# Should I mate or should I wait? The morphology of sperm storage, and its consequences for sperm viability and mating strategies in a temperate skink species, *Niveoscincus ocellatus*.

by

Mathieu Russell

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To a new chapter…

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This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by the way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledge is made in the text, nor does the thesis contain any material that infringes copyright.

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Mathieu Russell

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## Abstract:

Sperm storage is a central phenomenon to the reproductive cycle of a wide range of vertebrate species. Although the morphological mechanisms of sperm storage and its consequences on sperm viability have been described separately in many taxa, there is a paucity of studies which have combined detailed descriptions of morphological variation with accurate estimates of sperm viability and its links to male and female mating strategies in the wild. This is particularly true in reptiles, despite the relatively high occurrence of sperm storage within this group. This thesis addresses these shortcomings by investigating seasonal morphological changes in sperm storage organs, along with temporal variation in sperm viability in epididymal sperm and its links to mating patterns in *Niveoscincus ocellatus*, a temperate-climate reptile with both male and female sperm storage. I show that *N. ocellatus* sperm viability varied throughout the mating period, being most viable early in the mating season before decreasing in viability later. In accordance with these changes, mating patterns also varied across the mating season. Specifically, copulations were more abundant early in the season (when sperm were most viable and abundant) compared to late in the season when copulation frequency was significantly lower. In contrast, I found no evidence for variation in mate choice across the mating season; males preferred large, unmated females to small, promiscuous ones throughout. In addition, I observed structural changes in sperm production and storage organs (e.g., the germinal epithelium and seminiferous tubules of the testes, the epithelium and lumen of the epididymal ducts, and the musculature, ciliation, lumen, and secretory activity of oviductal segments) across the mating season. In most cases these changes are closely linked to the variation observed in reproductive cycles and mating patterns reported here and elsewhere. Specifically, the epididymides varied in size in accordance with sperm abundance and usage and closely corresponded with the observed mating patterns. The cauda epididymis swelled in size as sperm were transferred into this storage location, and decreased in size as the mating season progressed and sperm abundances decreased. Additionally, the female oviduct underwent significant morphological changes in conjunction with vitellogenesis, and secretory activity was

heightened during periods of mating activity and sperm storage, increasing in concentration at the start of the mating season. This work also generated several additional observations. For example, I found that oviductal sperm storage occurs in different locations to those previously described for skinks (i.e. the anterior and posterior vagina, as opposed to the infundibulum), supporting recent suggestions that morphological mechanisms of sperm storage vary between species much more than once believed. Furthermore, I found that spermatogenesis begins in late autumn, but a long refractory period halts sperm production until late summer, when spermatogenesis resumes in preparation for the beginning of the mating season in early autumn. This expands on previous work which implied that spermatogenesis began in late summer. Finally, I found a rise in live sperm proportions in conjunction with a significant decrease in sperm abundance in spring, suggesting that, dead or defective sperm are either evacuated, or leak out of the epididymides to increase mating success from copulations occurring in their second period of mating activity.

In summary, my results confirm previous descriptions of the reproductive cycle of male and female *N. ocellatus* and provide further information as to the progression of spermatogenesis, and the location of sperm storage in the female reproductive tract. Many of these changes correlate with changes in sperm production, usage and viability, and ultimately variation in mating patterns. This thesis thus provides an insight into the consequences of sperm storage in temperate-climate reptiles and the morphological features underlying it. In doing so, I provide an opportunity for further research to investigate the consequences of mating strategies with respect to sperm storage, for fertilization success, offspring phenotype and ultimately reproductive success.

# CHAPTER ONE

## General Introduction:

Sperm storage describes the retention of live sperm within the reproductive tract of either males or females, and has evolved across a wide range of taxa (Parker, 1970; Birkhead and Møller, 1993; Olsson and Madsen, 1998; Holt and Lloyd, 2009). Sperm storage can last for as little as a few days in some mammals, to weeks or months in bats, fish and amphibians, and several years in some reptiles (Birkhead and Møller, 1993; Holt and Lloyd, 2009). Although many studies have described the occurrence of sperm storage, its duration, and its adaptive advantages, reviews have highlighted the paucity in our understanding of the mechanisms involved in sperm storage, and have emphasized the need to study sperm storage in more species (Girling, 2002; Holt and Lloyd, 2009).

Since its discovery, many hypotheses have been generated to explain why animals store sperm (Parker, 1970; Birkhead and Møller, 1993; Simmons, 2005; Holt and Lloyd, 2009). Two hypotheses are generally accepted to account for the majority of occurrences of sperm storage. The first suggests that sperm storage arose as a fertility insurance mechanism. This has been suggested for systems in which mate encounter rates are low. In such systems, sperm storage would improve the chances of viable sperm occurring in the oviduct at time of fertilization and allow for the temporal separation of copulation from ovulation (Connor and Crews, 1980; Saint Girons, 1982; Birkhead and Møller, 1993). Alternatively, it has been suggested that sperm storage is the result of temperature-dependent optimisation of gamete production. Specifically, spermatogenesis requires body temperatures above 20°C and a minimum of 8 to 10 weeks of favourable conditions whereas vitellogenesis is dependent on stored fat reserves (Aldridge, 1975; Joly and Saint Girons, 1975; Saint Girons, 1985; Jones et al., 1997), allowing male and female reproductive cycles to become disassociated and thus, resulting in a protracted mating season which requires long-term sperm storage (Jones et al., 1997; Murphy et al., 2006). This explanation has been suggested to be particularly relevant to reptiles with high encounter rates.

Irrespective of the ultimate mechanisms underlying it, the evolution of sperm storage has a number of important consequences for adaptive evolution. The temporal separation of male and female reproductive cycles, for example, requires sperm to be stored throughout the mating season which spans the interval between spermatogenesis and ovulation. As sperm degrade with age (Millar, 1972; Vishwanath and Shannon, 1997; Jones, 2004; Pizzari et al., 2008a), this can have significant consequences for individual fitness. Variation in sperm quality as a result of sperm storage can have significant implications for sperm viability, and in turn act as a strong selective force underlying mating strategies and mate choices in order to circumvent the consequences of sperm degradation during storage (Preston et al., 2001; Olsson et al., 2004; Kelly and Jennions, 2011). Males should optimise their mate choices to maximise the likelihood of fertilizing ova, and ultimately their reproductive success. Indeed, males are known to show preference for female fecundity in a wide range of taxa in order to maximise the number of ova they fertilize (Olsson, 1993; Andersson, 1994; Jones et al., 2001). Additionally, males should also optimise their mate choices for less promiscuous females as well; this would minimise sperm competition risks and increase siring success (Parker, 1970; Trivers, 1972). Furthermore, if sperm quality is compromised as the season progresses, males should also be choosier early in the season when sperm viability is highest, and less discriminating later, when sperm viability is lower (Hardling et al., 2008; Cornwallis and O'Connor, 2009; Morse, 2010; Kelly and Jennions, 2011). Despite this, the majority of studies on sperm storage have focussed on the evolution of anatomical and physiological adaptations for sperm storage, and few have considered the consequences of sperm storage for mate choices (Sever and Hopkins, 2004; Demont et al., 2011).

The need for spermatozoa to be viable at the time of fertilization has selected for a variety of different mechanisms to sustain it over lengthy periods of time (Birkhead and Møller, 1993; Girling, 2002; Holt and Lloyd, 2009). In females, sperm storage is known to occur in the lumen of the vagina, cervix, or uterus of mammals, as well as in specialized sperm storage tubules in some birds or reptiles (see Holt and Lloyd, 2009). Fish have also developed several other mechanisms such as direct storage within ovarian follicles to maintain sperm viability between mating and fertilization (Potter

and Kramer, 2000; Vila et al., 2007; Storrie et al., 2008). In contrast, males tend to all store sperm in a similar manner: once sperm are produced in the seminiferous tubules of the testes, they travel through the epididymides and are stored in its posterior portion until they are ejaculated (Marion, 1982; Flemming, 1993; Van Wyk, 1995; Ibargüengoytía, 2004). The different sperm storage mechanisms result in variation in the quality of sperm available at fertilization, which in turn is likely to act as a strong proximate mechanism underlying male and female mating patterns, such as when an individual mates (Parker, 1970; Trivers, 1972; Galvani and Johnstone, 1998; Preston et al., 2001; Sato et al., 2006; Cornwallis and O'Connor, 2009). Importantly, the timing of copulation will determine the length of time sperm are stored by each sex, and should be optimised to maximise the viability of sperm at fertilization. The timing of copulation could thus have important repercussions on sperm quality at fertilization, offspring phenotype, and ultimately reproductive success (Olsson et al., 2007; Pizzari et al., 2008b; Olsson et al., 2009). Despite this, few studies have compared the morphological mechanisms associated with sperm storage in males and females across a mating season, let alone the consequences of sperm storage for mating strategies.

Despite the high occurrence of sperm storage in reptiles, there is a paucity of studies which have investigated sperm storage and its consequences in this taxon. Since mate encounter rates are generally high in many reptiles (Uller and Olsson, 2008) it is suggested that sperm storage has evolved as a result of temperature-dependent spermatogenesis being temporally separated from fertilization due to environmental constraints (Saint Girons, 1985; Birkhead and Møller, 1993). Since females commonly synchronize their biological processes with favourable environmental conditions in order to maximise offspring survival and their reproductive success (Heatwole and Pianka, 1993), their reliance on environmental temperatures has resulted in geographical variation in their reproductive cycles (Heatwole and Taylor, 1987; Wapstra et al., 1999). In Australia, for example, 11 different reproductive cycles have been described, differing in the time of year when spermatogenesis and ovulation take place, as well as the duration of their mating season, and thus, the length of time for which sperm needs to be stored (Heatwole and Taylor, 1987). In males, by contrast, reproductive cycles are much more diverse than in females. Heatwole and Taylor

(1987) divided their 11 reproductive cycles into two main groups: species with spring ovulation and spring spermatogenesis (Type I), and species with spring ovulation and autumn spermatogenesis (Type II). As reproductive cycles have evolved to vary in response to the environment, there have been concurrent requirements for sperm storage duration, and a large variation in mechanisms allowing for the storage of sperm. The females of some species, for example, store sperm in their infundibulum, whereas others do so in their vagina, and some do so multiple areas (see Sever and Hamlett, 2002). There is also considerable morphological variation within these regions as well: some species store sperm in special storage tubules whereas others store sperm in crypts, glands, epithelial folds or in loose bundles in the lumen (Bou-Resli, 1981; Saint Girons, 1973; Connor and Crews, 1980; Perkins and Palmer, 1996; Girling et al., 1997). Furthermore, some species have also been reported to produce acidic secretions in sperm storage areas as well, perhaps in aid of sperm survival within the female reproductive tract (Girling, 2002; Sever and Hamlett, 2002; Holt and Lloyd, 2009). Despite considerable work having documented variation in sperm storage mechanisms between species, however, the reasons for this interspecific variation, and the consequences of sperm storage on mating patterns remain unknown.

This thesis investigates the influence of sperm viability on the timing of copulations in a temperate-climate reptile in conjunction with mate choices, and also provides a morphological description of sperm storage mechanisms. In doing so, I aim to better understand the consequences of sperm storage for mating patterns, and the morphological patterns that underlie them. *Niveoscincus ocellatus* is a small viviparous lizard (adult snout-vent-length (SVL) = 55-85 mm) endemic to Tasmania, a temperate island south of Australia, occurring in rocky outcrops between sea level and 1200m of elevation (Wapstra and Swain, 2001). This species has temporally separated gonadal cycles with spermatogenesis occurring in late-summer and ovulation in late spring (Jones et al., 1997; Wapstra et al., 1999), and long-term sperm storage between these two reproductive events. Consequently, *N. ocellatus* also have a protracted mating season; they begin mating in early autumn and end in late spring, creating a large period of time in which mating can occur (Jones et al., 1997; Wapstra et al., 1999). Epididymal sperm storage takes place prior to copulation, and sperm storage by

females follows and lasts until females ovulate and fertilization occurs (Jones et al., 1997; Wapstra et al., 1999; While and Wapstra, 2009). All female *N. ocellatus* copulate in autumn, prior to winter, and later, 30-40% of females copulate again in spring, after emergence from winter hibernation (Jones et al., 1997; Wapstra et al., 1999). Thus, the timing of copulation determines the duration of epididymal and oviductal sperm storage in this species; females receiving copulations early in the year store sperm for more time than females that copulate later do so, and these latter receive sperm that has been stored longer in the epididymides. Despite this, we do not yet know the reason for this variation in copulation phenology. Furthermore, despite *N. ocellatus'* spermatogenic cycle being described as occurring prior to the mating season (Type II, Heatwole and Taylor, 1987; Jones et al., 1997; Wapstra et al., 1999), detailed descriptions of sperm storage locations, structures and morphological mechanisms involved in sperm storage do not exist for either sex of this species.

This species' dissociated gonadal cycles and lengthy sperm storage across a protracted mating season make it ideal for studying the consequences of variation in sperm viability on mating patterns in a temperate-climate reptile, as well as how their morphological mechanisms for sperm storage compare to other, previously documented squamate reptiles. The purpose of this thesis is thus two-fold. Firstly, to present anatomical descriptions of the testes, epididymides and oviducts of *Niveoscincus ocellatus* throughout their mating season in order better understand the morphological mechanisms involved in sperm storage in this species. Secondly, to conduct analyses of epididymal sperm viability in conjunction with field-based documentations of mating patterns in order to better understand the consequences of sperm storage for sperm viability and the resulting mating patterns.

N.B.

The following two chapters have been formatted for independent submission to peer-reviewed journals and therefore, some repetition exists between chapters. Personal pronouns have also been changed from first person singular (I) to first person plural (we) due to co-authors being involved in their creation.

# CHAPTER TWO

# Temporal variations in mating phenology in relation to sperm viability in *Niveoscincus ocellatus*, a temperate-climate reptile with long-term sperm storage.

**Mathieu Russell, Geoff While, Susan M. Jones, and Erik Wapstra**

*School of Zoology, University of Tasmania. Private Bag 5, Hobart, Tas 7001, Australia*

**Abstract**

Sperm storage is an intrinsic component of the mating systems of a wide range of vertebrate species. Although the effects of sperm age and degradation on fertilization potential and offspring phenotype are well documented, we lack an understanding of the influence of sperm storage on mating strategies and reproductive success. We investigated the patterns of viability of stored sperm in conjunction with the timing of copulations in *Niveoscincus ocellatus*, a temperate-climate reptile with a protracted mating season and sperm storage in both sexes. Analyses of sperm viability revealed sperm viability to be high at the beginning of the mating season, and to gradually decrease throughout it. Interestingly we noticed a rise in live sperm proportions late in the mating season which suggests the presence of a process that removes dead sperm from the epididymides. Additionally, *N. ocellatus* copulated most often when sperm viability was highest, and males also showed preferences for larger females, and unmated females over smaller or more promiscuous ones. Thus, copulation

patterns and mate choices in *N. ocellatus* were driven by sperm viability and the likelihood of fertilizing ova; however, their effects on reproductive success still require further investigation.

