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Research Article

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Herbicides Mixture with Potent Endocrine-Disrupting Properties In Goldfish: As An Early Warning At Environmentally Relevant Concentration

Masroor Fatima*, Fazle Rasool, Farheen Saba

School of Sciences (Zoology), Maulana Azad National Urdu University, Gachibowli, Hyderabad, Telangana-500032, India

***Corresponding Author**
Masroor Fatima, School of Sciences (Zoology), Maulana Azad National Urdu University, Gachibowli, Hyderabad, Telangana-500032, India.

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Abstract

For a meaningful interpretation of sub-lethal effect on fish, we have conducted a series of studies with a relevant environmental concentration of a mixture of herbicides; Atrazine, simazine, diuron and isoproturon (ASDI) to observe endocrine-disruption in goldfish under controlled laboratory conditions. Carassius auratus were exposed to 50µg/l of ASDI mixture for 12 weeks. Recorded mid-term assays shows, significant increased levels of cortisol and aromatase after 4-weeks. Reduction in aromatase activity at 8 and 12 weeks is followed by estradiol (E2) showing a positive correlation between them. Hence, 11-Keto reduced significantly at 4-8 weeks and enhanced at 12 weeks. Remarkable reduction in E2 and 11-Keto may be correlated with the reduced egg production in stressed fish by these herbicides. However, no remarkable change in testosterone is observed at 4 and 12 weeks as compared to the control. ASDI mixture treatment induced remarkable induction in vitellogenin (Vtg) biomarker protein in male stressed fish. Furthermore, it may confer that modulation in the pathway of the reproductive cycle may alter the gamete quality, quantity and consequently may disrupt spawning and mating behaviour in fishes even at a realistic level of pollutant in the environment.

Keywords: Cocktail, Atrazine, Simazine, Diuron and Isoproturon, Steroid Hormones.

Introduction

Low-level chemicals exposure may adversely affect several ecosystems whose mode of action may disrupt the normal endocrine function. The organic chlorine compounds like pesticides, fungicides and herbicides are in particular responsible to load ponds, wetlands and other aquatic habitats through runoff from agricultural fields and industrial settings. There are some extensively used categories of pesticides affecting aquatic organisms and humans directly or indirectly [2]. Among these popular pesticides are the herbicides that are suspected to induce reproductive dysfunction in aquatic animals [20,46]. Therefore, the possible harmful consequences of these chemicals are hormonal disruption and subsequent impairment in reproduction and development by interfering with the function of enzymes involved in steroid synthesis and inhibition [33]. Steroid regulates various biological processes including reproductive development, immune and stress responses. Moreover, they are well known to regulate immune response by steroid-receptor dependent and steroid-receptor independent mechanism in different types of fish immune cells. Habitants of the aquatic environment are adversely affected by the ubiquitous presence of these xenobiotics at low level for a long duration. Their mode of action is to disrupt endocrine function and imposes a challenge to the sexual development of animals. It is of great concern for ecotoxicological researchers to avoid their hazardous and retrogressive effect due to their function as hormone mimic compounds [31]. As well researchers should focus on studies whether they act in an additive, synergistic, or antagonistic manner [22]. However, environmental concentrations of these pesticides in the freshwater system are declining due to the restriction taken to limit their use and discharge in the aquatic system. But low degradation rate of studied herbicides caused their presence above the permissible level in the drinking water of Belgium (EAEW report, 1998) and aquatic environments despite restriction policies [27,22].

