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A rapid zebrafish embryo behavioral biosensor that is capable of detecting environmental β-blockers☆

Patrick T. Gauthier, Mathilakath M. Vijayan*

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

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ABSTRACT

β-Blockers (BB) are one of the most commonly prescribed pharmaceuticals used for treating cardiovascular and acute anxiety-related disorders. This class of drugs inhibit β-adrenoceptor signalling and given their growing, widespread use, BB are routinely detected in surface waters at nM concentrations. This is concerning as trace levels of BB impart developmental and reproductive dysfunction in non-target aquatic organisms, with potential for ecological risks. To date, environmental pharmaceutical risks to non-target animals are not part of the monitoring framework due to the lack of bioassays for assessing their biological effects. Behavioral endpoints have the advantage of a systems-level integration of multiple sensory signals and motor responses for toxicity screening; however, they are not currently used for risk assessment of environmental contaminants. The zebrafish (*Danio rerio*) embryo photomotor response (zfPMR) has been used in high-throughput behavioral screenings for neuroactive drug effects at high, therapeutic concentrations. Our objective here was to examine if we could utilize the zfPMR for screening environmental levels of BB. Embryos were placed into 96-well plates, exposed to chemicals and/or municipal wastewater effluent (MWWE), and their zfPMRs were measured with video-analysis. To specifically target BB, embryos were co-treated with isoproterenol, a β-adrenergic agonist that stimulates the zfPMR, and the inhibition of isoproterenol-induced response was used as a biomarker of BB exposure. Our results reveal that the inhibition of isoproterenol-stimulated zfPMRs can be used as a biosensor capable of detecting BB in the parts-per-billion to parts-per-trillion in water samples, including diluted MWWE. The method developed detects BB in spite of the presence of other neuroactive compounds in water samples. This systems level approach of rapid screening for BB effects provides the most promising evidence to date that behavioral neuromodulation can be potentially applied for environmental effects monitoring of pharmaceuticals.

1. Introduction

Beta-adrenoceptor antagonists (β-blockers; BB), including propranolol, metoprolol and atenolol are heavily prescribed (IMS HEALTH, 2013; QuintilesIMS. Pharmaceutical Trends, 2017) cardioprotective, antihypertensive, and psychotropic anxiolytic drugs (Helfand et al., 2009; Cecchi et al., 2002; Gorman and Dunn, 1993; Morilak et al., 2005; Bremner et al., 1996). BB are found in surface waters (Godoy et al., 2015) at concentrations sufficient to exert neurotoxic and reproductive dysfunction in aquatic organisms (Franzellitti et al., 2011; Feiner et al., 2014; Huggett et al., 2002; Lorenzi et al., 2012; Mitchell and Moon, 2016; Hedgesperth et al., 2016). Their effect on non-target animals (Wiklund et al., 2011; Ding et al., 2015) has restructured species composition in aquatic food webs (Oskarsson et al., 2014), entailing large-scale impacts on aquatic ecosystem health (Fent et al., 2006; Corcoran et al., 2010). Indeed, pharmaceuticals that modify behavior and reproduction have led to the collapse of entire fish populations in the wild (Kidd et al., 2007). These examples emphasize a real need to improve environmental effects monitoring of pharmaceuticals released into our environment, but currently there are no bioassays to monitor BB effects on aquatic species. Although BB target cardiovascular function in fish (Larsson et al., 2006), monitoring cardiac output in fish is neither easy nor sensitive enough to be applied as a bioindicator of environmental BB effects (Larsson et al., 2006). However, BB mediate psychotropic effects in fish at trace, environmentally-

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* Corresponding author.
E-mail address: matt.vijayan@ucalgary.ca (M.M. Vijayan).

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relevant concentrations (Lorenzi et al., 2012; Mitchell and Moon, 2016; Hedgesperth et al., 2016), suggesting the possibility of using non-invasive behavioral bioassay for screening the effects of environmental levels of BB in fish.

The zebrafish (*Danio rerio*) has garnered considerable interest for its application as a model organism for ecotoxicological applications, including neurotoxicity (Bambino and Chu, 2017). We recently proposed that zebrafish embryo and larval behaviors are suitable models for rapid screening of environmental contaminants (Gauthier and Vijayan, 2018). The zebrafish embryo photomotor response (zfPMR) has been used for high-throughput screening of psychotropic drugs for therapeutic use (Kokel et al., 2010; Copmans et al., 2016), including chemicals that bind to adrenoceptors (Gauthier and Vijayan, 2018; Kokel et al., 2010; Copmans et al., 2016). The zfPMR is an acute twitching of the embryo induced by photostimulation of opsin-based photoreceptors that lasts approximately 10 s and occurs between 30 and 40 h post-fertilization (hpf; Gauthier and Vijayan, 2018; Kokel et al., 2010; Kokel et al., 2013; Copmans et al., 2016). At this stage of development, the zebrafish hindbrain is innervated with aminergic neurons (McLean and Fetcho, 2004; Guo et al., 1999), with adrenergic, serotonergic, and dopaminergic neurons located in the hindbrain, midbrain, and forebrain, respectively (Guo et al., 1999). Furthermore, stimulation of neuronal innervations specifically in the hindbrain (i.e., location of adrenergic neurons) has been shown to promote the zfPMR (Kokel et al., 2013). Consequently, this positions the zfPMR as an ideal behavioral model for screening adrenoceptor activity. Moreover, Schwerte et al. (2006) demonstrated that adrenergic stimulation of cardiac function develops only later (4 dpf) in zebrafish, suggesting that the adrenergic stimulation in the brain is chiefly responsible for the zfPMR.

Although studies have examined the utility of zfPMR as a screening tool for drug discovery (Kokel et al., 2010; Copmans et al., 2016), these studies used high therapeutic concentrations of pharmaceuticals (Kokel et al., 2010; Copmans et al., 2016). There has been no study to date that has examined the effect of environmental levels of pharmaceuticals in municipal wastewater effluent on the zfPMR. These points led us to test if the zfPMR can be used as a biosensor for screening environmental levels of BB. The concept is that BB will affect the zfPMR, and the associated phenotype can be refined for use as a biosensor for detecting environmental levels of BB. This biosensor would eventually be linked with long-term performance dysfunction, so that changes in zfPMR will have predictive value for assessing long-term adverse outcomes. However, before undertaking this initiative, we needed to establish, as a proof-of-concept, whether the zfPMR was sensitive in detecting neuroactive effects of environmental levels of BB. To this end, we optimized the data acquisition of the zfPMR for a rapid screening platform and quantified the changes in zfPMR in response to major neuromodulatory compounds (e.g., aminergic and cholinergic) using the optimized protocol. A phenotypic biomarker for BB effect on the zfPMR was established, and this was further tested with environmentally-realistic BB concentrations. This zfPMR biosensor was successfully applied for detecting BB in environmentally-realistic dilutions of MWWE. Our results demonstrate the potential application of a fish behavior-based biosensor platform for detecting BB in environmental samples.