## Introduction

Sexual selection is a consequence of variance in male and female reproductive success and the diverse mating strategies employed by each sex to maximize the quantity of fit offspring they produce (Shuster and Wade, 2003). Therefore, if we are to understand how sexual selection operates within a system, we need to understand the mechanisms underlying variance in male and female mating strategies as well as the consequences of this for reproductive success. One of the key mechanisms likely to influence male and female mating strategies is the quantity of viable sperm available (Galvani and Johnstone, 1998; Preston et al., 2001; Sato et al., 2006; Sato and Goshima, 2007; Cornwallis and O'Connor, 2009). For example, as fertilization success has been shown to be strongly influenced by sperm quantity and quality (Millar, 1972; Vishwanath and Shannon, 1997; Jones, 2001; Lewis and Aitken, 2005; Simon et al., 2010), variation in the quality and quantity of the sperm that males and their rivals have should influence when, and with which females, males choose to mate (Preston et al., 2001; Olsson et al., 2004; Cornwallis and O'Connor, 2009; Kelly and Jennions, 2011). Therefore, factors which influence the availability of viable sperm are likely to act as strong proximate mechanisms underlying male and female mating strategies and should be studied further in order to understand their ultimate effect on variance in male and female reproductive success (Trivers, 1972; Parker, 1970; Galvani and Johnstone, 1998).

It is widely accepted that sperm cells degrade and die with age, and as a result, sperm age is tightly correlated with sperm viability (Millar, 1972; Vishwanath and Shannon, 1997; Jones, 2004; Pizzari et al., 2008a). Indeed, sperm age has been shown to

influence fertilization success, offspring morphology, and ultimately reproductive success in a range of species (Lewis and Aitken, 2005; Pizzari et al., 2008b; Olsson et al., 2007; Simon et al. 2010). This has obvious implications when species display sperm storage: an obligatory component of the reproductive cycle of a wide range of taxa where sperm are stored for an extended period of time (Birkhead and Møller, 1993; Sever and Hamlett, 2002). There is evidence that variation in the duration of sperm storage can have significant implications for sperm viability, and as a consequence, both fertilisation success and offspring phenotype (Olsson et al. 2007; Pizzari et al., 2008b; Olsson et al., 2009). The effectiveness of sperm storage within males, prior to copulation, should therefore act as a strong selective force underlying mating strategies. For example, variation in sperm quality as a result of sperm storage may select for variation in both mating effort *per se,* as well as mating phenology and mate choices (Preston et al., 2001; Olsson et al., 2004; Kelly and Jennions, 2011). Thus far, however, the majority of studies on sperm storage have focussed on the evolution of anatomical and physiological adaptations that maximize storage capabilities of females, and few have studied sperm storage in males; field-based studies investigating the consequences of sperm storage on mating patterns in the wild are lacking (Sever and Hamlett, 2002; Demont et al., 2011).

Sperm storage is particularly prominent in reptiles, with the females of certain species storing sperm for up to seven years (Birkhead & Moller 1993; Sever & Hamlett 2002). Two main hypotheses have been suggested to explain the occurrence of sperm storage in reptiles. The first suggests that reptiles have relatively low encounter rates, which would favour sperm retention mechanisms to reduce sperm deterioration

during the time between mate encounters (Birkhead and Moller 1993; Gist and Congdon 1998, Pearse and Avise, 2001); however, this seems an unlikely explanation given that encounter rates for the majority of reptile species are high (Uller & Olsson 2008). A more plausible explanation is that sperm storage is a consequence of temperature- dependent optimisation of gamete production. Specifically, spermatogenesis requires body temperatures above 20°C and a minimum of 8 to 10 weeks of favourable conditions (Aldridge, 1975; Joly and Saint Girons, 1975; Saint Girons, 1985; Jones et al., 1997). Since vitellogenesis is less dependent on thermal conditions, in some species, the different requirements of gamete maturation in males and females can result in dissociation between their reproductive cycles, a protracted mating season, and long-term sperm storage (Jones et al., 1997; Murphy et al., 2006). This is particularly the case in temperate reptilian species, for which their climate limits the time available for spermatogenesis. In many temperate species, males mate in both autumn and spring but only produce sperm once in early autumn and then store sperm: sperm storage eliminates the need for males to emerge from hibernation prior to females in spring and allows the same sperm stores to be used during both mating periods (Smyth and Smith, 1968; Heatwole and Taylor, 1987; Olsson et al., 1999). Such systems have the added facet of sperm being stored in both the male epididymides and the female reproductive tract; epididymal sperm storage occurs from spermatogenesis to when copulation occurs, and oviductal sperm storage takes place henceforth, until females ovulate. Importantly the timing of mating will determine the length of sperm storage by each sex, and should be optimised to maximise reproductive success. Additionally, males should also optimise their mate choices for more fecund and less promiscuous females to increase siring success and

minimise sperm competition risks (Parker, 1970; Trivers, 1972). Indeed, males show preference for female fecundity and favourable mating history in many reptile species (King, 2000; Olsson et al., 2002; Olsson et al., 2004). Despite how sperm storage and reproductive success can be influenced by male mating strategies, most studies on sperm storage have concentrated on female sperm storage (Fox, 1956; Saint Girons, 1962; Birkhead and Møller, 1993; Laloi *et al.,* 2004; Sever and Hopkins, 2004); few studies have focused on sperm storage in males, or on the consequences on mate choices or when copulations occur (see Sever and Hamlett, 2002).

Here we examined variation in male sperm viability and its consequences for male mating strategies within a temperate lizard species, *Niveoscincus ocellatus*. *N. ocellatus* has dissociated gonadal cycles by which spermatogenesis occurs in mid- to late-summer and females ovulate in mid-spring (Jones et al. 1997; Wapstra et al. 1999). As a result, males exhibit obligate epididymal sperm storage in autumn, prior to copulation, and females exhibit oviductal sperm storage post-copulation, until ovulation (Jones et al., 1997; Wapstra et al., 1999). Mating occurs in both autumn and spring, with a period of inactivity over the dividing winter (Jones et al., 1997; Wapstra et al., 1999). The lengthy sperm storage, long mating season, and division of the mating season into two distinct periods make this species ideal for examining how sperm viability may influence male mating strategies in species with prolonged sperm storage.

Specifically, this study had two aims. Firstly, to document the mating patterns of female *N. ocellatus* throughout the 2011 reproductive cycle and determine whether

females of a certain phenotype received more copulations than others. Secondly, to analyze the viability of epididymal sperm throughout the same reproductive cylce by means of dissections and microscopy in order to better understand the gonadal cycle and sperm storage mechanism of male *N. ocellatus*. Results from these observations would allow us to better understand the influence of changes in epididymal sperm viability on mating patterns in natural populations of *N. ocellatus*. We hypothesized that larger females would receive more copulations than smaller females, and that promiscuous females with many previous copulation scars would receive fewer subsequent copulations than females with fewer previous copulation scars. Additionally, we also hypothesized that sperm would degrade with time within the male epididymis and that as it did so, males would adjust their mate choices. We hypothesized that larger females would receive more copulations than smaller ones early in the mating season and that females with few previous copulation scars would receive more copulations than promiscuous females later in the mating season.

## Methods

### STUDY SPECIES

*Niveoscincus ocellatus* is a small viviparous lizard (adult snout-vent-length (SVL) = 55-85 mm; mass = 3-12 g) endemic to Tasmania, occurring in rocky outcrops between sea level and 1200 m of elevation (Wapstra and Swain, 2001). In this species, mating begins in early March, following a major peak in testes volume (Jones et al., 1997). The mating season continues until mid-May, when individuals go into torpor as a result of cold winter temperatures. Both sexes emerge from torpor in mid-August and the mating season resumes until females ovulate around October 1$^{st}$ (Wapstra et al.,

1999). Females mate with multiple partners over the course of the two mating periods (Atkins 2007). Although copulations are rarely observed, males bite the females during mating, leaving clear scars on the females` undersides. These marks are blue when fresh and darken over time; they last up to six months, and are useful indicators of female mating history (Fig 2.1, also see Jones et al., 1997, Wapstra et al., 1999; While and Wapstra, 2009).

**MATING PHENOLOGY**

Adult *N. ocellatus* females were captured between March 10th and 20th, August 18th and September 15th, and October 12th and 26th 2011 at a well-established study site in Eastern Tasmania (Wapstra et al., 1999; While and Wapstra 2009; Cadby et al. 2010; Pen et al., 2010). The chosen sampling times allowed us to determine how many copulations individual females received in early (Austral) autumn, late autumn, and spring respectively. Animals were captured by noosing and mealworm fishing and each individual's SVL (±1 mm) and mass (± 0.1g) were measured. Only mature females (i.e., > 55mm SVL) were used in this study; all smaller females were released immediately. Each adult female's copulation history was measured by recording the number, location, colour, and clarity of copulation marks on her underside. Females that had not been previously marked were permanently marked using toe-clipping, and then released at their site of capture within minutes of being caught. As these subject lizards are part of a larger life history study which has taken place across eleven consecutive reproductive seasons, 2000/2001 to 2010/2011 (see Wapstra et al., 1999; While and Wapstra 2009; Cadby et al. 2010; Pen et al., 2010), the majority of individuals had already been permanently marked previous to this study. Thus only

unmarked females were toe-clipped during this study (n=24, representing 10% of captures).

**EPIDIDYMAL SPERM STORAGE**

Adult *N. ocellatus* males were captured at an additional site in Eastern Central Tasmania (41°59S, 146°44E) using the same methods described above in order to not remove individuals from a long-standing field study (Pen et al., 2010). Specifically, eight adult males were sampled in each of six sampling periods (n = 48) during their nine-month reproductive cycle (1, early in the autumn mating season; 2, mid-way through the autumn mating season; 3, pre-hibernation; 4, mid-hibernation; 5, mid-way through the spring mating season; and 6, post-ovulation). Maturity was determined by size (SVL): males smaller than 55mm SVL, were released immediately. At each time period, adults were brought back to the terrestrial ecology laboratory at the University of Tasmania and housed communally in plastic terraria with a basking light at one end and a shelter at the other to allow individuals to thermoregulate. Food and water were available *ad libitium*. Within 48 hours of capture, males were euthanized by intraperitoneal injection of sodium pentobarbital (1ml/2kg diluted 1:100). Since collection of animals from the wild is impossible during winter hibernation, however, individuals required for the mid-hibernation sampling period were collected in the period prior to it instead and transferred to semi-natural outdoor enclosures until they were needed. Following euthanasia, a longitudinal incision was made along the ventrum of each animal and one epididymis was randomly chosen and dissected out intact. The epididymis was placed on a glass slide and 15µL of sperm were extracted from it by gently palpating the posterior end of the cauda (Fig. 2.2) with a blunt probe.

The second epididymis was also removed, along with both testes, for a concurrent histological study of these organs (see Chapter 3).

Each 15µL sperm sample was diluted with 90 µL of reptile Ringer's solution (0.112M NaCl, 5.097mM KCl, 5.605mM CaCl2, 2.21mM NaHCO3, pH 7.4) and 90 µL of HEPES-buffered saline solution containing Bovine Serum Albumen (10mM HEPES, 150mM NaCl, 10% BSA, pH 7.4) as a proxy for their natural storage environment (C. Sherman, pers. comm). A 15 µL aliquot of each dilution was stained with fluorescent dyes (SYBR 14 and Propidium Iodide from a Live/Dead® Sperm Viability Kit from Molecular Probes ®) and deposited on a glass slide. These stains make live sperm fluoresce green and dead sperm fluoresce red under ultraviolet light. Fluorescent microscopy (Axioskop 2 Plus Zeiss microscope) was then performed at 400 x using a filter set which accommodates for the emission and excitation wavelengths of both stains at once (Zeiss Filter Set 009). Live and dead sperm counts were taken from five fields of view for each sperm sample in order to calculate mean live sperm proportion as a proxy for sperm viability. These counts were made at two time intervals, after 30 minutes (Time 1), and after 120 minutes (Time 2) in order to determine sperm survival.

**STATISTICAL ANALYSIS**

To assess the time of year when copulations took place, only data for those females that were captured in all three sampling periods were used (n=25). We measured both the total number of copulations a female had received throughout the entire mating season (i.e., the cumulative number of copulations received across all sampling periods) as well as the number of copulations females received within each given

mating period (i.e. the number of fresh copulations in each sampling period). We calculated the latter by subtracting the number of copulatory scars visible in one mating period from the total number visible in the previous period (i.e., fresh copulations in late autumn = total copulations in late autumn – total copulations in early autumn). The length of the early autumn mating period was calculated as the number of days between the start of the mating season (March 1$^{st}$) and the date of first capture. The length of the late autumn mating period was calculated as the number of days between each female`s first and second capture, subtracted by 91 days to account for winter inactivity (May 16$^{th}$ to August 15$^{th}$). The end of the mating season was taken to be October 1$^{st}$, which is the date upon which females of this species are assumed to ovulate (Wapstra et al., 1999; Uller and Olsson, 2008; While and Wapstra, 2009). Thus the length of the spring mating period was the same for all females (spring = 45 days; August 15$^{th}$ to September 30$^{th}$). Female body condition was calculated using the residuals from regressions of mass over SVL in each mating period (early autumn, $r^2 = 0.70$, $p < 0.0001$; late autumn, $r^2 = 0.75$, $p < 0.0001$; spring, $r^2 = 0.77$, $p < 0.0001$).

The variation in the number of copulations females received in early autumn, late autumn, and spring was analyzed using a mixed logistic model (PROC GLIMMIX) with mating period as a nominal classification variable, female SVL and female body condition as covariates, and female identity as a random repeated factor. We also included the number of days between captures in order to control for differences in time available for females to acquire copulations in each mating period. Since the data were multinomial, the mixed logistic model was set to a Poisson distribution with

a logit link function (PROC GLIMMIX). We also examined the relationship between the number of copulations a female received across the entire mating season (total copulations) and a female's phenotypic traits (SVL and body condition) using a Spearman's correlation (PROC CORR). Finally, to examine whether male mate preference was influenced by a female's mating history, we examined the relationship between the number of fresh copulations a female received in each mating period and the total number of copulations each received in the previous mating period (PROC CORR). This latter test was only carried out for the late autumn and spring mating periods, since no copulations took place prior to the early spring period.

