Can Naphthalene Exposure Stress Alter Brain Biogenic Amine Levels Before and during Vitellogenesis in Liza klunzingeri Fish?

Zahra Yarahmadi1, Abdolali Movahedinia1, Ahmad Savari1, Morteza Behnam Rassouli1, Mohammadreza Sahraeian2, Hossein Pasha Zanoosi3 and Sara Rastgar*1

1Department of Marine Biology, Faculty of Marine Sciences, Khorramshahr University of Marine Science and Technology, Khozestan, P.O.Box: 669, Iran
2Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Iran
3Emam Khomeini Marine Fish Research Station, Khozestan, Iran

*Corresponding author: Sara Rastgar, Department of Marine Biology, Faculty of Marine Sciences, Khorramshahr University of Marine Science and Technology, Khozestan, P.O. Box: 669, Iran, Tel: +00980751335767; E-mail: bio.rastgar@gmail.com

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Abstract

In this study, effects of naphthalene (NAP) exposure on plasma levels of 17-β estradiol and concentrations of neurotransmitters in different female regions of female Klzunziingeri’s mullet, at the previtellogenic and vitellogenic stages were examined. Studied neurotransmitters were noradrenalin (NA), serotonin (5-HT), dopamine (DA), and 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxy-3-indoleacetic acid (SHIAA). In the short- term stress experiment, fish were i.p. injected with 2 μl/g vegetable oil alone (control) or oil containing NAP (50 mg/kg body weight). To study the long-term effect of NAP an amount of (50 mg/kg NAP +10 μl/g coconut oil) as i.p. implant and 10 μl/g coconut oil alone for control group. After long-term exposure, 17-β estradiol level in the plasma decreased in both stages fish. Monoamines analyses showed changes induced by NAP which depended upon the type of neurotransmitter and exhibited a marked brain regional selectivity. According to the results, NAP had more disturbing effects at the previtellogenic than the vitellogenic stages. Serotonergic system responded to the NAP exposure rapidly whereas dopaminergic system changed during the chronic naphthalene stress. The mentioned disturbing effects of naphthalene on the brain monoaminergic systems may cause some endocrine disruptions especially in the hypothalamus-pituitary-gonadal axis and finally affect the vitellogenesis process and final oocyte maturation. Reduction in the plasma levels of 17-β estradiol during the naphthalene exposure probably are related biological feedbacks of neurotransmitters on the pituitary.

Keyword: Naphthalene; Stress; Neurotransmitters; 17-β estradiol; Liza klunzingeri.

Introduction

Generation requires a successful reproduction in living organisms. In mature ovaries of female fish vitellogenesis is an important physiological process during which ova store yolk [1]. This process is started by gonadotropin hormones released from pituitary into the blood stream. The conversion of androgens into 17β- estradiol (E2) is accelerated by gonadotropins which then promotes the growth of granular cells of follicles and maturation of ovum [2]. Furthermore, in liver E2 stimulates the synthesis of vitellogenin protein (VTG), a precursor of yolk production in ovum [2,3]. The xenobiotics induced endocrine disorders may have destructive effects on health and survival of living animals, and in fish reproductive system, can be distinguished by several indices such as estradiol (E2) which is important in yolk production process [4]. Blood E2 level alterations can be related to the degree of environmental pollution [4] so that, pollutants lower E2 level [5,6], inhibit plasma vitellogenin [6] and early vitellogenesis [5] and finally disturb sexual maturation [4]. These malfunctions may be attributed by the effects of pollutants on the hypothalamo- pituitary- gonadal (HPG) axis [7].

Polycyclic aromatic hydrocarbons (PAHs), as environmental pollutants, inhibits estrogen biosynthesis and inactivate estrogen receptors (ER), by aryl hydrocarbon receptors (AhRs) activation [8] and therefore may lead to estrogen imbalance in target tissues [9]. AhR activation, on the other hand, alters the neurophysiological processes such as, synthesis and secretion of neurotransmitters in specific neural tissue [10]. Since in bonefish, gonadotropin secretion is control by aminergic pathways [11] therefore it seems that neurotransmitters are sensitive to organic pollutants. Naphthalene (NAP) is a simplest PAH compound. NAP and its methylated forms (alkyl naphthalene), as two hydrocarbon constituents of crude oil [12], considered as the most effective environmental pollutant [13] are very toxic for marine organisms [14]. During the sexual maturation, activation of neuroendocrine systems fluctuate the blood level of gonadotropins. Alternatively, gonadotropins secretion is under the control of serotonergic and dopaminergic pathways [15]. Despite of many reports which focused on the negative effects of PAHs on reproduction [9,16,17] the exact mechanism of the pollutant effects is not elucidated, so far. The aim of the present research was to investigate the effects of NAP on the level of neurotransmitters engaged in HPG at previtellogenesis and early vitellogenesis stages in fish. Measuring of the levels of neurotransmitters in fish brain may lead to better understanding of physiological and behavioral complexities in encountering pollutant stress during reproduction.