2. Materials and methods

2.1. Zebrafish culturing and embryo collection

Adult zebrafish were cultured in 10 L polypropylene tanks at 28.5 °C, pH 7.6, and 740 μS conductivity on recirculating systems (Pentair Aquatic Habitats, Apopka, FL). The recirculating systems were housed in an animal care facility at the University of Calgary with a 14-:10-h light:dark daily light cycle. Animals were fed with Ziegler™ adult zebrafish diet (Pentair) and live Artemia (San Francisco Bay Brand. Newark, CA) in the morning and evening, respectively. Zebrafish were bred in-system by transferring adults to 3 L tanks fitted with breeding inserts (Pentair) the evening before embryo collection. Embryos were collected the following morning and transferred to petri dishes filled with HEPES buffered E3 embryo medium consisting of 1.5 mM HEPES, 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and 0.31 μM methylene blue (Nüsslein-Volhard and Dahm, 2002). Methylene blue was added as an antifungal agent. Embryos were incubated at 28.5 °C in an air-jacketed incubator (VWR International, Edmonton, AB). A 50% embryo medium change was carried out the evening of embryo collection and following morning, and dead/unfertilized embryos were removed. 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.

2.2. Embryo zfPMR assay

The Zebrabox (Viewpoint Life Sciences, Montreal, QC) is an ideal behavioral system for high-throughput applications because it can simultaneously stimulate and quantify zfPMRs from all wells of a 96-well plates in only 30 s (Kokel et al., 2010). We 3D-printed a custom light emitting diode (LED; Super Bright LEDs, St. Louis, MO)-array for use with the ZebraBox to provide a light intensity of ca. 57,295 lux as per Kokel et al. (2010). Embryos were dechorionated at 24 hpf by agitation in a 10 mL solution of 1 g L^{-1} Pronase (Sigma-Aldrich, Oakville, ON; Westerfield, 2007) in a Petri dish until ~50% of embryos were dechorionated (ca. 6 min). Embryos were then transferred to a 200 mL glass beaker and rinsed 5 times with system water. Rinsing served to dechorionate the remaining chorionated embryos and wash off Pronase. Dechorionated and rinsed embryos were transferred to petri dishes filled with HEPES buffered E3 and embryos that were not developing normally were removed. At 28 hpf, embryos were transferred to the center 48 wells of a 96-well plate (Greiner, Sigma-Aldrich). Final well volume for all trials was 300 μL. Only the center wells were used for embryo behavioral tests to maximize magnification and resolution for video acquisition. Thus, each plate had 8 columns with 6 wells per column, and treatment groupings were randomly assigned to columns. For chemical trials, a minimum of 6 control wells (i.e., no chemical treatment) were included on every 96-well plate. Twenty min before behavioral trials, embryos were transferred to the ZebraBox and maintained at 28.5 °C. zfPMR trials were 30 s and were carried out in total darkness, with the exception of two 1 s light pulses at 10 and 20 s (Gauthier and Vijayan, 2018). Videos were recorded at 30 frames per second and activity was quantified as Δ pixel intensity from each frame using ZebraLab (Viewpoint).

2.3. Optimization of the zfPMR assay

We determined the ontogeny of the zebrafish embryo zfPMR between 29 and 34.5 hpf at 30 min intervals to determine peak responsiveness. Photomotor responses were measured using 4, 6, and 9 embryos per well, which were either chorionated or dechorionated. We compared chorionated and dechorionated embryos to determine whether the lack of the chorion may be better suited to increase the sensitivity of the bioassay. We also tested whether strain differences might affect the sensitivity of zfPMR by comparing 32 hpf TL, AB, and WIK strains of zebrafish.

2.4. Exposure to single neuroactive compounds

Stock solutions of all chemicals (Sigma-Aldrich) were prepared in de-ionized water (Millipore, Etobicoke, ON), and were kept at −80 °C and replaced weekly. Exposure durations ranged from 30 to 150 min for time-series experiments. Exposure durations for all other assays were 30 min. For all chemical exposures, 75 μL aliquots of chemical stocks were added to each well to achieve final nominal treatment concentrations. For control treatments, 75 μL aliquots of E3 were added. Dechorionated embryos were exposed independently to a range of concentration for isoproterenol (0.1, 1, 10, 20, and 100 μM) and propranolol (5, 50, and 100 μM), phenylephrine (100 μM), serotonin (100 μM), apomorphine (0.01, 0.1, and 25 μM), chlorpromazine (1 and 10 μM), acetylcholine (0.1, 1, and 100 μM), atropine (0.1, 1, and 100 μM), and mecamylamine (0.1, 1, 10, and 100 μM).

2.5. Isoproterenol inhibition exposures

Inhibition experiments were carried out as described above with the inclusion of binary mixtures of isoproterenol, BB, and BB mixtures. We chose concentrations of isoproterenol that elicited clear and robust stimulation of the zfPMR based on the concentration-series experiments described above. For initial characterization of BB inhibition, chorionated embryos were exposed to isoproterenol (100 μM), a range of atenolol (10,100, and 200 μM), metoprolol (1, 10, and 100 μM), nadolol (1, 10, and 100 μM), propranolol (0.01, 0.1, 1, 10, and 100 μM), and sotalol (10, 100, and 200 μM), and binary mixtures of isoproterenol (100 μM) with atenolol, metoprolol, nadolol, propranolol, or sotalol at each of the respective above mentioned concentrations. We then characterized the inhibition of isoproterenol by propranolol with dechorionated embryos exposed to isoproterenol (20 μM), propranolol (0.01, 0.1, 1, 10, and 100 μM) and binary mixtures of isoproterenol (20 μM) with propranolol (0.01, 0.1, 1, 10, and 100 μM). A simulated mixture of environmental BB concentrations was produced based on the environmental concentrations of BB reported previously (Godoy et al., 2015). The mixture contained atenolol (41.3 nM), metoprolol (38.3 nM), propranolol (2.28 nM), nadolol (0.2 nM), and sotalol (3.14 nM). The mixture summed to 87.7 nM propranolol equivalents based on molar mass of propranolol, and this molar equivalency was used to compare predictions from the propranolol concentration response curve to the effect of the BB mixture. We also tested if apomorphine would have an inhibitory effect on isoproterenol, as initial range-finder experiments suggested that apomorphine may supress the zfPMR. Therefore, we tested whether apomorphine inhibits isoproterenol-induced zfPMR by exposing dechorionated embryos to isoproterenol (20 μM), apomorphine (0.01 and 0.1 μM), and binary mixtures of isoproterenol (20 μM) and apomorphine (0.01 and 0.1 μM).

2.6. Exposure to MWWE

We obtained 24 h composite samples of City of Calgary MWWE on August 1st, 2nd, and 3rd, 2017. A 1 L subset of each sample was shipped on ice to SGS AXYS Analytical (Sydney, BC) for detection of acid soluble positive ESI pharmaceuticals and personal care products with LC MS/MS according to their MLA-075 method for List 5 compounds. Another 100 mL subset was retained for embryo exposures. Dechorionated embryos were exposed to isoproterenol (20 μM), MWWE (90% diluted), propranolol (1 μM), binary mixtures of isoproterenol (20 μM), MWWE (90% diluted), and propranolol (1 μM), and a tertiary mixture of isoproterenol (20 μM), MWWE (90% diluted), and propranolol (1 μM).