For the males, changes in sperm viability were analyzed using a two-way repeated measures ANOVA in which sperm viability was the dependent variable, sampling period (1 - 6) was the between individual factor, and time interval (Time 1 and Time 2) was the within individual (repeated) factor (PROC GLM). Tukey's post-hoc pairwise comparisons were undertaken in order to compare sperm viability measurements from each sampling period (1 - 6) against those of other sampling periods. These post-hoc comparisons were carried out for Time 1 and Time 2 separately.

All models were run including all interaction terms, and non-significant interactions were subsequently eliminated backwards at $p > 0.25$ (Quinn and Keough, 2002). For mixed models, the significance of fixed effects was tested using F-tests and degrees of freedom were calculated using the Satterthwaites approximation. Data were scrutinized for violations of assumptions but no data required transformations. All statistical analyses were carried out in SAS V9.2.

## Results

### MATING PHENOLOGY

A total of 251 *N. ocellatus* female captures were made over the course of three sampling periods (early autumn n = 72, late autumn n = 91, and spring n = 88). Of those captured, 25 females were caught in every sampling period. Data from these 25 females showed that the number of copulations a female received differed significantly across the three sampling periods ($F_{2, 69} = 4.94$; $p = 0.0099$) and females did not have equal probabilities of receiving copulations in all mating periods. Specifically the majority of copulations took place in late autumn (n=91, $\bar{x}=3.64 \pm 1.35$, $p<0.0001$) and relatively few took place in early autumn (n=16, $\bar{x}=0.64 \pm 0.81$) and Spring (n=15, $\bar{x}=0.60 \pm 0.76$); females were mated with an average of $0.045 \pm 0.012$ times per day in early autumn, $0.051 \pm 0.003$ times per day in late autumn, and $0.016 \pm 0.005$ times per day in spring. There was no effect of female snout-vent length or body condition on the number of copulations females received within any of the mating periods independently (SVL, $F_{1, 69} = 1.97$, $p = 0.1649$; body condition, $F_{1, 69} = 0.45$, $p = 0.51$). However, when we examined the number of copulations females received across the entire mating season as a whole (i.e. the total number of copulations a female received between March 1$^{st}$ and September 30$^{th}$) we found that female SVL was positively correlated with total number of copulations ($r = 0.46$, $p = 0.02$; Fig. 2.3a), but this result was not replicated for body condition ($r = 0.35$, $p = 0.08$; Fig. 2.3b). Additionally, we found a negative correlation between the number of copulations a female received in any individual mating period and the number of copulations she had received in previous mating periods. This was true for both the

late autumn (r = -0.37; p = 0.07), and spring (r = -0.51; p = 0.009) mating periods (Fig. 2.3c), although the former failed to reach statistical significance.

**EPIDIDYMAL SPERM STORAGE**

Each field of view observed for live and dead sperm counts included 40 ± 12 sperm. Sperm viability varied significantly across the mating season ($F_{5,42} = 35.35$, $p < 0.001$). Specifically, sperm viability was highest in autumn (March and April), then decreased into early winter (May) and remained low throughout winter before rising again in spring (October; $p < 0.0001$; Fig. 2.4). Sperm survival was similar throughout all 6 sampling periods ($F_{5,42} = 1.03$, $p = 0.41$; Fig. 2.4).

**Discussion:**

The quantity and quality of sperm available for mating are thought to drive male mating strategies in a variety of taxa, including squamate reptiles (Olsson et al., 2004; Preston et al., 2001; Jones et al., 2004; Sato et al., 2006, Kelly and Jennions, 2011). This study investigated the relationship between sperm viability and two male mating strategies (i.e. copulation phenology and mate choice) in *Niveoscincus ocellatus*, a lizard species with long-term sperm storage. We show that patterns of mating phenology varied over the course of the reproductive period, and were in general accordance with patterns of sperm viability, with the highest number of copulations occurring when sperm viability was highest. Below we discuss these results within the context of the factors which influence mating strategies in *N. ocellatus*.

Previous work on the reproductive cycle of *N. ocellatus* has revealed that individuals copulate in both autumn and spring, and that males have a large peak in testes volume in late summer (Jones et al, 1997; Wapstra et al., 1999), but until now, there has been no examination of how patterns of sperm production and survival may be correlated to mating patterns. The present study shows that the number of copulations females received vary across the mating period, being highest in autumn and lowest in spring. These patterns were in accordance with our data on sperm viability which showed that although proportions of live sperm were high in both autumn and spring, a decrease in sperm viability occurred between autumn and early spring. Females acquired almost three times as many copulations per day when sperm viability was highest (autumn), compared to when sperm viability was lower (early spring). These results, although correlative, suggest that sperm viability may be a key factor influencing mating behaviour in this species. Indeed, work on other systems has shown that male fertilization success, as well as offspring morphology, and consequently reproductive success, is influenced by sperm viability (Birkhead and Møller, 1998; Vishwanath and Shannon, 1997; Blount et al., 2001; Olsson et al., 2004; Jones, 2004, Lewis and Aitken, 2005). Therefore, males (and females) may concentrate their mating efforts early in the mating season when sperm viability is highest as a means of increasing the likelihood of fertilization. Our results support this hypothesis: all females were copulated with prior to winter, but fewer than a third of these females (32%) also received copulations in the spring. This suggests a possible advantage of mating in the autumn, when sperm in the epididymides are most viable. The occurrence of matings during the spring despite reduced sperm viability, however, shows that although influenced by sperm viability, mating strategies in *N. ocellatus* may also be mediated

by the cost of additional matings (Uller and Olsson, 2008). Although this study investigated the influence of sperm viability on mating patterns, these may also be influenced by the quantity of sperm available within epididymides. Future work examining the consequences of the timing of copulations on male and female reproductive success will greatly enhance our ability to elucidate the function of sperm quantity and quality on the mating behaviour of this species. Furthermore, manipulative studies which control male access to females at different times of the year could help disentangle the effects of sperm viability and mating opportunity on male mating strategies in *N. ocellatus.*

Any benefits of mating early when sperm viability is highest will be traded-off against the consequences of long-term sperm storage within the female reproductive tract. In this species, ovulation does not occur until early October (Wapstra et al., 1999), and as a result, sperm can spend up to seven months within the female reproductive tract. As the mating phenology observed in the wild is likely a result of selection to maximise sperm viability, the concentration of matings early in the season suggests that the female oviduct plays a crucial role as a sperm storage site and may be better suited for long-term storage than the epididymis. Indeed female sperm storage occurs widely across all vertebrate groups and is especially well developed in reptiles: in some species, sperm are successfully stored for up to seven years; Birkhead and Møller, 1993; Sever and Hamlett, 2002; Holt and Lloyd, 2000). Unfortunately, despite attempts to remove sperm from the female oviduct and measure its viability, we were unable to develop a reliable method of flushing sperm from all mated females, and were unable to determine whether the sperm collected was an accurate subsample of all the sperm

within the oviduct. We were thus unable to compare the proportions of live/dead sperm between the male and female sperm storage organs in *N. ocellatus*.

Sperm expenditure theory suggests that the quantity and quality of sperm should not only influence male and female mating effort *per se,* but could also influence strategies related to male mate choice (Trivers, 1972). For example, if sperm quality is compromised as the season progresses, we would predict mate choice to be stricter early in the season when sperm viability is highest (Preston et al., 2001; Bonduriansky, 2001; Hardling et al. 2008; Cornwallis and O'Connor, 2009; Morse 2010; Kelly and Jennions, 2011). In contrast, towards the end of the season, when sperm viability is lower, males may become less discriminating (Bonduriansky, 2001; Preston et al, 2001; Kelly and Jennions, 2011). In many species, male preference for female phenotypes is based largely on fecundity (King, 2000; Bonduriansky, 2001; Olsson et al., 2002; Olsson et al., 2004; Demont et al., 2011; Siepielski et al., 2011). In accordance with this, in *N. ocellatus*, female size is correlated with fecundity (Wapstra and Swain, 2001), and previous research has shown that female body size is a strong predictor of the number of copulations females received (Wapstra et al., 2009). However, despite female SVL being correlated to the number of copulations a female received over the mating season as a whole, we failed to identify variation in the strength of this relationship between individual mating periods; SVL did not influence the number of fresh copulations a female received within any specific mating period.

Despite the lack of strong selection for female SVL or body condition, the number of copulations females received was not completely non-random. Indeed, there was a

strong negative correlation between the number of copulations a female received within a given mating period and the number of copulations she had received in previous ones. Females with many copulation scars received fewer fresh copulations than those with fewer copulation scars. These results correspond with empirical studies which show that males adjust sperm investment in order to maximize fitness (Preston et al., 2001; Bonduriansky, 2001; Sato et al., 2006; Hardling et al. 2008; Cornwallis and O'Connor, 2009; Morse 2010; Kelly and Jennions, 2011). By mating with unmated females and forgoing mating with promiscuous females, males may be able to reduce the risks associated with sperm competition and increase the probability that their sperm, as opposed to a competing male's, will fertilize a female's ova (Birkhead and Møller, 1998; Engqvist and Reinhold 2006; Ball and Parker 2007). Although we were not able to determine the number of partners females had, we know that *N. ocellatus* individuals mate with multiple partners per year (Atkins, 2007), and thus, females with many copulation scars still suggest a higher risk of sperm competition, and thus, a lower chance of fertilization success than females with fewer. The combination of these results suggests mate preferences in *N. ocellatus* are complex, and male mating strategies can be influenced by female phenotype as well as female mating history.

Finally, although sperm viability decreased between autumn and winter, it increased significantly in spring: this unexpected result warrants further discussion. Given that we know that male *N. ocellatus* produce sperm only once per year, in late summer (Jones et al., 1997), this rise in live sperm proportion is intriguing. One possible explanation is that it is the result of a process of epididymal cleansing which rids the

epididymides of dead sperm (Jones, 2004). Such processes have been previously documented in several taxa (Millar, 1972; Lincoln 1974; Jones, 2004): they remove dead and defective sperm from the storage organ in preparation for the next mating event and occur in conjunction with low plasma androgen concentrations. Interestingly, *N. ocellatus* exhibit low plasma testosterone concentrations over winter (Jones *et al.* 1997), suggesting that such a mechanism is possible. Unfortunately, we were unable to estimate total sperm abundances so we cannot confirm the existence of this process in *N. ocellatus*. Ongoing histological work will examine the quantity of sperm within the epididymides across the reproductive cycle in order to determine whether epididymal cleansing occurs in *N. ocellatus*.

In summary, our work shows that the mating strategies of *Niveoscincus ocellatus* males are influenced by sperm viability, as well as by female phenotype and female mating history. However, future work needs to investigate how these relationships we have described influence male and female reproductive success before we fully understand the consequence of long term sperm storage for mating strategies.

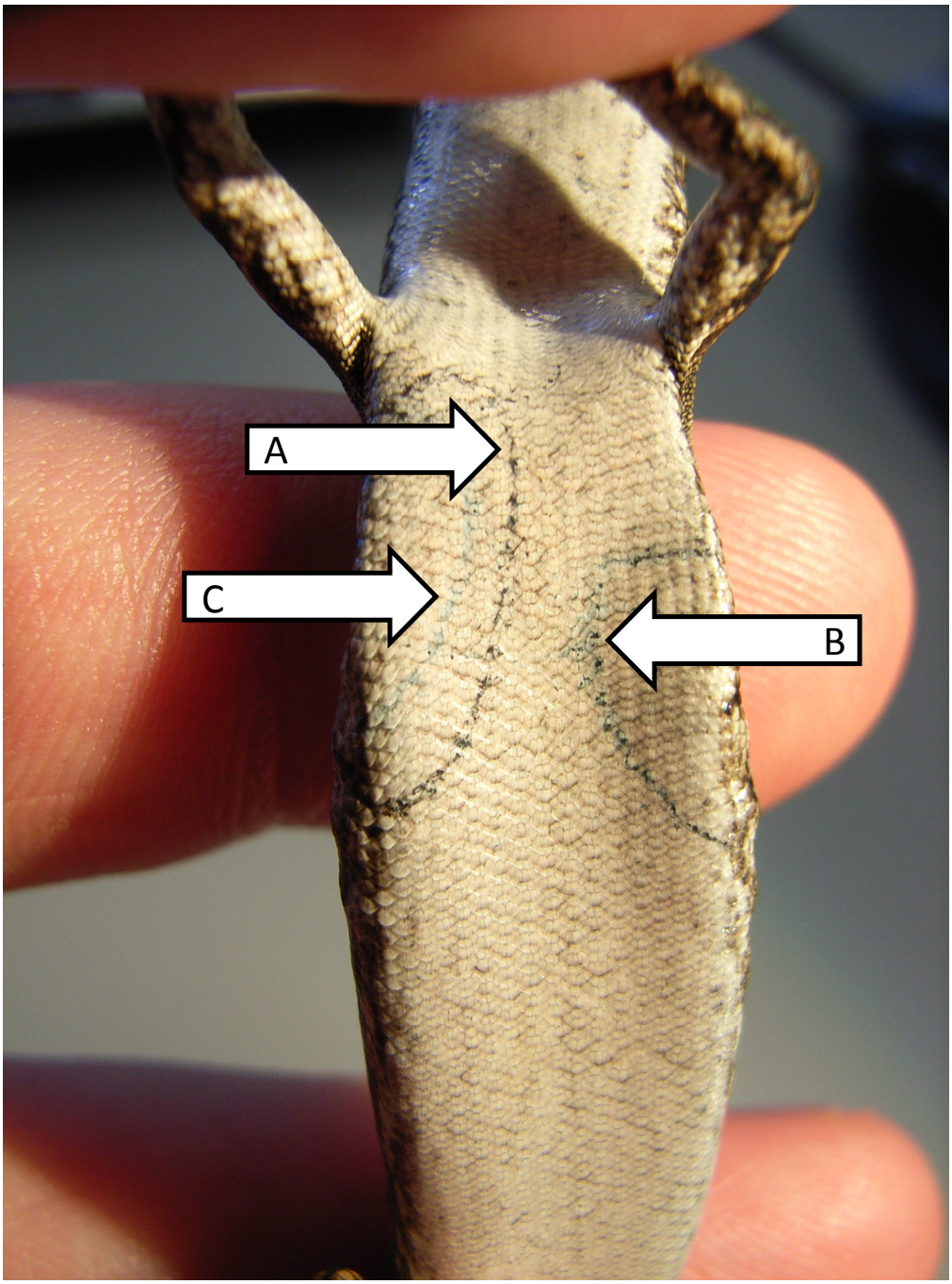


Figure 2.1 The ventrum of a female *N. ocellatus* with three copulation scars of differing colour and clarity. Scar A is darkest and most obvious, meaning that it happened prior to scars B and C. By comparison, Scar C is light blue and faint, meaning it happened recently, and the colour and clarity of scar B is between that of scars A and B, meaning it happened prior to C, but after A.

