Theriogenology 191 (2022) 141–152

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Recovering an endangered frog species through integrative reproductive technologies

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ARTICLE INFO

Article history:
Received 5 July 2022
Accepted 7 July 2022
Available online 12 July 2022

Keywords:
Ex situ breeding programs (EBPs)
Assisted reproductive technologies (ARTs)
Genetic management
Conservation breeding
Ultrasound
Hormone monitoring
Amphibian
Translocation

ABSTRACT

The establishment and management of *ex situ* breeding and assurance populations around the globe are meant to provide short-term solutions to the formidable loss of amphibian diversity presently occurring. Large multi-scaled facilities, such as zoos and aquariums, can provide the infrastructure to safeguard species and populations. However, often even large, economically viable facilities lack the knowledge to efficiently cater to the plethora of environmentally controlled physiological strategies that amphibians possess. Anurans present a class of amphibians that have often been viewed as easy to maintain *ex situ*. However, while adult survival may be relatively successful it is rarely accompanied by good reproductive output, health, and fitness. Even more conspicuous is the low survivorship of offspring produced *ex situ* once they are translocated back into the wild. The mountain yellow-legged frog (*R. muscosa) ex situ* breeding program EBP is a prime example of the challenges that amphibians EBPs face. Although more research is needed, the *R. muscosa* program has increased reproductive output and health of its colony by incorporating reproductive technologies and strategic genetic management in conjunction with a greater understanding of the species' natural history, to produce and translocate viable animals each year. This paper highlights the EBPs past decade of research featuring the program's contribution to building empirical, multidisciplinary approaches that boost the robustness of an endangered species, by safeguarding existing genetic diversity and maximizing fitness and survival outcomes.

1. Introduction

1.1. Amphibian ex situ breeding programs and their significance in the zoo industry

Twenty twenty-one marked the re-assessment of global priorities surrounding the environmental and biodiversity crisis the world faces. The profound failure of the world to address or attenuate the loss of species has only highlighted how critical intensive management of flora and fauna is to safeguard biodiversity. For some taxonomic groups, the integration of *ex situ* and *in situ* management has finally merged into a comprehensive conservation approach designed to preserve, augment, and manage the genetic diversity of at-risk species. Zoological institutions are heavily involved in the management of these assurance populations with explicit goals to provide high quality care and welfare, to safeguard genetic diversity and to optimize *ex situ* reproduction to produce individuals for population creation and augmentation.

However, species conservation is a race against the clock and recovery efforts often occur in the absence of a comprehensive understanding of the target species' biology. Over the last three decades, implementation of assisted reproductive technologies (ARTs) has increased dramatically in *ex situ* breeding programs (EBPs). Although incorporating a species' natural history would arguably allow you to create a more holistic EBP, ARTs may help overcome limited breeding and maximize offspring production, effectively creating new populations of at-risk species or bolstering existing ones when wild numbers are precariously low [1,2].

Additionally, as with other taxonomic groups, amphibian reproduction *ex situ* combined with genome resource banking of *in-* and *ex situ* populations is becoming increasingly important to capture and preserve remaining genetic diversity [2–4]. Inclusive of these advances is the establishment of the IUCN's Amphibian

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https://doi.org/10.1016/j.theriogenology.2022.07.003
0093-691X/

Specialist Group's Assisted Reproductive Technologies and Biobanking Working Group, the development of a specialist ARTs and Biobanking chapter in the new edition of the Amphibian Conservation Action Plan (ACAP) and, increased support from the wider conservation community to increase amphibian representation in genome resource banks (Calatayud et al., In review).

In this review we use the endangered mountain yellow-legged frog, *Rana mucosa*'s, EBP to illustrate the importance of a holistic approach to species recovery. The goal of the *R. muscosa* conservation breeding and translocation program is to produce and head start fit individuals *ex situ*, while preserving remaining genetic diversity that allows for adaptation to environmental challenges, in order to support and restore self-sustaining populations in the wild through translocation. With recent advancements in genome sequencing and ARTs the *R. muscosa* program is moving forward strategically through improved genetic screening, reproductive management, and procurement of genetic diversity through biobanking. Here we review the reproductive and genetic research associated with the breeding program: (1) the technologies used for monitoring and managing *R. muscosa* reproduction, (2) the incorporation of ARTs, where appropriate, for the acquisition of gametes for biobanking, (3) the integration of single nucleotide polymorphic genome sequencing to improve genetic management and, (4) incorporating new technologies to expand our understanding of reproductive behavior.

2. Mountain yellow-legged frog, *Rana muscosa*, as a case-study for amphibian *ex situ* breeding colonies (EBPs)

The southern California populations of *R. muscosa* form a distinct population segment that is listed as endangered by the California Department of Fish and Wildlife and the U.S. Fish and Wildlife Service. Historically prevalent in the streams and tributaries of four southern California mountain ranges, *R. muscosa* in this region are now extinct in most locations. Surveys suggest that remaining populations are actively declining and that a few may have recently become extirpated [5]. Historic declines have been attributed to numerous factors including invasive predators (brown trout, bullfrogs), airborne pollutants contributing nitrogen deposition in streams, extreme drought and wildfires, and infection with the amphibian chytrid fungus (USGS, unpublished), which has contributed to amphibian declines globally. With fewer than 200 adults detected in the wild during annual surveys, *R. muscosa* is highly vulnerable to extinction.

The continued precariousness of wild *R. muscosa* populations requires a management strategy that includes human managed assurance populations and augmentation and/or reestablishment of wild populations [6]. For the species to recover, it is vital that existing small populations be supplemented to increase genetic diversity and resilience, and that new populations are established to (1) reduce the risk that all populations become extirpated (i.e., extinction), (2) increase connectivity among isolated populations so that locally extirpated populations can be recolonized by natural emigration and the effects of inbreeding depression can be mitigated, and (3) maintain genetic representation in redundant systems to increase resilience.

San Diego Zoo Wildlife Alliance (SDZWA) has been actively involved in *R. muscosa* recovery since 2005. Tadpoles were salvaged from two drying pools within the San Jacinto population and transferred to our *ex situ* facility to develop methods for husbandry, breeding and translocation. Because the *R. muscosa* program began with a salvage effort and the initial goal was to develop husbandry and breeding protocols for the species, we did not incorporate genetic management (see Section 6) into the program until wild populations declined to the point at which our *ex situ* populations needed to serve as insurance against extinction. Since 2009, we have produced hundreds to thousands of eggs annually, resulting in the translocation of nearly 8,500 *R. muscosa* at various life stages across populations, including the near-extirpated San Bernardino population [7,8]. Our *ex situ* propagation and release efforts have yielded some short and medium-term success with evidence of released individuals surviving to sexual maturity in the San Bernardino population (Stage 1: survival and growth of individuals) and successful reproduction of released San Jacinto individuals (Stage 2: reproductive success) [9]. However, until our reestablishment efforts yield population growth (Stage 3) and ultimately produce self-sustaining populations (Stage 4), San Bernardino populations will remain functionally extinct in the wild making our *ex situ* breeding efforts critical for recovery *R. muscosa*.

As a recovery program, the *R. muscosa* is a prime example of the challenges that *ex situ* breeding amphibians face and the extensive knowledge gaps that remain. From the data collected for this species over the last fifteen years, we have learned that (1) a varied diet is critical for growing healthy frogs [10], (2) brumating frogs *exsitu* improves reproduction and short-term post-release survival [11,12], (3) mate choice improves captive fitness [13], (4) assisted reproductive techniques are most effective during the breeding season [13], (5) *ex situ* San Jacinto and San Bernardino populations have low sperm concentrations [10,13], and (6) genetic diversity of our captive San Jacinto population is low compared to that found in the wild [14]. Yet despite these advances in our understanding, southern California *R. muscosa* continues to decline, and reproduction rates and survival of the *ex situ* population are highly variable. As with any species, simply producing and reintroducing *R. muscosa* is not sufficient. To adaptively manage and address this suite of challenges, more research is urgently needed to boost production of genetically diverse *R. muscosa*, to safeguard existing genetic diversity through a strategically implemented biobanking approach, while addressing ongoing threats in the wild.

