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Acute exposure to offshore produced water has an effect on stress- and secondary stress responses in three-spined stickleback *Gasterosteus aculeatus*

Anne Christine Knag [a,*], Annette Taugbøl [b]

[a] Department of Biology, University of Bergen, P.O. Box 7803, N-5020 Bergen, Norway
[b] Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biology, University of Oslo, P.O. Box 1066, Blindern, N-0316 Oslo, Norway

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ABSTRACT

Pollution is one of today's greatest problems, and the release of contaminants into the environment can cause adverse changes in vitally important biological pathways. In this study, we exposed three-spined stickleback *Gasterosteus aculeatus* to produced water (PW), i.e. wastewater from offshore petroleum production. PW contains substances such as alkylphenols (APs) and aromatic hydrocarbons (PAHs) known to induce toxicant stress and endocrine disruption in a variety of organisms. Following exposure to PW, a standardized confinement treatment was applied as a second stressor (PW-stress), testing how fish already under stress from the pollutant would respond to an additional stressor. The endpoint for analysis was a combination of blood levels of cortisol and glucose, in addition to transcribed levels of a set of genes related to toxicant stress, endocrine disruption and general stress. The findings of this study indicate that low doses of PW do not induce vitellogenin in immature female stickleback, but do cause an upregulation of cytochrome (CYP1A) and UDP-glucuronsyltransferase (UDP-GT), two biomarkers related to toxicant stress. However, when the second stressor was applied, both genes were downregulated, indicating that the confinement exposure had a suppressive effect on the expression of toxicant biomarkers (CYP1A and UDP-GT). Further, two of the stress related genes, heat shock protein 90 (HSP90) and stress-induced phosphoprotein (STIP), were upregulated in both PW- and PW-stress-treatment, but not in the water control confinement treatment, indicating that PW posed as a larger stress-factor than confinement for these genes. The confinement stressor caused an increased level of glucose in both control and PW-treated fish, indicating hyperglycemia, a commonly reported stress response in fish.

1. Introduction

The everyday operation of offshore oil and gas production is associated with large amounts of produced wastewater (PW). PW is a complex mixture that involves formation water from the reservoir in addition to man-added chemicals, and consists of thousands of compounds. The chemical composition varies both between oil- and gas fields and with time as the field matures but in general the main components are organic acids, alkylphenols (AP) and polyaromatic hydrocarbons (PAHs)(Neff, 2002; Neff et al., 2011). The water-soluble fraction of PW and its main components are known to disrupt steroidogenesis in vitro (Knag et al., 2013). The PW-components are quickly diluted in the marine environment, but especially PAHs and APs have been found to induce biological effects such as modifications of the endocrine system, also at low concentrations (Meier et al., 2007, 2011).

APs are a family of organic compounds known to have estrogenic effects on fish through various suggested mechanisms, for example by binding to the estrogenic receptor (ER), and thereby causing endocrine disruption. APs have been found to stimulate changes in important life-history traits in fish, such as inducing the egg yolk precursor protein vitellogenin (VTG) in male Atlantic cod *Gadus morhua*, decrease the levels of sex steroids in the blood of both male and females and delay ovary and testis development (Meier et al., 2007). APs are also known to cause transcriptional responses of VTG, for example in rainbow trout *Oncorhyncus mykiss* (Arukwe et al., 2001), that again can be linked to a reduced lifespan in the worm *Caenorhabditis elegans* (Murphy et al., 2003) and fathead minnow *Pimephales promelas* population decrease (Kidd et al., 2007).

PAHs are one of the most widespread organic pollutants and many naturally occurring PAHs are known to have mutagenic and carcinogenic effects, as well as possessing estrogenic and antiestrogenic activity (Santodonato, 1997). A commonly used biomarker of PAH exposure is upregulation of the enzyme cytochrome P450 1A (CYP1A), a protein

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; BT, β-tubulin; CYP, cytochrome P450 monooxygenase enzymes; EF, elongation factor; GAPDH, glyceraldehyde 3-phosphate dehudrogenase; GR, glucocorticoid receptor; HSP, heat shock protein; HPI, hypothalamic-pituitary-interrenal axis; PLA, phospholipase A2; PW, produced water; RIA, radioimmunoassay; rtPCR, real time polymerase chain reaction; SULT, sulfotransferases; STIP, stress-induced phosphoprotein; UDP-GT, UDP-glucuronosyltransferases; VTG, vitellogenin.

* Corresponding author. Tel.: +47 970 46 302; fax: +47 5558 4450.
E-mail address: anne.knag@bio.uib.no (A.C. Knag).

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that catalyzes the oxidation of a number of organic chemicals and is considered a sensitive biomarker for oil pollution, as previously reviewed by van der Oost et al. (2003).

Exposure to toxicants and altered physical conditions can evoke non-specific stress responses in fish (Barton, 2002). Activation of the hypothalamic-pituitary-interrenal (HPI) axis is one of the primary responses to stress, culminating in the release of the corticosteroid hormone cortisol, commonly used as an indicator of the degree of stress experienced by fish (Gamperl et al., 1994). One of the functions of cortisol is to increase the blood concentration of glucose, providing extra energy for a potential "fight or flight" reaction and enabling the fish to cope with increased energy demands associated with stress. Several studies describe pollutants to have the potential to modulate the HPI stress axis in fish, as reviewed by Pottinger (2003).

Secondary responses to a stressor are complex but include transcriptional upregulation of "stress proteins". Stress proteins are a group of proteins that are synthesized in response to stressors, and include glucose-regulated proteins and heat-shock proteins (HSP) (Moseley, 2000).

