Chemosphere 209 (2018) 857–865

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Changes in thyroid status of *Menidia beryllina* exposed to the antifouling booster irgarol: Impacts of temperature and salinity

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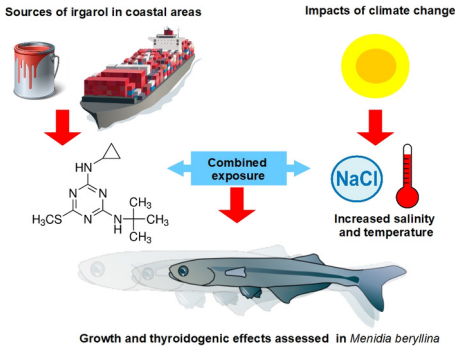
HIGHLIGHTS

- Thyroid effects of irgarol assessed in *Menidia beryllina*.
- Condition factor of *M. beryllina* affected by irgarol.
- Thyroid targets of irgarol affected by temperature and salinity.
- Potential Adverse Outcome Pathway for irgarol is provided.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 March 2018
Received in revised form
1 June 2018
Accepted 24 June 2018
Available online 26 June 2018

Handling Editor: Jim Lazorchak

Keywords:
Biocides
Climate change
Estuary
Endocrine disrupting chemicals
Fish endocrinology
Inland silversides

ABSTRACT

The triazine-based herbicide irgarol is widely used in antifouling systems as an algicide and has been detected recently in multiple coastal environments. Studies evaluating sub-lethal responses of fish following exposure to irgarol are limited. Moreover, impacts of climate change on fish endocrinology may also contribute to the sublethal toxicity of irgarol. We assessed the effects of irgarol on thyroid endpoints in juveniles of *Menidia beryllina* under two different treatments of salinity (10 and 20 ‰) and two temperatures (10 and 20°C). Condition factor coefficients (K) of animals were significantly affected by 0.1 to 10 μg/L of irgarol at the higher temperature. Levels of T3 were changed in whole body homogenates from both temperatures at 10‰ following exposure to 1 to 10 μg/L. T4 levels were altered only at 10°C when animals were treated with 1 to 10 μg/L (10 ‰), and in 0.1 and 10 μg/L (20 ‰). Increased transcripts of deiodinase enzymes at 10 °C may be impacted by salinity and alter thyroid hormone homeostasis. Impact on gene expression of thyroid (α and β) and growth hormone receptors were also determined. Our results highlight the relevance of environmental variable that may impact the ecological risk of irgarol in estuarine systems.

1. Introduction

Antifouling compounds are used in coating systems to prevent biocorrosion and unwanted development of biofouling on man-

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https://doi.org/10.1016/j.chemosphere.2018.06.152
0045-6535/

made surfaces including boats, vessels, off-shore formation and other constructions in aquatic environments (Chambers et al., 2006; Thomas and Brooks, 2010). Due to regulatory restrictions of organotin paints (OT), the use of OT-free antifouling systems has increased as an alternative to reduce the toxicity of antifouling systems to non-target organisms (Hall et al., 2009). Such paints contain biologically active compounds known as "organic boosters', which includes different biocides that are incorporated into the coating formulation formulations (Mochida and Fujii, 2009).

Since the mid-1980s the triazine-based herbicide irgarol (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine) has been widely used in antifouling systems as an algaecide (Hall et al., 2009; Mochida and Fujii, 2009). Since usage has increased, detection of irgarol has been observed in coastal environments (Bao et al., 2013). Irgarol is the most common detected antifouling booster worldwide, with environmental concentrations ranging from <1 ng/L to 1 μg/L (Konstantinou and Albanis, 2004). In Southern California for example, levels of irgarol in water were measured up to 254 ng/L in sites nearby recreational marinas (Sapozhnikova et al., 2013). Irgarol has a reported half-life of 100–350 days (7 ± 3 days for estuarine waters), with moderate water solubility (7 mg/L) and lipophilicity (log K_{ow} = 3.95) (Thomas et al., 2002; Konstantinou and Albanis, 2004; Sapozhnikova et al., 2009, 2013).

The mode of action of irgarol is similar to other triazine herbicides, in that it blocks electron transport during photosynthesis by inhibiting the energy transfer of photosystem II (PSII), reducing both CO_2 uptake and carbohydrate production, which causes the starvation of the plant (Ebert et al., 1976). The toxicity of irgarol in estuarine and marine organisms is well documented in the literature and most studies have focused on acute and chronic effects endpoints in bioassays for algae, invertebrates and fish (Konstantinou and Albanis, 2004; Bao et al., 2011; Castro et al., 2011; Perina et al., 2011; Mai et al., 2013). However, studies on sub lethal effects are limited with one study showing immunosuppression in the ascidian *Botryllus schlosseri* (Cima and Ballarin, 2012) and changes in the fatty acid composition of Asian sea-bass *Lates calcarifer* (Ali et al., 2015). Regarding endocrine responses, the anti-estrogenic activity of irgarol was reported using a yeast-based in vitro assay (Westlund and Yargeau, 2017). Recent studies demonstrate that the antifouling booster diuron, which also acts in PSII, affect the thyroid system, growth, and development of aquatic organisms, including tadpoles American bullfrog *Lithobates catesbeianus* and the estuarine fish *Menidia beryllina* (Freitas et al., 2016; Moreira et al., 2018).

