

**Assessing species-specific reproductive variation to inform assisted reproductive  
technologies for Canadian snakes**

By

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A thesis submitted to the Graduate Program in Biology

in conformity with the requirements for the

Degree of Master of Science

Queen's University

Kingston, Ontario, Canada

April 2025

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## Abstract

Assisted reproductive technologies (ART), frequently used to manage domestic livestock, are increasingly incorporated into conservation programs for species-at-risk. Species-specific insights on reproductive biology, including seasonal variation and endocrine function, improve ART outcomes. Despite representation in many zoological collections, snakes are underrepresented in the ART literature and research is needed before it can be broadly implemented in conservation. My research characterizes temporal variation in spermatozoa quality and sexual steroids for two endangered Canadian snakes to facilitate gamete cryo-preservation and artificial insemination. These focal species have different life history strategies: an oviparous viperid, the eastern massasauga rattlesnake (*Sistrurus catenatus*) and oviparous colubrid, the gray ratsnake (*Pantherophis spiloides*). I collected spermatozoa samples monthly from May to October in 2023 and 2024 from individuals of both species held in captive populations. Spermatozoa samples were quantified for concentration, motility, membrane integrity and rapid forward progression of spermatozoa. I predicted the highest quality spermatozoa would be produced during the period corresponding to the mating season in wild population; late July through August in rattlesnakes, and late May through June in ratsnakes. I found species-specific variation in quality and concentration of spermatozoa, but not during the intervals predicted. To characterize the reproductive hormone cycle, faeces from captive rattlesnakes was collected from March 2023 to September in 2024. Sexual steroid faecal metabolites of progesterone (FPM) and testosterone (FTM) were quantified using commercial enzyme-linked immunosorbent assay kits. I predicted FTM concentrations would be highest from July to August, corresponding to spermatogenesis and the peak in mating behaviour. I predicted that FPM would increase in May and June during vitellogenesis, be expressed in the highest concentrations annually from May to August and remain at high concentration through to parturition in gravid females. I found annual variation in FTM and FPM, but not in the patterns which I had predicted. My research provides novel insights into the reproductive physiology of two Canadian at-risk species and facilitates efforts to develop

ART in managed population. The integration of this information into conservation programs will improve fecundity, ensuring that captive population can best contribute to conservation.

## Lay Abstract

My research focused on the development of assisted reproductive technologies (ART) for Canadian snakes at risk of extinction. Assisted reproductive studies aim to increase fecundity or reproductive success of focal species and include techniques such as artificial insemination. While ART are well developed for human applications and agriculture, fewer studies centre on wild species and fewer still focus on augmenting snake reproductive success. As species have evolved a diverse array of reproductive traits to optimize reproductive success in the wild, detailed species-specific knowledge of reproductive function and annual variation is essential to ART development. My project focused on assessing annual variation of species-specific reproductive characteristics in two snake species. The eastern massasauga rattlesnake, which is live-bearing, and the gray ratsnake, which is egg-laying, are both species at risk in Canada for which the federal government has recommended immediate intervention. My goals included characterizing annual variation in spermatozoa of captive snakes produced over the active season in wild population in Canada (May to October). I predicted that the best spermatozoa would be collected during each species mating season. My results did confirm species-specific annual variation; however, the highest quality spermatozoa were collected in October for both massasauga and ratsnake. I also attempted to quantify sexual steroid output of Canada's eastern massasauga rattlesnake *ex situ* mating population using non-invasive faecal samples. I predicted annual variation would result in sex steroids being expressed at the highest concentration when snakes are developing egg and spermatozoa cells. My results indicate annual variation for males and females within the collection surveyed, but not in the predicted pattern. The integration of this information into conservation mating programs will help to improve reproductive success, ensuring that captive population can best support conservation of these two species at risk in Canada.

## **Co-Authorship**

Dr. Amy Chabot; African Lion Safari, Cambridge, ON, and Queen's University, Kingston, ON;

Conceptualization, Analysis, Writing - review and editing.

Dr. Stephen Lougheed; Queen's University, Kingston, ON; Conceptualization, Analysis, Writing - review and editing.

Dr. Drew Sauve; African Lion Safari, Cambridge, ON; Analysis.

## Acknowledgements

*A sincere thank-you to my co-supervisor, Dr. Amy Chabot, who encouraged and supported my research aspirations. I would not be the scientist, nor the person I am today without your guidance. Thank you for the support and understanding as I navigate the academic world and for providing me with numerous personnel development opportunities as I begin my professional career. I will remain eternally grateful.*

*A huge thank-you to my co-supervisor, Dr. Stephen Lougheed, for his support and guidance throughout this endeavor. I am immensely grateful to the opportunities provided to me throughout this project. My development as a scientist has been improved by the exposure to diverse researchers and colleagues that I have been privy to as a member of the Lougheed Lab.*

*Thank you to my committee member, Dr. Sarah Yakimowski, for providing support and mentorship throughout this project. Your questions and feedback were of immense help during this work, and I am incredibly grateful to have had your input.*

*I also want to take the time to thank African Lion Safari for the unending support of this project and my development as a researcher. Thank-you to Trish Gerth and Mike Tackas for supporting this research project, taking a chance on new species and allowing me to continue my professional development journey throughout my education. Further, thank you to Dr. Drew Sauve, as without your assistance I would not have half the comprehension of statistics I do now; and of course, Hana Thompson, as there is not a chance, I would have collected half the data I did without your assistance.*

*This work could not have been performed without the numerous dedicated animal keepers and support staff at each zoological facility that assisted with this project. Thank you to each and every person at Little Ray's Nature Center, Science North, Sciensational Sssnakes!!, Toronto Zoo, and Scales Nature Park for their help. Your time and contribution have been crucial to this project. The care and welfare of the animals was a top priority and made possible by the dedication of project partners.*

*I would not have been able to pursue my graduate research without the unending support from my partner, Christopher. He has been my biggest cheerleader and never wavered in his confidence in the importance and success of this project.*

*Last but not least, thank you to my parents, for taking me out of the city as a child, fostering a love of the forest and its many creatures.*

## Land and Environmental Impact Acknowledgements

It is important to recognize the colonial influences still prevalent in Western education as we work towards increased understanding and reconciliation on Turtle Island. This place which we currently call Canada, is the ancestral home of many peoples who stewarded the land since time immemorial. The work described in this manuscript was conducted across multiple treaty lands in the traditional territories of many nations, including the Huron-Wendat, Ojibway/Chippewa, Mississauga's of the New Credit, Attawandaron, Haudenosaunee, and Anishnaabeg peoples. I recognize my privilege as a white colonist descendant and in being able to attend higher education. I am thankful for the experiences I have had in this place I call home which have influenced me to protect and steward the land and its waters. I aspire to keep furthering my understanding and listening to the people, animals, and plants on this land.

While this work is being conducted to expand on conservation methodologies, research generates substantial waste products (Monson et al., 2024). The largest environmental impacts from this project were gas mileage spent commuting between partner facilities and single use plastic waste. To reduce future gas incurrences the validated techniques are being taught to partner facility staff so work can be done in house. This further supports the partner facilities' research skills development and contributes to conservation outcomes. Reducing the use of plastic in research while maintaining contamination protocols is difficult but not impossible. One change that the African Lion Safari lab has made is to employ repeater pipettes when protocol allows, facilitating a single repeater-pipette tip being used for multiple aliquots as opposed to using a new tip for each aliquot. To take it a step further, in my personal life my use of single-use plastics is extremely limited, and I aspire to ensure my needs are fulfilled in ecologically sustainable ways.

Monson, E. A., Rutter, S., Reimann, C. C., Bueno-Pedraz, A., Vella, C., Pearce, X. G., ... and Fanson, K. V. (2024). The future of scientific labs: how we are making my research more sustainable. *Immunology and Cell Biology*, 103(2): 105-110.

<https://doi.org/10.1111/imcb.12840>

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## List of Abbreviations

AIC <sub>c</sub>	Akaike information criteria corrected for small sample sizes
ART	Assisted reproductive technologies
CI	Confidence interval
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
DOY	Day of year
ELISA	Enzyme linked immunosorbent assay
FTM	Faecal testosterone metabolites
FPM	Faecal progesterone metabolites
ID	Identification
PBS	Phosphate Buffered Saline
RFP	Rapid forward progressive motility
SE	Standard error
Temp.	Temperature
TEST	Tes-Tris Yolk Buffer
uL	Microliter

## Chapter 1: Introduction

### 1.1 Assisted reproductive technologies

Assisted reproductive technologies (ART) include methods developed to overcome reproductive limitations and manipulate conception (Herrick, 2019). Broadly, these include technologies that improve the fecundity of an individual, such as hormone treatments, artificial insemination, *in-vitro* fertilization and genome resource banking (Bolton et al., 2022). ART have been extensively developed for agricultural animals and human family planning (Howard et al., 2015; Herrick, 2019). In humans, the ART market accounts for over 25 billion US dollars spent annually, facilitating continued research and refinements improving the successful implementation of ART (Aderaldo et al., 2023). ART have been proposed as tools to enhance the success of *ex situ* conservation programs and improve efforts to maintain genetic diversity (Bolton et al., 2022). For example, endangered species, including black footed ferret (*Mustela nigripes*) and white rhinoceros (*Ceratotherium simum*), have been the focus of species-specific research resulting in the development of novel ART to improve and recover genetic diversity (Mackie et al., 2020; Biasetti et al., 2022).

For livestock species and laboratory rodents, ART are now a key component of management practices (Herrick, 2019). The value of these methods to management of agricultural species suggests similar ART applied to zoological population would improve the outcome of conservation efforts (Herrick, 2019). Evidence demonstrates effective application of *in situ* conservation, wherein actions to conserve the species are conducted within the range, and *ex situ* conservation, wherein actions to conserve the species are conducted in human care, can assist in halting and reversing population declines (Pizzutto, Colbachini, and Jorge-Neto, 2021; Lees et al. 2021). The intention of ART when applied in a conservation program is not to replace natural mating, but rather to provide population managers with tools when natural mating alone cannot maintain self-sustaining population (Meuffels-Barkas et al., 2023). Benefits of ART technologies include limiting inbreeding depression, reducing loss of genetic diversity, providing

means of exchange of gametes between *in situ* and *ex situ* population as well as among *ex situ* population, and optimizing resource allocation (Clulow et al., 2022).

To date, ART development for exotic animals, including genome resource banking, has been focused largely on mammalian species at risk (Clulow et al., 2022). ART developed for agricultural species, such as horses, provided model systems by which to begin ART development for related at risk species (Meuffels-Barkas et al., 2023). However, broad scale applications of ART are limited due to the need for species-specific knowledge on reproductive systems. Research shows that even among closely related species, small differences in reproduction affect successful conception of viable young (Dziekonska and Partyka, 2023).

Reptiles are widely held *ex situ* in the pet trade, for conservation, and for biomedical research (Clulow and Clulow, 2016; Vieira et al., 2024). The incorporation of ART into *ex situ* reptile population management could reduce or eliminate the need for further *in situ* animal collection and alleviate genetic pressures on existing population (Clulow and Clulow, 2016; Herrick 2019). Further, ART could assist with numerous reptile conservation programs that incorporate *ex situ* management as a recovery action, but which often struggle with small founder population and low fecundity (Herrick, 2019). Within reptiles, ART development has begun for all orders (Clulow et al., 2022). However, most reptile ART published literature focuses on members of the order Squamata and the saltwater crocodile (*Crocodylus porosus*) (Clulow et al., 2022).

### *1.2 Squamate reproduction*

Reptiles encompass the evolutionary descendants of the clade Sauria which includes Aves (birds), crocodylians, testudines (turtles), squamates, and rhynchocephalians (tuataras) (Gauthier, Kluge, and Rowe, 1988). The largest group within the class Reptilia is the order Squamata comprising three paraphyletic suborders, including the Lacertilia (lizards), Serpentes (snakes), and Amphisbaenia (worm

lizards) (Ossiboff, 2018). Squamates currently represent the second largest order of living vertebrates, second only to Perciforms (bony fishes) (Ossiboff, 2018). Extant reptiles can be found on every continent except Antarctica, inhabiting a wide variety of habitat types (Pough, 2017). From an evolutionary standpoint, squamates are highly successful, which may in part be a result of the highly diverse reproductive strategies that have evolved to facilitate maximum productivity across differing environments (Pough, 2017). Unique to squamates is the evolution of the hemipenis in males, a pair of intromittent organs held internally that are everted to reproduce (Pough, 2017). The hemipenis is found in all squamates but is highly diverse morphologically (Andonov et al., 2017).

Squamate reproduction typically involves sex in nature; however, certain species are known to be unisexual, reproducing only by parthenogenesis; e.g. the Laredo striped whiptail (*Aspidoscelis laredoensis*) (Barley et al., 2022). Squamate species that display obligate parthenogenesis are limited in number and largely found only within lizards (Booth and Schuett, 2016). The only obligate parthenogenic snake so far described is the Brahminy blind snake (*Indotyphlops braminus*) (Booth and Schuett, 2016). In some squamates, sexual and asexual reproduction has co-evolved allowing for facultative parthenogenesis (Booth and Schuett, 2016). Research into the drivers of facultative parthenogenesis excluding the effects of resource limitation have been conducted in pit vipers (cottonmouth, *Agkistrodon piscivorus*, and copperhead, *A. contortrix*) (Booth et al., 2012). Discoveries of facultative parthenogenesis are often opportunistically observed in *ex situ* population where detailed individual life histories are known (Booth and Schuett, 2016; Booth et al., 2024). However, recent molecular advancements, such as polymerase chain reaction and microsatellite genotyping, have facilitated the ability to assess parentage and identify parthenogenetic events in nature (Cubides-Cubillos et al., 2020; Booth et al., 2024).

When sexual reproduction is employed, females can be monogamous or polygamous in nature. Long-term monogamous pairings are rare in squamates, though have been observed in some species like the long-lived Australian shingleback (*Tiliqua rugosa*) (Murray and Bull, 2004). Polygamy, on the other hand, is common among squamates, frequently resulting in clutches with multiple paternity (Gangloff et

al., 2021; Friesen, Kahrl, and Olsson, 2020). Polygamous mating systems have led to the development of both pre- and post-copulatory competition among conspecific male squamates and facilitated pre- and post-copulatory female choice (Gangloff et al., 2021; Friesen, Kahrl, and Olsson, 2020; Kahrl, Cox, and Cox, 2016). Pre-copulatory competition includes male-male combat, mating balls, and guarding to prevent females from mating with other males (Kahrl, Cox, and Cox, 2016). Male post-copulatory selection encompasses spermatozoa competition, seminal plugs, increased testis size, and long-term spermatozoa storage (Kahrl, Cox, and Cox, 2016).

Long-term spermatozoa storage has been recorded in various vertebrate species (Sever and Hamlett, 2001). Long-term spermatozoa storage describes a state wherein viable spermatozoa is maintained in vertebrate reproductive tract for an extended period after copulation (Levine, Schuett, and Booth, 2021). This allows individuals to optimize the timing of gestation and parturition to coincide with optimal environmental conditions and provides time for post-copulatory female choice (Birkhead and Møller, 1993). Hypotheses of possible drivers for oviductal long-term spermatozoa storage in vertebrates include seasonal changes (Birkhead and Møller, 1993). The longest confirmed vertebrate offspring containing paternal deoxyribonucleic acid (DNA) from long-term spermatozoa storage to date was observed in a female western diamond-backed rattlesnake (*Crotalus atrox*) after 71 months of isolation from males (Levine, Schuett, and Booth, 2021).

Squamate gestation and parturition encompass multiple strategies that have likely evolved to increase offspring survival rates (Orrell and Kunz, 2016; Lind, Taylor, and DeNardo, 2024). Squamates can develop their young either via oviparity, or ovoviviparity (Vitt and Caldwell, 2009; Orrell and Kunz, 2016). In oviparous species, eggs develop in the oviduct and are laid in a suitable nest site for further incubation (Vitt and Caldwell, 2009). Maternal care of eggs is not common but has been recorded in booid snakes including Children's python (*Antaresia childreni*) and Southern African python (*Python natalensis*) (Alexander, 2018; Lind, Taylor, and DeNardo, 2024). Environmental sex determination, in which environmental conditions determine the sex of offspring, has been observed in oviparous reptiles

(Janzen and Paukstis, 1991). Environmental sex determination is less common in squamates than in either testudines or crocodylians but has been confirmed in the family Gekkonidae (geckos) (Janzen and Paukstis, 1991; Pensabene, Kratochvíl, and Rovatsos, 2020). In ovoviviparous reproduction, young develop in the female's oviduct, acquiring energy from the egg yolk (lecithotrophy) until they 'hatch' internally and parturition of young occurs (Vitt and Caldwell, 2009; Van Dyke and Griffith, 2018). Maternal care has been recorded in a variety of squamates that have live-birth (Lind, Taylor, and DeNardo, 2024). Studies of vipers have demonstrated that the mother will often remain with their clutch through to their first shed providing protection from predators (Greene et al., 2002).

Reproductive seasonality refers to the tendency of a species to reproduce when the environmental conditions favour fecundity and offspring survival (Aldridge et al., 2020). Seasonal reproduction in squamates is observed in species experiencing both temperate and tropical climates because of environmental variation (Brown and Shine, 2006). To optimize energy allocation, squamates can start and stop spermatozoa production throughout the year (Aldridge et al., 2020). A review of North American colubrid snakes found that in most studied species, females conduct vitellogenesis - the production of vitellogenin (egg yolk) in oocytes - and mating in the spring (Aldridge et al., 2009), whereas male spermatogenesis occurs in the summer and mating the following spring (Aldridge et al., 2009). This suggests that in regions of North America where colubrid species spend the winter season in a state of brumation, individuals arrest spermatogenesis to conserve energy (Saint Girons, 1982). However, recent research into the reproduction of rattlesnakes in the United States have shown that viperids in the same environments have widely variable reproductive modes (DeNardo and Taylor, 2011).

Reproductive activities can be triggered both by endogenous cues from sexual steroids and exogenously due to environmental triggers. Research on squamates have demonstrated sexual steroid hormones including androgens, estrogens, and progestogens, are involved in the development and maintenance of gametes, initiation of reproductive behaviours (i.e. male-male combat and sexual intercourse), gravidity, parturition, and in regulating pheromone production (Lind, Taylor, and DeNardo,

2024; Parker and Mason 2014). Whereas environmental cues including temperature, photoperiod, social cues (i.e. conspecific pheromones), and rainfall, are correlated with changes in behaviour and sexual steroid production (Van Dyke, 2015; Lind, Taylor, and DeNardo, 2024). Further, evidence in temperate species suggests exposure to cold temperatures resulting in metabolic changes to facilitate overwinter survival, hereafter brumation, facilitate reproductive function (Van Dyke, 2015).

The terms associated and dissociated have been used to identify the timing of gametogenesis in relation to mating season (Lind, Taylor, and DeNardo, 2024). However, many squamates have multiple mating seasons in a single year, not all of which can be described in such a dichotomise fashion (DeNardo and Taylor, 2011). In a recent review of snake reproduction, Lind, Taylor, and DeNardo (2024) propose a method of describing snake reproductive variation that accounts for both gametogenesis and hormonal variation. The relationship of gametogenesis and mating can more correctly be categorized as pre-nuptial, post-nuptial, or continuous, while the relationship of hormones and mating can be identified as associated or dissociated (Lind, Taylor, and DeNardo, 2024).

