

This is a self-archived version of an original article. This version may differ from the original in pagination and typographic details.

Author(s): Vehniäinen, Eeva-Riikka; Haverinen, Jaakko; Vornanen, Matti

Title: Polycyclic aromatic hydrocarbons phenanthrene and retene modify the action potential via multiple ion currents in rainbow trout *Oncorhynchus mykiss* cardiac myocytes

Year: 2019

Version: Accepted version (Final draft)

Copyright: © 2019 SETAC

Rights: In Copyright

Rights url: <http://rightsstatements.org/page/InC/1.0/?language=en>

Please cite the original version:

Vehniäinen, E.-R., Haverinen, J., & Vornanen, M. (2019). Polycyclic aromatic hydrocarbons phenanthrene and retene modify the action potential via multiple ion currents in rainbow trout *Oncorhynchus mykiss* cardiac myocytes. *Environmental Toxicology and Chemistry*, 38(10), 2145-2153. <https://doi.org/10.1002/etc.4530>

Eeva-Riikka Vehniäinen ORCID iD: 0000-0001-6061-9014

Running head: PAHs affect cardiac ion currents in rainbow trout

Polycyclic aromatic hydrocarbons phenanthrene and retene modify
the action potential via multiple ion currents in rainbow trout
Oncorhynchus mykiss cardiac myocytes

Eeva-Riikka Vehniäinen^{1*}, Jaakko Haverinen², Matti Vornanen²

1 University of Jyväskylä, Department of Biological and Environmental Sciences, P.O.
Box 35, FI-40014 University of Jyväskylä, Finland

2 University of Eastern Finland, Department of Environmental and Biological Sciences,
P.O. Box 111, 80101, Joensuu, Finland

* Address correspondence to eeva-riikka.vehniainen@jyu.fi

Acknowledgement: The authors would like to thank Anita Kervinen for technical assistance. Kontiolahti fish-farm is acknowledged for the donation of the fish. This research was supported by the Academy of Finland (projects 285296, 294066 and 319284 to ERV).

Disclaimer:

Data Accessibility Statement: Data available from authors.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/etc.4530.

This article is protected by copyright. All rights reserved.

Statement of Protocol Approval for Animal Research: The fish used in the experiments fall under the laboratory animal permission of ESAVI/2832/04.10.07/2015.

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in aqueous environments. They affect the cardiovascular development and function in fishes. The three-ring PAH phenanthrene has recently been shown to impair cardiac excitation-contraction coupling by inhibiting Ca^{2+} and K^{+} currents in marine warm-water scombrid fishes. To see if similar events take place in a boreal freshwater fish, we studied if the PAHs phenanthrene and retene (an alkylated phenanthrene) modify the action potential (AP) via effects on Na^{+} (I_{Na}), Ca^{2+} (I_{CaL}) or K^{+} (I_{Kr} , I_{Kl}) currents in the ventricular myocytes of the rainbow trout *Oncorhynchus mykiss* heart. Electrophysiological characteristics of myocytes were measured using whole-cell patch-clamp. Micromolar concentrations of phenanthrene and retene modified the shape of the ventricular AP, and retene profoundly shortened the AP at low micromolar concentrations. Both PAHs increased I_{Na} , and reduced I_{CaL} and I_{Kr} , but retene was more potent. Neither of the PAHs had an effect on I_{Kl} . Our results show that phenanthrene and retene affect the cardiac function in rainbow trout by a mechanism that involves multiple cardiac ion channels, and the final outcome of these changes (shortening of AP) is opposite to that observed in scombrid fishes (prolongation of AP). The results also show that retene, and aryl hydrocarbon receptor (AhR) agonist, has an additional mechanism of toxicity besides the previously known AhR-mediated, transcription-dependent one.

Key words: aquatic toxicology, cardiotoxicity, mode of action, polycyclic aromatic hydrocarbons (PAHs)

1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants that occur as complex mixtures in aquatic environments. They originate from petrogenic or pyrogenic sources, and they enter the waters via atmospheric deposition, oil accidents, municipal and industrial effluents, and urban runoff. Individual PAHs as well as PAH mixtures (such as oil) affect the development and function of the heart in several fish species (Billiard et al., 1999, Incardona et al., 2004, Incardona et al., 2006, Incardona et al., 2009, Incardona et al., 2011, Dubansky et al., 2013, Incardona et al., 2014, Brette et al., 2017, Raine et al., 2017).

Phenanthrene and retene (1-methyl-7-isopropyl phenanthrene) are three-ring PAHs. Phenanthrene is common in both petrogenic and pyrogenic mixtures of PAHs, and it causes reversible bradycardia and atrioventricular conduction block in zebrafish (*Danio rerio*) and slight increase in heart rate and reduction of circulation in marine medaka (*Oryzias melastigma*) (Incardona et al., 2004, Mu et al., 2014, Sun et al., 2015, Cypher et al., 2017). In Pacific bluefin tuna (*Thunnis orientalis*), phenanthrene affects the cardiac action potential (AP) and ion currents (Brette et al., 2017). Retene is an alkylated phenanthrene, and it has been found in sediments downstream from pulp and paper mills, in landfills, and in oil sands produced water (Leppanen and Oikari, 1999a, Leppanen and Oikari, 1999b, Legler et al., 2011, Cheng et al., 2018). Retene is an aryl hydrocarbon receptor (AhR) agonist, and it activates the AhR and causes changes in the transcription

of several genes, leading to developmental defects in the cardiovascular system (Billiard et al., 1999, Scott et al., 2011, Vehniainen et al., 2016).

Contraction of the vertebrate heart is triggered by cardiac AP, which originates from the primary pacemaker center at the border zone between the sinus venosus and the atrium (Yamauchi and Burnstock, 1968, Haverinen and Vornanen, 2007). From there AP spreads throughout the atrium, and via the atrioventricular canal further to the ventricular wall thereby triggering sequential contractions of atrium and ventricle (Sedmera et al., 2003). Cardiac AP is generated by the complex interaction between several voltage-gated ion currents in the sarcolemma of cardiac myocytes. In fish ventricular myocytes, there are two major inward currents, the fast Na^+ current (I_{Na}) and L-type Ca^{2+} current (I_{CaL} ; long-lasting), and two major outward K^+ currents, the fast component of the delayed rectifier K^+ current (I_{Kr}) and the background inward rectifier K^+ current (I_{K1}) (Vornanen, 2016). Besides these major ion currents, fish ventricular myocytes may have T-type Ca^{2+} current (I_{CaT} ; transient) and the slow component of the delayed rectifier K^+ current (I_{Ks}) (Nemtsas et al., 2010, Hassinen et al., 2011, Abramochkin et al., 2018, Haverinen, Hassinen, Dash et al., 2018). The shape of cardiac AP is regulated by time- and voltage-dependent opening and closing of the Na^+ , Ca^{2+} and K^+ channels. Electrical excitability of cardiac myocytes, i.e. the ease with which the cardiac AP can be triggered, is dependent on the antagonistic effects of I_{Na} and I_{K1} on membrane potential and an important factor in uninterrupted propagation of cardiac AP (Varghese, 2016, Vornanen, 2016).

The aim of this work was to study if phenanthrene and retene modulate the four major ion currents of the rainbow trout *Oncorhynchus mykiss* ventricle, which could reveal novel toxic effects of these PAHs on fish heart.

2 MATERIAL AND METHODS

2.1 Animals

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*) (73.43 ± 11.69 g, n=18) were obtained from the local fish farm (Kontiolahti, Finland). In the animal facilities of the University of Eastern Finland, the trout were maintained in 500 L metal aquaria for a minimum of 3 weeks before used in the experiments and fish were fed aquarium fish food (Ewos, Finland) at least five times a week. Water temperature was regulated at $14 \pm 0.5^\circ\text{C}$ (Computec Technologies, Joensuu, Finland) and oxygen saturation was maintained by aeration with compressed air. Ground water (average pH 8.0, conductivity $13 \mu\text{S}/\text{cm}$) was constantly flowing through the aquaria at the rate of 150-200 L per day (permission ESAVI/2832/04.10.07/2015).