Silversides from the species *M. beryllina*, are the most common fish originated from West Atlantic inhabiting coastal environments from marshes to estuaries, and was introduced to Pacific coastal ecosystems (Middaugh and Hemmer, 1992). The species is a secondary consumer feeding on zooplankton and represents an important link in the marine food web as a prey item of other fish from higher links (Gleason and Bengtson, 1996). *M. beryllina* is also a eurythermic (tolerance range from 2.9 to 30 °C) and euryhaline (tolerance range from 0 to 35‰) (USEPA, 2002), and recently it has been selected as a model for estuarine fish in the assessment of endocrine disrupting chemicals (EDCs) in coastal zones (Brander et al., 2016; Cole et al., 2016).

Shifts in environmental conditions including those induced by climate change events can also affect the toxicity of contaminants (Schiedek et al., 2007; Keller et al., 2015). In California's San Francisco Estuary-Watershed (SFEW) for example, climate change projections indicate extreme conditions of summers and winters (Cloern et al., 2011; Wagner et al., 2011). Such models project the decrease of freshwater input combined with seawater intrusion, as a result of sea level rising, increasing thus water temperature and salinity in the estuarine system. Considering this scenario, we assessed the effects of irgarol on thyroid function of estuarine fish using *M. beryllina* as a model under two conditions of temperature and salinity. Thyroid hormone (TH) endpoints were evaluated in juveniles and genes involved in TH pathways such as deiodinases enzymes (Dio1 and Dio3), growth (GHR) and thyroid hormone receptors (TRα and TRβ) and were evaluated in fish's body homogenates following treatments. Molecular responses were combined to condition factor to determine if an Adverse Outcome Pathway could be formulated for irgarol.

2. Materials and methods

2.1. Animals and experimental design

We used sexually immature juveniles with 50 days of age, with 11.42 ± 1.27 mm length and 0.016 ± 0.006 g of weight. Animals were obtained from commercial cultures (Aquatic BioSystems Inc., Fort Collins, CO) and kept for 10 days of acclimation in experimental conditions of control preceding the experiments:10‰ of water salinity, 10 °C of temperature and photoperiod of 12:12 h light:dark. Fish were fed brine shrimp nauplii once per day at 5:00 p.m. (USEPA, 2002). After the 10-day acclimation period, a semi-static exposure was performed at 10 °C to evaluate the thyroidogenic effects of irgarol in *M. beryllina*. We tested two factors in each exposure: (I) salinity: 10‰ and 20‰, in order to assess salinity changes in oligohaline waters and (ii) irgarol: solvent control and three concentrations (0.1, 1 and 10 μg/L). Such concentrations were selected based on levels detected in samples from marinas of Southern California and other areas of East Coast from Florida to North Carolina (Hall et al., 2005; Sapozhnikova et al., 2013). Three months later, a new batch of fish with 50 days of age was purchased and a second exposure was performed at 20 °C with the same salinities and levels of irgarol, based on variations in estuarine conditions tolerated by estuarine fish and temperature changes predicted by SFEW models (Cloern et al., 2011).

Analytical grade irgarol (Sigma-Aldrich) was purchased for preparing stock solutions in ultra-pure water and using 0.01% MeOH as a solvent. Irgarol exposure systems were assembled in 4L tanks settled in a water bath (100 L capacity) with control of temperature performed *via* water-cooled chillers connected to thermostats. Sea salt aliquots were dissolved in dechlorinated water to achieve the selected salinity and then irgarol at nominal concentrations was added in a final volume of 3L. Two tanks *per* treatment were set up and kept in a cycle of 12–12 h (light-dark) with constant aeration. Following the 10 days of acclimation, 10 fish *per* tank were assigned to the respective treatment and exposed to irgarol for 15 days. Fish were fed on brine shrimp nauplii once *per* day at 5:00 p.m. at least 2h preceding the water changes (90% of total volume), which were performed every 48 h. Water pH (7.5 ± 0.5) and NH3 (below 0.01 mg/L) were monitored before water changes. No changes in feeding rate or mortality were observed during the experimental exposures and after that, fish were euthanized using 300 mg/L of tricaine methanesulfonate (MS-222). Measurements of total length and wet weight were recorded and animals were frozen on liquid nitrogen to be stored at -80°C until the analysis. These procedures followed the Animal Use Protocol (AUP), approved by UC Riverside Institutional Animal Care and Use Committee (IACUC).

2.2. Condition factor and hormone concentration analysis

Condition factor (K) was applied as the index of fish health following the exposure to irgarol. The equation $K = W/L^b$, indicated for allometric growth was selected using measurements of weight (W), length (L) and the constant b (0.14), obtained from the slope of W x L regression of acclimated fish (Bolger and Connolly (1989).

Levels of triiodothyronine (T3) and thyroxine (T4) were determined in homogenates of individual fish selected from each treatment (n = 5) by enzyme-linked immunosorbent assay (ELISA) using specific kits (GenWay Biotech Inc., San Diego). For this analysis, a lysis solution was prepared containing Tris-Base (50 mM), Deoxycholate (0.5%), EDTA (1 mM), NaCl (50 mM), SDS (0.1%) and PMSF (1 mM). The buffer was added to each fish (1:5 dilution) to a subsequent incubation for 30 min at 4°C. Then, tissue samples were disrupted using a mechanical homogenizer, centrifuged for 20 min (15,000 g at 4°C) and the supernatant separated and stored at -20°C. The extracts were diluted again during the assay to fit the respective standard curves, ranging from 0 to 7.5 ng/mL of T3 and 0 to 250 ng/mL of T4. The intra-assay coefficient of variability (CV) of T3 duplicates was calculated at 7.62% and 9.26 for T4 (n = 100). Inter-assay CV was estimated from control samples in each plate (n = 3) and were 10.93% for T3 and 11.73% for T4. Both levels were expressed in ng/g of wet weight after the normalization by the weight of fish.