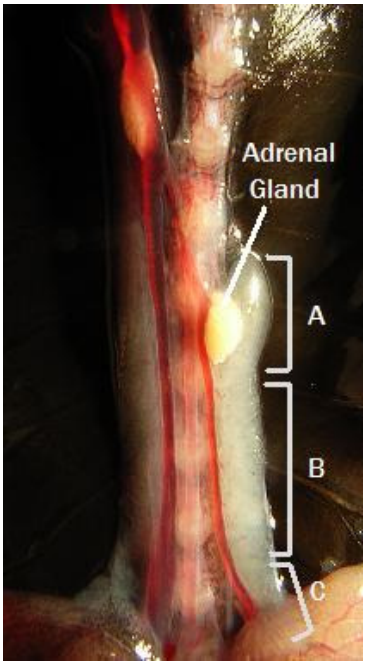


**Figure 2.2** The Caput (A), Corpus (B), and Cauda (C) of the epididymis of *N. ocellatus.* Since the Corpus was unable to be differentiated from the Caput and Cauda in *N. ocellatus*, however, only these latter segments were investigated.

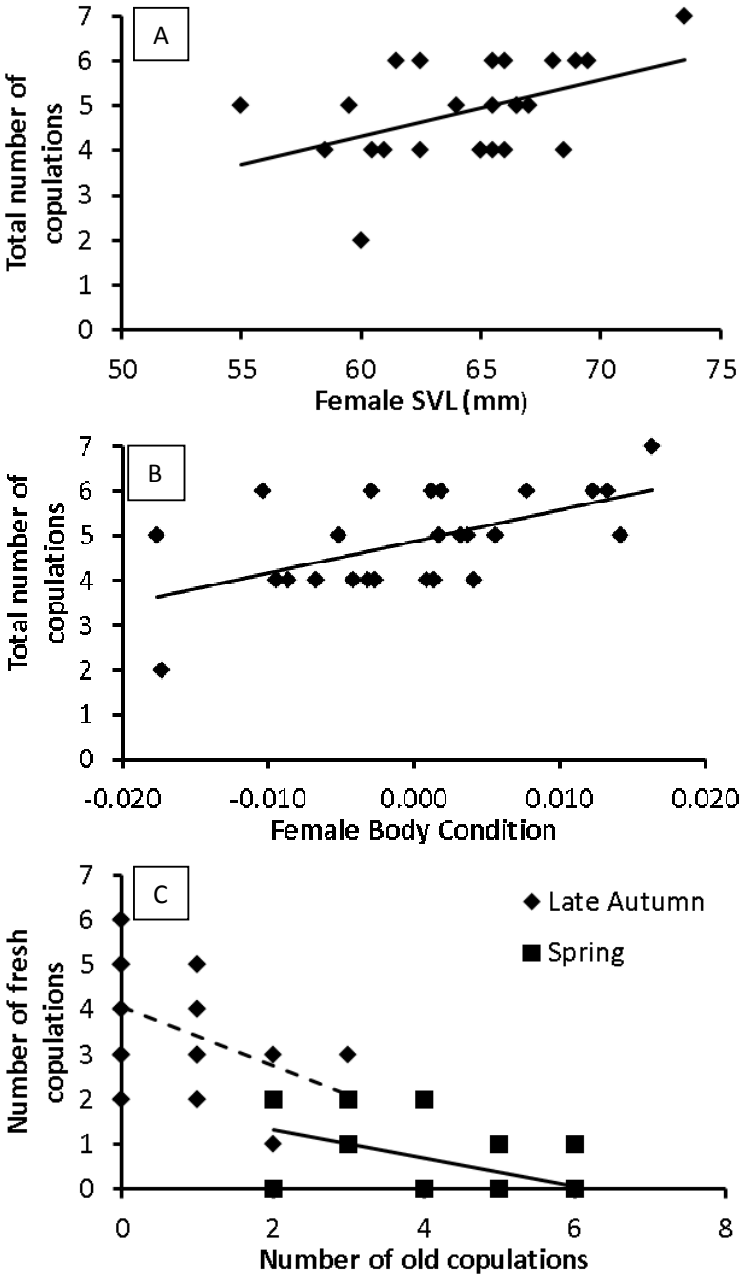


**Figure 2.3.** The positive relationships between the total number of copulations acquired over the entire breeding season by *N. ocellatus* females and snout-vent-length (SVL) (A), and body condition (B), as well as the negative effect of a female's prior mating history, and the number of fresh copulations she acquires (late autumn -solid line, and spring-dashed line) (C).

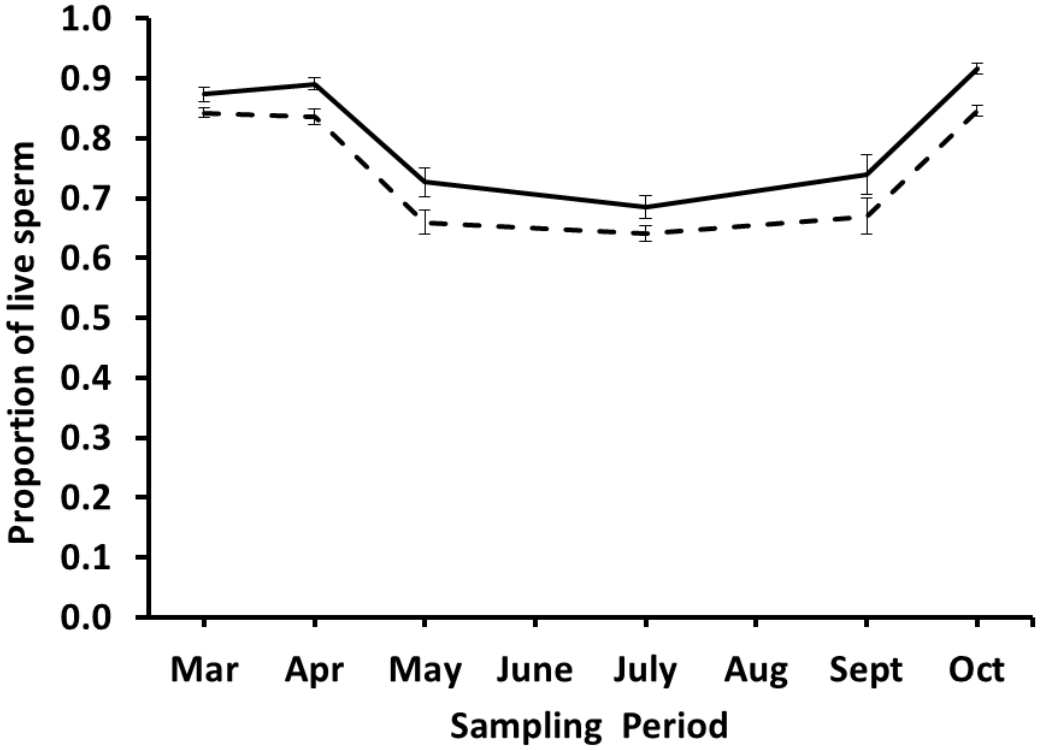


**Figure 2.4** Live sperm proportions in male *N. ocellatus* epididymides measured at two sampling times (Time 1=30 minutes and Time 2=120 minutes), throughout the 2011 mating season.

# CHAPTER THREE

## Seasonal variation in the reproductive morphology of a temperate skink species, *Niveoscincus ocellatus.*

**Mathieu Russell, Geoff While, Susan M. Jones, and Erik Wapstra**

*School of Zoology, University of Tasmania, Private Bag 5, Hobart, Tas 7001, Australia*

**Abstract:**

Sperm storage has been studied in a variety of vertebrate species; however, the wide interspecific variation in mechanisms involved in storing sperm is still poorly understood. We investigated the structure and seasonal changes of sperm storage organs in male and female *Niveoscincus ocellatus,* a temperate-climate reptile with a protracted mating season and sperm storage in both sexes. Morphological analyses revealed that males began spermatogenesis in late autumn, and a long refractory period halted the process from late autumn through to the end of the following summer, when spermatogenesis resumed and sperm were transferred to the epididymis. Sperm abundance was highest in the testes in January, and decreased during autumn, as sperm were transferred into the epididymides, moved through the caput, and were stored in the cauda until ejaculation. Analysis of the female oviduct revealed that after copulation, sperm were stored in the lumen and epithelial folds of the anterior and posterior vagina. Sperm in the lumen were organized in bundles, but had their heads in contact with, or partially embedded in the epithelium in the folds.

Furthermore, acid muccopolysaccharide secretions increased in concentration during the mating season in the vagina and the infundibulum, suggesting they played a role in maintaining sperm viability during storage in the oviduct. This study confirms previous descriptions of the reproductive cycle of male and female *N. ocellatus* and provides further information as to the progression of spermatogenesis, the location of sperm storage in the female reproductive tract, and the seasonal changes which take place within both sexes' reproductive organs throughout an entire mating season.

**Introduction:**

Sperm storage is a common phenomenon in a wide range of vertebrate species (Heatwole and Pianka, 1993) and has been shown to have significant implications for sperm viability (Jones, 2004; Pizzari et al., 2008a). Two main hypotheses have been suggested to explain the evolution of sperm storage. Firstly, it has been suggested to arise because of a scarcity of mating opportunities, thereby increasing the chances of viable sperm occuring in the oviduct at the time of fertilization (Birkhead and Møller, 1993; Gist and Congdon, 1998; Pearse and Avise, 2001). Alternatively, sperm storage has been suggested to result from temperature-dependent optimisation of male and female gamete production. Specifically, males require body temperatures above 20°C and a minimum of 8 to 10 weeks of favourable conditions for spermatogenesis whereas females depend on stored fat reserves for vitellogenesis (Aldridge, 1975; Joly and Saint Girons, 1975; Saint Girons, 1985; Jones et al., 1997). This latter hypothesis is particularly relevant in reptiles due to their dependence on environmental temperatures. In some temperate species constrained by their climate, the difference in requirements of male and female gamete production has resulted in the temporal separation of spermatogenesis and ovulation, in turn requiring sperm viability to be maintained for sometimes extended periods of time (Saint Girons, 1985; Heatwole and Taylor, 1987; Birkhead and Møller, 1993; Jones et al., 1997; Holt and Lloyd, 2009.

Some species with protracted mating seasons can store sperm in males and females, depending on when mating takes place; epididymal sperm storage occurs from the time of spermatogenesis until copulation occurs, and oviductal sperm storage takes place henceforth, until females ovulate. In such species, we would predict the

anatomical evolution of both the male and female reproductive tracts in order to maximise sperm viability during the mating season, and ultimately their reproductive success. Indeed, there exists a variety of different mophological traits associated with sperm storage (Birkhead and Møller, 1993; Girling, 2002; Murphy, 2006; Holt and Lloyd, 2009). In females, for example, sperm storage locations appear to differ across species: some species store sperm in the infundibulum, whereas others do so in the vagina, and some do so in both (see Sever and Hamlett, 2002). There is also considerable variation between species within these regions, with some species storing sperm in special tubules and others storing sperm in crypts, glands, epithelial folds or in bundles in the lumen (Bou-Resli, 1981; Saint Girons, 1973; Connor and Crews, 1980; Perkins and Palmer, 1996; Girling et al., 1997). Additionally, females have also been reported to produce secretions such as acid muccopolysaccharides in sperm storage areas in some species (Sever and Ryan, 1999; Picariello et al., 1989), which may play a role in sperm storage, nourishment, and maintenance (Hamlett et al., 2002; Storrie et al., 2008). Compared to the immense literature documenting the variation in female sperm storage mechanisms, few studies have investigated epididymal storage. Of those that have, similar morphological changes have been described throughout: once sperm is produced in the seminiferous tubules of the testes, it travels through the epididymis and is stored in the posterior epididymal segments until it is ejaculated (Marion, 1982; Flemming, 1993; Van Wyk, 1995; Ibargüengoytía, 2004). Despite knowledge of patterns, prevalent reviews have emphasized the need to study sperm storage in more species before the evolutionary significance of the interspecific variation can be understood (Girling, 2002; Sever and Hamlett, 2002, Holt and Lloyd, 2009).

Here we examine the morphological structure of sperm storage organs, and their seasonal variation in *Niveoscincus ocellatus,* a temperate skink species. *Niveoscincus ocellatus* has temporally separated gonadal cycles, with spermatogenesis occurring in late summer and ovulation in late spring (Type II sensu Heatwole and Taylor, 1987; see also Jones et al., 1997; Wapstra et al. 1999), resulting in a long protracted mating season. As a result, obligate epididymal sperm storage takes place in autumn prior to copulation, and sperm storage by females occurs after copulation takes place (Jones et al., 1997; Wapstra et al., 1999; While and Wapstra, 2009). Additionally, low temperatures completely halt all reproductive activity over winter and divide their mating season into two distinct mating periods: autumn and spring (Wapstra et al., 1999; While and Wapstra, 2009). The gonadal cycles and lengthy sperm storage of this species make it ideal for documenting sperm production and storage. The purpose of this study is to document the morphological changes of the testes, epididymides and oviducts of *N. ocellatus* throughout a mating season, with specific attention to times of sperm production and locations of sperm storage in order to better understand the mechanisms involved.

## Methods:

### STUDY SPECIES

*Niveoscincus ocellatus* is a small viviparous lizard (adult snout-vent-length (SVL) = 55-85 mm; mass = 3-12g) endemic to Tasmania. It is geographically widespread and occurs in rocky outcrops between sea level and 1200m of elevation throughout the state (Wapstra and Swain, 2001). Its life-history traits and reproductive cycles are described elsewhere with specific reference to geographic and annual differences in

life history and reproductive traits (e.g., Jones et al., 1997; Wapstra et al., 1999; Wapstra and Swain, 2001; Atkins, 2007; Wapstra et al., 2009; Cadby et al., 2010), and only relevant information is repeated here. Spermatogenesis takes place in late summer, concurrently with major peaks in testes volume and plasma testosterone levels (Jones et al., 1997). The start of the mating season follows in early March, and lasts through to when females ovulate, around the beginning of October (Jones et al., 1997; Wapstra et al., 1999; Wapstra et al., 2010). Their mating season is halted over winter (mid-May to mid-August) when all individuals to go into torpor. All females copulate prior to winter, during the autumn mating period, and 30-40% of females copulate again in the spring after their emergence from hibernation (Wapstra et al., 1999). Both sexes mate with multiple partners over the course of the mating season (Atkins, 2007). The length of time sperm is stored by each sex is determined by the timing of copulation. Sperm transferred in autumn copulations will be stored by females for longer periods than sperm that is transferred in spring copulations. Although this species has been the subject of studies describing its gestation (Thompson et al., 2001; Stewart and Thompson, 2004; Wapstra et al., 2008; While and Wapstra, 2009; Cadby et al., 2010;), and general patterns of its reproductive cycle (Jones et al., 1997; Wapstra et al., 1999) detailed descriptions of their sperm storage mechanisms do not exist.

