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Alterations of larval photo-dependent swimming responses (PDR): New endpoints for rapid and diagnostic screening of aquatic contamination

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ABSTRACT

Detection and toxicity assessment of waterborne contaminants are crucial for protecting human health and the environment. Development of easy-to-implement, rapid and cost-effective tools to measure anthropogenic effects on watersheds are critical for responsible management, particularly in times of increasing development and urbanization. Traditionally, environmental toxicology has focused on limited endpoints, such as lethality and fertility, which are directly affecting population levels. However, more sensitive readings are needed to assess sub-lethal effects. Monitoring of contaminant-induced behavior alterations was proposed before, but is difficult to implement in the wild and performing it in aquatic laboratory models seem more suited. For this purpose, we adapted a photo-dependent swimming response (PDR) that was previously described in zebrafish larva. We first asked if PDR was present in other aquatic animals. We measured PDR in larvae from two freshwater prawn species (*Macrobrachium rosenbergii*, MR, and *Macrobrachium carcinus*, MC) and from another fish the fathead minnow (FHM, *Pimephales promelas*). In all, we found a strong and reproducible species-specific PDR, which is arguing that this behavior is important, therefore an environmental relevant endpoint. Next, we measured PDR in fish larvae after acute exposure to copper, a common waterborne contaminant. FHM larvae were hyperactive at all tested concentrations in contrast to ZF larvae, which exhibited a concentration-dependent hyperactivity. In addition to this well-accepted anxiety-like behavior, we examined two more: photo-stimulated startle response (PSSR) and center avoidance (CA). Both were significantly increased. Therefore, PDR measures after acute exposure to this waterborne contaminant provided as sensitive readout for its detection and toxicity assessment. This approach represents an opportunity to diagnostically examine any substance, even when present in complex mixtures like ambient surface waters. Mechanistic studies of toxicity using the extensive molecular tool kit of ZF could be a direct extension of such approaches.

1. Introduction

Water resources are continuously and increasingly stressed by human populations. Monitoring of contaminants in drinking water and watersheds represents an essential service of environmental public health (https://www.cdc.gov/nceh/ehs/10-essential-services/index.html), which is crucial for human health and for the environment (Watts, 2011; Halder and Islam, 2015; Torres et al., 2016; Gavrilescu et al., 2015; Brooks et al., 2009). Toxicity assessment of anthropogenic compounds has been performed with battery of *in-vitro* (Dreier et al., 2015; Judson et al., 2010) and *in-vivo* tests (Sipes et al., 2011; Padilla et al., 2012; Okamoto et al., 2015; Ong et al., 2014; Mesnage et al., 2015). This helps regulatory agencies to establish toxicity thresholds. However, most thresholds for specific chemicals are based on endpoints, like mortality and fertility, which have an extreme impact on a species survival (Padilla et al., 2012; Kovrižnych et al., 2013; Guilhermino et al., 2000; Martin and Young, 2001). Increasingly, new endpoints associated with more specific potential adverse outcomes have been examined, such as embryonic spontaneous movements (Wu et al., 2014), larval heartbeat and hatching rates (Huang et al., 2010),

Abbreviations: PDR, photo-dependent swimming response; CEC, chemicals of emerging concern; ZF, zebrafish; FHM, fathead minnow; MC, Macrobrachium carcinus; MR, Macrobrachium rosenbergii; PSSR, photo-stimulated startle response; CA, center avoidance; MCLG, maximum contaminant level goal; L, light; D, dark; dpf, days post-fertilization

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shelter seeking (Valenti et al., 2012), and molting (Zou, 2005) that allow detection of more subtle developmental disruptors. The needs for such tests is growing as new classes of chemicals are identified, often called contaminants of emerging concern (CECs), which have the potential to produce adverse effects even at nanomolar concentrations. Detection of CECs and other contaminants has advanced greatly with current analytical methods (Gavrilescu et al., 2015) and more adequate monitoring of aquatic environments (Noguera-Oviedo and Aga, 2016), but read-outs for physiological adverse effects are still lacking. Chronical contaminant exposure probably causes ecological and physiological alterations at concentrations far lower than the presently established thresholds. Thus, it is crucial to develop sensitive assays able to detect more subtle effects of aquatic contaminants.

Alterations of the fauna behavior has been proposed as an alternative to survival monitoring (Robinson, 2009). Contaminant-induced behavioral changes may serve as a more subtle readout for physiological and ecological fitness (Gerhardt, 2007). Within the broad spectrum of behaviors, anxiety-like behaviors are amongst those that can be quantified more readily, and numerous animal assays that were established for biomedical purposes could be adapted for environmental toxicology and diagnostic applications (Maximino et al., 2010; Clark et al., 2011). Increased anxiety-like behaviors are likely to impact survival in the wild by affecting key responses like escaping a predator, avoiding a threat, parenting, or searching for food (Brodin et al., 2014). Similarly, social interactions necessary for mating could be compromised (Pottinger and Carrick, 2001; Colwill and Creton, 2011; Ruiz-Gomez et al., 2008). Recently, innovative work showed altered behavior in adult wild perch exposed to psychiatric drugs because of uncontrolled urbanization (Brodin et al., 2013). However, these types of studies on animals chronically exposed in their natural habitat are extremely challenging and not easily amenable to diagnostic applications. Reproducing chronical exposure in a laboratory setting comes with the same shortcomings. Therefore, behavioral assays detecting changes after acute exposure would present clear advantages for rapid and cost-effective pollution assessment.