2.7. Statistical analyses

We used asymmetric Lorentzian mixed-modelling to characterize the zfPMR and contrast chemical-induced changes (Gauthier and Vijayan, 2018). The effects of treatments on the zfPMR were compared in terms of total embryo activity as well as temporal features of the response (Gauthier and Vijayan, 2018). Total embryo activities for each well from 96-well plates represent the summed Δ pixel intensity from frame to frame of the video recording for the zfPMR (i.e., between 10 and 20 s of the 30 s trial; Gauthier and Vijayan, 2018). Nonlinear mixed-modelling (nlme) was carried out using the 'nlme' function of the 'nlme' package in R 3.4.1 (Gauthier and Vijayan, 2018; Bates et al., 2015; Pinheiro and Bates, 2000; Pinheiro et al., 2017; R Core Team, 2018). An ANOVA was applied for each nlme model as an omnibus test to confirm the effect of treatment, and thereafter, Dunnett-style contrasts were used to distinguish treatment effects from control. For isoproterenol inhibition data, interaction terms were included in the nlme to detect interactive chemical effects (i.e., inhibition). The estimated interactive effects were then used to calculate % inhibition of isoproterenol's stimulation of the zfPMR. For statistical comparisons, columns on the 96-well plates were considered as replicates. For example, experiments involving 4 treatments had 2 columns per plate dedicated to each treatment (i.e., n = 2 per plate). For individual behavioral trials, all treatment groups were included on each plate, and plate was specified as a random effect in the nlme modelling (Gauthier and Vijayan, 2018). Estimated total activities during the zfPMR for each treatment group were further analysed with log-logistic mixed-models, where possible, with inter-plate variability included as a random effect. Single chemical exposure data are reported as % of control total activity ± standard error of mean (s.e.m). Inhibition data are reported as % inhibition of isoproterenol-stimulated total activity ± s.e.m.

3. Results and discussion

3.1. Optimizing the zfPMR for rapid measurements

We took several steps to optimize measurements of the zfPMR with the Zebrabox and considered embryo age, zebrafish strain, presence/absence of the chorion, the density of embryos in each well, and the type of data analysis which would be best suited for our intended purpose of developing a rapid bioassay. Ultimately, we found that changes in total activity (i.e., area under the modelled asymmetric Lorentzian curve) was the most reliable and sensitive metric in terms of β-adrenergic modulation of the zfPMR (Fig. S1). Beginning our zfPMR trials no earlier than 32 hpf assured a robust zfPMR, while still allowing for numerous plates to be screened on the same day (Fig. 1a and b). Increasing the well density from 4 to 6 embryos per well increased total activity of the zfPMR; however, increasing the well density from 6 to 9 offered no improvement (Fig. 1e–h). Variability in total activities among replicates was homogenous and distributed evenly about the mean (Fig. S2). Removing the embryo chorion increased the measurable magnitude of the zfPMR between 6- to 16-fold compared with chorionated embryos (Fig. 1e-l), which was most likely attributed to a greater range of movement for tail contractions in unconfined embryos, and clearer image acquisition due to the lack of a physical, albeit transparent, barrier (i.e., chorion). Dechorionation allowed us to obtain greater activity measurements, while also eliminating uncertainty regarding chorion permeability (Mandrell et al., 2012), and allowed for clearer and quicker assessment of embryo development for quality control. We found no significant differences among zfPMRs with different strains of zebrafish, including TL, AB, and WIK (Fig. 1c and d). Based on these results we used 6

