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Sex-biased regulation of respiratory burst, phagocytic activity and plasma immune factors in lined seahorse (*Hippocampus erectus*) after subchronic benzo[*a*]pyrene exposure

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ABSTRACT

Both wild and aquacultured seahorses are currently under great threat from marine pollution, notably from the potent contaminant and carcinogen benzo[*a*]pyrene (BaP). However, very little data are available regarding the immunomodulating effects of BaP in seahorses. Therefore, in this study, we exposed lined seahorses (*Hippocampus erectus*) for 7 d to BaP at three dosages (0.5, 5, and 50 μg/L) to evaluate sexual dimorphism in immune response. We measured eight immune parameters in the blood, including respiratory burst (RB), phagocytic activity (PA), monocytes/leucocytes, immunoglobulin M, complement 3, complement, interferon-a, and interleukin-2. Male seahorses had significantly higher parameters than females, except in terms of monocytes/leucocytes ($P < 0.05$). Although flow cytometry showed that RB and PA variation per BaP dose were roughly similar across sexes, RB and PA exhibited distinct patterns. Additionally, fluorescence intensity and leucocyte percentage were positively correlated in PA but not RB for all treatment and sex combinations. Through ELISA, we showed that the other six parameters had complex responses that nevertheless varied in a BaP-dosage and sex-dependent manner. Overall, adult male seahorses had higher immunocompetence than females before BaP exposure, and sexual dimorphism continued to be apparent during BaP exposure. Furthermore, all eight parameters were sensitive to BaP. Based on these results, we highly recommend *H. erectus* as a sentinel species for crude contamination, whereas PA and RB are valuable bioindicators of marine contaminants such as BaP.

1. Introduction

Seahorses are small teleosts with highly specialized morphology and life history (e.g., male pregnancy, viviparity, and monogamy) [1], making them charismatic icons to biologists and aquarium hobbyists. Unfortunately, this popularity has resulted in the exhaustion of wild populations worldwide [2–7], with millions of seahorses caught and traded across 93 countries [4]. Aquaculture is a promising strategy for restoring wild stock while addressing consumer demand for seahorses [8,9]. Coupled with the rapid development of aquaculture [4,10,11], however, is the finding that pollution has become the number one threat to wild seahorse stock, exceeding the negative effect of fishing by 1.5 times [12]. In particular, marine pollution from petroleum and its derivatives is a serious threat to nearshore wild seahorses [13–15], probably as well as offshore seahorse aquaculture.

Polycyclic aromatic hydrocarbons (PAHs) are the most concerning petroleum derivative because of their elevated toxicity in aquatic environments and immunoregulatory effects on fishes [14,16]. Benzo[*a*]pyrene (BaP) is a representative PAH and known human carcinogen, found at high concentrations in polluted water [17]. Existing evidence in fish suggests that BaP exposure suppresses phagocytic activity [18,19], resistance to bacterial infection [16,20,21], lymphocyte proliferation [19,20,22], antibody-forming cell count [19,22], and blood lymphocyte numbers [21]. However, available data are inconsistent

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Abbreviations:

PAHs	polycyclic aromatic hydrocarbons
BaP	benzo[*a*]pyrene
RB	respiratory burst
PA	phagocytic activity
IgM	immunoglobulin M
C3	complement 3
C4	complement 4
IFN-α	interferon-α
IL-2	interleukin-2
FI	fluorescence intensity
LP	leucocyte percentage
FIR	FI of RB
LPR	LP of RB
FIP	FI of PA
LPP	LP of PA
PBS	phosphate buffered saline
H2DCF-DA	2′,7′-dichlorofluorescin-diacetate
FCM	flow cytometry
GALT	gut-associated lymphatic tissue

with regard to the exact mechanism of action of BaP in fish. A study on tilapia (*Oreochromis niloticus*) showed that BaP concentration is directly correlated with up- or down-regulation of B-cell-mediated immunity [23]. In rainbow trout (*Oncorhynchus mykiss*), BaP exposure decreases blood B- and T-lymphocytes, as well as spleen B-lymphocytes and myeloid cells, while increasing B-lymphocytes in head kidney [21]. Yet another study, however, suggests that B cells in head kidney decreases in a BaP dose- and time-dependent manner [16].

