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Effects of Bisphenol A on the Expression of CYP1A Transcripts in Juvenile False Clown Anemonefish (*Amphiprion ocellaris*)

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ABSTRACT

Bisphenol A (BPA), used in the manufacturing of various plastic products, is widely distributed in the marine environment and significantly impacts aquatic wildlife. In this study, juvenile false clown anemonefish (*Amphiprion ocellaris*) was used as a model species for low-dose BPA exposure, and to examine the cytochrome P450 1A gene (*cyp1a*) as a potential biomarker. Fish were exposed to BPA at a range of concentrations for up to 48 hours. Quantification of BPA in the exposed animals revealed that accumulation was significantly higher in the liver than in the muscle. Therefore, liver tissue was selected for further study. In addition to this, the selection of an appropriate reference gene for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was investigated. Our results showed that ribosomal protein l8 gene (*rp18*) was the most stable reference gene, compared to 18S ribosomal RNA gene (18S *rRNA*) and elongation factor 1α gene (*EF-1α*). This was used as an internal control in the qRT-PCR experiments. Following exposure to BPA, *cyp1a* expression significantly increased in a dose- and time-dependent manner: after 24 hours exposure to 50 ng/l BPA, it increased 2.82-fold; and after 12 hours exposure to 100 ng/l BPA, it increased 3.44-fold. A low dose of BPA rapidly induces c*yp1a* expression in this sentinel species. Thus, *cyp1a* could be used as a potential biomarker for BPA exposure.

Keywords: biomarker; BPA; clownfish; *cyp1a*; qRT-PCR

INTRODUCTION

Aquatic pollution, as a result of human activities discharging harmful substances into the marine environment, is an issue affecting many countries globally (Soyano *et al*., 2010). One significant pollutant is bisphenol A (BPA) (Environmental Protection Agency, 1992; Erler & Novak, 2010). BPA, an endocrine-disrupting chemical (EDCs), is used as a monomer in the production of polycarbonate plastic (PC). PC is used in various materials such as food containers, baby bottles, and epoxy resins, as well as a protective lining for canned food and beverages (Li *et al*., 2017: Mirzajani *et al.,* 2017). The European Union and Canada banned the use of BPA in food and beverage containers after 1 March 2011, mainly targeting baby bottles. In humans, BPA can mimic female hormones and lead to adverse effects such as developmental delays in infants and children, reproductive disorders, and an increased risk of cancer (Erler & Novak, 2010). BPA is also toxic to aquatic life, inducing the synthesis of vitellogenin in multiple fish species (Canesi & Fabbri, 2015). It has been shown to cause reduced sperm quality, growth suppression, delayed or inhibited ovulation, and delayed hatching in the brown trout (*Salmo trutta f. fario*) (Lahnsteiner *et al*., 2005) and juvenile rainbow trout (*Oncorhynchus mykiss*) (Aluru *et al.,* 2010). Many researches have reported that BPA is acutely toxic to aquatic organisms in concentrations as low as 100 µg/l, in both freshwater and marine species (Alexander *et al.,* 1988; Kang *et al.,* 2006). BPA exposure could result in changes to aquatic populations through alteration of sex ratios (Drastichová *et al*., 2005; Chen *et al*., 2015).

Aquatic systems are the end-destination of a variety of EDCs, including BPA. Recent studies have revealed the distribution and accumulation of BPA in rivers and coastal oceans: BPA in coastal waters of China, Japan, and the Netherlands, was found in concentrations between 12 and 608 ng/l (Flint *et al*., 2012; Xu *et al*., 2015). In the polluted coastal area of the Gulf of Thailand, Wonapha beach, Chonburi, it was 37.13 ± 2.70 ng/l (Ocharoen *et al*., 2018). Accumulation of BPA occurs in various marine bivalve

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species (i.e., *Perna viridis, Mytilus galloprovincialis*) (Ocharoen *et al.*, 2018), and teleost fish (i.e., *Oryzias latipes* and *Pimephales promelas*) (van der Oost *et al*., 2003; Lee *et al*., 2015). Therefore, BPA is potentially transferred to humans through bioaccumulation via the food web (van der Oost *et al*., 2003).

