HISTOLOGY REVEALS TESTICULAR OOCYTES AND TREMATODE CYSTS IN THE THREATENED OREGON SPOTTED FROG (*RANA PRETIOSA*)

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ABSTRACT—The Oregon Spotted Frog (*Rana pretiosa*) is endemic to the Pacific Northwest and was recently listed as threatened under the Endangered Species Act. We tested the hypothesis that reproductive or physiological stress and parasitic disease may be contributing to the decline of this species. We histologically examined gonads and kidneys of newly metamorphosed wild-caught *R. pretiosa* to confirm sex and search for evidence of abnormal reproductive development and parasites. A subset of these specimens were also cleared and stained for examination of their skeletal morphology to identify potential skeletal malformations. The sex ratio did not differ significantly from 1:1, and we found no skeletal abnormalities. Trematode metacercarial parasites were present in the kidneys of all Spotted Frogs examined. We also report, for the first time, oocytes developing in the testes of 5 out of the 11 newly metamorphosed male Spotted Frogs examined. Further study into gonadal development of this species is necessary to investigate the significance of testicular oocytes in developing *R. pretiosa* and to identify whether these gonadal abnormalities are related in any way to their decline.

Key words: amphibian, *Echinostoma*, extinction, Oregon Spotted Frog, testicular oocytes, Pacific Northwest, parasites, *Rana pretiosa*, Ranidae

The Oregon Spotted Frog (*Rana pretiosa*) is a highly aquatic ranid frog residing in wetlands and slow-moving streams of the Pacific Northwest (Hayes 1997). The historic range of *R. pretiosa* sensu stricto is from northern California to southwestern British Columbia (Hayes 1994; Jennings and Hayes 1994); however, this species is thought to have been extirpated from western Oregon, much of Washington, northern California, and portions of British Columbia (Leonard and others 1993; McAllister and others 1993; Green and others 1997; Hayes 1997; Hammerson and Pearl 2004; USFWS 2010). Growing concern about its apparent decline has recently led to the listing of *R. pretiosa* as a threatened species under the United States Endangered Species Act (ESA; USFWS 2014) and an endangered species under the Canadian Species At Risk Act (SARA; COSEWIC 2011).

Multiple factors are likely contributing to the decline of this and other anuran species, including hydrological alterations, predation and competition from invasive Bullfrogs (*Rana catesbeiana*; Yuan and others 2016) and Brook Trout (*Salvelinus frontinalis*), and disease (Nussbaum and others 1983; Hayes and Jennings 1986; Marco and others 1999; Pearl and others 2007, 2009; USFWS 2010; COSEWIC 2011). Previous research has shown that herbicides, pesticides, and other chemical pollutants have had, or potentially have, detrimental effects ranging from malformation and endocrine disruption to mortality on both juvenile and adult amphibians (for example, Boyd and others 1963; reviewed in

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Carey and Bryant 1995; Ouellet and others 1997; Gardiner and Hoppe 1999; Sparling and others 2001; Hayes and others 2002; Relyea 2005; Bernal and others 2009; Belden and others 2010). Though exposure to some agricultural pesticides has not been shown to be necessarily detrimental to *R. pretiosa* survival or development (such as the herbicide imazapyr; Yahnke and others 2013), some populations of *R. prestiosa* exhibited increased mortality after exposure to 1,1,1-trichloro-2,2-bis-(p-chlorophenyl) ethane (DDT, an agricultural pesticide; Kirk 1988). It is also thought that decreases in reproductive fitness, immunosuppression, and other signs of stress could be the result of either direct or indirect effects of pesticides, singly or in combination (Carey 1993; Kiesecker 2002; Forson and Storfer 2006). To investigate potential causes for decline in this species, we looked for evidence of reproductive or physiological stress using whole-mount clearing and staining to look for skeletal abnormalities, and histological analysis of gonads and kidneys to look for evidence of reproductive peculiarities and parasitic disease, in a sample of newly metamorphosed *R. pretiosa* from a single locality in Oregon.