Triazine (atrazine and simazine) and phenyl urea (diuron and isoproturon) are among the most widely used herbicides in the world. Several studies have been conducted in the past few years to address the gonadal abnormalities due to altered sex steroid concentration by atrazine [6,37,40], simazine [24,38,42], diuron

and isoproturon [15, 12,10,16]. For a meaningful interpretation of toxic effects in fish, knowledge of sub-lethal events is essential and for this reason, we have conducted a series of studies with a relevant environmental concentration of a mixture of triazine and phenyl urea herbicides; Atrazine, simazine, diuron and isoproturon (ASDI) pollutants and using immune and endocrinological changes as principal endpoints under controlled laboratory conditions [9]. Goldfish (*Carassius auratus*) were exposed to a cumulative concentration of 50µg/l of ASDI mixture for 12 weeks which was reported above the permissible level in the drinking water of Belgium (EAEW report, 1998) and nearby aquatic environments, confirming some of these herbicides still persistence despite restriction policies [1,27]. Because there is increasing evidence that supports a bi-directional relationship between the endocrine and immune systems in fish [34]. Of particular interest is the mixture of herbicides ASDI at their realistic level in the environment causing endocrine disruption at the biochemical level. There is a growing awareness that these chemicals may function as hormone mimic and effect on the endocrine system. Further the present study focused on potential and sensitive marker like Vtg in goldfish to explore the potential biomarkers for combined effect of herbicides at low realistic concentration biomonitoring in aquatic environments. As well studies should focus on the cocktail of these pollutants at their realistic concentration in the water bodies and investigate their deleterious effects on the fish population.

Methods

Chemical Preparation

To observe the mode of action of 4-herbicides mixture i.e., Atrazine (A), Simazine (S), Diuron (D) and Isoproturon (I) were purchased from Sigma (Aldrich) for the experiment. Each herbicide was first dissolved in acetone to give a final concentration of 50µg/l. The acetone concentration-1µl/ml was maintained in the exposure media. Then the solution was mixed with 1L-distilled water at 37-40 °C with continuous stirring to evaporate acetone. Prior to exposure of fish in experimental tanks, the degradation rate of each experimental herbicide (ASDI) in water recirculation tanks was evaluated by high pressure liquid chromatography (HPLC). Degradation rate and half-life values of these herbicides were evaluated different through HPLC. Therefore, the treatment of ASDI mixture was decided to refresh twice a week to maintain the optimal desired concentration of each experimental chemical in the experimental tanks. Water samples were collected twice a week from both groups to maintain the desired constant levels of each herbicide. These water samples were analysed by HPLC during the exposure duration too. The recorded average value for degradation of Atrazine (42±9.5), Simazine (41±7.8), Diuron (41.51±8.4) and Isoproturon (47±8.4) were observed. The mixture of these herbicides solution was finally poured in re-circulatory system of tanks made available for these studies.

Maintenance of Experimental Fish

The experiment was performed at department of Biology, University of Namur, Belgium for the treatment of ASDI, Goldfish (*Carassius auratus*) of 18.1 ± 2.7 cm length and weight 71.6 ± 33.3 g were used in the present study collected from rearing university pond. They were maintained in 100 l capacity PVC tanks. Experimental fish were acclimatized for three weeks and fed commercial pellets (Cyprico, 4949, Coppens International, The Netherlands). Thirty fish per tank were randomly allotted to series of six tanks which were equipped with well-aerated, re-circulated water, passing through a mechanical filter. Goldfish were provided with 16h light: 8h dark photoperiod. Water quality was monitored for unionised ammonia, nitrate, nitrite, pH, dissolved oxygen, and temperature at least twice a week throughout the experiment to maintain its optimal level. The average values (±S.D.) of dissolved oxygen: 8.61±0.67; 8.14±0.86, temperature: 19.55±2.03; 20.15±1.997, ammonia: 0.35±0.2; 0.29±0.12, nitrate: 4.35±1.83; 4.65±1.71, nitrite: 0.006±0.01; 0.01±0.4, were measured in control and ASDI-treated tanks, respectively throughout the experimental duration.

ASDI Exposure and Analytical Procedures

Thirty fish of mixed sex per tank were allocated to four tanks representing two replicated groups of both control and ASDI-treated fish. After acclimatization, the fish were exposed to 50µg/l-ASDI for 12 weeks which have given noticeable results in our immunological investigations [9]. From the same group of goldfish, three fish from each tank (12 fish per treatment) at 4, 8, and 12 weeks were used to determine plasma hormones. Fish were anaesthetized with 120 mg/l amino-benzoic acid (Sigma-Aldrich, Belgium) before drawing the blood by caudal puncture in heparinized syringes. Blood samples were centrifuged at 10,000g for 10 min and separated plasma frozen at -20°C until assayed for steroids (radioimmunoassay). After measurement of fish length and weight, brain and gonads were dissected out, weighed and immediately kept in liquid nitrogen and frozen at -80 °C until used.