The main quantitative analytical techniques for determining BPA concentrations, both in aquatic systems and animal tissues, are High-Performance Liquid Chromatography (HPLC) and Liquid Chromatograph-Mass Spectrometry (LC-MS). These two techniques can achieve high precision and sensitivity; however, they cannot determine the effects on the organism itself. Therefore, biological indicators, or biomarkers, are more suitable analytical tools for monitoring environmental pollutants (van der Oost *et al*., 2003; Zheng *et al*., 2014). Biomarkers can be used to determine the effects of contamination at the level of tissues, proteins, and/or genes. The most sensitive method to quantify gene expression changes is quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) (Stephenson, 2016). This technique measures either absolute- or relative-quantification of gene expression. The latter requires at least one reference gene with a constant expression level to minimize errors in the comparative analysis (Hellemans *et al*., 2007; Kim *et al*., 2008; Boonphakdee *et al*., 2019). The expression of a gene relies on external factors, such as the type, duration, and route of chemical exposure (Boonphakdee *et al*., 2019), and biological factors such as the species, age, and sex of the organism (Vandesompele *et al*., 2002; Nicot *et al*., 2005; Boonphakdee *et al*., 2019). Another aim of this study, therefore, is to find a suitable reference gene for the qRT-PCR from the commonly used candidate housekeeping genes; 18S ribosomal RNA (18S *rRNA*), elongation factor-1α (*EF-1α*), and ribosomal protein l8 (*rpl8*).

The *cyp1a* gene is widely used as a biomarker of metabolic response to many toxicants. Numerous studies have used *cyp1a* expression as a biomarker for pollution in fish species such as Japanese medaka (*Oryzias latipes*) (Kim *et al*., 2008), marine medaka (*O. melastigma*) (Kim *et al*., 2013), Atlantic salmon (*Salmo salar*) (Olsvik *et al*., 2017), and common carp (*Cyprinus carpio*) (Fisher *et al*., 2006; Salvo *et al*., 2012; Karaca *et al*., 2014). Previous researches have predominantly used freshwater fish as model species, while marine fishes are only occasionally studied. However, BPA accumulation in coastal water is significantly higher than in freshwater systems (Ocharoen *et al*., 2018). Of the marine teleosts, the false clown anemonefish (*Amphiprion ocellaris*) is a native species to Thai coastal waters. This study focuses on using *A. ocellaris* as a model species to evaluate candidate gene transcripts, potentially expressed in response to BPA. The advantages of using this fish include its small size; being an undifferentiated hermaphrodite during immature stages; easy to breed in captivity; and easily maintained in the laboratory (Madhu *et al*., 2012). Therefore, this study uses *cyp1a* expression in the false clown anemonefish as a potential biomarker of BPA exposure and a model study of the BPA pathway from the environment to aquatic wildlife.

MATERIALS AND METHODS

Experimental animals

Juvenile (3 months old) *A. ocellaris* (*n = 150*) were purchased from the Coastal Aquaculture Research and Development Regional Center (Samutsakhon, Thailand). They were transported to a hatchery facility within the Department of Aquatic Science, Faculty of Science, Burapha University. They were then acclimatized for one week in a 500-L flow-through seawater tank before the start of the experiment. The light cycle was set for 12 hours of light and 12 hours of dark. The water salinity was 30 ppt, the temperature was maintained at 25 ± 2°C, and water pH was between 8 and 8.5 throughout the experiment. Fish were fed twice daily with commercial fish diet pellets (NRD G8, INVE Aquaculture, Thailand). Health monitoring, feeding, tank siphoning, and oxygen measurements were performed daily. The animal care and protocols used in this study were reviewed and approved by the Research Ethics Committee, Burapha University (Approval ID # IACUC 040-2561).

