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Using reproductive technologies to assess the development of secondary sexual characteristics, ovarian senescence and hermaphroditism in the endangered mountain yellow-legged frog *Rana muscosa*

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Abstract. Anurans can display a host of intriguing sexual syndromes, including hermaphroditism and sex reversal. Using a multifaceted approach for diagnosing and characterising hermaphroditism in the endangered anuran species *Rana mucosa*, we tracked changes in female reproductive status using hormone monitoring, ultrasound examinations, individual life history, fertilisation records and post-mortem findings. Seven individuals originally sexed as females developed secondary male sexual characteristics, behaviour and hormone profiles and, in some cases, had testicular tissue despite having previously laid eggs. Our results suggest that reproductive technologies can shed light on life history patterns and reproductive anomalies that may affect endangered anuran survival.

Keywords: captive breeding, *ex situ*, hormones, mass spectrometry, nuptial pads, sex reversal, ultrasound.

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Introduction

Intersexuality, including hermaphroditism, describes the phenomenon in which animals born with primary sex characteristics of one sex develop atypical secondary sex characteristics. Researchers have documented these anomalous sexual phenomena in a variety of non-human animals, including mammals, birds, molluscs and reptiles, but have rarely examined these phenomena in amphibians (Parhon and Parhon 1922; Wadsworth *et al.* 1978; Jenner 1979; Chaffaux *et al.* 1980; Haibel and Rojko 1990; Reeder *et al.* 1998; Avise *et al.* 2004; Padilla *et al.* 2005; Crespo *et al.* 2013). In addition, the presence of intersex phenotypes in adult (reproductively mature) anurans has only previously been documented in animals that were exposed to chemical contaminants at larval and metamorphic life stages (Reeder *et al.* 1998; Orton and Tyler 2015). As amphibians continue to decline worldwide (Grant *et al.* 2016), the recovery of at-risk amphibian species is increasingly dependent on conservation breeding programs. To optimise reproductive output and produce numerous viable offspring for reintroduction into the wild (Harding *et al.* 2016), a more holistic understanding of amphibian reproduction, including anomalies like hermaphroditism, is needed. Many conservation breeding and *ex situ* programs enhance and track reproduction through the use of assisted reproductive technologies (ARTs), including exogenous hormone-stimulated gamete production, assisted fertilisation and sperm cryopreservation (Kouba and Vance 2009; Upton *et al.* 2018; Silla *et al.* 2019; Della Togna *et al.* 2020); however, the potential of ARTs to monitor and evaluate reproductive health in amphibians is underutilised.

This study describes, for the first time, the loss of reproductive capabilities and the onset of reproductive anomalies in an aging *ex situ* population of the mountain yellow-legged frog *Rana muscosa*, a state-, federal- and International Union for Conservation of Nature (IUCN)-listed endangered species endemic to southern California (Backlin *et al.* 2015). After the first few successful years of breeding within the Conservation Breeding and Reintroduction Program for *R. muscosa* at San Diego Zoo Wildlife Alliance, Beckman Center for Conservation

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Research, several individuals from the founder population that had bred as females developed male sexual characteristics. *R. muscosa* is a sexually dimorphic species. Males can be distinguished from females morphologically by the presence of nuptial pads on their thumbs, which develop at the onset of sexual maturity (roughly 2–3 years after metamorphosis). Because chromosomal sexing is not currently available for this species, in our conservation breeding program we rely on a combination of visual sexing, ultrasound, hormone measurement and breeding history to determine an individual's sex. Here we use less-documented ARTs (hormone measurements and ultrasound), along with breeding records and necropsies, to describe the internal and external changes that may be occurring in females that lead to the development of male morphological characteristics and behaviours. Combined, these methods may shed light on drivers influencing reduced fertility, including hermaphroditism, intersex and senescence in at-risk *ex situ* amphibian populations.

Materials and methods

Study animals

The *R. muscosa* females ($n = 10$), males ($n = 14$) and nuptial pad females (NPF; $n = 7$) used in this study were all housed at San Diego Zoo Wildlife Alliance, Beckman Center for Conservation Research (Escondido, CA, USA), as described previously by Calatayud *et al.* (2020; see also Table S1). Here we define NPF as individuals who previously exhibited female reproductive behaviours (e.g. egg development and oviposition) but later developed nuptial pads and male reproductive behaviour (e.g. amplexus) during the breeding season.

All procedures were approved by San Diego Zoo Wildlife Alliance's Institutional Animal Care and Use Committee (No. 15-001 and 16-005).

Reproductive monitoring

As part of our *ex situ* conservation breeding program, we quantified the number of eggs laid and documented fertilisation and pair information each year. To evaluate whether there were differences in ova produced between females that developed nuptial pads and those that did not, we compared average ova produced for each female over the course of their breeding history.

