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Variation of fitness and reproductive strategy in male *Bufo raddei* under environmental heavy metal pollution

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ABSTRACT

Environmental pollution is known to adversely affect amphibian reproduction and survival, however, the knowledge of environmental heavy metal pollution on fitness of male amphibian is largely unknown. The present study aimed to explore the variation in fitness of male *Bufo raddei*, a widespread anuran in northwest China, subjected to long-term heavy metal stress in Baiyin (BY) city. BY is heavily polluted by heavy metals mainly copper, zinc, lead and cadmium; meanwhile, Liujiaxia (LJX), a relatively unpolluted area, was chosen as control. Differences in advertisement call, larynx size, breeding glands size, as well as forearm force during courtship and amplexus in male *B. raddei* between the two populations were analyzed. The results revealed a competitive advantage in advertisement call in BY population, together with larger breeding glands size and forearm force, which demonstrated a relatively higher fitness. Using skeletochronological analysis, we found that more than 40% of males from BY began to breed at 2 years old, which was only 6.93% for males from LJX. Correspondingly, the average age for all males participate in breeding was younger from BY than from LJX. Not surprisingly, males from BY showed a relatively lower body condition. All these results illustrated males from BY invested more in reproduction to increase fitness at the cost of health and survival. This reproductive trade-off might ultimately influence stability of *B. raddei* population because of the environmental heavy metal pollution.

1. Introduction

The skin of amphibians is highly permeable, allowing environmental pollutants to easily permeate epidermis, which would lead to growth and development delay, or even reproductive failure. Meanwhile, reproductive failure has been determined to be an important contributor to the population decline of amphibians (Blaustein et al., 2003; Sun et al., 2018). Currently, research about toxic effects of environmental pollutants on reproduction of male amphibians mainly focused on tissue damage of testis, yet toxic effects of pollutants on male fitness, particularly courtship display and secondary sexual traits, have rarely been reported.

Amphibians produce distinctive advertisement calls to convey necessary biological information regarding their reproductive conditions and territorial boundaries to ensure reproductive success, whereas females choose mates mostly based on the call characteristics (Mc et al., 1998; Guerra et al., 2014; Huang et al., 2015). Pollutants, such as endocrine disrupting chemicals, have been documented to negatively influence the calling behavior of amphibians. For example, long-term exposure to low level of cadmium (Cd) at environmentally relevant concentrations could adversely affect the characteristics (call latency, call duration and call rate) of advertisement call and the proportion of individuals that responded to first movement of male *Pelophylax nigromaculata* (Huang et al., 2015). Vocalization composition could be altered in vinclozolin-treated male *Xenopus laevis*, and vinclozolin is a fungicide used in agriculture (Hoffmann and Kloas, 2010). In addition, 17α-ethinylestradiol, a synthetic estrogen the most commonly used in a contraceptive drugs, which concentration in the environment is still increasing, could increase the proportion of repelling calls and decrease the advertisement calls of *X. laevis* (Hoffmann and Kloas, 2012).

The larynx is the main organ responsible for sound production in amphibians. The process of laryngeal development in amphibians is sexually dimorphic and the formation of a laryngeal capable of male calling behavior is androgen-dependent. It is expected that environmental pollutants, especially environmental hormone-like pollutants, would affect the development of larynx. However, according to current studies, effect of environmental pollutants on larynx of male amphibian is varied, even in the same species exposed to the same pollutants with similar concentrations. For example, atrazine, an commonly used herbicide, was reported a decrease of laryngeal muscle size and testosterone level in male *X. laevis* under worldwide exposure (Hayes et al., 2010), however, no significant difference was found in the same species

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exposed to similar concentrations of atrazine in Carr et al.' s research (Carr et al., 2003). Sub-acute exposure to heavy metals, like Cd treatment also could decrease laryngeal size in the same species (Duan and Huang, 2016).

In addition to advertisement call , nuptial pads play key roles in sexual display to increase mating opportunity for males. Nuptial pads are specialized dermal structures on the ventral side of digits in the forelegs, which enlarge during mating season in sexually dimorphic male anurans. Nuptial pads contain keratinized hooks and saccular glands, called male breeding glands, which are mostly recognized as secondary male characteristics (Fujikura et al., 1988). The breeding glands in nuptial pads of amphibians are androgen-dependent secondary sexual characteristics associated with the breeding biology (Rastogi and Chieffi, 1971; Emerson et al., 1999; Van Wyk et al., 2003). The exact function of the secretions of these male breeding glands may be related to sexual behavior, especially the mating behavior (Kurabuchi, 1993; Emerson et al., 1999). Various natural and xenobiotic chemicals such as anti-androgen flutamide, octylphenol, nonylphenol, 17β-estradiol and fungicide vinclozolin could reduce the cross-sectional areas of breeding glands in male *X. laevis* (Van Wyk et al., 2003). Nevertheless, whether environmental heavy metal pollution could affect characteristics of advertisement call, area of larynx and structure of nuptial pads is currently unclear.

