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GROWTH AND METAMORPHOSIS INHIBITING EFFECTS OF EXOGENOUS HYDROCORTISONE ON *DUTTAPHRYNUS MELANOSTICTUS* (SCHNEIDER, 1799) TADPOLES: A PRELIMINARY LOOK TO GLOBAL POPULATION DECLINE OF AMPHIBIANS

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ABSTRACT

Amphibian metamorphosis is regulated under the influence of several hormones, including glucocorticoids. It is well documented that during critical stages of amphibian metamorphosis, exposure to environmental stressors can affect the later physiological functions via neuro-endocrine signaling pathway. We investigated how hydrocortisone, a potential water corticoid contaminant, could affect the pre-metamorphic stage in amphibians. We exposed tadpoles of the Asian common toad (*Duttaphrynus melanostictus* Schneider, 1799) from two different clutches to 5 μg/ml hydrocortisone solution separately for a period of 13 days. By recording tadpole body lengths as a measure of growth, we were able to observe growth inhibitory effects of hydrocortisone. Average lengths of treated tadpoles were 3.4 mm and 3.2 mm shorter than untreated control tadpoles respectively. By day 13, hind limbs appeared in all control tadpoles while only 15 % of treated tadpoles showed this character. Further in contrast to 50 % control tadpoles, not a single test tadpole entered metamorphic climax, indicating that exposure to elevated levels of hydrocortisone during early larval stage in amphibians has a negative effect on growth and developmental rate. These findings suggested that hydrocortisone, when applied in a sustained manner during critical developmental stages, resulted in delayed development.

Key Words: *Duttaphrynus melanostictus*, Hydrocortisone, Metamorphosis, Glucocorticoids, Growth inhibitors, Trace organic contaminants

List of abbreviations: ACTH = adrenocorticotropic hormone; CRH = corticotropic releasing hormone; GC = glucocorticoid; TH = thyroid hormone; TRH = thyroid releasing hormone; TSH = thyroid stimulating hormone; TrOCs = trace organic contaminants

INTRODUCTION

Amphibians are important in ecosystem as being secondary consumers in many food chains. Adult amphibians contribute to control vertebrate disease vectors, for example by eating mosquitoes and their larvae, frogs can control the spread of diseases like avian malaria and dengue fever (Blaustein *et al*., 2004; Brodman and Dorton, 2006; DuRant and Hopkins, 2008; Rubbo *et al*., 2011; Valencia-Aguilar *et al*., 2013).

Amphibians are also regarded good ecological indicators (Hopkins, 2007) and either during the tadpole stage or as adults exhibit a high degree of sensitivity in response to very slight change in the environment (Rowe *et al*., 2003).

In the past two decades, population decline have been recorded worldwide affecting many species of amphibians including frogs, toads and salamanders (Stuart *et al*., 2004; Daszak *et al*., 2005; Hayes *et al*., 2010; Whiles *et al*., 2013). Several causes (e.g. habitat destruction, pollution, environmental changes and viral, fungal or bacterial infections) have been pointed out for this global amphibian crises (Bishop *et al*., 2012; Searle *et al*., 2014).

As amphibians live in aquatic environments and toxic substances may readily be absorbed through permeable skin, thus factors affecting water quality can in turn affect amphibians (Rowe *et al*., 2003). Amphibian species from different aquatic habitats have been reported to show developmental malformations (e.g. missing, malformed and extra limbs) and increased mortality attributed to chemical contaminants in water (Johnson *et al*., 2001b; Blaustein and Johnson, 2003). These chemical contaminants termed "trace organic contaminants"(TrOCs), include pharmaceuticals, natural and synthetic steroid hormones, pesticides and personal- care products that are discharged into river waters via effluents from sewage treatment plants (Anumol *et al*., 2013; Ammann *et al*., 2014; Sun *et al*., 2015).