Materials and Methods

The fish under study

During January 2012 some 160 healthy Liza klunzingeri female
fish, weighted 96.7 ± 2.77 g, were caught at Khowr-e-Musa Estuary, located at the northern littoral of Persian Gulf (Khuzestan, Iran) and transferred to the Marine Fish Research Center in Imam Khomeini Port. To adapt to normal light and temperature, they were kept in 300 liter tanks for one week and fed for 1% of their body weight up to 24 hours before sampling.

**Experimental design**

In the experiment, an injection and implant of a certain dose of pollutants was used. Dose selection was based upon the previous studies conducted to evaluate PAHs toxicological effects in which, after PAH injection, fish bile PAHs contents were similar to those that naturally exposed to PAH [18-21]. To study the short-term effect of NAP, 80 fish were divided into control and test groups (n=40). Under anesthesia (0.2% 2-phenoxyethanol), test fish were weighed and then injected (ip) 50 mg/kg NAP soluble in 2 μl/g sunflower oil. The control fish were injected 2 μl/g sunflower oil. After 3 hours, the blood, brain, and gonads of all fish were sampled. In order to study the long-term effect of NAP, control and test groups (n=40) were anesthetized, weighed and then, in each fish, an amount of 50 mg/kg NAP soluble in 10 μl/g coconut oil was implanted (ip). The control fish were implanted 10 μl/g coconut oil, as the same manner. After 3 days, the blood, brain, and gonads of all fish were sampled.

**Sampling procedure**

In order to determine the plasma E2 level, fish anesthetized and their bloods were pulled out from caudal vein, using a heparinized syringe, centrifuged (6000 rpm for 7 minutes) and then plasma samples rapidly freeze in liquid nitrogen and kept at -80°C until use. Then all fish were decapitated and their brains dissected out. From the removed brains the pituitary, telencephalon (except olfactory lobe), pre-optic area (included optic tract) and hypothalamus [11,22] were separated, weighted and then rapidly freeze in liquid nitrogen and then kept at −80°C until neurotransmitter measurements. To determine the previtellogenic and early vitellogenic phases, the ovaries were also removed, fixed in Bouin’s solution and processed for histological preparation. The paraffin blocks were cut, stained (hematoxiline-eosin) [23] and examined under light microscope.

**Plasma 17β-estradiol measurement**

The plasma concentration of E2 was measured by ELISA technique [1,24].

**Assessment of neurotransmitters levels**

To measure the levels of dopamine, serotonin, noradrenalin, 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxy-3-indoleacetic acid (5HIAA) Waters 2695 HPLC technique was used. The HPLC system was equipped with Waters 2465 Electrochemical Detector (Waters Associates). The procedure was as proposed by Miguez et al. and modified by Gesto et al.

The HPLC liquid phase was prepared as a solution made up by 63.9 mM NaH₂PO₄, 0.1 mM Na₂EDTA (Sigma), 1.63 mM sodium 1-octanosulfate (Merck), and 14.9% methanol (Merck). The solution was filtrated and degasified before use.

In order to prepare tissue samples for HPLC, each sample was separately homogenized by an MSE 100 W ultrasonic homogenizer. Then, a volume of liquid phase solution equal to 0.1 ml for pituitary and 0.5 ml for hypothalamus, telencephalon, and the pre-optic area was added to the vial of the tissue, homogenized again, centrifuged at 16000 rpm and the supernatant was separated. After that they were diluted by liquid phase solution (1:1 for telencephalon and pre-optic area and 1:2 for hypothalamus, pituitary tissue did not diluted) [25-27]. Each injection volume was 30 μl, each sample run time was 15 minutes, each noise was 3, and the isocratic flow rate was 1.1 ml/min at room temperature.