3. Understanding the environmental challenges faced by the *R. muscosa* EBPs

The creation of naturalistic environments is important for the overall health of amphibians in EBPs [12]. For *R. muscosa*, substantial evidence suggests that the manipulation of artificial environments which mimic those experienced in the wild have important implications for reproduction and translocation success [11,12,15]. As such, husbandry practices developed for the *R. muscosa* program have sought to increase the *ex situ* population's exposure to critical biotic and abiotic factors present at translocation sites. Using data collected year-round at release sites, environmental parameters of the laboratory are adjusted where possible to mimic seasonally fluctuating abiotic factors such as temperature, both atmospheric and aquatic, and seasonally appropriate photoperiod (Fig. 1 [16]).

As a high elevation temperate species, wild *R. muscosa* are exposed to extreme weather and brumate (overwinter) for at least three to six months a year [12,15]. Initially, brumation was avoided under *ex situ* management because it was thought to slow growth, compromise immunity, and/or increase morbidity of amphibians under human care. However, research has shown that brumating *R. muscosa* is essential to reproduction and growth in this species [11,12]. Santana et al. (2015) demonstrated that artificial brumation in refrigerators improved reproductive output of adult *R. muscosa* however, the complex relationship between ovarian function and environmental factors requires further exploration in *R. muscosa*. For example, ongoing research using ultrasounds to monitor reproduction, have revealed a great degree of variability in female reproductive cycling consistent with other species. For example,

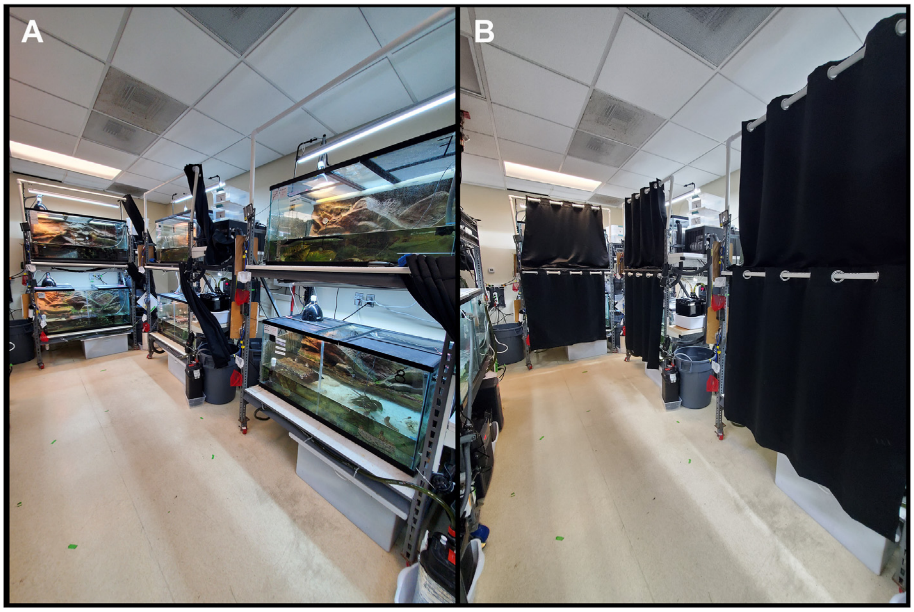

Fig. 1. A. Enclosure set-up post-brumation with UV basking lamps set to on, LEDs and open curtains. B. Brumation set-up with closed, thermal black-out curtains, no UV basking lamp and minimal in-tank lighting.

similar to *Rana temporaria*, *Bufo bufo* and *Bufo vidris*, ovulation in *R. muscosa* can occur both during brumation or in response to amplexus during breeding indicating that females can enter the winter period at different stages of vitellogenesis [17,18]. Anecdotal observations suggest that females that exit brumation before ovulation can remain in amplexus for days or weeks before ovipositing. Thus, amplexus may influence final maturation in females that have not completed vitellogenesis or follicular maturation prior to entering brumation. Though unconfirmed in this species, final maturation leading to ovulation could occur through a gradual increase in GnRH sensitivity [17,18]. Therefore, while the influence of brumation on reproduction in *R. muscosa* is apparent, further research is required to understand the relationships between foraging, somatic growth, vitellogenesis, maturation and ovulation in animals under human care.

The benefits of exposing *R. muscosa* to an overwintering period extend beyond reproduction. In a subsequent study Calatayud et al. (2020) evaluated the effect of brumation on developing metamorphs and showed that while brumated metamorphs exhibit slower growth rates than non-brumated controls, compensatory growth in the months following brumation yielded individuals of the same size as controls [12]. Perhaps more importantly, short-term post-release survival assessments indicated that there may be a fitness advantage to brumation [12]. As a physiologically important phenomenon, *R. muscosa* brumation coincides with other temperate species [18–20]. For high elevation, temperate female anurans, physiological resources are divided between current and future reproduction and growth in response to environmental stressors and resource availability [11,18–22].

The environments within which amphibians have evolved have shaped their reproductive plasticity. For example, variability in clutch sizes in *Rana temporaria,* have been correlated to body size as well as the number of follicles recruited to vitellogenesis, while egg size is correlated to body mass [23]. Body size and mass in turn, correlate, along with genetic factors, with foraging resource availability and duration [24]. In our *R. muscosa* EBP, we consistently provide a high quality diverse diet to our *ex situ* population and on average we have recorded between 300 and 500 eggs per clutch with the largest clutch recorded comprised of 1,349 eggs [13]. In the wild, insect availability is diverse but likely more variable and accounts of average clutch size report 100 to 350 eggs per egg mass with a single report of up to 800 eggs per single female recorded [25–28]. To date, there is no data comparing egg or clutch size of wild *R. muscosa* with that of females in the *ex situ* population and the mechanisms controlling the number of follicles recruited per cycle remains unexplained in this or other species' to the author's knowledge [18,23]. However, it is possible that when consistently abundant food resources were provided, *R. muscosa* females in our EBP, invested energetic resources into larger clutches with smaller (less yolk) eggs rather than larger (more yolk heavy) eggs [23].

What remains unclear is why fertility is highly variable in *ex situ* populations. Despite high egg production in our EPB, fertility continues to be low (average 20–30%). This could be in part to management practices that lead to overproduction, a lack of understanding of seasonal and age related hormone fluctuations and/or potential desynchronization of gamete deposition during breeding, age class (juvenile (1–3 year olds) vs. adult (4–8 year olds) vs. senior (9+ year old) age classes), and the influence of males on reproductive output. What is clear is that amphibians rely on a host of abiotic factors to facilitate breeding, making the mimicking of these factors in EBP environments critical not only for the health and wellbeing of the *ex situ* population, but for successful reproduction. Other abiotic features such as water cycling and quality and environmental enrichment; are now being considered as important in promoting natural behaviors, but their precise influence is still under investigation [12,24,29].

