < 0.0001$) and 5% ($t_{120} = 6.8$; $p < 0.0001$) dilutions, flavourless nicotine VAE increased basal activity. However, at 10% dilutions embryos were severely hypoactive ($t_{120} = 9.5$; $p < 0.0001$). These effects on basal activity were absent in flavourless nicotine-free VAE (figure 1a–c), confirming that nicotine, and not constituents in the e-liquid, was causing the neurobehavioural effects. Similar biphasic activity has been observed in early life stage zebrafish exposed to nicotine, with acute exposures causing hyperactivity in embryos and prolonged exposures causing eventual paralysis due to disruption of neuromuscular development [27]. However, the hypoactivity we observed may not be related to neuromuscular dysfunction, as the embryos were exposed to nicotine for only 30 min. Also, prolonged exposures to

nicotine at concentrations as high as 50 μM had no effect on survival or spontaneous tail twitching in 25 hpf zebrafish [28]. As the highest concentration of nicotine VAE we tested contained approximately 80 μM nicotine, our results suggest that this concentration may be acutely toxic to zebrafish embryos leading to neuromuscular dysfunction, while the mechanism remains to be determined.

Exposures to flavourless VAE with nicotine also produced a concentration-dependent decrease in photosensitivity (figure 1*a–c*) that was absent in flavourless nicotine-free VAE (figure 1*a–c*). Photosensitivity was dulled in embryos exposed to 5% dilutions of flavourless nicotine VAE ($t_{120} = 3.8$, $p = 0.0047$), and completely abolished by 10% dilutions ($t_{120} = 9.4$, $p < 0.0001$). This outcome was distinct from the biphasic effect nicotine had on basal activity, most notably at the 5% dilutions where embryos became hyperactive but also had a dulled photosensitivity. These data suggest that dulled photosensitivity was not simply due to motor impairment, but rather indicative of impaired sensory perception [16]. Nicotine had a biphasic response on basal activity, and impaired sensory perception; these results highlight the utility of the PMR as a robust screen for potential neurotoxicity [11].

We next tested the neurotoxic potential of nicotine-free flavoured vapes on zebrafish embryos. Both blue raspberry (10%) and cinnamon (5% and 10%) VAEs resulted in an increase in basal activity following 30 min exposures; with 10% cinnamon being notably more potent than blue raspberry or flavourless nicotine (figure 1*a–c*). Neither blue raspberry nor cinnamon flavours produced the biphasic response to basal activity seen with nicotine, alluding to possible differences in the mode of action of nicotine and the flavourants. Both blue raspberry and cinnamon VAEs also decreased photosensitivity, as seen with nicotine (figure 1*a–c*). Exposure to blue raspberry VAE decreased photosensitivity in a concentration-dependent manner, whereas cinnamon VAE was effective at 10% dilutions, and it completely abolished the PMR ($t_{120} = 9.1$, $p < 0.0001$). Nicotine-induced hypoactivity was partly and completely abolished by the hyperactive effect of blue raspberry ($t_{120} = 5.2$, $p = 0.0002$) and cinnamon ($t_{120} = 6.3$, $p < 0.0001$) flavourants, respectively. The inclusion of nicotine along with these flavourants generally produced an additive effect for both activity and photosensitivity. To confirm whether pure flavouring compounds also show a neurobehavioural response, we exposed embryos to cinnamaldehyde, a common cinnamon flavourant used in vapes. Cinnamaldehyde reduced basal activity ($F_{4,89} = 37.4$, $p < 0.0001$) and dulled photosensitivity ($F_{4,89} = 34.9$, $p < 0.0001$). Embryos exposed to 0.01% v/v (0.76 mM) cinnamaldehyde for 30 min were hyperactive ($t_{89} = 6.6$, $p < 0.0001$) and had reduced photosensitivity ($t_{89} = 8.7$, $p < 0.0001$; figure 2*a–c*), demonstrating that pure flavourants at concentrations measured in vapes [29,30] may be neurotoxic to developing embryos. At 0.1 (7.5 mM), 1% (75.6 mM) and 10% (756.6 mM) cinnamaldehyde concentrations, embryos were severely hypoactive and their PMRs were abolished, which may be the result of a combination of neurobehavioural dysfunction and acute toxicity [30].