The testes and epididymides of 64 adult male *Niveoscincus ocellatus* and the oviducts of 42 reproductively active female *Niveoscincus ocellatus* were examined between January and October, and between March and November of the 2010 reproductive cycle respectively (Table 3.1). Animals were caught by mealworm-fishing and noosing

from a field site on the southern shore of Great Lake, near Miena (41°59S, 146°44E). This site comprises a large hydroelectric dam with multiple rocky slopes and a population of over 5000 *N. ocellatus*. The maturity of individuals was determined by measuring their size (SVL) and those smaller than 55mm (SVL) were released immediately (Wapstra et al., 1999; Wapstra and Swain, 2001). Once captured, all mature animals were returned to the terrestrial ecology laboratory at the University of Tasmania and housed in plastic terraria with a basking light at one end and a shelter at the other to allow individuals to thermoregulate. Food and water were available *ad libitium*. Within 48 hours of capture, individuals were euthanatized by intraperitoneal injection of sodium pentobarbital (1ml/2kg diluted 1:100). A longitudinal incision was then made along the ventrum in order to remove the oviducts from females, and the testes and epididymides from males. Both oviducts were removed from each female, along with both testes and one epididymis from each male. The second epididymis of each male was used in a concurrent study of sperm viability (see Chapter 2). All excised tissues were weighed and testes volume was calculated by measuring their length and width with digital callipers and using the following equation: $V = \left(\frac{4}{3\pi}\right)\left(\frac{1}{2}length\right)\left(\frac{1}{2}width\right)^2$. Since collection of animals from the wild was impossible during winter hibernation, individuals required for the mid-hibernation sampling period were collected in the period prior to it and transferred to semi-natural outdoor enclosures until they were needed.

Tissues were then fixed in Bouin's solution before being stored in 70% ethanol. Preserved tissues were later embedded into wax using a Tissue-Tek® automated embedding machine and sectioned 7µm. Tissue sections were then stained with

Haematoxylin and Eosin for general histological viewing, as well as Alcian Blue to detect acid muccopolysaccharides, a secretion that some suggest is involved in sperm nourishment and maintenance during storage (Hamlett et al., 2002; Storrie et al., 2008). Light microscopy (AxioLab Zeiss microscope) was performed at 400x in conjunction with Leica Application Suite® to take detailed structural measurements of the reproductive organs of both sexes and describe their structure and changes within them throughout the reproductive cycle. Tissues were also photographed using a Leica DF 425 microscope-mounted digital camera for comparisons of sperm abundances in the testes, epididymides and oviducts.

In males, size measurements were taken from the testes (luminar and exterior diameters of seminiferous tubules, the thickness of the germinal epithelium, and abundances of spermatids and spermatozoa) and from the epididymides (luminar diameter of the ductus epididymis, the thickness of the epithelium and the muscularis, and sperm abundance). These traits are commonly used to describe the morphological structure of sperm production and storage organs, and their seasonal variation (e.g., Marion, 1982; Flemming, 1993; Gribbins, 2011). The epididymis consists of three segments (caput, corpus and cauda), but the corpus unable to be differentiated from the caput and cauda. Thus, five measurements of each morphological variable were taken from the these latter segments, using different tissue sections for each individual measurement. Sperm abundances were compared using digital photographs and the highest density of epididymal sperm was given a score of 5 (very dense with sperm), and the lowest density was given a score of 1 (no sperm), other abundances were scored relatively to the highest and lowest densities.

In females, size measurements (i.e. luminar diameter, the depth of epithelial folds, and the thickness of the epithelium and the muscularis, and the surrounding serosa) were taken from all five segments of the oviduct (i.e. infundibulum, uterine tube, uterus, anterior vagina and posterior vagina) in the same manner as described above (see also Girling et al., 1997; Girling, 1998) in order to determine how oviductal areas differed from each other and changed during the reproductive cycle. Digital photographs were used to compare sperm abundances between oviductal segments, and score them on a relative scale from 1-5, with areas very dense with sperm being given a score of 5, areas without sperm being given a score of 1, and other areas being scored comparatively. Ciliar density and Alcian Blue staining intensity were also scored on a relative scale , with the highest ciliar density and staining intensity being given a score of 5 (very dense with cilia or secretions), the lowest ciliar density and staining intensity being given a score of 1 (no cilia or secretions), and all others scored comparatively.

**STATISTICAL ANALYSIS**

Male morphological measurements (i.e. testes volume, the thickness of the germinal epithelium, the diameter of the seminiferous tubules, epithelial cell height, and the size of the lumen of the caput and cauda) were analyzed with general linear models (Proc GLM, SAS Institute, Cary, NC, USA) with post-hoc Tukey's tests to investigate their changes across sampling periods. Each male trait was entered as the dependent variable with period as the predictor variables. For female morphological measurements (i.e. the thickness of the serosa, the epithelium, and the muscularis, and the depth of epithelial folds) we took measurements across oviductal areas to

compare how oviductal segments changed and differed from each other within each sampling period. Therefore, to analyse variation in female morphology we used a general linear mixed model (Proc Mixed, SAS Institute, Cary, NC, USA). Each female trait was entered as the dependent variable, period and area were entered as predictor variables, and female identification nested within period was entered as a random repeated factor. Where there was an interaction between period and area we re-ran that analysis for each area separately. Data were scrutinized for violations of assumptions and distribution was assessed using box-plots. Only one variable required transformation: oviductal serosa thickness data were positively skewed and square-root transformed to return them to a normal distribution for analysis. Ordinal variables measured on relative scales 1-5 (i.e. stain intensity, ciliar density, and sperm abundances in the oviduct, testes and epididymides) were analyzed with Kruskal-Wallis one-way analyses of variances (Proc NPAR1WAY, SAS Institute, Cary, NC, USA), followed by post-hoc mated-pair rank total tests to compare changes between sampling periods (Langley, 1971). Since these latter tests are non-parametric, control for female identification was not possible.

## Results:

### MALE REPRODUCTIVE CYCLES

The testes of *N. ocellatus* males contained a convoluted seminiferous duct composed of primary and secondary spermatocytes (germinal epithelium) surrounded by a thin serous membrane (Fig. 3.1). Male SVL was not correlated to testes size in any of the mating periods (all $r < 0.57$, all $p > 0.10$), and thus, SVL was not used in further analyses. The volume of the testes changed significantly throughout the course of the

reproductive cycle ($F_{6,55}$ = 38.35, $p < 0.001$ ). Testes volume peaked in January, then decreased significantly over the course of the autumn mating period (Jan-Apr, $p < 0.05$, Fig. 3.2A) and remained low throughout winter and the rest of the mating season. As the testes changed in volume, seasonal variation also occurred in the exterior diameter of the seminiferous tubules ($F_{6,55}$ = 32.01, $p < 0.001$) and the thickness of the germinal epithelium within them ($F_{6,55}$ = 17.18, $p < 0.001$; Fig. 3.2B). The germinal epithelium and seminiferous tubules were both at their largest in January, when testes were at peak volume, then decreased significantly in size over the course of the autumn mating period (seminiferous tubules, Jan-May, $p < 0.05$; germinal epithelium, Jan-Apr, $p < 0.05$; Fig. 3.2B). The seminiferous tubules then remained at this reduced size throughout the rest the mating season (May-October, $p > 0.05$), but the germinal epithelium grew significantly in size again at the end of autumn, prior to winter (Apr-May, $p < 0.05$; Fig. 3.2B). In addition, the lumen inside the seminiferous tubules also changed significantly in size over the mating season ($F_{6,56}$ = 49.69, $p < 0.001$; Fig. 3.2B); it was largest in size in March and decreased significantly in size at the beginning of winter (Jan-May, $p < 0.05$) and remained at this decreased size for the rest of the mating season.

Changes in testes size occurred concurrently with changes in the abundance of sessile spermatids and free-swimming spermatozoa within them (spermatids, $\chi^2_{6,56}$ = 52.38, $p < 0.001$; spermatozoa, $\chi^2_{6,56}$ = 45.36, $p < 0.001$; Figs 3.2A and 3.3). Spermatids were most abundant in January, prior to the mating season, and then decreased significantly in number between January and May ($p < 0.05$). As spermatid abundances decreased, free-swimming spermatozoa abundances rose concurrently (Jan-Apr; $p < 0.05$), before

decreasing significantly between April and May as they moved into the epididymides ($p < 0.05$). Spermatid and spermatozoa abundances then remained low from May onwards.

The epididymis of *N. ocellatus* males consisted of a convoluted duct similar to the seminiferous tubules, and was separated into two segments, the caput (anterior) and the cauda (posterior), but these sections were too similar to differentiate structurally (Figs. 3.4 and 3.5). As spermatids developed into spermatozoa and were transferred to the epididymides for storage, the lumen of the caput and cauda both underwent significant seasonal changes in luminar diameter (caput lumen, $F_{6,53} = 14.18$, $p < 0.001$; cauda lumen, $F_{6,53} = 13.18$, $p < 0.001$; Figs. 3.4 and 3.5). The lumen of the caput began to increase in diameter in January, peaked in size in in April (Jan-Apr, $p < 0.05$), and stayed large throughout winter before diminishing significantly in size over spring (Jul-Oct, $p < 0.05$; Fig. 3.7). The cauda also swelled in size in January, but reached its maximum size in May (Jan-May; $p < 0.05$), after the caput, and like the anterior segment, then reduced in size in the spring (Jul-Oct, $p < 0.05$; Fig. 3.7). The cauda, however, was larger than the caput throughout all periods of reproductive activity (Fig. 3.7). By comparison, however, the serosa surrounding the epithelium of the epididymis did not change in thickness over the course of the mating season. As with the testes, the morphological changes in the epididymides occurred in conjunction with significant changes in sperm abundances (i.e. in the caput, $\chi^2_{6,51} = 33.78$, $p < 0.001$, and the cauda, $\chi^2_{6,51} = 35.42$, $p < 0.001$). Sperm within the caput was most abundant in January, and then decreased steadily until spring, when it declined significantly (Sept-Oct, $p < 0.05$; Fig. 3.7). Posteriorly, sperm grew in abundance within

the cauda from January onwards, reaching its peak in April instead (Jan-Apr, $p < 0.05$), and decreased significantly at the end of the autumn mating period (Apr-May, $p < 0.05$, Fig. 3.7) and again sharply in late spring (Sept-Oct, $p < 0.05$; Fig. 3.7).

**FEMALE REPRODUCTIVE CYCLES**

The oviduct of female *N. ocellatus* was structurally composed of three layers, the serosa, the muscularis and the epithelium, and was separated into five different segments based on their function and location, anterior to posterior: the infundibulum, the uterine tube, the uterus, the anterior vagina, and the posterior vagina (Fig. 3.8; see also Girling, 2002; Sever and Hopkins, 2004). These segments differed in the thicknesses of their serous layer ($F_{4,55.9} = 148.45$, $p < 0.001$), their muscular layer ($F_{4,53.9} = 191.35$, $p < 0.001$) and the thickness of their epithelium ($F_{4,94} = 3.53$, $p = 0.0099$), the depth of their epithelial folds ($F_{4,147} = 51.49$, $p < 0.001$), the ciliar density of their epithelium ($\chi^2_{4,194} = 72.69$, $p < 0.001$), and the intensity with which they were stained by Alcian Blue ($\chi^2_{4,195} = 132.87$, $p < 0.001$; Fig. 3.9).

The infundibulum was funnel-shaped, flaccid, and convoluted, and it had the thinnest serous layer and least ciliated epithelium of all oviductal segments. The epithelium of the infundibulum was stained least intensely of all oviductal segments (Fig. 3.9). Posteriorly, in the uterine tube, the serous layer was thicker and the epithelium was significantly more ciliated. Further along, in the uterus, the epithelium was arranged in deep, regular folds, and stained more intensely for acid muccopolysaccharides than any other segment (Fig. 3.9). This segment was more muscular and had a thicker serous layer than both segments anterior to it (Fig. 3.9). The epithelium of the uterus

was also similarly ciliated to that of the uterine tube, but was stained less intensely by Alcian Blue, and had shallower folds (Fig. 3.9). Additionally, the epithelium of the uterus also contained tubular glands which were more numerous posteriorly than anteriorly. Posterior to the uterus, the vagina was the most muscular region of the oviduct and had a serous layer of similar thickness to that of the uterus. The anterior portion of the vagina resembled anterior segments in several ways, it had an epithelium that was similarly ciliated to the uterine tube and uterus, and that was arranged in folds of similar depths to the uterine tube, deeper than in the uterus (Fig. 3.9). The anterior vagina's epithelium was also stained more heavily by Alcian Blue than that of the uterus, but not as intensely as in the uterine tube (Fig. 3.9). The posterior vagina's epithelium had the deepest folds of all oviductal segments and was similarly ciliated and stained to that of the anterior vagina (Fig. 3.9).

In addition to the structural differences between oviductal areas listed above, changes also occurred in the thickness of the muscularis, the serosa, and the epithelium of these areas over the course of the 2010 reproductive cycle (muscularis, $F_{24,53.9} = 4.27$, $p < 0.0001$; serosa, $F_{24,55.9} = 8.91$, $p < 0.0001$, epithelium, $F_{24,94.1} = 5.09$, $p < 0.0001$), but these changes were dependent on where in the oviduct we measured . Notably, in the uterus and posterior vagina, the muscularis grew significantly in thickness between April and July, and subsequently decreased in thickness between July and October (uterus, $F_{6.13} = 4.46$, $p = 0.0115$; posterior vagina, $F_{6,15} = 14.42$, $p < 0.0001$). Also, the thickness of the epithelium increased between April and July in both vaginal segments, then decreased between July and September and rose again between September and November (anterior vagina, $F_{6,25} = 9.65$, $p < 0.0001$; posterior vagina, $F_{6,25} = 2.90$, $p =$

0.0275). The epithelium also fluctuated in thickness in the uterine tube, increasing between March and April, before decreasing between April and July, increasing once more between July and October, and subsequently decreasing again between October and November (uterine tube, $F_{6,23} = 7.67$, $p < 0.0001$). The serosa changed in thickness as well, growing to a peak thickness between March and July, and subsequently decreasing again in thickness between July and October in the uterine tube and uterus (uterine tube, $F_{6,14} = 80.23$, $p < 0.001$; uterus, $F_{6,13} = 14.81$, $p < 0.0001$). The thickness of the serosa also fluctuated in the anterior vagina, growing in thickness between March and April, before decreasing again between April and May, then rising once more between May and July, and decreasing again between July and October (anterior vagina, $F_{6,15} = 3.9$, $p = 0.0139$).