2.3. Gene expression

For mRNA measurements, whole body total RNA was isolated from individual fish per treatment (n = 5) by using Qiazol and the purification kit RNeasy Mini® that doesn't require DNase treatment (Qiagen, Germany). The purity and concentration of RNA were checked in a spectrophotometer (NanoDrop®, Technologies Inc.) and the synthesis of cDNA was performed following the Quick Protocol A3500 (Promega Reverse Transcription System kit, Wisconsin, USA).

Quantitative polymerase chain reaction (qPCR) was conducted using a CFX Connect® (Bio-Rad) Real-Time PCR Detection System. Primers were designed for *M. beryllina* using Primer Quest platform (www.idtdna.com) based on sequences provided by Brander et al. (2016) (Table 1). The qPCR protocol included the reaction mixture containing the binding dye SsoAdvanced Universal SYBR® (Bio-Rad, Hercules, CA). The parameters for qPCR were fixed as 0.5 min (95 °C) followed by 40 cycles of 10s (95 °C) and 30s for the steps of annealing and elongation (55 °C). The comparative delta-delta model ($\Delta\Delta C_t$) proposed by Pfaffl (2001) was used to determine relative gene expression, based in the fold-differences of threshold cycle values (C_t) between the target gene and housekeeping genes. The efficiency of target genes was used to normalize the relative fold changes, expressed as the ratio of gene expression of each exposure group relative to its respective group of vehicle control.

2.4. Data analysis

Results of K, hormone analysis and gene expression were assessed by Shapiro-Wilk's and Cochran's tests for normal distribution and homoscedasticity, respectively. For K, the confidence interval (CI) range was estimated in 5 to 95% and outliers were excluded by interquartile range criteria (Tukey, 1977). Results from the two tanks were analyzed by *t*-test aiming to determine the possible influence of pseudoreplication and no significant effects were observed. Then, each fish was considered as sampling unit and for each temperature experiment, we analyzed significant effects of irgarol levels *versus* salinity. Responses from K and ELISA were tested using a two-way ANOVA with fixed factors and four levels of exposure (0.01% MeOH, 0.1, 1 and 10 μg/L of irgarol) and two levels for salinity (10‰ and 20‰). Bonferroni method of multiple comparisons was used to test differences in K results from different treatments due to a different n. Dunnett's test was used for ELISA and qPCR results to compare the responses observed in the exposure groups with its respective solvent control ($\alpha = 0.05$).

3. Results and discussion

Results of K are presented in Fig. 1. Significant decreases in K were observed at 10 °C and 10‰ for 0.1 and 10 μg/L irgarol, with no interaction found between salinity and compound (p = 0,45). At 20‰, irgarol affected the K of fish in the 0.1 μg/L treatment. At the condition of higher temperature (20 °C), the interaction of irgarol and salinity (p = 0.0008) decreased K of animals exposed in all treatments (10‰ and 20‰). The herbicide diuron, which is also an inhibitor of PSII, impaired the growth and K of *M. beryllina* (Moreira et al., 2018). These findings corroborate our results indicating that irgarol produced responses on growth and they are consistent with effects induced by other triazine compounds in other aquatic vertebrates. Tadpoles of *Silurana tropicalis* chronically exposed for 28 days to 7 s-triazines: ametryn (0.2 mg/L), prometryn (0.25 mg/L), dimethametryn (0.03 and 0.3 mg/L), simazine (0.8 mg/L), atrazine (0.1 and 1 mg/L), propazine (0.1 and 1 mg/L), and cyanazine (0.05 and 0.5 mg/L) exhibited reduced growth assessed by total length and body mass, and delayed developmental stages (Saka et al., 2018). In fish, juveniles of Atlantic salmon (*Salmo salar*) exposed for 21 days to 100 μg/L of atrazine had a significant reduction of feeding rate and growth (Nieves-Puigdoller et al., 2007). In contrast, 90 days of exposure to environmental concentrations of simazine (0.06–4 μg/L) did not affect the length, body weight and

Table 1
Primer sequences of genes assessed in *M. beryllina* by qPCR analysis.

