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Effects of water accommodated fractions (WAFs) of crude oil in two congeneric copepods *Tigriopus* sp.

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ABSTRACT

Oil pollution has deleterious effects on marine ecosystems. However, the toxicity of crude oil towards Antarctic marine organisms has not been well studied. We compared the deleterious effects of water accommodated fractions (WAFs) of crude oil on reproduction, intracellular reactive oxygen species (ROS) levels, and antioxidant enzymatic activity in Antarctic (*Tigriopus kingsejongensis*) and temperate (*Tigriopus japonicus*) copepods. Reproductive rates of *T. kingsejongensis* and *T. japonicus* were significantly reduced ($P < 0.05$) in response to WAFs. Furthermore, *T. kingsejongensis* showed elevated levels of ROS and higher antioxidant enzyme (glutathione peroxidase [GPx]) activity than *T. japonicus* in response to WAFs. *CYP* genes from congeneric copepods were identified and annotated to better understand molecular detoxification mechanisms. We observed significant up-regulation ($P < 0.05$) of *Tk-CYP3024A3* and *Tj-CYP3024A2* in response to WAFs, suggesting that *CYP* genes may contribute to the detoxification mechanism in response to WAF exposure. These finding also suggest that WAFs may induce oxidative stress, leading to reproductive impairment in copepods. Furthermore, *Tk-CYP3024A3* and *Tj-CYP3024A2* genes can be considered as potential biomarkers of WAF toxicity in the congeneric copepods *T. kingsejongensis* and *T. japonicus*. This study will be helpful for enhancing our knowledge on the harmful effects of WAFs in Antarctic and temperate copepods and provides insight into the underlying molecular mechanisms.

1. Introduction

Antarctica is one of the most pristine and unpolluted marine ecosystems on earth. However, petroleum-related human interventions in recent years have increased concerns about the deleterious effects of oil pollution on Antarctic marine ecosystems (Aislabie et al., 2004; Oil Tanker Spill Statistics, 2009; Australian Maritime Safety Authority, 2012). Many accidental oil spills have occurred, and have resulted in severe damage to ecosystems (Cripps and Priddle, 1991; Ansaldo et al., 2005; Bargagli, 2008; Alexander et al., 2017).

Crude oil is a heterogeneous mixture and contains several compounds (i.e. polyaromatic hydrocarbons [PAHs], alkylated PAHs, and non-hydrocarbons) (Volkmann et al., 1994; Salar Amoli et al., 2006). Of these, PAHs are considered to be carcinogenic, and therefore, that can cause serious damage to diverse marine organisms (Xue and Warshawsky, 2005). Furthermore, PAHs can lead the formation of reactive oxygen species (ROS) that induce oxidative stress (Parti et al., 2009). For example, ROS generation was observed in the liver tissue of the fish *Carassius auratus* in response to phenanthrene (Yin et al., 2007). In the copepod *T. japonicus*, ROS was significantly increased in response to β-naphthoflavone (βNF) (Rhee et al., 2015). This stress has been identified as one of the reasons for its adverse effects on the normal physiologies (e.g. growth and reproduction) of marine organisms in laboratory studies (Peterson et al., 2003; Salar Amoli et al., 2006; Jernelöv, 2010). Water accommodated fractions (WAFs) of crude oil have been used in the laboratory setting to study the harmful effects of crude oil on model organisms. The acute toxicity of WAFs was evaluated in the key Arctic species; the copepod *Calanus glacialis*, juvenile Arctic cod (*Boreogadus saida*), and larval sculpin (*Myoxocephalus* sp.), and found that the relative sensitivity was similar without much discrepancies among the species (Gardiner et al., 2013). Furthermore, WAFs have been found to have adverse effects on survival, growth, and reproduction with alteration of cellular molecular responses in marine organisms like the marine medaka *Oryzias melastigma* (Mu et al., 2014), copepods *Calanus finmarchicus*, C. *glacialis* (Hansen et al., 2011), *Tigriopus japonicus* (Hwang et al., 2017), and *Paracyclopina nana* (Puthumana et al., 2017a). These studies suggest that WAFs have effects at multiple biological and ecological levels from molecules to

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individuals. More detailed information on oil toxicity and associated physiological and molecular alterations in marine invertebrates have been published in recent years (reviewed by Han et al., 2017). However, no previous study has compared the toxic effects of WAFs between Antarctic and temperate marine invertebrates.