METHODS

Newly metamorphosed *R. pretiosa* (snout-to-vent-lengths, SVL, ranging from 29 to 35 mm) were collected from a non-agricultural site: Lake Aspen, Deschutes County, Oregon, USA (UTM: Zone 10U 624781 4860424; 1268 m elevation) in August and September 2010. We examined a total of 27 *R. pretiosa*. All individuals were euthanized humanely by immersion in a buffered 0.3% tricaine methane sulfonate solution (MS222; Torreilles and others 2009). Gonads and kidneys of all specimens were removed using a standard dissection kit. Kidneys, located dorsally to the gonads, were removed with gonads still attached to prevent damage to the much smaller gonads. Kidney and gonad specimens were dehydrated in an ethanol series and xylene, then embedded in paraffin wax. Paraffin-embedded tissues were serially sectioned using a rotary microtome (AO® American Optical) every 10 μm and mounted on glass slides. Slides were stained using Ehrlich's hematoxylin and Eosin Y solutions, prepared in-house, following a protocol modified from Humason (1962). Gonadal and renal abnormalities were observed and quantified for each individual with the use of a compound microscope and SPOT digital camera with SPOT Advanced Software®. Sex of each individual was determined by superficial morphology and general histological structure of the gonads. Sex ratios were tested for significance using a χ^2 analysis, performed manually. We cleared tissue and stained the bones and cartilage of 10 *R. pretiosa* specimens, following a protocol modified from Hanken and Wassersug (1981) and Sessions and Ruth (1990), to look for the presence of skeletal abnormalities. All specimens used in this study are archived at the Amphibian Research Laboratory, Department of Biology, Hartwick College (Oneonta, NY USA).

RESULTS

Of the 27 *Rana pretiosa* specimens we investigated, 16 were female and 11 male. This was not significantly different from a 1:1 sex ratio ($\chi^2 = 0.889$; $P = 0.346$). All *R. pretiosa* specimens exhibited trematode metacercariae (cysts) in their kidneys (Fig. 1). We found a total of 285 cysts with a range of 1 to 39 cysts per specimen ($\bar{x} = 10.6$ cysts per frog). We found 168 and 117 cysts in females and males, respectively, which was not significantly different when corrected for total number of each sex ($P = 0.9597$, *t*-test). We tentatively attribute these trematode infections to *Echinostoma* spp. due to the small and spherical nature of the metacercariae and their presence in the kidneys (Olsen 1986). We did not identify any skeletal abnormalities in the cleared and stained specimens (Fig. 2). Superficially, gonads did not exhibit any abnormalities; however, histological analysis of the gonads showed that 5 of the 11 males (45.5%) exhibited early vitellogenic oocytes in their testes (Fig. 3). Oocytes were identified on the basis of the size of the cells and the presence of an enlarged germinal vesicle (oocyte nucleus). Each testicular oocyte occupied part of a testicular lobule (Fig. 3). The surrounding cells were non-meiotic spermatogonia. We found no visible abnormalities in the ovaries of female *R. pretiosa*.

DISCUSSION

The only notable apparent reproductive abnormality we report is the presence of testicular oocytes in approximately half the males examined from this locality. This is the 1st documented report of testicular oocytes in *R. pretiosa*. The