Tissue Preparation/Homogenate

Brain was carefully removed, weighed, homogenized in nine times its weight in ice-cold phosphate buffer (KPO_4, pH 7.4). The tissue was homogenized for 30 seconds by up-and-down strokes at 400 rpm in the Potter-Elvehjelm homogenizer. The homogenate was distributed in two Eppendorf tubes and kept in -80 ˚C until assay. All tissue preparation steps described above were carried out at 0-4˚C. The brain aromatase (P450 aromatase) activity was measured as the specific release of tritiated water accompanying the conversion of 1β-3H-androstenedione to estrone. Protein was quantified according to Lowry et al., (1951).

Chemicals and Materials

Androst-4-ene-3, 17-dione, [1β-3H(N)]- was purchased from ICN Biomedicals Inc., β-NADPH and Charcoal from Sigma. All Radioactive hormones were purchased from Amersham Pharmacia Biotech Inc. (NJ).

Steroid hormone extraction from Plasma

Samples of 50µl of plasma for each steroid at 4, 8 and 12 weeks were extracted twice with Cyclohexane/ethyl acetate (V/V), evaporated under nitrogen and reconstituted in Phosgel buffer (0.01

M NaH2PO4, 99% NaCl, 1g/L gelatin, pH 7.25). Testosterone (T), estradiol-17β (E2), 11-Ketotetosterone (11-KT) concentrations were determined by Radio-immunoassay (RIA).

Aromatase Activity in Brain

50mM KPO4, 250mM sucrose, 10mm dithiothreitol, pH 7.4 reagents were added to 100µl brain homogenate in duplicate as follows: KPO4, 25nM 3H-androstenedione (250µCi/µmol; ref: NET 926, NEN Life Science Product), β-NADPH (Sigma). All reactions were started by the addition of NADPH and terminated by the addition of 5% TCA. Incubation took place at 0˚C and 30˚C in a shaking water bath. After 1h the products and remaining substrate were extracted with 1ml ethyl acetate by vertexing for 5 min in a multitube vortex. After centrifugation for 10 min at 1000rpm, an aliquot of the solvent was evaporated and the residues were kept in -80 ˚C until use. During sex steroid hormones and cortisol estimation, the dried residues were dissolved in a small amount of ethanol containing unlabeled reference steroids. Values of each radioactive steroid were expressed in percentages, with 100% being the total radioactivity recovered from the blank. From this, the Pico mol conversion of the substrate to the relevant products was calculated.

Radioimmunoassay (RIA)

Plasma sex steroid content for cortisol, T, 11-KT, E2 were measured by RIA according to the method as described by Spano et al., (2004). The concentrated charcoal solution was used for all assays to reduce non-specific binding.

Vitellogenin (Vtg) Estimation

Plasma vitellogenin (Vtg) was determined following the method of Spano et al., (2004).

Data Analysis

Data of the present experiment are presented as mean ± S.D. Significant differences between the control and ASDI treated group was analyzed by using computer package STATISTICA followed by the analysis of variance to test homogeneity of all data by MANNOVA / ANOVA. Duncan's post hoc test was used to calculate the significant differences among the experimental groups. Differences were considered at $P< 0.05$.

Results

Effect of ASDI on brain Aromatase and Plasma Hormones

The cortisol data expressed in (Fig. 1) indicated that its level in plasma was increased significantly ($p<0.05$) following 4 weeks of treatment with mixture of ASDI having the concentration 50µg/l. Hence, in the extended exposure duration of ASDI to goldfish, plasma cortisol level responded in a linear manner to change at 8 and 12 weeks which were not significant at $p<0.05$.