As with many amphibians held in artificial environments, clutch and egg size may not translate into the appropriate adaptive and fitness traits necessary for survival in the wild [22,30,31]. For example, in *Rana limnocharis* and *Rana latestei,* increased egg size was correlated with maternal investment and led to an increase in hatching success, and larval size and survival although the effect of maternal identity cannot be ruled out [31,32]. While, in other species — such as *Bombina occidentalis*— egg size was correlated with survival and depended on environmental conditions. In *B. occidentalis,* smaller eggs and hatched larvae were more likely to survive than larger eggs in warmer temperatures, but larger eggs survived better than smaller eggs at cooler temperatures [30]. Since *ex situ* conditions are unable to replicate all aspects of wild environments, it is difficult to determine the cause of poor fertility. Based on the "maternal match" hypothesis, which predicts that maternal environment (e.g., high density) results in phenotypic variation transmitted to the offspring (e.g., reduced per capita yolk investment), the extensive energetic investment in reproduction by females may require an annual or biannual rest from breeding [18–20,33]. Moreover, the incorporation of a more variable diet, and the addition of extra vitamins and minerals [34,35] are likely critically important to promote reproduction by increasing nutrients critical to gamete development and quality. More research is needed on how aspects of diet (e.g. quantity, frequency, and/or breadth) affect maternal reserves and investments and the resulting offspring fitness.

Maternal investment theory, is one of many possible explanations for the loss of fecundity and may relate to the disadvantage of life in EBPs, increasing domesticity and lowering fitness through a host of genetic, environmental, developmental, and phenotypic factors [36].

4. *R. muscosa* natural and assisted breeding

Despite the implementation of multiple abiotic and biotic factors mentioned previously, reproductive failure in EBPs is common and, in these cases, may require additional intervention using assisted reproductive technologies (ARTs) [37]. One challenge with *ex situ* programs, however, is managing and maintaining adaptive potential (genetics) that will increase population resilience to future environmental challenges resulting in self-sustaining wild and captive populations [38–40]. With this in mind, programs designed to breed species in EBPs should make every effort to understand the natural history of the target species as well as reproductive viability and fitness and its relationship to long-term *ex situ* management and induction using ARTs. These techniques can also facilitate genetic management in a low cost and spatially conservative manner while integrating previously uncaptured genetics into populations, generations at any time, as effectively as if the individuals were present [41].

The most commonly documented ARTs applied in amphibian conservation are: the administration of exogenous hormones, use of artificial fertilization (AF) and sperm cryopreservation. The purpose of these techniques is to increase reproductive productivity, facilitate population augmentation, and preserve genetic diversity that can be stored in Genome Resource Banks. However, reproductive monitoring should be conducted before proceeding with the use of ARTs [2]. In amphibian EBPs, reproductive monitoring is vastly overlooked as a means to assess reproductive health or status, and little information has been published linking reproductive monitoring directly to the development of ARTs protocols. For *R. muscosa*, reproductive monitoring —including ultrasounds, hormone profiles, and fertility assessments— is conducted each year in an effort to increase our understanding of reproductive capacity, timing, and fitness of breeding individuals (and their offspring) as well as their overall reproductive lifespan. These reproductive parameters are of particular importance for this species, given its highly endangered status coupled with extremely limited genetic diversity [14] which, in turn, might be tightly linked to its survival and recovery in the wild. In the case of the *ex situ R. muscosa* population, reproductive monitoring was introduced as part of standard husbandry practices in 2014. Despite the successes seen in the *R. muscosa* conservation breeding program at SDZWA, gamete production, cleavage rates and embryo survivorship remain highly variable across both the San Jacinto and San Bernardino populations. The implementation of ARTs to enhance breeding, improve fertilization and provide hormone protocols that can be used to induce gametes independently is ongoing.

4.1. Induction of breeding behaviors after hormone administration

Previous research on this species indicates that obtaining viable sperm and ova are reliant on temporal factors [10,13]. In males, administration of commercially available hormones, gonadotropin release hormone (GnRH) or human chorionic gonadotropin (hCG) or a combination of the two can induce spermiation. However, preliminary research on hormone induction of egg laying and enhancement of breeding behaviors has only examined the effects of Amphiplex as a hormonal induction protocol and results suggest that further optimization is required [42]. In 2015, 18 paired *R. muscosa* produced an average of 321 eggs after receiving the following treatments: group 1 (control), males and females were injected with a placebo (amphibian ringer's solution (SAR)), group 2 had control males paired to an Amphiplex-treated female (control male/Amphiplex female), group 3 had an Amphiplex-treated male paired with a control female (Amphiplex male/control female) and in group 4 both males and females were treated with Amphiplex (Amphiplex pair). Overall, the results suggest that treatment had no effect on egg laying (though a trend was observed in which group 4 had a decreased number of eggs per clutch laid (Amphiplex pair T-value = −1.978, P = 0.0666) (Fig. 2). When examined separately, the influence of the female was greater than that of the male regardless of hormone administration suggesting, not surprisingly, that the gravidity of the female has a greater influence of the probability of egg laying than the hormone treatment itself (F-value = 3.6483, P = 0.03718).

Consequently, in accordance with the previous results, there was no effect of hormones on the number of embryos cleaved (control male/Amphiplex female: T-value = 0.441, P = 0.6631; Amphiplex male/control female: T-value = 0.785, P = 0.4403; Amphiplex pair: T-value = −1.312, P = 0.2024). However, because pairs that included non-treated control males produced embryos that cleaved indicates that the role of male fertility requires further exploration (see section 6 below). Unfortunately, sperm production was not quantified in this study, thus it is impossible to compare spermiation rates in the control versus Amphiplex-treated males. Furthermore, there is no documented information on the quality of sperm in the *ex situ* population and coupled to gamete synchronization, these factors need careful dissection to better understand the factors that limit breeding. Much about male fertility requires further study. For example, it is unclear whether hormone treatment (with Amphiplex or any other hormone) affects the amount or duration of spermiation or sperm quality including the asychronity of reproduction, i.e. the rates at which males are producing sperm at the time egg laying occurs.

5. *R. muscosa* female reproduction

5.1. Female breeding histories

Given the ambiguity of the results obtained from the aforementioned breeding study, we conducted a closer examination of the individual reproductive histories of the *ex situ* population's breeding females. From 2015 to 2017, 48% of founder females oviposited just once in five years, 40% in two consecutive years, 8% oviposited eggs in two consecutive years, skipped a year, and then oviposited again in two consecutive years, and 4% oviposited in three consecutive years, suggesting that breeding may not occur annually [10]. However, in more recent years (2018–2021), subsequent generations of F1+ females have shown an increase in annual

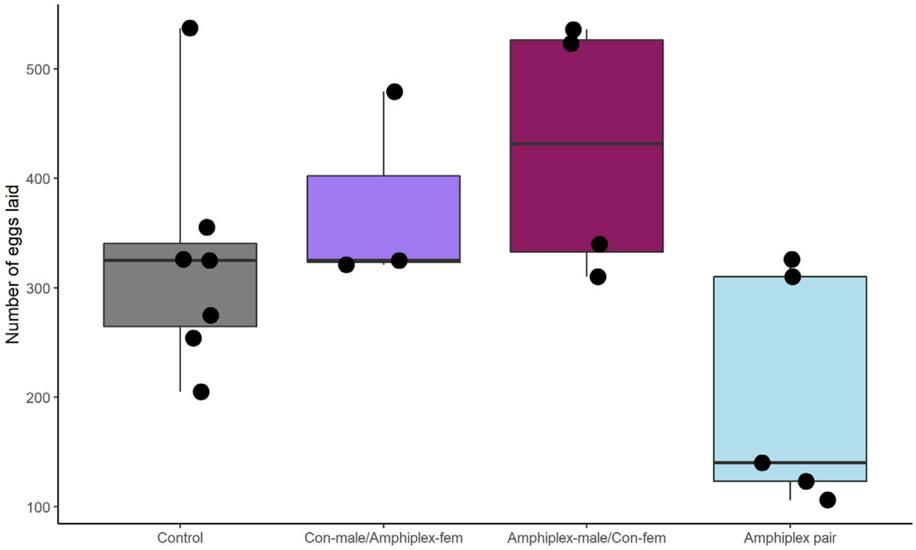

Fig. 2. Number of eggs laid by *R. muscosa* breeding pairs after administration of Amphiplex. Treatment groups included male and female *R. muscosa* were tested in four distinct groups: control pair (where male and females were injected with a placebo saline amphibian ringer (SAR) injection, control male and hormone-treated female (Con-male/Amphiplex-fem), hormone-treated male and control female or, hormone-treated pair (Amphiplex pair). Statistical method and experimental approach summary described in supplementary/appendix figure (https://github.com/natlebug/CANDES.git).