### *1.3 ART in snakes*

#### *1.3.1 Gamete collection*

Spermatozoa collection is a common requirement for ART and is a necessary first step toward the development of artificial insemination techniques (Mattson et al., 2007; Coeti et al., 2020). The digital manipulation method for spermatozoa collection and artificial insemination using freshly collected spermatozoa in a snake was first published by Mengden et al., (1980). This technique has since been successfully used for a wide range of snake taxa (Mattson et al., 2007; Coeti et al., 2020; Oliveri et al., 2018; Sandfoss et al., 2022), but artificial insemination has not been broadly attempted (Sandfoss et al., 2024; Roberts et al., 2024; Coeti et al., 2020; Oliveri et al., 2018; Langlada, Santos, and Ferreira, 1994). Use of a local anesthetic, to relax the muscles around the cloaca, in conjunction with digital manipulation

has been used on venomous species and large constrictors with varying success (Zacariotti et al., 2007; Blank et al., 2022). However, the use of anesthesia during spermatozoa collection has coincided with reports of abnormal ejaculates in domestic animals (Tourmente et al., 2007).

Collection of viable gametes from female snakes, oocytes, is complicated by morphology, seasonal reproduction, and egg development (Perry, 2021). Unlike spermatozoa, oocytes are not expelled by snakes during reproduction (Zug and Dowling, 2024). Thus, oocytes would have to be recovered through surgical procedures or during post-mortem studies. I found no investigations on snake oocyte recovery in the published literature.

### 1.3.2 *Species-specific reproduction*

Collection of gametes from males and females is optimized when species-specific reproductive parameters are considered (Comizzoli, Paulson, and McGinnis, 2018). As snake reproduction is seasonal in nature, gamete collection may be affected by reproductive quiescence (Shine, 2003; Lind, Taylor, and DeNardo, 2024). Traditionally, radioimmunoassay was used for endocrinology; however, these methods were costly and required radioactive materials (Taylor, Patel, and Bourne, 1983). Development of enzyme-linked immunoassay (ELISA) has facilitated broader use of endocrine analysis (Taylor, Patel, and Bourne, 1983). Endocrine profiles of the sexual steroids, progesterone, estrogen, and testosterone have been optimized for many species as these steroids are responsible for reproductive traits and behaviours to varying extents among all extant vertebrates (Taylor, DeNardo, and Jennings, 2004; Vieira et al., 2024; Lind, Taylor, and DeNardo, 2024). Previous studies of endocrine expression in snakes predominantly focus on red-sided gartersnake (*Thamnophis sirtalis parietalis*), and North American vipers (families *Crotalus*, *Vipera*, and *Agkistrodon*) (DeNardo and Taylor, 2011).

Investigations of snake reproduction using ultrasound methodology have proven successful across taxa (Bertocchi et al., 2018; Vieira et al., 2024). Technological advancements have resulted in portable

ultrasonography equipment that can be used both *in situ* and *ex situ* (Sassoè-Pognetto, Acierno, and Roatta, 2022). The images produced via ultrasonography allow researchers to track the development of follicles and embryo within a female snake's oviduct (Bertocchi et al., 2018). This work facilitates the development and successful implementation of ART by improving the timing of artificial fertilization procedures (Vieira et al., 2024). Further, in male snakes, ultrasonography images of the kidney have revealed that an area contributing to ejaculate, known as the sexual segment of the kidney, increases in size during spermatogenesis (Garcia and de Almeida-Santos, 2021).

### 1.3.3 Artificial fertilization

Artificial fertilization procedures have been developed to circumvent issues arising when sexual reproduction is unsuccessful or cannot occur; e.g. males and females are housed in separate zoological collections (Silva et al., 2020). Artificial fertilization can include *in vivo* fertilization such as gametic intrafallopian transfer or intrauterine insemination, hereafter collectively known as artificial insemination (AI), wherein male gametes are introduced to the female reproductive tract (Silva et al., 2020). In addition, there is *in vitro* fertilization, where male and female gametes are introduced *ex vivo* to form a viable embryo which is subsequently implanted in a female (Silva et al., 2020). For *in vitro* fertilization to be successful, females must be in a reproductive state that can support the development of viable young when procedures occur (Silva et al., 2020). I found no published studies on *in vitro* fertilization in snake species. Artificial fertilization development in snakes is further complicated by the diverse reproductive systems within the taxa. Research on these techniques must validate the success of trials using genetic data as females of many species are capable of both long-term spermatozoa storage and facultative parthenogenesis (Mattson et al., 2007). Developing further knowledge on the species-specific reproductive capabilities of females will facilitate efforts to successfully develop and implement artificial insemination and *in vitro* fertilization (Vieira et al., 2024).

Cloacal morphology varies little between snake species and thus artificial insemination techniques may be broadly replicable (Oliveri et al., 2018). I found only eight published investigations on the development of artificial insemination in snakes (Mengden et al., 1980; Quinn, Blasedel, and Platz, 1989; Langlada, Santos, and Ferreira, 1994; Mattson et al., 2007; Hoser, 2008; Oliveri et al., 2018; Roberts et al., 2024; Sandfoss et al., 2024). Of these, six confirmed female subjects produced viable offspring after artificial insemination (Quinn, Blasedel, and Platz, 1989; Langlada, Santos, and Ferreira, 1994; Mattson et al., 2007; Oliveri et al., 2018; Roberts et al., 2024; Sandfoss et al., 2024). Only Quinn, Blasedel, and Platz, (1989) used females born *ex situ* and isolated from males since birth to ensure exclusion of long-term spermatozoa storage; however, no exclusion of parthenogenesis was made. Only three of these published studies verified successful implementation of artificial insemination using genetic data to confirm the introduced gametes were used to produce young (Mattson et al., 2007; Roberts et al., 2024; Sandfoss et al., 2024). In confirmed cases of successful artificial insemination with fresh and cooled spermatozoa, colubrid species were used, and multiple artificial insemination procedures were conducted over consecutive days for individual females (Mattson et al., 2007; Roberts et al., 2024). The birth of the first Louisiana pine snake (*Pituophis ruthveni*) from frozen-thawed spermatozoa suggests that low quality spermatozoa, when used in sufficient quantities, may be sufficient for fertilization (Sandfoss et al., 2024).

#### 1.3.4 *Preservation and storage of spermatozoa*

Artificial fertilization can use fresh, cooled, or cryopreserved-thawed spermatozoa, whereas genome resource banking requires cryopreservation (Bolton et al., 2022). Previous studies in domesticated species have demonstrated that ART using freshly collected ejaculate (maximum of 5-6 hours following collection) has the highest success rates as spermatozoa are unaffected by cold-shock and have limited exposure to the effects of the *ex vivo* environment (Linde-Forsberg, 1995; Bailey, Bilodeau, and Cormier, 2000). However, resources and animal access do not always facilitate the immediate use of collected

gametes. Development of preservation techniques to facilitate transport and storage of viable gametes is therefore essential to development of ART (Young, Ravida, and Durrant, 2021). Short-term storage is facilitated by refrigeration and extender/holding medium, hereafter extender, which facilitates survival *ex vivo* by providing nutrients to the spermatozoa and protecting them from the effects of cold shock (Bustani and Baiee, 2021). Extenders vary in composition and can include a range of lipoproteins including egg-yolk, bovine sera, and milk, to protect spermatozoa, as well as various antibiotics and nutrient sources (Brinsko et al., 2011). Cooled spermatozoa storage methodologies are variable from species to species, though do not often exceed seven days of storage in agricultural ART (Wiebke et al., 2022). Cooled spermatozoa samples decrease in quality over time; however, better rates of fertilization are achieved with cooled spermatozoa as opposed to thawed-cryopreserved spermatozoa (Linde-Forsberg, 1995; Bailey, Bilodeau, and Cormier, 2000).

Cryopreservation of spermatozoa allow samples to be stored for decades (Bolton et al., 2022). Crucial to long-term applications of ART is the development of methods to cryopreserve viable reproductive materials that can be recovered from genome resource banks when needed (Ballou et al., 2023). In combination with ART, genome resource banking has allowed for unique applications of genetic rescue within efforts to conserve biodiversity (Bolton et al., 2022). However, the process of cooling spermatozoa to  $-196^{\circ}\text{C}$  for cryopreservation places the cells under extreme physiological stress; when water freezes and expands inside the cell, the cell membrane can rupture resulting in death (Ozimic, Ban-Frangez, and Stimpfel, 2023). Cryo-preserved agents are added to spermatozoa samples to prevent this effect; however, in high concentrations they have demonstrated toxic effects on spermatozoa (Ozimic, Ban-Frangez, and Stimpfel, 2023). These effects can be reduced if the spermatozoa extender provides protection from cryo-preserved agent toxicity and can assist with recovery post thawing from cryopreservation (Bustani and Baiee, 2021). Cryo-preserved agents used in a wide range of species include glycerol and dimethyl sulfoxide (Raju et al., 2021).

Research investigating snake spermatozoa morphology to date reveals that the gross morphology and concentrations of spermatozoa in ejaculate are comparable to the related class Aves (birds) (Clulow and Clulow, 2016). The wealth of research applied to the agriculturally significant bird species (e.g. chickens, quail) may provide insights into development of spermatozoa handling and genome resource banking ARTs for snakes. To date, limited research on snake spermatozoa cold storage at 4°C have suggested that samples can remain viable for 48 hours when diluted in extender (Fahrig et al., 2007; Silva et al., 2021). Snake spermatozoa stored in cold storage at 4°C from 48 to 120 hours were not considered viable in previous studies (Fahrig et al., 2007).

Cryopreservation of spermatozoa is being developed for all reptile orders; however, there have been no published attempts to cryopreserve reptile oocytes (Clulow et al., 2022). This could be because the yolk content interferes with cryopreservation, as is seen in chicken (*Gallus gallus*) (Clulow et al., 2022; Golkar-Narenji et al., 2023). In response, cryopreserving primordial germ cells has been suggested as an alternative for squamates (Golkar-Narenji et al., 2023).

I have found nine published accounts of spermatozoa cryopreservation experiments in the suborder Serpentes (Mengden et al., 1980; Mattson et al., 2007; Zacariotti et al., 2011; Young, Ravida, and Durrant, 2021; Young et al., 2021; Sandfoss et al., 2021; Sandfoss et al., 2022; Blank et al., 2022; Sandfoss et al., 2024). To date glycerol and dimethyl sulfoxide along with dimethylacetamide, dimethylformamide, ethylene glycol, and methanol have been investigated as cryo-preserved for snake spermatozoa (Mengden et al., 1980; Mattson et al., 2008; Zacariotti et al., 2011; Young, Ravida, and Durrant, 2021; Young et al., 2021; Sandfoss et al., 2021; Sandfoss et al., 2022; Blank et al., 2022). Results from the published studies show species-specific effects of cryo-preserved agents on cryopreserved spermatozoa; dimethyl sulfoxide, glycerol, and dimethylformamide are recommended most frequently for snakes (Mengden et al., 1980; Mattson et al., 2008; Zacariotti et al., 2011; Young, Ravida, and Durrant, 2021; Young et al., 2021; Sandfoss et al., 2021; Sandfoss et al., 2022; Blank et al., 2022).

#### 1.4 Focal species

My research focuses on refining existing ARTs, adapting those that have been tested on snake species globally for use with endangered Ontario snake species. Among Canadian snake species, gray ratsnake (*Pantherophis spiloides*), eastern massasauga rattlesnake (*Sistrurus catenatus*), queensnake (*Regina septemvittata*) and blue racer (*Coluber constrictor foxii*) have been identified as potentially benefiting from *ex situ* conservation intervention (Environment and Climate Change Canada, 2020; Parks Canada, 2015; Environment Canada, 2015; Winton et al., 2021). Following a meeting of native taxon experts in 2019, the *ex situ* conservation roles of insurance population and reintroduction were only suggested for eastern massasauga rattlesnake (Winton et al., 2021). The use of *ex situ* animals as a source for population reinforcement was further identified as potentially benefitting both the gray ratsnake and eastern massasauga rattlesnake (Winton et al., 2021). While population of the gray ratsnake and eastern massasauga rattlesnake do not currently co-occur in the wild, historical distributions overlapped in Carolinian Canada (COSEWIC 2012; 2018).

The gray ratsnake is a large oviparous colubrid native to North America (COSEWIC, 2018; Figure 1.1). The Canadian population occurs only in Ontario and is grouped by COSEWIC into two Designatable Units with no connectivity between them or population in the United States (COSEWIC, 2018). Designatable Units are a tool for diagnosing the conservation status of population of organisms in Canada and can represent separate groups within a species if they have attributes that make them both discrete and evolutionarily significant (COSEWIC, 2023b). The Carolinian Designatable Unit is classified nationally as Endangered, while the Great Lakes/St. Lawrence Designatable Unit on the Frontenac Arch is classified as Threatened (COSEWIC, 2018). Population declines in both regions have been attributed to habitat destruction and fragmentation, road mortality, and human persecution (COSEWIC, 2018). Recovery strategies for this species in Canada highlight a need to investigate approaches to augmenting the gray ratsnake Carolinian Designatable Unit (Environment and Climate Change Canada, 2020). An *ex situ* population of this species exists across multiple organizations in Southern Ontario, Canada (Winton

et al., 2021), these animals have not been formally managed as a source for translocation but rather are used for education, training, and research (Winton et al., 2021). Studies on the Canadian population reproductive biology in the wild are limited, with most of what is known based on anecdotal observation or through use of molecular genetic methods in the Great Lakes / St. Lawrence Designatable Unit (Blouin-Demers, Prior, and Weatherhead, 2000; 2002; Blouin-Demers, Gibbs, and Weatherhead, 2005).

In Ontario, the northernmost region where gray ratsnake is found, brumation typically occurs between October and April, with animals emerging from hibernacula and dispersing into surrounding habitat in late May (Blouin-Demers, Prior, and Weatherhead, 2002). Gray ratsnake are believed to mate in this region between late-May and mid-June, with females laying their clutches in July and early August (COSEWIC, 2018). Both males and females mate with multiple partners during a single mating season and females can produce clutches with multiple paternity (Kraus et al., 2010). Long-term spermatozoa storage has not been investigated in gray ratsnake. Within their Canadian range, the species is thought to reach sexual maturity between seven and ten years of age (COSEWIC, 2018). Females produce clutches of between eight to 15 eggs every two to three years in the wild (Blouin-Demers, Prior, and Weatherhead, 2000; 2002; Blouin-Demers, Gibbs, and Weatherhead, 2005). Survivorship of young has been difficult to quantify in Ontario due to the secrecy of this species prior to when they join communal hibernaculum as they approach sexual maturity (COSEWIC, 2018).



**Figure 1.1** Adult male gray ratsnake, *Pantherophis spiloides*, from Scales Nature Park © Helen Toner

The eastern massasauga rattlesnake is a small bodied, ovoviviparous viperid native to North America (COSEWIC, 2012; Figure 1.2). Within its Canadian range, which is restricted entirely to Ontario, it is the only remaining venomous snake (COSEWIC, 2012). Timber rattlesnake (*Crotalus horridus*) have not been seen in the province since 1941 and were officially designated by the Committee on the Status of Endangered Wildlife in Canada as extirpated from Canada in May of 2001 (COSEWIC, 2023a). Historically the Canadian eastern massasauga rattlesnake population is thought to have once been continuous from the Great Lakes / St. Lawrence area south through Carolinian Ontario (COSEWIC, 2012). Today the remaining eastern massasauga rattlesnake are restricted to two separate regions with no or little dispersal between the Designatable Units (Parks Canada Agency, 2015). The Carolinian Designatable Unit is classified as federally Endangered, whereas the Great Lakes/St. Lawrence Designatable Unit in the Great Lakes / St. Lawrence area is classified as Threatened (COSEWIC, 2012). Threats to the two designatable units are similar, with both impacted by habitat fragmentation, road mortality and frequent persecution (Parks Canada Agency, 2015). A formal *ex situ* conservation mating program has been established for the species (Winton et al., 2021), with the *ex situ* population being used

to address federal and provincial recovery goals, increasing the genetic resiliency of Canadian population generally, and protecting the species from extirpation in the Carolinian Designatable Unit (Parks Canada Agency, 2015).

Eastern massasauga rattlesnake is at the northern limit of its range in Ontario (COSEWIC, 2012). The species overwinters in hibernacula from October to April (Smolarz et al., 2018). The eastern massasauga rattlesnake reproductive season in Canada is known to be from late July to early September, though mating has been noted in the spring post-brumation (COSEWIC, 2012; S. Marks, personal communication, 2024). In Illinois, USA spermatogenesis occurs from June to September and peaks during late July and early August corresponding to when mating *in situ* has been observed (Aldridge et al., 2008). Testosterone expression in vertebrates is often associated with spermatogenesis; however, the sexual segment of the kidney in eastern massasauga rattlesnake remains hypertrophied throughout the year (Aldridge et al., 2008). Sexual steroid metabolites of eastern massasauga rattlesnake have not been investigated in detail; but enzyme linked immunosorbent assays have been used to detect steroid hormone metabolites of the glucocorticoid corticosterone, using faeces and shed skin samples (Berkvens et al., 2013).

Sexual maturity in eastern massasauga rattlesnake is thought to occur between three to six years of age *in situ* (COSEWIC, 2012). In captivity, females will gestate 65 to 70 days before producing a litter of six to 18 young (AZA Eastern Massasauga Rattlesnake SSP, 2013). In the wild, females are believed to be capable of producing a clutch of offspring every other year if resources are sufficient (COSEWIC, 2012). Eastern massasauga rattlesnake male-male combat behaviour has been observed *in situ* and in artificial environments within the genus (pygmy rattlesnake, *S. miliarius*) (Perelman, Lutterschmidt, and Reinhert, 2022). Long-term spermatozoa storage by females is known in other rattlesnake species, but it is unknown if the eastern massasauga exhibits this trait (Stedman et al., 2016). Whether male eastern massasauga rattlesnake can store viable spermatozoa through brumation in the vas deferens or whether

spring mating produces fertile ova is unknown; however, multiple paternity has been confirmed in wild population (Stedman et al., 2016).



**Figure 1.2** Eastern massasauga rattlesnake, *Sistrurus catenatus*, held in a Canadian conservation mating center. © Ginger Elliott

### *1.5 Thesis aims and objectives*

The overarching goal of my research is to provide some foundational information on the reproductive physiology of two at-risk snake species. I address two hypotheses:

- 1. Difference in life history traits (i.e. ovi- vs ovovivi- parous, post-nuptial vs. pre-nuptial spermatogenesis) and reproductive phenology affects spermatozoa concentration and quality produced in gray ratsnake and massasauga rattlesnake.** I predicted that spermatozoa collected would be of higher concentration and quality during the species mating season. By producing the best spermatozoa during this period, males would optimize their chance of successful fertilization at a time when females are willing to mate. In Ontario, the mating seasons for these two species occur at different times of year; however, both gray

ratsnake and eastern massasauga rattlesnake females become gravid in spring (May or June). As gray ratsnake are seen mating in the wild from late May into June, I predicted that the highest quality and concentration spermatozoa for this species would be collected between Julian dates 134 to 181. Eastern massasauga rattlesnake are seen mating in the wild from late July through August; thus, I also predicted the highest quality and concentration spermatozoa for this species would be collected between Julian dates 195 to 243.