Ultrasound examinations

We performed ultrasound examinations on all females in the colony from 2014 to 2018, paying specific attention to three time points: (1) during brumation (before breeding; October–January); (2) during breeding (March–April); and (3) after breeding (June–July). We assessed reproductive cycles and compared females with and without nuptial pads. As an additional point of comparison, we examined results of male ultrasound examinations during the breeding season to highlight morphological differences between NPF and females. We classified ovarian cycle stages for females and NPF by examining images from both the left and right coelom (for more detail about ultrasound staging methods, see Calatayud *et al.* 2019).

Hormone quantification

Serum samples were collected from adult individuals (Table S1) across three different time points (May, July and November 2017), as described by Calatayud *et al.* (2019). We used a blank matrix sample and a spiked-matrix sample (matrix: SeraSub; CST Technologies) as control samples to assess contamination and extraction efficiency. Samples were diluted 13-fold and hormones were extracted using 100% methanol (Fisher Scientific), homogenised for 1 min by vortexing and then centrifuged at 12 000*g* for 2 min at 10 °C. The supernatant was split to dansylate one aliquot for enhanced oestrogen ionisation and quantified as described previously (Meredith 2018). Briefly, testosterone and oestrogens (β-oestradiol and oestriol) were quantified using an Agilent 1260 liquid chromatograph coupled to an Agilent 6460 mass spectrometer. Chromatographic separation was performed using an Agilent Poroshell 120 EC-C18 column (inside diameter 2.1 mm × 50 mm; 1.8 µm) maintained at 40 °C. We ionised hormones using electrospray ionisation in positive mode with an auxiliary gas (N_2), a source temperature of 150 °C and 300 °C and a gas flow rate of 13 and 11 L min^{-1} for oestrogens and testosterone respectively. Optimised multiple reaction monitoring conditions are provided in Table S2.

Post-mortem analyses

As part of our *ex situ* program, board-certified pathologists necropsied all individuals at the time of death. A gross examination was performed, and tissues (fixed in 10% buffered formalin) were collected for histopathology. Sex was first determined based on the appearance of gonads on gross examination and confirmed with histopathology. A total of 15 necropsies was performed on individuals from founders collected from the wild.

Behaviour

We assigned mating pairs each year based on a combination of pedigree and genomic data. For each chosen pair, we documented breeding behaviour by quantifying amplexus (amphibian breeding position in which the male clasps the female from behind, positioning his cloaca close to hers for external fertilisation of deposited eggs). In addition, we recorded any attempted breeding occurring outside of assigned breeding pairs.

Statistical analyses

All statistical analyses were conducted in the R programming environment (Version 3.6.0; R Core Team 2019). We constructed generalised linear mixed models (GLMMs) using the lme4 package (Bates *et al.* 2007). Hormone concentrations were log transformed before analysis to ensure the data met model assumptions (e.g. normality and homoscedasticity of residuals). Three separate models were constructed to characterise patterns in testosterone, β-oestradiol and oestriol. In each model, we included sex (categorical variable; male, female or NPF) and month (categorical variable; May, July, November) as fixed effects and individual identity as a random effect. For models of oestrogens, we also included an interaction term between sex and month; this interaction term could not be included in the

testosterone model without violating GLMM assumptions. The DHARMa package (Hartig 2020) evaluated model residuals and confirmed that all models met GLMM assumptions. We used the Satterthwaite approximation to estimate degrees of freedom and to test for the significance of each fixed effect using the lmerTest package (Kuznetsova *et al.* 2017). The code generated during this study is available online (https://github.com/clw224/Rana_muscosa_NPF).

Results and Discussion

R. muscosa breeding, ova produced and fertility have been highly variable since the inception of the *ex situ* breeding program in 2006. Individual variation in ova produced is high for both NPF and females; however, on average, NPF produced fewer ova than their female counterparts (mean (±s.e.m.) 282.7 ± 77.8 vs 417.4 ± 75.2 respectively; $W = 46$, $P = 0.0079$, Wilcoxon rank sum test). For some females, nuptial pads appeared a year after their first breeding season, whereas other females did not develop nuptial pads until 5–8 years after their first breeding season. For a majority of NPF, breeding ceased after the emergence of nuptial pads. In human care, *R. muscosa* have been documented to breed from 2 to 12 years of age. NPF may have been experiencing a form of reproductive senescence. Although not necessarily the case here, because some females reproduced only once at 4 years of age, this phenomenon cannot be ruled out. To our knowledge, no research has been done on post-reproductive amphibians.