Metamorphosis in amphibians

The anuran metamorphosis is controlled by the hypothalamo - hypophyseal - thyroid axis involving actions of several hormones including thyroid–releasing hormone (TRH), thyroid – stimulating hormone (TSH) and thyroid hormone (TH) (Huang *et al*., 2001; Furlow and Neff, 2006). An increased concentration of TH has been reported to accelerate metamorphosis of anuran tadpoles (Page *et al*., 2008). Prolactin is also important in regulating the anuran larval development and metamorphosis (Takada and Kasai, 2003).

Anuran larval development is divided into three specific periods of Pre-metamorphosis, Pro-metamorphosis and Metamorphic climax:

1) Pre-metamorphosis, during which embryogenesis and early tadpole growth and development occurs in the absence or very low TH (Tata, 2003; Saha and Gupta, 2011);

2) Pro-metamorphosis, identified by increasing concentration of TH with rapid extensive growth of hind limbs (Rojas *et al*., 2003);

3) Metamorphic climax, when most of the larval characters are lost with complete resorption of tail and development of structures and functions of an adult due to high concentration of TH (Hall and Larsen, 1998; Mc Diarmid and Altig, 1999).

In amphibians, corticosterone also plays a crucial role to regulate metamorphosis by directly mediating genotype – environment interactions in tadpoles by synergizing with TH to cause early metamorphosis under environmental stresses (Glennemeier and Denver, 2002a; Hossie *et al*., 2010). However, the chronic elevated levels of corticoids in pre-metamorphic tadpoles have negative effects on growth and development (Glennemeier and Denver, 2002b; Belden *et al*., 2005; Ledón- Rettig *et al*., 2009).

Environmental stressors

As mentioned before, environmental factors may act as stressors, leading to elevated plasma corticosterone, which is the main glucocorticoid (GC) hormone in amphibians (Yao and Denver, 2007), involved in timing and initiation of metamorphosis (Belden *et al*., 2005). It is generally elevated during late larval stage, reaching to peak at metamorphic climax then slowly declined (Wingfield and Romero, 2001; Chambers *et al*., 2011). But persistent elevated corticosterone concentration, in response to environmental stress stimuli, can result in altered growth and development (Sapolsky, 2002; Moore and Jessop, 2003; Crespi and Warne, 2013).

Studies have demonstrated that the aquatic environment is contaminated with chemicals (Sparling *et al*., 2010), particularly endocrine disruptors, including natural and synthetic steroid hormones (Sumpter and Jobling, 1995; Gutendorf and Westendorf, 2001; Körner *et al*., 2001). Steroid hormones and their metabolites are not only excreted by human and livestock but many drugs, especially synthetic GC and progesterone used in human and veterinary therapy, can be discharged into environmental water through effluents from sewage treatment plants and pharmaceutical manufacturing plants (Chang *et al*., 2007). In a study of 45 river and 13 discharging sites, water samples analysis detected the presence of GC (including hydrocortisone, cortisone, dexamethasone, methylprednisolone, prednisolone and prednisone) up to 52 ng/ l and 390 ng/ l respectively (Chang *et al*., 2009). Because of the low removal percentage of GC in an aerobic unit of sewage treatment plant (2 – 36 %), GC concentration proportions were much higher in naturally attenuated untreated sewage as compared to other hormones. A study reported the prevalence of GC and androgen activity in water sources from 14 different geographic locations in United States (Stavreva *et al*., 2012). Other analytical studies on water samples from different sources (including wastewater treatment plants, ground water, river waters and drinking water treatment plants) detected significant low (42± 2 ng/ l) concentration of GC along with several TrOCs (Fan *et al*., 2011; Anumol *et al*., 2013; Ammann *et al*., 2014), as well as various glucocorticoids in the range of 13- 1900 ng/ l (Schriks *et al*., 2010). However, the occurrence of detectable levels of GC in various water sources, even in trace amounts may have adverse effects to aquatic life by acting as stressor during critical transitional stage of development.