- 2. Circulating reproductive hormones vary throughout the year in a pattern that correlates with gametogenesis.** I predicted that male faecal testosterone metabolite concentration of eastern massasauga rattlesnake would be highest during the mating season, late July-early August (Julian dates 195 to 243), which should correspond to the peak in spermatogenesis. Second, as progesterone is an essential hormone involved in maintaining gestation of vertebrate species, I predicted females faecal progesterone metabolite concentration of eastern massasauga rattlesnake would increase in May (Julian dates 121 to 151) and June (Julian dates 152 to 181) during vitellogenesis, be expressed in the highest concentrations annually from May to August (Julian dates 121 to 243) and remain at high concentration through to parturition in gravid females.

My work contributes to the growing field of squamate ART and genome resource banking in efforts to preserve global biodiversity. Development of ART should focus on endangered species, in which application of the technology would help to slow and eventually reverse the extinction vortex (Herrick, 2019). My research provides insights on the variation in physiology associated with reproductive seasonality for gray ratsnake and eastern massasauga rattlesnake at the northern edge of their range. Further, my research facilitates broader understanding of how ART methodologies transfer across taxa. By integrating the results of our research into *ex situ* management, managers may be able to improve species-specific husbandry, and thus increase our ability to preserve genetic diversity, and fecundity of managed population.

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## **Chapter 2:**

**Do divergent life history traits predict spermatozoa concentration and quality in gray ratsnake and eastern massasauga rattlesnake?**

## 2.1 Abstract

Assisted reproductive technologies (ART) have proven key to propagation of domesticated animals for agriculture and pet trade. When used to help manage *ex situ* population of species-at-risk, ART lead to increased fecundity and have helped to better manage genetic diversity. ART can include assisted insemination, *in vitro* fertilization, and gamete preservation among other technologies. The use of ART in squamates to date is limited, partly because of insufficient knowledge of species-specific reproductive function. We hypothesized that difference in life history traits (ovi- vs ovovivi- parous, post-nuptial vs. pre-nuptial spermatogenesis) and reproductive phenology will affect spermatozoa concentration and quality over the active season for both gray ratsnake and eastern massasauga rattlesnake, with higher concentration and quality during the mating season of each species. We collected spermatozoa samples from gray ratsnake and eastern massasauga rattlesnake monthly from individuals in captive collections. We assessed spermatozoa concentration, percent motility, rapid forward progressive motility, and membrane integrity. We found that both gray ratsnake and eastern massasauga rattlesnake exhibit species specific annual variation in quality but not concentration of spermatozoa, but this did not coincide with patterns expected from wild population. Gray ratsnake samples did not vary in concentration through the active season and were most likely to contain high quality spermatozoa well after spermatogenesis, during the month of October. Eastern massasauga rattlesnake samples did not vary in concentration through the active season and were most likely to contain high quality spermatozoa well after spermatogenesis, during the month of October. Our results will facilitate development of more complex ART and generally expand our understanding of the reproductive biology of gray ratsnake and eastern massasauga rattlesnake at their northern range limits.

## 2.2 Introduction

As biodiversity declines around the globe, new strategies are being incorporated into species recovery plans nationally and internationally (McGowan et al., 2017). *Ex situ* programs, in which species are held in accredited zoos, aquariums, and conservation partners for initiatives such as public education, reintroduction, and reinforcement programs, are increasingly common (McGowan et al., 2017). Today, zoos and aquariums are tasked with maintaining healthy, sustainable *ex situ* population and in many cases, use these animals in managed mating programs to support *in situ* population recovery (Herrick, 2019). Unfortunately, not all species held *ex situ* breed readily when introduced to an appropriate conspecific (Gray et al., 2022). For many animals, facilitating natural mating *ex situ* can pose challenges when species-specific reproductive knowledge is limited (Bolton et al., 2022). Assisted reproductive technologies (ART) developed for use in domesticated species to improve reproductive success have been modified and optimized for use on a variety endangered species (Biasetti et al., 2022; Bolton et al., 2022). Detailed knowledge of species-specific reproductive annual variation is needed to inform efforts to develop and refine ART for *ex situ* conservation population (Vieira et al., 2024).

Maintenance of reptiles *ex situ* is common around the world. For example, the *ex situ* maintenance of many viperid species is crucial to production of medicines (e.g. anti-coagulants and blood thinners) and anti-venoms for human health care (Vieira et al., 2024). Yet while reptiles represent 16% of all globally threatened species currently assessed, ART for reptiles are significantly less developed than they are for more charismatic taxa (Clulow et al., 2022; IUCN, 2024). Within squamates, ART research has begun on a variety of oviparous and ovoviviparous species (Clulow et al., 2022; Sandfoss et al., 2024). Spermatozoa from snakes have been collected using a minimally invasive technique developed by Mengden, et al., (1980), which has been successfully replicated numerous times across a range of snake genera with slight modification (Mattson et al., 2007; Oliveri et al., 2018; Coeti et al., 2020; Sandfoss et al., 2021). However, to develop complex ARTs such as living cell cryopreservation for genome resource banking, gamete collection and handling techniques must first be refined for each species (Herrick, 2019).

Seasonality in reproduction occurs across animal and plant taxa, being most pronounced in regions that exhibit strong seasonality (Brown and Shine, 2006). Though patterns of reptilian reproduction are highly variable across the globe, preliminary studies have shown that spermatogenesis and fecundity in reptiles are a seasonally dependant physiological process (Moss and MacLeod, 2022; Hubert, Bentz, and Mason, 2023). Further, squamates have evolved a variety of reproductive strategies to compensate for short active seasons that necessarily occur for population in northern regions, including ovoviviparous and oviparous parturition, and reproductive quiescence (Aldridge et al., 2020).

The eastern massasauga rattlesnake (*Sistrurus catenatus*) and gray ratsnake (*Pantherophis spiloides*) both have ranges that encompass the eastern United States and Canada (COSEWIC, 2012; COSEWIC, 2018). In Canada, both species are found only in Ontario, and each has been subdivided into two Designatable Units (COSEWIC, 2012; COSEWIC, 2018). Designatable Units are a tool for diagnosing the conservation status of population of organisms in Canada and can represent separate groups within a species if they have attributes that make them both discrete and evolutionarily significant (COSEWIC, 2023). The eastern massasauga rattlesnake is an ovoviviparous species that brumate annually and typically mates in the late summer and early fall (COSEWIC, 2012); although mating has been seen in the spring *in situ* (S. Marks, personal communication, 2024). Previous studies of this species indicate that males display prenuptial spermatogenesis, commencing in July prior to peak mating behaviour in August (Aldridge et al., 2008). It is uncertain whether male eastern massasauga rattlesnake can store viable spermatozoa through brumation in the vas deferens as occurs in other North American vipers (Lind, Talyor, and DeNardo, 2024) or whether spring mating produces fertile ova. Gray ratsnake employ a different set of reproductive tactics, including oviparity (COSEWIC, 2018). In their Ontario range, the gray ratsnake brumate annually and mate in the spring, from late May into early June (COSEWIC, 2018). A review of North American snakes found most colubrid species undertake spermatogenesis in the summer (Aldridge et al., 2009). Thereafter colubrid males could store viable spermatozoa in the vas

deferens until the following spring mating season (Aldridge et al., 2009); an example of post-nuptial spermatogenesis (Lind, Talyor, and DeNardo, 2024).

Our research objectives were to take the first steps towards development of ART for these two endangered Ontario snake species. We hypothesized that different life history traits (oviparous vs ovoviviparous, post-nuptial vs. pre-nuptial spermatogenesis) affects annual variation of spermatozoa concentration and quality in gray ratsnake and eastern massasauga rattlesnake. We predicted that spermatozoa collected from both species would have highest concentration and quality during the respective mating seasons of wild gray ratsnake and eastern massasauga rattlesnake in Canada. Producing the best quality spermatozoa in the highest concentrations during the peak mating period would allow males to optimize their chance to fertilize ova at a time when females are willing to mate. In Ontario, the mating seasons for these two species occur at different times of year; however, both gray ratsnake and eastern massasauga rattlesnake females become gravid in early spring (May or June). Gray ratsnake breed in the wild from late May into June; therefore, we predicted the highest quality and concentration spermatozoa for this species would be collected between the 134 to 181 days of the year (COSEWIC, 2018). Eastern massasauga rattlesnake mates in the wild from late July through August; thus, we predicted the highest quality and concentration spermatozoa for this species would be collected between the 195 to 243 days of the year (COSEWIC, 2012).

## 2.3 Methods

### 2.3.1 Sample collection

All research was conducted according to approved Queen's University Animal Care Protocol #2021-2137 and Toronto Zoo, Permit 2024-02-01. A summary of the animals used for this study are noted in Table 2.1. The *ex situ* population of eastern massasauga rattlesnake used for this study is being formally managed for reinforcement and reintroduction of the species within Ontario, Canada under a Species Survival Plan (AZA Eastern Massasauga Rattlesnake SSP, 2013; Ontario Ministry of Natural Resources

and Forestry, 2016). The gray ratsnake used in the study are involved in *ex situ* programs for education, training, and research, but do not comprise a single, formally managed population (Environment and Climate Change Canada, 2020). Animals from *ex situ* population that were sampled for this study include those found at Scales Nature Park, Barrie, Ontario, Canada; Little Ray’s Nature Center, Hamilton, Ontario Canada; Scienstational Ssnakes!!!, Guelph, Ontario, Canada, and the Toronto Zoo, Toronto, Ontario, Canada. All animals are maintained with ad libitum water. At Scales Nature Park and Toronto Zoo, rattlesnakes are housed separately unless paired for mating. At Scales Nature Park, ratsnakes are held individually, in male:male, and in male:female groups. At Little Rays Nature Center, animals are housed individually. At SS, animals are housed in sex specific groups.

**Table 2.1** Summary of study animals, including sample sizes, age range, and morphometrics, from which spermatozoa were collected and assessed.

Location	Common Name	Number of individuals	Age Range	Snout-vent length (range/mean)	Weight (range/mean)
Scales Nature Park	Eastern massasauga rattlesnake	11	3-4 years	53cm-79cm / 62cm	203g-362g / 278g
Toronto Zoo	Eastern massasauga rattlesnake	10	2-10 years	59cm-76cm / 67cm	236g-496g / 441g
Scales Nature Park	Gray ratsnake	3	8-12 years	106cm-153cm / 130cm	613g-814g / 736g
Scienstational Ssnakes!!!	Gray ratsnake	7	3-16 years	80cm-177cm / 130cm	303g-1847g / 1050g
Little Rays Nature Center	Gray ratsnake	8	5-6 years	102cm-170cm / 139cm	456g-1725g / 1161g

Spermatozoa collection occurred once per month throughout the snakes’ wild counterparts Canadian active season, following and prior to brumation (May to October). Prior to spermatozoa collection, male skin and scale condition were assessed. Animals that were nearing shed, or that had shed recently, were not sampled to avoid damaging scales and skin. Mass (grams) and snout-vent-length (centimeters) measurements, room and cloacal temperature (Celsius) were recorded.

We used the ventral/caudal massage developed by Mengden et al. (1980). The technique requires the researcher to repeatedly ventro-laterally stroke the musculature of the caudal third of the body in a downward motion from head to tail and massage of the region of the tail containing hemipenes (Mengden et al., 1980). Any urates, faeces, and musk produced were rinsed off with phosphate buffered saline solution. The sample was then collected from the cloaca using a micropipette (Coeti et al., 2020). In this study, animals were manipulated for a maximum of ten minutes or until ejaculate was collected. If no ejaculate was collected after ten minutes the collection attempt was considered a failed attempt and the animal returned to its respective housing.

The change in temperature spermatozoa samples were exposed to when collected from the cloaca was determined by subtracting the room temperature at the time of collection from cloacal temperature prior to spermatozoa collection. Body condition indices were calculated for every individual at each collection event following previously published methods for snakes (Dillon et al., 2024; Weatherhead and Brown, 1996).

### 2.3.2 *Sample assessment*

All analysis was performed immediately following sample collection. Ejaculate total volume was estimated via micropipette. As snake ejaculate is highly concentrated, proper assessment requires some initial manipulation of raw samples (Brito et al., 2016; Blank et al., 2022). In brief, spermatozoa samples were divided into aliquots and diluted in one of three extenders or a control solution (Table 2.2). The extenders TL HEPES (Agtech, Inc, Kansas, USA) with 10% fetal bovine sera (ThermoFisher Scientific) and TES-TRIS Yolk Buffer (Irvine Scientific CA USA) were chosen based of a review of the existing related literature (Sandfoss et al., 2021; 2022; Young et al., 2021), whereas INRA-96 (IMV Technologies, East Gen, Guelph ON Canada), was chosen based on discussion with experts (Tullis Matson, personal communication, 2022). Assessments of spermatozoa were completed using a SWIFT SW380T compound

microscope at magnifications of x250, x400, and x1000 (Swift Microscopes, Online Science and Technology Co., Ltd., Fujian, China). Sperm motility is an indicator of fertilization potential for artificial insemination of livestock (Waberski, Suarez, and Henning, 2022). Motility assessment followed the methods for livestock, estimated by calculating the percent of motile versus non-motile spermatozoa cells among a total of 100 total cells (Fahrig et al., 2007). Membrane integrity, hereafter viability of samples was assessed using an eosin-nigrosine live-dead stain (Jorvet Stain, Jorgensen Laboratories, Inc., Loveland, CO, USA) and calculated as the percentage of live versus dead spermatozoa out of 100 cells (Sandfoss et al., 2022). Dead cells were identified as those where the head became stained, or possessed gross morphology defects, such as tail deformities (Blank et al., 2022). We determined concentration of spermatozoa in the sample using a Neubauer hemocytometer (Hausser Scientific, Horsham, PA, USA) and standard methods (World Health Organization, 2021). Rapid forward progressive motility (RFP) was determined by a single observer, scoring the direction and progress of cell movement (Silva et al., 2015; Blank et al., 2022; Sandfoss et al., 2022). We assigned scores of 1 to 5, where 1 denoted high levels of progressively motile spermatozoa and 5 denoted the lowest levels of progressively motile spermatozoa (Blank et al., 2022; Table 2.3).

**Table 2.2** Spermatozoa extender mediums used in the study and their associated base.

<b>Extender</b>	<b>Base</b>
Phosphate Buffered Saline ph 7.4 (ThermoFisher Scientific)	Control
INRA-96 (IMV Technologies, Spermex, Guelph ON Canada)	Milk-base
TEST Yolk Buffer (Irvine Scientific CA USA)	Yolk-base
HEPES with 10% fetal bovine sera (ThermoFisher Scientific)	Sera-base

**Table 2.3** Rapid forward progressive motility scoring chart.

<b>Spermatozoa progressive movement discretion</b>	<b>Score</b>
Rapid forward linear movement of spermatozoa	1
Forward linear movement of spermatozoa	2
Forward movement of spermatozoa	3
Spermatozoa moves in place	4
Spermatozoa exhibits movement infrequently	5
No movement	NA

### 2.3.3 *Brumation effect on spermatozoa viability.*

In 2024, brumation trials of *ex situ* snakes were undertaken to assess potential effects of physiological cooling on spermatogenesis. The brumation protocols were developed via discussion with species experts and veterinary staff at partner facilities. Brumation of eastern massasauga rattlesnake took place at Toronto Zoo and at Scales Nature Park, while brumation of gray ratsnake occurred at Scales Nature Park only (Table 2.4). Non-brumated gray ratsnake environmental maintenance did not change throughout the year. Non-brumated eastern massasauga rattlesnake stayed in their captive habitats, but the environment was gradually cooled, and daily lighting ('day length') was decreased to simulate the photoperiod they would be subjected to in Southern Georgian Bay as per staff and facility capability. During the months of December-April, Toronto Zoo animal holdings are cooled to 18-20°C and Scales Nature Park holdings to 20-22°C. The animals were offered food biweekly and provided with access to a heat lamp during the daylight hours.

#### 2.3.3.1 *Eastern massasauga rattlesnake*

Snakes selected for brumation were not fed for three weeks prior to cooling. Temp Sticks (Ideal Sciences, Bountiful, UT) were used to monitor humidity and temperature in each location throughout the brumation period. All animals were housed individually. Health assessments to determine whether each male was fit to enter brumation consisted of a visual assessment (body condition: muscle and fat distribution), righting test and respiratory assessment (no bubbles, audible wheezing or whistling). Brumation holding and lighting was changed on the day animals started cooling; lighting was decreased by one hour every two days over a period of six days, then decreased one hour per day until total darkness was achieved. Temperature control varied by facility. Toronto Zoo used an unheated outbuilding that would cool to 6-10°C over the brumation period. Scales Nature Park used a lockable refrigeration unit (272 Bottle Single Zone Black Full Glass Door Wine Refrigerator, Eurodib, NY, USA) and temperature was decreased by 2°C every other day until animals could be maintained between 6-10°C during brumation. Near the end of the over-winter period, the ambient temperature for snakes in brumation gradually increased. At Toronto Zoo, warming coincided with the natural spring temperature increase and at Scales Nature Park, animals were warmed 1°C per day to 10°C, then 2°C per day to 20°C. Lighting for all animals at Toronto Zoo and Scales Nature Park was increased by one hour per day for six days and then two hours per day for three days. Throughout the brumation period eastern massasauga rattlesnake were visually assessed whenever possible to monitor health status.

#### 2.3.3.2 *Gray ratsnake*

Gray ratsnake selected for brumation were not fed for three weeks prior to cooling. Healthy individuals were transferred to brumation holding and housed in a single group for the brumation period. Males were offered water during the brumation period and housed on newspaper. All gray ratsnake were submitted to

hands-on health checks monthly during brumation. The brumation holding area for gray ratsnake consisted of an unheated room with no lighting, but temperature was never allowed to drop below 6°C.

**Table 2.4** Summary of brumation study animals, location, and duration of brumation trials.

<b>Species</b>	<b>Location</b>	<b>Duration</b>	<b>n</b>
Eastern massasauga rattlesnake	Toronto Zoo	January to March	4
Eastern massasauga rattlesnake	Scales Nature Park	March	5
Gray ratsnake	Scales Nature Park	December to March	2

#### 2.3.4 Statistical Analysis

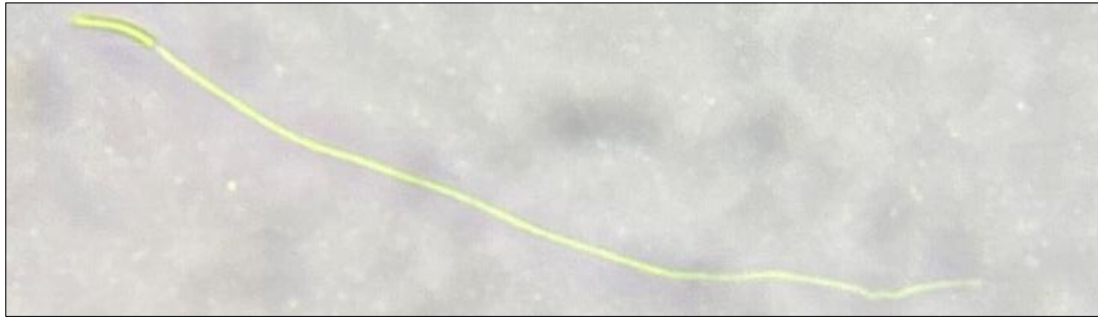
Data analyses were conducted in R (version 4.2.2, R Core Team 2022). The workspace in R was formatted using the “tidyverse” (Wickham et al., 2019) R package. We tested for associations between variables (motility, concentration, RFP, viability) using Pearson correlations. Analysis was conducted separately for each species. A linear regression of body mass (grams) against snout-vent-length (centimeters) was calculated using the data collected throughout the project, with residuals for each snake at each sampling point was used as a proxy of its body condition (Weatherhead and Brown, 1996). Residuals were added to the data set using the “modelr” (v0.1.11, Wickham, 2023) R package. Outliers were excluded using the 1.5 interquartile range method, where upper outliers were identified as anything lying above the *Interquartile range + 1.5 \* Quartile 3* and lower outliers were calculated as anything below the *Interquartile range - 1.5 \* Quartile 1*.