2.2 Myocyte isolation

All experiments were conducted *in vitro* on enzymatically isolated ventricular myocytes. The fish were killed by a cranial concussion and pithing, and the heart was rapidly excised. Ventricular myocytes were isolated using the retrograde perfusion of the heart and the standard concentrations of hydrolytic enzymes as reported for the method developed in our lab (Vornanen, 1997). Cell isolation was conducted at room temperature

(20-22°C). Isolated myocytes were used in the experiments within 10 hours from isolation.

2.3 Whole-cell patch clamp

The whole-cell current-clamp recordings were made by using an Axopatch 1D amplifier (Axon Instruments, Saratoga, USA). Clampex 9.2 software was used for data acquisition, and off-line analysis of the recordings was done using the Clampfit 10.4 software package. During the experiments, myocytes were continuously superfused with the external saline solutions at the rate of 1.5-2 ml min⁻¹. The temperature of the external solution in the recording chamber was regulated at 14°C by using a Peltier device (CL-100, Warner Instruments, CT, USA or HCC-100A, Dagan, MN, USA), and continuously recorded on the same file with electrophysiological data. Patch pipettes were pulled (PP-83, Narishige, Tokyo, Japan) from borosilicate glass (King Precision, Claremont, CA) and had a resistance of 2.7 ± 0.06 M Ω when filled with the internal saline solutions. After gaining a giga ohm seal, the membrane under the pipette tip was ruptured by a short voltage pulse (zap) to get access to the cell, transients due to series resistance (7.3 ± 0.26 M Ω) and pipette capacitance were cancelled, and capacitive size of ventricular myocytes was determined.

For recording of APs and K⁺ currents the external saline solution contained (mmol l⁻¹) 150 NaCl, 5.4 KCl, 1.2 MgCl₂, 1.8 CaCl₂, 10 HEPES, 10 glucose and pH was adjusted with NaOH to 7.6 at 20°C (giving a pH of 7.68 at the experimental temperature). The composition of the pipette (internal) solution was as follows (mmol l⁻¹): 140 KCl, 4

MgATP, 1 MgCl₂, 0.03 Tris-GTP, 10 HEPES (pH adjusted with KOH to 7.2 at 20°C). To elicit APs, ventricular myocytes were stimulated with current pulses of constant duration (4 ms) and with increasing amplitude. The initial stimulus strength was 200 pA and it was raised with 20 pA increments until an all-or-none AP was elicited (Badr et al., 2018). The stimulation frequency was 1 Hz. The following AP parameters were analysed off-line: resting membrane potential (V_{rest} , mV), threshold potential of AP (V_{th} , mV), threshold current (I_{th} , pA), critical depolarization ($CD = V_{th} - V_{rest}$, mV), AP overshoot (OS, mV), AP amplitude (AMP, mV), AP duration at 50% repolarization level (APD_{50} , ms), maximum rate of AP upstroke ($+dV/dt$, mV ms⁻¹) and the maximum rate of AP repolarization ($-dV/dt$, mV ms⁻¹) (Fig. 1). V_{th} , I_{th} and CD are measures for electrical excitability of ventricular myocytes, i.e. the ease with which AP can be triggered by depolarizing current.

Voltage-dependency of the rapid component of the delayed rectifier K⁺ current (I_{Kr}) and the inward rectifier K⁺ current (I_{K1}) were measured using standard stimulation protocols (Vornanen, Ryökkyneen et al., 2002) from the holding potential of -80 mV. When recording I_{K1} , the external saline included 2 μM E-4031 (1-[2-(6-methyl-2-pyridyl)ethyl]-4-(4-methylsulfonyl-aminobenzoyl)piperidine), 0.5 μM tetrodotoxin (TTX, Tocris Cookson, Bristol, UK) and 10 μM nifedipine to block I_{Kr} , I_{Na} and I_{CaL} , respectively. I_{Kr} was recorded in the presence of TTX (0.5 μM), nifedipine (10 μM) and 0.2 mM BaCl₂ (to block I_{K1}).

The fast Na⁺ current (I_{Na}) was measured under a reduced Na⁺ gradient (20 mM [Na⁺]_o, 5 mM [Na⁺]_i) across the sarcolemma to obtain good control of the membrane voltage. The

composition of the external saline was (mmol l^{-1}): 20 NaCl, 120 CsCl, 1 MgCl_2 , 0.5 CaCl_2 , 10 glucose and 10 HEPES at pH 7.7 (adjusted with CsOH at 20°C) (Haverinen and Vornanen, 2004). Nifedipine ($10 \mu\text{mol l}^{-1}$) was included in the external solution to block I_{CaL} . The pipette solution consisted of (in mmol l^{-1}) 5 NaCl, 130 CsCl, 1 MgCl_2 , 5 EGTA, 5 Mg_2ATP and 5 HEPES (pH adjusted to 7.2 with CsOH at 20°C). I_{Na} was elicited from the holding potential of -120 mV with established stimulus protocols (Haverinen and Vornanen, 2006, Haverinen, Hassinen, Korajoki et al., 2018).

The composition of the external saline solution for recording I_{CaL} was as follows (mmol l^{-1}): 150 NaCl, 5.4 CsCl, 1.8 CaCl_2 , 1.2 MgCl_2 , 10 HEPES and 10 glucose (pH adjusted to 7.6 at 20°C with CsOH). TTX ($0.5 \mu\text{M}$) was included in this saline to block Na^+ current (I_{Na}) (Vornanen, 1998). Since Cs^+ may flow through the Erg K^+ channels, $2 \mu\text{M}$ E-4031 was included in the external solution to prevent contamination by I_{Kr} . The pipette solution contained (mmol l^{-1}) 130 CsCl, 15 TEACl (tetraethylammonium chloride), 5 MgATP , 1 MgCl_2 , 5 oxaloacetate, 10 HEPES and 5 EGTA (pH adjusted to 7.2 at 20°C with CsOH) (all chemicals from Sigma). I_{CaL} was elicited from the holding potential of -80 mV to $+10 \text{ mV}$ at the frequency of 0.2 Hz . Recording of I_{CaL} is complicated by time-dependent run-down (decline) of the current. To minimize the effect of run-down on results, time-dependent changes in I_{CaL} were monitored after getting access to the whole configuration. For the same reason the analysis of PAH effects was limited to two highest concentrations. Only those cells were accepted for analysis, where I_{CaL} stabilized within about 5 minutes from the start of recording.

2.4 PAHs

The stocks of phenanthrene (Sigma-Aldrich, Steinheim, Germany) and retene (MP Biomedicals LLC, Illkirch, France) were made in DMSO at 20 mM. Test solutions at the concentrations 0.3, 1.0, 10 and 30 μ M for phenanthrene and 0.1, 1.0 and 10 μ M for retene were made daily in external saline solutions. Effects of the highest DMSO concentration in the experimental solutions on AP parameters and ion currents were tested in separate experiments. No statistically significant effects were noticed.

2.5 Statistical analyses

After checking normality of distribution and equality of variances, one-way ANOVA (with Tukey's or Dunnett's T3 post hoc test) or nonparametric test (with Friedman's test) were used for evaluating the effect of different PAH concentrations on AP parameters and maximum ion currents. All the statistical tests were performed using SPSS (IBM; version 21.0) software. Data are presented as mean \pm SEM and $P < 0.05$ was considered statistically different.

3 RESULTS

3.1 Phenanthrene and retene differentially modify the action potential in rainbow trout ventricular myocytes

Phenanthrene had no effect on the duration of AP at the level of 50% repolarization (APD₅₀) (Fig. 2A, E), but shortened it at the zero voltage level (APD₀) at 30 μ M (Fig. 2A). Phenanthrene increased the maximum upstroke velocity (+dV/dt) at 1 and 10 μ M,

and accelerated the maximum rate of AP repolarization ($-dV/dt$) at 10 and 30 μM (Fig. 2C). Retene had more pronounced effects on APs than phenanthrene. The duration of the AP (APD_{50} and APD_0) was strongly shortened at 1 and 10 μM concentrations (Fig. 3A, E). Retene augmented the AP amplitude at 10 μM and increased the overshoot at 1 and 10 μM (Fig. 3B). The maximum rate of AP upstroke became faster at all concentrations of retene, but the effect on $-dV/dt$ was significant only at 10 μM (Fig. 3C). Excitability of ventricular myocytes was decreased at the highest (10 μM) retene concentration, as CD needed to elicit AP was about 18% higher than in the control (Fig. 3B).