In this preliminary study we investigate the effects of exogenous GC (hydrocortisone) as an environmental stressor, looking at its impacts on the growth and development of Asian common toad, *Duttaphrynus melanostictus* (Schneider, 1799) tadpoles. We also discuss environmental contamination as a cause of global accelerated population declines of amphibian species.

MATERIALS AND METHODS

Experimental design

In August 2016, we collected *Duttaphrynus melanostictus* (Schneider, 1799) tadpoles from two different temporary ponds at Karachi University Campus (Sindh, Pakistan). The tadpoles were then brought to the laboratory and divided into groups of equal number. Individuals were given refined suspension of parboiled spinach as food (Saidapur and Girish, 2001; Hasan and Ahmad, 2012) and allowed to acclimatize for two days. All of them were at Nieuwkoop and Faber developmental stage 52, i.e. long before physiological connections between pituitary and hypothalamus were established (Nieuwkoop and Faber, 1994). The tadpoles were selected with the help of a binocular microscope. After measurement, individuals of the same size were randomly divided into comparable groups of one control and 4 treatments respectively for two simultaneous experiments A and B.

Tadpole crowding has been reported to accelerate metamorphosis (Saidapur and Girish, 2001; Davis and Maerz, 2009). Therefore, tadpoles measured for both experiments were randomly divided into 5 groups (10 tadpoles / container) and kept in 30 x 10 x 10 cm separate plastic containers filled with 2 liters aged water (Saidapur and Girish, 2001).

Husbandry and monitoring

Growth recording experiments require special care and maintenance of proper environmental conditions of healthy surroundings (Warkentin, 2002), clean water (Berzins and Bundy, 2002), constant temperature and regular feeding (Saidapur and Hoque, 1995; Morey and Reznick, 2000; Nicieza *et al*., 2006). Therefore, to keep the concentration of hydrocortisone constant (5µg/ml) all containers belonging to treatment and control groups were cleaned daily and filled with 2 liters of aged tap water. Tadpoles were fed daily parboiled spinach suspension with leaves cut into small pieces of uniform size before boiling. The amount of food provided was 1 ml suspension per tadpole in all containers and was always in slight excess. In addition, the tadpoles were kept under fairly constant temperature (28 $\pm$ 1°C) during the entire experiment (Saidapur and Hoque, 1995).

Drug information and administration

Hydrocortisone is a GC, and as such, it affects every system of the body to provide energy by metabolizing the stored sugar, fat, and protein for combating physical or emotional stress (Skarlandtov′a *et al*., 2010). Hydrocortisone is used therapeutically and is administered in tablets, intravenous or intramuscular injections or ointment; is used as anti-inflammatory, anti-allergic medication; as treatment of certain kinds of autoimmune diseases, skin conditions, asthma and other lung conditions; to treat variety of cancers such as leukemia, lymphoma and multiple myeloma, also to treat nausea and vomiting associated with some chemotherapy drugs (Shaikh *et al*., 2012).

Due to difficulties involved in intramuscular injections for small tadpoles, hydrocortisone solution (containing 100 mg Hydrocortisone sodium succinate U.S.P. with more than 99 % solubility in water, made by Cirin Pharmaceuticals Pvt. Ltd., Pakistan) was added to the 2 liters rearing water of the treatment containers to a final concentration of 5µg/ml. The selected concentration of hydrocortisone was based on previous studies (Saxena, 1996; Belden *et al*., 2005; Hasan and Ahmad, 2012) where differentiation and growth of tadpoles, reared in 5 µg/ml corticoids solution was inhibited. Hydrocortisone is absorbed from external sites of local application. Therefore, absorption by skin was considerably sufficient to cause systemic effects (Brühl *et al*., 2011).

Hydrocortisone has a short biological half-life of 8-12 hours. It seems that its relative anti-inflammatory and Na^+ retaining potencies remain stable during the period of absorption (Hardman and Limbird, 2001). Therefore, we provided the same dose of fresh hydrocortisone solution with the daily water change to the treatment groups.