We created generalized linear mixed models to test for effects of Julian date (DOY) on seminal parameters (concentration, motility, RFP, and viability) using the “lme4” (Bates et al., 2015) R package

with the “glmmTMB” R package applied to account for mixed beta regression models (Brooks et al., 2017). Fixed effects investigated for both gray ratsnake and eastern massasauga rattlesnake included DOY, age, body condition index (body condition index), brumation, extender media, and the difference in temperature between room and cloaca (d.t.). Mixed models allow for the introduction of random effects into the models which assist the model in accounting for inference about the fixed effects (Harrison et al. 2018). Mixed models were used to include random effects of individual animals and their relative husbandry (location) (Zuur et al., 2009). Using the R package “MuMIn” (v1.47.5; Bartoń, 2023), we used the dredge function to test for significant fixed effects on each male spermatozoa characteristic using generalized linear regression mixed models (Sandfoss, Reichling, and Roberts, 2023). We employed AIC<sub>c</sub> model selection, identifying all best model(s) as those with the lowest AIC<sub>c</sub> value and models lying within  $\Delta 2$  AIC<sub>c</sub> of it (Burnham and Anderson, 2002). Plotting was assisted by the “ggpubr” R package (v0.6.0; Kassambara, 2023).

#### *2.4 Results*

We found no correlation between concentration of samples and motility, viability nor RFP measures. We found a moderate negative correlation between the rapid forward progression of samples and percent motility ( $r=-0.57$ ,  $p = < 2.2e^{-16}$ , CI = -0.64 to -0.49). Research to date suggests that the gross morphology of spermatozoa is conserved among squamate species (Oliver, Jamieson, and Scheltinga, 1996). Our results support this hypothesis, under magnification we found both gray ratsnake and eastern massasauga rattlesnake spermatozoan were filiform in shape, with identifiable head pieces but little differentiation between tail and midpiece preventing clear identification (Figure 2.1).



**Figure 2.1** Gray ratsnake spermatozoan in phosphate buffered saline photographed under x400 magnification compound light microscopy.

The data set included 120 eastern massasauga rattlesnake samples and 438 gray ratsnake samples collected over 279 sampling events performed on 39 snakes in four locations across Southern Ontario (Table 2.1). Only one gray ratsnake male was sampled every month of the study in both 2023 and 2024. The average number of collection procedures performed on any male in the study was seven (range = 3 to 12). The average temperature at time of collections was  $24.88^{\circ}\text{C} \pm 0.99$  SE (range =  $18.7^{\circ}\text{C}$  to  $32.1^{\circ}\text{C}$ ).

#### 2.4.1 *Gray ratsnake*

Sampled gray ratsnake ( $n=18$ ) ranged in SVL from 80 to 177 cm (mean =  $134.4 \pm 0.4$  SE) and in mass from 303 to 1847g (mean =  $1007.3 \pm 8.1$  SE) (Table 2.1). Body mass index for gray ratsnake ranged from -1.13 to 0.74 (mean =  $4.23 \times 10^{15} \pm 0.01$  SE). One gray ratsnake was removed from the study after its first spermatozoa collection procedure due to congenital morphological abnormality that interfered with the manual collection. The average cloacal temperature of gray ratsnake was  $26.83^{\circ}\text{C} \pm 0.10$  SE (range =  $20.5^{\circ}\text{C}$  to  $32.1^{\circ}\text{C}$ ). We were successful in obtaining spermatozoa for visualization via microscopy 79% of the time ( $N = 162$ ). Sample volume ranged from 0-60 $\mu\text{L}$ , with on average, 15 $\mu\text{L}$  ( $\pm 1$  SE) available for assessment. The average concentration of spermatozoa samples collected from gray ratsnake was  $1.81 \times 10^4$  cells/ $\mu\text{L}$  ( $\pm 0.89 \times 10^4$  SE) spermatozoa per microliter of ejaculate. gray ratsnake spermatozoa

collected had an average motility of 78.12% ( $\pm$  1.18 SE) and average membrane integrity of 53.95% ( $\pm$  1.71 SE).

AIC<sub>c</sub> selection identified four top models for gray ratsnake motility (Appendix A). Age of the animals and temperature change were included in all four top models (Appendix A). The effect of brumation was included in three of four top models, while the effects of extender were included in three of four top models, and the effects of body condition index in one of four top models (Appendix A). The model with the lowest AIC<sub>c</sub> (-289.5), hereafter the best model, was a beta mixed model with the random effects of animal ID and location as well as the fixed effects donor age, extender media, brumation, and temperature change (Table 2.5). The best model predicts lower motility ejaculate to be collected from donors hatched in 2014 (-1.98,  $p = 3.28 \times 10^{-4}$ ) and when samples experience larger temperature changes upon collection (-0.13,  $p = 9.30 \times 10^{-4}$ ; Table 2.5). Further, the best model predicts gray ratsnake spermatozoa can be collected with at least 60% motility throughout the active season with the likelihood of collecting samples with at least 70% motility in October (DOYs 274 to 304; Figure 2.2A).

**Table 2.5** Beta model results, including fixed and random effects, best predicting spermatozoa motility in gray ratsnake. The model predicts lower motility ejaculate to be collected from donors hatched in 2014 (-1.98,  $p = 3.28 \times 10^{-4}$ ) and when samples experience temperature change upon collection (-0.13,  $p = 9.30 \times 10^{-4}$ ). One asterisk indicates that the p-value is less than 0.05 and three indicate the p-value is less than 0.0001.

Term	Estimate	Std. Error	z value	P value	Variance	Std. Deviation
Random effects						
Animal					$6.12 \times 10^{-10}$	$2.49 \times 10^{-5}$
Location					$1.78 \times 10^{-13}$	$4.22 \times 10^{-7}$
Fixed effects						
(Intercept)	0.45	0.47	0.95	0.34		
Hatch 2011	-0.11	0.52	-0.20	0.84		
Hatch 2012	0.45	0.47	0.96	0.34		
Hatch 2014	-1.98	0.55	-3.59	$3.28 \times 10^{-4}$ ***		
Hatch 2015	-0.46	0.44	-1.05	0.30		
Hatch 2017	-0.60	0.52	-1.14	0.26		
Hatch 2018	0.07	0.41	0.18	0.86		
Hatch 2020	0.41	0.46	0.91	0.36		
Hatch unknown	0.49	0.47	1.06	0.29		
Brumated 2024	-0.48	0.27	-1.75	0.08		
Temp. change	-0.13	0.04	-3.31	$9.30 \times 10^{-4}$ ***		
HEPES	0.78	0.37	2.10	0.04 *		
INRA	0.87	0.42	2.10	0.04 *		
PBS	0.50	0.36	1.37	0.17		
TEST	1.06	0.43	2.46	0.01 *		

AIC<sub>c</sub> selection identified two top models for gray ratsnake spermatozoa viability (Appendix A). Age was difficult to include in the models due to its near-zero variance in random effect levels, which led to singular fits. We thus removed age from the model along with the interaction of age and brumation. The effects of DOY, brumation, and temperature change were included in both top models (Appendix A). The effect of body condition index was included in one of the two top models (Appendix A). The best model is a beta mixed model with the random effects of animal ID and location and fixed effects DOY, brumation, and temperature change (Table 2.6). Significant effects on the model predictions were exhibited by DOY (-0.13,  $p = 8.24 \times 10^{-5}$ ) and temperature change (0.01,  $p = 2 \times 10^{-16}$ ) experienced by spermatozoa, with larger temperature fluctuations resulting in lower percent viable samples (Table 2.6). Animals that were brumated in the previous winter season had improved spermatozoa percent viability (1.01,  $p = 8.24 \times 10^{-5}$ ; Table 2.6). The best model predicts that gray ratsnake spermatozoa viability increases from May through to July before plateauing in August (Figure 2.2B). The likelihood of collecting a sample with a percent viability above 50% is maintained through August, after which the model indicates spermatozoa viability will increase through September and October (Figure 2.2B).

**Table 2.6** Beta model results, including fixed and random effects used to predict spermatozoa viability in gray ratsnake. Significant effects on the model predictions were exhibited by DOY ( $-0.13$ ,  $p = 8.24 \times 10^{-5}$ ), temperature change ( $0.01$ ,  $p = 2 \times 10^{-16}$ ) and in animals that were brumated in the previous winter season ( $1.01$ ,  $p = 8.24 \times 10^{-5}$ ). Three asterisks indicates that the p-value is less than 0.0001.

Term	Estimate	Std. Error	z value	P value	Variance	Std. Deviation
Random effects						
Animal					$9.54 \times 10^{-2}$	0.31
Location					0.21	0.46
Fixed effects						
(Intercept)	-3.09	0.50	-6.16	$7.43 \times 10^{-10}$ ***		
Brumated 2024	1.01	0.26	3.94	$8.24 \times 10^{-5}$ ***		
DOY	-0.13	0.04	-3.39	$6.93 \times 10^{-4}$ ***		
Temp. change	0.01	$1.65 \times 10^{-3}$	8.59	$< 2 \times 10^{-16}$ ***		

AIC<sub>c</sub> selection identified six top models for gray ratsnake concentration (Appendix A). The effects of DOY and extender were included in all six top models (Appendix A). The effect of brumation was included in three of six top models, the effect of temperature change was included in two of six top models, and the effect of age in two of six top models (Appendix A). The best model, with the lowest AIC<sub>c</sub> (6059.9), indicates a gamma log linked mixed model with the random effects of animal ID and location and fixed variables DOY, temperature change, and extender, effecting the concentration of gray ratsnake spermatozoa (Table 2.7). Only the effects of body condition were excluded from the other top model. DOY significantly affected concentration of spermatozoa ( $5.18 \times 10^{-3}$ ,  $p = 2.65 \times 10^{-4}$ ) collected from gray ratsnake (Table 2.7). The best model predicts extenders TEST ( $-1.29$ ,  $p = 7.78 \times 10^{-4}$ ) and INRA-96 ( $-1.11$ ,  $p = 2.53 \times 10^{-3}$ ) significantly impact concentration (Table 2.7). The model indicates that the concentration of spermatozoa from gray ratsnake increased throughout the collection period (Figure 2.2C). Of the four sperm characteristics measured, the best model for concentration is the least certain in it's predictions indicated by the large standard error (Figure 2.2C)

**Table 2.7** Gamma model results, including fixed and random effects used to predict spermatozoa concentration gray ratsnake. The best model predicts DOY ( $5.18 \times 10^{-3}$ ,  $p = 2.65 \times 10^{-4}$ ) and the extender medias TEST ( $-1.29$ ,  $p = 7.78 \times 10^{-4}$ ) and INRA-96 ( $-1.11$ ,  $p = 2.53 \times 10^{-3}$ ) significantly impact concentration. Two asterisks indicate the p-value is less than 0.001 and three indicate the p-value is less than 0.0001.

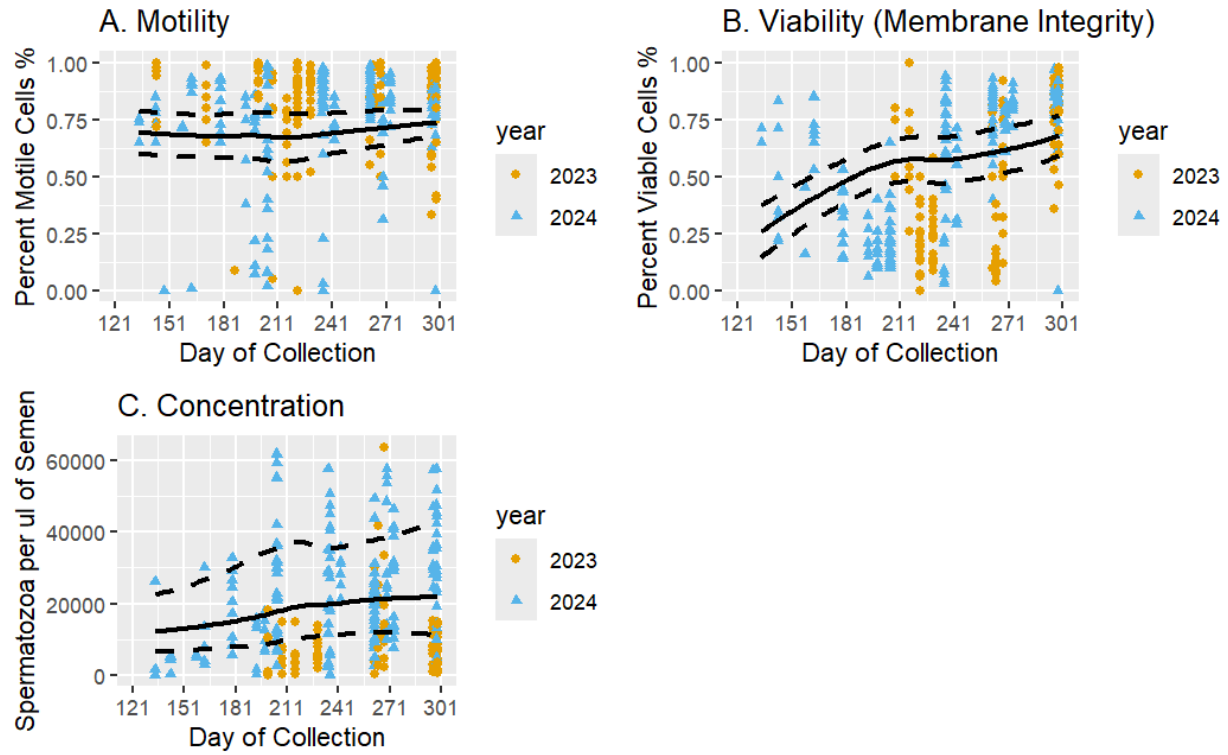
Term	Estimate	Std. Error	T value	P value	Variance	Std. Deviation
Random effects						
Animal					$1.81 \times 10^{-2}$	0.13
Location					$2.63 \times 10^{-8}$	$1.62 \times 10^{-4}$
Residual					$5.80 \times 10^{-1}$	0.76
Fixed effects						
(Intercept)	8.90	0.44	20.04	$< 2 \times 10^{-16}$ ***		
Temp. change	0.06	0.04	1.66	0.10		
DOY	$5.18 \times 10^{-3}$	$1.42 \times 10^{-3}$	3.65	$2.65 \times 10^{-4}$ ***		
HEPES	-0.48	0.32	-1.53	0.13		
INRA	-1.11	0.37	-3.02	$2.53 \times 10^{-3}$ **		
PBS	-0.43	0.31	-1.38	0.17		
TEST	-1.29	0.38	-3.36	$7.78 \times 10^{-4}$ ***		

AIC<sub>c</sub> selection identified four top models for gray ratsnake RFP (Appendix A). The effects of DOY and extender were included in all four top models (Appendix A). The effect of body condition index was included in three of four top models and the effect of temperature change was included in three of four top models (Appendix A). The effect of brumation were only included in one of four top models (Appendix A). The best model was a gamma log linked mixed model with the random effects of animal ID and location and fixed effects including DOY, body condition index, temperature change and the extender media (AIC<sub>c</sub> = 590.8; Table 2.8). The extender media PBS ( $0.44$ ,  $p = 3.86 \times 10^{-3}$ ) and DOY (-

$2.17 \times 10^{-3}$ ,  $p = 2.94 \times 10^{-5}$ ) significantly affected RFP collected from gray ratsnake (Table 2.8). When scoring RFP motility, gray ratsnake spermatozoa samples were classified as “1” in 62.79% of assessments; “2” in 16.70% of assessments; “3” in 7.80% of assessments; “4”, in 2.70% of assessments; “5” in 0.90% of assessments; and “NA” in 9.13% of assessments.

**Table 2.8** Gamma model results, including fixed and random effects used to predict spermatozoa rapid forward progressive motility gray ratsnake. The extender media PBS ( $0.44$ ,  $p = 3.86 \times 10^{-3}$ ) and DOY ( $-2.17 \times 10^{-3}$ ,  $p = 2.94 \times 10^{-5}$ ) significantly affected RFP collected from gray ratsnake. Two asterisks indicates that the p-value is less than 0.001 and three indicate the p-value is less than 0.0001.

Term	Estimate	Std. Error	T value	P value	Variance	Std. Deviation
Random effects						
Animal					$9.93 \times 10^{-3}$	$9.97 \times 10^{-2}$
Location					$3.46 \times 10^{-3}$	0.06
Residual					0.22	0.47
Fixed effects						
(Intercept)	0.60	0.20	3.03	$2.43 \times 10^{-3}$ **		
Body condition index	0.27	0.14	1.91	0.06		
Temp. change	0.03	0.01	1.88	0.06		
DOY	$-2.17 \times 10^{-3}$	$5.20 \times 10^{-4}$	-4.12	$2.94 \times 10^{-5}$ ***		
HEPES	0.05	0.15	0.31	0.75		
INRA	0.32	0.17	1.95	0.05		
PBS	0.44	0.15	2.89	$3.86 \times 10^{-3}$ **		
TEST	-0.04	0.17	-0.26	0.80		



**Figure 2.2** Gray ratsnake spermatozoa concentration and quality measurements with predictive models represented by the solid line and associated standard error represented by the dashed lines. **A.** Percent motile spermatozoa. The best model predicts spermatozoa can be collected with at least 60% motility throughout the active season with the likelihood of collecting samples with at least 70% motility in October (DOYs 274 to 304) **B.** Percent viable spermatozoa. The best model predicts the highest percent viable spermatozoa will be collected in October (DOYs 274 to 304) and the lowest in May (DOYs 121 to 151). **C.** Concentration spermatozoa in a microliter of ejaculate. The model indicates that the concentration of spermatozoa from gray ratsnake increased throughout the collection period, predicting the highest concentration samples will be collected in October.