output without the breeding breaks observed in the founding population. In fact, for the F1+ generation eleven of twelve females have produced a clutch annually following sexual maturity. Clutch sizes have also differed between founding and F1+ females. Founding females have produced clutches of 286 eggs on average while F1+ females' average clutch size was 508. This preliminary information warrants more exploration as it highlights a lack of knowledge pertaining to the reproductive viability of females in the *ex situ* population. Moreover, other confounding factors that occurred at the inception of the program may also have played a significant role in female reproduction. For example, incorporation of brumation protocols to husbandry practices and dietary changes (both of which are discussed in further detail in section three). These preliminary results highlight the variability of reproduction and reproductive health of *R. muscosa* and the need for incorporation of better assessment tools such as annual ultrasound and hormone monitoring into conservation breeding.

5.2. *Assessing female reproductive viability using ultrasound*

Determining the reproductive readiness of female anurans has most commonly been assessed visually, by palpation and trans-illumination [43]. More recently, preovulatory status (gravidity), in several species has also been identified by ultrasound [40–42] and hormone monitoring [33,43–47]. Staging ovarian development has been previously described in *R. muscosa* and broadly categorized into three stages [43]. Females that are categorized as Grade 1 show the presence of echogenic dots that are assumed to correspond to a developing ovary containing pre-vitellogenic follicles, while females categorized as Grades 2 and 3 have more prominent looking oocytes suggestive of vitellogenic development (Fig. 3). Historically, breeding strategies have dictated that all females be introduced to males at the beginning of breeding seasons regardless of the categorization by ultrasound. However, the variability observed in the number of eggs deposited and the time to deposition for females of different categories suggests that the stage of ovarian development may influence the amount of time a female needs to remain in amplexus before oviposition. Although these observations require further research, if female ovarian status translates to egg production, the implications of this would be numerous. First, females in earlier stages of ovarian development may not need to be bred that year. Second, requiring females in early ovarian development to breed may have long-term deleterious effects on the quality of gametes and embryos produced due to over harvesting. Third, males placed with females that require weeks of amplexus to promote final egg development may experience burn out before females are ready to deposit eggs resulting in a lack of gamete synchronization.

5.3. *Assessing female reproductive viability and endocrinology using liquid gas chromatography and mass spectrometry (LC/MS_MS)*

Knowledge of the basic underlying mechanisms of endocrinology is key to monitoring the functional reproductive status and performance of *ex situ* populations [44,45]. Unlike other taxonomic groups, amphibian species housed in zoological institutions seldomly receive the attention that other more charismatic groups receive. To date, no reports of long-term endocrine monitoring are available for any amphibian species, a critical oversight of amphibian *ex situ* programs. Moreover, the wide gap in our knowledge of reproductive health and status for this taxonomic group makes it challenging to optimize ARTs [4]. As such, mass spectrometry protocols are under development to track annual changes in estriol, estrogen, progesterone, testosterone, and corticosterone in *R. muscosa*, to elucidate the naturally fluctuating annual hormone levels in males and females [46,47]. The aims of studying endocrinology in this species is three-fold, to, (1) determine the seasonal fluctuations of key reproductive hormones to better manage breeding, (2) understand how exogenous hormones exert their action during artificial gamete induction and, (3) optimize exogenous hormone protocols and timing of administration for this species.

Using serum samples collected at eight different time points, from 74 males and females (juvenile (n = 26 female and 10 male); adult (n = 6 female and 4 male), and senior (n = 9 female and 19 male)) over two-years, LC/MS_MS protocols were developed that would enable analysis of multiple hormones (8+) with minimal

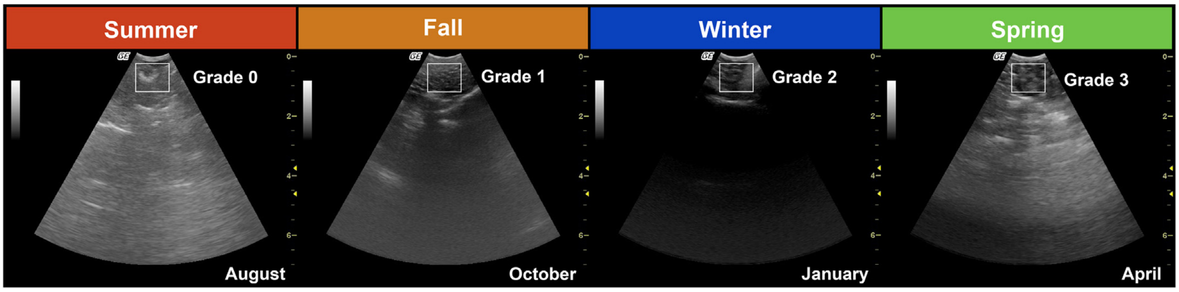

Fig. 3. Ultrasound images depicting the seasonality of the female reproductive system from post-reproduction (Summer) to the start of brumation (Fall) throughout brumation (Winter) and directly before the end of brumation and reproduction (Spring). Boxes highlight the growth of follicular development; grade 0 (Summer) with no follicular development, grade 1 (Fall) minimal follicular development, grade 2 (Winter) with moderate follicles, and grade 3 (Spring) with highly developed follicles.

amount of serum (25 μL). Though preliminary, hormone analysis showed clear sexual dimorphism in hormone concentrations regardless of age class. But more importantly, as reported in other discontinuous breeders, an expected seasonal fluctuation in testosterone, estrogens, progesterone, and corticosterone (Fig. 3) [18,46,48] coincided with breeding periods (April–July), post-reproductive quiescence (August–September) and pre-brumation recrudescence (October–December; Table 1; Fig. 4). The most prominent hormones detected were: estradiol, estriol and testosterone. Estradiol and testosterone levels increased in late summer and fall coinciding with periods of follicular development (yolk accumulation) and resurgence and acceleration of spermatogenic cell divisions [49] (Fig. 3). Progesterone and corticosterone also varied across seasons however, at extremely low concentrations, a likely artefact of limited sampling missing rise and peaks. In seasonal amphibians, testosterone would be expected to increase during breeding season with access to females, while increased corticosterone levels should also increase with the energetic requirements of mating [50,51] (see Fig. 5).