Bisphenol A (BPA) exposure

The chemical BPA (> 98% purity) was purchased from Sigma-Aldrich, Switzerland. *A. ocellaris* (0.34 ± 0.07 g body weight) were transferred to glass aquarium tanks (11 x 19 x 13 cm, five fish per tank, 10 tanks per treatment level, 30 tanks total) filled with water appropriate for their needs (as mentioned above), and given three days to acclimate in order to minimize potential variables. This is to test the degree of BPA uptake in liver and muscle, as well as the effect of exposure time on expression of *cyp1a* at three different concentrations (0, 50, 100 ng/l). A random sampling of six fish (three per tank) were removed from each set of BPA concentration at 0, 6, 12, 24, and 48 hours. After each sampling, the fish

were sacrificed with a lethal dose of anesthesia (100 µg/l MS-222; Sigma-Aldrich, Switzerland). Total length (mm) and body weight (g) were measured for each individual. Liver and muscle samples were excised and then immediately flash frozen on ice and stored at -80˚C until extraction of BPA and RNA. Of those exposed to 0 and 100 ng/l of BPA for 6 hours, liver and muscle tissues were collected and analyzed for BPA concentration by HPLC analysis. The time intervals chosen were selected to determine a full and detailed curve of the degree of BPA uptake after initial exposure. The higher BPA accumulated tissue was then subjected to be used in BPA induced *cyp1a* expression analysis.

BPA accumulation analysis

The BPA content was measured in the liver (0.09 g ww) and muscle (0.3 g ww) of *A. ocellaris* using a method previously reported by Gatidou et al. (2010). Briefly, dissected tissue samples were submerged in ultrapure water (3 ml), and methanol (5 ml) then boiled for 30 min at 50˚C. After centrifugation, the supernatant was collected and diluted with ultrapure water to a final volume of 100 ml. After the extraction of BPA from fish tissue, 100 ml was added to the solid phase extraction (SPE) cleanup using a C18 Sep-Pak cartridge (Waters Corp., Milford, MA, USA), which was activated before use with 6 ml of methanol and 4 ml of ultrapure water. The flow rate of the water sample was adjusted to 5 ml/min. The cartridges were washed with 5 ml of 5% methanol and dried under a nitrogen gas stream for 5 min. Then, the cartridge was eluted with 3 ml of methanol and injected into the HPLC for BPA analysis.

The concentration of BPA in the tissue samples was measured in triplicate by HPLC (Waters Alliance® e2695 Separations Module, Milford, MA, USA). The method was the modified protocol of Zhou *et al*. (2017). The BPA extract (10 µl) was injected into SunFire C18 reverse phase columns (4.6 × 250 mm, 5 µm; Waters Corporation, Ireland). The column temperature was held at 30˚C, and the UV detection wavelength was set at 280 nm. The mobile phase was a mixture of methanol and ultrapure water (75:25 v/v), with a constant flow rate of 1 ml/min. Data was collected using the Empower 2.0® software package (Waters, Milford, MA, USA). The standard calibration curve was performed using a series of six concentrations (0.5, 1, 5, 10, 100, and 500 ng/ml) prepared from the analytical standard. The correlation coefficients (r^2) of the calibration curve was linearity, and the acceptable value was greater than 0.995. Statistical differences between experimental groups were assessed by one-way analysis of variance (ANOVA) (Bewick *et al.*, 2004), followed by Duncan's *post-hoc* test (McHugh, 2011). All experimental data are shown as the mean ± standard error of the mean (SEM). Differences were considered statistically significant at $p < 0.05$.