The intensity of Alcian Blue staining also varied seasonally in the anterior vagina, the posterior vagina and the uterine tube ($\chi^2_{6,45} = 34.85$, $p < 0.001$, $\chi^2_{6,43} = 17.26$, $p = 0.008$ and $\chi^2_{6,45} = 26.63$, $p < 0.001$ respectively). All three segments were stained more intensely during the mating season than they were prior to, or after it (Nov-Mar; Fig. 3.10). Moreover, the tubular glands present in the uterus also grew in size in mid-autumn and remained large throughout the rest of the mating season, before reducing in size after ovulation (Fig 3.8H and I).

Large quantities of sperm were found in the posterior vagina between April and September and similar quantities were found in the anterior vagina between May and July but none in other segments. Sperm were present in the lumen and epithelial folds of the anterior and posterior vaginal segments. Sperm were arranged in disorganized clumps in the lumen, and were more organized in the epithelial folds, with their heads

up against or partially imbedded within the epithelium (Fig. 3.8G). No sperm were found in any oviductal segment after ovulation.

**Discussion:**

In temperate reptiles, females of most species typically have late spring ovulation and spring to summer oviposition for oviparous species, or summer parturition for viviparous species (Duvall et al, 1982; Saint Girons, 1985; Shine, 1985; Wither and O'shea, 1993; Murphy et al., 2006); however, there is more variation among species in the timing of key events in the male reproductive cycle (Fitch, 1970; Saint Girons, 1982; James and Shine, 1985; Heatwole and Taylor, 1987). The mechanisms underlying sperm storage are also known to differ significantly between species (Girling, 2002; Sever and Hamlett, 2002; Murphy et al., 2006). Here we provided detailed morphological descriptions of the structure of male and female sperm storage organs, and described changes within them during the reproductive cycle.

**MALE MORPHOLOGICAL CHANGES**

The seasonal changes in the morphology of the testes and epididymides observed here were comparable with previous descriptions of sperm production cycles in this species (Jones et al., 1997). As spermatids matured following peak testes volumes, they separated from the seminiferous epithelium and were released into the lumen of the seminiferous tubules as free-swimming spermatozoa and the lumen expanded accordingly. Spermatozoa then travelled into the epididymides, with the majority travelling through the caput before being stored in the cauda. These patterns are similar to those observed in many taxa, including several other temperate lizard

species (Marion, 1982; Flemming, 1993; Van Wyk, 1995; Ibargüengoytía, 2004). Sperm were only present in the seminiferous tubules of the testes in early autumn, providing evidence that *N. ocellatus* has a single period of sperm production, as has been previously suggested on the basis of gross changes in testes volume and testosterone cycles (Jones et al., 1997). In addition, the reductions in sperm abundance in the caput and cauda in spring (Fig. 3.7) suggest that males either used up all of their sperm during the mating season, or they expelled the unused sperm at this time. Similar mechanisms, whereby unused sperm is either evacuated or leak out slowly at the end of the mating season have been noted in a variety of amphibian species (Sever and Bart Jr., 1996; Sever, 1997). Other mechanisms, however, evacuate only dead and defective sperm in order to maximise sperm viability prior to a secondary reproductive period (Millar, 1972; Lincoln 1974; Jones, 2004). Although the decrease in sperm abundance suggests the presence of such a mechanism in male *N. ocellatus*, this cannot be confirmed without investigating whether live or dead sperm are being removed from the epididymides in spring. Sperm viability analyses from Chapter 2, however may offer a better understanding of the mechanisms resulting in this decrease in sperm abundance by determining whether live or dead sperm are being removed from the epididymides at this time.

Despite previous work having associated peaks in testes sizes and changes in androgen levels in late summer with spermatogenesis (Jones et al., 1997), our work suggests that spermatogenesis begins in late autumn. The proliferation of secondary spermatocytes and thickening of the germinal epithelium within the seminiferous tubules in late autumn, suggests that the gametogenic cycle of male *N. ocellatus* began

then with Mitosis and Meiosis I (early spermatogenesis). The testes then entered a refractory period and did not resume activity again until the end of the following summer with Meiosis II and the differentiation of spermatozoa (late spermatogenesis). Such refractory periods of spermatogenic activity are common to many bird (Marshall, 1951; Donham, 1979) and temperate reptile species (Marion, 1982; McKinney and Marion, 1985; Angelini et al., 1983; Gribbins, 2011). Although spermatogenesis was previously described to be triggered by a peak in plasma testosterone levels in *N. ocellatus* (Jones et al., 1997), the refractory period we revealed did not occur at the same time of year as any previously described peaks in plasma testosterone levels. We also revealed that variation in testes size was not associated with the refractory period either; the peak in testes size previously described to be associated with spermatogenesis (Jones et al., 1997) was in fact representative of luminar growth in the testes in preparation for spermatozoa differentiation in late spermatogenesis, not early spermatogenic proliferation of secondary spermatocytes.

**FEMALE MORPHOLOGICAL CHANGES**

Epididymal sperm storage tends to be similar in most species; however, considerable interspecific variation exists in female sperm storage mechanisms. Several reviews have described variation in the location of sperm storage within the oviduct, as well as the morphological structure of these locations between species (Girling, 2002; Sever and Hamlett, 2002; Sever and Hopkins, 2004). In skinks, for example, despite females having been previously described as storing sperm exclusively in the infundibulum (Saint Girons, 1973; Sever and Hamlett, 2002), sperm has also been found in other segments of the oviduct (Smyth and Smith, 1968; Schaefer and Roeding, 1973; Sarkar

and Shivananadappa, 1989). In support of this, we found that female *N. ocellatus* stored sperm in the anterior and posterior portions of the vagina rather than the infundibulum. These sperm were stored in loose bundles in the vaginal lumen, and in an organized fashion inside vaginal epithelial folds with their heads in contact, or partially imbedded in the epithelium. This type of organization has been previously observed in several squamate reptile species (Bou-Resli an Al-Zaid, 1981; Adams and Cooper Jr., 1988; Sever and Ryan, 1999; Sever and Hopkins, 2004), and interestingly, some authors have described it to reduce degradation of the sperm (Bou-Resli and Al-Zaid, 1981; Sever and Hopkins, 2004). This suggestion is further supported by vaginal sperm storage having previously been reported in other cool-climate reptiles as well (i.e. the tuatara and geckos; Saint Girons and Newman, 1987; Girling et al., 1997). Additionally, sperm were also found further anteriorly in May than in April, suggesting that sperm migrated anteriorly within the vagina in autumn. Similar sperm migration has been noted in the oviducts of other squamate reptiles, and is suggested to be mediated by ciliary action within the female reproductive tract (Halpert et al., 1982; Perkins and Palmer, 1996). Furthermore, no sperm were visible in the female reproductive tract after ovulation, revealing that female *N. ocellatus* do not store sperm for multiple seasons. Unfortunately, however, degradation rates of sperm in the oviduct were not investigated and live sperm migrations were not observable in this study. The orientation and embedding of sperm within the epithelial folds, and mechanisms by which sperm migrate anteriorly, however, warrant future SEM investigations of the interaction between sperm and the female oviduct and its consequences for sperm survival.

The locations of sperm storage in *N. ocellatus* females differed from those described in other species; however, the seasonal changes that occurred within them also warrant discussion. Firstly, the muscularis was thickest in the uterus and posterior vagina mid-hibernation. Although this period corresponded with times of female sperm storage, these changes resembled the pre-vitellogenic peaks in musculature in which have been previously described in the uterus of other reptiles (Palmer and Guillette Jr., 1990; Corso et al., 2000). The vagina, however, is typically suggested to function as a sphincter to facilitate embryo retention during gestation or gravidity (Girling, 1997; Girling, 2002), but the slight pre-ovulatory increase in musculature in the anterior vagina did not reach significance. Secondly, the rise in height in the vaginal epithelium during winter hibernation has also been previously noted in other reptiles, whereby epithelial cells reach their maximum height during vitellogenesis (see Girling et al., 1997). These changes, along with similar changes in the uterine tube, have been described to be controlled by reproductive hormones (Callard et al., 1978; Mead et al., 1981; Sarkar et al., 1996; also see Girling et al., 1997). Accordingly, rises in progesterone and estradiol levels have previously been reported in *N. ocellatus* during vitellogenesis (Jones et al., 1997). Furthermore, the serosa increased in thickness in the anterior vagina, uterine tube, and uterus during winter hibernation as well. Although these changes occurred in conjunction with winter sperm storage, since the blood vessels associated with the oviductal tissues reside in the serosa, the seasonal changes in serosa thickness are likely better explained to coincide with the female gonadal cycle (i.e. vitelleogenesis) rather than sperm storage.

The intensity with which the oviduct was stained by Alcian Blue, and thus, the quantity of acid muccopolysaccharides secreted, also changed over the reproductive cycle. Alcian Blue staining intensity peaked at the beginning of the mating season and remained high throughout periods of reproductive activity and sperm storage, before decreasing in late spring, when females ovulate. These increased concentrations of acid muccopolysaccharide secretions in areas of sperm storage suggest that they provide a benefit for sperm survival, and indeed, muccopolysaccharide secretions were previously hypothesized to provide a type of nourishment for stored sperm (Holt and Lloyd, 2009). These secretions, however, are also typical of the epithelial cells of the trachea and intestine. In addition, the lack of sperm in the area which stained most intensely (the uterine tube), further contradicts this hypothesis and more research into the influence of acid muccopolysaccharides on sperm survival is needed in order to ascertain their function in the oviduct. Moreover, the growth of tubular glands in the uterus during the mating season, and their subsequent degradation after ovulation, would suggest they are also involved during the mating season, but they were not present in areas of sperm storage. Tubular glands are common in the posterior end of the uterus and the anterior vagina of many reptile species (Palmer and Guillette Jr., 1992; Sarkar et al., 1995; Girling, 2002), but are typically associated with egg-shell production in oviparous species (Girling, 1998; Girling, 2002). Since *Niveoscincus ocellatus* have been previously described to produce yolk masses covered by a thin shell membrane (Stewart and Thompson, 2004), the seasonal changes in oviductal tubular glands are likely due to this latter explanation.

**Conclusion:**

Our histological analyses provided support for previous descriptions of spermatogenesis and female sperm storage, and revealed new information about spermatogenic progression and sperm storage locations and mechanisms in females. The testes began spermatogenesis in late autumn, many months prior to the eventual release of sperm into the epididymides. They then entered a long refractory period and resumed spermatogenesis in late summer, when spermatozoa were released into the epididymides for use during the mating season that follows. As the mating season progressed and males used some of their sperm, the female oviduct secreted acid muccopolysaccharides, and tubular glands in the uterine epithelium grew in size. Following emergence from hibernation, sperm abundance decreased significantly in the epididymides.

Although this study supports some previous descriptions of oviductal, epididymal, and testicular structure and function, sperm storage locations in *N. ocellatus* differ from those previously documented in other skink species. The histological analyses of sperm storage in male and female *N. ocellatus* provided by this study support the wide variation in sperm storage mechanisms noted in previous reviews (Girling, 2002; Sever and Hamlett; 2002, Holt and Lloyd; 2009). Despite the large number of studies documenting sperm storage across a wide range of taxa, certain aspects of oviductal structure (i.e. acid muccopolysaccharides) still lack understanding and closer investigations are needed. Further histological studies comparing the morphological mechanisms of sperm storage in species with different gonadal cycles are necessary in

order to better understand the function of these seasonal changes in the oviduct, as well as the reason for the variation in sperm storage mechanisms documented so far.

Table 3.1. Sampling times of male and female *N. ocellatus* during the 2010 breeding season.

| Date | Period of the Reproductive Season | Number of Individuals |
|---|---|---|
| ***Males*** | | |
| January | Prior to spermatogenesis | 8 |
| March | Late spermatogenesis | 8 |
| April | Early autumn mating period | 8 |
| May | Post-autumn mating period | 8 |
| July | Prior to the spring mating period | 8 |
| September | Late spring mating period | 8 |
| October | Post breeding season | 8 |
| ***Females*** | | |
| March | Prior to the autumn mating period | 6 |
| April | Early autumn mating period | 6 |
| May | Prior to hibernation | 6 |
| July | Middle of hibernation | 6 |
| September | Prior to ovulation | 6 |
| October | After ovulation | 6 |
| November | Early gestation | 6 |

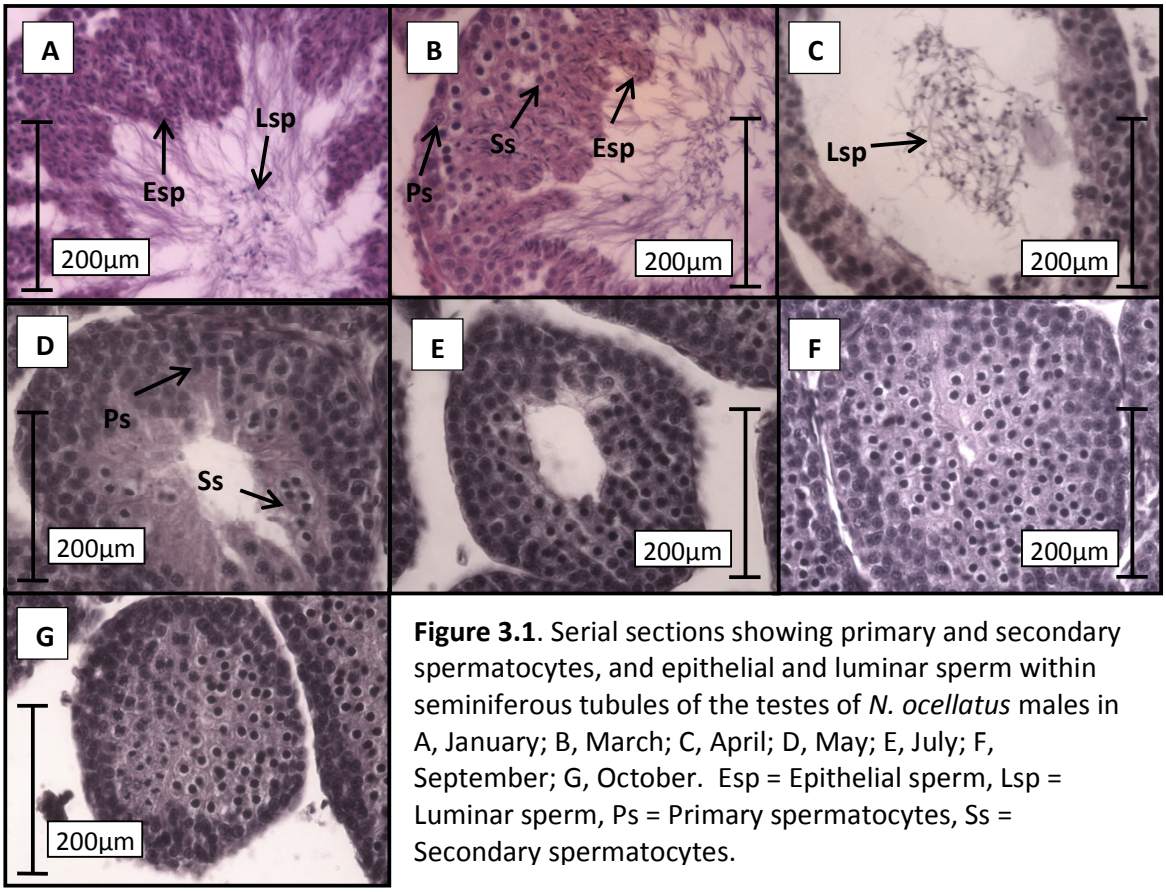


**Figure 3.1**. Serial sections showing primary and secondary spermatocytes, and epithelial and luminar sperm within seminiferous tubules of the testes of *N. ocellatus* males in A, January; B, March; C, April; D, May; E, July; F, September; G, October. Esp = Epithelial sperm, Lsp = Luminar sperm, Ps = Primary spermatocytes, Ss = Secondary spermatocytes.