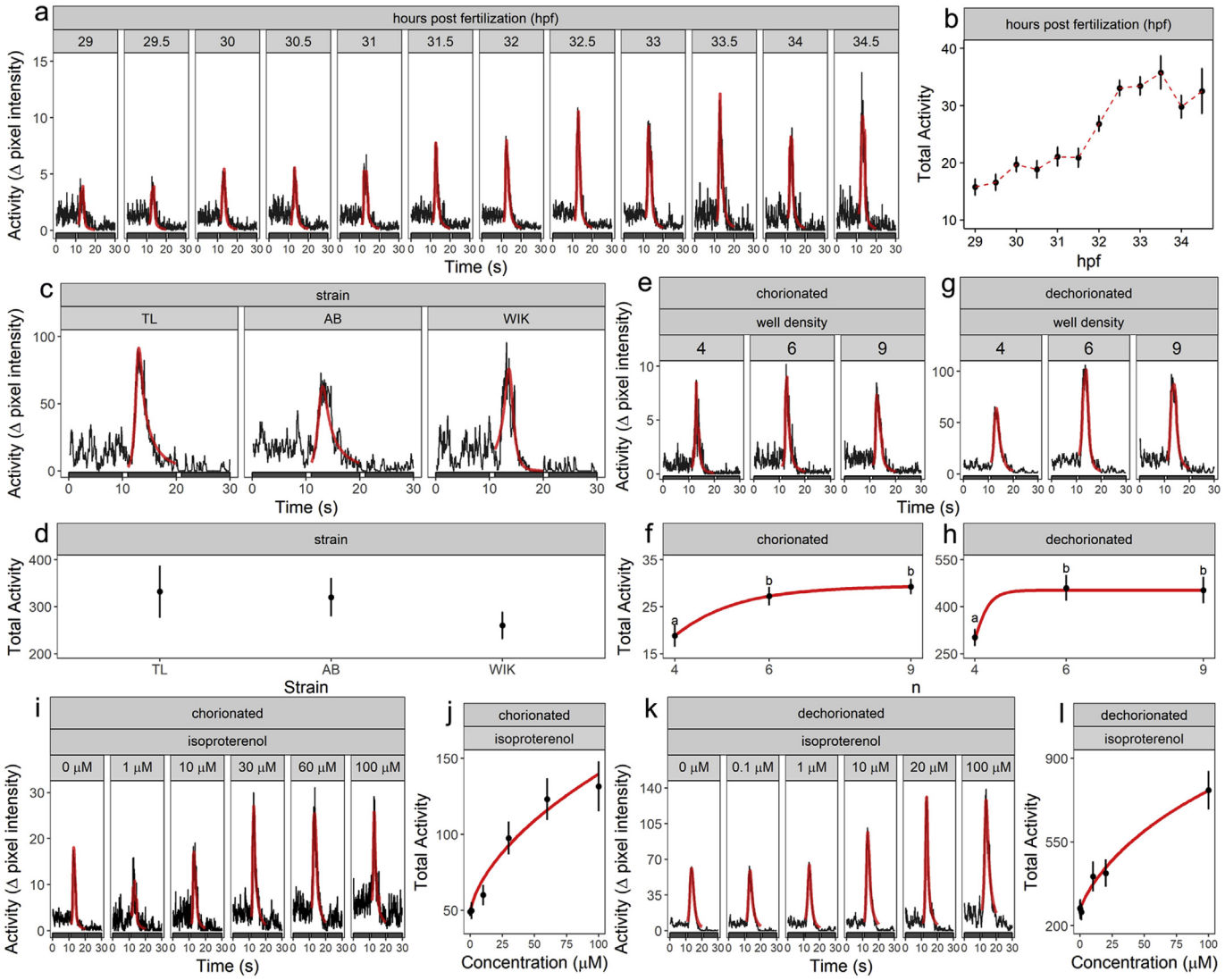

Fig. 1. Optimization of zebrafish embryo photomotor response (zfPMR) assays. a, b, The ontogeny of the zfPMR from 29 to 34.5 h post fertilization (hpf, n = 8 to 12). **c, d,** zfPMRs from different strains of zebrafish. There were no significant differences in total activity during the zfPMR among the strains ($F = 0.354$; $p = 0.70$; $n = 6$ to 10). **e, f, g, h,** Measured total activity during the zfPMR between wells with different densities of chorionated ($F = 5.39$, $p = 0.0046$; $n = 6$) and dechorionated embryos ($F = 8.77$, $p = 0.0002$; $n = 6$). Increasing embryo density from 4 to 6 increased measurable total activity proportionally (i.e., by ca. 1.5-fold; $t_{chorionated} = 2.59$, $p_{chorionated} = 0.0097$; $t_{dechorionated} = 3.76$, $p_{dechorionated} = 0.0002$). **i-l**, Stimulation of the zfPMR by isoproterenol for chorionated ($F = 9.93$; $p < 0.0001$; $n = 3$ to 9) and dechorionated ($F = 14.66$, $p < 0.0001$, $n = 3$ to 6) embryos. Measured total activities were 6-fold higher in dechorionated embryos. Grey (lights off) and white (lights on) bands represent the lighting stages of the PMR trials (a, c, e, g, i, k). Red curves represent nonlinear model predictions of activity during the zfPMRs (a, c, e, g, i, k), and log-logistic model predictions of total activity during the zfPMRs (f, h, j, l). Closed circles indicate mean total activities during the zfPMR ± s.e.m (b, d, f, h, j, l). Lettering indicates differences among densities at $p \leq 0.05$, t-tests on densities.

dechorionated TL strain embryos per well for developing the zfPMR biosensor. With these specifications, variability in zfPMRs from controls was acceptable over the span of experimental testing (i.e., within 2 standard deviations; Fig. S3).

3.2. β-adrenergic signalling modulates the zfPMR

To validate the reliability of β-adrenoceptor mediated effects on the zfPMR, we characterized the β-adrenergic stimulation and suppression on the zfPMR in concentration- and time-dependent experiments. Stimulation of β-adrenergic signalling by isoproterenol ($\beta_{1,2}$-adrenoceptor agonist) led to a stimulation of the zfPMR at concentrations > 1 μM, with concentrations of 100 μM nearly tripling the total activity of the zfPMR (Fig. 2a and b). Suppression of β-adrenergic signalling by propranolol ($\beta_{1,2}$-adrenoceptor antagonist) suppressed the zfPMR at concentrations > 5 μM (Fig. 2e and f). The suppression of isoproterenol-induced zfPMR by propranolol confirmed that β-adrenoceptor signalling stimulates the zfPMR (Fig. 2a, b, e, f). Isoproterenol (Fig. 2c and d) and propranolol (Fig. 2g and h) also acted rapidly at stimulating or supressing the zfPMR, respectively (Table S1). There was no clear benefit to exposing embryos for longer than 30 min, as clear effects were observed at 30 min. Moreover, exposing embryos after the zfPMR has already developed eliminates potential effects on development that may accelerate or delay the ontogeny of the zfPMR. Thus, shorter exposure durations ensured that our observations were indeed associated with a direct neuromodulatory effect of the drug on the zfPMR. The benefits of a short exposure duration lend perfectly to the high-throughput potential of the zfPMR in detecting BB, as environmental samples can be screened in as short as 30 min.

Our goal was to produce an assay that specifically detects the neuromodulation of the zfPMR by BB from complex mixtures,