Gene	Symbol	Function	Amplicon length	Efficiency (%)	Primer sequence
60s ribosomal protein l7	RPL7	Reference	76	92	F – aacttcttgtggccgttcaag R – tcgcctccctccacaaagt
Thyroid hormone receptor alpha	TRα	Hormone receptor	51	94	F – tgtcggacgccatattcgat R – cctcggtgtcatccaagttga
Thyroid hormone receptor beta	TRβ	Hormone receptor	108	90	F – cgaagggaagtgcgttatc R – ctgtcatccaacaccaagtc
Growth hormone receptor beta	GHR	Hormone receptor	60	90	F – tcaagaaaggcaagcttcgag R – cggccttaaatcaagaggacc
Iodothyronine deiodinase type I	Dio1	Thyroid hormone metabolism	217	91	F – cttgccagaagaggctattac R – ggaagaactgactcccattg
Iodothyronine deiodinase type III	Dio3	Thyroid hormone metabolism	205	90	F – gcatctctgactccaacaag R – gagcagctgccaaagtta

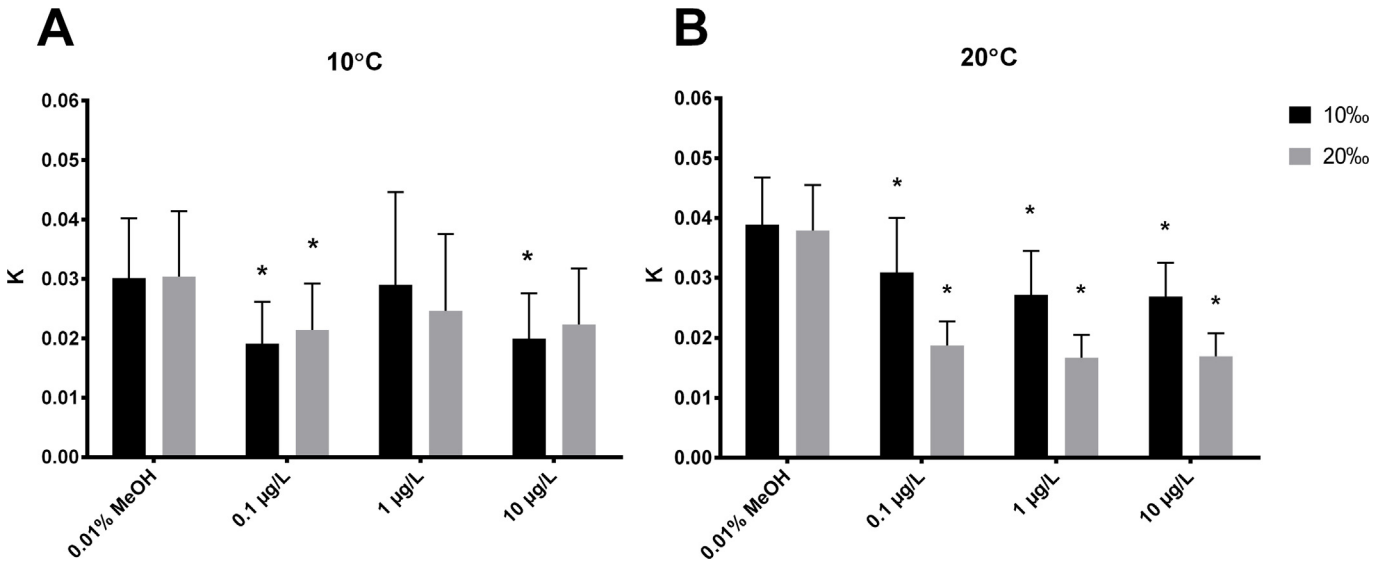

Fig. 1. Results of K assessed in juveniles of *M. beryllina* treated with irgarol for 15 days at 10 °C (A) and 20 °C(B). Bar plots represent the mean + SD (n = 20). Data analyzed by two-way ANOVA followed by Bonferroni's test comparing each group with control (*p < 0.05).

Fulton's condition factor of one-year-old common carp *Cyprinus carpio* L. (Velisek et al., 2012a). For the same species, fertilized eggs exposed for 36 days to a range of 0.2–2 mg/L of terbutryn exhibited delayed larval development and decreased in condition factor (Velisek et al., 2012b).

The early development of the central and peripheral nervous system, the control of growth and basal metabolism in juveniles fish are regulated by actions of THs (Power et al., 2001). The combined influence of ontogenic development with environmental conditions stimulate the hypothalamus to release thyrotropin-releasing hormone (TRH) which controls the pituitary release of thyroid stimulating hormone (TSH) (MacKenzie et al., 2009). TSH causes the thyroid glands to produce T4, which is activated into T3 form by deiodinases (Power et al., 2001; Blanton and Specker, 2007; Jarque and Piña, 2014).

In the present investigation, the function of HPT axis was evaluated by TH levels in homogenates of *M. beryllina* treated with irgarol. At 10°C the effects were more pronounced and significant interactions were observed between compound and salinity ($p = 0.0001$ for T3 and T4). Fish exposed at 10‰ presented high levels of T3 in 1 µg/L while T4 increased in the 1 and 10 µg/L treatments. Exposure at 20‰ increased the content of T3 in animals from all exposures and T4 increased significantly in 0.1 and 10 µg/L. Higher temperature resulted in elevated levels of T3 only in animals treated with 1 and 10 µg/L of irgarol at 10‰ (significant interaction, $p = 0.0001$) and no changes in T4 levels were found (Fig. 2). Similar effects of enhanced T3 and T4 were reported in *M. beryllina* exposed to diuron at the same conditions temperature and salinity (Moreira et al., 2018), indicating that both photosynthetic inhibitors impact TH homeostasis.