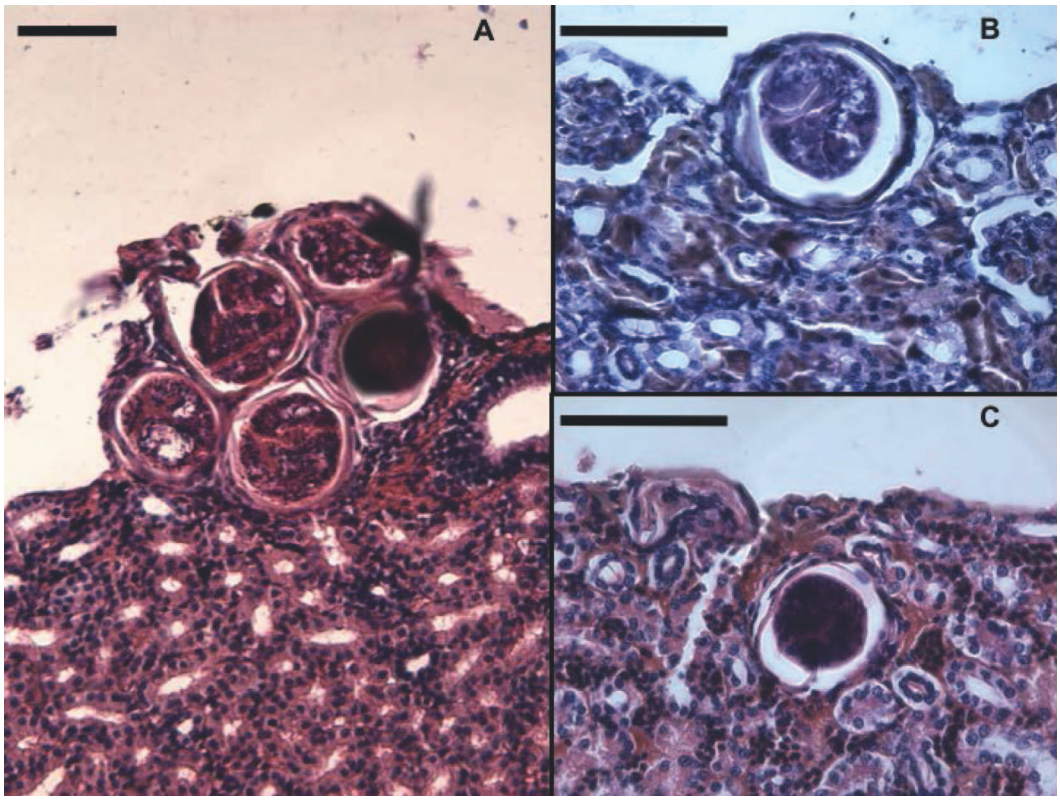

FIGURE 1. Coronal sections of *Rana pretiosa* kidneys exhibiting trematode metacercariae. Hematoxylin and eosin stain. Scale bars = 100 μm.

significance of this finding depends on the normal frequency of testicular oocytes in newly metamorphosed frogs in other populations of *R. pretiosa* as well as other frog species. To our knowledge, such data are scarce. The presence of oocytes in the testes of adult male frogs has been attributed to the herbicide atrazine and other environmental contaminants (Reeder and others 1998; Hayes and others 2002, 2003, 2010; McDaniel and others 2008; Papoulias and others 2013). Murphy and others (2006) and Papoulias and others (2013) both found that atrazine concentrations were not significantly associated with hermaphroditism in ranid frogs, but that testicular oocyte incidence was significantly greater in juvenile frogs collected from agricultural sites versus non-agricultural sites. This contrasts with the findings of Skelly and others (2010) who reported higher prevalence of testicular oocytes in *R. clamitans* (Green Frog) from suburban and urban sites but no positive correlation with agricultural lands. Storrs-Méndez and Semlitsch (2010) reported intersex gonads in multiple anuran species and suggested that this appears to be a normal aspect of gonad development in these species.

Prior to the use of modern pesticides, there are several early reports of hermaphroditism and feminization in, but not limited to, the ranid frogs *Rana pipiens* (Northern Leopard Frog; King 1910), *Rana sylvatica* (Wood Frog; Cheng 1929), *Pelophylax esculentus* (Edible Frog; Mitrophanow 1894), *Pelophylax ridibundus* (Marsh Frog; Friedmann 1898), and *Rana temporaria* (European Common Frog; Bourne 1884; Marshall 1884). In some frogs, most pre-metamorphic tadpoles have female gonads, and eventually a subset of these individuals develop into hermaphrodites until terminating their sexual development as males (Witschi 1929; reviewed in Eggert 2004). Given this ontogenetic plasticity, it is not surprising that factors other than exposure to