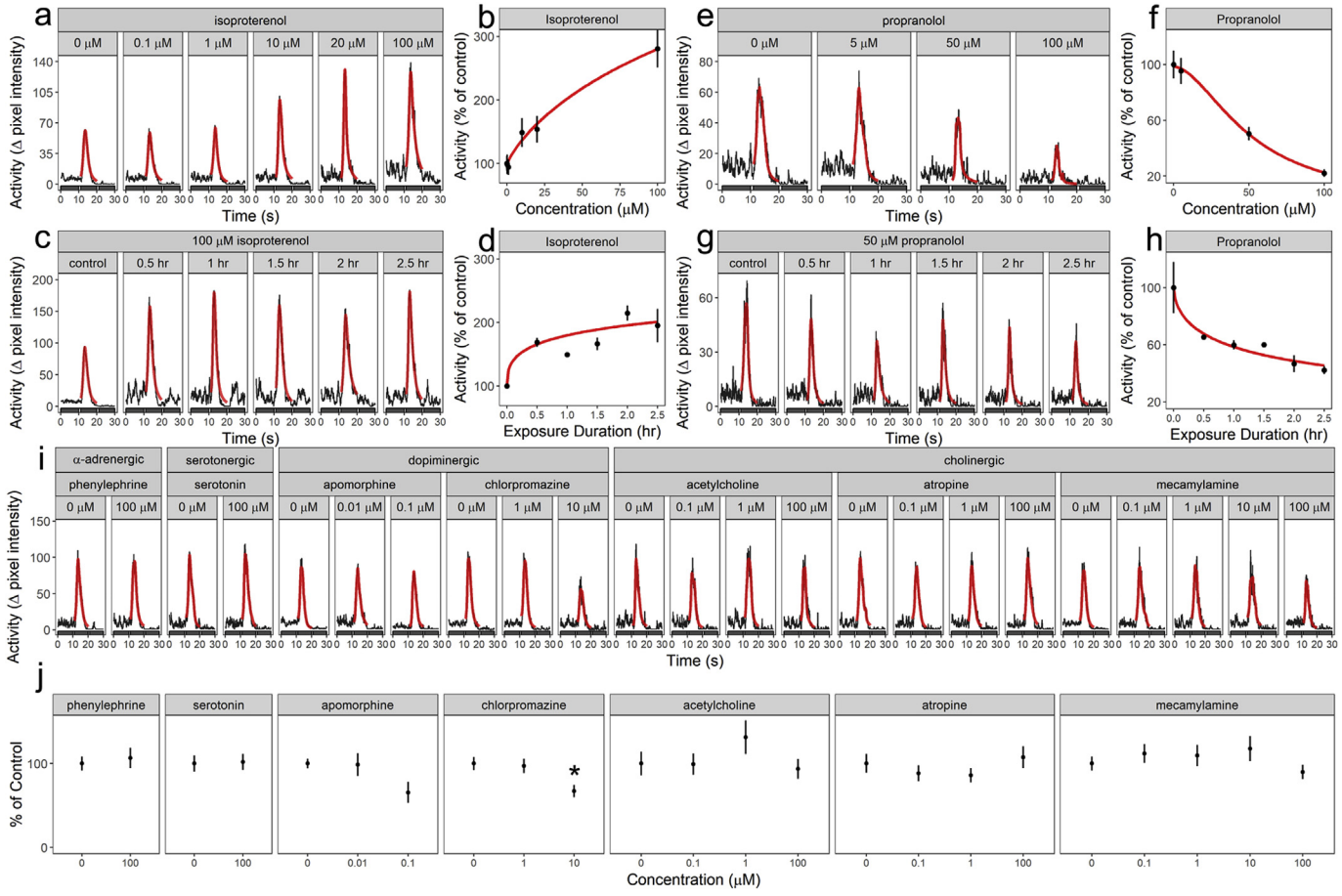

Fig. 2. Role of aminergic and cholinergic receptors in the zebrafish embryo photomotor response (zfPMR). a, b, e, f, Isoproterenol ($F = 14.66$, $p < 0.0001$, $n = 3$ to 6) and propranolol ($F = 27.47$; $p < 0.0001$; $n = 4$) concentration-dependent stimulation and suppression, respectively, of the zfPMR. **c, d, g, h,** Isoproterenol ($F = 28.12$, $p < 0.0001$, $n = 4$ to 8) and propranolol ($F = 8.37$, $p < 0.0001$, $n = 2$ to 3) time-dependent stimulation and suppression, respectively, of the zfPMR. Isoproterenol was effective following 30 min of exposure ($t = 5.65$, $p < 0.0001$) (**c, d**). Propranolol affected the zfPMR following 30 min of exposure ($t = -3.47$, $p = 0.0005$) (**g, h**). **i, j,** zfPMRs following treatment with aminergic and cholinergic compounds ($F = 2.09$, $p = 0.002$, $n_{serotonin} = 4$; $n_{phenyleprine} = 4$; $n_{apomorphine} = 4$; $t_{chlorpromazine10} = -2.25$, $p_{chlorpromazine10} = 0.025$, $n_{chlorpromazine} = 3$ to 5; $n_{acetylcholine} = 2$; $n_{atropine} = 4$; $n_{mecamylamine} = 3$ to 6). Grey (lights off) and white (lights on) bands represent the lighting stages of the behavioral trial (**a, c, e, g, i**). Red curves represent nonlinear model predictions of zfPMRs (**a, c, e, g, i**), and log-logistic model predictions of total activity during the zfPMRs (**b, d, f, h**). Closed circles indicate mean total activity during the zfPMR ± s.e.m (**b, d, f, h, i**).

including MWWE, that will undoubtedly contain other neuroactive compounds. Prior work has identified that numerous compounds that target a diversity of receptor classes, including adrenergic, dopaminergic, cholinergic and serotonergic, affect the zfPMR (Gauthier and Vijayan, 2018; Kokel et al., 2010; Copmans et al., 2016). Thus, it was necessary to demonstrate that the presence of other compounds would not mask the detection of BB. Although zebrafish hindbrain during the zfPMR window does not contain serotonergic (McLean and Fetcho, 2004) or dopaminergic (Guo et al., 1999) neurons, their stimulation may still affect the zfPMR downstream of the required neuronal stimulation in the hindbrain. Moreover, cholinergic stimulation, particularly at neuromuscular junctions, will also affect the zfPMR (Kokel et al., 2010; Copmans et al., 2016), but to what extent and how rapidly these effects will be observed remains unclear. Thus, we explored the influence of aminergic and cholinergic stimulation/suppression on the zfPMR. In addition to prior testing of β-adrenergic compounds, embryos were treated with serotonin (serotonergic receptor agonist), phenylephrine (α_1-adrenergic receptor agonist), apomorphine (dopaminergic receptor agonist), chlorpromazine (dopaminergic receptor antagonist), acetylcholine (cholinergic receptor agonist), atropine (muscarinic-cholinergic receptor antagonist), and mecamylamine (nicotinic-cholinergic receptor antagonist) for 30 min to explore the rapid effects of these compounds, with an emphasis on their effects at low concentrations. Serotonergic, α-adrenergic, and dopaminergic stimulation, and cholinergic stimulation/suppression did not influence the zfPMR after 30 min of exposure (Fig. 2i and j), and dopaminergic antagonism supressed the zfPMR only at high concentrations (Fig. 2i and j; Table S2). These trials further support a prominent role for β-adrenergic signalling in stimulating the zfPMR (Gauthier and Vijayan, 2018; Kokel et al., 2013).

3.3. The zfPMR is highly sensitive to the effects of BB

To demonstrate the sensitivity of the zfPMR to BB modulation, the potency of five commonly reported BB in the environment were characterized (Table S3). We were specifically interested in BB effects at environmentally realistic concentrations, as the ultimate goal of the assay is to screen environmental samples for BB. Atenolol, metoprolol, and sotalol had no effect on the zfPMR, and nadolol and propranolol were only effective at concentrations ≥1 (nadolol) and 10 (propranolol) μM. (Fig. 3a). However, BB have been designed specifically to antagonize β-adrenergic signalling. Consequently, rather than examining the direct effect of BB on the zfPMR, we hypothesized that BB are more potent at inhibiting the isoproterenol-stimulated zfPMR. To quantify and test the