Fishes are important models for evaluating potential risks to aquatic environments for two reasons. First, they are representative aquatic organisms, and second, they are highly sensitive to low concentrations of genotoxic substances and possess the ability to efficiently metabolize and accumulate chemical pollutants [24,25]. Seahorses are unlike other teleosts in being largely sedentary, anchored on substrates and rarely venturing away to pursue prey or colonize new areas [26,27]. Their low mobility and wide distribution readily expose seahorses to toxic substrates [14]. In *Hippocampus reidi*, for example, petroleum exposure increases blood genetic toxicity and DNA damage, while also causing gill hyperplasia [15]. In the highly sensitive long-snouted seahorse (*H. guttulatus*), PAHs accumulate in the tissue and significantly influence survival [13,28]. Understanding seahorse response to PAH contamination has promising implications both for their conservation and evaluations of environmental risk.

Sexual dimorphism in immune response and anti-parasite defense is common among animals [29,30]. The predominant explanation for this phenomenon is Bateman's principle: females have a longer lifespan and different reproductive strategies, thus requiring an immune system more efficient than that of males [31]. Owing to their sex role reversal (male parental care is typical), syngnathids provide an intriguing opportunity for studying sexual dimorphism in immune response [32,33]. However, very few studies have been performed on this topic using syngnathids [32,34–36].

In this study, we evaluated eight immune parameters of separately cultured adult male and female seahorses (*H. erectus*) exposed to different BaP doses. All parameters are demonstrated to reflect immunocompetence [34,37,38]. In addition, two subparameters, fluorescence intensity (FI) and leucocyte percentage (LP), and their correlation were evaluated for better presenting leucocytes RB and PA by FCM. Using these data, we addressed the following questions. 1) How do blood immune parameters respond to BaP exposure? 2) Is there a sex difference in immunocompetence before and after BaP exposure? 3) Does FI and LP correlate with each other as proxies of RB and PA? Finally, 4) which parameters are most suitable as bioindicators of seahorses to monitor marine BaP contamination?

2. Material and methods

2.1. Animals

Lined seahorses were maintained and treated in accordance with the guidelines of the animal ethics approval for experimentation granted by the Animal Care and Use Committee of the Ludong University.

Lined seahorses were cultured in ponds connected to a central circulation system with mechanical and biological filtration, ultraviolet sterilization, and protein skimmer that continuously aerated water (salinity: 31.5 ± 0.5‰, temperature: 25.5 ± 0.5 °C, pH: of 8.2 ± 0.1) in Wendeng seahorse center of the Ludong University. Five months old healthy seahorses (males [n = 150]: body height = 13.2 ± 0.34 cm, wet body weight = 14.60 ± 0.40 g; females [n = 150]: body height = 11.7 ± 0.5 cm, wet body weight = 12.7 ± 0.5 g) were selected and cultured in the seahorse center at Ludong University. Males and females were maintained in separate tanks for 2 weeks prior to the commencement of the experiments under the same culturing conditions as above. Plastic plants were provided as holdfasts. Fish were fed three times per day (08:00, 12:00, and 16:00) with frozen *Mysis*. Residual feed and feces were siphoned out 2 h after each feeding.

2.2. Exposure experiments and sampling

Stock BaP (SIGMA-ALDRICH, St. Louis, Missouri, USA) solution was prepared in DMSO (Amresco Commercial Finance LLC, Boise, Idaho, USA). Seahorses were divided into five 55 L tanks representing treatment groups: seawater, solvent, 0.5, 5, and 50 μg/L BaP. Once corresponding concentrations of BaP in each tank were set, the circulation system was ceased and the culture tanks were maintained the concentrations in 0.5, 5, and 50 μg/L BaP. After fully mixed by aerated water, fish were gently transferred into the experimental tanks as fast as possible. To maintain stable concentrations during the 7 days' exposure, fish were moved into the new tanks with corresponding BaP doses every day. Blood samples were taken on day 0, 1, 3, 5 and 7 before fish transfer among the tanks.

Six seahorses (three males and three females) in each treatment group were sampled randomly and placed into a bucket containing a solution of 0.035% MS-222 (Sigma-Aldrich, Castle Hill, NSW, Australia) in seawater before daily fish transfer for minimizing the effects on the immune system, and anaesthetized for 2 min, then 1/3 of the tail was cut for blood sampling [34].