#### 2.4.2 *Eastern massasauga rattlesnake*

We collected samples from 21 eastern massasauga rattlesnake, ranging in SVL from 53 to 79 cm (mean =  $61.8 \pm 0.4$  SE) and mass from 203 to 496g (mean =  $287 \pm 4.2$  SE) (Table 2.1). Body mass index for eastern massasauga rattlesnake ranged from -0.48 to 0.39 (mean =  $6.63 \times 10^{15} \pm 0.02$  SE). The average cloacal temperature of eastern massasauga rattlesnake was  $28.06^\circ\text{C} \pm 0.18$  SE (range =  $22.6^\circ\text{C}$  to  $31.1^\circ\text{C}$ ). One eastern massasauga rattlesnake was removed from the study prior to the second collection attempt as decreased mobility was observed during the restraint process, which was later diagnosed as spinal lesions due to salmonella.

Spermatozoa collection with eastern massasauga rattlesnake were successful in obtaining spermatozoa for visualization via microscopy 45% of the time (N = 117 sampling events). Total volume collected ranged from 0-60  $\mu\text{L}$  (average =  $10 \mu\text{L} \pm 0.77$  SE). The average concentration of spermatozoa collected from eastern massasauga rattlesnake was  $64.06 (\pm 8.32)$  SE) spermatozoa per microliter of ejaculate. Collected eastern massasauga rattlesnake spermatozoa had an average motility of  $46.50\% (\pm 4.92)$  SE) and average membrane integrity of  $32.42\% (\pm 5.33)$  SE).

AIC<sub>c</sub> selection identified three top models for eastern massasauga rattlesnake motility (Appendix A). Both age and body condition index were each included in one of three top models (Appendix A). The best model with the lowest AIC<sub>c</sub> (-556.9) is a beta mixed model with the random effects of animal ID and location as well as the fixed effects of DOY and temperature change (Table 2.9). As predicted, the best model predicts DOY significantly affects motility of samples ( $0.01$ ,  $p = 0.02$ ; Table 2.9); however not in the predicted pattern (Figure 2.3A). The model rather indicates eastern massasauga rattlesnake spermatozoa with the highest percent motility were collected in October (DOYs 274 to 304; Figure 2.3A). Further, the best model predicts that samples which have experienced larger temperature changes will have lower motility ( $-0.27$ ,  $p = 0.01$ ; Table 2.9).

**Table 2.9** Beta model results, including fixed and random effects used to predict spermatozoa motility eastern massasauga rattlesnake. DOY (0.01,  $p = 0.02$ ) and temperature change (-0.27,  $p = 0.01$ ) are predicted to influence sample motility. One asterisk indicates that the p-value is less than 0.05 and two indicate the p-value is less than 0.001.

Term	Estimate	Std. Error	z value	P value	Variance	Std. Deviation
Random effects						
Animal					$5.56 \times 10^{-9}$	$8.46 \times 10^{-5}$
Location					$2.13 \times 10^{-11}$	$4.62 \times 10^{-6}$
Fixed effects						
(Intercept)	-1.54	0.78	-1.99	0.05 *		
DOY	0.01	$3.56 \times 10^{-3}$	2.25	0.02 *		
Temp. change	-0.27	-0.10	-2.77	0.01 **		

AIC<sub>c</sub> selection identified two top models for eastern massasauga rattlesnake viability (Appendix A). The model with the lowest AIC<sub>c</sub> was a beta model which only included the random effects of individual and location indicating that none of the fixed effects tested have significant effects on spermatozoa viability (Table 2.10). The second top model included random effects of individual and location as well as the fixed effect of brumation on eastern massasauga rattlesnake spermatozoa viability (Appendix A). Predictions of the best model indicate spermatozoa viability is lowest in May (DOYs 121 to 151) and late July, early August (DOYs 196 to 226; Figure 2.3B). Whereas spermatozoa viability peaked between August 31 and September 28 (DOYs 243 to 271; Figure 2.2B).

**Table 2.10** Beta model results, including fixed and random effects used to predict spermatozoa viability eastern massasauga rattlesnake. Only the random effects of individual and location are included in the best model indicating that none of the fixed effects tested have significant effects on spermatozoa viability. One asterisk indicates that the p-value is less than 0.05.

Term	Estimate	Std. Error	Z value	P value	Variance	Std. Deviation
Random effects						
Animal					$3.19 \times 10^{-1}$	$5.65 \times 10^{-1}$
Location					$1.83 \times 10^{-10}$	$1.36 \times 10^{-5}$
Fixed effects						
(Intercept)	-0.84	0.35	-2.39	0.02 *		

Selection using  $AIC_c$  identified three top models for eastern massasauga rattlesnake spermatozoa concentration (Appendix A). The effects of body condition index and age were each included in one of three top models (Appendix A). The best model with the lowest  $AIC_c$  is a gamma log link mixed model in which concentration is best predicted by the random effects animal and location ( $AIC_c = 216.0$ ; Table 2.11). The best model does not indicate the variables tested have significant effects on spermatozoa concentration (Table 2.11). The concentration of spermatozoa in eastern massasauga rattlesnake samples collected throughout the year is expected to remain lower than 75 cells based on results of the best model (Figure 2.3C). Our results predict samples may be slightly higher in concentration in the month of June (DOYs 152 to 181) but overall are collected in consistent concentrations (Figure 2.3C).

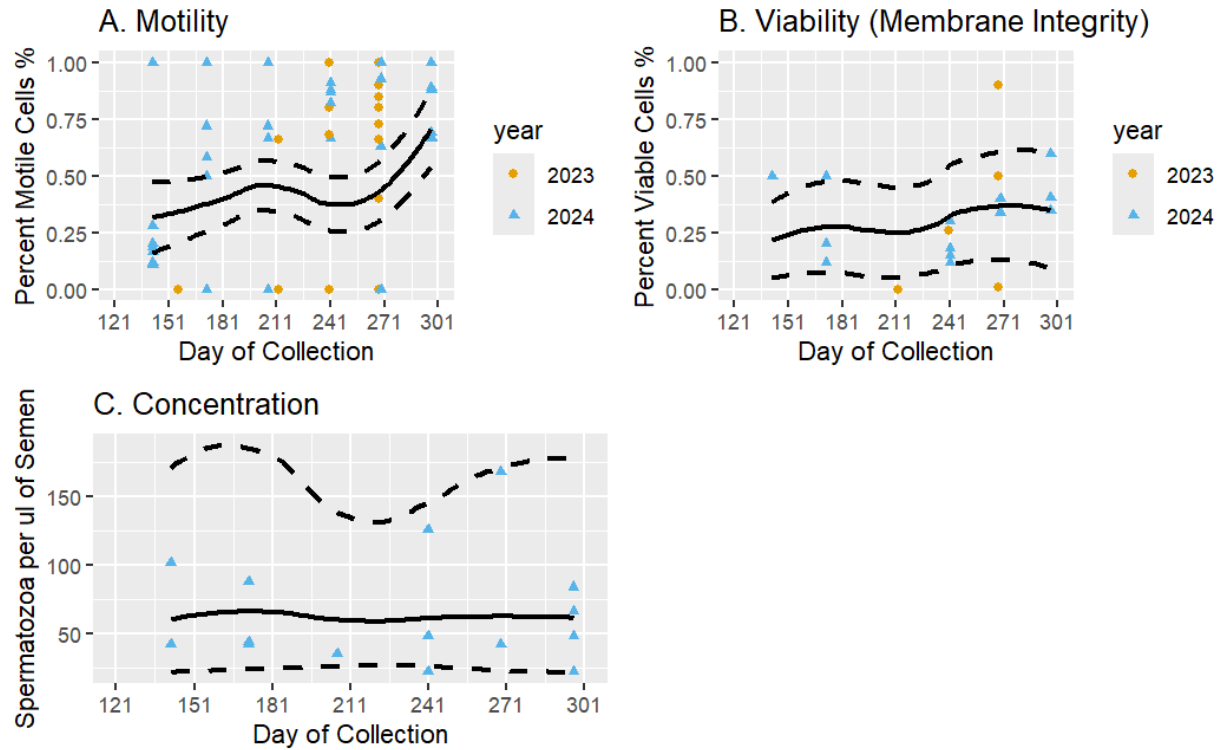
**Table 2.11** Gamma model results, including fixed and random effects used to predict spermatozoa concentration eastern massasauga rattlesnake. Only the random effects of individual and location are included in the best model indicating that none of the fixed effects tested have significant effects on spermatozoa concentration. Three asterisks indicate the p-value is less than 0.0001.

Term	Estimate	Std. Error	T value	P value	Variance	Std. Deviation
Random effects						
Animal					0.03	0.18
Location					0.00	0.00
Residual					0.32	0.57
Fixed effects						
(Intercept)	4.11	1.15	26.8	<2x10 <sup>-16</sup> ***		

AIC<sub>c</sub> selection identified three top models for eastern massasauga rattlesnake RFP (Appendix A). The effect of brumation and extender media were each included in one of three top models (Appendix A). The best model is a linear mixed model in which RFP of eastern massasauga rattlesnake spermatozoa is best predicted by the random effects of animal and location and the fixed effect of body condition index (Table 2.12). The best model results did not indicate significant effects (Table 2.12). When scoring RFP motility for eastern massasauga rattlesnake, spermatozoa samples were classified as “1” in 7.50% of assessments; “2” in 20.0% of assessments; “3” in 18 - 15% of assessments; “4” in 8.33% of assessments; “5” in 0.83% of assessments; and “NA” in 48.33% of assessments.

**Table 2.12** Linear model results, including fixed and random effects used to predict spermatozoa rapid forward progressive motility eastern massasauga rattlesnake. The best model results did not indicate significant effects.

<b>Term</b>	<b>Estimate</b>	<b>Std. Error</b>	<b>T value</b>	<b>Variance</b>	<b>Std. Deviation</b>
Random effects					
Animal				9.42x10 <sup>-7</sup>	9.70x10 <sup>-4</sup>
Location				2.30x10 <sup>-9</sup>	4.80x10 <sup>-5</sup>
Residual				1.10	1.05
Fixed effects					
(Intercept)	2.60	0.16	16.34		
body	-2.28	1.19	-1.92		
condition					
index					



**Figure 2.3** Eastern massasauga rattlesnake spermatozoa concentration and quality measurements with predictive models represented by the solid line and associated standard error represented by the dashed lines. **A.** Percent motile spermatozoa. The model indicates eastern massasauga rattlesnake spermatozoa with the highest percent motility were collected in October (DOYs 274 to 304). **B.** Percent viable spermatozoa. Predictions of the best model indicate spermatozoa viability is lowest in May (DOYs 121 to 151), late July, early August (DOYs 196 to 226; Figure 2.3B) and highest between August 31 and September 28 (DOYs 243 to 271; Figure 2.2B). **C.** Concentration spermatozoa in a microliter of ejaculate. The concentration of spermatozoa in eastern massasauga rattlesnake samples collected throughout the year is predicted to remain low.

## 2.5 Discussion

The quality of spermatozoa collected from gray ratsnake and eastern massasauga rattlesnake varied within and between species. Our results indicate gray ratsnake spermatozoan quality is highest in October, rather than during the mating season (May-June) as we had originally predicted. Eastern massasauga rattlesnake spermatozoa with the highest quality was also collected in the month of October, rather than during the mating season (July-August) as originally predicted.

### 2.5.1 Gray ratsnake

Our analyses suggest that gray ratsnake spermatozoa is of highest quality (motility, viability, and RFP) at the end of the active season in October. Gray ratsnake breed in the spring after emergence from brumation and it is thought spermatogenesis commences in summer when resources are plentiful (COSEWIC, 2018; Aldridge et al., 2009; Lind, Talyor, and DeNardo, 2024). Thereafter, gray ratsnake males must store spermatozoa in the vas deferens during brumation, as has been demonstrated in other temperate colubrid species (Aldridge et al., 2009; COSEWIC, 2018). Spermatozoa are therefore stored from seven to 11 months prior to being used during mating events. Thus, for gray ratsnake, spermatozoa collected in May and June prior to gametogenesis is older than spermatozoa collected in October post-gametogenesis. Long-term spermatozoa storage by males of up to a year has been described in various snake species including South American rattlesnake (*Crotalus durissus terrificus*), jararaca (*Bothrops cotiara*), and Neotropical rattlesnake (*C. durissus*) (Almeida-Santos et al., 2004; Barros, Rojas, and Almeida-Santos, 2017; Carvalho, de Avelar, and de Resende, 2024). However, the lifespan and degradation of viable squamate spermatozoa prior to ejaculation is not fully understood.

The average concentrations of spermatozoa in samples collected from gray ratsnake were less than previously reported for a *Pantherophis* species (*P. guttata*, Fahrig et al., 2007). Characterization of cornsnake (*P. guttata*) spermatozoa across the mating season (May and June) produced samples with an

average concentration of  $8.25 \times 10^5 \pm 5.85 \times 10^5$  cells/ $\mu\text{L}$ . Our study investigated spermatozoa across the entire active season (May to October;  $1.81 \times 10^4$  cells/ $\mu\text{L} \pm 0.09 \times 10^4$  SE); however, within the gray ratsnake mating season (May and June) the average concentration was  $1.29 \times 10^4$  cells/ $\mu\text{L}$  ( $\pm 0.15 \times 10^4$  SE) (COSEWIC, 2018). In Fahrig et al.'s (2007) analysis of cornsnake spermatozoa, only individuals that had previously bred were included. It is possible that, although all males included in our study were considered sexually mature based on size, some individuals were not yet reproductive. Four of the gray ratsnake males within the study had never been exposed to mating opportunities, eight males had unknown reproductive histories, and six had been exposed to opportunities to sire offspring; however, parentage confirmation analysis were not conducted on the resulting offspring. This calls into question the assumption that size of reptiles can be used as a proxy for maturity/age.

Successful artificial insemination and cryopreservation experiments in snakes use high concentrations of high-quality spermatozoa (Mattson et al., 2007; Roberts et al., 2024; Sandfoss et al., 2024). However, the baselines for minimum quality spermatozoa capable of fertilization via artificial insemination varies among papers (Mattson et al., 2007; Roberts et al., 2024; Sandfoss et al., 2024). Motility characterization of samples prior to insemination procedures by Mattson et al. (2007) and Roberts et al. (2024) were variable ranging between 60% and 95% motile (2007; 2024). Yet, Sandfoss et al. (2024) were able to successfully produce viable young when using samples with a lower percent motility ranging from 16% to 39% (2024). Each study also scored the progressive movement of samples, with Mattson et al. (2007) and Roberts et al. (2024) using samples with scores in the top three movement classifications (2007; 2024), and Sandfoss et al. (2024) using samples within the third and fourth movement classifications. Future, research to characterize annual variation exhibited by female gray ratsnake would improve optimization of ART procedures such as artificial fertilization. Further, our results indicate gray ratsnake spermatozoa can be collected throughout the active season in sufficient high concentration motile samples to begin genome resource banking.

### 2.5.2 *Eastern massasauga rattlesnake*

Contrary to predictions, our results suggest that spermatozoa quality is highest in eastern massasauga rattlesnake in October. Eastern massasauga rattlesnake spermatogenesis begins in July and can continue through September (Aldridge et al., 2008), which corresponds with the species mating season across its range. Thus, eastern massasauga rattlesnake males are thought to conduct pre-nuptial spermatogenesis (COSEWIC, 2018; Lind, Talyor, and DeNardo, 2024). However, the possibility of a second, but earlier mating season in May, as occurs in some North America *Crotalus* species (i.e. western diamondback rattlesnake, *C. atrox* and Mojave rattlesnakes, *C. scutulatus*), cannot be eliminated since eastern massasauga rattlesnake have been observed mating *in situ* after emergence from brumation (Taylor and DeNardo, 2011; S. Marks, personal communication, 2024). Our results do not support the hypothesis that the highest quality spermatozoa is available for fertilization when females are receptive to mating, regardless of whether a spring mating season exists. However, the effects of the female reproductive system on the function of snake spermatozoa have yet to be investigated. Spermatozoa motility and RFP could change upon exposure to signals produced by reproductively viable females, as has been seen in Japanese fire-bellied newt (*Cynops pyrrhogaster*; Watanabe et al., 2010). Future investigations into how eastern massasauga rattlesnake spermatozoa characteristics are affected by female reproductive signals (i.e. oviduct secretions) would benefit assisted insemination development.

Eastern massasauga rattlesnake spermatozoa characteristics differed from those reported in previous studies of snake spermatozoa concentration and quality. To date, no other *Sistrurus* species spermatozoa has been characterized; however, descriptions of spermatozoa from other viperids are available (Almeida-Santos et al., 2004; Zacariotti et al., 2007; Mozafari, Shiravi, and Todehdeghan, 2012; Moshiri, Todehdeghan, and Shiravi, 2014; Coeti et al., 2020; Blank et al., 2022). Quality metrics are not comparable inter-species as eastern massasauga rattlesnake spermatozoa was collected in numbers insufficient for proper analysis. Eastern massasauga rattlesnake spermatozoa was consistently of significantly lower concentration than reported for the *Crotalus*, *Micrurus*, and *Bothrops* genera

(Zacariotti et al., 2007; Coeti et al., 2020; Blank et al., 2022). Average spermatozoa concentration collected from Brazilian rattlesnake (*Crotalus durissus terrificus*) and painted coral snake (*Micrurus corallinus*) were  $1.38 \times 10^6$  ( $\pm 0.13 \times 10^6$  SE) cells per microliter and  $1.30 \times 10^6$  cells per microliter, respectively (Zacariotti et al., 2007; Coeti et al., 2020). Analysis of spermatozoa collected from six Neotropical species (*Bothrops jararaca*, *B. jararacussu*, *B. atrox*, *B. moojeni*, *B. leucurus*, and *C. durissus*) revealed spermatozoa from all the examined species could be collected in concentrations of at least  $1.0 \times 10^8$  ( $\pm 6.0 \times 10^7$ ) cells per microliter (Blank et al., 2022). However, none of the collection attempts with eastern massasauga rattlesnake were successful in retrieving ejaculate in concentrations exceeding 175 cells per microliter. Potentially, this genus has quantities minimizing the energy to produce spermatozoa cells (Rotimi et al., 2024). Alternatively, the sampling methodology that we employed might have been unsuitable for eastern massasauga rattlesnake.

While the digital manipulation method for spermatozoa collection has been shown to be effective across numerous taxa, an alternative spermatozoa collection procedure using local anesthetic has been used on venomous snake species (Zacariotti et al., 2007; Blank et al., 2022). Local anesthetic administered via injection in advance of using Mengden et al.'s (1980) digital manipulation method for collecting spermatozoa relaxes the muscles around the cloaca, facilitating access of the genital papilla to collect clean samples (Zacariotti et al., 2007). We choose to use methods that would minimize the invasiveness of live-animal procedures, in accordance with the three R's (replacement, reduction, and refinement) advocated by the Canadian Council on Animal Care (2017), opting to forego local anesthetic when handling eastern massasauga rattlesnake to decrease physical intervention and handling time. Unfortunately, the concentration of spermatozoa collected from eastern massasauga rattlesnake was frequently insufficient for analysis, preventing assessment of spermatozoa quality.

Previous research into drivers of poor-quality spermatozoa have suggested that inbreeding depression is correlated with male gamete performance (Fitzpatrick and Evans, 2009). A semi-quantitative risk assessment of the “*North American Eastern Massasauga Rattlesnake Species Survival Plan*”, identified

male contribution to mating as a limiting factor in the captive population reproductive output (Choquette, 2024). However, records from the *ex situ* program of eastern massasauga rattlesnake in North America indicate that female eastern massasauga rattlesnake produce unfertilized ova at a high rate considering the average litter size of *in situ* females (Choquette, 2024). Our results suggest that low reproductive output in females may be a result of limited spermatozoa availability as fertilization success correlates with concentration of motile spermatozoa (Tourmente et al., 2007). Investigations of the genetic diversity of eastern massasauga rattlesnake, particularly inbreeding and inbreeding depression within the captive population may facilitate better understanding of our results.