Clearly more research is needed to understand changes in hormone levels in this species throughout the year. The application of LC/MS-MS will provide an efficient tool for screening multiple hormones from a single small sample volume and new protocols are being developed that will allow non-invasive saliva sampling for hormone analysis. The variability seen across the time points for the hormones sampled are only single snapshots in time and as such indicate a great degree of variability that needs further evaluation. With reproduction as the focus of this research, it is also likely that the panel of hormones screened should be widened to include other candidates such as dihydrotestosterone, prolactin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Finally, hormone monitoring can help us understand the reproductive lifespan of amphibians in *ex situ* populations. It is common for the lifespan of animals to increase in EBPs compared to the wild. As such, there is an expectation that ex-situ managed animals will eventually become reproductively senescent. *Ex situ*, *R. muscosa* males reach sexual maturity between 2 and 3 years while females are sexually mature between 3 and 4 years of age (younger than estimated in the wild) and both sexes are capable of living over 10 years (founder animals are currently 15 years old). Initial hormone profiles indicate that when categorized into three main groups, juveniles, adults, and seniors, male and female *R. muscosas* show significantly different levels of estradiol, estriol and testosterone (Fig. 6, Table 2). Preliminary data indicates that levels of corticosterone and progesterone do not differ between seniors and juveniles, however protocols for these hormones may require further optimization. Nevertheless, discerning hormonal cycles in animals of different ages will help us understand aspects of reproduction such as age-related loss of viability and the endocrinological mechanisms associated with other pathologies such as hermaphroditism [46].

In 2021, Jacobs et al., reported on the anomalous development of nuptial pads in a small group of older, F0 females [46]. Seven individuals that had previously been identified as breeding females began developing nuptial pads (male secondary sexual characteristic) and exhibiting male amplectant behaviors one to eight years post sexual maturity [46,52]. Of the seven individuals, five had a history of egg laying and embryonic development highlighting that at one time these animals were reproductively viable as females. Females that developed nuptial pads and displayed male behaviors displayed male characteristics in all aspects of examination. They had hormone profiles more similar to males than females, had no follicular development in ultrasounds and ceased breeding as females following the emergence of nuptial pads. Finally, as these

Table 1
Statistical differences in male and female juvenile, adult and senior *R. muscosa* hormone concentrations detected over time reflect seasonal variations.

		Corticosterone		Progesterone		Testosterone		Estrone (e3)		17β-Estradiol (e2)	
		z.ratio	p-value	z.ratio	p-value	z.ratio	p-value	z.ratio	p-value	z.ratio	p-value
Female	Autumn 2016 - winter 2016	−2.845	0.103	−0.028	1.00	6.469	<0.0001	5.527	<0.0001	−5.241	<0.0001
	Winter 2016 - spring 2017	−0.957	0.990	0.222	1.00	−16.571	<0.0001	−1.829	0.663	5.583	<0.0001
	Spring 2017 - summer 2017	0.971	0.988	0.006	1.00	10.124	<0.0001	−4.47	0.000	−35.843	<0.0001
	Summer 2017 - winter 2017	−5.762	<0.0001	−0.016	1.00	−7.740	<0.0001	6.336	<0.0001	10.091	<0.0001
	Winter 2017 - spring 2018	−0.662	0.999	3.098	0.051	5.841	<0.0001	1.153	0.936	2.484	0.240
	Spring 2018 - autumn 2018	7.782	<0.0001	−3.554	0.011	5.678	<0.0001	3.410	0.019	5.570	<0.0001
Male	Autumn 2016 - winter 2016	−2.849	0.083	0.000	1.000	−8.092	<0.0001	5.532	<0.0001	−5.243	<0.0001
	Winter 2016 - spring 2017	−0.971	0.978	0.000	1.000	−16.564	<0.0001	−1.282	0.906	−10.994	<0.0001
	Spring 2017 - summer 2017	0.967	0.979	0.000	1.000	10.125	<0.0001	16.491	<0.0001	−35.826	<0.0001
	Summer 2017 - winter 2017	−5.755	<0.0001	0.000	1.000	−7.737	<0.0001	1.038	0.969	10.109	<0.0001
	Winter 2017 - spring 2018	−0.302	1.000	3.093	0.042	8.951	<0.0001	1.148	0.946	2.418	0.203
	Spring 2018 - autumn 2018	6.142	<0.0001	−3.566	0.009	5.491	<0.0001	0.259	1.000	5.571	<0.0001

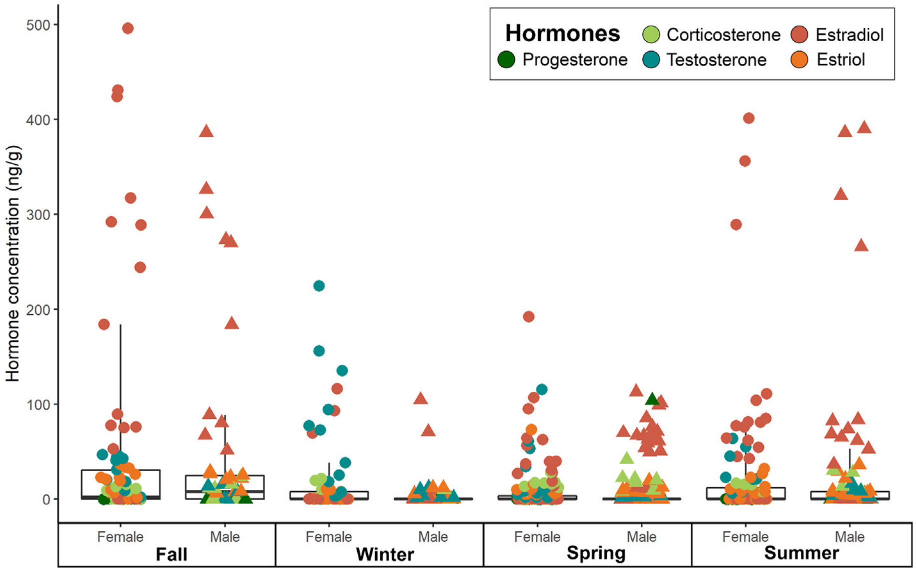

Fig. 4. Hormone profiles quantified by mass spectrometry in male and female *R. muscosa* shows preliminary indications of seasonality. Estrogens were the most prominent hormones detected with a substantial rise in levels in the Summer and Fall months coinciding with gamete growth and development. Testosterone levels in both sexes were detectable but show variation in seasonal fluctuations. Corticosterone and progesterone had low levels at all time points collected.

animals began dying of natural causes, post-mortem findings identified these individuals as hermaphrodites, some with active spermatogenesis with at least one individual reportedly fertilizing a clutch at least once [46]. This phenomenon highlights the importance of reproductive monitoring to assess reproductive health of and help elucidate the factors contributing to reduced reproduction in amphibians.

6. *R. muscosa* male reproduction

6.1. Determining male reproductive fitness

As previously mentioned in section four, understanding the reproductive contribution of both sexes is not only important to reproductive outcomes but necessary for informing the