2.3. Leucocyte isolation and immune parameter assays

Blood was first kept on ice for 10 min, mixed with 120 μL of 0.01 M phosphate buffered saline (PBS, Solarbio Science & Technology Co., Ltd, Beijing, China), and centrifuged at 840 ×*g* and 4 °C for 10 min. Plasma samples were stored at −80 °C before being used for IgM, C3, C4, IFN-α, and IL-2 determinations.

A leucocyte suspension using Percoll (SIGMA-ALDRICH, Co.,) was prepared from the centrifugation precipitate. After resuspension in 2 mL of 0.01 M PBS, the mixture was centrifuged at 100 ×*g* and 4 °C for 10 min. The supernatant was then slowly added into a 15 mL sterilized centrifuge tube, filled in advance with 3 mL of Percoll at 1.070 g/cm^3 density and 3 mL of Percoll at 1.020 g/cm^3 density. After centrifugation (840 ×*g* and 4 °C for 15 min), ~3.5 mL of leucocyte-containing liquid between scale range of 2–6 mL was collected and transferred to a FCM

tube. Leucocytes were rinsed twice with 0.01 M PBS to remove Percoll and centrifuged at 400 ×*g* and 4 °C for 5 min. Leucocytes were re-suspended in 900 μL PBS and adjusted to a concentration of 1×10^7 cells/mL for further analysis of respiratory burst and phagocytic activity.

Plasma IgM, C3, C4, IFN-α, and IL-2 were measured with commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following manufacturer protocol.

2.4. Analysis of RB, PA, and monocytes/leucocytes

A BD FACS Calibur flow cytometer was used for analysis of 20 000 cells per sample (Fig. S1A). Parameters RB and PA were represented with FI and LP (Fig. S1 B and C). Using 2′,7′-dichlorofluorescin-diacetate (H2DCF-DA, SIGMA-ALDRICH, Co.,), an ROS curve was determined to evaluate RB. Briefly, 2 μL H2DCF-DA was added into the FCM tube containing previously PBS-diluted leucocytes. To collect leucocytes, the tube was gently shaken for 45 min at 100 rpm and 25 °C in the dark, then centrifuged at 400 ×*g* for 5 min. After a 500 μL PBS wash and centrifugation, leucocytes were re-suspended in 300 μL PBS in preparation for FCM analysis.

Analysis of PA was performed using 1 μm Fluoresbrite yellow green carboxylate microspheres (Polysciences, Inc., Warrington, Florida, USA), diluted to 1×10^6 microspheres/μL with PBS. Diluted microspheres (10 μL) were added into the FCM tube with PBS-diluted leucocytes. The mixture was incubated in a dark shaker (100 rpm) for 1 h and 25 °C. After centrifugation at 400 ×*g* for 5 min, leucocytes were washed once with 500 μL PBS. Leucocytes were re-suspended in 300 μL PBS for further analysis.

Monocytes/leucocytes were evaluated according to cell size (FSC) and granularity (SSC). Leucocytes and monocytes were represented as G1 and G2, respectively (Fig. S1A).

2.5. Statistical analysis

All data were presented as means ± SD and analyzed in SPSS (version 22.0, Chicago, Illinois, USA). Student's *t*-test was used to determine significant differences of immune parameters in the following pairwise comparisons: male seawater control vs. female seawater control, male seawater control vs. male solvent control, female seawater control vs. female solvent control, male solvent control vs. female solvent control, male solvent control vs. male treatment, female solvent control vs. female treatment, and male treatment vs. female treatment. Pearson's correlations were used to analyze relationships between FI and LP of RB (FIR and PLR), as well as between FI and LP of PA (FIP and LPP) for both sexes.

Table 1
Correlations between FI and LP of RB and PA in seahorses treated with three BaP doses.

Parameter	Correlation coefficient					
	0.5 μg/L BaP		5 μg/L BaP		50 μg/L BaP	
	Female	Male	Female	Male	Female	Male
RB	0.431	0.310	0.855**	0.605*	−0.080	0.622*
PA	0.767**	0.876**	0.887**	0.914**	0.891**	0.925**

FI, fluorescence intensity; LP, leucocyte percentage; RB, respiratory burst; PA, phagocytic activity; BaP, benzo[*a*]pyrene; * significant differences at $P < 0.05$; ** significant differences at $P < 0.01$.