As presented in (Fig. 2a), the aromatase (AA) activity was significantly reduced at week-8 and 12 with increasing duration of exposure. In the testes and ovary, no significant variability observed in aromatase activity between males and females as shown in (Fig. 2b) fish respectively. Aromatase is an important enzyme used in the synthesis of estrogen.

Experimental goldfish exposed to approximately realistic concentration of ASDI mixture in drinking water (50µg/l) did not show any significant difference in classical active androgen of fish-testosterone (T) in comparison to control group of fish at 4 and 12-week time duration of exposure (Fig. 3). Remarkable reduction in hormone T concentration at $p<0.05$ in the stipulated time period of ASDI exposure was observed at 4-week. Whereas, in the similar experiment, the level of 11-ketotestosterone (11-K) was stable in reference fish throughout the experiment with reduction in treated group of experimental fish at 4 and 8 weeks which is significant at $p<0.05$. However, its activity was peaking in the last week i.e., 12 weeks of exposure as highlighted in (Fig. 4).

The analysis of 17β-estradiol as shown in (Fig. 5) depicted a significant decreased level in terms of ng/ml of E2 in the blood plasma of ASDI-treated *C. auratus* (352.00±200 and 334.16±87.75, n=12) at 4 and 12 weeks respectively of ASDI-exposure in comparison to control group of experimental fish. The different values are highly significant at $p<0.05$. For the vitellogenin a remarkable difference observed in male goldfish exposed to ASDI. Such biomarker protein Vtg significantly ($p<0.05$) increased in exposed male fish in comparison to female (Fig. 6).

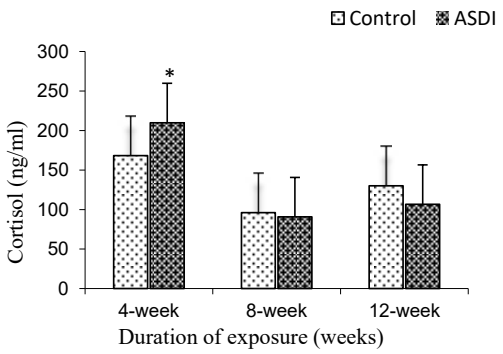

Fig. 1 ASDI-exposure duration dependent effect on cortisol (pg/ml) activity in goldfish

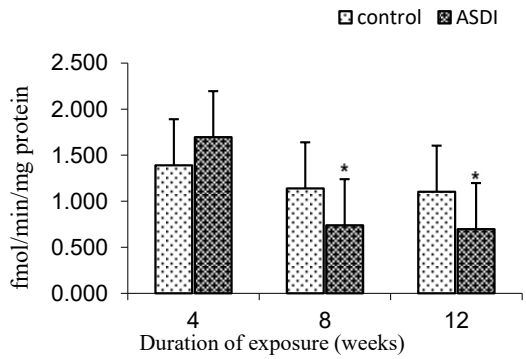

Fig.2a ASDI-exposure duration dependent effect on aromatase activity in goldfish

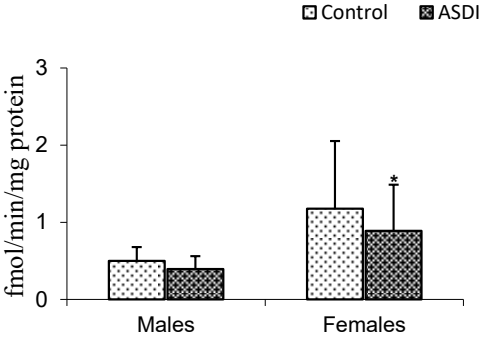

Fig. 2b Aromatase activity in male and female goldfish after ASDI-exposure

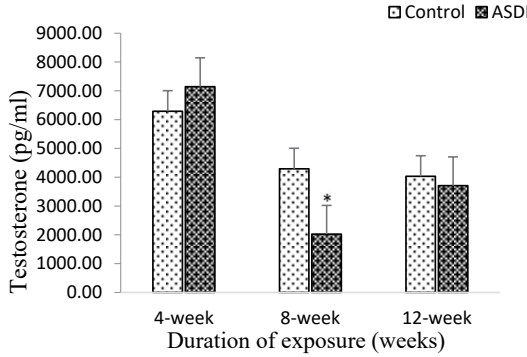

Fig. 3 ASDI-exposure duration dependent effect on testosterone (pg/ml) activity in goldfish