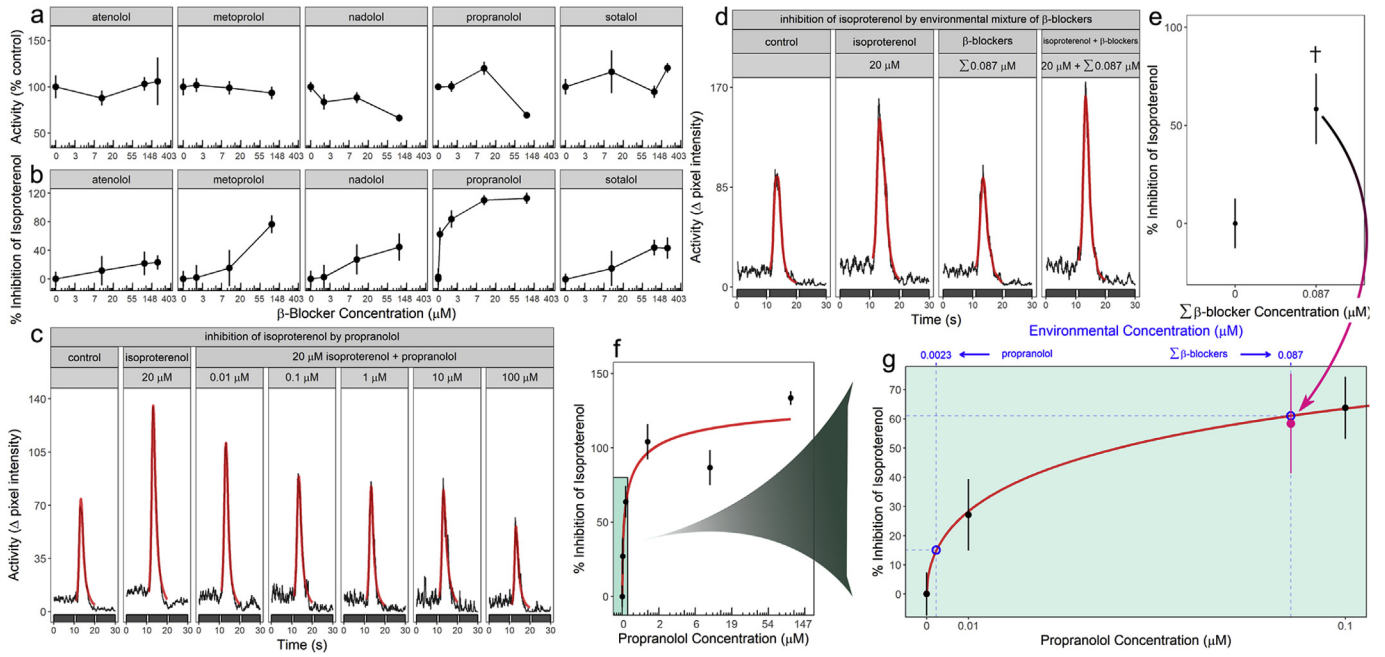

Fig. 3. Effect on β-blockers (BB) on the zebrafish embryo photomotor response (zfPMR). **a,** Direct effects of various BB on the total activity of the zfPMR ($n_{atenolol} = 4$; $n_{metoprolol} = 6$ to 10; $n_{nadolol} = 3$ to 7; $n_{propranolol} = 13$; $n_{sotalol} = 3$ to 8). **b,** BB concentration-dependent inhibition of isoproterenol-induced stimulation of the zfPMR ($n_{atenolol} = 12$; $n_{metoprolol} = 11$; $n_{nadolol} = 9$; $n_{propranolol} = 20$; $n_{sotalol} = 9$). Propranolol was the most potent inhibitor of isoproterenol. The lowest concentrations of propranolol we tested (0.01 μM) inhibited the isoproterenol-induced zfPMR by 35.7 ± 5.6% ($t = 4.19$, $p < 0.0001$). At 1 μM, propranolol was ~200 fold more potent at inhibiting isoproterenol than directly modulating the zfPMR. **c,** Inhibition of isoproterenol-induced stimulation of the zfPMR by propranolol ($F = 28.65$, $p < 0.0001$, $n = 19$). **d,** Characterization of the inhibition of isoproterenol by an environmentally realistic mixture of BB ($F = 7.12$, $p = 0.0077$, $n = 9$). **e,** The BB mixture inhibited the effect of isoproterenol-induced stimulation of the zfPMR (58.4 ± 17.7%). **f,** Propranolol concentration-dependent inhibition of isoproterenol-induced stimulation of total activity during the zfPMR. **g,** Magnified representation of propranolol concentration-dependent inhibition data with environmentally reported concentrations reported in blue on the upper x-axis. Inhibition of isoproterenol-induced stimulation of total activity during the zfPMR is overlain on top of the predicted inhibition data (pink closed circle ± s.e.m., **g**). Grey (lights off) and white (lights on) bands represent the lighting stages of the zfPMR (**c, d**). Red curves represent nonlinear model predictions of activity during the zfPMRs (**c, d**), and log-logistic model predictions of total embryo activity during the zfPMR (**f**). Closed circles indicated mean total activity as % of control ± s.e.m (**a**), and mean % inhibition of isoproterenol-induced stimulation of the zfPMR ± s.e.m (**b, e, f**). †$p \leq 0.05$, *t*-test on interactive effect (**e**).

inhibition of isoproterenol-stimulated zfPMR, we included interaction terms in the nonlinear mixed-models (Gauthier and Vijayan, 2018), and used the resulting estimated interactive effects to calculate % inhibition of the isoproterenol-induced zfPMR. Treatment with all the BB led to a concentration-dependent inhibition of the isoproterenol-stimulated zfPMR (Fig. 3b). Propranolol was the most potent BB tested and completely abolished the isoproterenol-induced zfPMR at 1 μM (Fig. 3a and b), and was ~200 fold more effective at inhibiting isoproterenol's effect on the zfPMR compared to its direct suppression of the zfPMR. Propranolol's relative potency may be related to its comparatively high logP; however, the chemical potency as a function of logP for all the chemicals we tested suggests logP may not be universally important in terms of effects on the zfPMR of dechorionated embryos at the concentrations and exposure durations we tested (Fig. S4). The potency of propranolol was further assessed by modelling the inhibition data to produce a predicted inhibition curve (Fig. 3f). Modelling predictions indicated that propranolol can inhibit the isoproterenol-induced zfPMR in the low μM to nM range (Fig. 3c, f), well within the ranges of concentrations that have been detected in environmental samples (Fig. 3g; Table S3). As environmental samples seldom contain BB in isolation (Godoy et al., 2015), we tested BB mixture based on concentrations reported in surface waters (Godoy et al., 2015), to determine their combined effect on the isoproterenol-stimulated zfPMR. The BB mixture inhibited the isoproterenol-induced zfPMR with similar potency as predicted (Fig. 3d, e, g; Table S4), proving that the zfPMR is sensitive at detecting BB mixtures at environmentally-relevant levels.

All other studies involving the zfPMR have involved observing global changes to the zfPMR induced by chemicals at pharmacological levels (Kokel et al., 2010; Copmans et al., 2016). However, we specifically quantify and qualify the interactive effects of mixtures of chemicals on the zfPMR (Gauthier and Vijayan, 2018). Moreover, only one study has attempted to test the sensitivity of the zfPMR to contaminants at environmental levels (Reif et al., 2016). Our results demonstrate the sensitivity of the zfPMR to a specific group of contaminants, BB, at concentrations reported in environmental samples. The use of antagonism of isoproterenol-stimulated zfPMR as our biomarker was critical to increasing the sensitivity of the biosensor to specifically detect BB in complex environmental samples. While other compounds present in environmental samples may influence the zfPMR (although this seems unlikely given our screening of major neuromodulators), the antagonism of the isoproterenol-mediated response is highly specific to BB (Pupo and Minneman, 2001) and forms the basis for our biosensor. Our inference on the presence of BB is based solely on supressing isoproterenol's effect (i.e., interactive effect), and the effect of samples/contaminants on their own (i.e., main effects) are accounted for when testing for interactive effects. The majority of the compounds we tested did not affect the zfPMR following rapid 30 min exposures, and our mixture studies with apomorphine revealed no inhibitory effects on isoproterenol-induced zfPMR (Fig. S5). This suggests that non-BB compounds at environmentally relevant concentrations likely do not interfere with the isoproterenol-induced zfPMR biosensor for detecting BB in environmental samples.

3.4. The zfPMR can detect trace BB effects in municipal wastewater effluents

Given that the BB at environmentally-relevant levels inhibited the isoproterenol-stimulated zfPMR, we moved forward with our testing and validated the response using a tertiary-treated MWWE. This undiluted MWWE contained a complex mixture of pharmaceuticals and personal care products, including the BB metoprolol (0.80 ± 0.01 nM) and propranolol (0.15 ± 0.003 nM; Table 1). A 90% diluted MWWE inhibited the isoproterenol-stimulated zfPMR by 34 ± 12% (Fig. 4a, c; Table S5). Based on the summed concentrations of metoprolol and propranolol that were measured in the undiluted MWWE, the zfPMR detected BB effects at concentrations as low as 0.095 nM. Because MWWEs may also contain other neuroactive compounds (Table 1), we compared the potency of 1 μM propranolol in inhibiting isoproterenol-stimulated zfPMR either in the absence or presence of 90% diluted MWWE. The inhibition of the isoproterenol-stimulated zfPMR by 1 μM propranolol was not affected by the MWWE (Fig. 4c, Table S5), suggesting that the concentrations of other chemicals in the diluted MWWE may not offset the BB effect, and that the zfPMR has the potential as a biomonitoring tool to detect BB in complex whole effluent samples.