3. Results

3.1. Effects of BaP exposure on RB and monocytes/leukocytes

Exposure to BaP strongly affected FIR and LPR, with significant sex differences ($P < 0.05$) at most time points (Fig. 1 upper panel). Males had significantly higher ($P < 0.05$) basal (solvent and seawater controls) FIR and LPP than females (Fig. 1 and S2A, B). The two sub-parameters generally exhibited a same up-and-down variation pattern in both sexes when exposed to BaP, especially at 0.5 and 5 μg/L. We did not find a significant correlation between FIR and LPR in either sex, except under 5 μg/L BaP (Table 1). Monocytes/leukocytes were also heavily affected by BaP exposure, but no consistent variation trend between sexes was identified.

3.2. Effects of BaP exposure on PA

Males had significantly higher ($P < 0.05$) basal (pre-BaP) FIP and

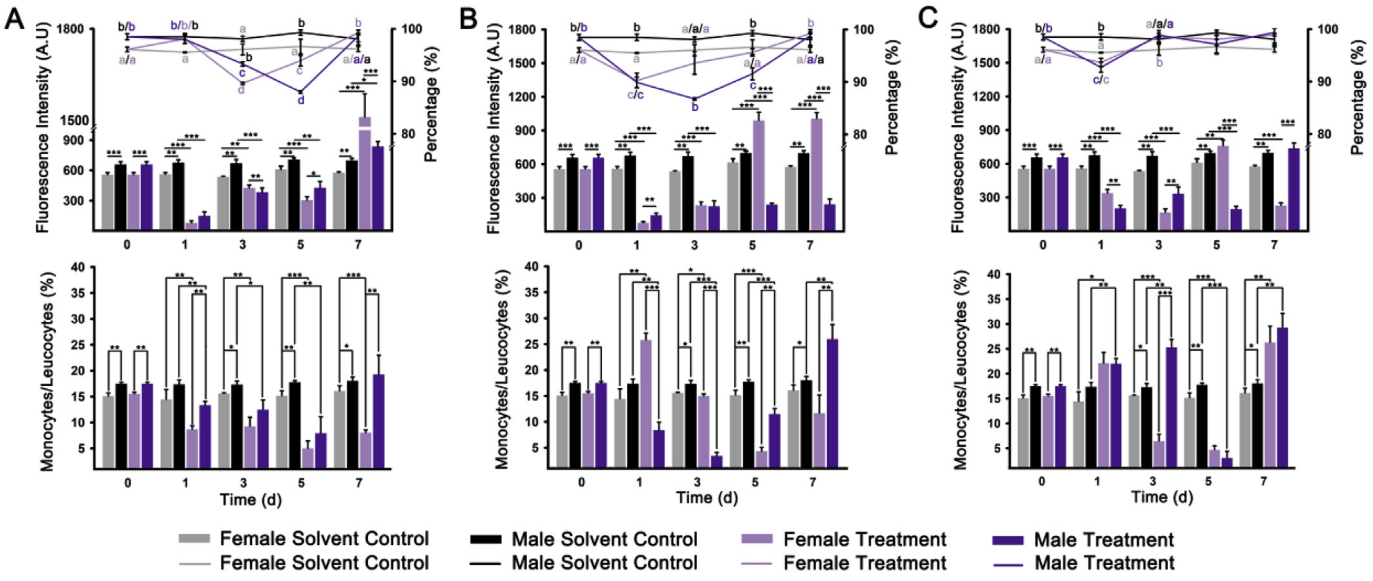

Fig. 1. RB (FIR and LPR) variation in blood leukocytes and monocytes/leukocytes sampled from male and female lined seahorses (*Hippocampus erectus*) across 7 d exposure to 0.5 μg/L (A), 5 μg/L (B), and 50 μg/L (C) BaP. Values are means ± SD, n = 3. *represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$. Lowercase letters indicate significant differences ($P < 0.05$) between treatment and sex combinations. RB, respiratory burst; FIR, fluorescence intensity of RB; LPR, leucocyte percentage of RB; BaP, benzo[*a*]pyrene.

PLP than females (Fig. 2 and S2B). The two subparameters were positively correlated ($P < 0.01$) in all treatment and sex combinations (Table 1). They also followed roughly the same pattern in both sexes, initially increasing and before a significant decrease, except in the case of females treated with 0.5 μg/L BaP (Fig. 2).