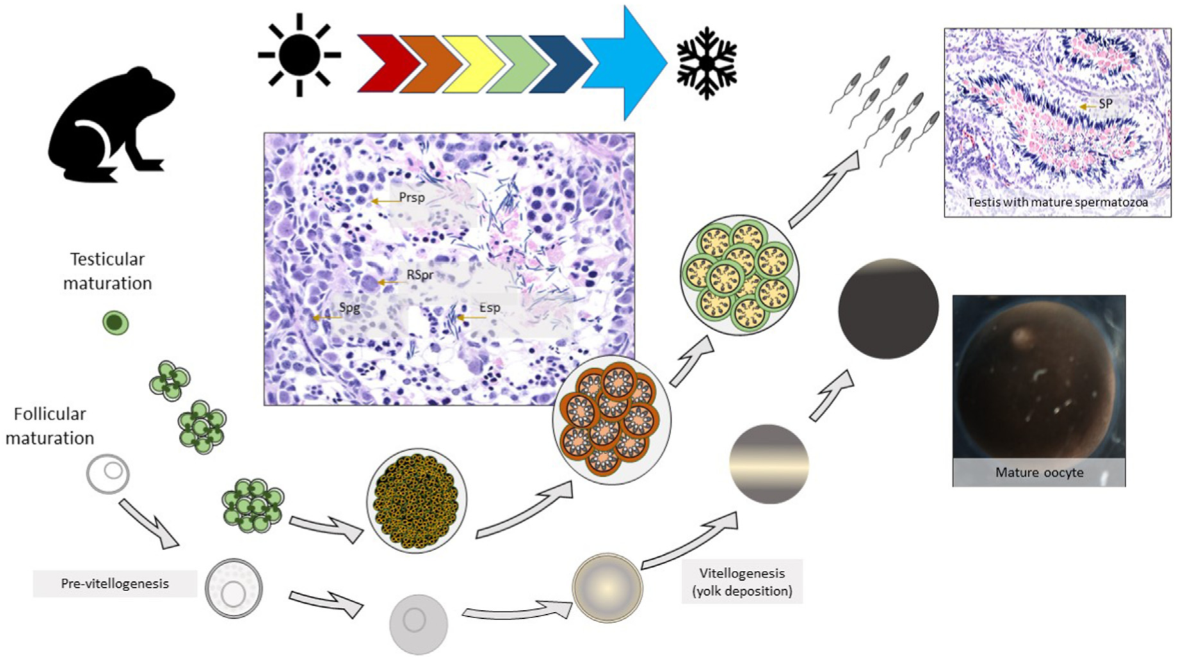

Fig. 5. Circadian and environmental influences seasonal variation and gamete development in discontinuous breeders via interactions with endocrinological and genetic mechanisms. In males, germ cell development progresses through several stages including, spermatogonium, (Spg), primary spermatocyte, (Prsp), round spermatids, (RSpr), elongating spermatids, (Esp) and finally, mature spermatozoa (SP). In females, oocyte development progresses from the end of summer to post-spawning into early recruitment and follicular growth transitioning from previtellogenic to vitellogenic follicles, accumulating yolk. Recruitment and growth will continue from fall into winter as animals undergo brumation. Final mutation involves additional biochemical changes that render mature oocytes ready for fertilization.

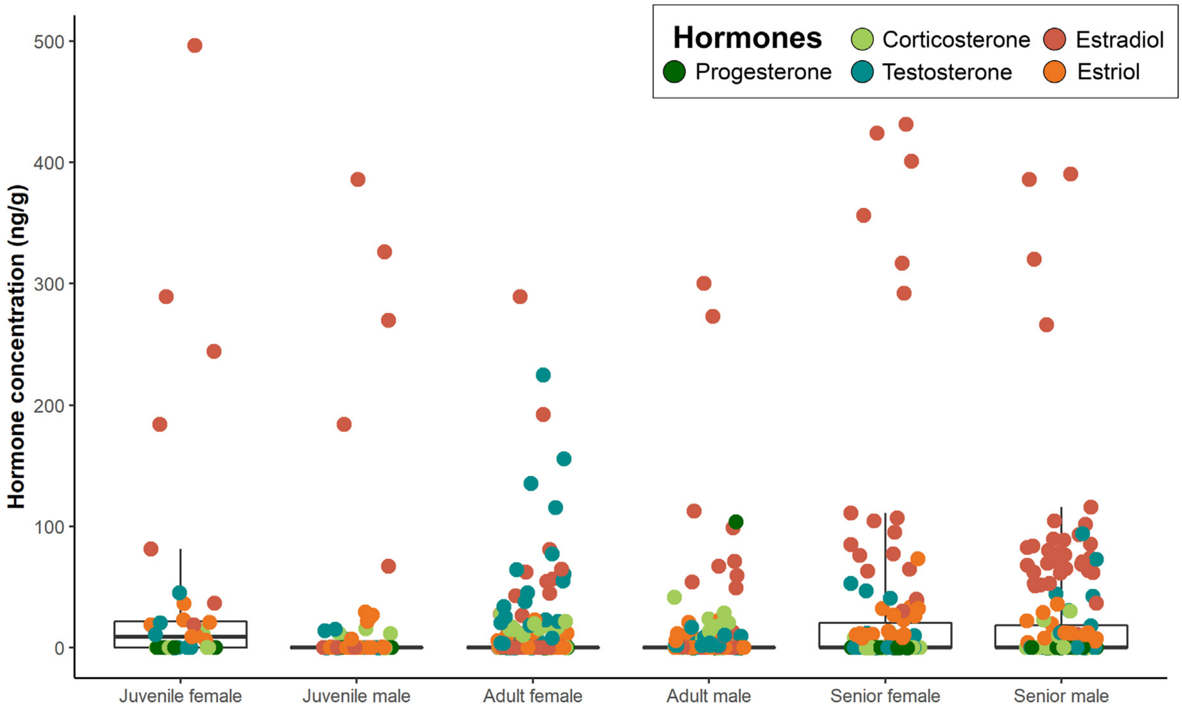

Fig. 6. Hormone analysis by mass spectrometry shows sexually dimorphic differences levels in three age groups, juvenile (1–3 years), adult (4–8 years) and senior (9–11 years).

implementation of ARTs. As with females in the colony, questions relating to male reproductive viability and fitness are ongoing and warrant the inclusion of ARTs not only as a means to increase reproduction but to, examine sperm production, quality and variability individually and seasonally, the influence of age, the synchronization of gamete release during breeding and the effects of inbreeding and outbreeding (if any) in this species (see reviews by Della Tonga et al., 2020; Silla and Byrne, 2019; [4,53]). As with other species, preliminary studies on the efficacy of hormonally induced spermiation and oviposition indicate that *R. muscosa* respond to hormonal induction when treated with human chorionic gonadotropin (hCG), Gonadotropin-releasing hormone (GnRH) or a combination of the two and Amphiplex (GnRH (0.4 μg/g body weight with metoclopramide 10 μg/g) [13].

6.2. Development and optimization of exogenous hormone protocols for sperm collection

Beginning in 2015, hormone induction experiments using two commercially available hormones (Gonadotropin-releasing hormone (GnRHa) and human Chorionic Gonadotropin (hCG)) at various concentrations were examined to determine the most effective hormone concentration to induce spermiation in *R. muscosa*. Overall, ten treatments were prescribed, and three sperm quality parameters were recorded: overall concentration, motility, and speed of progression (SOP). In addition, a sperm response curve was generated from the following data: time taken to initiate sperm production, time to peak concentration and total duration of spermiation response. Hormone trials began in October of 2015 and temporal variation of sperm production was recorded by injecting and analyzing males monthly up until 2017. Preliminary results indicate that male *R. muscosa* do respond to both hormones, GnRH and hCG, however, which cocktail, or concentration of hormone was most effective is still being examined. Individual male variation in sperm production, concentration and motility was high, irrespective of hormone treatment. The average time taken for spermiation to begin was not significantly different between hormones or month, although sperm production ceased

Table 2
Differences in hormone concentrations between juvenile (1–3 years), adult (4–8 years) and senior (9–11 years) *R. muscosa* males and females. Preliminary results indicate differences in corticosterone and progesterone levels between the juvenile and senior frogs compared to adult individuals. The steroid hormones were significantly different across the three age classes.