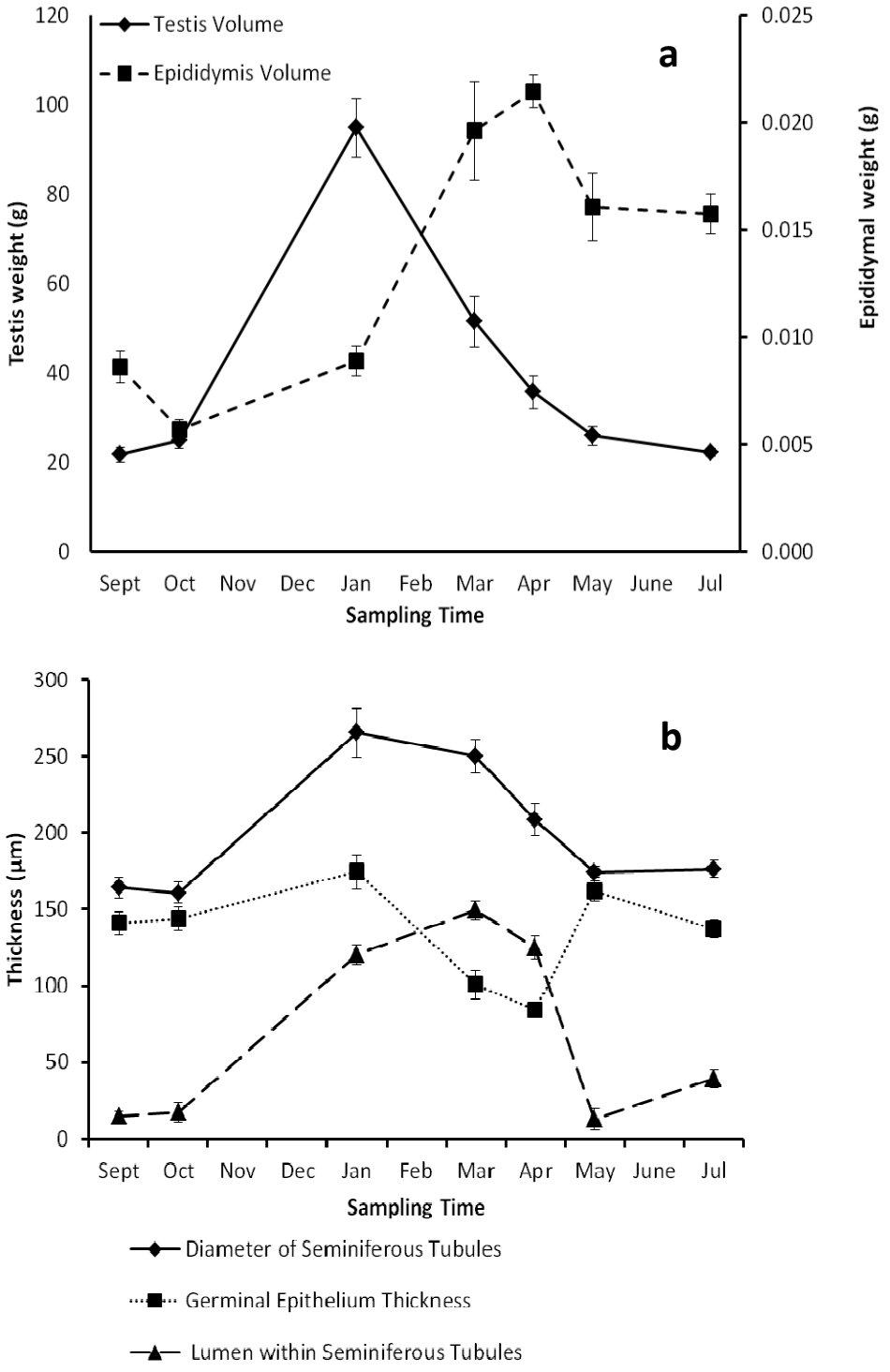


**Figure 3.2.A**. Changes in the mean weights of testes and epididymides, and **B.** the mean diameter of seminiferous tubules, the mean diameter of the lumen, and the mean thickness of the germinal epithelium within them, in relation to mean weight of a testis in male *N. ocellatus* in 2010.

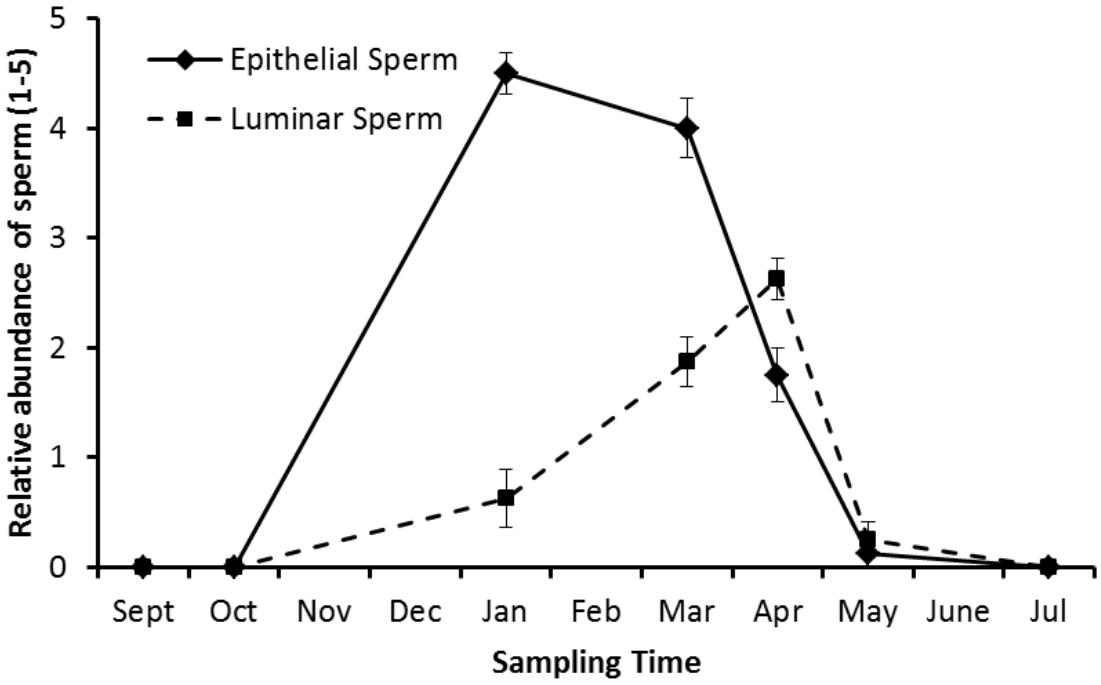


**Figure 3.3**. Changes in the mean relative quantities of sperm in the lumen, and embedded in the germinal epithelium in the testes of *N. ocellatus* males in 2010.

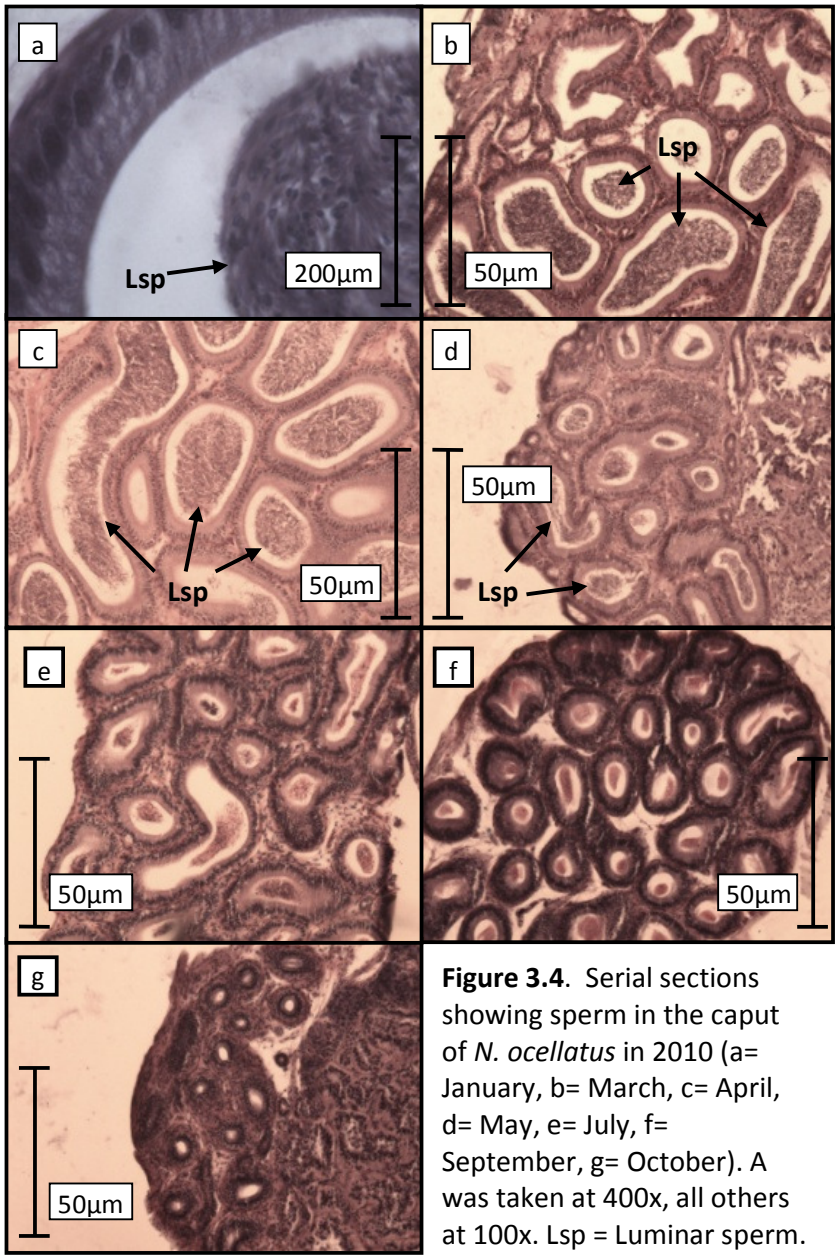


**Figure 3.4**. Serial sections showing sperm in the caput of *N. ocellatus* in 2010 (a= January, b= March, c= April, d= May, e= July, f= September, g= October). A was taken at 400x, all others at 100x. Lsp = Luminar sperm.

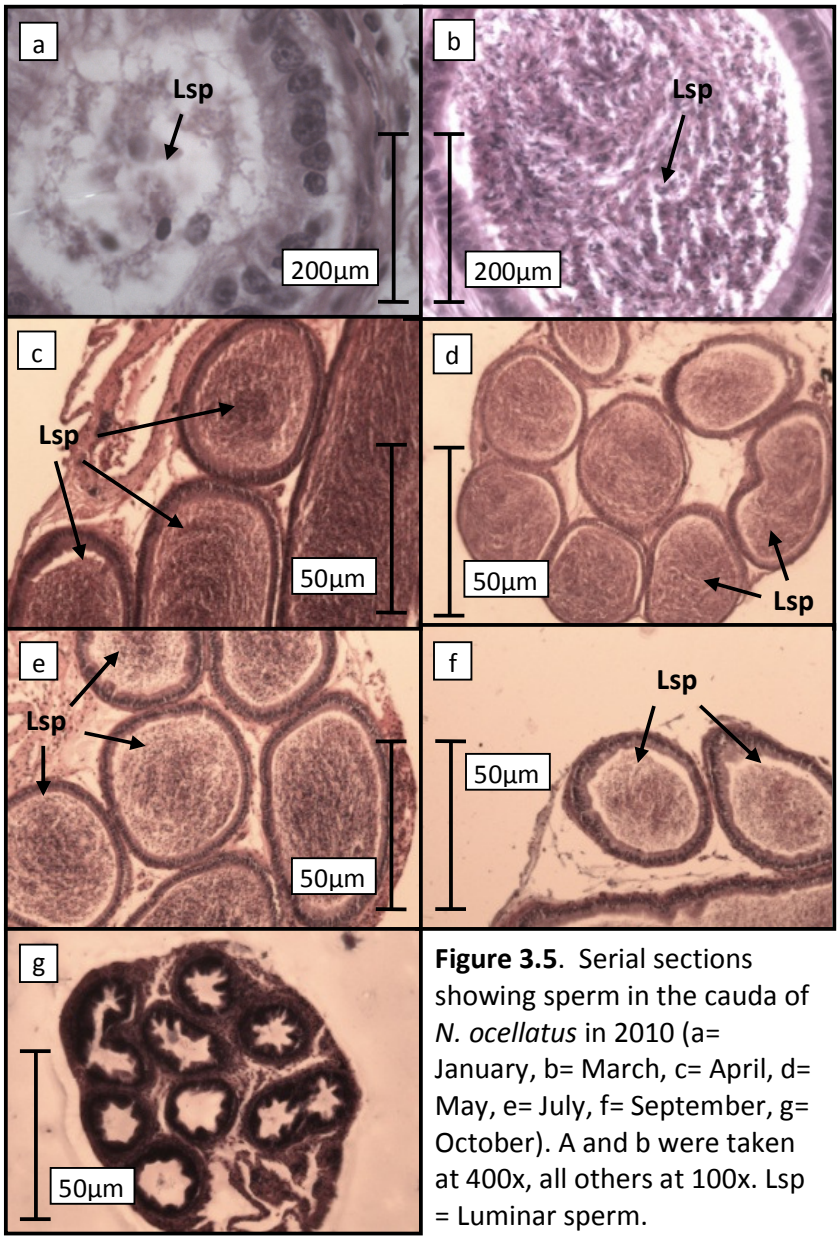


**Figure 3.5**. Serial sections showing sperm in the cauda of *N. ocellatus* in 2010 (a= January, b= March, c= April, d= May, e= July, f= September, g= October). A and b were taken at 400x, all others at 100x. Lsp = Luminar sperm.