The massasaugas used in our study had one of three origins: captive bred, sourced from the Great Lakes / St Lawrence Designatable Unit as hatchlings, or born in captivity from gravid mothers sourced from the Carolinian Designatable Unit and brought into a temporary holding facility until parturition. The current genetic diversity within these population is unknown. However, previous genomic studies of eastern massasauga rattlesnake have shown that this species has lower genetic diversity than related *Sistrurus* species (Ochoa and Gibbs, 2021). Further, small captive population may be at risk of higher levels of inbreeding simply due to numbers (Schulte-Hostedde and Mastromonaco, 2015). Both massasauga Designatable Unit's in Canada contain extremely fragmented population where anthropogenic features impeded gene flow (COSEWIC, 2018). As a result, the founders of the captive population may inherently have lower genetic diversity.

### 2.5.3 *Brumation*

Studies of various captive mating programs have shown that husbandry practices can influence the reproductive success of reptiles (Mariais, and Morgan, 1990). In North America, reptiles demonstrate reproductive sensitivity to annual environmental variation (Brown and Shine, 2006). Preventing an animal from completing all events in a normal life cycle, such as brumation, can have negative effects on

offspring production in squamates including the eastern collared lizard (*Crotaphytus collaris*) (Louth and Heatley, 2020). Conversely, when management was adapted to mimic natural cycles as closely as possible, the offspring production in narrow-headed garter snake (*Thamnophis rufipunctatus*) was improved (Blais et al., 2023). Our results could not determine whether brumation significantly affected the quality (motility, viability, RFP) of eastern massasauga rattlesnake ejaculate; however, the concentration of samples was found to be unaffected by brumation. Our limited sampling success in eastern massasauga rattlesnake likely impacted our findings. The model results did suggest that brumation impacted gray ratsnake spermatozoa motility and viability. However, the results should be interpreted with caution due to small sample size (gray ratsnake n=2; eastern massasauga rattlesnake n=5).

#### 2.5.4 Conclusions

Our results reveal species-specific variation in spermatozoa quality in our study species. Gray ratsnake spermatozoa exhibited annual variation in concentration, viability, and RFP but not motility. Eastern massasauga rattlesnake spermatozoa did not exhibit statistically significant annual variation in any of the investigated parameters; however, the low concentration samples and low sampling success rate impeded our ability to assess spermatozoa characteristics. These data further our understanding of squamate spermatozoa and support the observation that key aspects of spermatozoa like morphology are conserved across taxa, while others vary among species, presumably to increase reproductive success or offspring survival within each taxon. Our results provide novel insights into the reproductive function of two at risk species of snake found in Ontario, facilitating future efforts to develop ART and genome resource banking. Specific to conservation mating programs, our results suggest that both gray ratsnake and eastern massasauga rattlesnake spermatozoa be collected in October (DOYs 274 to 304).

## *2.6 Acknowledgements*

This project was made possible by the support of multiple animal keepers and managers. We sincerely thank Hana Thompson, Kurtis Marleau, Tom Eles, Cheryl Sheridan, Jenny Pearce, Sarah-Jane Stanger Guy, Jeff Hathaway, Rick Vos, and all the partner facility staff and volunteers that brought this initiative to life. Your passion for species conservation and herpetofauna in Canada inspires us to work harder every day.

## *2.7 Data accessibility statement*

R code and the full data set have been deposited in the Open Science Framework at the following link:

[https://osf.io/fr2wy/?view\\_only=a43487e6fb2446b190f3f89eba63328b](https://osf.io/fr2wy/?view_only=a43487e6fb2446b190f3f89eba63328b)

## *2.8 Conflict of interest*

None.

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### **Chapter 3:**

**Do *Sistrurus catenatus* sexual steroids in captive snakes mirror reproductive seasonality in the wild?**

### 3.1 Abstract

The eastern massasauga rattlesnake (*Sistrurus catenatus*) is a small venomous snake found in isolated patches of suitable habitat across Eastern North America. The species is threatened with global extinction and continues to decline because of persecution and habitat loss. Conservation efforts include a multi-disciplinary program incorporating *in situ* and *ex situ* initiatives. However, there is a need to improve fecundity in the *ex situ* population to ensure long-term sustainability and so that the program can reinforce and augment wild population. Information on species-specific reproductive variation would help to improve captive mating outcomes. We hypothesized that circulating reproductive hormones would vary throughout the year reflecting patterns of gametogenesis and the annual cycle of wild population. Thus, we predicted that faecal testosterone metabolite (FTM) concentration would be highest late July-early August (Julian dates 195 to 243) during spermatogenesis. We predicted that faecal progesterone metabolites (FPM) concentration would increase in May (Julian dates 121 to 151) and June (Julian dates 152 to 181) during vitellogenesis, be expressed in the highest concentrations annually from May to August (Julian dates 121 to 243) and remain at high concentration through to parturition in gravid females. Faecal samples were collected from massasauga rattlesnake in the Canadian *ex situ* population during routine care between March 2023 and September 2024. FTM and FPM were assessed in males and females, respectively, via commercial enzyme-linked immunosorbent assay (ELISA). We found that both sexual steroid metabolites of testosterone and progesterone displayed annual variation but not as predicted. Rather we found age of rattlesnakes and husbandry practices may affect sexual steroids required for reproductive function. Our results provide novel insights on the relationship between size and sexual maturity in snakes. Knowledge gained from this research may facilitate efforts to increase reproductive output within the *ex situ* mating population of massasauga rattlesnake.

### 3.2 Introduction

The preservation of species outside their native environment in human care, hereafter, *ex situ* conservation, has helped to reverse declines for multiple species of animals worldwide (Che-Castaldo, Grow, and Faust, 2018). The most successful of these initiatives have access to detailed knowledge about species-specific reproductive characteristics improving animal husbandry and reproductive management (Comizzoli, Paulson, and McGinnis, 2018). Information on endocrine profiles, specifically characterizing sexual steroid hormones, can help animal managers to improve population management (i.e. monitor contraception methods; Picone et al., 2024) and increase reproductive output (Vieira et al., 2024). Traditionally, circulating hormone concentrations were quantified through acquisition of blood samples; however, this technique typically requires immobilization or restraint of the animal in question and can negatively impact animal welfare (Zemanova, 2020). The use of non-invasively collected samples in which endogenously secreted steroid-metabolites are excreted from the animal (i.e., hair, faeces, shed skin, saliva, etc.) can alleviate welfare concerns (Heistermann, 2010).

Previous work describing snake reproductive modalities in North America have identified that the timing of gametogenesis, mating, and fertilization events can be uncoupled and differ among species (Aldridge et al., 2009). Seasonality in reproduction can be observed across squamates and appears most pronounced in regions that exhibit strong seasonal environmental variation (Brown and Shine, 2006). Even in tropical climates, many snake species reproduce during specific times of year (Brown and Shine, 2006). In northern regions, where seasonal differences are more extreme, reproduction is greatly influenced by brumation – a period of dormancy in ectotherms usually during winter (Saint Girons, 1982). Characterization of captive and free-ranging viperid hormone profiles for Mojave rattlesnakes (*Crotalus scutulatus*), cottonmouth (*Agkistrodon piscivorus*), black-tailed rattlesnake (*C. molossus*), among other taxa show how species can limit aspects of reproductive processes, including production of circulating sex steroids, through the coldest months of the year (but see timber rattlesnake, *C. horridus*) (Taylor and DeNardo, 2011; Lutterschmidt et al., 2009).

Testosterone and progesterone are steroid hormones critical to the reproduction of vertebrate animals that evolved within basal chordates (Baker, 2019). In North American rattlesnake species, increased circulating testosterone in males has been linked to spermatogenesis, which appears restricted to summer months (Taylor and Booth, 2016; Lind et al., 2010). Testosterone's relationship with rattlesnake mating and male-male competitive behaviours is less definitive and varies among species (Taylor and Booth, 2016). Studies of progesterone in female rattlesnakes suggest that it occurs in low concentrations within non-reproductive females but increases dramatically during reproduction, appearing essential to gestation (Taylor and Booth, 2016; Vieira et al., 2024; Lind et al., 2010; Taylor, DeNardo, and Jennings, 2004). Descriptive studies characterizing circulating progesterone during reproduction in northern pit vipers (*Bothrops atrox*) indicate an increase in concentration at the end of vitellogenesis, with progesterone concentrations remaining high throughout gestation until prior to parturition (Vieira et al., 2024).

The eastern massasauga rattlesnake (*Sistrurus catenatus*) is a species threatened with extinction throughout its range in Canada and the United States (COSEWIC, 2012). Eastern massasauga rattlesnakes at their northern range limit in Ontario, Canada undergo an extended period of brumation, with a correspondingly short reproductive season (COSEWIC, 2012). It is the only venomous snake currently found in Ontario, the most densely populated province in Canada (Statistics Canada, 2024). Eastern massasauga rattlesnake have experienced steep declines in densely human populated areas due to intense persecution that is compounded by road mortality and habitat fragmentation (COSEWIC, 2012). Efforts to conserve the species have taken a "One Plan Approach" (Byers et al., 2013) where recovery integrates *in situ* efforts, including habitat restoration and preservation, and *ex situ* management action – where animals have been taken into human care to support *in situ* actions. The *ex situ* population of eastern massasauga rattlesnake is used both as an insurance population, and to produce snakes that can be used to re-establish the species in southwestern Ontario (Ontario Ministry of Natural Resources and Forestry, 2016). However, the program has been impeded by low reproductive output (Choquette, 2024).

Here we characterize annual variation of endogenously secreted testosterone (FTM) and progesterone (FPM) in faeces throughout the eastern massasauga rattlesnake reproductive cycle. We hypothesized that circulating reproductive hormones vary throughout the year in a pattern that correlates with gametogenesis. Thus, we predicted that FTM concentration would be highest during the mating season, late July-early August (Julian dates 195 to 243), which should correspond to the peak in spermatogenesis. Second, as progesterone is an essential hormone involved in maintaining gestation of vertebrate species, we predicted FPM concentration would increase in May and June (Julian dates 121 to 181) during vitellogenesis, be expressed in the highest concentrations annually from May to August (Julian dates 121 to 243) and remain at high concentration through to parturition in gravid females.

### 3.3 Methods

#### 3.3.1 Animals

An *ex situ* population of eastern massasauga rattlesnake has been established using founders from wild Canadian population. Snakes were collected in their first year of life prior to brumation in 2020 and 2021. The goal of the *ex situ* population is to act as insurance against extinction, and to produce animals that can be used to reinforce and reintroduce the species within Ontario, Canada (Ontario Ministry of Natural Resources and Forestry, 2016). We collected faecal samples from animals in the *ex situ* population that are under the care of Scales Nature Park, a non-governmental conservation organization that maintains captive collections in Oro-Medonte, Ontario, Canada (Ontario Ministry of Natural Resources and Forestry, 2016). All animals were handled according to approved Queen's University Animal Care Protocol #2021-2137.

All individuals are housed separately unless paired for mating. During the months of December-April, animals are cooled to 20-22°C, whereas from May-November the habitats are maintained between 22–32 °C (AZA Eastern Massasauga Rattlesnake SSP, 2013). The light cycle is varied to simulate the

photoperiod they would be subjected to in Southern Georgian Bay as per staff and facility capability, such that animals received UVA and UVB for ~9 hours at winter solstice to ~15.5 hours at summer solstice (National Research Council Canada, 2020). Details regarding the provenance of *ex situ* animals from which samples were collected can be found in Table 3.1.

**Table 3.1** Summary of eastern massasauga rattlesnake from which faecal samples were collected, including sample sizes and origin.

Sex	Number of individuals	Great Lakes /	
		St. Lawrence	Carolinian
Male	7	3	4
Female	4	4	0

### 3.3.2 *Brumation*

In 2024, brumation trials of male eastern massasauga rattlesnake were undertaken to assess the effect of physiological cooling on FTM expression. A protocol was developed in consultation with species experts and veterinary staff at *ex situ* facilities. Animals were brumated in a lockable refrigeration unit (EUR-USF328S, Eurodib, NY, USA). We used smart sensors (Temp°Stick, Ideal Sciences, Bountiful, UT) to monitor humidity and temperature in each location throughout the brumation period. Two healthy mature male snakes were selected for brumation. Food was restricted starting three weeks prior to cooling, as snakes are unable to digest at low temperatures (AZA Eastern Massasauga Rattlesnake SSP, 2013). Individuals were then transferred to refrigeration units and gradually cooled until they could be maintained between 6-10°C during a period of brumation. Lighting was changed on the day animals started cooling: light cycles were decreased by one hour every two days for six days, then one hour per

day until total darkness was achieved (24 h). Brumation conditions were maintained for four weeks. At the end of the ‘over-winter period’, the ambient temperature for snakes in brumation gradually increased, as follows: one degree per day to 10°C, then two degrees per day to 20°C. Lighting was increased by one hour per day for six days and then two hours per day for three days.

The remaining five males and all females in the hormone study stayed in their captive housing ‘habitats’ and the environment was gradually decreased to an ambient temperature of 20-22 °C. The light cycle is varied to simulate the photoperiod they would be subjected to in Southern Georgian Bay as per staff and facility capability, such that animals received UVA and UVB for ~9 hours at winter solstice to ~15.5 hours at summer solstice (National Research Council Canada, 2020). Individuals were offered food biweekly and provided with a heat lamp during the daylight hours.

### *3.3.3 Sample collection, hormone extraction, and assays.*

Faecal samples were collected during routine habitat maintenance by animal care staff from March 2023 till September 2024. Samples were labeled with animal identification (ID) and date at the time of collection and stored at -20°C until extraction. Faecal hormone extraction was conducted using previously published methods (Mondol et al., 2020) as follows. Samples were dried for 12 to 24 hours in a London Sunshine Premium Food Dehydrator (London Sunshine®; Concord, ONT). Sub-samples were weighed to the nearest 0.000g and diluted with 1.0ml of 80% ethanol prior to vortexing and then placed on a shaker overnight. After a minimum of 12 hours, samples were spun in the centrifuge at 179g for 20 minutes. The aqueous solution was transferred to an evaporation proof vial and stored for up to three months at -20°C (Kalbitzer and Heistermann, 2013). We used commercially available DetectX® Progesterone ELISA Kits and DetectX® Testosterone ELISA Kits (Arbour Assays, MC, USA) assays. Assay protocols followed the manufacturer’s instructions. Kits were each validated for species use through a series of parallelism, spike and recovery, and repeatability experiments; details can be found in Appendix B.

### 3.3.4 Statistical Analysis

Data visualization and statistical analyses were done in R (version 4.2.2, R Core Team 2022). The workspace in R was formatted using “tidyverse” (Wickham et al., 2019). Data were subdivided into sex-specific data sets. Outliers were excluded from both data sets following the 1.5 interquartile range method. Upper outliers were calculated as those above the *Interquartile range + 1.5 \* Quartile 3* and lower outliers were calculated as those below the *Interquartile range - 1.5 \* Quartile 1*. To ensure data only included sexually mature individuals, snakes were included in the analysis based on two factors. First, individuals had to have achieved the size of a sexually mature adult from *in situ* Canadian population. Second, as this captive population does not enter brumation they experience double the growing season of their wild counterparts; therefore, all males had to be a minimum of two years of age (24 months of sustained growth approximate to four year-old male *in situ*), and all females had to be a minimum of three years of age (36 months of sustained growth approximate to six year-old female *in situ*) (COSEWIC, 2012). Using the R package “MuMIn” (v1.47.5; Bartoń, 2023), we used dredge to test for an effect of independent variables including, Julian day (DOY), age, brumation, Designatable Unit, (Carolinian or Great Lakes / St. Lawrence; COSEWIC, 2012) and intraspecific contact (mating) to investigate the effects of each on hormone metabolite excretion. Mixed models were investigated to include random effects of individual animals using the “lme4” R package (Bates et al., 2015; Zuur et al., 2009). Mixed models allow for the introduction of random effects into the models which assist the model in accounting for inference about the fixed effects (Harrison et al. 2018). We employed AIC<sub>c</sub> model selection, identifying the best model(s) as those with the lowest AIC<sub>c</sub> value and top models as any within  $\Delta 2$  AIC<sub>c</sub> (Burnham and Anderson, 2002). Plotting was assisted by the “ggpubr” R package (v0.6.0; Kassambara, 2023).

### 3.4 Results

We sampled 11 mature eastern massasauga rattlesnake in total; seven males and four females (Table 3.1). The data set included 149 faecal samples collected from March 2023 to September 2024. An average of 13 samples (range = 6 to 18) were collected per animal in the study. Mature male data totaled 93 samples and mature female data included a total of 56 samples.

#### 3.4.1 Testosterone

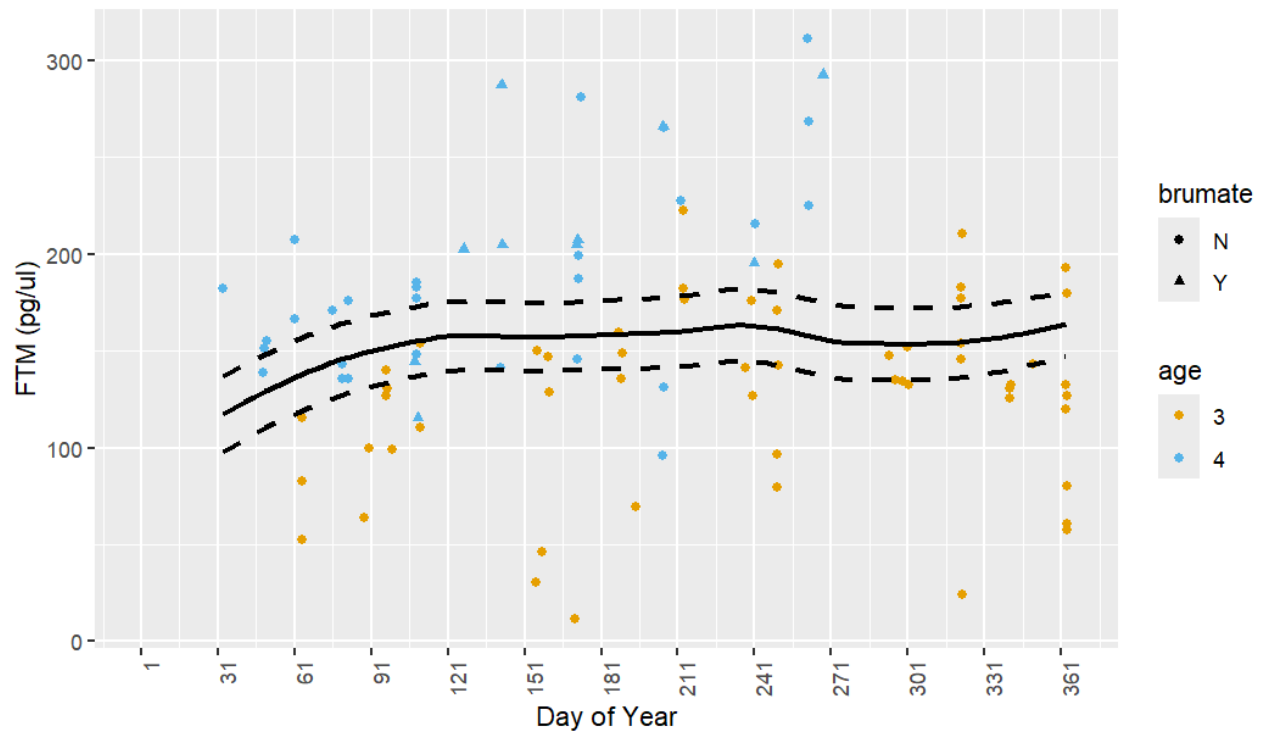
Faecal testosterone metabolite expression was not detected at significantly higher concentrations in July or August as we had predicted but displayed annual variation (Figure 3.1). Two models for FTM were identified by AIC<sub>c</sub> selection (Table 3.2). Based on results from linear regression models, FTM was significantly affected by Julian date (0.79,  $p = 1.08 \times 10^{-3}$ ) and age (79.30,  $p = 1.22 \times 10^{-10}$ ; Table 3.3). Whether males had been able to brumate the season prior was included in one of the two top models (Table 3.2). The best model indicates an increase in FTM concentration between Julian days 31 (January 31) and 121 (May 1), which levels off until Julian day 241 (August 29) where levels begin to drop (Figure 3.2). The results further indicate FTM concentration increases in the winter season from about Julian day 316 (November 11) until the end of the calendar year (Figure 3.2).