RNA extraction and cDNA synthesis

Total RNA was extracted from the liver using Trizol® Reagent (Invitrogen, USA) following the standard procedure. The RNA was treated with DNase (Thermo Fisher Scientific, USA) to eliminate genomic DNA contamination. Total RNA was quantified by light absorption wavelengths at 230, 260, and 280 nm ($A_{230/260}$, $A_{260/280}$) using a spectrophotometer (Eppendorf, Germany). RNA with an $A_{230/260}$ ratio of 2 or higher, and an $A_{260/280}$ ratio between 1.9 and 2.2 were used in further analyses. After RNA quality was determined, cDNA was synthesized from 500 ng of total RNA using a RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions, and stored at -20˚C until use.

qRT-PCR analysis

PCR reactions were conducted in 96-well plates using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). Each reaction contained 1 x SYBR® master mix (Thermo Fisher Scientific, USA), 1 µl of cDNA template, 0.2 µM primer for *cyp1a*, and 0.5 µM primer for 18S *rRNA*, *EF-1α*, and *rpl8* in a final volume of 15 µl. Primer sequences used in the qRT-PCR reactions are shown in Table 1. The primer pairs amplified PCR products between 102 and 348 bp long. The thermal profile of the reaction was as follows: initial incubation for 10 min at 95˚C, followed by 40 cycles of 15 sec denaturation at 95˚C, 20 sec annealing at 55˚C, and 30 sec at 72˚C. A melt curve analysis was performed to confirm the specific products after the amplification cycles were completed under the following conditions: 95˚C for 15 sec, 60˚C for 1 min, and 95˚C for 15 sec. To check the size and purity of the obtained amplicons, 3 µl aliquot of each PCR product was loaded onto a 0.8% agarose gel containing ethidium bromide (EtBr) and visualized on a UV detector (Water, USA). The threshold cycle (Ct) value for each gene was exported and analyzed using the StepOnePlus™ software (Applied Biosystems, USA). In addition to the melt curve analysis, standard curves for each gene were constructed using serially diluted plasmid DNA

templates. This ensures that each gene has similar and near 100% amplification efficiencies and whose expression is not affected by the study's PCR reaction components. The amplification efficiency (E) of each gene was calculated according to the equation: $E = (10^{(-1/slope)}-1) \times 100$ (Brankatschk *et al*., 2012). Linear curves produced for each gene had efficiencies of 90 – 105% and $R^2 = 0.99$.

Table 1 List of primer sequences used for the qRT-PCR analysis in this study**.**

Gene	primer sequence (5'-3')	Product size (bp)
18S *rRNA*	Fw: CGAGGAATTCCCAGTAAG	102
	Rw: CTCACTAAACCATCCAATC	
EF-1α	Fw: GGCTGGTATCTCCAAGAACG	240
	Rw: GTCTCCAGGATGTTGTCWCC	
rpl8	Fw: CCGAAACCAAGAAGTCCAGA	199
	Rw: ACAGGGTTCATAGCCACACC	
cyp1a	Fw: ACAAGGACAACATTCGTGACA	348
	Rw: TGCAGTGAGGGATAGTGAAGG	

Expression stability analysis of the genes

The threshold cycle (Ct) data of three reference genes (18S *rRNA*, *EF-1α*, and *rpl8*) was used to analyze the gene expression stability. Gene ranking was determined using three approaches: *BestKeeper* (Pfaffl *et al*., 2004), delta Ct (ΔCt) method (Silver *et al*., 2006), and *NormFinder* (Andersen *et al*., 2004). The Ct data entered in *NormFinder* was previously transformed to relative quantities (RQ) following $RQ = (1+E)^{\Delta Ct}$ and ΔCt = lowest Ct value of all samples - Ct value of the specimen (Livak & Schmittgen, 2001). The *BestKeeper* and ΔCt method used untransformed Ct values. Recommended rankings were obtained using geometric averages of the rankings generated for individual approaches.

***cyp1a* transcription level analysis**

mRNA levels were determined relative to the transcription levels of the reference gene, taken from the most stable gene in the expression stability analysis. The quantification of the *cyp1a* transcripts was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Data were statistically analyzed with the normality and homogeneity of variances, using the Kolmogorov-Smirnov one-sample test and Levene's test, respectively. A one-way ANOVA was used to examine mean differences between more than two groups, followed by Duncan's *post-hoc* test. The critical value for statistical significance was $p < 0.05$.