Environmental variables including water temperature and salinity are important to regulate metabolism and osmoregulatory responses of estuarine fish by the action of THs (Peter, 2011). Elevated levels of T4 were reported in the climbing perch *Anabas testudineus* acclimated in higher salinity for 7 days (Rejitha et al., 2009). For the same species, Peter et al. (2011) described a modulatory mechanism of salinity stimulating T3 activation regulating thus the activity of Na^+,K^+-ATPase in gill as a consequence of the hyperosmotic condition. Regarding the effects of triazine compounds on TH regulation, juveniles of *S. salar* treated with 100 µg/L of atrazine for 21 days at 10 °C were submitted to hyperosmotic stress (freshwater to saltwater, 30‰) and exhibited a significant increase in plasma cortisol, Cl^-, Mg^{2+}, Na^+, Ca^{2+} followed by a decrease in T4 and T3, indicating effects on ionoregulatory response and inhibition of TH by atrazine (Nieves-Puigdoller et al., 2007).

Temperature also influenced the effects of irgarol on TH by increasing T3 at 10‰. The activation of T4 into T3 occurs via deiodinase enzymes type I or type 2 (Dio1 and Dio2). T4 and T3 are inactivated and converted into rT3 and T2 (3,3′-diiodothyronine) by Dio1 and Dio3, respectively. Dio1 and Dio 2 transform rT3 in T2 (Orozco and Valverde, 2005; Jarque and Piña, 2014). We also assessed the transcripts of genes involved in TH deiodination in *M. beryllina* from treatments related to environmentally relevant levels of irgarol (0.1 and 1.0 µg/L). Expression of Dio1 increased in all temperatures at 10‰. The combination of irgarol at higher salinity (20‰) resulted in a 4–5-fold increase in the expression of deiodinase type 3 (Dio3) (Fig. 3). Treatments at 20 °C changed the expression in 10‰ treatments by increasing the fold change of Dio1 in 0.1 and 1.0 µg/L of irgarol in up to two orders of magnitude compared to 10 °C, indicating a temperature effect combined with the herbicide in such response. The decreased expression for Dio 1 and Dio3 was observed in 20‰ treatments at 0.1 µg/L and 1 µg/L, respectively (Fig. 4).

An induced activity of deiodinase was found associated with the somatic growth of the Atlantic cod *Gadus morhua* kept for 10 months at the increased temperature from 2 to 4°C (Cyr et al., 1998). The importance of TH function on thermal acclimation was also described in zebrafish (*Danio rerio*) acclimated in warm water compared with animals acclimated to cold water (Little et al., 2013). Previously studies have reported the higher expression of Dio1 and Dio3 in larval stage of killifish *Kryptolebias marmoratus* (Kang et al., 2017), zebrafish embryos (Dong et al., 2013) and *Gobiocypris rarus* (Chen et al., 2017) treated with the BDE-47 and 2,4-dichloro-6-nitrophenol (DCNP). For *M. beryllina*, diuron induced the expression of Dio3 in combination with higher salinity, and the expression of Dio1 was also increased as a response to higher temperature (Moreira et al., 2018). In the current study, the augmented levels of