Among marine invertebrates, copepods function as a bridge between producers and consumers, allowing transfer of energy and toxicants. They are therefore considered promising model species for environmental genomic and marine ecotoxicology (Theilacker and Kimball, 1984). The copepod genus *Tigriopus* is distributed worldwide, and the *T. japonicus* (temperate species) has been widely used as an appropriate laboratory model species of the marine environment due to the small size (< 1 mm), short life cycle (< 2 weeks), and easy maintenance (Raisuddin et al., 2007). Recently, the Antarctic copepod *T. kingsejongensis* (Copepoda: Harpacticoida: Harpacticidae) was considered as a suitable model species for Antarctic ecophysiological and ecotoxicological research (Park et al., 2014; Kim et al., 2016a, 2016b; Lee et al., 2016). In our previous study, we found that the Antarctic and temperate copepods *T. kingsejongensis* and *T. japonicus* had different susceptibilities to UV radiation by examining the expression of genes such as glutathione *S*-transferase (*GST*) and heat shock proteins (*Hsps*) (Han et al., 2016). Comparative toxicity studies of these congeneric species will likely provide a better understanding of molecular and ecological responses to marine pollution in these two climatic regions.

Cytochrome P450 (CYP) enzyme families are large and diverse enzyme groups associated with Phase I detoxification mechanisms in living organisms. They can recognize a wide variety of endobiotics and xenobiotics (e.g. drugs, chemicals, and hormones) as substrates (Snyder et al., 1998; Chaty et al., 2004). Furthermore, in the previous studies, *CYP* genes were used as biomarkers for environmental monitoring to detect oil spill and organochlorine pollution, as they were associated with detoxification mechanisms in marine invertebrates such as the copepods *T. japonicus* (Han et al., 2014a), *C. glacialis*, *C. hyperboreus* (Hoekstra et al., 2002) and *P. nana* (Han et al., 2015; Dahms et al., 2016) and the rotifer *B. koreanus* (Won et al., 2016). Thus, understanding the molecular response of *CYP* genes in Antarctic and temperate marine invertebrates to oil pollution is important to uncover the underlying detoxification mechanism and to determine if CYPs are suitable biomarkers for oil pollution in the polar region.

In this study, to determine if there was any difference in the toxic effects of WAFs on Antarctic versus temperate copepods, we investigated the reproduction rate and measured the cellular ROS level and enzymatic activity of GPx in *T. kingsejongensis* and *T. japonicus*. Furthermore, to evaluate the feasibility of using *CYP* genes as biomarkers to detect oil pollution in the Antarctic region, we identified 30 *CYP* genes from *T. kingsejongensis* and analyzed transcription of *Tk-CYP3024A3*, which was significantly elicited ($P < 0.05$) by WAFs (Han et al., 2014a). This comparative study will increase our understanding of the mechanistic effects of crude oil in Antarctic copepods.

2. Materials and methods

2.1. Culture and maintenance of Tigriopus kingsejongensis and T. japonicus

The copepods *T. kingsejongensis* (kindly provided by Dr. Sanghee Kim, Korea Polar Research Institute, Incheon, South Korea) and *T. japonicus* (collected from Haeundae beach (Busan, South Korea) were maintained at 8 and 25 °C for *T. kingsejongensis* and *T. japonicus*, respectively (Han et al., 2016). The detailed explanation of the culture condition and maintenance of *Tigriopus kingsejongensis* and *T. japonicus* are incorporated in the Supporting Information as described in our previous studies (Han et al., 2016). Species identity was confirmed by morphometric analysis followed by molecular characterization of the universal marker cytochrome oxidase 1 (*COI*) gene (Park et al., 2014).