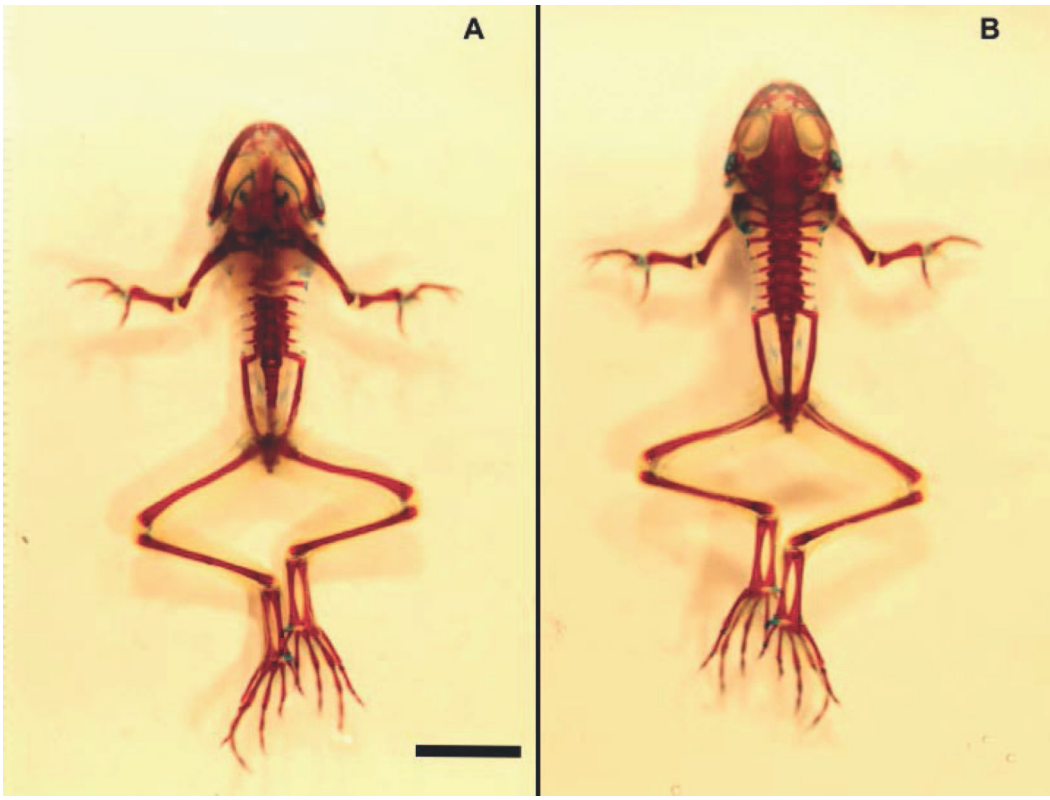

FIGURE 2. Ventral (A) and dorsal (B) views of a cleared and stained *Rana pretiosa* specimen. No osteological malformations are visible. Scale bar = 1 cm.

environmental contaminants can affect sex determination in amphibians. The hyperoliid frog *Hyperolius viridiflavus* (Common Reed Frog) has been shown to exhibit sex change from female to male in captivity, likely due to low male density (Grafe and Linsenmair 1989). Although there are no known cases of temperature-dependent sex determination in wild populations of amphibians, temperature extremes under experimental conditions can cause sex reversal in developing embryos. Ranids such as *R. sylvatica*, *R. japonica* (Japanese Brown Frog), and *R. catesbeiana* exhibit masculinization at high temperatures (Witschi 1929; Yoshikura 1963; Hsu and others 1971), with *R. temporaria* exhibiting both masculinization at high temperatures and feminization at low temperatures (Witschi 1914; Piquet 1930). Polyploid *Xenopus laevis* (African Clawed Frog), however, exhibit the opposite changes, being feminized at high temperatures and masculinized at low temperatures (Kobel 1996). Thus, sex determination in amphibians could potentially be vulnerable to the effects of extreme climate change. Without baseline knowledge of the natural occurrence of testicular oocytes in gonadal development of *R. pretiosa*, we believe it is premature to speculate on the developmental and reproductive significance of testicular oocytes in our samples and the possible impact on reproductive success or population dynamics. If demasculinization and feminization is occurring in wild populations of *R. pretiosa*, shifts in the sex ratio resulting in fewer successfully reproducing males could result in population declines in this already endangered species (Hayes 2005; Blaustein and others 2011). However, we found no evidence of skewed sex ratio in our sample.

Although trematode cysts have been linked to limb malformations in a variety of amphibian species (for example, Sessions and Ruth 1990; Johnson and others 1999; Sessions and others