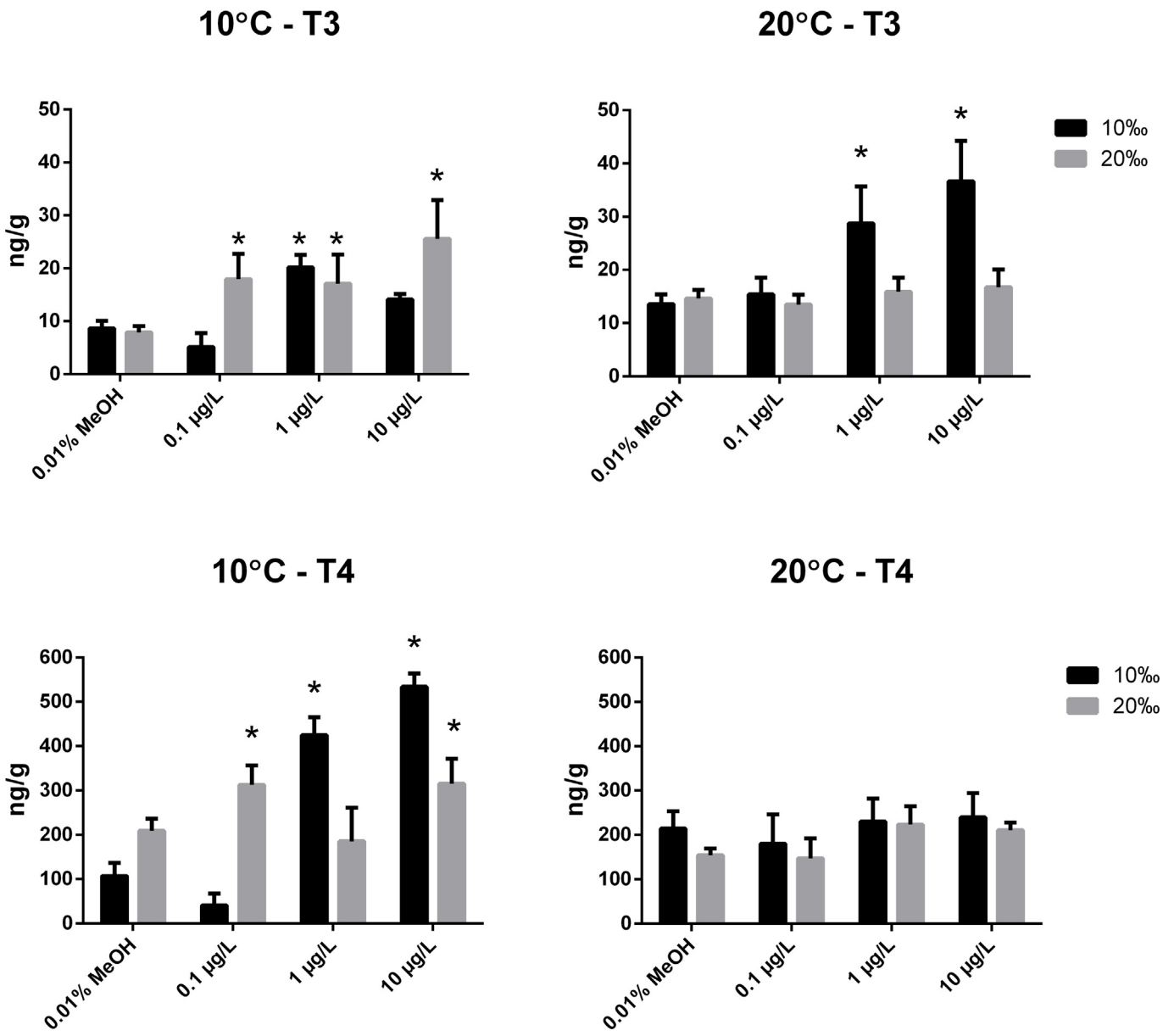

Fig. 2. Results of ELISA of T3 and T4 in juveniles of *M. beryllina* treated with irgarol for 15 days. Bar plots represent the mean + SD (n = 5). Data analyzed by two-way ANOVA followed by Dunnett's test comparing each group with 0.01% MeOH (*p < 0.05).

T4 may enhance formation of T3 by Dio1 in the peripheral tissues, while the increased transcripts of Dio3 at 20‰ can be associated with the T3 catabolism in order to restore TH homeostasis. In the treatments at 20°C the upregulated expression of Dio1 may explain the increase of T3 (Bianco and Kim, 2006; Jarque and Piña, 2014).

The expression of thyroid (α and β) and growth hormone receptors were analyzed. Treatments at 10 °C and 10‰ reduced the transcripts of TRα at 0.1 µg/L and GHR at 1.0 µg/L, while the exposure at 20‰ decreased the transcripts of TRα and GHR in both concentrations (Fig. 3). Treatments with irgarol at 20 °C changed the expression at 10‰ increasing TRβ (1.0 µg/L) and Dio1 (0.1 and 1.0 µg/L). Irgarol at 0.1 µg/L combined with 20‰ of salinity increased the expression of TRα, GHR and decreased TRβ. Fish exposed to 1 µg/L of irgarol reduced the expression of GHR (Fig. 4).

The actions of THs are mediated by binding to their specific nuclear thyroid receptors (TRs) which act as specific transcription factors of the thyroid system (Yamano and Miwa, 1998; Nelson and Habibi, 2009). In the same way, growth hormone (GH) has an essential role controlling fish growth and is produced by the adenohypophysis after binding to growth receptors (GHR) in target tissues (Gabillard et al., 2003; Deane and Woo, 2009). Gene expression of TRα and TRβ were increased in larvae of *K. marmoratus* exposed to BDE-47 as a compensatory effect caused by the balancing feedback of TH homeostasis (Dong et al., 2013). On the other hand, decreases in the transcription of both TRs was reported in larval stage of the big head carp *Hypophthalmichthys nobilis* treated with arsenite (AsIII) and arsenate (AsV) (Sun et al., 2016) and in zebrafish created with the fungicide triadimefon (Liu et al., 2011). Exposure of Japanese medaka *O. latipe* for five months to 100 mg/L of perchlorate at the higher temperature (from 26 to 33°C) decreased the transcripts of TRs as an effect of T4 depletion (Lee et al., 2014). Similar patterns of TR and GHR