**Statistical analysis**

To compare serum E2 level and neurotransmitters concentration in control and treatment groups at previtellogenic and early vitellogenic phases, the Two-way ANOVA was applied. For multiple comparisons, Student-Newman-Keuls was used. The safety coefficient is %95 (P<0.05) for the test. Moreover, Sigma plot ver. 11 was used for analyzing the data and drawing the diagrams.

**Results**

The *Liza klunzingeri* fish seemed healthy during short and long-term Exposure to NAP and no difference was observed in their swimming behaviors. Also, no stress-borne mortality happened. In light microscopy examination of the ovaries, previtellogenic and early vitellogenic stages can be identified.

**17β-Estradiol**

In short-term exposure the plasma levels of E2 showed no significant difference between control and treatment groups in both previtellogenic and early vitellogenic stages. However, in long-term Exposure with NAP, a significant decrease was observed in treatment group (P<0.05) (Figure 1).

**Measurement of Neurotransmitters Concentrations**

**Serotonin**

At previtellogenic stage: In response to short-term NAP exposure, serotonine content of telencephalon and hypothalamus increased (P ≤ 0.05), (Table 1) and 5-HT content of pituitary decrease significantly (P ≤ 0.05), (Table 1). In long-term NAP exposed fishes, there was a significant decrease in serotonine content of pre-optic area and hypothalamus (P ≤ 0.05), (Table 2). Also, significant decrease of 5-HIAA was observed in the four regions at pervitellogenic stage during the short-term NAP exposure while during the long-term exposure, 5-HIAA level was significantly decreased in pituitary and increased in hypothalamus and the pre-optic area (P ≤ 0.05), (Table 1 and 2).

**Figure 1:** Effect of naphthalene treatment on the response of plasma 17β estradiol to Short-term (3hr) or long-term (72hr) stress stimuli. P: previtellogenic stage. V: early vitellogenesic stage. White bars: control. Shaded bars: naphthalene treatment. *: indicatea significant difference from the control group (p<0.05).
**Vitellogenic stage:** In response to short-term NAP exposure, 5-HT content of telencephalon increased (P ≤ 0.05), (Table 1). The pre-optic area, in long-term exposure, 5-HIAA level showed a significant decrease (P ≤ 0.05), (Table 2).

**Dopamine**

At previtellogenic stage: The results showed a significant increase of dopamine concentration in telencephalon and the pre-optic area after short-term exposing with NAP (P<0.05), (Table 1). Moreover, three days after exposing with NAP, there was a significant decrease in dopamine concentration in telencephalon and the pre-optic area. In contrast, a significant increase of this monoamine was observed in hypothalamus and pituitary during the long-term exposuring with NAP (P<0.05), (Table 2). Dopamine concentration showed a significant increase in pre-optic area during the short-term stress (P<0.05), (Table 1). The average DOPAC concentration had a significantly increased in all areas of telencephalon, the pre-optic area, hypothalamus, and pituitary during the long-term exposing with NAP (P<0.05), (Table 2).

**Vitellogenic stage:** This neurotransmitter showed no significant difference in all areas in short-term exposing (P>0.05), (Table 1). For the long-term exposing, the average dopamine concentration showed a significant increase only in hypothalamus (P<0.05), (Table 2). The results of studying the long-term stress in the fish at vitellogenic stage showed a significant decrease in oxidized dopamine metabolite concentration in telencephalon, and its significant increase in hypothalamus (P<0.05), (Table 2).

**Noradrenalin:** Noradrenalin showed the least amount of change, and its changes were limited to hypothalamus and pituitary at previtellogenic stage. The results revealed a significant increase of noradrenalin in hypothalamus at previtellogenic stage in both exposure with NAP (P ≤ 0.05), (Table 1 and 2). However, at previtellogenic stage, significant decrease of noradrenalin was observed in pituitary during short and long-term (P ≤ 0.05), (Table 1 and 2).

**Discussion**

**Estradiol**

The results of the present research showed a significant decrease of 17β-estradiol levels at both previtellogenic and vitellogenic stages after the long-term NAP stress. Similar findings are reported on the effects of beta-naphthoflavon on Coho salmon [28] and BaP on Flander fish [9], rainbow trout [1] and Atlantic croaker [4]. PAHs are lipophilic compounds that may be stored in the tissues enriched in lipids, e.g., gonads and brain; therefore, they can have many effects on these organs [29,30]. The effect of NAP on 17β-estradiol may be due to either its interaction with the HPG axis or changes in E2 synthesis.