RESULTS

BPA accumulation in tissues of false clown anemonefish

A. ocellaris were exposed to BPA at a concentration of 100 ng/l for six hours. In the control group (no BPA exposure), BPA concentration in the muscle of the fish was 28.44 ± 1.61 ng/g, and liver samples were below detection limits of the HPLC (< 1.25 ng/g). In the BPA-exposed group, the liver and muscle accumulation were 116.52 ± 3.15 and 54.49 ± 1.98 ng/g, respectively. There was a significant increase in the concentration of BPA in the liver and muscle of the fish exposed to BPA, compared to the control group ($p < 0.05$; Figure 1). The concentration of BPA in the liver was higher than the muscle; therefore, we selected the liver for the gene expression study.

Amplification specificity and efficiency of the target gene and reference genes

Agarose gel electrophoresis showed the PCR products of the three candidate reference genes and the target gene, *cyp1a*, as a single band of the expected size (Figure 2). The efficiency of qRT-PCR calculated for each gene ranged from 92 to 105%, and all genes showed R^2 values of 0.99. The average melt curve values of 18S *rRNA*, *EF-1α*, *rpl8* and *cyp1a* were 81.04 ± 0.13, 83.76 ± 0.22, 84.16 ± 0.14 and 81.07 ± 0.12, respectively (data not shown).

Expression stability of the reference genes

18S *rRNA* exhibited the lowest Ct values (7.08 to 8.58), followed by *EF-1α* (20.39 to 22.22), and *rpl8* showed the highest (21.19 to 22.27) (data not shown). Expression stability was analyzed using three methods: *BestKeeper*, the delta Ct method and *NormFinder*, Summary rankings are shown in Table 2. According to geomean ranking, the overall order, from most to least stable, was: *rpl8* > 18S *rRNA* > *EF-1α.* Therefore, *rpl8* was selected as an internal reference gene for this study.

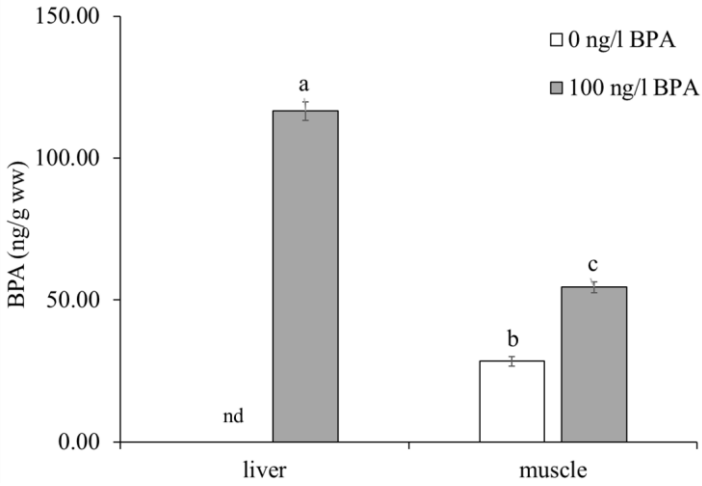

Figure 1 Average levels of bisphenol A (BPA) accumulation in the livers and muscles of juvenile false clown anemonefish (*Amphiprion ocellaris*) exposed to 0 (control) and 100 ng/l of BPA for 6 hours. The values are expressed as mean ± standard error of the mean (SEM). Nd; not detected. The different lowercase letters (a-c) indicate significant difference ($p < 0.05$) among tissues and concentrations of BPA. Differences exist among the means, the one-way analysis of variance (ANOVA), followed by Duncan's *post-hoc* test were performed.