**Table 3.2** Top models selected by AIC<sub>c</sub> for eastern massasauga rattlesnake faecal testosterone metabolite indicating day of year and age as significant predictors in both top models. The effects of animals being subjected to artificial brumation were included in one of two models.

Beta mixed effects structure	AIC <sub>c</sub>	ΔAIC <sub>c</sub>
Testosterone ~ DOY + I(DOY <sup>2</sup> ) + age	981.1	0.0
Testosterone ~ DOY + I(DOY <sup>2</sup> ) + age + brumate	982.7	1.6

**Table 3.3** Model results, used to predict faecal testosterone metabolite concentration of eastern massasauga rattlesnake. Based on results from linear regression models day of year (0.79,  $p = 1.08 \times 10^{-3}$ ; I(DOY<sup>2</sup>),  $-1.43 \times 10^{-3}$ ,  $p = 1.09 \times 10^{-2}$ ), and age (79.30,  $p = 1.22 \times 10^{-10}$ ) have significant effects. One asterisk indicates that the p-value is less than 0.05; two indicate the p-value is less than 0.001 and three indicate the p-value is less than 0.0001.

Term	Estimate	Std. Error	T value	Pr(>  t )
(Intercept)	34.98	22.50	1.55	0.12
DOY	0.79	0.23	3.38	$1.08 \times 10^{-3}$ **
I(DOY <sup>2</sup> )	$-1.43 \times 10^{-3}$	$5.51 \times 10^{-4}$	-2.60	$1.09 \times 10^{-2}$ *
Age 4	79.30	10.89	7.29	$1.22 \times 10^{-10}$ ***

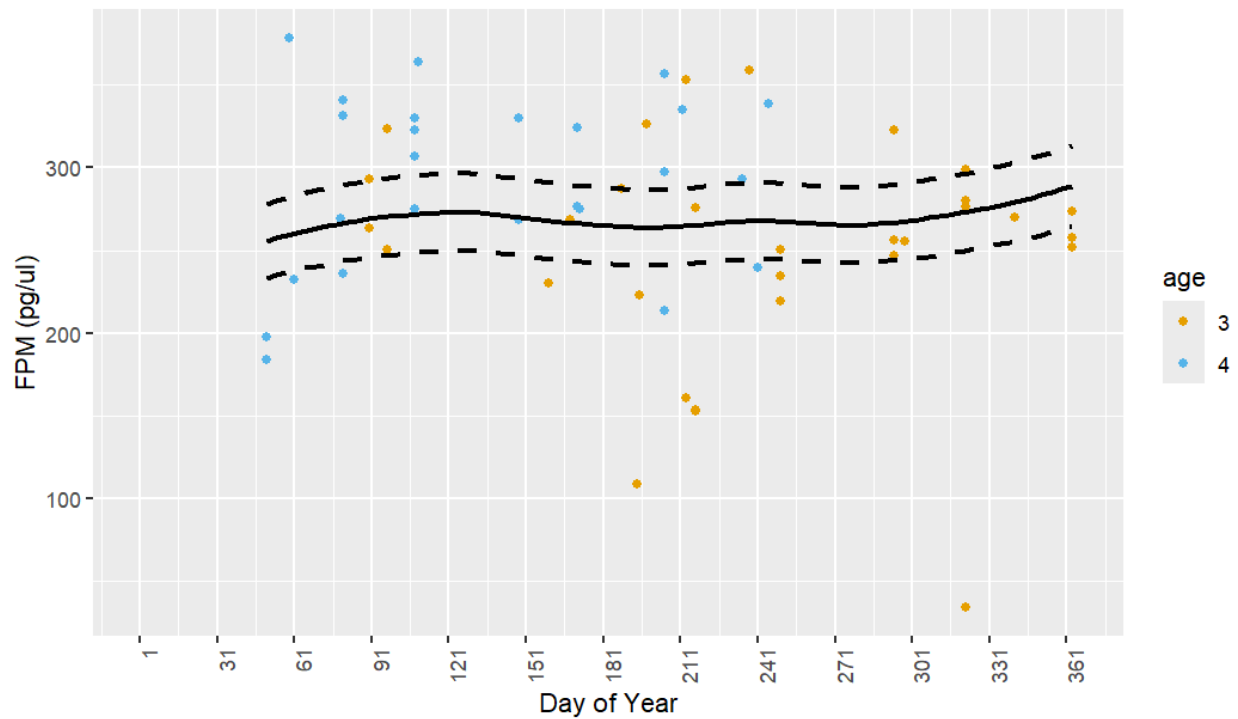


**Figure 3.1** Eastern massasauga rattlesnake faecal testosterone metabolite concentration over the Julian calendar. The solid line represents the best fit model predicted by day of year and age and the dashed lines indicate the calculated standard error. The best model indicates an increase in FTM concentration between DOY 31 and DOY 121, which levels off until DOY 241. The data represents seven mature males opportunistically sampled from March 2023 to September 2024, producing approximately 13 samples (range = 6 to 18) per male. All samples from brumated individuals were collected in 2024.

### 3.4.2 Progesterone

Faecal progesterone metabolite expression was not detected at higher concentrations in May as we had predicted, but did display annual variation (Figure 3.2). One model for FPM was identified by AIC<sub>c</sub> selection (Table 3.4). Based on results from linear regression models, FPM was affected only by snake age (42.46,  $p = 1.47 \times 10^{-2}$ ; Table 3.4). Only one female became gravid during the study period preventing

investigation of the effect of gravidity on results. The best model indicates an increase in FPM concentration between Julian days 46 (February 15) and 121 (May 1) and again between Julian days 287 (October 14) and 361 (December 27); however, the standard error exceeds the change in FPM (Figure 3.2). The results further indicate FPM concentration decreased in the summer season from June 30 to October 13 (DOY 181 to 286; Figure 3.2).



**Figure 3.2** Eastern massasauga rattlesnake faecal progesterone metabolite concentration over the Julian calendar. The solid line represents the best fit model predicted by age and the dashed lines indicate the calculated standard error. The best model indicates an increase in FPM concentration between DOYs 46 and 121 and again between DOYs 287 and 361. The data represents four females sampled from March 2023 to September 2024, on average 14 samples (range = 13 to 15) were collected per female.

**Table 3.4** Model results, used to predict faecal progesterone metabolite concentration of eastern massasauga rattlesnake. Based on results, only by snake age (42.46,  $p = 1.47 \times 10^{-2}$ ) affected faecal progesterone metabolites. One asterisk indicates that the p-value is less than 0.05; two indicate the p-value is less than 0.001 and three indicate the p-value is less than 0.0001.

Term	Estimate	Std. Error	T value	Pr(>   t   )
(Intercept)	250.18	11.26	22.23	$< 2 \times 10^{-16}$ ***
Age 4	42.46	16.85	2.52	$1.47 \times 10^{-2}$ *

### 3.5 Discussion

Faecal testosterone and progesterone metabolite concentrations varied across the annual cycle in captive eastern massasauga rattlesnake but not as we had expected. However, our results indicate that while both FTM and FPM increased with age, only FTM is affected by the Julian date. Our analyses further suggest that husbandry influences FTM. Eastern massasauga rattlesnake housing, including their exposure to mating opportunities and brumation, may trigger endogenous cues that increase circulating testosterone, resulting in increased expression of faecal steroid metabolites. In our analysis, the random effect of individual animal could not be estimated; we found the parameter values given for the best models of both FTM and FPM were comparable between mixed and non-mixed models.

We observed that FTM concentration model predictions are affected by Julian date (DOY) in eastern massasauga rattlesnake held in captivity at a mating center in Southern Ontario. FTM have not been quantified for any other snake species in the published literature, and thus we cannot gain insight from other species. Up to a month can pass between faecal excretions and thus faecal analysis may not be a suitable to assess acute increases in circulating testosterone (Palme, 2019). However, using blood

samples, previous analysis of viperids suggest that elevated testosterone is sustained across the mating season in many species (Schuett et al., 1997; Taylor, DeNardo, and Jennings, 2004; Schuett et al., 2006; Lind et al., 2010). An exception is the timber rattlesnake (*C. horridus*), which was extirpated from Canada in the early 1940's (COSEWIC, 2023b). Timber rattlesnakes do not demonstrate annual variation in testosterone (Lutterschmidt et al., 2009); however, this study used a small sample of individuals, which may have affected the conclusions. Unlike mammals, many snake species do not defecate on a regular basis (Lillywhite, de Delva, and Noonan, 2002). Excretion pathways for eastern massasauga rattlesnake include faecal and urate, shed skin, saliva and venom. Thus, an alternative explanation for the variation seen in our analysis is that the bulk of testosterone metabolites are excreted from the body through another pathway, such as saliva (Palme, 2019). In preliminary experiments, shed skin samples were sourced for enzyme linked immunosorbent assay; however, we found the concentration of testosterone metabolites in shed skin was insufficient to be detected.

FPM concentrations were not significantly affected by Julian date in eastern massasauga rattlesnake held in captivity. For many species, increased circulating progesterone during gestation may assist in maintaining pregnancy (Taylor and DeNardo, 2011). In previous reproductive studies of snakes, circulating progesterone increased during gestation and dropped either prior to or post parturition (DeNardo and Taylor, 2011). However, in viperids studied to date, females do not enter a period of reproductive activity each year (Lind, Taylor, and DeNardo, 2024), as described in wild eastern massasauga rattlesnake, where biennial reproduction is common (COSEWIC, 2012). Our results are consistent with what has been previously reported for females of the North American western diamondback rattlesnake (*C. atrox*); in this species progesterone levels are sustained throughout the year in non-reproductive females (Taylor, DeNardo, and Jennings, 2004). One noted exception is the red-sided gartersnake (*Thamnophis sirtalis paretalis*), which exhibits low levels of circulating progesterone through to late gestation (Whittier, Mason, and Crews, 1987).

Age of an individual appears to affect both FTM and FPM. In both sexes, increased age resulted in increased sexual steroid metabolite concentrations, as detected in faecal samples. In Canada near their northern range limit, eastern massasauga rattlesnake are thought to reach sexual maturity between five and six years after hatching (COSEWIC, 2012); whereas in the captive collection successful parturition has occurred with sires and dams under four years of age. As determining the exact age of snakes *in situ* is not possible, researchers frequently assess sexual maturity based on an animal's size (Petersen et al., 2024). However, multiple endogenous and exogenous factors affect sexual maturity, and this assumption may not hold true among all snake species especially at their northern range limits (Petersen et al., 2024). The population of animals used for our study were sourced from an *in situ* population as hatchlings; prior to our study these eastern massasauga rattlesnake had not been subjected to brumation. Further, they had access to sufficient resources throughout the entire year, as opposed to being restricted to a five or six month active season in the wild. As a result, individuals held in human care exhibited faster growth than *in situ* counterparts and reached the size expected for sexual maturity at a younger age. Our results imply that eastern massasauga rattlesnake used in our study may have not reached sexual maturity. Continued research of these animals through to the known age of sexual maturity *in situ* and its endogenous triggers would facilitate a better understanding of the maturity-size relationship and improve efforts to manage the species *ex situ*.

Male representatives from both the Great Lakes / St Lawrence and Carolinian Designatable Units comprised the captive collection under study, facilitating investigation into potential source population effects. Designatable Units are a tool for diagnosing the conservation status of population of organisms in Canada and can represent separate groups within a species if they have attributes that make them both discrete and evolutionarily significant (COSEWIC, 2023a). Our analyses implies that FTM concentration in individuals from Great Lakes / St. Lawrence Designatable Unit snakes may be higher than in individuals from the Carolinian Designatable Unit. This is opposite what we would expect considering the latitudes from which these individuals originated. The Carolinian Designatable Unit snakes derive from a

lower latitude inferring a longer active season and potential to achieve sexual maturity earlier than in the Great Lakes / St. Lawrence Designatable Unit (Amat and Meiri, 2018). The results could be a consequence of the severe population decline and reduced dispersal resulting from threats to the species in the Carolinian Designatable Unit, including habitat fragmentation, which has led to inbreeding depression, that could impact reproductive function (Achermann et al., 2002).

Our analysis also suggested that males that experienced brumation were more likely to have higher FTM concentrations than non-brumated individuals. In 2024, two males underwent brumation to attempt to increase mating success. Previous studies of snake conservation mating programs have shown that exposing the captive population to brumation that reflects actual conditions in wild population increased reproductive success of a viviparous temperate species, *T. rufipunctatus* (Blais et al., 2023). Current management practice with the eastern massasauga rattlesnake include a slight cooling period over the winter months when the species is known to brumate in the wild (AZA Eastern Massasauga Rattlesnake SSP, 2013). This process was thought to be sufficient for promoting reproduction within the species as young have been produced in captivity; however, our results suggest that this is not true. Changes in husbandry protocols in other squamate conservation mating programs including those in the smooth greensnake (*Ophedrys vernalis*) and eastern collared lizard (*Crotaphytus collaris*) have improved reproductive success via formalized brumation protocols (Sacerdote-Velat et al., 2014; Louth and Heatley, 2020). Notably, in the first successful artificial insemination of reptile using frozen-thawed ejaculate, both male and female study subjects underwent brumation (Louisiana pinesnake, *Pituophis ruthveni*; Sandfoss et al., 2024).

In summary, our results suggest that the captive eastern massasauga rattlesnake do exhibit annual variation in FTM and FPM and that age of rattlesnakes and potentially husbandry practices affect sexual steroids required for reproductive function. Thus, the reproductive capacity of these animals may be improved by facilitating brumation. Such considerations enrich insights into the reproductive function of this species in captivity but also suggest that future study should focus on investigating the relationship

between age, size and sexual maturity. Continuing to develop a broader understanding of the species-specific endocrinology through to sexual maturity would improve our ability to adapt management to realize improved mating outcomes and ART development, which would, in turn, facilitate efforts to meet recovery goals for this and other species (Vieira et al., 2024).

### *3.6 Acknowledgements*

This project could never have been completed without the dedicated keeper staff maintaining the eastern massasauga rattlesnake at Scales Nature Park. Thank you to everyone who took time to collect samples for the project, we are eternally grateful for your efforts.

### *3.7 Data accessibility statement*

R code and the full data set have been deposited in the Open Science Framework at the following link:

[https://osf.io/gzaxm/?view\\_only=367aece529454f2186d9662f3273e4a1](https://osf.io/gzaxm/?view_only=367aece529454f2186d9662f3273e4a1)

### *3.8 Conflict of interest*

The authors declare no conflict of interest.

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## Chapter 4: General Discussion and Conclusions

### 4.1 Overview

I researched species-specific annual variation in reproductive function and explored the development of assisted reproductive technologies and genome resource banking initiatives for the under-represented squamate order. I assessed the strengths and limitations of my findings and suggested potential avenues for future research that would help improve interpretation of the results. I provide a summary of the contributions that I believe my research has made to animal management and *ex situ* conservation.

### 4.2 Chapter Two

My first data chapter (Chapter 2) evaluates species-specific annual variation in spermatozoa concentration and quality in two at-risk snake species, gray ratsnake (*Pantherophis spiloides*) and eastern massasauga rattlesnake (*Sistrurus catenatus*). I used 39 snakes (n = 18 gray ratsnake, n = 21 eastern massasauga rattlesnake) from four *ex situ* facilities in Southern Ontario that were sampled in 2023 and 2024 (Table 2.1). Between May and October, we attempted to sample each animal monthly, with our data comprising 279 sampling events and producing 558 samples. Analysis of sample concentration and quality revealed species-specific annual variation throughout the active season (May-October). I found that the concentration of spermatozoa collected from gray ratsnake and eastern massasauga rattlesnake was unlikely to vary significantly across the active season as I had predicted. In contrast, snake spermatozoa quality (motility, viability, and RFP) was likely to vary throughout the annual cycle of gray ratsnake and eastern massasauga rattlesnake, but not in the pattern I had originally predicted. These results suggest that gray ratsnake and eastern massasauga rattlesnake spermatozoa quality is highest in October. However, spermatozoa collected from eastern massasauga rattlesnake was substantially below the concentration and quality parameters published for other viperid species (Almeida-Santos et al., 2004;

Zacariotti et al., 2007; Mozafari, Shiravi, and Todehdehghan, 2012; Moshiri, Todehdehghan, and Shiravi, 2014; Coeti et al., 2020; Blank et al., 2022).

In the effort to combat global biodiversity loss, banking of viable reproductive cells and tissues is considered essential. A significant first step to cryopreservation methodologies for this purpose is knowing when to collect gametes (Bolton et al., 2022; Coeti et al., 2020). With the knowledge gained from my research, we can now begin to optimize cryopreservation protocols by identifying the best periods during which to focus spermatozoa collection. Spermatozoa samples high in both concentration and quality are essential to the development of both assisted reproductive technologies and genome resource banking, as spermatozoa begin to decrease in fertilization capability from the moment they are collected (i.e. during the cryopreservation process) (Graham, 2001). This chapter provides a vital first step in developing gamete handling protocols and facilitating the development of assisted reproductive methodologies. Further, the information gained from my research broadens our understanding of the reproductive biology of two at-risk snake species in Canada.