3.3. Effects of BaP exposure on plasma immune molecules and cytokines

Male seahorses had significantly higher ($P < 0.05$) basal plasma immune molecules (IgM, C3, C4) and cytokines (IFN-α, IL-2) than females (Figs. 3 and 4 and S3-4). All five molecules responded to BaP exposure and exhibited significant sex differences ($P < 0.05$) for most of the experimental period.

4. Discussion

In this study, we exposed *H. erectus* to different BaP doses and determined the responses of major blood immune parameters, including whether sex differences existed. Our research follows an increasing interest in the effects of marine pollution of wild and cultured seahorse stock [12–15,28]. We demonstrated that males had significantly elevated levels of most measured immune parameters (leucocyte RB and PA; plasma IgM, C3, C4, IFN-α, and IL-2) compared with females. Our findings indicate that male seahorses have higher immunocompetence, supporting the idea that parental investment—rather than sex per se—has a stronger effect on sexual dimorphism in the immune system. Specifically, the sex that maximizes fitness through longevity (i.e., those engaging in parental care) may require a stronger immune response [32,34]. Our results are in line with existing evidence in conventional and sex-role reversed species [32,34,39].

We found that FI and LP of both sexes were positively correlated in all trials of PA, as well as in 5 μg/L BaP trial of RB (Table 1). In *Oncorhynchus mykiss*, intraperitoneal injection of BaP significantly reduces PA (LPP in present study) without changing the phagocytic index (FIP in present study) of blood leucocytes [18]. Taken together, these results imply that both FI and PL are indispensable for FCM evaluation of RB and PA.

In juvenile European sea bass (*Dicentrarchus labrax*), intraperitoneal injection of 20 mg/kg BaP significantly suppressed spleen RB, without affecting spleen PA or kindney RB and PA [40]. In tilapia (*Oreochromis niloticus*), 50 but not 5 or 25 mg/kg BaP significantly suppressed pronephros macrophage RB without affecting PA [41]. In contrast with these earlier studies, we did not observe any dose or organ-dependent variation in RB and PA; instead, leucocyte RB and PA responded to BaP exposure at all tested doses, even though final BaP accumulation in *H. erectus* was ~1000 times lower than in other fishes (data not shown). This difference implies high sensitivity in lined seahorses, similar to findings in *H. guttulatus* and *H. reidi* [13,14,42]. Thus, *H. erectus* and other seahorses are strong candidates for a bioindicator of BaP exposure. Unlike the fluctuating pattern of RB (Fig. 1 upper panel), *H. erectus* PA was generally elevated on the first day and suppressed thereafter (Fig. 2). This pattern differs from the consistent BaP-induced suppression of blood PA (followed by an increase after 7 d of treatment) in *Epinephelus coioides* [43]. Characteristics of immune system and metabolic capability of BaP among fish species may mainly attribute to such discrepancy, even though different treatment methods may also affect the results. The lack of spleen and gut-associated lymphatic tissue (GALT) endowed seahorses with a partial but natural "deficient immune system" [44,45], which may greatly affect their responses to BaP exposure. On the other hand, metabolic capability varies among teleosts [46,47], since half-lifes of BaP were found different in *D. labrax* and *Pimephales promelas* [47,48].

Paralichthys olivaceus head kidney cells and macrophages experienced a significant downregulation of IgM after BaP treatment for 48 h [49]. Similarly, BaP treatment caused plasma IgM in *E. coioides* to fall in the first 4 d, before gradually increasing in the following 10 d [43]. We did not observe such consistent trends in IgM variation under different doses of BaP exposure (Fig. 2 upper panel).