3.5. Environmental application

Our results underscore the zfPMR as an excellent behavioral model for screening the neuroactive effect of BB on non-target organisms in our waterways. We have developed a workflow that has potential for high-throughput applications (Fig. 5), and optimized the age, embryo density, and exposure duration to provide the best signal to noise ratio in a 96-well plate format (Fig. 1). With this platform, water samples can be screened in 30 min to determine whether the BB in the samples can exert a neuromodulatory effect (i.e., capacity to alter fish behavior) at the systems level. In the context of effects monitoring, the capacity to assess neuromodulatory behavioral alterations offers substantial improvement over chemical analyses of environmental samples, which only inform the researcher of the presence or absence of a list of compounds. Moreover, chemical analyses require highly precise extraction processes catered to the chemical properties that differ among BB, whereas the zfPMR assay simply uses the water samples directly.

The agonist/antagonist interaction model we applied allowed for greater sensitivity and specificity in detecting BB in comparison to observing their direct effects, and was essential for detecting environmental BB concentrations. Isoproterenol is a non-selective β-adrenergic agonist and binds with both β_1 and β_2 receptors, and the BB we tested were either non-selective (e.g., propranolol, nadolol, and sotalol), or selective β_1-antagonists (e.g., atenolol and metoprolol). We found no clear evidence that the induction of the zfPMR was specifically modulated by either β_1 and β_2 receptor types, as all the BB we tested inhibited the isoproterenol-stimulated zfPMR. Moreover, there are virtually no pharmaceuticals with therapeutic application that are β_3 receptor specific. In this sense, the agonist/antagonist model is versatile in that it is not limited by receptor specificity of BB found in the environment. Moving forward, focussing on agonist/antagonist interactions may prove just as useful for screening environmental samples for different classes of pharmaceuticals.

Table 1
Municipal wastewater effluent composition. Measured concentrations of acid extractable positive ESI detection pharmaceuticals and personal care products, including β-blockers, in tertiary-treated municipal wastewater effluent (MWWE).

Compound	Minimum Detection Limits ± se (ng L^{-1})	Concentration (ng L^{-1})		
		MWWE 1	MWWE 2	MWWE 3
Alprazolam	0.92 ± 0.018	–	–	–
Amitriptyline	0.95 ± 0.0088	62.3	51.3	54.4
Amlodipine	4.62 ± 0.092	13.2	13.8	15.8
Benzoylecgonine	0.93 ± 0.0023	13.5	13.5	14.5
Benztropine	1.54 ± 0.029	–	–	–
Betamethasone	4.62 ± 0.092	–	–	–
Cocaine	0.48 ± 0.0079	2.31	2.79	2.81
DEET	2.46 ± 0.047	97	65.5	57.9
Desmethyldiltiazem	0.46 ± 0.0092	50	54.3	46.9
Diazepam	0.92 ± 0.018	–	–	–
Fluocinonide	18.47 ± 0.35	–	–	–
Fluticasone propionate	6.16 ± 0.12	–	–	–
Hydrocortisone	184.67 ± 3.53	–	–	–
10-hydroxy-amitriptyline	0.46 ± 0.0092	4.06	4.06	3.61
Meprobamate	12.33 ± 0.23	–	–	–
Methylprednisolone	12.33 ± 0.23	–	–	–
Metoprolol	19.17 ± 4.18	208	217	218
Norfluoxetine	4.62 ± 0.092	–	–	–
Norverapamil	0.46 ± 0.0092	2.45	2.68	3.11
Paroxetine	12.33 ± 0.23	–	–	–
Prednisolone	28.87 ± 4.24	–	–	–
Prednisone	61.57 ± 1.19	–	–	–
Promethazine	1.23 ± 0.023	–	–	–
Propoxyphene	0.92 ± 0.018	–	–	–
Propranolol	6.16 ± 0.12	40.6	38.4	41.4
Sertraline	1.23 ± 0.023	26.4	29.1	29.8
Simvastatin	61.57 ± 1.19	–	–	–
Theophylline	184.67 ± 3.53	–	–	–
Trenbolone	12.33 ± 0.23	–	–	–
Trenbolone acetate	0.92 ± 0.018	–	–	–
Valsartan	12.33 ± 0.23	141	120	137
Verapamil	0.46 ± 0.0092	17.4	12.8	21.3

'-' denotes none detected.