The physiological stress reaction is considered beneficial as it initiates advantageous behavioral responses, and thereby ensures that the individual again reaches homeostasis. However, if the intensity of the stressor(s) is overly severe or long lasting, the physiological response(s) themselves may become detrimental and affect overall survival of the individual through reduced growth and condition. Studies have shown that fish exposed to chronic chemical stressors display a decreased corticosteroid response to a second acute stressor (Hontela et al., 1992, 1995, 1997; Hontela, 1998; Cunningham et al., 2000), suggesting that pollutants may lead to an exhaustion of the cortisol-producing endocrine system, possibly as a result of prolonged hyperactivity of the system.

The aim of this study was to assess effects of exposure to PW by measuring both genetic and blood parameters as an indication of exposure to both pollution and stress. The three-spined stickleback was chosen as the model organism as it is a promising bioindicator species for ecotoxicity studies (Katsiadaki et al., 2007; Sanchez et al., 2007; Gao et al., 2011) and has recently been used in studies investigating the effect of pollutants on stress response in the field (Pottinger et al., 2013). The objectives of our study were to (i) assess how specific genes and blood parameters were affected by PW exposure, (ii) assess how specific genes and blood parameters were affected by an additional stressor-represented here as confinement stress, and (iii) assess the combined effect of PW and stress. By including a second stressor after exposure of PW we could test if PW-exposure caused a dysfunctional response in the established pathways, implying that fish would still be capable of responding to an additional stressor when in a PW-polluted environment. A short exposure time of 72 h was chosen, as exposure to produced water in the environment is not continuous, but rather chronic pulsed exposures of shorter durations.

2. Materials and methods

2.1. Fish and maintenance conditions

Adult three-spined stickleback (*Gasterosteus aculeatus*) were captured in local ponds in Bergen, Norway, in September 2011. After capture, the fish were taken into the lab and acclimatized to holding conditions for three months prior to the experiment in a large fiberglass holding tank. The fish were kept in flow-through sea water (34‰) at a constant 11 °C and the light was set to 12:12 h light:dark with in-between dimming. Oxygen saturation, salinity and temperature were constantly monitored throughout the experiment. The fish were fed two times a day with either frozen artemia or red mosquito larvae (Aleds aquarium AB, Sweden) throughout the acclimation and exposure period. The average length of the fish used in this study was 4.2 cm, implying that all fish were immature (<1 year old).

2.2. Experimental design and protocol

PW samples were obtained directly from the Oseberg C oil production platform in the Norwegian sector of the North Sea (60° 36′ N). On arrival at the University of Bergen (UiB) the PW was immediately subdivided into 10 L containers and frozen at −20 °C.

When starting the experiment, fish that appeared healthy were sorted into four 30 L glass aquaria supplied with flow-through seawater (SW) with two duplicated treatments: SW control (600 mL SW/h) or SW added 1% PW (600 mL SW/h and 7 mL PW/h), as shown in Fig. 1(a). Peristaltic pumps (Watson-Marlow 323) provided accurate delivery of SW and PW and the pumps were also manually controlled once every day during the experiment.

After 72 h of exposure, approximately half of the 40 fish in each aquarium were quickly netted out for sampling. These were the unstressed groups. The rest of the fish were transferred to group confinement beakers (180 mL) to experience confinement stress for 60 min (oxygenated and with continuous water monitoring with a water quality sensor) before sampling (see Fig. 1(b)).

Previous work on stickleback has confirmed that confinement activates the stress response (Pottinger et al., 2013) and is used here as a general activator of the stress axis (pilot testing of stickleback in confinement for 1 h had plasma cortisol levels up to 421.7 ng/mL compared to control fish taken directly from the holding tank where the same levels were 14.6 ng/mL, unpublished results). The fish were treated in strict compliance with the Animal Welfare Act under the Regulation of the Norwegian National Animal Research Authority, and there was no mortality during the period of the experiment.

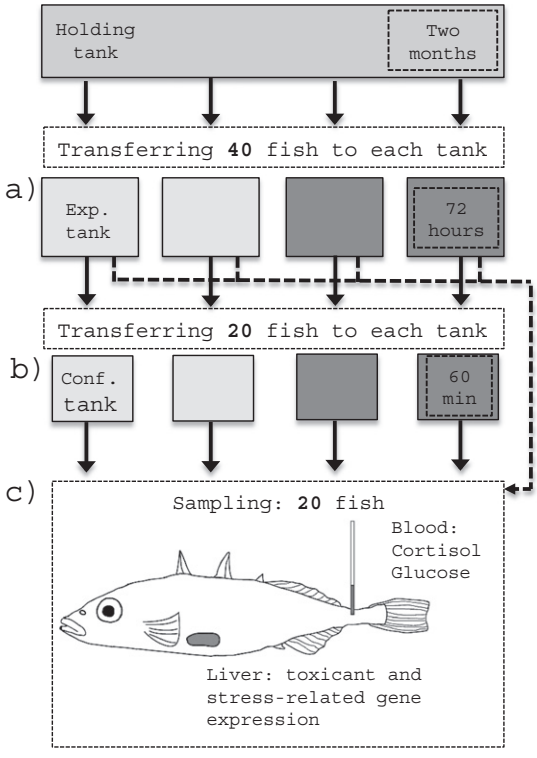

Fig. 1. Setup for experiment. Acclimated, wild caught sticklebacks were exposed to either salt water (light gray box) or 1% PW (dark gray box) for three days (a). The fish were then separated (b) into unstressed and stressed groups as the unstressed were sampled directly and the fish that were to become stressed were placed in confinement treatment for an additional 60 min. Sampled fish (c) was either harvested for blood (directly) or for mRNA (snap frozen). Further, the blood was analyzed for cortisol and glucose and eight genes related to AP, PAH and stress pathways were quantified from mRNA isolated from the liver.