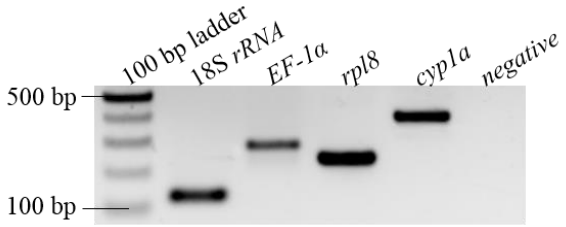

Figure 2 Agarose gel electrophoresis showing a single band of the potential reference and target genes of the amplification products, compared to the negative control.

Table 2 Ranking of candidate reference genes according to their stability value (SV) using *BestKeeper*, delta Ct method and *NormFinder* analysis.

Rank	Geomean ranking		*BestKeeper*		delta Ct		*NormFinder*	
	gene	SV	gene	SV	gene	SV	gene	SV
1	*rpl8*	1.26	18S *rRNA*	1.18	*rpl8*	2.86	*rpl8*	1.25
2	18S *rRNA*	2.08	*rpl8*	2.15	*EF-1α*	3.09	*EF-1α*	2.16
3	*EF-1α*	2.29	*EF-1α*	2.97	18S *rRNA*	3.46	18S *rRNA*	2.98

BPA effect on *cyp1a* expression

Expression of *cyp1a* in the liver of *A. ocellaris* increased significantly following exposure to 50 ng/l BPA to 1.64-fold at 12 hours, and up to a maximum of 2.82-fold after 24 hours. At 48 hours, it decreased to 1.13-fold. The higher concentration of BPA (100 ng/l) had a more rapid effect, causing a 1.89-fold increase in expression after 6 hours, to a maximum rise of 3.44-fold higher expression after 12 hours. The expression then decreased at 24 hours to 0.79-fold (Figure 3).