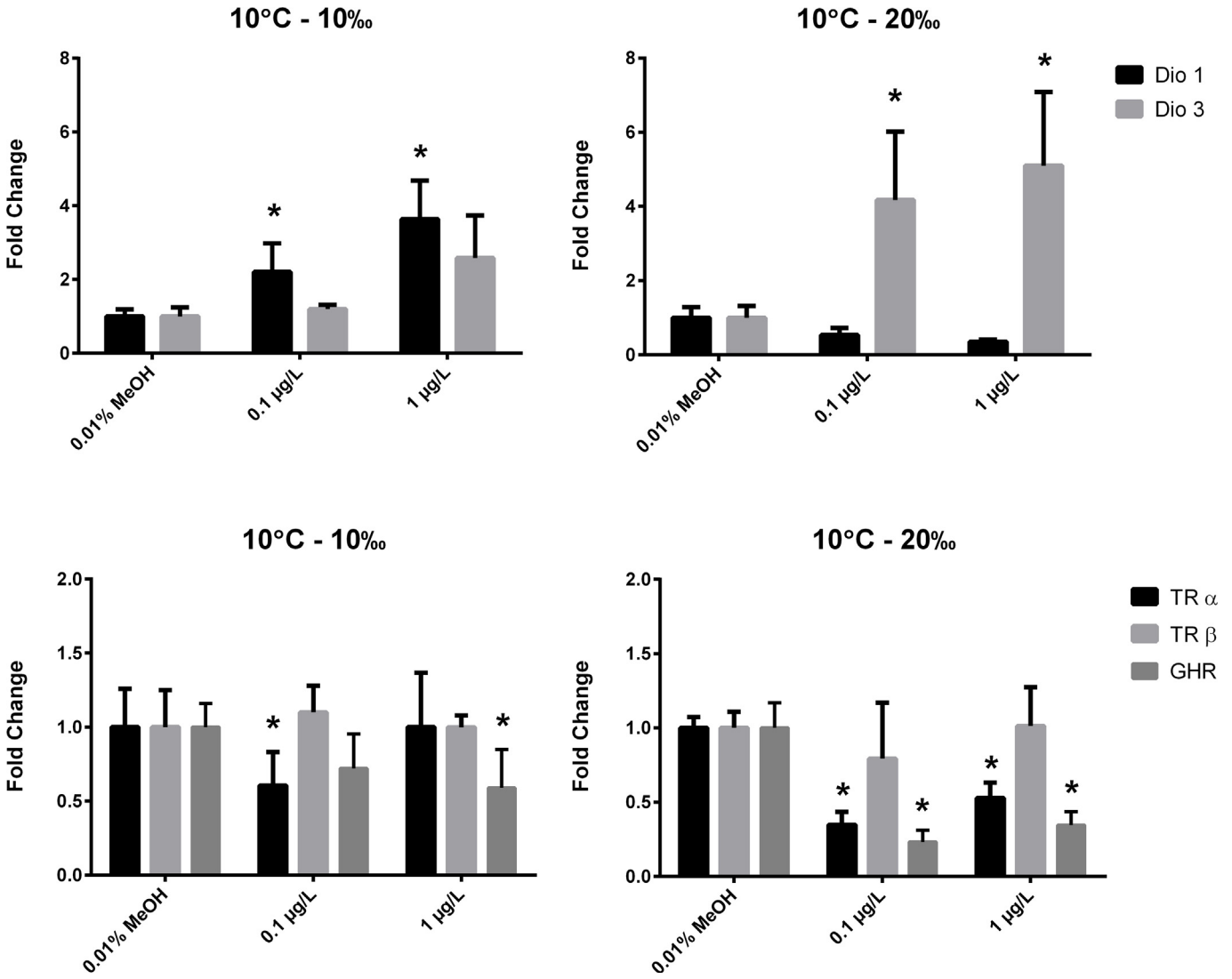

Fig. 3. Results of qPCR targeting deiodinases enzymes (Dio1 and Dio3), thyroid (TRα and TRβ) and growth hormone receptors (GHR) in juveniles of *M. beryllina* treated with irgarol for 15 days at 10 °C. Data are expressed as fold change relative to 0.01% MeOH and bar plots represent the mean + SD (n = 5). Data analyzed by one-way ANOVA followed by Dunnett's test comparing each group with 0.01% MeOH (*p < 0.05).

expression was also reported in *M. beryllina* after an exposure to diuron at the same levels and experimental conditions of this study (Moreira et al., 2018). For these studies it was hypothesized that the down-regulation of these receptors may be related to a response to restore the TH balance and highlighted the importance of further studies exploring such mechanism(s).

Fig. 5 summarizes the responses observed in K and the thyroidogenic responses in *M. beryllina* exposed to irgarol in a diagram based in the adverse outcome pathway (AOP) approach (Ankley et al., 2010). In the present study, irgarol augmented THs in both salinities and temperature affected only T3 levels. Deiodination pathways were triggered and TH and GH receptors were down-regulated as a response to balance TH levels. Condition factor was also affected following the effects on TH. These findings are similar with the responses caused by diuron in *M. beryllina* (Moreira et al., 2018) suggesting that these two biocides, which act by blocking PSII, induce the same mode of action in the thyroid system. Regarding the ecological relevance of the responses, silversides are a relevant group in estuarine and marine ecosystems due to their limited period of lifespan and rapid development, with annual life cycles. Juveniles present accelerated somatic growth reaching almost 30% of their biomass during the first 4 months of age (Conover and Ross, 1982; Huber and Bengtson, 1999; Chizinski et al., 2007). Silverside populations are numerous and spend most of its early life stages in shallow inland waters and after that, they migrate to coastal waters. Rapid growth coupled with this migration strategy indicates that this species is a primary source of biomass and energy exported from inner areas of the estuary to coastal zones (Conover and Ross, 1982). Consequently, impairment of K and growth may alter this ecological dynamic in estuarine systems.

4. Conclusions

Impacts on thyroid hormone homeostasis were observed in *M. beryllina* induced by the biocide irgarol at two salinities (10 and 20‰) and temperatures (10 and 20 °C). Since the thyroid system controls development and growth of fish, thyroid disrupting effects caused by Irgarol in *M. beryllina* can affect not only individual fish but also the population and its ecological function. Our results also