NPF appeared to exhibit more masculine traits based on reproductive hormones, ultrasound visualisations, external phenotype and amplexing behaviours. For example, testosterone concentrations were significantly lower in NPF than in phenotypically normal females but did not differ from that in males (Table S3; Fig. 1). Sex-based differences in oestrogen profiles were seasonally dependent, with phenotypically normal females (but not males) differing from NPF in July but not in other months (GLMM: sex (female) × month (July), d.f. = 52, $t = 2.17$, $P = 0.03$ for both β-oestradiol and oestriol; Tables S4, S5; Fig. 1). Hormonal profiles align with ultrasound results, which showed a lack of follicle development at any time point in NPF compared with breeding females (e.g. before, during or after breeding; Fig. 2). Behaviourally, three of seven NPF amplexed other females during the breeding season after nuptial pad emergence. Only one NPF successfully bred as a male 3 years after nuptial pad emergence. Although average fertilisation was low (0.425%), this confirmed spermatogenesis.

Differences in hormone profiles between females and NPF could be associated with the role of oestrogens and testosterone, which are critical for follicle development and oviposition. A seasonal rise in oestrogens at the end of the reproductive period (July) observed in females compared with NPF likely indicates the reinitiation of oogenesis, because oestrogens are required for oocyte growth and yolk deposition (Ogielska and Bartmanska 2009). The resumption of follicle growth may cause the secretion of oestrogens required for hepatic synthesis of vitellogenin (Fernandez and Ramos 2003), whereas an increase in testosterone observed in November in reproductive females compared with NPF may indicate the end of follicle growth before the

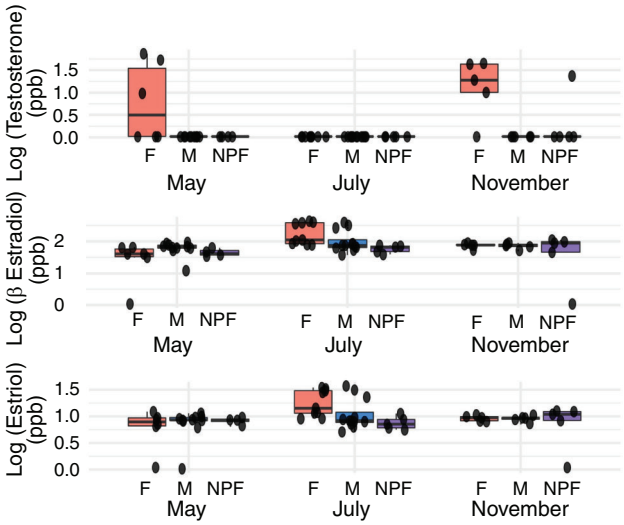

Fig. 1. Box and whiskers plots showing log-transformed hormone concentrations in parts per billion (ppb) detected by mass spectrometry. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range. Overlaid points indicate individual values. F, female; M, male; NPF, nuptial pad female. For statistical analyses of these results, see Tables S3–S5.

onset of brumation (Fernandez and Ramos 2003). Overall, our results indicate a lack of normal ovarian activity (or ovaries) in NPF, whereas phenotypically normal females exhibited high levels of follicle and oocyte development before and during the breeding season.

Histological examination of six of the seven necropsied NPF revealed true hermaphroditism or at least male pseudohermaphroditism. Of the six NPF, five had active testes with spermatogenesis, mature oviducts and suspect remnant degenerate follicles (Fig. 3), and one had an ovotestis along with large, mature oviducts, confirming true hermaphroditism (Table S1). Furthermore, breeding records show that four of the six NPFs had previously oviposited, indicating female reproductive capability. Hermaphroditism in amphibians is generally associated with exposure to chemical contaminants during the larval period (Orton and Tyler 2015). All the individuals that exhibited these phenomena or female nuptial pad emergence were collected from the wild. To date, we have not observed female nuptial pad development, ovarian senescence or hermaphroditism in any *ex situ*-produced individuals. Therefore, it is possible that the hermaphroditism we documented may be related to chemical exposure from agricultural practices (Hayes *et al.* 2002). Protogynous hermaphroditism (sex change from female to male) is also a possibility, although typically restricted to cases where it maximises an individual's lifetime reproductive success (Grafe and Linsenmair 1989).

The appearance of nuptial pads in females remains an intriguing phenomenon that is not yet fully understood. Combined across our analyses, NPF appear to be more similar to males than females, as indicated by hormone concentrations (Fig. 1), ultrasound examinations in which images of NPF reproductive tissue were almost indistinguishable from those observed in males