Measurements and data analysis

It is well known that growth follows the expression of size and weight. Measurement of body size has been shown to serve as a basis for the study of growth. In order to measure each tadpole, nylon net was used to introduce tadpoles into a glass Petri dish of 6 cm diameter, devoid of water. As soon as the tadpole ceased its wriggling movements, the Petri dish was tilted slightly towards the head side of tadpole. Due to this, the tadpole body was manipulated into a straight line. The Petri dish was, then, arranged on a millimeter grid and the length from head to tip of the tail was noted.

SPSS version 21 was used for statistical analysis, the data was subjected to Repeated Measures ANOVA and Bonferoni's post- hoc test with statistical level of significance of $p<0.05$.

RESULTS

Duttaphrynus *melanostictus* (Schneider, 1799) tadpoles maintained in 5 µg/ml solution of hydrocortisone for a period of 13 days showed a retarded growth (Table 1 and 2).The results of both experiments A and B showed a significant difference between mean length of tadpoles in control and treated groups [F (4, 60) = 2.748094, $p < 0.05$ and F (4,60) = 2.57363, $p < 0.05$, respectively]. After the adjustments made by Bonferoni's corrections for multiple comparisons, a significant smaller mean lengths of treated tadpoles was observed from day 7 to day 13.

Table 1. Effects of 5 µg/mL Hydrocortisone on the growth of pre-metamorphic *Duttaphrynus melanostictus* tadpoles - experiment with clutch #1.

Days after treatment	**Control (mm)†**	**Test (mm)‡**
1	8.0 ± 0.0	8.0 ± 0.0
2	8.6 ± 0.01	8.3 ± 0.04
3	9.5 ± 0.01	8.7 ± 0.01
4	11.2 ± 0.09	9.8 ± 0.0
5	11.4 ± 0.0	10.3 ± 0.04
6	12.8 ± 0.01	10.4 ± 0.0
7*	13.9 ± 0.01	11.0 ± 0.0
8	14.9 ± 0.01	11.3 ± 0.01
9	15.7 ± 0.01	11.9 ± 0.04
10	16.3 ± 0.01	12.3 ± 0.01
11	16.4 ± 0.0	13.0 ± 0.01
12	17.0 ± 0.01	13.6 ± 0.01
13	17.4 ± 0.04	14.0 ± 0.01

†Each value is the mean of 10 measurements with ± SD.
‡ Each value is the mean of 40 measurements with ± SD.

* All test values from day 7 onwards are significant ($p<0.05$).

As a result of treatment, by day 13 tadpoles of both experiments A and B were 3.4 mm and 3.2 mm shorter than their counterparts, respectively. In 25 % of control tadpoles, hind limbs appeared by day 9; by day 10 they appeared in 40 %; by day 11 in 75 % and by day 13, in 100 % of the control tadpoles. Whereas, in treated tadpoles hind limbs appeared in 5 % and 15 % by day 11 and 13, respectively. Fore limbs erupted by day 11 in the control tadpoles and 50 % of them entered metamorphic climax while, all treated tadpoles even failed to enter pro-metamorphic period.

Moreover, test animals presented a lot of variation in their sizes. Some remained small and some much smaller than their counter parts indicating the individual sensitivity to the same dose of hydrocortisone. Hydrocortisone inhibited not only the growth of limbs and tail, but also retarded the growth of head and produced much diminished eyes. The pigmentation of treated tadpoles was also badly affected and exhibited a yellowish skin.

DISCUSSION

Although TH is important for the initiation and completion of metamorphosis, GC released via hypothalamo–pituitary–interrenal axis through corticotropic releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) can modulate the advancement of metamorphic process (Hu *et al*., 2008; Burraco *et al*., 2015). Thus tadpoles' pituitaries are stimulated to secrete TSH in response to CRH (Boorse and Denver, 2003).

In the present study tadpoles selected belonged to early pre-metamorphic stage. It is a well-established fact that timing and progression of amphibian metamorphosis is affected by various extrinsic and intrinsic factors (Boorse and Denver, 2003; Saha and Gupta, 2011; Bekhet *et al*., 2014). In view of this, all control and treated groups were kept in a controlled laboratory setting (Schank and Koehnle, 2009).