It is has been suggested that NAP inhibit the synthesis of the three enzymes involved in 17β-estradiol production [9], has anti-estrogenic activities and compete for bonding with ERs, and probably by prevention of expression of genes related to aromatase enzyme and by disturbance in yolk production reduce vitellogenin [31]. Even more, the results obtained from the present research indicate that NAP-induced E2 reduction is more remarkable at previtellogenic stage. Therefore, it can be concluded that previtellogenic stage is more susceptible to chemical stress than vitellogenic stage [27,32].

**Serotonin**

The cell bodies of serotonergic neurons were mostly located in diencephalon and mesencephalon, being more concentrated in hypothalamus [11]. Therefore, it can be expected that, when treated with NAP, more turbulence in serotonin system is observed at these areas. In telencephalon and hypothalamus, an increase in serotonin, a decrease in its metabolite concentration, and a decrease in 5-HIAA/5-HT ratio were occurred at previtellogenic stage after a 3-hour exposure with NAP (Figure 2). To explain this suggestion, the ratio of the amount of main metabolite of a transmitter to the amount of transmitter can be applied as an index for estimating the amount of neurotransmitter release. This ratio is an index of synaptic activity; high ratios indicate high resorption and release or high activity of aminergic neurons, and low ratios show low resorption and release or low activity of aminergic neurons [33-37]. It seems that decrease in serotonin activity is due to the decrease in serotonin catabolism. It is likely that non functional MAO enzyme inhibit serotonin convergence into 5-HIAA [38,39]. Since, in short-term NAP exposure at previtellogenic stage, a decrease in serotonin metabolite and also in 5-HIAA/5-HT ratio occurred at the pre-optic area (Figure 2), with no change in serotonin content it can be concluded that the activity of serotoninergic neurons may prevented by NAP [21]. NAP may have a preventive effect on serotonin catabolism at the pre-optic area.

At previtellogenic, 72 hours after treatment with NAP, a decrease in hypothalymus serotonin content occurred without any change in its metabolite and in the ratio of 5-HIAA/5-HT (Figure 2). Any disorder in the function of the enzymes synthesizing serotonin, e.g., tryptophan hydroxylase due to NAP [40].

Since NAP has narcotic characteristics [41], and the transporters of biogenic amines, such as serotonin, are the primary targets of narcotics in neurons [11,42,43], these compounds commonly bond with transporters and prevent the activity of neurotransmitters. Therefore, they may change the concentration of neurotransmitters.

**Figure 2:** Effect of naphthalene treatment on the response of 5-HIAA/5-HT to Short and long- term stress stimuli. P: previtellogenic stage. V: early vitellogenic stage. White bars: control. Shaded bars: naphthalene treated. *: indicate a significant difference from the control group (P<0.05).

and disturb the normal equilibrium of monoamine neurotransmitters [44]. Hence, the decrease of serotonin concentration and 5-HIAA/5-HT ratio in pituitary compared to control samples at previtellogenic stage [44]. Hence, the decrease of serotonin concentration and 5-HIAA/5-HT ratio in pituitary compared to control samples at previtellogenic stage. Thereafter, it might be caused by the disturbance in function of serotonin vesicular transporters during treatment with NAP (Figure 2). Although it is likely that NAP decreases the efficiency of the enzymes synthesizing serotonin, including tryptophan hydroxylase [45], the occurrence of each possibility may decrease serotonin metabolite and serotonin activity following a decrease in serotonin content.

**Dopamine**

Since, in vertebrate, reproduction is under the control of HPG axis and amine system indirectly regulates gonadal functions [15, 35], disfunctions of amine systems may disturb vitellogenesis [27, 46]. After 72 hours, the amount of DA decreased in telencephalon and the pre-optic area at previtellogenic stage during exposuring with NAP. There are a few possibilities for decreasing DA: (1) as tyrosine convert into catecholamine dopamine by tyrosine hydroxylase enzyme after absorption in neurons, it is probable that NAP decreases dopamine production by preventing from its activity [47], and (2) NAP may decrease DA vesicular absorption in presynaptic neuron through its agonistic effect on DA transporters [48]. Since catabolism rate increases simultaneously with DA decrease, the occurrence of each possibility seems quite rare. In fact, DA decreasing, when it exposure with NAP, results from increasing the absorption rate and consumption of this neurotransmitter. In other words Increased dopaminergic activity in these areas (Figure 2).

