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

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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 regions of female Klunzinger’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 (SHI). 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 hypothalano-pituitary-gonadal (HPG) axis [7].

Polycyclic aromatic hydrocarbons (PAHs), as environmental pollutants, inhibits estrogen biosynthesis and inactivate estrogen receptors (ER), by aryl hydrocarbon receptors (AhR) activation [8] and therefore may lead to estrogenic 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 amineamic 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 naphthalenes), 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
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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 prev-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).

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.

Figure 1: Effect of naphthalene treatment on the response of plasma 17β estradiol to Short-term (3hr) or long-term (72hr) stress stimulus. P: previtellogenic stage. V: early vitellogenic stage. *: indicates a 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 (P ≤ 0.05), (Table 2). DOPAC 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-naphthoflaven 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-esterogenic 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 amineergic neurons, and low ratios show low resorption and release or low activity of amineergic 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 serotonergic 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 hypothalamic 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 vitellogenesnic stage. White bars: control. Shaded bars: naphthalene treatment. *, indicate a significant difference from the control group (p < 0.05).
Telencephalon, Hypothalamus, Hypophysis, Preoptic area

production by preventing from its activity [47], and (2) NAP may absorption in neurons, it is probable that NAP decreases dopamine into catecholamine dopamine by tyrosine hydroxylase enzyme after pre-optic area at previtellogenic stage during exposing with NAP. After 72 hours, the amount of DA decreased in telencephalon and the and aminergic system indirectly regulates gonadal functions [15, 35] occurrence of each possibility may decrease serotonin metabolite and synthesizing serotonin, including tryptophan hydroxylase [45], the serotonin vesicular transporters during treatment with NAP (Figure 2). stage in short-term may be induced by disturbance in the function of HT ratio in pituitary compared to control samples at previtellogenic [44]. Hence, the decrease of serotonin concentration and 5-HIAA/5-HT significantly increased in compare with control, ↓ significantly decreased in compare with control.

Table 1: Alterations of transmitter’s content in certain regions of brain in female fish exposed to NAP.

|       | Telencephalon | Hypothalamus | Hypophysis | Preoptic area | Telencephalon | Hypothalamus | Hypophysis | Preoptic area |
|-------|---------------|--------------|------------|---------------|---------------|--------------|------------|---------------|
| 5-HT  | C 572.96 ± 66.53 1834.02 ± 21.81 55.77 ± 4.25 295.34 ± 3.1 249.33 ± 1.85 1351.66 ± 15.89 | 24.01 ± 0.55 238.78 ± 2.14 | Table 2: Alterations of transmitter’s content in certain regions of brain in female fish exposed to NAP.

|       | Telencephalon | Hypothalamus | Hypophysis | Preoptic area | Telencephalon | Hypothalamus | Hypophysis | Preoptic area |
|-------|---------------|--------------|------------|---------------|---------------|--------------|------------|---------------|
| 5-HT  | C 638.72 ± 1.54 1831.06 ± 20.72 53.96 ± 2.09 295.66 ± 3.19 | 24.01 ± 1.10 239.23 ± 2.45 | | | | | | |
| 5-HIAA| C 579.46 ± 64.89 1787.36 ± 2.43 | 157.56 ± 19.19 | 281.50 ± 2.27 | 1386.10 ± 2.41 | 16.43 ± 0.16 234.81 ± 0.53 | | | |
| DA    | C 370.93 ± 4.38 191.16 ± 3.72 | 106.79 ± 2.57 | 213.43 ± 1.82 | 124.75 ± 1.05 | 73.76 ± 0.77 223.03 ± 1.03 | | | |
| DOPAC | C 187.56 ± 2.27 1787.36 ± 2.43 | 106.79 ± 2.57 | 213.43 ± 1.82 | 124.75 ± 1.05 | 73.76 ± 0.77 223.03 ± 1.03 | | | |
| NA    | C 679.68 ± 5.52 678.69 ± 5.73 | 207.33 ± 2.30 | 604.56 ± 2.73 | 190.22 ± 5.82 | 117.03 ± 1.06 198.56 ± 0.78 | | | |
|       | E 753.53 ± 97.70 695.82 ± 3.22 | 229.83 ± 2.08 | 593.03 ± 3.02 | 674.92 ± 1.79 | 203.66 ± 2.47 198.56 ± 0.78 | | | |

Table 2: Alterations of transmitter’s content in certain regions of brain in female fish exposed to NAP.

and disturb the normal equilibrium of monoamine transmitters [44]. Hence, the decrease of serotonin concentration and 5-HIAA/5-HT ratio in pituitary compared to control samples at previtellogenic stage in short-term may be induced by disturbance in the 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 aminergic system indirectly regulates gonadal functions [15, 35] disfunctions of aminergic systems may disturb vitellogensis [27,46]. After 72 hours, the amount of DA decreased in telencephalon and the pre-optic area at previtellogenic stage during exposing 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 simultaneous 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 various species, difference in NAP storage pattern, and consequently in its effects on the area, seems possible.

It may be said that long-term exposure with NAP has decreased dopamine system activity through stimulating synthesis and resorption
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 noradrenalin in hypothalamus increased at previtellogenic stage within 3 and 72 hours after exposure 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 noradrenalin through its stimulating effect on its synthesis. Moreover, as DA is the precursor of noradrenalin synthesis and amino acids are bounded between these two neurotransmitters [49], it may be said that noradrenalin changes follow dopamine changes in hypothalamus after 72 hours. As the cellular masses of noradrenalin neurons are located at locus coeruleus in brainstem and they extend into pituitary [11,50], it appears that noradrenalin decrease is induced by NAP at both 3 and 72 hour exposures preventive effect on noradrenalin 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 noradrenalin, 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 aminergic 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 aminergic neurotransmitters related to hypothalamus, pituitary, and the pre-optic area are involved in pituitary endocrine activities such as regulating GTH hormone, synchronization of important occurrence in reproduction [11,35]. The results indicate most changes in the aminergic 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 aminergic systems which alternatively alter the output of HPG axis. Disturbance of yolk production endangers the successful reproduction in fish.
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