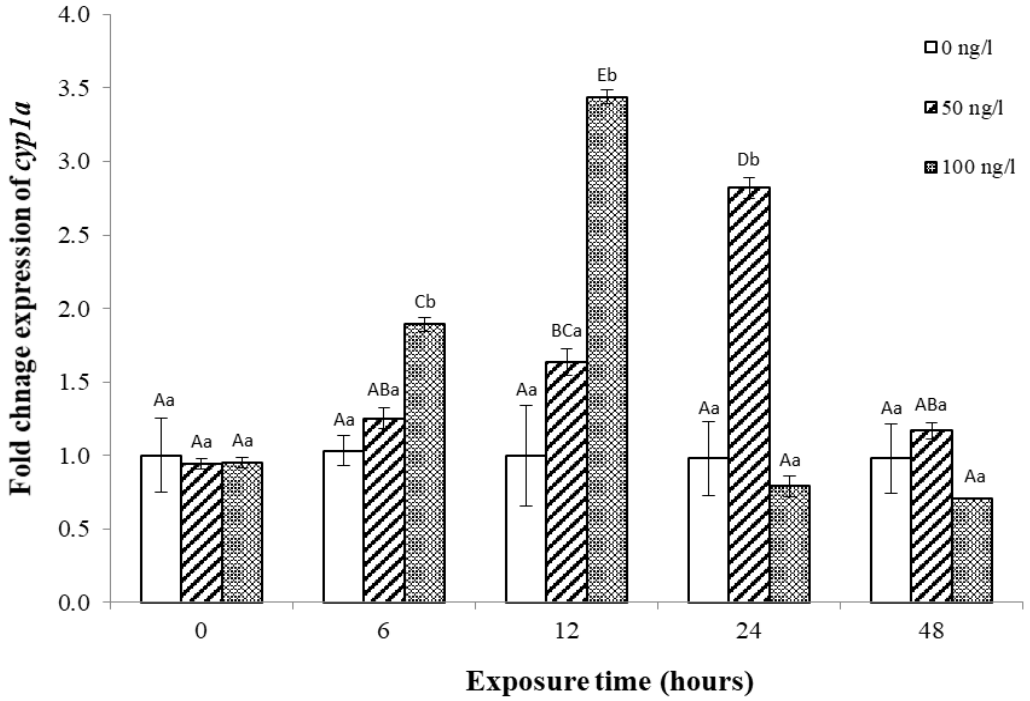

Figure 3 Bisphenol A (BPA) affects *cyp1a* transcript levels in juvenile false clown anemonefish (*A. ocellaris*). *A. ocellaris* was exposed to 0, 50, and 100 ng/l of BPA for 0, 6, 12, 24, and 48 hours. The values are expressed as mean ± standard error of mean (SEM); $n = 6$. The different capital letters (A-E) indicate significant differences ($p < 0.05$) among conditions at the corresponding concentration and time of exposure to BPA. The lowercase letters (a and b) within the same time of exposure indicate significant difference ($p < 0.05$) among concentrations. Differences exist among the means, the one-way analysis of variance (ANOVA), followed by Duncan's *post-hoc* test were performed. The results are represented as gene expression fold changes of *cyp1a* comparative to the reference gene ribosomal protein l8 *(rpl8*).

DISCUSSION

BPA accumulation in false clown anemonefish tissues

The concentrations of BPA accumulated in the liver and muscle tissues of *A. ocellaris* were examined following exposure to 100 ng/l BPA for 6 hours as the basis to access BPA accumulation in the tissues after initial exposure. These were chosen to effectively screen the maximum *cyp1a* expression within a short period of exposure in this experimental model. BPA was found in the liver higher than that in the muscle. This result is consistent with the study by Lindholst *et al.* (2000), which found that BPA levels in the liver were higher than those in the muscle of rainbow trout exposed to BPA concentrations of between 10 and 500 µg/l for 12 days. Likewise, BPA concentrations in the liver were higher than muscle concentrations in various fish species from the Tyrrhenian Sea (Mita *et al.*, 2011) and in flounder (*Platichthys flesus*) and bream (*Abramis brama*) collected from marine and estuarine areas in the Netherlands (Belfroid *et al.*, 2002). The liver is an essential organ for contaminant uptake and biotransformation; therefore, it generally contains higher concentrations of toxicants than those of any other tissues (Belfroid *et al.*, 2002). The liver is commonly selected for gene expression studies, such as this, as it can be a useful indicator of marine environmental pollution.

Despite the control animals not exposed to BPA, a low level of BPA was found in the muscle (28.44 ± 1.61 ng/g) (Figure 1). However, these BPA concentrations were significantly lower than those

found in the exposed fish (54.49 ± 1.98 ng/g), especially in the livers (116.52 ± 3.15 ng/g). This was due to the experimental fish we bought from the research center contained a low level of BPA. Even though we acclimated the fish prior to the experiment for seven days. This period could not eliminate the BPA completely from the tissues, especially those that were retained in the muscles. This was confirmed by investigation of the holding water of the control fish via HPLC, and no BPA was detected. In addition to this, we evaluated the recovery efficiencies of the BPA (spiking 100 ng/l authentic BPA into the water and left for 0 and 6 hours), which was almost 100% (105.99 ± 0.15, 98.47 ± 0.15 ng/l, respectively). Given that liver showed high systemic clearance and shorter time for BPA elimination than muscle, as reported by Yoo *et al.* (2000) and references therein. These findings were to be expected.

Expression stability of reference genes

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) is an effective method for analyzing gene expression (Belfroid *et al*., 2002; Muldoon & Hogan, 2016). The number of gene expression studies has increased exponentially, becoming an essential tool in several biological science areas over the last two decades (Nolan *et al*., 2006). However, selecting appropriate reference genes for these studies is crucial, as often the same reference genes are used for a number of different species. Factors such as age, gender, infection, stress, and toxicant exposure can cause members of the same species to have dramatically different expression levels of the same 'reference' genes. For example, *EF-1α* had been validated as an unstable reference gene in shrimp (*Penaeus vannamei*) infected with *Penstyldensovirus* 1 (PstDV-1) (Valenzuela-Castillo *et al*., 2017), as well as in fathead minnows (*Pimephales promelas*) exposed to EE_2 (Belfroid *et al*., 2002). This gene was found to be most stable in the embryonic stage of the zebrafish (*Danio rerio*) (Tang *et al*., 2007) and in salmon (*S. salar*) from different habitats (Olsvik *et al*., 2005). Panicz (2016) reported that different tissues of the tench (*Tinca tinca*) had variations in their expression of the reference genes: *rpl8* being the most stable in kidney and muscle tissues, and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) being the most stable in the liver.