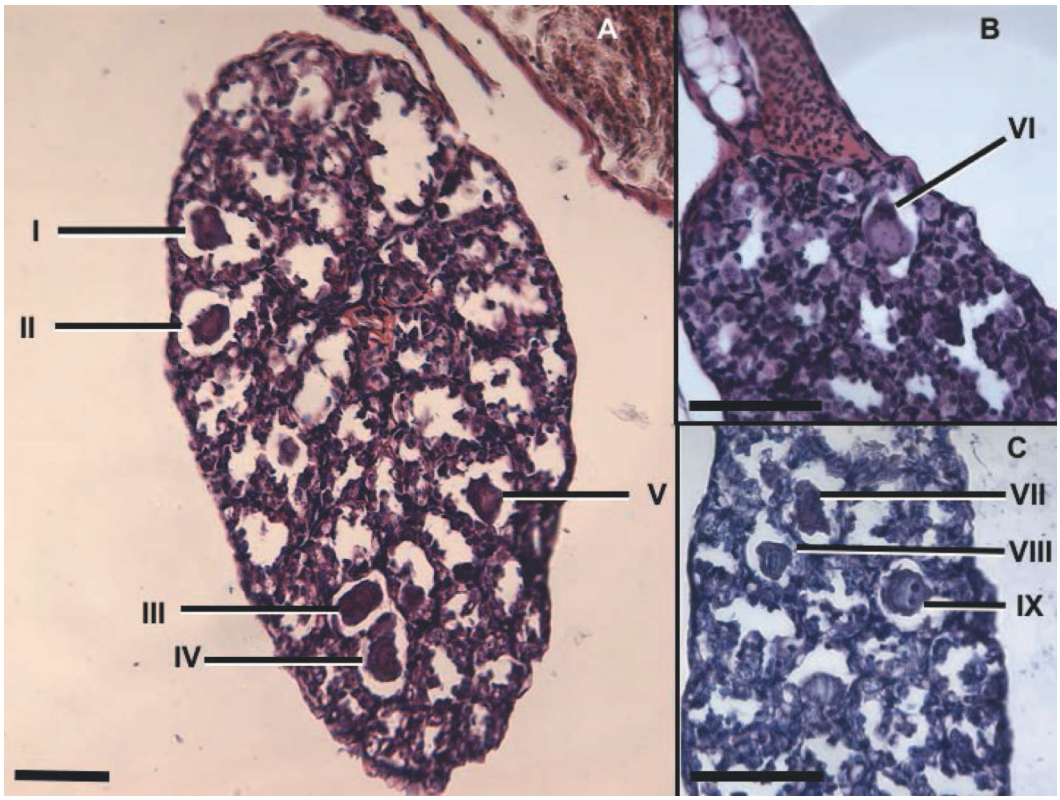

FIGURE 3. Coronal sections of *Rana pretiosa* testes exhibiting the development of oocytes (I–IX). Hematoxylin and eosin stain. Scale bars = 100 μm.

1999), including *R. pretiosa* (Bowerman and Johnson 2003), such abnormalities appear to be relatively rare and generally associated with the trematode *Ribeiroia ondatrae* (Sessions and others 1999; Johnson and others 2001; Stopper and others 2002). Wojdak and others (2014) demonstrated that there is a negative relationship in infection intensity between *Ribeiroia ondotrae* and *Echinostoma trivolvis* within individual tadpoles of *R. clamitans*. We did not find evidence of *R. ondatrae* infections in our samples, and observed no limb abnormalities, consistent with published accounts of background rates of malformations generally <5% (Johnson and others 2001, 2010; Eaton and others 2004). Although we found trematode cysts in 100% of our sample, identified as belonging to the Echinostome group, they were found in the kidneys rather than the cloacal-inguinal area where *R. ondatrae* appear to induce limb malformations (Sessions and Ruth 1990; Johnson and others 1999; Sessions and others 1999; Stopper and others 2002). Previous studies have shown that trematode infection in ranid frogs is more frequent in agricultural sites (Kiesecker 2002; Marcogliese and others 2009). We did not test water from Lake Aspen for the presence of agricultural chemicals during our study. However, this site receives water that flows adjacent to a golf course, approximately 3.6 km south of the Lake Aspen locality. The water in this system was tested for environmental contaminants in 2007 by the US Fish and Wildlife Service (USFWS; Materna and Buck 2009). Two agricultural chemicals were detected above reporting levels. Endosulfan I, an organochlorine insecticide, was detected at 0.15 μg L^{-1}. This concentration is below the Oregon Department of Environmental Quality (ODEQ) water quality acute criterion (0.22 μg L^{-1}), but above the chronic criterion (0.056 μg L^{-1}; ODEQ 2014). Carbaryl, a carbamate insecticide, was detected at 0.63 μg L^{-1}.

This concentration is above the recommended instantaneous threshold value of 0.02 μg L^{-1} (National Academy of Sciences and National Academy of Engineering 1973). Though there have been no changes in chemical usage or golf course management practices in recent years, this concentration of carbaryl is lower than concentrations reported as having adverse effects in amphibians (1.2–3.4 mg L^{-1}; Boone and Semlitsch 2002; Relyea 2003). It seems doubtful that these chemicals are contributing to the parasite load, since it is likely that the parasites would also be negatively affected (King and others 2007). Likewise, we have no evidence that these chemical contaminants cause the development of testicular oocytes, which could be physiological (normal) in newly metamorphosed *R. pretiosa*. Nevertheless, we think that these possibilities merit further chemical monitoring at the Lake Aspen locality.

Future research should include such careful monitoring of populations of *R. pretiosa* as well as other species of amphibians, to include comparing sites of low and high probability of exposure to environmental pollutants. Additionally, further investigation into the gonadal development of both lab-raised and wild-caught newly metamorphosed *R. pretiosa* is imperative to evaluate the biological significance of testicular oocytes. Federal and state restrictions now in place limit the collection and sacrifice of *R. pretiosa* and other threatened or endangered species. Therefore, active salvage, preservation, and examination of dead or dying frogs are required. There is also a need to find nondestructive methods that will allow examination of internal organs using, for example, ultrasound or microtomography.

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