		Corticosterone		Progesterone		Testosterone		Estrone (e3)		17β-Estradiol (e2)	
		z.ratio	p-value	z.ratio	p-value	z.ratio	p-value	z.ratio	p-value	z.ratio	p-value
Female	**Juvenile - adult**	10.806	<.0001	4.858	0.0001	18.821	<.0001	−7.346	<.0001	−26.57	<.0001
J = 26	**Juvenile - senior**	0.484	1.000	−0.518	1.0000	−7.058	<.0001	−6.168	<.0001	−34.97	<.0001
A = 6	**Adult - senior**	12.813	<.0001	6.226	<0.0001	16.651	<.0001	−14.58	<.0001	−9.38	<.0001
S = 9											
Male	**Juvenile - adult**	10.806	<.0001	4.858	0.0001	18.821	<.0001	−7.346	<.0001	−26.57	<.0001
J = 10	**Juvenile - senior**	0.484	1.000	−0.518	1.000	−7.058	<.0001	−6.168	<.0001	−34.97	<.0001
A = 4	**Adult - senior**	12.813	<.0001	6.226	<.0001	16.651	<.0001	−14.58	<.0001	−9.38	<.0001
S = 19											

Juvenile age range = 1–3 years; Adult age range = 4–8 years; Senior age range = +9 years.

for a majority of animals after 24 h. One major takeaway of the hormonal induction trials is that hormone efficiency in eliciting sperm release was strongly associated with the month of administration. Spermiation was not easily induced by hormone administration in the months following the reproductive season (August–October) with only 3.7% (3/81) of males responding to 3 out of 10 hormone treatments (1 male/hormone treatment). Responsive periods coincided with the species' expected reproductive season but was followed by a period of quiescence. In the months leading up to brumation (October–November) males once again became responsive (testicular recrudescence). This may be related to the start of sperm production and storage prior to brumation when metabolism and basic body functions are reduced [17,54,55]. Light and temperature, as well as the length of time since an individual male was reproductively active, may affect the testes' ability to produce a new spermiation response when hormonally stimulated. In other temperate frog species, wintry conditions correlate positively with an increase in the production of new sperm in the testes and a period of brumation has been linked to increased reproductive capability [17,56].

6.3. Sperm characterization and quality assessments

A common pitfall for cryopreservation and AF is the lack of multi-faceted quality assessments for produced gametes. Historically, concentration and motility were used as quality assessment parameters but with advancements in technologies and increased knowledge of the multitude of factors that can affect reproductive outcomes, more thorough assessment of gamete quality is critical. For example, unlike other taxonomic groups, amphibian sperm motility does not accurately reflect sperm viability, nor do forward progression and speed reflect fertilizing capability [57,58]. Instead, examining aspects of sperm functionality such as DNA and acrosome integrity and mitochondrial function may be more relevant parameters to assess sperm viability (Fig. 7). Coupled with these viability stains is the incorporation of technologies such as CASA and flow cytometry that provide more accurate quantification of sperm concentration and viability at one time using less volume and less time (see Fig. 8).

One of the limitations encountered when inducing and collecting sperm from *R. muscosa* is the poor concentrations and extremely low volumes of spermic urine obtained (2–100 μL/sample). When linked to seasonality and the limited times of year at which sperm can be collected, low volumes of spermic urine pose a significant obstacle to developing other ARTs in this species such as AF and cryopreservation. Consequently, developing protocols to assess multiple sperm characteristics using the least amount of sample quickly and accurately as possible is imperative when working with this species. Once developed, these protocols can help elucidate and monitor the reproductive readiness and viability of the colony's males and may help address concerns about low fertility and reproductive output for the animals in EBPs.

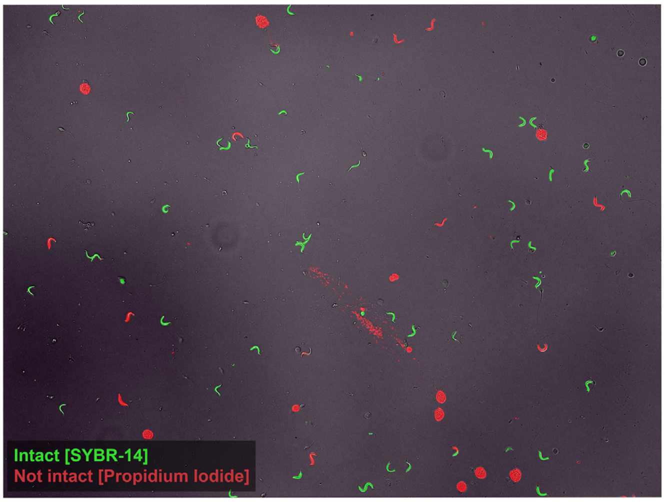

Fig. 7. Fluorescent imaging of *Xenopus laevis* sperm with a Live/Dead stain. Green sperm are nuclear membrane intact/live sperm while red sperm are membrane compromised/dead sperm. Photograph provided by L. Jacobs, personal communication. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

7. Use of genomics for improving management practices (genetic diversity and inbreeding)

The emergence of applied genomics as tools for conservation management have quickly evolved over the last decades. When applied, species management determinations of population structure, effective population size, inbreeding and genetic load can be made [59]. These estimates can be used to make informed decisions about breeding in EBPs, and translocation for genetic restoration in the wild [60]. Applied genomics in amphibian genetic management has seldom been implemented due to the large variation in genome size leading to high cost and challenging genome assembly [60,61]. However, the benefits of these types of approaches for improved management are indisputable. As part of the *R. muscosa* management plan, the genetics of the two populations of southern California *R. muscosa* bred at SDZWA were genetically screened using restriction site-associated DNA sequencing (RADseq) and targeted capture [61]. The San Jacinto and San Bernardino populations were genotyped at 6,619 SNP loci after filtering the data. The results of this genetic work uncovered a low level of heterozygosity in the managed populations overall which elucidated the need to supplement the breeding program with new imports from the wild to increase genetic diversity in the assurance population. Additionally, genome-wide estimates of relatedness within populations allowed clarifying gaps in the pedigree of the managed populations and relationship assumptions among founders, which is crucial for guiding breeding recommendation according to the lowest mean kinship values. Genetically informed breeding decisions will allow maximizing retention of genetic diversity and reduction of inbreeding in the assurance population, as well as the release of genetically diverse frogs back in the wild. The implications for this are critical to the *R. muscosa* recovery program as an already limited number of frogs are available for breeding. The application of new genetic management strategies based on genetic estimates of relatedness, and the continuation of genotyping efforts will aim to maintain sustainable *R. muscosa* populations for future translocations.

Supplementing and preserving diversity through strategic genetic management of *R. muscosa* will require additional intervention through ARTs including biobanking of gametes. The integration of frozen founder sperm and the recovery of sperm collected and cryopreserved from wild populations could provide significant genetic benefits to *R. muscosa*'s EBP through:

1. Maintenance of gene flow between the EBP and *in situ* populations [4,62] via “incremental introgression of new genes (via natural selection), without swamping locally adapted alleles” and without the need to remove individuals from the wild [66,67].
2. Artificial manipulation of genetics where the breeding of desirable individuals is not effective through more naturalistic breeding.