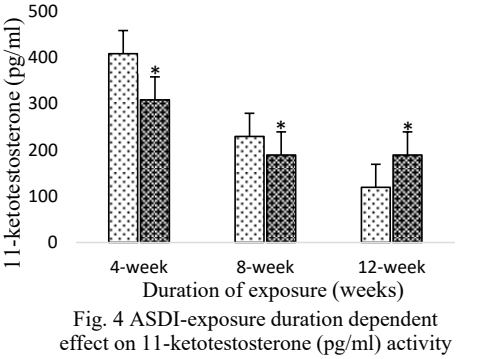

Fig. 4 ASDI-exposure duration dependent effect on 11-ketotestosterone (pg/ml) activity in goldfish

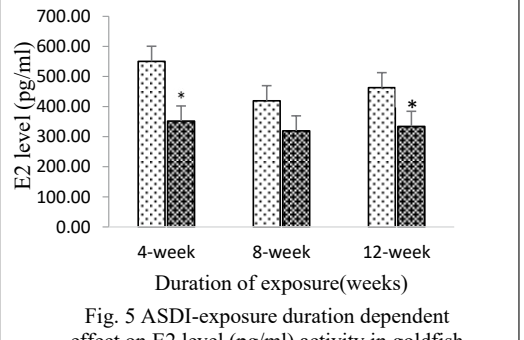

Fig. 5 ASDI-exposure duration dependent effect on E2 level (pg/ml) activity in goldfish

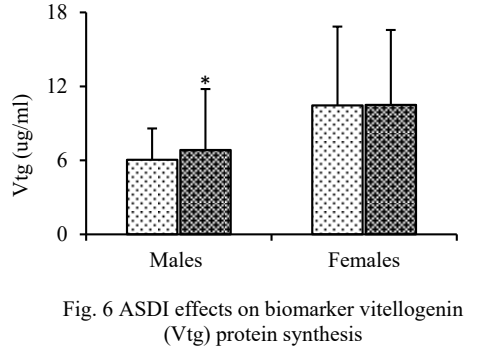

Fig. 6 ASDI effects on biomarker vitellogenin (Vtg) protein synthesis

Discussion

The hormone is cortisol which has been proposed as a key factor associated with environmental stresses to reproductive issues in teleost fish [13]. Moreover, it acts as a regulatory factor for physiological functions and known to suppress reproductive function, as a part of rapid physiological adjustments at the time of exposure to stressors [23]. In the present study, cortisol was increased in ASDI-stressed fish immediately after 4-weeks. Hence, afterwards no significant changes occurred compared to control group of fish throughout the experiment showing the changes in habitat stressed the exposed fish that released cortisol as a chemical factor in high quantity which is commonly used as a primary response to stressors such as metals and pesticides [11]. In fish, it is noted that cortisol dramatically rise during stress and seems to play key role in stress associated responses [15]. Boscolo et al., (2018) also found increased cortisol after exposure to diuron metabolites to Nile Tilapia and concluded that these herbicides alter the production of neurotransmitters related to modulation in behaviour and in consequence caused anti-androgenic effects on fish [26]. Impaired level of cortisol after the treatment of atrazine-simazine based herbicides caused hampered metabolism and altered ionic responses in fish fingerlings as reported by Koakoski et al., (2014). Variation in cortisol activity shows that how the fish mobilize energy substrate to cope with the stress, the increased catabolism of cortisol may have an adverse effect on the performance of fish to stressful situations [28].

