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Plasma sex steroid and thyroid hormones profile in male water frogs of the *Rana esculenta* complex from agricultural and pristine areas

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Abstract

Some chemical compounds used in intensive agriculture have been found to induce estrogenic effects; therefore a histological analysis of the testes and an evaluation of plasma levels of sex steroid, thyroid hormones, and vitellogenin were carried out in adult male water frogs of two coexisting taxa (*Rana lessonae* and the hemiclonal hybrid *Rana esculenta*) sampled in agricultural and pristine areas. Differences in seasonal profiles of hormones were found in water frogs living in the agricultural area where the presence of endocrine disrupting compounds was suspected on the basis of a previous study. In *R. esculenta*, sampled in the pristine area, high androgen levels were found in May; the opposite trend was found for *R. esculenta* sampled in agricultural areas in which the highest androgen levels were found in September, significantly lower compared with those found in *R. esculenta* sampled in the pristine area. Low androgen levels were also recorded in *R. lessonae* males sampled both in pristine and agricultural areas, while the highest levels were found in September. Regarding the trend of estradiol-17β, an increase of this hormone was found in July both in *esculenta* and *lessonae* sampled in the agricultural area, and in the same month an estradiol-17β peak, even though lower, was also found both in *esculenta* and *lessonae* males captured in the pristine area; detectable vitellogenin was found neither in males captured in the agricultural area, nor in those sampled in the pristine one. Moreover, while no significant changes of thyroid hormones were found either in the *esculenta* or *lessonae* males sampled in the pristine area, increased T3 and T4 titers were found in July in both *esculenta* and *lessonae* captured in the agricultural area. Morphological differences of the testes in males of parental species captured in the agricultural area were also observed. These findings indicate alterations in endocrine and reproductive function in frogs in the agricultural area, that could suggest the presence of endocrine disrupting compounds.

Keywords: *Rana esculenta* complex; Estrogen; Androgens; Thyroid hormones; Vitellogenin; Agriculture

1. Introduction

Over the past 30 years, many amphibian species have suffered population decline throughout the world. Mass mortality has frequently been reported, and in several instances infectious diseases appear to be the major cause of death (Berger et al., 1998; Carey et al., 1999; Daszak et al., 1999). Environmental compounds could play a role in these die-offs; in particular, endocrine disrupting chemicals (EDCs) should be taken into account, since they interfere with the endocrine and immune systems by mimicking the effects of

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endogenous hormones (reviewed in Colborn et al., 1993). EDCs have been associated with developmental and reproductive abnormalities, alterations of immune functions and of endogenous hormone levels in laboratory and wildlife amphibian species (Bevan et al., 2003; Guex et al., 2001; Gutleb et al., 1999; Hayes et al., 2002, 2003; Hopkins et al., 1997; Kiesecker, 2002; Kloas et al., 1999; Levy et al., 2004; Mosconi et al., 2002; Noriega and Hayes, 2000; Pickford and Morris, 2003; Qin et al., 2003).

Among EDCs, environmental estrogens induce feminization processes in male fish and amphibians, as evidenced by applying validated biomarkers, such as the presence of plasma vitellogenin (VTG), and by evaluating liver estrogen receptor expression in the males (Polzonetti-Magni et al., 2004). In all oviparous vertebrate species, VTG is a female-specific protein precursor of yolk protein components, synthesized in the liver upon multihormonal control, and estradiol-17β is considered to be an important trigger acting through hepatic estrogen receptor interactions (Carnevali et al., 2002).

The *Rana esculenta* complex includes several species as well as natural hybrid lineages that reproduce hemiclonally, transmitting only one of the two parental genomes to gametes (reviewed by Graf and Polls Pelaz, 1989). In the germ line of *R. esculenta*, which is a natural hybrid between *Rana ridibunda* and *Rana lessonae*, the *lessonae* chromosomes are excluded before meiosis, the remaining *ridibunda* chromosomes are endoreduplicated, and two apparently normal meiotic divisions result in genetically identical haploid gametes containing an unrecombined *ridibunda* genome (Ogielska, 1994; Tunner and Heppich-Tunner, 1991). Hybridity in these lineages is restored through fertilization of these gametes by gametes from the syntopic *R. lessonae*; therefore, *R. esculenta* can be indicated as 'a reproductive parasite' of its host.

Previous research carried out on a population of the *Rana esculenta* complex living in a small agricultural area of central Italy showed an alteration of the taxon composition, an increased occurrence of infectious diseases and anomalies in reproduction and development (Fagotti et al., 2005; Pascolini et al., 2003; Pereira et al., 2005). In addition, the tissues of the two taxa contained low levels of bioaccumulated organochlorine compounds (Fagotti et al., 2005).

Since chemical compounds, such as pesticides and herbicides, used in intensive agriculture have been found to induce estrogenic effects (Hayes et al., 2002, 2003; Noriega and Hayes, 2000), histological analyses of the testes, and sex steroid and thyroid hormone plasma levels, together with those of vitellogenin were evaluated in adult male frogs sampled in agricultural and pristine areas; the xenoestrogenic impact was determined after species determination.

2. Materials and methods

2.1. Sampling locations

Two sites of comparable altitude and latitude were studied in the Lake Trasimeno district (Umbria, central Italy). The frogs were collected from ponds and streams within a 500-m radius in each site. One site, characterized by intensive agricultural land use, mainly cereal growing, was selected due to the presence of pesticides used in cereal production that were likely to run off into catchment ponds and streams encircled by the cultivated fields. The other site is characterized by wild environmental conditions. Both sites feature a bioclimatic low-hill plane and colluvial-argillaceous soil.

2.2. Animals

Water frog capture and sampling were performed at the same time (in the afternoon) in both sites in May, June, July, and September 2003. In the reproduction cycle of water frogs belonging to the *R. esculenta* complex, spawning occurs in May, and then in post-reproductive summer months, during which a kind of refractoriness intervenes in spite of favorable environmental conditions, such as temperature, food availability and day length (Polzonetti-Magni et al., 1990).

Adult male water frogs were collected from both the sites. In each site and for every sampling a larger number (more than 20) of frogs, thought to belong to the *R. lessonae* or *R. esculenta* taxa on the basis of a morphological screening, was caught. After general anesthesia with 0.05% solution of 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222; Sigma Chemical Company, St. Louis, MO), blood samples were taken using a heparinized syringe, and testes were removed; blood samples were centrifuged at 2000*g* for 15 min at 4 °C to separate out the plasma fraction. Plasma was stored at −80 °C until further analyses. A toe phalanx was also taken and conserved in ethanol (80%) for species determination. For each sampling, the measurement of plasma hormones and vitellogenin was carried out on ten specimens of *R. lessonae* (snout-vent length 54–58 mm; wet weight 16–18 g) and ten of *R. esculenta* (snout-vent length 61–64 mm; wet weight 23–25 g), selected after taxon identification by molecular analysis.

2.3. Histological analysis

Testes of male water frogs collected in pristine and agricultural areas were removed and fixed in freshly prepared 4% paraformaldehyde for histological examination. Following dehydration in graded ethanol and embedding in paraffin, serial sections (5 μm) were stained