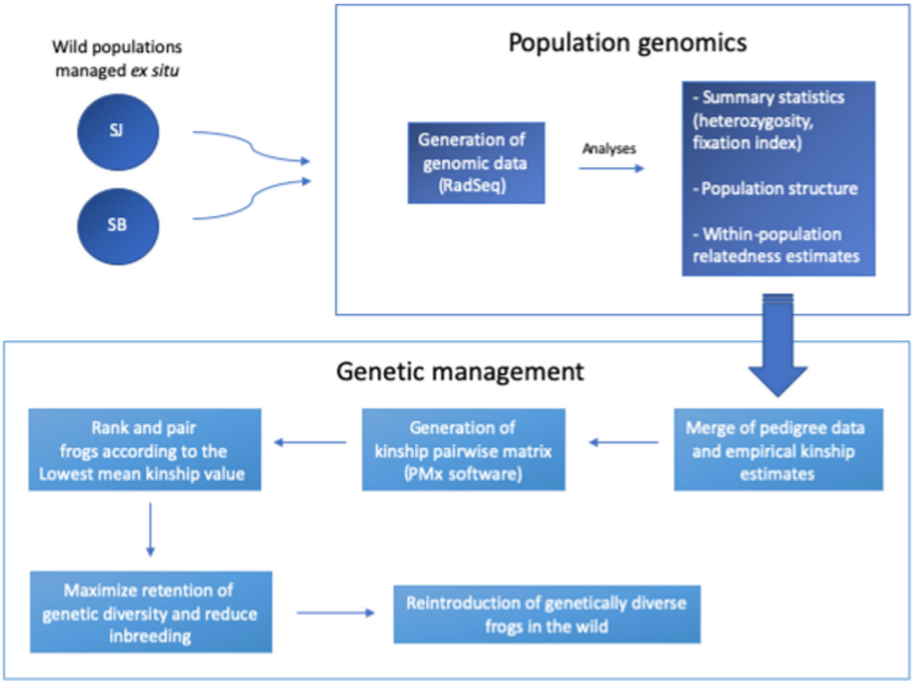

Fig. 8. Workflow of the population genomic analyses and genetic management strategy used in the *R. muscosa* EBP. Genomic data, in the form of reduced-representation or RadSeq loci (restriction site-associated DNA sequencing), were collected from both populations managed *ex situ*, San Jacinto (SJ) and San Bernardino (SB). Population genetic analyses were conducted to determine genetic diversity, population structure and relatedness. Empirical estimates of kinship were used to generate a pairwise kinship matrix that also included pedigree information from the breeding program. The lowest mean kinship values were used to rank and pair individuals for breeding (using the PMx software) in order to maximize genetic diversity and reduce inbreeding *ex situ*, but also reintroduce the most genetically diverse frogs in the wild.

3. Reduction of financial and spatial constraints on the recovery program, preserving genetics through biobanking [34, 35, 68–72].

Future research must clarify the impact of inbreeding and outbreeding, if any, on *R. muscosa* and whether genetic incompatibility between populations is a problem for this species in case of interbreeding [67]. To date, there is limited research on inbreeding vs. outbreeding depression in amphibians. Studies suggest that in some cases, mate selection is increasingly skewed to occur between related individuals posing inbreeding depression as a greater risk to long-term fitness [66]. Questions of inbreeding and outbreeding depressions are relevant to *R. muscosa* management due to its extremely low genetic diversity. Currently no data examining possible links between reproduction, offspring viability, fitness, and genetic diversity exists. Therefore, a greater understanding of fitness in subsequent generations produced through natural mating (through mate choice) compared to breeding between genetically desirable combinations or those created using ARTs and artificial fertilization (AF), will be critical if these animals are to be returned to the wild [71].

8. Future directions

There are many other factors that may influence reproductive success (e.g. reproductive behavior) that are beyond the scope of this review. Each year following brumation, males and females are paired for breeding, and several aspects of reproductive behavior, including amplexus, time to amplex as well as duration of breeding event for each pair are recorded. This information may be important to assess the receptivity of both males and females to breeding as well as the viability of the eggs laid. Amplexus and breeding behaviors are physically demanding for males as well as females and the length of amplexus may impact the production of viable embryos. Additionally, mate choice is a known reproductive event that has an enormous impact on breeding success in a variety of amphibians [73]. While mate choice has been examined in our *ex situ R. muscosa*, the scope which mate choice plays on overall success of breeding remains unknown. Preliminary findings indicate that mate choice may be an important aspect of *R. muscosa* reproduction and can lead to higher output of viable embryos [11].

Using non-traditional reproductive technologies, such as analytical chemistry and microbial methods may help illuminate some of these often cryptic behaviors. For example, many animals, including amphibians are known to communicate through the excretion of volatile compounds, such as pheromones (reviewed by [74,75]). These chemicals may signal receptivity of females and allow for the initiation of reproductive behaviors in males. Previously, we identified 96 volatile molecules associated with *R. muscosa* using solid-phase microextraction fibres analyzed by gas chromatography-tandem mass spectrometry. In this precursory analysis, we found significant differences between animals of different age groups ($P = 0.018$, PERMANOVA, unpublished data), but much more work is necessary to distinguish differences between animal sex and changes in volatile composition between seasons.

Interestingly, sex-specific differences in amphibian pheromones are associated with microbially-derived molecules [76]. We have many microbiome projects underway. To date, our primary focus has been on understanding interactions between skin microbiota and chytrid fungus. However, it appears that host-microbe symbiosis may extend beyond that of pathogen mitigation or exclusion on skin. Microbiota have been linked to host endocrine function, including reproduction and behavior for a broad range of animal taxa (reviewed by Williams et al., 2020 [77]). Further work is also needed in this area to determine the potential role of microbiota in creating volatile compounds that may drive amphibian reproduction. For a species with limited audible calls, the use of chemical signalling may be the key to initiate breeding and allow for the successful locating of suitable mates in the wild [78]. Unlocking this system could lead to many novel avenues for ARTs. For example, if we discover what pheromone signals female receptivity, it is possible that exogenous exposure to this pheromone could be used to stimulate males to spermiate without the need for exogenous hormone administration. Therefore, further work is needed to integrate new microbiological and analytical chemistry methods, such as hormone monitoring mentioned previously, along with analysing volatile compounds to better understand how hormones and pheromones shift seasonally, driving reproduction in a cryptic species like *R. muscosa*.

9. Conclusions

While there are still many aspects of reproductive physiology, ecology, behavior and genetics that warrant examination, the *R. muscosa* conservation breeding and reintroduction program is a prime example of how a multi-faceted, integrated EBP can function to maintain a sustainable assurance population that aims not only to contribute to population augmentation but the management of genetic diversity and evolutionary potential of the species. Although the last decade has increased our understanding of the challenges associated with the conservation of *R. muscosa*, some major milestones have been achieved. To date, consistent reproduction in the EBP has provided hundreds of metamorphs for reintroduction annually. Coupled with more accurately informed genetic management, the future of *R. muscosa* recovery efforts will continue to build on strategies to increase EBP-driven augmentation of the wild population whilst capturing diversity from the distinct populations through ARTs.

Acknowledgements

The authors would like to thank Dr. Ron Swaisgood, Dr. Barbara Durrant, Dr. Talisin Hammond and Ms. Michelle Curtis for their involvement in SDZWAs *R. muscosa* conservation breeding and reintroduction program, their efforts in the field and, in the laboratory, which are part of the greater project for which the data contained in this manuscript was collected. Calatayud would like to thank Dr. Patricia Gaffney for providing histological images used in Fig. 4. We would also like to thank Ms. Ashley Meredith, Dr. Abbey Wilson, and Dr. Mabel Gonzalez for their assistance with volatile chemical analyses. Additionally, Calatayud would like to thank Explotadora de Inmuebles, S.A. (EISA) for awarding financial aid for her first two years as a postdoctoral fellow and the financial support awarded by the Disney Conservation Fund AZA/AZH (Conservation Grants Funds File Number: 17–1433) for *R. muscosa* genomic sequencing and genetic management project. Thanks to our partners in the *R. muscosa* recovery program including the United States Fish and Wildlife Service, California Department of Fish and Wildlife, United States Forest Service, United States Geological Survey, the Los Angeles Zoo and Omaha's Henry Doorly Zoo. Finally, and most importantly, we acknowledge that our research takes place on the traditional lands of numerous Indigenous Peoples. We would therefore like to respectfully acknowledge the traditional custodians of the land on which this work was conducted and pay our respect to Elders both past, present and emerging. Animal Ethics number (just in case): IACUC permits#19-003 *R. muscosa* Federal permit number too just for good form: TE76006B-2.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2022.07.003.

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