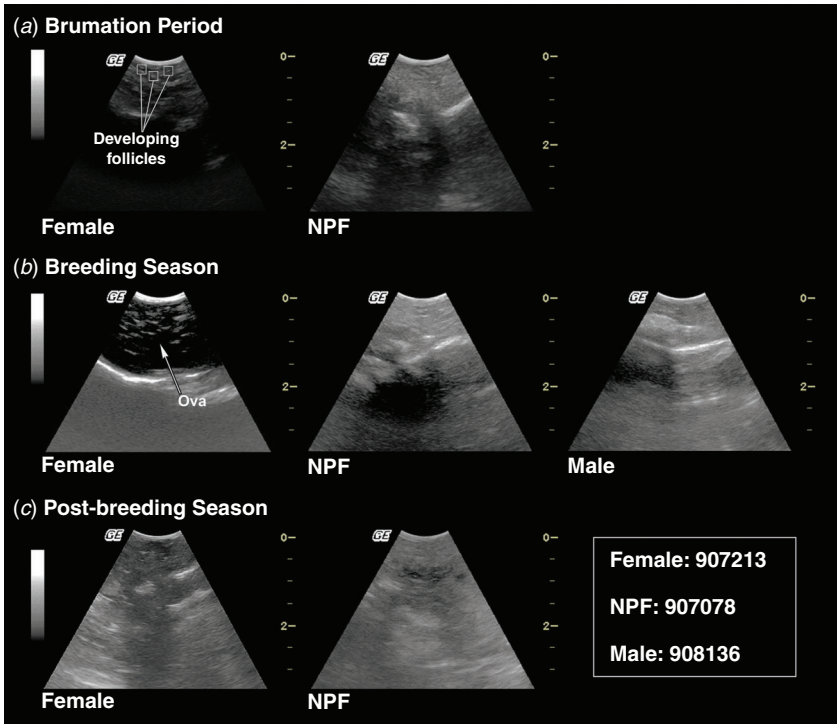

Fig. 2. Ultrasound images showing (*a*) females during brumation (before breeding) with echogenic dots indicating follicle development (left) and nuptial pad females (NPF) during brumation with no visible follicle development (right); (*b*) females during the breeding season (March–April) with developing oocytes (arrow; left), males during the breeding season (middle) and NPF during the breeding season with a lack of ovarian development (right); and (*c*) females (left) and NPF (right) after the breeding season (June–July).

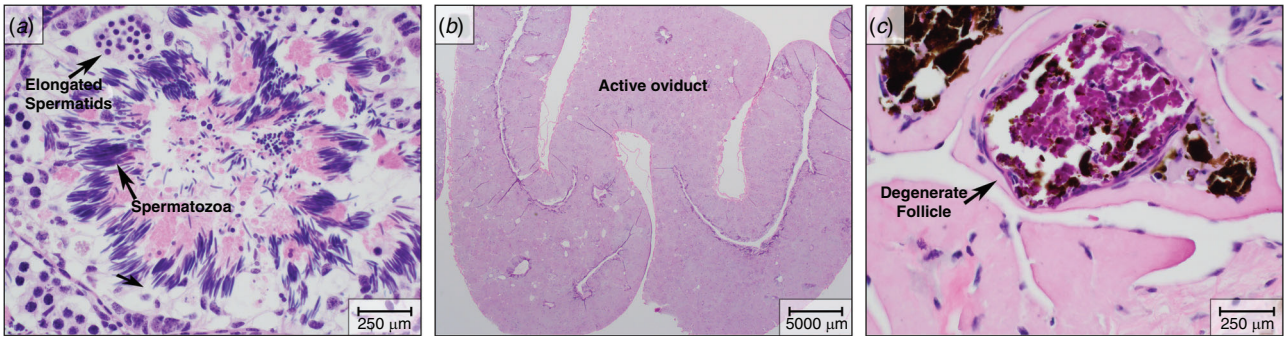

Fig. 3. Histological staining of tissues from (*a*) a nuptial pad female (NPF) with testicular tissue showing active spermatogenisis, and (*b*, *c*) a NPF that exhibited both mature, active oviducts (*b*) and a rare suspect remnant, degenerate follicles (*c*). Tissues were stained with haemotoxylin and eosin.

(Fig. 2) and post-mortem histological analysis (Fig. 3). We initially hypothesised that this phenomenon was related to aging and post-reproductive senescence or sex reversal at the larval stage. However, necropsies revealed that most these animals were hermaphrodites. More research on aging in amphibians and hermaphroditism is needed. *Ex situ* breeding programs are an ideal venue to collect these data, providing opportunities to apply physiological and medical knowledge to enhance and better understand amphibian reproduction. Incorporating ARTs, including ultrasound and hormone profiling, into conservation breeding programs will produce a more holistic understanding of the life history, aging and reproduction of amphibian species. This knowledge will enhance *ex situ* reproductive success while allowing for more informed management decisions.

Conflicts of interest

Natalie Calatayud is a guest Associate Editor of this Journal issue. She did not at any stage have editor-level access to this

manuscript while in peer review, as is the standard practice when handling manuscripts submitted by an editor of this Journal. The authors have no further conflicts of interest to declare.

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