3.2 Phenanthrene and retene modulate cardiac I_{Na} , I_{CaL} and I_{Kr} currents but have no effect on I_{K1}

Phenanthrene and retene affected all studied ventricular ion currents except I_{K1} , but retene caused the effects at lower concentrations than phenanthrene. Under exposure of 10 μM phenanthrene or 1 μM retene, the peak density of I_{Na} was increased by 12 and 17 %, respectively (Fig. 4A, B). The effects of the highest concentrations (30 μM phenanthrene, 10 μM retene) were slightly less and statistically non-significant (Fig. 4B).

After getting electrical access to the cell, there was a clear increase in the amplitude of I_{CaL} due to the buffering of intracellular free Ca^{2+} by EGTA of the pipette solution (removal of Ca^{2+} -dependent inactivation of I_{CaL}). Then the current stabilized and enabled the recording of drug effects on I_{CaL} (Fig. 5A, B). Phenanthrene reduced I_{CaL} , but the effect was statistically significant only at the highest concentration tested, 30 μM (Fig. 5C). Retene diminished I_{CaL} at 1 and 10 μM (Fig. 5C).

Whereas phenanthrene attenuated I_{Kr} at 10 and 30 μM , retene was effective even at the lowest test concentration (0.1 μM) (Fig. 6). Both phenanthrene and retene decreased the I_{Kr} tail currents at all voltages, where the tail current was activated (Fig. 6 C, D). The maximum inhibition of I_{Kr} tail at +40 mV was 79.3 and 59.2% for phenanthrene and retene, respectively. During the depolarizing prepulse, phenanthrene and retene inhibited I_{Kr} ($I_{Kr\text{activ}}$) in the voltage range between 0 and +20 mV, but did not have any effect at +40 and +60 mV (Fig. 6 E, F). This suggests that there is a phenanthrene- and retene-resistant current underlying I_{Kr} , probably the slow component of the delayed rectifier K^+ current, I_{Ks} . Neither of the PAHs had an effect on the background inward rectifier, I_{K1} (Fig. S1 A, B).

4 DISCUSSION

4.1 Effects on action potential

Both three-ring PAHs affected ventricular AP of the rainbow trout heart, but retene was a much stronger AP modifier than phenanthrene. Phenanthrene did not change V_{rest} or AP amplitude consistently with the findings from bluefin tuna cardiomyocytes (Brette et al., 2017). V_{rest} is maintained by the I_{K1} , which remained untouched by phenanthrene. Phenanthrene had only minor effects on APD; APD was slightly reduced at the zero-voltage level but remained unchanged at 50% repolarization level. In this respect, rainbow trout clearly differs from bluefin tuna, where phenanthrene lengthened ventricular APD (Brette et al., 2017). APD is regulated by a delicate balance between influx of Ca^{2+} via I_{CaL} and efflux of K^+ via I_{Kr} , I_{Ks} and I_{K1} (Grant, 2009). Since the resistance of the sarcolemma at AP plateau is high (Ca^{2+} and K^+ fluxes are small), small

changes in the amplitude and activation/inactivation rate of Ca^{2+} and K^{+} currents will affect APD (Zaza, 2010). Shortening of AP at the zero-voltage level suggests that in the early plateau I_{CaL} is reduced more than I_{Kr} by phenanthrene. Our results show a small but clear increase in $+\text{d}v/\text{d}t$ in rainbow trout with 10 and 30 μM phenanthrene. Phenanthrene also steepened the rate of repolarization ($-\text{d}v/\text{d}t$). These are novel actions of PAHs on AP of the fish heart. Collectively, the present findings show that phenanthrene has partly different effects on cardiac APs in rainbow trout and bluefin tuna.

In contrast to phenanthrene, retene strongly shortened APD and the effect occurred at the lower drug concentrations (1 and 10 μM). The strong shortening of APD suggest that the relative effect of retene on I_{CaL} is stronger than its effect on I_{Kr} , the main repolarizing current. The effects of retene differ from those of phenanthrene in that AP amplitude and overshoot were enhanced by retene but not by phenanthrene. This difference may be associated with slightly stronger stimulation of I_{Na} by retene. Notably, the highest retene concentration (10 μM) attenuated excitability of ventricular myocytes, as CD was increased. *In vivo*, the decrease in excitability could cause interruptions in AP propagation between ventricular myocytes (Vornanen, 2016).

4.2 Effects on cardiac ion currents

The Na^{+} current (I_{Na}) is active during the upstroke of the AP causing the depolarization of the sarcolemma by fast and large influx of Na^{+} . Both phenanthrene and retene increased the peak I_{Na} density in the ventricular myocytes of rainbow trout. Retene was more potent than phenanthrene in enhancing I_{Na} , which is in line with its larger effect on $+\text{d}v/\text{d}t$ and overshoot of the AP. At the level of intact tissue the larger I_{Na} means a faster propagation

of AP in the ventricular wall. To our knowledge, there is no earlier data on the effects of PAHs on fish cardiac I_{Na} . However, in the bluefin tuna ventricular myocytes, phenanthrene did not affect the upstroke velocity of the AP, thus suggesting species-specific differences in PAH modulation of I_{Na} .

I_{CaL} and I_{Kr} are the main determinants of the long AP plateau. They are counteracting currents, as I_{CaL} is depolarizing and I_{Kr} repolarizing. The net outcome of the inhibition of these currents can be seen as changes in APD. Notably, both PAHs caused shortening of APD, but retene was much more powerful than phenanthrene. Strong shortening of APD by retene indicates that the net charge influx via I_{CaL} is inhibited more than the K^+ efflux via I_{Kr} . The final phase 3 repolarization is accelerated by the background inward rectifier I_{K1} . The increase in the rate of $-dV/dt$ by PAHs is probably attributable to the resistance of I_{K1} to retene and phenanthrene whereby the uninhibited I_{K1} overwhelms the reduced I_{CaL} .

The lowering of Ca^{2+} influx in phenanthrene-treated rainbow trout cardiac myocytes is in line with previous research showing that phenanthrene decreased Ca^{2+} transients in ventricular myocytes of scombrid fishes, even though scombrids may be more dependent on intracellular Ca^{2+} stores for contractile activation (Brette et al., 2017). The magnitude of I_{CaL} inhibition by phenanthrene seems to differ between tuna and rainbow trout: Whereas 5 μ M phenanthrene decreased I_{CaL} by ~30 % and 25 μ M phenanthrene by ~75 % in bluefin tuna, in rainbow trout 10 and 30 μ M phenanthrene attenuated I_{CaL} only by ~20 and ~40 %, respectively (Brette et al., 2017). On the other hand, in tuna the reduction of I_{CaL} should be partly compensated by the prolonged AP plateau. Brette et al. (2017) did

not report whether the run-down of I_{CaL} was taken into account, which might have also affected their results. In rainbow trout, retene was more potent than phenanthrene in attenuating I_{CaL} , as the same reduction of I_{CaL} by ~20 and ~40 % was achieved with lower concentrations of retene, 1 and 10 μ M, respectively.