In this study, we investigated a set of three reference genes (18S *rRNA*, *EF-1α*, and *rpl8*) for use as the internal control in the qRT-PCR assay. We verified that the most stables reference gene in the liver of *A. ocellaris* exposed to BPA was *rpl8*. The *rpl8* gene encodes a ribosomal protein component of the large 60S subunit (Nissan *et al*., 2002). This gene was found to be suitable as a reference in an experiment of tench (*T. tinca*) fed with poultry by-products (Panicz, 2016), as well as in fathead minnows and abalone (*Haliotis discus hannai*) exposed to 17α-ethynylestradio (EE_2) (Filby & Tyler, 2007; Lee & Nam, 2016). Muldoon and Hogan (2016) also used *rpl8* as the reference gene in their study on brook stickleback (*Culaea inconstans*) exposed to EE_2.

Effect of BPA on *cyp1a* expression

Biomarkers are useful tools in understanding the health effects and physiological responses of organisms to environmental contamination. Cytochrome P450 plays a role in the metabolism of many compounds, such as polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs), which include BPA (Sarasquete & Segner, 2000). Many researchers use the *cyp1a* gene as a biomarker for aquatic pollution. For example, exposure to crude oil has been shown to stimulate the expression of *cyp1a* in zebrafish (*D. rerio*), anemonefish (*A. ocellaris*), and marine medaka (*Oryzias melastima*) (Anjos *et al*., 2011; Kim *et al*., 2013). Likewise, *cyp1a* expression significantly increased in common carp (*C. carpio*) exposed to pesticide contamination (Salvo *et al.,* 2012; Karaca *et al.,* 2014).

In this study, the expression of *cyp1a* in *A. ocellaris* was used as a biomarker for BPA exposure. In the time-course experiment, *cyp1a* expression significantly increased 24 and 12 hours after exposure to 50 and 100 ng/l of BPA, respectively. These results show that expression of *cyp1a* by BPA is dose-dependent, with higher amounts of BPA (100 ng/l) significantly increased expression after 12 hours exposure, compared to 24-hour exposure at 50 ng/l. At greater concentrations, BPA binds with more receptors that stimulate the synthesis of *cyp1a* (Anjos *et al*., 2011). After 24 hours (50 ng/l) and 12 hours (100 ng/l), *cyp1a* expression levels decreased due to the fish adapting and metabolizing the BPA. This is similar to the results seen in marine medaka exposed to crude oil (Kim *et al*., 2013) and guppies (*Jenynsia multidentata*) exposed to beta-naphthoflavone (Pinto *et al*., 2015). Also, *cyp1a* expression levels were consistent with BPA accumulation in the liver tissue. BPA accumulation in the liver resulted in higher levels of *cyp1a* expression. The expression of *cyp1a* was sensitive to BPA exposure, even at low concentrations. Therefore, *cyp1a* expression can be

used as a potential biomarker of BPA exposure in marine fish. It could potentially evaluate the biological effects of BPA contamination in coastal areas and be used as an early warning indicator of contamination.

CONCLUSIONS

The results from this study indicated that: (i) BPA accumulated in greater concentration in the liver, compared to muscle; (ii) *rpl8* was the most stable reference gene in the qRT-PCR analysis of BPA exposure; and (iii) measuring BPA exposure using *cyp1a* gene expression in the liver of the juvenile false clown anemonefish was sensitive to low doses of BPA contamination. We have demonstrated that *cyp1a* expression was highly responsive to short-term BPA exposure and that, therefore, *cyp1a* can be used as a biomarker of BPA exposure in the marine environment.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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