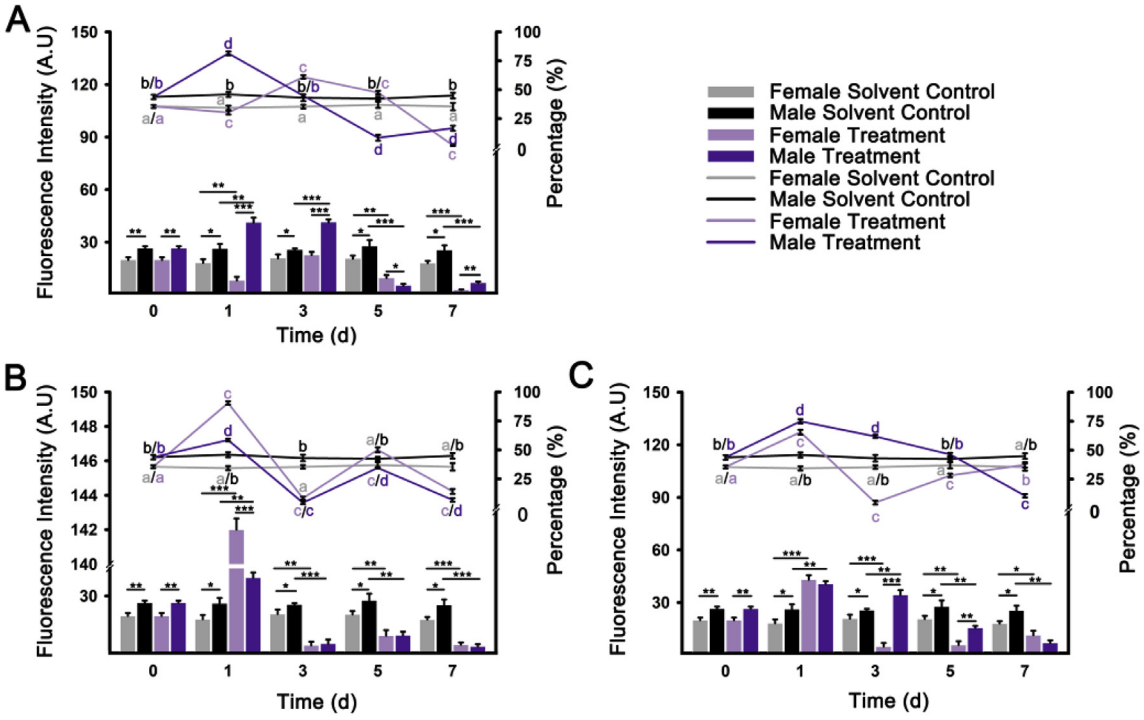

Fig. 2. Leucocyte PA (FIP and LPP) variation in male and female lined seahorses (*Hippocampus erectus*) exposed for 7 d to 0.5 μg/L (A), 5 μg/L (B) and 50 μg/L (C) BaP. Values are means ± SD, n = 3. *represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$. Lowercase letters indicate significant differences ($P < 0.05$) between treatment and sex combinations. PA, phagocytic activity; FIP, fluorescence intensity of PA; LPP, leucocyte percentage of PA; BaP, benzo[*a*] pyrene.