The strong shortening of APD and inhibition of I_{CaL} by retene is expected to have a strong reducing effect on the intracellular free Ca^{2+} concentration. In trout ventricular myocytes, the activation of contraction is largely dependent on the sarcolemmal Ca^{2+} influx during the AP plateau, as about 2/3 of the activator Ca^{2+} is estimated to come from the extracellular space (Vornanen, Shiels et al., 2002). Inhibition of I_{CaL} and shortening of the plateau means that Ca^{2+} influx is smaller and there is less time for Ca^{2+} entry. In the intact ventricle, this should appear as reduced force of contraction. Indeed, exposure to PAHs or oil reduces atrial and ventricular contraction and diminishes cardiac stroke volume in larval fish (Incardona et al., 2013, Jung et al., 2013, Edmunds et al., 2015, Esbaugh et al., 2016, Sørhus et al., 2016, Khursigara et al., 2017, Perrichon et al., 2018). In rainbow trout yolk sac larvae, retene causes pericardial and yolk sac edemas (Billiard et al., 1999, Scott et al., 2011, Vehniainen et al., 2016), phenomena often seen with PAH and oil exposures, and proposed to be caused by reduced cardiac output (Incardona and Scholz, 2016). Taken together, inhibition of I_{CaL} and shortening of AP plateau would compromise contractility and cardiac output of the heart with the outcome of reduced physical performance level and fitness of the fish. These effects would be particularly strong under the intoxication by retene.

I_{Kr} channels are notorious about their susceptibility to inhibition by low concentrations of various small molecule compounds (Sanguinetti and Tristani-Firouzi, 2006). The wide pore cavity of the channel allows access of small molecules to the pore (Vandenberg et al., 2001). Therefore, it is no surprise that also PAHs can block these channels in fish cardiac myocytes. In rainbow trout ventricular myocytes, 10 and 30 μM phenanthrene reduced I_{Kr} by 43 and 75 %, respectively. This is slightly less than the inhibition in bluefin tuna, where 5 and 25 μM phenanthrene decreased I_{Kr} by 60 and over 85 %, respectively (Brette et al., 2017). In rainbow trout, the effect of retene on I_{Kr} was more pronounced, as 10 μM retene caused a 60 % reduction in I_{Kr} (Fig. S2).

In mammalian heart, blockade of I_{Kr} by many drugs is shown to be proarrhythmic and able to induce chaotic ventricular tachycardia, *torsades de pointes* (Vandenberg et al., 2001). However, if both I_{Kr} and I_{CaL} are inhibited simultaneously and at similar drug concentrations, the effect is antiarrhythmic, even when drugs prolong, shorten or triangulate ventricular APs (Kramer et al., 2013, Obejero-Paz et al., 2015). A typical example is verapamil, a useful human cardiovascular medicine, which inhibits human I_{Kr} and I_{CaL} at similar concentrations (Shetuan et al., 1999, Kang et al., 2012). As PAHs inhibit both I_{Kr} and I_{CaL} at similar micromolar concentrations, they should not be proarrhythmic in fish ventricle. However, I_{Kr} and I_{CaL} are essential components of the cardiac pacemaker, which determines the rate and rhythm of the heartbeat (Schram et al., 2002). Half-maximal inhibition of I_{Kr} by E-4031 is known to reduce the beating rate of rainbow trout sinoatrial preparations, and therefore inhibition of I_{Kr} might explain the PAH-induced bradycardia of larval fish (Haverinen and Vornanen, 2007). Inhibition of I_{CaL} is likely to affect impulse generation and conduction of the nodal tissues (sinoatrial

pacemaker and atrioventricular canal), since I_{CaL} is the main determinant for the rate of AP upstroke and impulse conduction (I_{Na} is absent or small in nodal cells)(Schram et al., 2002). Inhibition of I_{CaL} might therefore appear as atrioventricular block and ventricular bradycardia, phenomena seen in larval fish exposed to PAHs or oil (Incardona et al., 2004,Zeltser et al., 2004,Incardona et al., 2005,Incardona et al., 2009,Incardona et al., 2011,Perrichon et al., 2016,Perrichon et al., 2018). However, care must be taken when applying results from mature fish to embryos or larval fish.

As retene is quite hydrophobic ($\log K_{ow} \sim 6$), the actual concentrations in the test chamber have most probably been lower than nominal. It must also be borne in mind that retene is quickly metabolized by CYP1A in fish, and this may lower the concentration of parent retene that reaches cardiac myocytes *in vivo* (Hawkins et al., 2002). In nature, however, fish are exposed to PAH mixtures that frequently contain CYP1A inhibitors, which in turn decrease the metabolism of PAHs and thus increase the concentration of parent compounds (Hawkins et al., 2002).

Retene is an AhR agonist, and it disturbs the cardiovascular development in fish via activating AhR and altering transcription (Scott et al., 2011,Vehniainen et al., 2016). Our study shows that in addition to this transcriptional route, retene has a direct effect on cardiac function via modulating voltage-gated ion channel activity. As normal cardiac function is important for cardiovascular development (Glickman and Yelon, 2002,Incardona et al., 2015), as well as the development of other tissues and organs (Incardona et al., 2004), retene may cause developmental defects also independent of

AhR. The ability to modulate the activity of cardiac ion channels also means that in addition to early-life stages, retene may be cardiotoxic to juveniles and adults.

5 CONCLUSION

Three-ring PAHs phenanthrene and retene differentially modified ventricular APs in rainbow trout cardiomyocytes. Retene was more potent and strongly reduced the duration of ventricular AP. Although phenanthrene and retene had qualitatively similar effects on ion currents, phenanthrene only slightly affected AP duration, probably due to its weaker inhibition of I_{Kr} and I_{Ca} in comparison to retene. Furthermore, the effects of phenanthrene on ventricular AP differed from those reported earlier for the marine warm-water scombrid fish bluefin tuna. These results suggest that different PAHs may have different direct effects on cardiac function, and that these effects may be partly species-specific. This further complicates the environmental risk assessment of PAHs.

REFERENCES

- Abramochkin, D.V., Hassinen, M., Vornanen, M., 2018. Transcripts of Kv7.1 and MinK channels and slow delayed rectifier K^+ current (IKs) are expressed in zebrafish (*Danio rerio*) heart. *Pflügers Archiv - European Journal of Physiology* 470, 1753-1764.
- Badr, A., Abu-Amra, E., El-Sayed, M.F., Vornanen, M., 2018. Electrical excitability of roach (*Rutilus rutilus*) ventricular myocytes: effects of extracellular K^+ , temperature, and pacing frequency. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* 315, R303-R311.

Billiard, S., Querbach, K., Hodson, P., 1999. Toxicity of retene to early life stages of two freshwater fish species. *Environ. Toxicol. Chem.* 18, 2070-2077.

Brette, F., Shiels, H.A., Galli, G.L.J., Cros, C., Incardona, J.P., Scholz, N.L., Block, B.A., 2017. A Novel Cardiotoxic Mechanism for a Pervasive Global Pollutant. *Scientific Reports* 7, 41476.

Cheng, I., Wen, D., Zhang, L., Wu, Z., Qiu, X., Yang, F., Harner, T., 2018. Deposition Mapping of Polycyclic Aromatic Compounds in the Oil Sands Region of Alberta, Canada and Linkages to Ecosystem Impacts. *Environ. Sci. Technol.*

Cypher, A.D., Consiglio, J., Bagatto, B., 2017. Hypoxia exacerbates the cardiotoxic effect of the polycyclic aromatic hydrocarbon, phenanthrene in *Danio rerio*. *Chemosphere* 183, 574-581.

Dubansky, B., Whitehead, A., Miller, J.T., Rice, C.D., Galvez, F., 2013. Multitissue Molecular, Genomic, and Developmental Effects of the Deepwater Horizon Oil Spill on Resident Gulf Killifish (*Fundulus grandis*). *Environ. Sci. Technol.* 47, 5074-5082.

Edmunds, R.C., Gill, J.A., Baldwin, D.H., Linbo, T.L., French, B.L., Brown, T.L., Esbaugh, A.J., Mager, E.M., Stieglitz, J., Hoenig, R., Benetti, D., Grosell, M., Scholz, N.L., Incardona, J.P., 2015. Corresponding morphological and molecular indicators of crude oil toxicity to the developing hearts of mahi mahi. *Scientific Reports* 5, 17326.