When suffering from environmental pressure, animals will face a trade-off between reproduction and survival. Increased resource allocation into one life-history trait will ultimately preclude the opportunity to other traits. One of the most well studied trade-off situations is resulted from the cost of reproduction, in which a trade-off arises when investment to reproduction reduces longevity, growth, future fertility or immune function (Kotiaho and Simmons, 2003; Reniers et al., 2015). Reproduction investment of male amphibian includes courtship (Cordts and Partridge, 1996; Kokko, 1998), development of secondary sexual traits (Brooks, 2000; Hunt et al., 2004) and mating (Fedorka et al., 2004). Furthermore, for amphibians suffering from long-term environmental pollution, extra energy allocated to detoxification is needed to ensure their health or survival. With intense agricultural and industrial production, prevalence of heavy metals in surface water has increased. Heavy metals could induce antioxidant defense, inhibit the growth and delay the development of amphibian larvae through disrupting thyroid hormone pathway, which would ultimately affect survival of amphibian (Wang et al., 2016; Guo et al., 2017). Meanwhile, heavy metals could inhibit amphibian reproduction through oxidative stress and DNA damage in testes at extremely low levels (Zhang et al., 2012). In addition, Cd, as an endocrine disrupting chemical, could adversely affect male amphibian reproduction, such as leading to the reduction of testosterone level, inhibiting laryngeal development and call behavior (Sinha et al., 2008; Huang et al., 2015; Duan and Huang, 2016). Our previous study has shown that heavy metal pollution varied female reproduction investment in amphibians (Zhang et al., 2018). Giving the importance of reproductive strategy for males under pollution, however, to the best of our knowledge, no study has been reported about the effect of environmental heavy metal pollution on fitness, particularly the trade-off between reproduction investment and survival in male amphibian.

The present study aims to: 1) investigate the effect of long-term heavy metal pollution on fitness of male amphibian, including advertisement call characteristics, larynx, nuptial pads and forearm force during courtship and amplexus; 2) provide insights into the trade-off between reproductive investment and survival in male amphibian under environmental heavy metal stress.

2. Materials and methods

2.1. Animal ethics

Animal studies and experiments were carried out according to "Guidance of the Care and Use of Laboratory Animals" approved by Ethic Committee of Experimental Animals of Lanzhou University, China.

2.2. Study areas and study species

In the present study, a downstream area of the Dongdagou stream (E104°23′, N36°25′) in Baiyin (BY) was selected as the experimental sample area, which has long been contaminated by heavy metals mainly copper (Cu), zinc (Zn), lead (Pb) and Cd (Table S1). Concentrations of Zn, Pb and Cd in the water exceed the level of V guidelines in the environmental quality standards for surface water (GB 3838-2002). Correspondingly, a relatively unpolluted wetland (E103°15′, N35°56′) in Weijiachuan village in Liujiaxia (LJX) town, which has similar organism components and natural characteristics as those of BY, was chosen as the unpolluted area (Table S1). Straight-line distance from BY to LJX is about 114 km (Fig. S1).

Bufo raddei, a widespread anuran in northwest China, was selected as research object, its home range is within 1 km. Adult snout-vent length (SVL) for males is approximately 45–65 mm, and breeding toads are generally 3 and 4 years old in Gansu Province. Breeding typically occurs in water body from April to July. The breeding peak of *B. raddei* is in June in Gansu Province, toads always start to courtship at about 19:00 every day. The peak of amplexus happens between 20:00 and 22:00, and the amplexus lasts until the end of fertilization in the next morning.

2.3. Advertisement call and forearm force determination

In breeding season, advertisement call of male *B. raddei* was recorded by quadrat sampling units (50 m × 50 m) from the two areas, three quadrats were chosen in each area, the distance between every two quadrats is about 500 m. Ten minutes of advertisement call was recorded by voice recorder (ICDPX-470; SNOY, Japan) at 19:00–22:00 in May 2017, during which the air temperature was 22–25 °C, and the distance between male *B. raddei* and voice recorder was kept within 1–2 m. After that, males were captured and their body mass, SVL, head width, forearm width and nuptial pad size were measured. The longest phalanges from the right hind limb of the toads were snipped off and preserved in 10% formalin solution for skeletochronological analysis. Males were then marked by nylon thread with different colors, and put back to the capture site. Finally, to verify whether each toad successfully amplexus or not, all the marked males were checked every half hour between 19:00 and 22:00 every day, by line transect method. All the experiments were taken in the two sites during the same period.

Meanwhile, amplexus pairs were captured in a separate quadrat. Their forearm force was determined by a detector of arm force for amplexus male anuran, invented by our group (Patent number: 201710022860.9). After that, their body mass, SVL, head width, forearm width and nuptial pad size were measured. The longest phalanges from the right hind limb of the toads were snipped off and preserved in 10% formalin solution for skeletochronological analysis. In addition, 12 males of 3-year-old from BY and LJX were brought back to the laboratory separately for further analysis of secondary sexual traits and sex hormone levels.

2.4. Morphometric analysis (larynx and nuptial pads)

Study tissues were generated from *B. raddei* within 48 h after collection. The whole blood was collected by heart puncture into tube with an anticoagulant, and then, the toad was euthanized via double pithing. After incubated at room temperature for 10–20 min, blood tubes were centrifugated for 20 min at 2000 rpm, supernatant was carefully collected as plasma samples and stored at −80 °C. The third toes of the hind leg and toads were separately fixed with 10% formalin until analysis.