The flavoured VAEs affected PMRs with potency similar to that observed in flavourless nicotine vapes. Because neurobehavioural effects are biomarkers of potential neurotoxicity [11], this finding highlights the importance of considering neurodevelopmental consequences of using flavoured e-cigarettes while pregnant. The subtle differences in the concentration response profiles of blue raspberry and

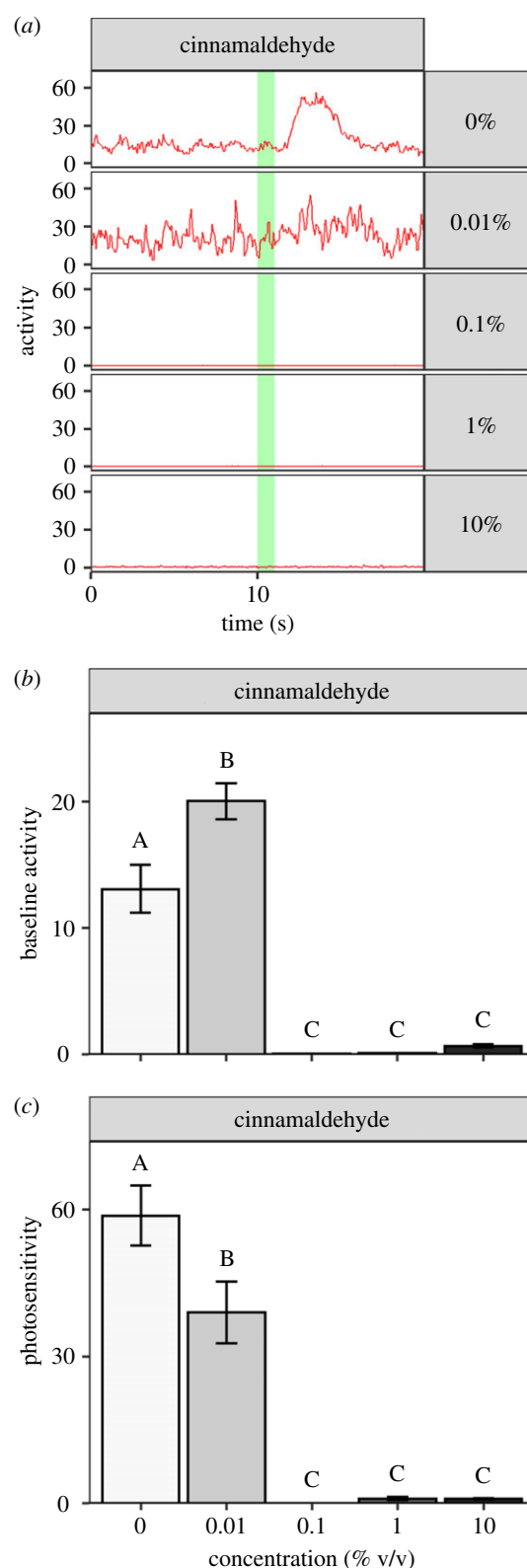


Figure 2. Neurobehavioural testing of cinnamaldehyde with the zebrafish embryo. (a) Representative images of the real-time basal activity measurement, and the photomotor response (PMR) in response to 1 s light pulse (green bar) at 10 s. The effects of volume/volume (v/v) dilutions of cinnamaldehyde on (b) basal activity $\pm$ standard errors (s.e.) and (c) photosensitivity $\pm$ s.e. (i.e. intensity of the PMR response above basal activity) at different concentrations following 30 min exposures. Bars with different letters are significantly different for basal activity ($F_{4,89} = 37.4$, $p < 0.0001$) and photosensitivity ($F_{4,89} = 34.9$, $p < 0.0001$).

cinnamon, particularly for photosensitivity, suggests further work should consider the potential diversity of neuroactive effects from specific flavourants resulting from vaping. Because

e-cigarette use during pregnancy is increasing, there is an urgent need to understand the potential neurotoxicity and long-term developmental impacts of fetal exposure to vapes. While there is evidence linking maternal smoking of conventional tobacco cigarettes containing nicotine and prenatal hyperactivity [19], applying prenatal activity as a biomarker of potential neurotoxicity is complicated by the need to expose and observe embryos *in utero*. In addition to other studies involving nicotine [27,31], we demonstrate that prenatal neurobehavioral changes in zebrafish embryos have potential use as a biomarker of neurotoxicity for vape flavourants. Results from this study provide the first evidence that the PMR may prove to be an ideal candidate for screening the greater than 7000 vape flavours for developmental neurotoxicity.