The genetic and developmental laboratory model, zebrafish (ZF, *Danio rerio*) is widely used for human disease modeling (Kalueff et al., 2014a, 2014b; Homberg et al., 2016; Brittijn et al., 2009; McCammon and Sive, 2015) and biomedical toxicological studies (Kalueff et al., 2016). Multiple parallel behavior measurements can be performed rapidly, thus enabling high-throughput screening (Reif et al., 2016). Statistical power is increased through adjusted sample size (Peng et al., 2015) and substances can be readily tested at multiple concentrations (Irons et al., 2010), which is indispensable for toxicological studies. Screens for small molecules (Rihel and Schier, 2012), neuro-active drugs (MacRae and Peterson, 2015), and mutations affecting locomotor (Behra et al., 2002) and visual systems (Neuhauss, 2003) were performed, but not applied to environmental research. Various photo-dependent swimming responses have been previously measured in ZF embryos and larvae (Kristofco et al., 2016; MacPhail et al., 2009; Burgess and Granato, 2007; Kokel and Peterson, 2011). In particular, a highly reproducible photo-dependent swimming response (PDR) was described in 6 day post fertilization (dpf) larvae (Kristofco et al., 2016; Torres-Hernández et al., 2016a). To be relevant for aquatic contamination and environmental adverse outcome detection, PDR should be a conserved behavior found in various aquatic species. To be a useful environmental diagnostic tool, PDR should be modulated by waterborne contaminants, especially after acute exposure.

To assess if PDR was a commonly found behavior in larval aquatic species, including in invertebrates, we measured it in two freshwater prawn species. *Macrobrachium rosenbergii* (MR) is extensively farmed and adults are routinely used in behavior studies (Vázquez-Acevedo et al., 2009; Kruangkum et al., 2015), thus they can be assimilated to a laboratory model (Muralisankar et al., 2016; Satapornvanit et al., 2009). *Macrobrachium carcinus* (MC), also called the big claw river prawn, is mostly found in the wild from Florida to southern Brazil, including most of the Caribbean islands (Holthuis and World, 1980). Next, we measured PDR in fathead minnow (FHM, *Pimephales promelas*) which is the model of choice for aquatic toxicology testing (Ankley and Villeneuve, 2006). We found a strong species-specific PDR in all animals tested, arguing that it is strongly conserved behavior thus, a physiological relevant endpoint usable for environmental contaminant toxicity assessment and detection.

To test PDR alteration after acute exposure to a waterborne contaminant, we exposed ZF and FHM larvae to copper (Cu), a common contaminant in watersheds. We found that PDR was significantly altered in both fish species. Animals were more active, FHM larvae at all concentrations tested and ZF larvae in a [Cu]-dependent manner. FHM seems more sensitive but ZF responses were strikingly more reproducible and robust, which would arguably make it the model of choice. Hyperactivity is a well-accepted measure of anxiety-like behavior (Peng et al., 2015), and so are photo-stimulated startle response (PSSR) (Burgess and Granato, 2007; Ray et al., 2009; Kimmel et al., 1974; Ellis et al., 2012) and center avoidance (CA) (Richendrfer et al., 2012; Ahmad and Richardson, 2013), which we also measured. PSSR and CA were also increased, therefore corroborating the observed hyperactivity.

Taken together, we established that PDR was a strong behavior found in two invertebrate and two vertebrate aquatic species. PDR was significantly altered by acute exposure to copper, in both fish species. Highly reproducible [Cu]-dependent hyperactivity in ZF larvae allowed us to further analyze two additional anxiety-like behaviors, PSSR and CA which were both significantly increased. Thus, contaminant-induced PDR-alterations are sensitive and relevant endpoints for diagnostic screening of aquatic contamination. This approach, when coupled with mechanistic studies of toxicity using the extensive molecular tool kit of ZF could be further used for understanding adverse outcomes of a contaminant present in complex mixtures like ambient surface waters.

2. Results

2.1. PDR is a highly reproducible larval behavior in crustacean *Macrobrachium rosenbergii* (MR) and *Macrobrachium carcinus* (MC)

To validate larval PDR as an appropriate and relevant endpoint for establishing an environmental diagnostic tool, we first asked if this behavior was present in other aquatic species. We tested two invertebrate species *Macrobrachium rosenbergii* (MR) and *Macrobrachium carcinus* (MC). We monitored the swimming activity of individual untreated crustacean larvae (stage 1 = 0–2-day post hatching) of MR (Fig. 1 orange lines and sur-imposed bars) and of MC (purple lines and bars). We recorded during 4 cycles of alternating light (L1 to L4, white boxes) and dark (D1 to D4, grey boxes) periods of 10 min each. We reported the mean travelled distance (in cm) per larva ($n = 72$) and per minute for 80 consecutive minutes, distinguishing two swim speeds: cruising (Fig. 1A and C) and bursting (Fig. 1B and D). Remarkably, both MR and MC showed a clear light dependent behavioral pattern, but inverted when compared to each other.

MR (orange lines) displayed in both swim speeds, high activity in the dark that was greatly lowered in the light. In dark (D1 to D4, dark boxes), MR larvae steadily increased activity to ~ 12 cm/min in cruising speed (Fig. 1A) and to ~ 1 cm/min in bursting speed (Fig. 1B). Activity was lowered in all light periods (L1–L4, light boxes) to ~ 8 cm/min in cruising (Fig. 1A) and ~ 0.5 cm/min in bursting (Fig. 1B) swim speeds. Remarkably, at all measured time points, there was a highly consistent response from larva to larva as illustrated by the small standard error (SEM, Fig. 1A and B orange error bars), which we also reported separately (Fig. 1C and D, superimposed orange bars), thus highlighting the strong reproducibility of this larval swimming behavior in MR.

MC (purple lines) displayed in cruising swim speed, high activity in