Esbaugh, A.J., Mager, E.M., Stieglitz, J.D., Hoenig, R., Brown, T.L., French, B.L., Linbo, T.L., Lay, C., Forth, H., Scholz, N.L., Incardona, J.P., Morris, J.M., Benetti, D.D.,

Grosell, M., 2016. The effects of weathering and chemical dispersion on Deepwater Horizon crude oil toxicity to mahi-mahi (*Coryphaena hippurus*) early life stages. *Science of The Total Environment* 543, 644-651.

Glickman, N.S., Yelon, D., 2002. Cardiac development in zebrafish: coordination of form and function. *Seminars in Cell & Developmental Biology* 13, 507-513.

Grant, A.O., 2009. Cardiac Ion Channels. *Circulation: Arrhythmia and Electrophysiology* 2, 185-194.

Hassinen, M., Laulaja, S., Paajanen, V., Haverinen, J., Vornanen, M., 2011. Thermal adaptation of the crucian carp (*Carassius carassius*) cardiac delayed rectifier current, IKs, by homomeric assembly of Kv7.1 subunits without MinK. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 301, R255-R265.

Haverinen, J., Hassinen, M., Dash, S.N., Vornanen, M., 2018. Expression of calcium channel transcripts in the zebrafish heart: dominance of T-type channels. *J. Exp. Biol.* 221, jeb179226.

Haverinen, J., Hassinen, M., Korajoki, H., Vornanen, M., 2018. Cardiac voltage-gated sodium channel expression and electrophysiological characterization of the sodium current in the zebrafish (*Danio rerio*) ventricle. *Progress in Biophysics and Molecular Biology* 138, 59-68.

Haverinen, J., Vornanen, M., 2007. Temperature acclimation modifies sinoatrial pacemaker mechanism of the rainbow trout heart. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 292, R1023-R1032.

Haverinen, J., Vornanen, M., 2006. Significance of Na⁺ current in the excitability of atrial and ventricular myocardium of the fish heart. *J. Exp. Biol.* 209, 549-557.

Haverinen, J., Vornanen, M., 2004. Temperature acclimation modifies Na⁺ current in fish cardiac myocytes. *J. Exp. Biol.* 207, 2823-2833.

Incardona, J.P., Carls, M.G., Day, H.L., Sloan, C.A., Bolton, J.L., Collier, T.K., Scholz, N.L., 2009. Cardiac arrhythmia is the primary response of embryonic Pacific herring (*Clupea pallasii*) exposed to crude oil during weathering. *Environ. Sci. Technol.* 43, 201-207.

Incardona, J.P., Carls, M.G., Holland, L., Linbo, T.L., Baldwin, D.H., Myers, M.S., Peck, K.A., Tagal, M., Rice, S.D., Scholz, N.L., 2015. Very low embryonic crude oil exposures cause lasting cardiac defects in salmon and herring. *Scientific Reports* 5, 13499.

Incardona, J.P., Carls, M., Teraoka, H., Sloan, C., Collier, T., Scholz, N., 2005. Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development. *Environ. Health Perspect.* 113, 1755-1762.

Incardona, J.P., Collier, T.K., Scholz, N.L., 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* 196, 191-205.

Incardona, J.P., Day, H.L., Collier, T.K., Scholz, N.L., 2006. Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. *Toxicol. Appl. Pharmacol.* 217, 308-321.

Incardona, J.P., Gardner, L.D., Linbo, T.L., Brown, T.L., Esbaugh, A.J., Mager, E.M., Stieglitz, J.D., French, B.L., Labenia, J.S., Laetz, C.A., Tagal, M., Sloan, C.A., Elizur, A., Benetti, D.D., Grosell, M., Block, B.A., Scholz, N.L., 2014. Deepwater Horizon crude oil impacts the developing hearts of large predatory pelagic fish. *Proc. Natl. Acad. Sci. U. S. A.* 111, E1510-E1518.

Incardona, J.P., Linbo, T.L., Scholz, N.L., 2011. Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. *Toxicol. Appl. Pharmacol.* 257, 242-249.

Incardona, J.P., Scholz, N.L., 2016. The influence of heart developmental anatomy on cardiotoxicity-based adverse outcome pathways in fish. *Aquatic Toxicology* 177, 515-525.

Incardona, J.P., Swarts, T.L., Edmunds, R.C., Linbo, T.L., Aquilina-Beck, A., Sloan, C.A., Gardner, L.D., Block, B.A., Scholz, N.L., 2013. Exxon Valdez to Deepwater Horizon: Comparable toxicity of both crude oils to fish early life stages. *Aquatic Toxicology* 142-143, 303-316.

Jung, J., Hicken, C.E., Boyd, D., Anulacion, B.F., Carls, M.G., Shim, W.J., Incardona, J.P., 2013. Geologically distinct crude oils cause a common cardiotoxicity syndrome in developing zebrafish. *Chemosphere* 91, 1146-1155.

Kang, J., Chen, X., Ji, J., Lei, Q., Rampe, D., 2012. Ca^{2+} channel activators reveal differential L-type Ca^{2+} channel pharmacology between native and stem cell-derived cardiomyocytes. *J. Pharmacol. Exp. Ther.* 341, 510-517.

Khursigara, A.J., Perrichon, P., Martinez Bautista, N., Burggren, W.W., Esbaugh, A.J., 2017. Cardiac function and survival are affected by crude oil in larval red drum, *Sciaenops ocellatus*. *Science of The Total Environment* 579, 797-804.

Kramer, J., Obejero-Paz, C., Myatt, G., Kuryshev, Y.A., Bruening-Wright, A., Verducci, J.S., Brown, A.M., 2013. MICE Models: Superior to the HERG Model in Predicting Torsade de Pointes. *Scientific Reports* 3, 2100.

Legler, J., van Velzen, M., Cenijn, P.H., Houtman, C.J., Lamoree, M.H., Wegener, J.W., 2011. Effect-directed analysis of municipal landfill soil reveals novel developmental toxicants in the zebrafish *Danio rerio*. *Environ. Sci. Technol.* 45, 8552-8558.

Leppanen, H., Oikari, A., 1999a. Occurrence of retene and resin acids in sediments and fish bile from a lake receiving pulp and paper mill effluents. *Environ. Toxicol. Chem.* 18, 1498-1505.

Leppanen, H., Oikari, A., 1999b. The occurrence and bioavailability of retene and resin acids in sediments of a lake receiving BKME (bleached kraft mill effluent). *Water Science and Technology* 40, 131-138.

Mu, J., Wang, J., Jin, F., Wang, X., Hong, H., 2014. Comparative embryotoxicity of phenanthrene and alkyl-phenanthrene to marine medaka (*Oryzias melastigma*). *Marine Pollution Bulletin* 85, 505-515.

Nemtsas, P., Wettwer, E., Christ, T., Weidinger, G., Ravens, U., 2010. Adult zebrafish heart as a model for human heart? An electrophysiological study. *J. Mol. Cell. Cardiol.* 48, 161-171.

Obejero-Paz, C., Bruening-Wright, A., Kramer, J., Hawryluk, P., Tatalovic, M., Dittrich, H.C., Brown, A.M., 2015. Quantitative Profiling of the Effects of Vanoxerine on Human Cardiac Ion Channels and its Application to Cardiac Risk. *Scientific Reports* 5, 17623.

Perrichon, P., Le Menach, K., Akcha, F., Cachot, J., Budzinski, H., Bustamante, P., 2016. Toxicity assessment of water-accommodated fractions from two different oils using a zebrafish (*Danio rerio*) embryo-larval bioassay with a multilevel approach. *Science of The Total Environment* 568, 952-966.

Perrichon, P., Mager, E.M., Pasparakis, C., Stieglitz, J.D., Benetti, D.D., Grosell, M., Burggren, W.W., 2018. Combined effects of elevated temperature and Deepwater Horizon oil exposure on the cardiac performance of larval mahi-mahi, *Coryphaena hippurus*. *Plos One* 13, e0203949.