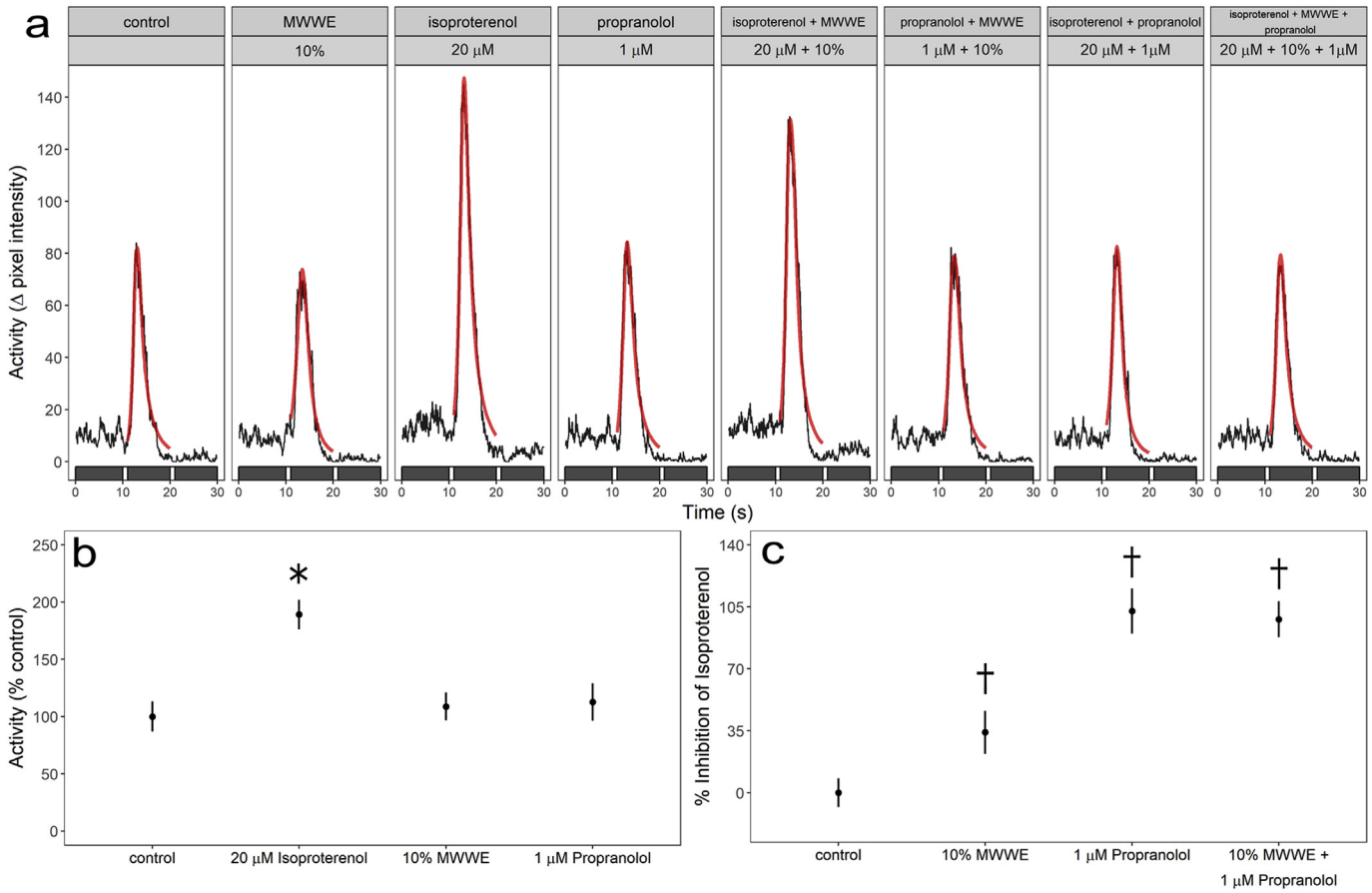

Fig. 4. Municipal wastewater effluent (MWWE) effect on zebrafish photomotor responses (zfPMRs). a, Characterization of the inhibition of isoproterenol by a 90% diluted MWWE ($n = 9$). **b,** The 90% diluted MWWE and 1 μM propranolol had no direct influence on the zfPMR ($F_{MWWE} = 0.88$, $p_{MWWE} = 0.35$; $F_{propranolol} = 1.84$, $p_{propranolol} = 0.18$). **c,** However, co-exposure to the 90% diluted MWWE inhibited the isoproterenol-induced stimulation of the zfPMR ($F = 6.30$, $p = 0.015$). Concurrent exposure to a binary and tertiary mixture of isoproterenol and propranolol ($F = 61.75$, $p < 0.0001$), and isoproterenol, propranolol, and a 90% diluted MWWE ($F = 4.29$, $p = 0.043$) also inhibited the isoproterenol-induced stimulation of the zfPMR, demonstrating that β-blockers (BB) are still effective in inhibiting isoproterenol when present in a complex environmental mixture. Results are from three independent experiments with three different MWWEs. Grey (lights off) and white (lights on) bands represents the lighting stages of the zfPMR (**a**). Red curves represent nonlinear model predictions of activity during the zfPMR. Closed circles indicate mean total activity as % of control ± s.e.m (**b**) and % inhibition ± s.e.m. (**c**). * *t*-test on individual effects. †$p \leq 0.05$, *t*-test on two- or three-way interactions.

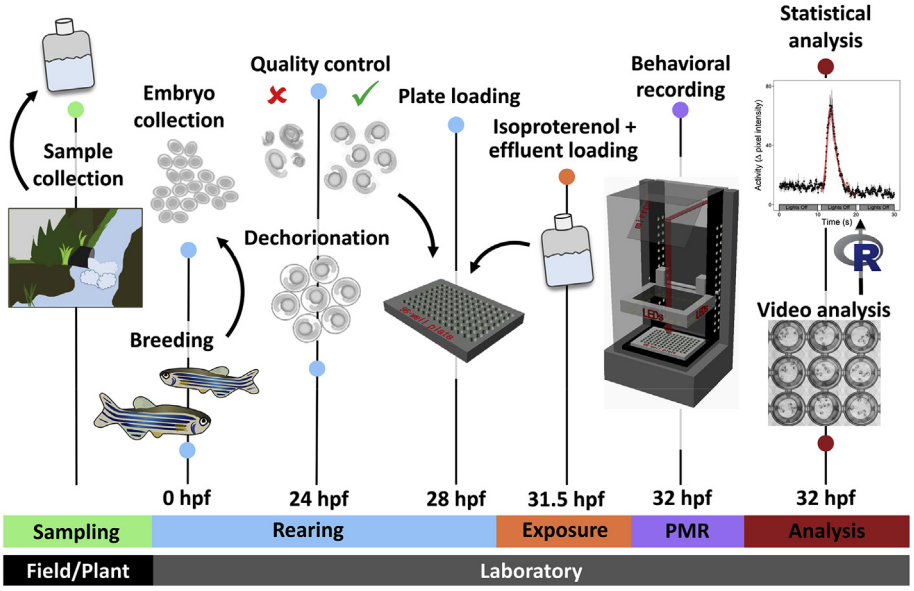

Fig. 5. Schematic of workflow for the rapid non-invasive β-blocker screening tool. Samples are collected in the field or from a plant (e.g., municipal wastewater treatment plant) and frozen or shipped immediately to the laboratory for analyses. Fish are bred and embryos are collected 24 h in advance of receiving the sample. At 24 h post fertilization (hpf), embryos are dechorionated and dead/malformed embryos are removed. At 28 hpf embryos are loaded into the 96-well plate with a density of 6 embryos per well. Embryos are exposed to isoproterenol and effluents for 30 m prior to photomotor response (zfPMR) trials, and transferred to the ZebraBox 20 min prior to zfPMR trials to acclimate in complete darkness. The zfPMR trials last 30 s and data can analysed within 10 m. With 20 m intervals between zfPMR trials, a total of 24 96-well plates per behavioural acquisition system can be screened within the 32–40 hpf zfPMR window.

3.6. Ecotoxicological relevance

Exposure to BB alters the behavior of fathead minnow (*Pimephales promelas*; Lorenzi et al., 2012) and European perch (*Perca fluviatilis*; Hedgesperth et al., 2016), two keystone species in North America and Europe. Behavioral changes in wild fish populations can have serious consequences for population health and sustainability (Kidd et al., 2007), emphasizing the need to increase our understanding and monitoring of BB toxicity. Here we show that BB can modulate zebrafish embryo behavior at nM concentrations in as little as 30min of exposure. Embryonic exposure to propranolol results in prolonged changes in the development of zebrafish catecholamine and behavioral responses into adulthood (Mitchell and Moon, 2016), indicating that exposure to BB during this critical stage of development may have long-term consequences in fish. Chemical modulation of the zfPMR has potential to predict developmental effects in larvae (Reif et al., 2016), positioning the zfPMR screening tool as an ideal model to screen for potential long-term consequences of BB exposure, as effective concentrations can be screened rapidly at ~30 h of development. The use of a systems level fish model to rapidly detect BB effects in complex environmental samples and provide predictions of long-term effects will be invaluable for effects monitoring and regulatory purposes. Thus, we intend to expand the predictive capacity of the zfPMR, and are currently working on linking whole lifecycle effects in zebrafish to the changes observed in the zfPMR.

We provide the most convincing evidence to date that behavioral endpoints can be molded to standardized toxicity protocols for ecotoxicological assessments of environmental levels of contaminants (i.e., BB). This work lays a solid foundation for continued development of behavioral models, specifically the zfPMR, as rapid screening tools for ecotoxicological applications (i.e., contaminant screening and effects monitoring). There are 1000's of compounds that effect the zfPMR at pharmacological doses (Kokel et al., 2010; Copmans et al., 2016), and the scope of the zfPMR as a screening tool for these compounds at environmentally-relevant levels remains to be determined.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2019.03.053.

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