Regarding the changes of dopamine system at the pre-optic area, during its 3-hour exposure with NAP at previtellogenic stage. Gesto et al. reported opposite results in immature rainbow fish. The reason for such different results may be attributed to fish sexual stage; because fish prepare to enter into yolk production at previtellogenic stage. Thus, in addition to NAP effect, hormonal feedbacks and physiologic changes in maturity may influence dopamine variations. Since the ecology and genetics of these species may differentiate the structure of the brain area in maturity may influence dopamine variations. Since the ecology and genetics of these species may differentiate the structure of the brain area in maturity may influence dopamine variations. Since the ecology and genetics of these species may differentiate the structure of the brain area in maturity may influence dopamine variations. Since the ecology and genetics of these species may differentiate the structure of the brain area in maturity may influence dopamine variations. Since the ecology and genetics of these species may differentiate the structure of the brain area in maturity may influence dopamine variations. Since the ecology and genetics of these species may differentiate the structure of the brain area in maturity may influence dopamine variations. Since the ecology and genetics of these species may differentiate the structure of the brain area in
and dopamine catabolism, or has decreased dopamine concentration by disordering the function of monoamine oxidase (MAO) enzymes and stimulating such enzymes for more dopamine oxidation [38,39].

In long-term NAP exposure, DA content and DOPAC metabolite increased in hypothalamus at both previtellogenic and vitellogenic stages. It means that the synthesis, release, and catabolism of dopamine were increased.

Noradrenalin

The amount of noradrenaline in hypothalamus increased at previtellogenic stage within 3 and 72 hours after exposing with NAP. Gesto et al. reported similar results when they studied the effects of NAP on brain neurotransmitters in rainbow trout. Since noradrenergic inputs reach mesencephalon (hypothalamus) from hindbrain, it appears that NAP increases the concentration of noradrenaline through its stimulating effect on its synthesis. Moreover, as DA is the precursor of noradrenaline synthesis and amino acids are bounded between these two neurotransmitters [49], it may be said that noradrenaline changes follow dopamine changes in hypothalamus after 72 hours. As the cellular masses of noradrenaline neurons are located at locus coeruleus in brainstem and their axons extend to pituitary [11,50], it appears that noradrenaline decrease is induced by NAP at both 3 and 72 hour exposures preventive effect on noradrenaline production in brainstem or its release from the axons of adrenergic neurons in pituitary. Despite the fact that intermediate metabolites are bounded between dopamine and noradrenaline, it seems that different mechanisms arising from NAP activity indirectly influence the metabolism of each catecholamine in pituitary neural terminals.

Treatment with NAP disturbed the balance of biogenic amines in some parts of fish brains at previtellogenic and vitellogenic stages. However, the changes were more evident at previtellogenic stage. The patterns of these changes may vary due to aminegic neurons variance, exposure duration, and sexual stages. The results show that NAP, as an organic pollutant, has a high potential for creating extensive changes in brain monoamine system. The complex of aminegic neurotransmitters related to hypothalamus, pituitary, and the pre-optic area are involved in pituitary endocrine activities such as regulating GH hormone, synchronization of important occurrence in reproduction [11,55]. The results indicate most changes in the aminegic system in this area.

The feedback effects of steroids on the hormonal task of pituitary-hypothalamus are well recognized [51]. The steroids released into the body may influence the increase or decrease of pituitary secretions, at least by positively or negatively affecting the controlling factors of these secretions, including neurotransmitters [15,52]. Since NAP treatment has caused changes in 17β-estradiol levels in plasma, it is likely that level changes of this hormone has led to the disturbance of brain monoamine system due to NAP treatment through positive or negative feedbacks. NAP may disturb yolk production process by directly influencing the gonadal tissue or the HPG axis.

Conclusion

The results of the present study indicate that, in previtellogenic stage of fish reproductive period, NAP may targets serotonergic and dopaminergic systems in short-term and long-term exposure, respectively. Moreover, NAP induced changes in 17β-estradiol probably is due to the consequence of the effects of NAP on aminegic systems which alternatively alter the output of HPG axis. Disturbance of yolk production endangers the successful reproduction in fish.

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