Raine, J.C., Turcotte, D., Tumber, V., Peru, K.M., Wang, Z., Yang, C., Headley, J.V., Parrott, J.L., 2017. The effect of oil sands tailings pond sediments on embryo-larval walleye (*Sander vitreus*). *Environmental Pollution* 229, 798-809.

Sanguinetti, M.C., Tristani-Firouzi, M., 2006. hERG potassium channels and cardiac arrhythmia. *Nature* 440, 463.

Schram, G., Pourrier, M., Melnyk, P., Nattel, S., 2002. Differential Distribution of Cardiac Ion Channel Expression as a Basis for Regional Specialization in Electrical Function. *Circ. Res.* 90, 939-950.

Scott, J.A., Incardona, J.P., Pelkki, K., Shepardson, S., Hodson, P.V., 2011. AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (*Danio rerio*) embryos exposed to retene. *Aquat. Toxicol.* 101, 165-174.

Sedmera, D., Reckova, M., deAlmeida, A., Sedmerova, M., Biermann, M., Volejnik, J., Sarre, A., Raddatz, E., McCarthy, R.A., Gourdie, R.G., Thompson, R.P., 2003. Functional and morphological evidence for a ventricular conduction system in zebrafish and *Xenopus* hearts. *American Journal of Physiology-Heart and Circulatory Physiology* 284, H1152-H1160.

Shetuan, Z., Zhengfeng, Z., Qiuming, G., Makielski, J.C., January, C.T., 1999. Mechanism of block and identification of the verapamil binding domain to HERG potassium channels. *Circ. Res.* 84, 989-998.

Sørhus, E., Incardona, J.P., Karlsen, Ø, Linbo, T., Sørensen, L., Nordtug, T., van, d.M., Thorsen, A., Thorbjørnsen, M., Jentoft, S., Edvardsen, R.B., Meier, S., 2016. Crude oil exposures reveal roles for intracellular calcium cycling in haddock craniofacial and cardiac development. *Scientific Reports* 6, 31058.

Sun, L., Zuo, Z., Chen, M., Chen, Y., Wang, C., 2015. Reproductive and transgenerational toxicities of phenanthrene on female marine medaka (*Oryzias melastigma*). *Aquatic Toxicology* 162, 109-116.

Vandenberg, J.I., Walker, B.D., Campbell, T.J., 2001. HERG K⁺ channels: friend and foe. *Trends in Pharmacological Sciences* 22, 240-246.

Varghese, A., 2016. Reciprocal modulation of IK₁–I_{Na} extends excitability in cardiac ventricular cells. *Frontiers in Physiology* .

Vehniainen, E., Bremer, K., Scott, J.A., Junttila, S., Laiho, A., Gyenesi, A., Hodson, P.V., Oikari, A.O.J., 2016. Retene causes multifunctional transcriptomic changes in the heart of rainbow trout (*Oncorhynchus mykiss*) embryos. *Environ. Toxicol. Pharmacol.* 41, 95-102.

Vornanen, M., 2016. The temperature dependence of electrical excitability in fish hearts. *J. Exp. Biol.* 219, 1941.

Vornanen, M., 1998. L-type Ca²⁺ current in fish cardiac myocytes: Effects of thermal acclimation and beta-adrenergic stimulation. *J. Exp. Biol.* 201, 533-547.

Vornanen, M., 1997. Sarcolemmal Ca influx through L-type Ca channels in ventricular myocytes of a teleost fish. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* 272, R1432-R1440.

Vornanen, M., Ryökkönen, A., Nurmi, A., 2002. Temperature-dependent expression of sarcolemmal K⁺ currents in rainbow trout atrial and ventricular myocytes. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* 282, R1191-R1199.

Vornanen, M., Shiels, H.A., Farrell, A.P., 2002. Plasticity of excitation–contraction coupling in fish cardiac myocytes. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 132, 827-846.

Yamauchi, A., Burnstock, G., 1968. An electron microscopic study on the innervation of the trout heart. *J. Comp. Neurol.* 132, 567-587.

Zaza, A., 2010. Control of the cardiac action potential: The role of repolarization dynamics. *Journal of Molecular and Cellular Cardiology* 48, 106-111.

Zeltser, D., Justo, D., Halkin, A., Rosso, R., Ish-Shalom, M., Hochenberg, M., Viskin, S., 2004. Drug-induced atrioventricular block: prognosis after discontinuation of the culprit drug. *Journal of the American College of Cardiology* 44, 105-108.

Figures

Figure 1. AP parameters measured in the current clamp experiments. (A) The first 150 ms of a rainbow trout ventricular action potential (AP) showing the parameters that were determined from the recordings. The continuous line shows the all-or-none AP and the dotted line the local passive response of the membrane. V_{rest} , resting membrane potential; CD, critical depolarization; V_{th} , threshold voltage; overshoot (OS) and amplitude (AMP) of AP. The maximum rate of depolarization (upstroke) ($+dV/dt$) and repolarization ($-dV/dt$) of AP were measured from the first derivative of the AP tracing. (B) The stimulus protocol of increasing current strengths (duration 4 ms) used to search the trigger level for APs. The strength of current just able to trigger an AP is the threshold current (I_{th}). (C) A typical response of a ventricular cell to increasing stimulus strength.

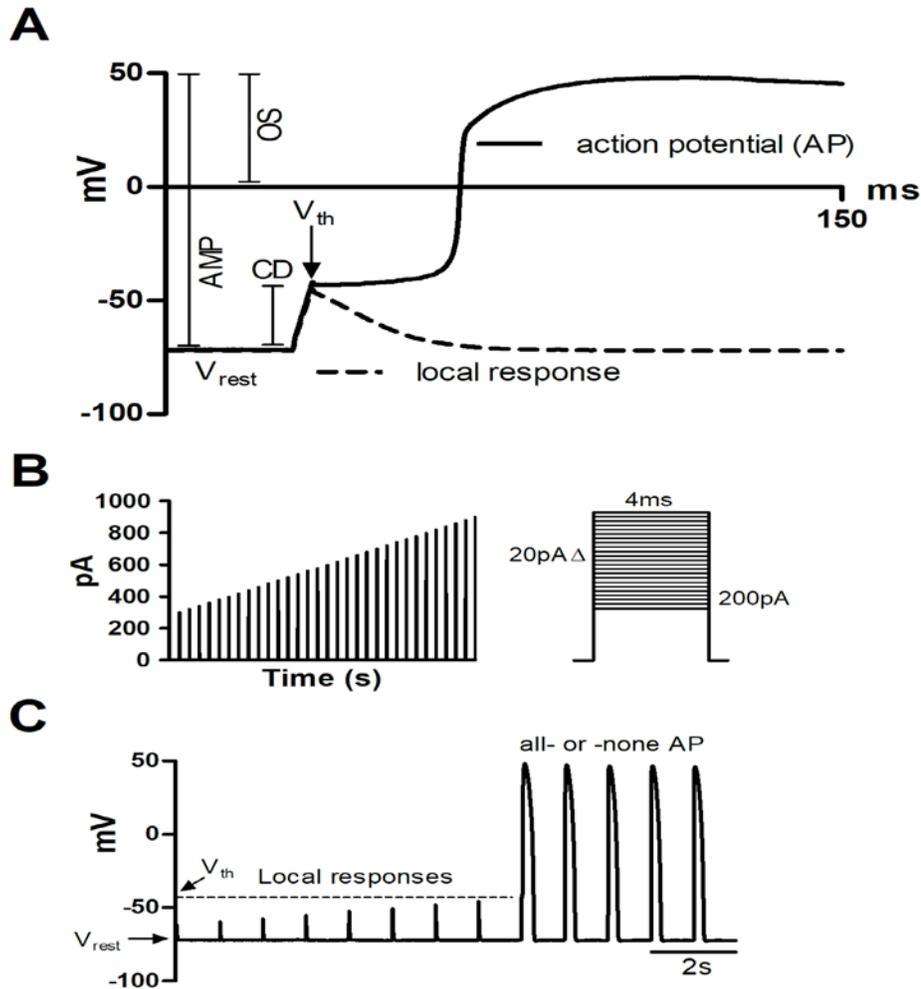


Figure 2. Phenanthrene modifies the shape of the action potential (AP) but has no effect on the AP duration in rainbow trout cardiac ventricle cells. (A) A representative experiment showing the effect of cumulatively increasing concentrations of phenanthrene on AP. (B,D,E) Phenanthrene has no effect on the resting membrane potential (V_{rest}), threshold voltage (V_{th}), AP amplitude (AMP), overshoot (OS), critical depolarization (CD), threshold current (I_{th}) and AP duration at 50% repolarization level (APD50). (C) Phenanthrene steepens both the maximum rate of AP depolarization ($+dV/dt$) and repolarization ($-dV/dt$). An asterisk indicates statistically significant difference from control.