The recent study of Castaneda-Cortes and Fernandino (2020) explained that the brain, through the hypothalamus detects the changes in the environment by increasing the level of cortisol and finally caused changes to the ratio of sex steroids. For the balanced production of androgen, cortisol directly may inhibit the aromatase activity which converts androgens into estrogens to maintain ovarian function resulting in the suppression of estrogen synthesis. Estrogen-E2 is produced via aromatization of T by aromatase enzyme and is responsible for inducing and maintaining the ovarian development in fish like other vertebrates. Environment relevant concentration of ASDI exposure to goldfish, *C. auratus* successfully inhibit aromatase activity in long duration leading to significant reduction in T and continuous reduction in E2. Furthermore, brain AA activity was positively and significantly correlated with plasma E2 during long-term exposure and shown remarkable 11-KT lower level than the level of T in the initial weeks of exposure. Aromatase is located in estrogen-producing cells in the adrenal glands, ovaries, testicles, adipose tissues and brain essentially the forebrain and specially implicated in reproduction [21,25,29]. Therefore, AA is known as regulating enzyme of brain function in teleost fishes and its level maintenance play a major role in maintaining the high-level sexual plasticity in fishes into adulthood [30]. Present study is in agreement that either the diuron and its metabolites individually or atrazine and isoproturon mixture at their realistic level in the environment targets behavior, metabolism and steroid biosynthesis in goldfish and other aquatic animals that is associated with impairment of the neuroendocrine and immune system of the fish [5,9,12,35,44]. Moreover, decreased level of steroid hormones like 11-keto and E2 due to the exposure of herbicides cocktail may be the indication of adverse reproductive effects in fishes including reduced number of eggs production by females [4]. Because during gametogenesis, eggs and sperm are arrested at specific developmental stages and their maturation is induced by steroids. Therefore, disruption in the synthesis of reproductive hormones like T, E2 and 11-keto levels may be associated with skewed sex ratios [3], and absence of reproductive behaviour [3,14]. Such abnormalities may result in declining the population of fishes [32, 4]. However, further studies are needed to monitor the deleterious effects of mixture of the pesticides having the low degradation rate and continuously reported in the aquatic impoundments.

Protein vitellogenin (Vtg) induction in male fish is a biomarker for exposure to xenoestrogen-chemicals that mimic steroid hormones through an interaction with the estrogen receptor. And if male fish are exposed to such exogenous estrogens like herbicides, may synthesize Vtg protein equivalent to a mature female fish [40]. In the present finding the ASDI-exposed male fish shown remarkable induction in plasma Vtg in comparison to control group of fish. The results from this study indicated that although ASDI cocktail induced significant effects at a lower concentration causing increased level of Vtg in male fish. The present result is in agreement to the finding of Sumpter and Jobling (2013) that the increased level of Vtg in male fishes is an early warning indicator to assess the water bodies contamination with endocrine disrupting chemicals. Besides that, high level of Vtg may also be responsible for the removal of calcium from the scales and bones as well damage the liver and kidney of exogenous estrogen stressed fish [7].

Conclusion

The findings of the present study revealed that the approximately realistic concentration of ASDI mixture (50µg/l) contamination in our experimental condition may alter the physiological and reproductive performances through decreased steroid hormones, egg production and semen quality. So, herbicides can carefully be suggested to use for weed controlling because their ambiguous presence in water may arrest metabolism and physiological success, influencing not only the gonadal differentiation but also the reproductive functions of fishes. Because the alteration caused by these xenoestrogen on gametogenesis and steroidogenesis of fishes ultimately affect the reproductive success and fish culture practices.

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Conflict of Interest

The authors declare that there is no conflict of interest concerning the research, authorship, and/or publication of this article. Both the authors read and approved the final manuscript.

Highlights of this paper:

1. Chronic exposure of environmental relevant concentration (50µg/l) of triazine-atrazine and simazine and phenyl urea-diuron and isoproturon (ASDI) cocktail treatment showed modulatory effects on goldfish steroid hormones.
2. Herbicides cocktail exposure to goldfish, C. auratus successfully inhibit aromatase (AA) activity in long duration
3. (ASDI) mixture caused remarkable reduction in testosterone (T), estradiol (E2) and 11-Ketotestosterone.
4. Brain AA activity was positively and significantly correlated with plasma E2 during long-term exposure
5. ASDI cocktail induced significant effect on male goldfish by increasing level of Vtg.

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