### *4.3 Chapter Three*

My second data chapter (Chapter 3) evaluated annual variation of sexual steroids, testosterone and progesterone, in eastern massasauga rattlesnakes (*Sistrurus catenatus*). I used faecal samples collected from seven male (n= 93) and four female (n=56) *ex situ* eastern massasauga rattlesnake between March 2023, and September 2024. I found annual trends in expression from males and females, respectively but not the trends as I had predicted. Male eastern massasauga rattlesnake exposed to brumation had increased FTM in the subsequent mating season. As expected, both FTM and FPM expression increased with age. My research provides deeper comprehension of the reproductive function within the *ex situ* eastern massasauga rattlesnake population that is part of the recovery effort in Ontario. Our results differ from other North American rattlesnake species implying that the animals sampled may not yet be sexually

mature despite their size (Petersen et al. 2024). Broadly, our results suggest husbandry practices may influence the reproductive function of eastern massasauga rattlesnake, as has been demonstrated in other temperate squamates (Sacerdote-Velat et al., 2014; Louth and Heatley, 2020). Chapter 3 is a vital first step in understanding the reproductive endocrinology eastern massasauga rattlesnake. Incorporating these insights into management of the *ex situ* population may improve efforts to produce offspring for conservation translocations, ensuring that the goals of this recovery activity can be achieved.

#### 4.4 Strengths and limitations

The strengths and limitations of my research are indicated in each chapter. Here, I expand on the relationship between integrated “One Plan Approach” (Byers et al., 2013) conservation programs, emphasizing how the results of my research can be applied to recovery of the eastern massasauga rattlesnake in Ontario. In December 2022, 196 countries adopted the Kunming-Montreal Global Biodiversity Framework at the UN Biodiversity Conference, which contains targets specifically outlining the need to adopt a One Plan Approach to conservation (CBD, 2022). The integration of *in situ* and *ex situ* conservation within the One Plan Approach can help to reverse species declines (McGowan et al., 2017). Moreover, *ex situ* research has made contributions to the fields of evolution, behaviour, reproductive physiology, genetics, and welfare, an observation that has been recognized for more than 30 years (Ryder and Feistner, 1995). A challenge to surmount is skepticism of the value of findings from such research on *in situ* applications (relative to data from wild population; e.g. see Miranda et al., 2023).

By conducting research on animals in *ex situ* facilities, detailed records of life history such as age, previous reproductive experience, and health can be used in analyses, data that are often not available from wild population (Minteer and Collins, 2013). Thus, we can explore how development of individual animals may affect reproduction. Further, using animals held *ex situ*, we can access the same animals for repeated measurements (Minteer and Collins, 2013). When conducting physiology studies, repeated

measures of an animal allow us to account for individual temporal variation and responses, which may confound species-level characterization in wild populations (Sullivan, 2008). Results from both data chapters suggest that the ontogeny of squamate species can be altered through husbandry practices with significant impact on reproduction (Sacerdote-Velat et al., 2014; Louth and Heatley, 2020; Blais et al., 2023).

Both gray ratsnake and eastern massasauga rattlesnake are at-risk in Ontario (COSSARO, 2023). Sampling individuals *in situ* requires significant investment of time to locate individuals (COSEWIC 2012; 2018). Research may be further confounded by the inability to reliably sex these species in the field (COSEWIC 2012; 2018). Both the predation of tracked snakes and the fossorial nature of many species limits recovery *in situ* (Smith, Schuett, and Schwenk, 2010; Lind et al., 2010). While tracking technologies (e.g. radiotracking) can facilitate relocating individual animals *in situ*, there is no assurance males will be able to be retrieved safely for repeated measures (Smith, Schuett, and Schwenk, 2010; Lind et al., 2010). The overall cost of field work can be prohibitive. As such, refining gamete handling procedures prior to expending resources *in situ* will help apportion funds among multiple conservation initiatives and ensure that, when *in situ* sampling is done for assisted reproductive technologies, high concentration and quality gametes can be retrieved.

#### *4.5 Implications for future research*

My research expands our understanding of reproductive variation in gray ratsnake and eastern massasauga rattlesnake but also suggest several future avenues of investigation. As I noted in Chapter 2, the results of digital manipulation spermatozoa collection procedures with eastern massasauga rattlesnake differed from what has previously been reported for vipers (Almeida-Santos et al., 2004; Zacariotti et al., 2007; Mozafari, Shiravi, and Todehdehghan, 2012; Moshiri, Todehdehghan, and Shiravi, 2014; Coeti et al., 2020; Blank et al., 2022). One possible reason is that the original protocol described by Mengden et

al., (1980) is not suitable to produce spermatozoa samples in eastern massasauga rattlesnake. Experiments with other live-viperids including *Bothrops* and *Crotalus* species have altered the protocol to include application of a local anaesthetic to the cloacal region (Zacariotti et al., 2007; Blank et al., 2022). Future experiments incorporating this revised methodology may improve collection of spermatozoa from eastern massasauga rattlesnake.

My results show that high concentration and quality (motility, viability, and RFP) gray ratsnake spermatozoa can be collected throughout the mating season, and thus we can begin to develop additional assisted reproductive technologies (e.g. artificial fertilization procedures). Fertilization via artificial insemination in the cornsnake (*Pantherophis guttata*) has been performed with limited success *ex situ* (Mattson et al., 2007; Oliveri et al., 2018). Regardless, this provides a method that can be refined and applied to gray ratsnake. Efforts to realize successful artificial fertilization would be facilitated by additional studies of species-specific female reproductive annual variation (Comozzoli et al., 2018) and use of augmented technologies. For example, ultrasound may prove useful to quantify vitellogenesis and egg development (Vieira et al., 2024).

While viable spermatozoa appears to be available throughout the mating season, my results highlight a specific period when optimal spermatozoa could be collected from gray ratsnake. This would improve the outcome of genome resource banking. The long-term maintenance of viable cells through cryopreservation require specialized spermatozoa handling techniques and holding media to protect cells from the effects of extreme cold (Ballou et al., 2023). Regardless of the techniques employed, some cell death is expected. As such, highly concentrated motile cells are preferred (Barbas and Mascarenhas, 2009). My results will facilitate the next step in genome resource banking development for the species – development of suitable cryopreservation protocols.

In Chapter 3, I found annual trends in FTM and FPM of eastern massasauga rattlesnake held at a conservation mating center in Southern Ontario but not in the expected pattern. Future studies should further quantify hormone profiles in these animals as they age through to the expected age of sexual

maturity of eastern massasauga rattlesnake *in situ*, regardless of body size (5-6 years; COSEIWC, 2012). Such research will help to more accurately correlate size and age in relation to reproductive function within the species. Future brumation trials on both male and female eastern massasauga rattlesnake would allow us to strengthen our conclusions on the effects of husbandry on reproductive function. Finally, targeted blood sample collection, though more invasive, would likely allow for a better comprehension of hormone concentration variation that is acute in nature and therefore may not be detected by faecal sample enzyme linked immunosorbent assay (Palme, 2019).

#### 4.6 Conclusion

My research focused on the refinement of known methodologies to monitor annual variation in the reproductive traits of two Canadian at-risk snake species. My work contributes to our understanding of gray ratsnake and eastern massasauga rattlesnake reproductive biology at their northern range limit and lays the foundation for further research to develop assisted reproductive technologies and genome resource banking to assist with conservation efforts of these and other snake species. My research highlights how species-specific annual reproductive variation in snakes affects spermatozoa recovery and that husbandry practices may affect reproductive function. My results imply that the reproductive capabilities of the *ex situ* eastern massasauga rattlesnake population may be limited by low spermatozoa counts. This could be a result of the young age of individuals within the *ex situ* population but tests for inbreeding and local adaptation in wild population would be valuable. Continuing long-term endocrine studies of this captive eastern massasauga rattlesnake population through to sexual maturity would facilitate a better understanding of my results. Regardless, integration of my findings into the conservation mating program will likely help to improve fecundity, ensuring that captive population can fully meet its goals for the species.

#### *4.7 Funding*

This work was funded by the African Lion Safari, Amphibian and Reptile Conservation Canada, Queen's University, Natural Sciences and Engineering Research Council of Canada Discovery Grant Program, and Mitacs Accelerate.

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## **Appendix A: Chapter two supplemental results: top statistical models identified in gray ratsnake and eastern massasauga rattlesnake spermatozoa analysis.**

For my second data chapter, we created generalized linear mixed models to test for effects of Julian date (DOY) on seminal parameters (concentration, motility, RFP, and viability) using the “lme4” (Bates et al., 2015) R package with the “glmmTMB” R package applied to account for mixed beta regression models (Brooks et al., 2017). Mixed models allow for the introduction of random effects into the models which assist the model in accounting for inference about the fixed effects (Harrison et al. 2018). Fixed effects investigated for both gray ratsnake and eastern massasauga rattlesnake included Julian date (DOY), age, body condition index (BCI), brumation, extender, and the difference in temperature between room and cloaca ( $\Delta$  temp.). Mixed models were used to include random effects of individual animals and their relative husbandry (location) (Zuur et al., 2009). Using the R package “MuMIn” (v1.47.5; Bartoń, 2023), we used the dredge function to test for significant fixed effects on each male spermatozoa characteristic using generalized linear regression mixed models (Sandfoss, Reichling, and Roberts, 2023). We employed AIC<sub>c</sub> model selection, identified all best model(s) as those with the lowest AIC<sub>c</sub> value, and reported them in the results section of Chapter 2. The following tables contain the results of models lying within  $\Delta 2$  AIC<sub>c</sub> of the best model reported in the main text (Burnham and Anderson, 2002).

Our analysis of gray ratsnake (*Pantherophis spiloides*) identified four top models for spermatozoa percent motility (Table A1); two top models for spermatozoa percent viability (Table A2); six top models for concentration (Table A3); and four top models for spermatozoa rapid forward progression (Table A4). Our analysis of eastern massasauga rattlesnake (*Sistrurus catenatus*) identified three top models for spermatozoa percent motility (Table A5); two top models for spermatozoa percent viability (Table A6); three top models for concentration (Table A7); and three top models for spermatozoa rapid forward progression (Table A8).

**Table A1.** Top four models selected by AIC<sub>c</sub> for gray ratsnake spermatozoa percent motility. Age of the animals and temperature change ( $\Delta$  temp.) were included in all four top models, the effect of brumation was included in three of four top models, while the effects of extender were included in three of four top models, and the effects of body condition index (BCI) in one of four top models.

Beta mixed effects structure	AIC <sub>c</sub>	$\Delta$ AIC <sub>c</sub>
Motility ~ age + brumation + extender + $\Delta$ temp. + (1   animal) + (1   location)	-289.5	0.0
Motility ~ age + extender + $\Delta$ temp. + (1   animal) + (1   location)	-288.6	0.85
Motility ~ age + brumation + extender + $\Delta$ temp. + (1   animal) + (1   location)	-287.9	1.58
Motility ~ age + BCI + brumation + $\Delta$ temp. + (1   animal) + (1   location)	-287.5	1.97

**Table A2.** Top two models selected by AIC<sub>c</sub> for gray ratsnake spermatozoa percent viability. The effects of Julian date (DOY), brumation, and temperature change ( $\Delta$  temp.) were included in both top models, while the effect of body condition index (BCI) was included in one of the two top models.

Beta mixed effects structure	AIC <sub>c</sub>	$\Delta$ AIC <sub>c</sub>
Viability ~ brumation + DOY + $\Delta$ temp. + (1   animal) + (1   location)	-89.0	0.0
Viability ~ BCI + brumation + DOY + $\Delta$ temp. + (1   animal) + (1   location)	-87.1	1.94

**Table A3.** Top six models selected by AIC<sub>c</sub> for gray ratsnake spermatozoa concentration (conc.). The effects of Julian date (DOY) and extender were included in all six top models; the effect of brumation was included in three of six top models, the effect of temperature change ( $\Delta$  temp.) was included in two of six top models, and the effect of age in two of six top models.

Gamma log linked mixed effects structure	AIC <sub>c</sub>	$\Delta$ AIC <sub>c</sub>
Conc. ~ DOY + extender + $\Delta$ temp. + (1   animal) + (1   location)	6059.9	0.0
Conc. ~ DOY + extender + $\Delta$ temp. + brumation + (1   animal) + (1   location)	6060.2	0.32
Conc. ~ DOY + extender + brumation + (1   animal) + (1   location)	6060.4	0.52
Conc. ~ DOY + extender + brumation + age + (1   animal) + (1   location)	6060.5	0.57
Conc. ~ DOY + extender + (1   animal) + (1   location)	6060.5	0.62
Conc. ~ DOY + extender + age + (1   animal) + (1   location)	6061.3	1.38

**Table A4.** Top four models selected by AIC<sub>c</sub> for gray ratsnake spermatozoa rapid forward progression. The effects of Julian date (DOY) and extender were included in all four top models. The effect of body condition index (BCI) was included in three of four top models and the effect of temperature change ( $\Delta$  temp.) was included in three of four top models and the effect of brumation were only included in one of four top models.

Gamma log-linked mixed effects structure	AIC <sub>c</sub>	$\Delta$ AIC <sub>c</sub>
RFP ~ DOY + extender + BCI + $\Delta$ temp. + (1   animal) + (1   location)	590.8	0.0
RFP ~ DOY + extender + BCI + (1   animal) + (1   location)	592.1	1.38
RFP ~ DOY + extender + $\Delta$ temp. + (1   animal) + (1   location)	592.2	1.45
RFP ~ DOY + extender + BCI + $\Delta$ temp. + brumation + (1   animal) + (1   location)	592.6	1.84

**Table A5.** Top three models selected by AIC<sub>c</sub> for eastern massasauga rattlesnake spermatozoa percent motility. Julian date (DOY) and temperature change ( $\Delta$  temp.) were included in all three top models. Both age and body condition index (BCI) were each included in one of three top models.

Beta mixed effects structure	AIC <sub>c</sub>	$\Delta$ AIC <sub>c</sub>
Motility ~ DOY + $\Delta$ temp. + (1   animal) + (1   location)	-556.9	0.0
Motility ~ DOY + $\Delta$ temp. + BCI + (1   animal) + (1   location)	-555.4	1.47
Motility ~ DOY + $\Delta$ temp. + age + (1   animal) + (1   location)	-555.1	1.75

**Table A6.** Top two models selected by AIC<sub>c</sub> for eastern massasauga rattlesnake spermatozoa percent viability. The model with the lowest AIC<sub>c</sub> only included the random effects of individual and location indicating that none of the fixed effects tested have significant effects on spermatozoa viability. Brumation is included in one of the two top models therefore cannot be definitively included or excluded as in the final model.

Beta mixed effects structure	AIC <sub>c</sub>	$\Delta$ AIC <sub>c</sub>
Viability ~ (1   animal) + (1   location)	-3.1	0.0
Viability ~ brumation + (1   animal) + (1   location)	-2.5	0.59

**Table A7.** Top three models selected by  $AIC_c$  for eastern massasauga rattlesnake spermatozoa concentration. The effects of body condition index (BCI) and age were each included in one of three top models each.

<b>Gamma log linked mixed effects structure</b>	<b><math>AIC_c</math></b>	<b><math>\Delta AIC_c</math></b>
Concentration $\sim$ (1   animal) + (1   location)	216.0	0.0
Concentration $\sim$ BCI+ (1   animal) + (1   location)	217.2	1.21
Concentration $\sim$ age + (1   animal) + (1   location)	217.3	1.25

**Table A8.** Top three models selected by  $AIC_c$  for eastern massasauga rattlesnake spermatozoa rapid forward progression. The effect of brumation, age, and extender were each included in one of three top models and the effects of body condition index (BCI) are included in two of three top models.

<b>Gaussian mixed effects structure</b>	<b><math>AIC_c</math></b>	<b><math>\Delta AIC_c</math></b>
RFP $\sim$ BCI + (1   animal) + (1   location)	138.3	0.0
RFP $\sim$ BCI + brumation + (1   animal) + (1   location)	138.8	0.56
RFP $\sim$ age + extender + (1   animal) + (1   location)	139.8	1.56

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## **Appendix B: Commercial enzyme-linked immunosorbent assay analytical validations for eastern massasauga rattlesnake.**

The analysis of steroid hormones testosterone, progesterone, and estradiol can provide insight into rattlesnake reproductive function (Taylor and Booth, 2016). However, enzyme-linked immunosorbent assays (ELISA) need to be validated on a species-by-species basis (Palme, 2019; Table B1). For this experiment, we began with three commercial ELISA kits: DetectX® Testosterone ELISA Kit, DetectX® Progesterone ELISA, and DetectX® Estradiol ELISA Kit (Arbour Assays, MI, USA). The choice to use commercially available kits was made as they can be sourced across North America and thus methods could be replicated by animal managers at any facility. All DetectX® kits are sold for multi-species use with faecal extract but require validation when used on a novel species. A summary of the validation processes we used are provided in Table B1 (Mondol et al., 2019; Palme, 2019; Enzo Biochem Inc. 2023; Berkvens et al., 2013; S. Yakimowski, personal communication, October 2024). Results of statistical validations can be found in Table B2. Eastern massasauga rattlesnake (*Sistrurus catenatus*) species specific validation was successful for testosterone and progesterone kits. However, detection experiments with the DetectX® Estradiol ELISA kit never yielded the required 50% binding over a 1:5 dilution. Previous research of circulating estrogen in North American rattlesnake species indicates concentrations peak during vitellogenesis otherwise remaining at low levels throughout the year (Taylor and Booth, 2016). Possibly, the faecal samples obtained were from individuals that never achieved this reproductive status, which would explain why a pooled faecal sample would not yield substantial levels of faecal estradiol metabolites to be analyzed by ELISA.

**Table B1.** ELISA analytical validations following previously published methods for detection (Mondol et al., 2019), parallelism (Palme, 2019), intra-assay validation (Enzo Biochem Inc. 2023), inter-assay validation (Enzo Biochem Inc. 2023), extraction efficacy (Berkvens et al., 2013), and repeatability (S. Yakimowski, personal communication, October 2024).

<b>Validation</b>	<b>Goal</b>	<b>Methods</b>
Detection	Sensitivity	Pooled faecal extract diluted in a series and run along side standard curve to determine which dilution results in 50% binding (Mondol et al., 2019).
Parallelism	Accuracy	Serial dilutions of faecal extracts run along side standard curve to determine if the resulting values are in parallel with the standard curve (Palme, 2019).
Intra-assay validation	Precision	Samples run on plate on mass and variation between results compared (Enzo Biochem Inc. 2023).
Inter-assay validation	Precision	Standard curve results from all plates used for each hormone study compared (Enzo Biochem Inc. 2023).
Extraction efficacy	Sensitivity	Pooled faecal samples spiked with known amount of hormone run along side un-spiked pooled sample, extraction efficacy calculated as amount observed/amount expected (Berkvens et al., 2013).
Repeatability	Accuracy	Faecal extracts run on the same samples a minimum of six months apart (S. Yakimowski, personal communication, October 2024).

**Table B2.** Results of statistical analysis for eastern massasauga rattlesnake faecal hormone validation experiments. Testosterone and progesterone ELISA kits were able to be validated for use when samples were diluted 1:30 on the assay plate. Estradiol validation was unsuccessful.

<b>Hormone</b>	<b>Detection result - Dilution</b>	<b>Parallelism result - Slope (R2)</b>	<b>Inter-assay CV</b>	<b>Intra-assay CV</b>	<b>Extraction Efficacy - MeOH</b>	<b>Extraction Efficacy - Faeces</b>	<b>Repeat of samples CV</b>
Testosterone	1:30	0.90	22.30	8.81	99%	24%	22.33
Progesterone	1:30	0.96	9.38	5.24	105%	50%	18.73
Estradiol	1:5*	NA	NA	NA	NA	NA	NA

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