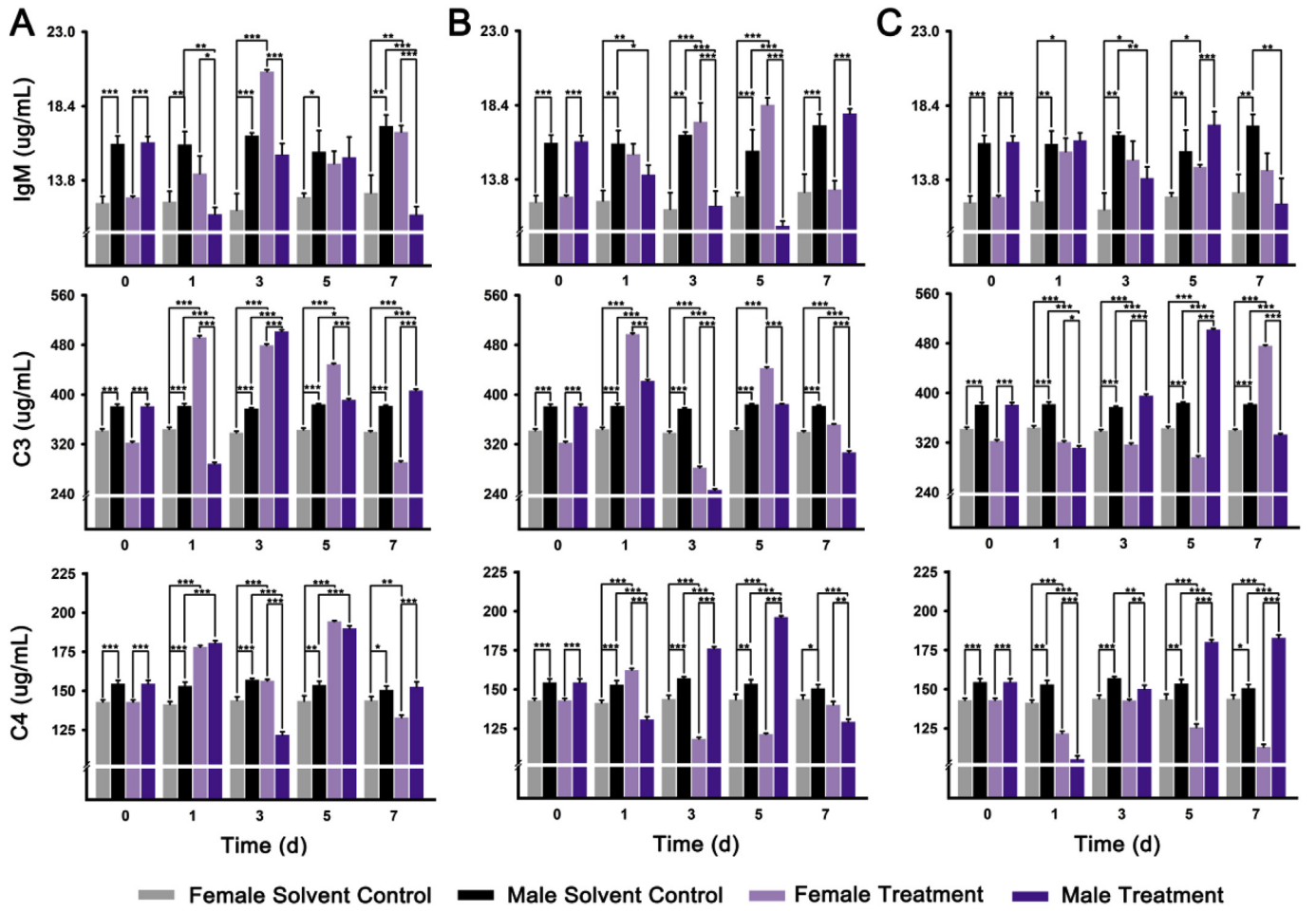

Fig. 3. Variation in plasma immune molecules IgM, C3, and C4 among male and female lined seahorses (*Hippocampus erectus*) exposed for 7 d to 0.5 μg/L (A), 5 μg/L (B), and 50 μg/L (C) BaP. Values are means ± SD, n = 3. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$. IgM, immunoglobulin M; C3, complement 3; C4, complement 4; BaP, benzo[*a*]pyrene.

The activity of the complement pathway was used for evaluating innate immune responses of fish challenged by marine contamination of oil and its derivatives [50–52]. Acute exposure to crude-oil-derived PAHs significantly increased ACH50 (alternative plasma complement pathway) activity in *D. labrax* after only 2 d, eventually returning to normal levels after 15 d of recovery [51]. Another *D. labrax* study testing BaP exposure found significant ACH50 induction after 4 h at two dosages (0.1 and 10 μg/L), but not after 24 h at all four dosages (0.001, 0.1, 10 and 1000 μg/L) [50]. While, in *O. mykiss*, no significant change of CH50 (classical plasma complement pathway) activity were detected after 6 weeks of exposure to diesel oil-based drilling mud extract [52]. Our results indicate C3 and C4 of the classical complement pathway may be better indicators because they can sensitively and continuously respond to a wider BaP dosage range (0.5–50 μg/L) over a longer period (7 d). In addition, their variation trends are dosage and sex-dependent (Fig. 2 middle and lower panel), similar to what we observed in monocytes/leucocytes, IFN-α, and IL-2 (Fig. 1 lower panel and 4). This study is the first to evaluate the response of these immune molecules to BaP exposure in lined seahorses. Future research should aim to uncover the mechanisms underlying sex dimorphism of our immune parameters during BaP exposure.

Based on previous studies, several teleost-derived molecules have been recommended as bioindicators for marine PAH monitoring, including mixed-function oxidase (MFO) enzymes, CYP1A1, vitellogenin, and metallothionein in rainbow trout [53,54], hepatic ethoxyresorufin-*O*-deethylase (EROD) in *E. areolatus* [55], as well as EROD and ethoxycoumarin-*O*-deethylase (ECOD) in *Acanthopagrus butcheri* [56]. In *H. reidi*, the micronuclei test and comet assay were found to be good biomarkers of petroleum contamination [42]. Here, we identified eight blood immune parameters that responded to three BaP doses, with significant between-sex variation. In particular, PA has more potential than RB as a biomarker for monitoring exposure to PAHs (including BaP) and crude oil.