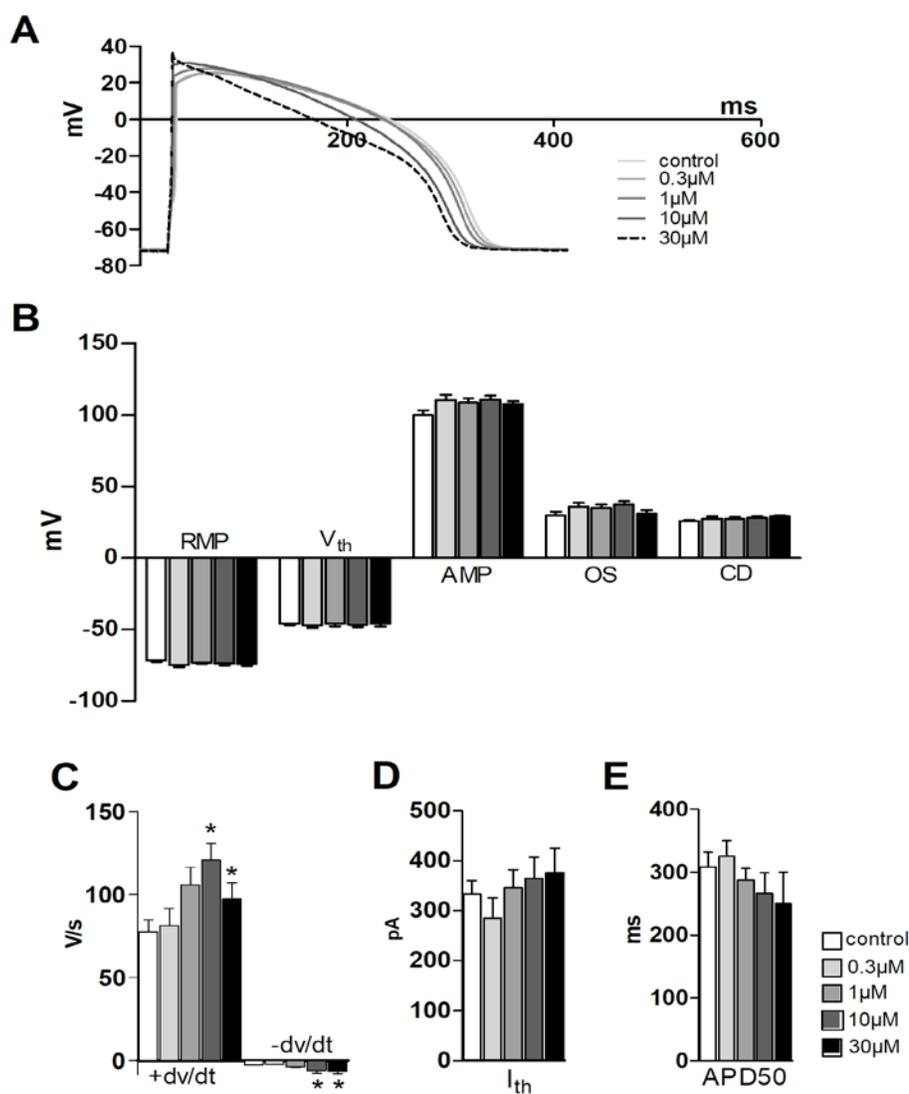


Figure 3. Retene shortens the action potential duration (APD) and modulates the shape of the AP in ventricular cardiomyocytes of the rainbow trout. (A) A representative experiment showing the effect of cumulatively increasing concentrations of retene on AP. (B,D) Retene has no effect on the resting membrane potential (V_{rest}), threshold voltage (V_{th}) and threshold current (I_{th}). (B) AP amplitude (AMP), overshoot (OS) and critical depolarization (CD) are increased by retene. (C,E) Retene shortens the AP duration (APD50) and steepens both the maximum rate of depolarization ($+dV/dt$) and the maximum rate of repolarization ($-dV/dt$) of the AP. An asterisk indicates statistically significant difference from control.

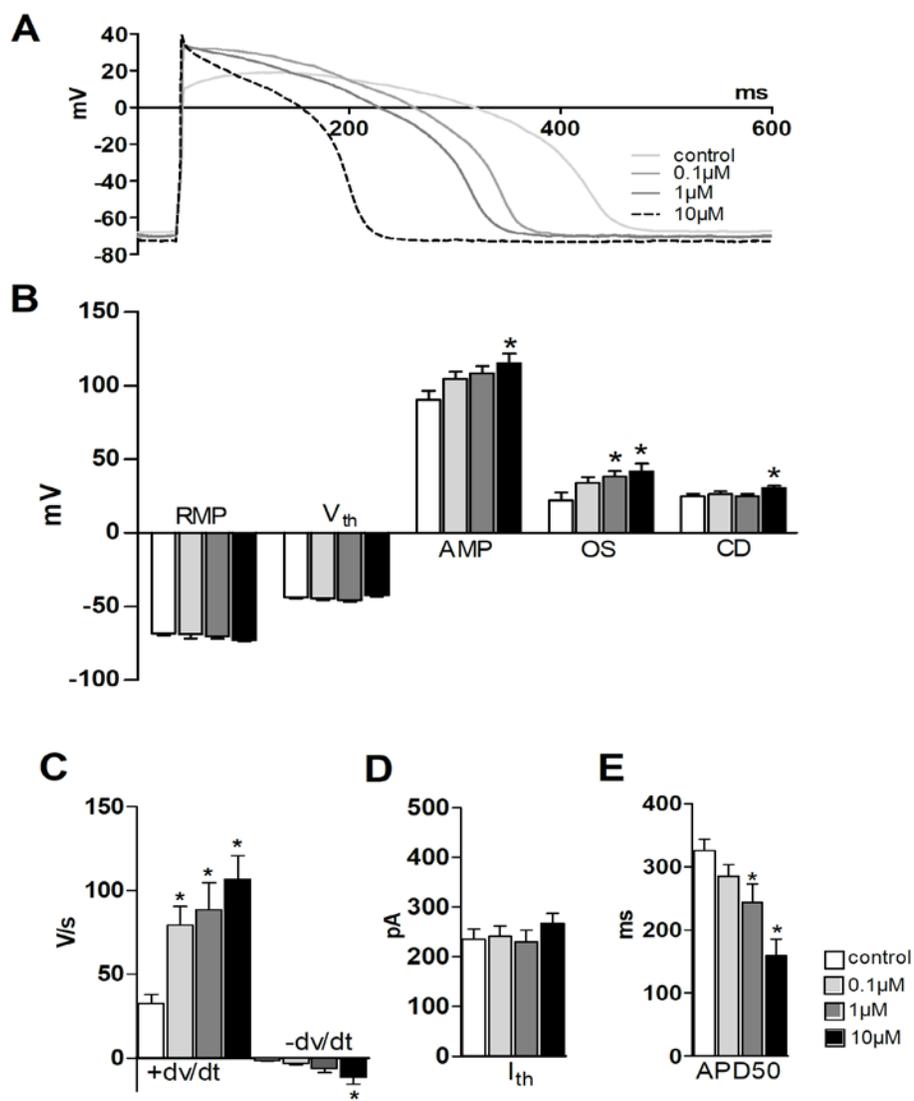


Figure 4. Phenanthrene and retene increase the fast Na^+ current (I_{Na}) in rainbow trout ventricular cardiomyocytes. (A) Current-voltage relationship of I_{Na} in the absence and presence of phenanthrene (left) and retene (right). The stimulus protocol is shown between the graphs. (B) Effects of phenanthrene (10, 30 μM) and retene (1, 10 μM) on the peak density of I_{Na} . The results are means \pm SEM of 12-14 myocytes from at least 3 animals. Groups denoted by the same letter do not differ significantly from each other.