In anuran tadpoles acute stress increases plasma GC (Wingfield and Romero, 2001; Belden *et al*., 2005; Hu *et al*., 2008; Dahl *et al*., 2012; Searle *et al*., 2014). However, during late larval stage GC levels increase up to a peak at metamorphic climax, followed by gradual decline (Kloas *et al*., 1997; Glennemeier and Denver, 2002a; Chambers *et al*., 2011). Therefore, in the present study the exposure to elevated levels of GC at larval stage resulted in shorter length of tadpoles in comparison to control tadpoles and failure of treated animals to enter into metamorphic climax.

Furthermore, in non-diseased amphibian larvae the elevated levels of endogenous and exogenous GC is advantageous to increase the chances of survival in life threatening situations and other stressful conditions, but chronic sustained exposure to GC (even a small dose of 5 µg/ml hydrocortisone solution) can negatively affect many fitness components such as reproductive ability (Glennemeier and Denver, 2002b; Moore and Jessop, 2003; Ellis *et al*., 2006; Crespi and Warne, 2013), increased susceptibility to infectious diseases (Belden and Kiesecker, 2005;

Reeve *et al*., 2013), as well as decreased circulating eosinophils and lymphocytes (Rollins-Smith *et al*., 1997; Belden and Kiesecker, 2005; Trottier *et al*., 2008; Davis and Maerz, 2010).

Table 2. Effects of 5 µg/mL Hydrocortisone on the growth of pre-metamorphic *Duttaphrynus* *melanostictus* tadpoles experiment with clutch #2.

Days after treatment	**Control (mm)†**			**Test (mm)‡**		
1	9.0	±	0.0	9.0	±	0.0
2	9.5	±	0.18	9.3	±	0.08
3	10.3	±	0.17	9.7	±	0.08
4	11.4	±	0.07	10.1	±	0.08
5	12.7	±	0.25	10.8	±	0.06
6	13.0	±	0.05	11.0	±	0.05
7*	13.5	±	0.0	11.4	±	0.07
8	14.0	±	0.09	11.9	±	0.01
9	14.7	±	0.10	12.1	±	0.06
10	15.2	±	0.08	12.7	±	0.02
11	16.3	±	0.0	13.0	±	0.04
12	16.8	±	0.08	13.8	±	0.05
13	17.7	±	0.01	14.5	±	0.01

†Each value is the mean of 10 measurements with ± SD.
‡ Each value is the mean of 40 measurements with ± SD.
* All test values from day 7 onwards are significant ($p<0.05$).

Our preliminary data suggest that exposure to hydrocortisone not only have growth inhibiting effect on the pre-metamorphic *Duttaphrynus* *melanostictus* tadpoles (Table.**1**and **2**) but also delayed the entry of test tadpoles into the pro-metamorphic period. This can be problematic since smaller size at metamorphosis may result in lower survival (Berven and Gill, 1983; Smith, 1987; Semlitsch *et al*., 1988; Morey and Reznick, 2001).

CONCLUSIONS

Present investigation showed inhibitory effects of exogenous hydrocortisone on growth and metamorphosis of tadpoles. Despite the fact that for any treatment large sample size with true replicates generates more reliable and accurate results (because of individual response), our experimental unit lacks treatment true replications due to unavailability of large number of animals combined with limitations of time and facilities to conduct research. Thus the recorded variations in response to the treatment could be due to slight environmental differences among containers rather than actual response of treatment. Further the concentration of hydrocortisone used in the experiment is, certainly, quite high when compared to what is present in various natural aquatic environments, wastewater sources and their treatment plants. However, the mixture of TrOCs including hydrocortisone may produce additive, synergistic or antagonistic effects that interfere with the cortico-signaling pathway and thus affect the amphibian metamorphosis at both larval and postembryonic life stages. In this regard and from a conservation angle, there is a need of further research efforts.

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