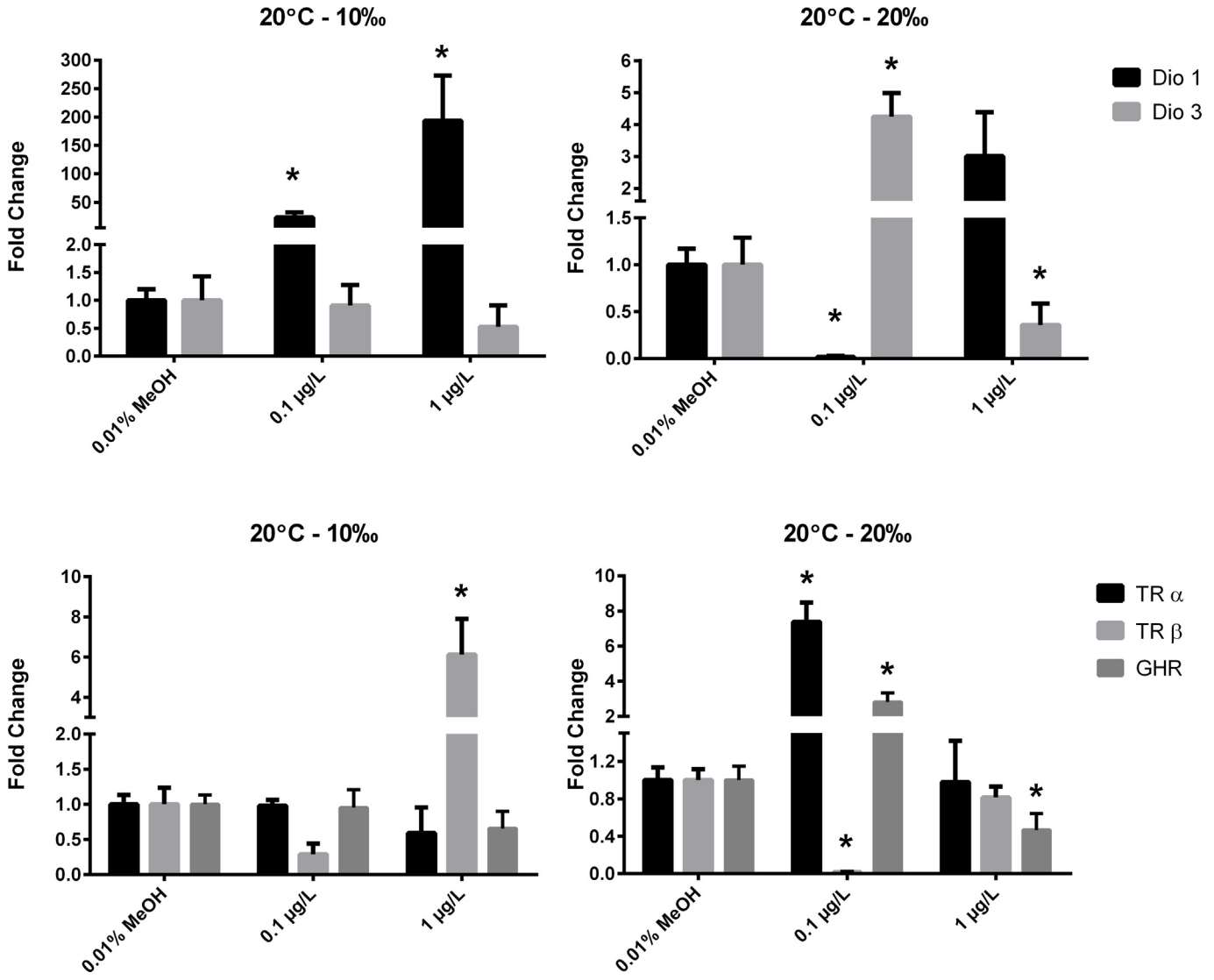

Fig. 4. Results of qPCR targeting deiodinases enzymes (Dio1 and Dio3), thyroid (TRα and TRβ) and growth hormone receptors (GHR) in juveniles of *M. beryllina* treated with irgarol for 15 days at 20 °C. Data are expressed as fold change relative to 0.01% MeOH and bar plots represent the mean + SD (n = 5). Data analyzed by one-way ANOVA followed by Dunnett's test comparing each group with 0.01% MeOH (*p < 0.05).

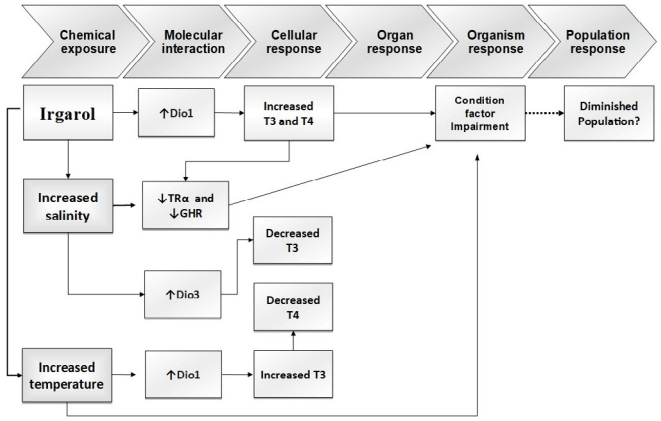

Fig. 5. Summary of the effects assessed in *M. beryllina* exposed to irgarol expressed as a potential adverse outcome pathway.

showed that natural variations in environmental variables or induced by climate change should be addressed in risk assessment of chemicals in systems impacted by climate.

Acknowledgments

This investigation was funded by São Paulo State Research Foundation (FAPESP, Brazil) [grant number #2016/01091-8] and also by the Research Allocation Program of the Agricultural Experiment Station (University of California Riverside). We kindly acknowledge the assistance of Dr. Susanne M. Brander and Dr. Richard E. Connon for contributing with the sequences used for primer design.

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