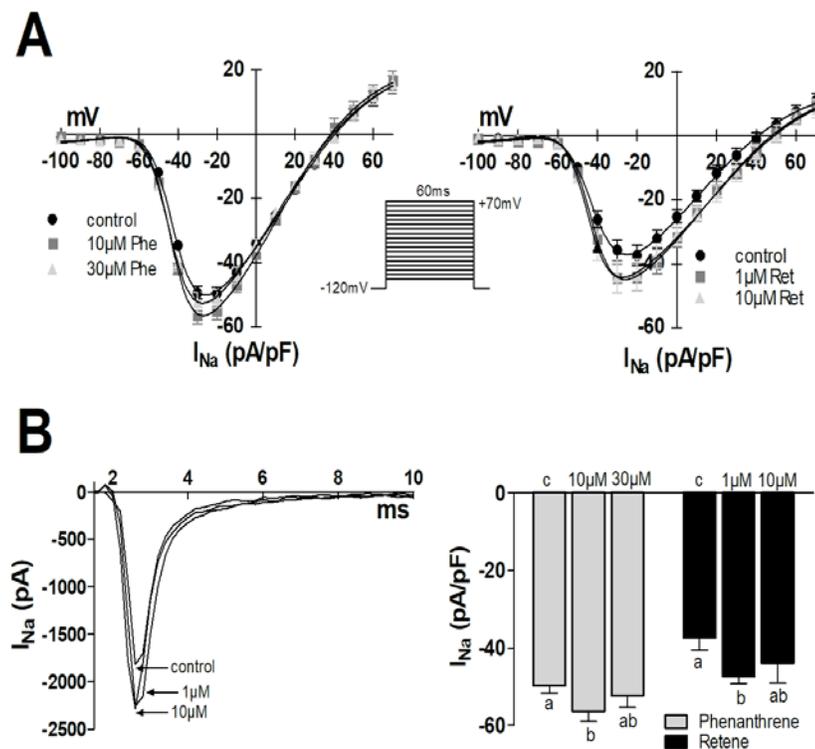


Figure 5. Phenanthrene and retene attenuate the L-type Ca^{2+} current (I_{CaL}) in rainbow trout ventricular cardiomyocytes. (A) A representative experiment on L-type Ca^{2+} current. Immediately after having access into the cell, I_{CaL} starts to increase due to the buffering of intracellular Ca^{2+} by EGTA (pipette solution is perfusing the cell from the inside). Then I_{CaL} stabilizes (b) and the cell is then cumulatively exposed to 1 (c) and 10 μM retene (d). (B) Fast time-based tracings of I_{CaL} under control conditions and in the presence of 1 and 10 μM retene at the positions shown by letters b, c and of the panel A. The stimulus pulse is shown below the tracings. (C) Effects of phenanthrene (10, 30 μM) and retene (1, 10 μM) on the peak density of I_{CaL} . The results are means \pm SEM of 12-16 myocytes from at least 3 animals. Groups denoted by the same letter do not differ significantly from each other.

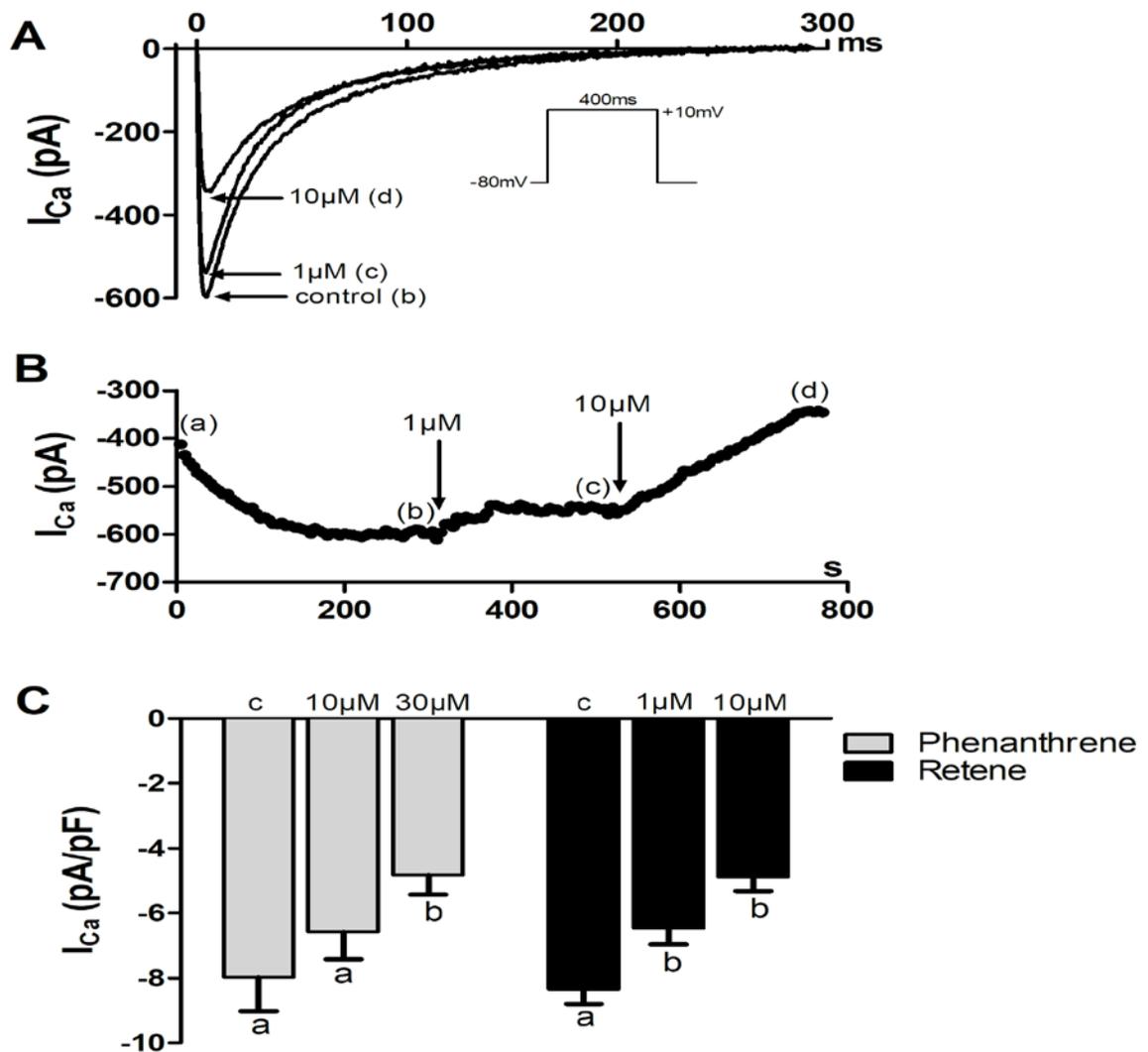


Figure 6. Phenanthrene and retene reduce the rapid component of the delayed rectifier K⁺ current (I_{Kr}) in rainbow trout ventricular cardiomyocytes. (A) A representative experiment showing the inhibitory effect of retene on I_{Kr} . The two-step stimulus protocol is shown on right. (C and D) Current-voltage relationship of the $I_{Kr,tail}$ during the repolarizing pulse at -20 mV in the absence and presence of phenanthrene (C) and retene (D). (E, F). Current-voltage relationship of the I_{Kr} during the depolarizing pulses from +80 to -60 mV in the absence and presence of phenanthrene (E) and retene (F). The results are means \pm SEM of 11-12 cells from at least 3 animals. An asterisk indicates statistically significant difference from control.