Ethics. The animal protocols were approved by the Animal Care Committee at the University of Calgary (protocol no. AC17-0079) and

were in accordance with the Canadian Council for Animal Care Guidelines.

Data accessibility. We have provided the data in the Dryad Data Repository at: <https://doi.org/10.5061/dryad.cnp5hqc2m> [26].

Authors' contributions. P.T.G. designed the study, carried out the experiments, data analysis and statistical analyses and drafted the manuscript. A.C.H. conceived the study, provided materials and critically revised the manuscript. M.M.V. conceived the study, coordinated the study, provided the materials and resources and critically revised the manuscript. All authors gave final approval for publication and agree to be held accountable for the work.

Competing interests. We declare we have no competing interests.

Funding. This study was supported by Canadian Institutes of Health Research (PJT-155981) and Natural Sciences and Engineering Research Council of Canada (RGPIN-2019-06291).

Acknowledgements. The authors would like to acknowledge the technical assistance of Sarah Kleiboer for developing the VAE extraction system and Sergio Raez-Villanueva for preparation of the VAEs for this study.

References

- Whittington JR, Simmons PM, Phillips AM, Gammill SK, Cen R, Magann EF, Cardenas VM. 2018 The use of electronic cigarettes in pregnancy: a review of the literature. *Obstet. Gynecol. Surv.* **73**, 544–549. (doi:10.1097/OGX.0000000000000595)
- Wickstrom R. 2007 Effects of nicotine during pregnancy: human and experimental evidence. *Curr. Neuroparmacol.* **5**, 213–222. (doi:10.2174/157015907781695955)
- Bahl V, Lin S, Xu N, Davis B, Wang Y, Talbot P. 2012 Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. *Reprod. Toxicol.* **34**, 529–537. (doi:10.1016/j.reprotox.2012.08.001)
- Muthumalage T, Prinz M, Ansah KO, Gerloff J, Sundar IK, Rahman I. 2018 Inflammatory and oxidative responses induced by exposure to commonly used e-cigarette flavoring chemicals and flavored e-liquids without nicotine. *Front. Physiol.* **8**, 1130. (doi:10.3389/fphys.2017.01130)
- Kaur G, Muthumalage T, Rahman I. 2018 Mechanisms of toxicity and biomarkers of flavoring and flavor enhancing chemicals in emerging tobacco and non-tobacco products. *Toxicol. Lett.* **288**, 143–155. (doi:10.1016/j.toxlet.2018.02.025)
- Wood PL, Khan MA, Kulow SR, Moskal JR. 2006 Neurotoxicity of reactive aldehydes: the concept of 'aldehyde load' as demonstrated by neuroprotection and hydroxylamines. *Brain Res.* **1095**, 190–199. (doi:10.1016/j.brainres.2006.04.038)
- LoPachin RM, Gavin T. 2014 Molecular mechanisms of aldehyde toxicity: a chemical perspective. *Chem. Res. Toxicol.* **24**, 1081–1091. (doi:10.1021/tx5001046)
- Zhu S-H, Sun JY, Bonnevill E, Cummins SE, Gamst A, Yin L, Lee M. 2014 Four hundred and sixty brands of e-cigarettes and counting: implications for product regulation. *Tob. Control* **23**, iii3–iii9. (doi:10.1136/tobaccocontrol-2014-051670)
- Klager S, Vallarino J, MacNaughton P, Christiani DC, Lu Q, Allen JG. 2017 Favoring chemicals and aldehydes in e-cigarette emissions. *Environ. Sci. Technol.* **51**, 10 806–10 813. (doi:10.1021/acs.est.7b02205)
- National Research Council. 1992 *Environmental neurotoxicology*. Washington, DC: The National Academies Press.
- Moser VC. 2011 Functional assays for neurotoxicity testing. *Toxicol. Pathol.* **39**, 36–45. (doi:10.1177/0192623310385255)
- Tilson HA, Moser VC. 1992 Comparison of screening approaches. *Neurotoxicology* **13**, 1–14. (doi:10.1016/0892-0362(92)90017-5)