2.2. Comparative effect of WAFs on reproduction

WAFs of Iranian crude oil were prepared in accordance with Aurand and Coelho (2005). To investigate the response of WAF exposure on the rate of reproduction in *T. kingsejongensis* and *T. japonicus*, one ovigerous female was transferred to 4 ml solution (0 [control], 20%, 40%, and 80% WAF), respectively in a 12-well cell culture test plate (SPL Life Sciences, Seoul, South Korea) and were maintained at 8 and 25 °C for *T. kingsejongensis* and *T. japonicus*, respectively. This procedure was performed for 10 replicates. Every 24 h, the number of newly generated nauplii was calculated using a stereomicroscope (SZX-ILLK200, Olympus, Tokyo, Japan) for 10 days. During the experiment time, 50% of the test solution was renewed and 10 µl of *Tetraselmis suecica* (~ 6×10^4 cells/ml) once per day were given as live feed.

2.3. Measurement of ROS and antioxidant enzyme activity

We measured ROS levels and glutathione peroxidase [GPx] activity in *T. kingsejongensis* and *T. japonicus* exposed to various concentrations of WAFs (0 [control], 20%, 40%, 60%, and 80%) for 24 h to assess WAF-induced oxidative stress. For the quantitative measurement, total protein was measured by the Bradford method (Bradford, 1976). The ROS was assessed by following our previous paper (Kim et al., 2011) and the detailed protocol is given as Supporting Information. Enzymatic activity of GPx was calculated by using a GPx cellular activity assay kit (Sigma–Aldrich Co, St. Louis, MO, USA) and the reduction in absorbance relative to the control was measured using a spectrophotometer (Ultrospec 2100 Pro, Amersham Biosciences, Cambridge, England).

2.4. Annotation and phylogenetic analysis of CYP genes

We searched RNA-Seq library of *T. kingsejongensis* (Kim et al., 2016a, 2016b) to obtain *CYP* gene sequences and the annotated genes were deposited in GenBank. To determine the phylogenetic position, we used amino acid sequences of CYPs from three copepods – *T. kingsejongensis*, *T. japonicus*, and *P. nana* – using ClustalW followed by phylogenetic analysis using MrBayes (WAG-model, version 3.1.2) and viewed by TreeView version 1.6.6 module of PHYLIP (Han et al., 2015; for detailed protocol see Supporting information).

2.5. Acute toxicity tests in response to WAFs

Acute toxicity test (96-h) was performed using 10 adult copepods and exposed to test solutions (4 ml in a dish) with various concentrations of WAFs (0 [control], 40%, 60%, 80%, and 100%) prepared in ASW in triplicate under confined laboratory conditions used for acclimation. Copepods were not fed during the experiments. Test solutions were renewed once every 48 h. Mortality was recorded once every 24 h. From the percentage mortality and WAFs concentration, LC10-96h and LC50–96h values were calculated using Probit analysis (ToxRat® Ver.2.09, ToxRat Solutions GmbH, Alsdorf, Germany).

2.6. Effect of WAFs on transcription of CYP genes

We exposed ~ 30 copepods to various concentrations of WAFs (0 [control], 40%, 60%, and 80%) for different periods of time (0, 6, 12, 24, and 48 h) to assess the mRNA transcription level of *Tk-CYP3024A3* gene. Extraction of total RNAs and quantitative real-time RT-PCR (q RT-PCR) were performed in accordance with our previous studies (Han et al., 2014a) and according to the manufacturer's instructions. Detailed protocols were appended as Supporting information. All qRT-PCR experiments were performed in triplicate and the primers used are given in Table 1. Relative expression patterns of *Tk-CYP3024A3* gene mRNA in terms of fold difference was evaluated using the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001).