5. Conclusions

In this study, we confirmed that our tested immune parameters were sensitive to BaP exposure. We also demonstrated clear sexual dimorphism in the response to BaP exposure. Furthermore, indices FI and PL are necessary for FCM evaluation of RB and PA. Our results lead us to recommend *H. erectus* as a sentinel organism, as well as PA and RB as molecular bioindicators of oil contamination. While the knowledge how seahorses differently response to BaP exposure compared to other teleosts and mechanisms underlying sexual dimorphism are still obscure. Take the advantage of the currently released genomic imformations seahorses [33, 57], RNA sequencing -based transcriptome analysis is a promising way to speed up the research processes.

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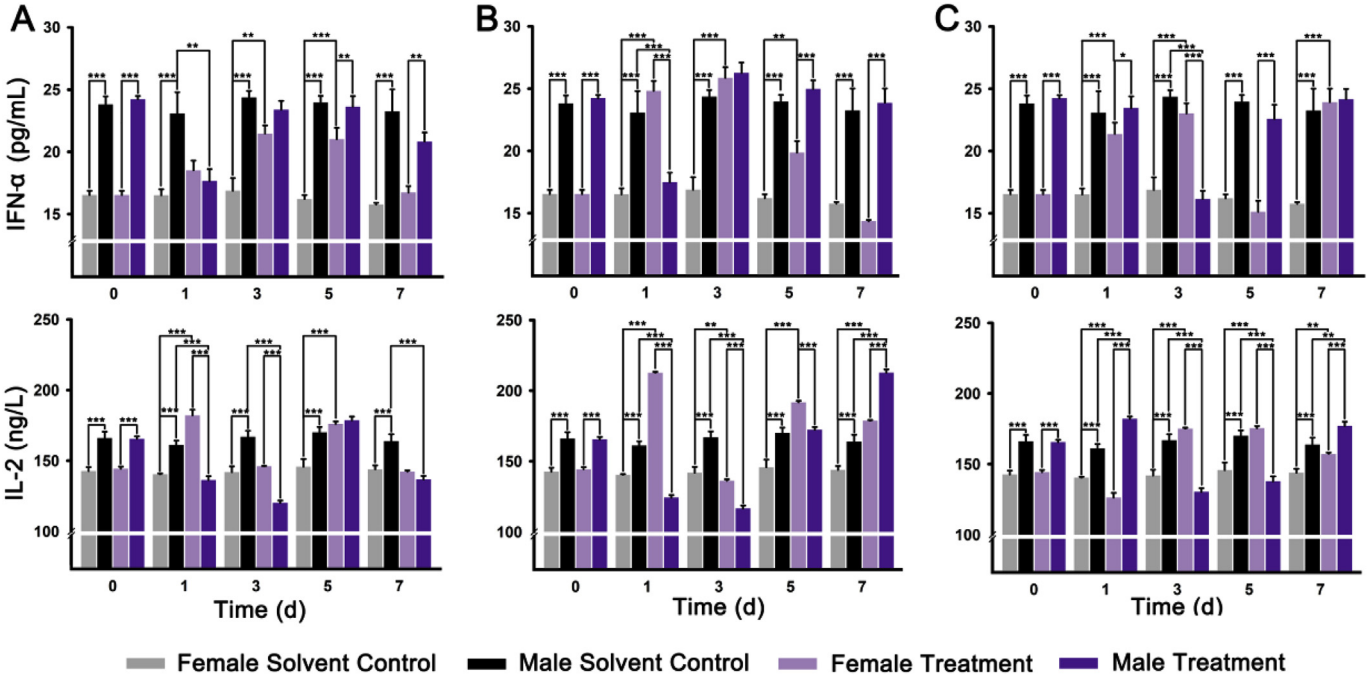

Fig. 4. Variation in plasma cytokines IFN-α and IL-2 among male and female lined seahorses (*Hippocampus erectus*) exposed for 7 d to 0.5 μg/L (A), 5 μg/L (B), and 50 μg/L (C) BaP. Values are means ± SD, n = 3. *represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$. IFN-α, interferon-α; IL-2, interleukin; BaP, benzo[*a*]pyrene.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.068.

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