- Klee EW, Ebbert JO, Schneider H, Hurt RD, Ekker SC. 2011 Zebrafish for the study of biological effects of nicotine. *Nicotine Tob. Res.* **13**, 301–312. (doi:10.1093/ntr/ntn010)
- Kokel D *et al.* 2010 Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nat. Chem. Biol.* **6**, 231–237. (doi:10.1038/nchembio.307)
- Kokel D *et al.* 2013 Identification of nonvisual photomotor response cells in the vertebrate hindbrain. *J. Neurosci.* **33**, 3834–3843. (doi:10.1523/JNEUROSCI.3689-12.2013)
- Tierney KB. 2011 Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish. *Biochim. Biophys. Acta* **1812**, 381–389. (doi:10.1016/j.bbadis.2010.10.011)
- Gauthier PT, Vijayan MM. 2018 Nonlinear mixed-modelling discriminates the effect of chemicals and their mixtures on zebrafish behavior. *Sci. Rep.* **8**, 1999. (doi:10.1038/s41598-018-20112-x)
- Gauthier PT, Vijayan MM. 2019 A rapid zebrafish embryo behavioral biosensor that is capable of detecting environmental β -blockers. *Environ. Pollut.* **250**, 493–502. (doi:10.1016/j.envpol.2019.03.053)
- Stroud L, McCallum M, Salisbury A. 2018 Impact of maternal prenatal smoking on fetal to infant neurobehavioral development. *Dev. Psychopathol.* **30**, 1087–1105. (doi:10.1017/S0954579418000676)
- Raez-Villanueva S, Ma C, Kleiboer S, Holloway AC. 2018 The effects of electronic cigarette vapor on placental trophoblast cell function. *Reprod. Toxicol.* **81**, 115–121. (doi:10.1016/j.reprotox.2018.07.084)
- Wild J, Kleiboer S, Raez-Villanueva S, Holloway AC, Britz-McKibbin P. 2017 Exploring the impact of flavoured electronic cigarette exposure on the placental metabolome with capillary electrophoresis-mass spectrometry. In *Canadian Society of Chemistry Annual Meeting, Toronto, ON, June 2017*.
- R Core Team. 2017 *R: a language and environment for statistical computing*, version 3.4.1. Vienna, Austria: R Foundation for Statistical Computing. See <http://www.R-project.org/>.
- Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. 2017 *nlme: linear and nonlinear mixed effects model*, version 3.1-131.
- Lenth R. 2019 emmeans: Estimated marginal means, aka least-squares means. R package version 1.4.2.
- Fox J, Weisberg S. 2018 *An R companion to applied regression*, 3rd edn. Thousand Oaks, CA: Sage.
- Gauthier PT, Holloway AC, Vijayan MM. 2020 Data from: Vape flavourants dull sensory perception and cause hyperactivity in developing zebrafish embryos. Dryad Digital Repository. (<https://doi.org/10.5061/dryad.cnp5hqc2m>)
- Svoboda KR, Vijayaraghavan S, Tanguay RL. 2002 Nicotinic receptors mediate changes in spinal motoneuron development and axonal pathfinding in embryonic zebrafish exposed to nicotine. *J. Neurosci.* **22**, 10 731–10 741. (doi:10.1523/JNEUROSCI.22-24-10731.2002)
- Rafferty TD, Isaacs GM, Yozzo KL, Volz DC. 2014 High-content screening assay for identification

- of chemicals impacting spontaneous activity in zebrafish embryos. *Environ. Sci. Technol.* **48**, 804–810. (doi:10.1021/es404322p)
29. Tierney PA, Karpinski CD, Brown JE, Luo W, Pankow JF. 2016 Flavour chemicals in electronic cigarette fluids. *Tob. Control* **25**, e10–e15. (doi:10.1136/tobaccocontrol-2014-052175)
 30. Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, Jaspers I. 2017 Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **313**, L278–L292. (doi:10.1152/ajplung.00452.2016)
 31. Thomas LT, Welsh L, Galvez F, Svoboda KR. 2009 Acute nicotine exposure and modulation of a spinal motor circuit in embryonic zebrafish. *Toxicol. Appl. Pharmacol.* **239**, 1–12. (doi:10.1016/j.taap.2008.08.023)