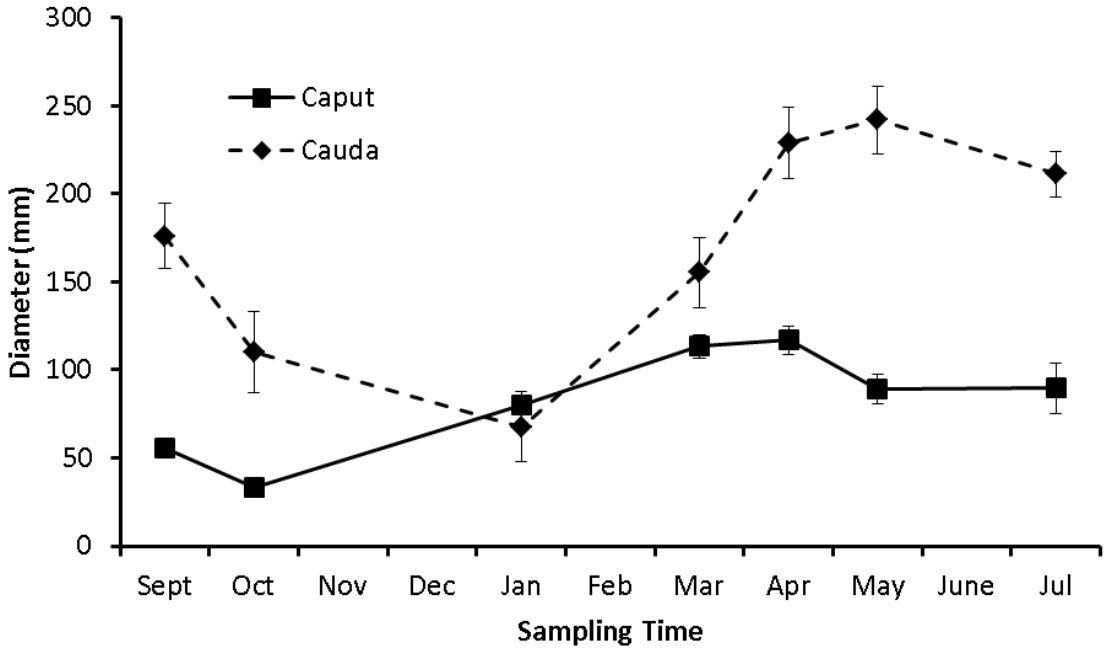


**Figure 3.6**. Seasonal changes in the mean luminar diameter of the caput and cauda epididymis of *N. ocellatus* males in 2010.

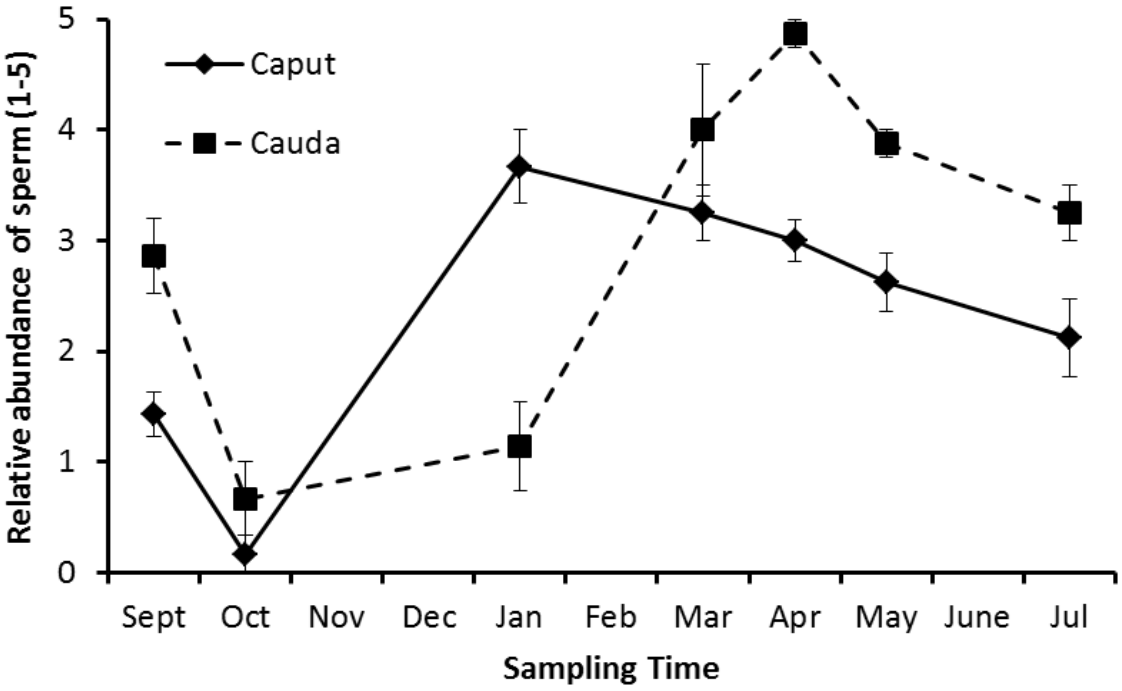


**Figure 3.7**. Changes in the mean relative quantities of sperm in the cauda and caput epididymis of *N. ocellatus* males in 2010.

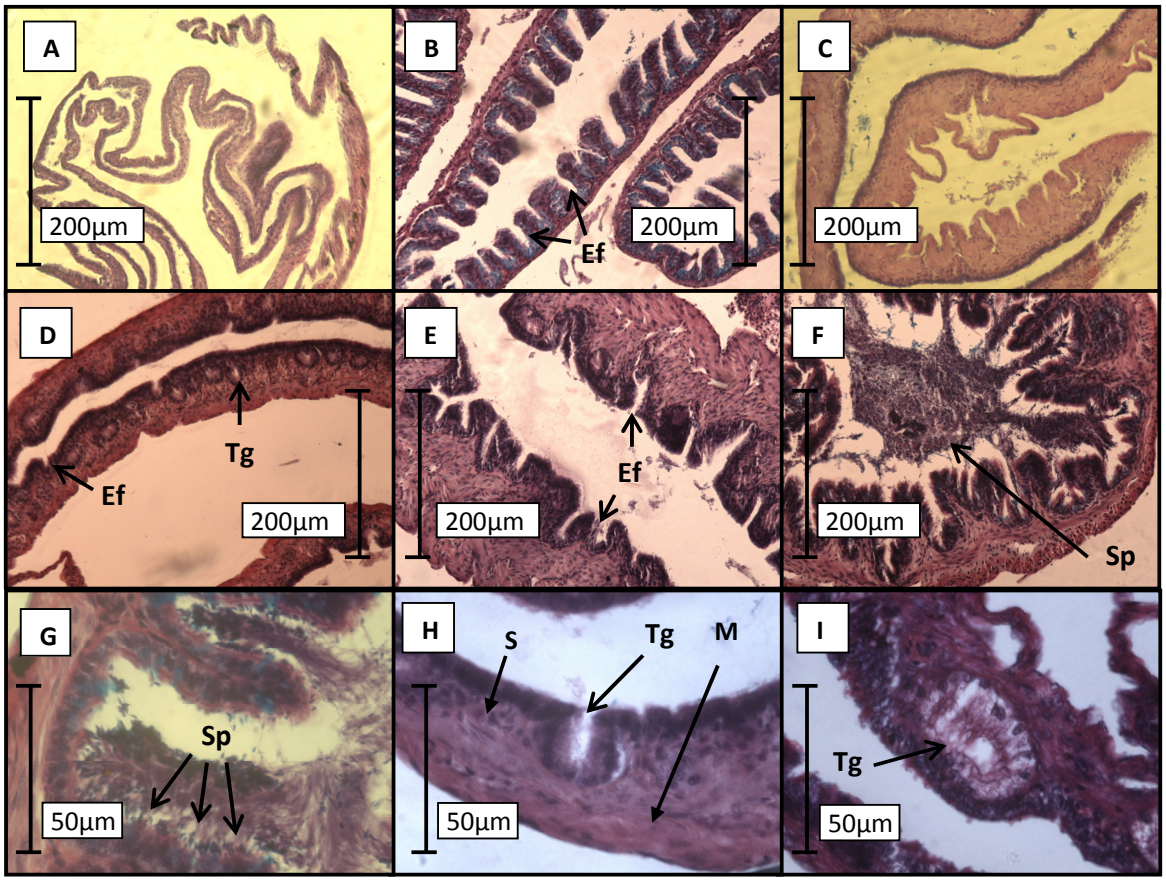


**Figure 3.8.** Serial sections showing areas of the oviduct of *N. ocellatus* females (A= Anterior Infundibulum, B= Uterine Tube, C= Anterior Uterus, D= Posterior Uterus, E= Anterior Vagina, F= Posterior Vagina), tubular glands (Tg), and sperm (Sp) stored within the lumen and epithelial folds (Ef). Image G shows sperm aligned with their heads facing the epithelial wall and images H, and I show tubular glands in the Uterus in April and October respectively. Alcian Blue staining can also be seen intensely in the Uterine Tube (image B) whereas it is absent from the Uterus (images C and D, and mildly in the Vagina (images F and G). The muscularis (M) and serosa (S) are also pointed out in Image H

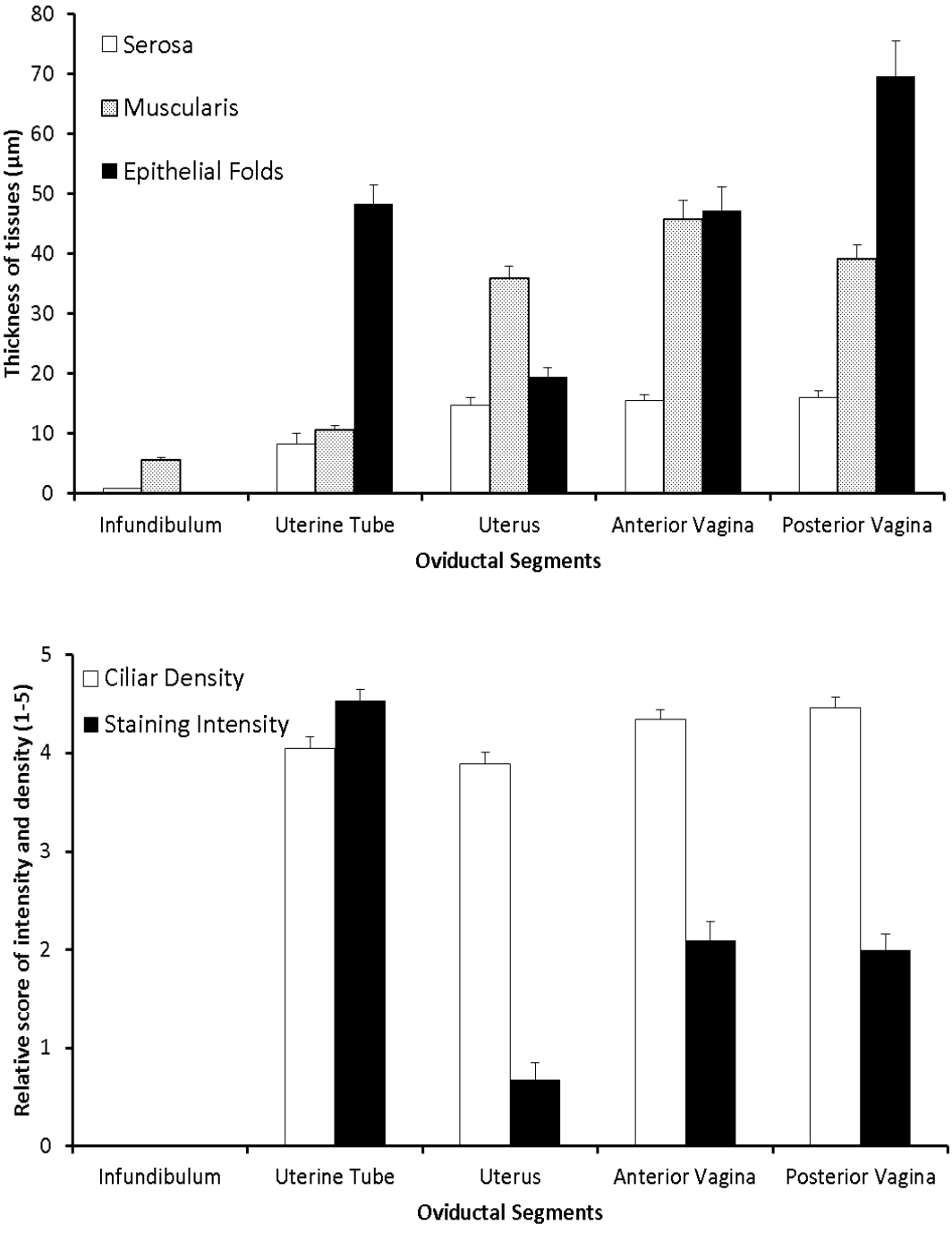


**Figure 3.9.** Differences in the mean thicknesses of the serosa and the muscularis, and the depth of epithelial folds (A), as well as the intensity of Alcian Blue staining, and the ciliar density (B) in the five oviductal segments of *N. ocellatus* females.

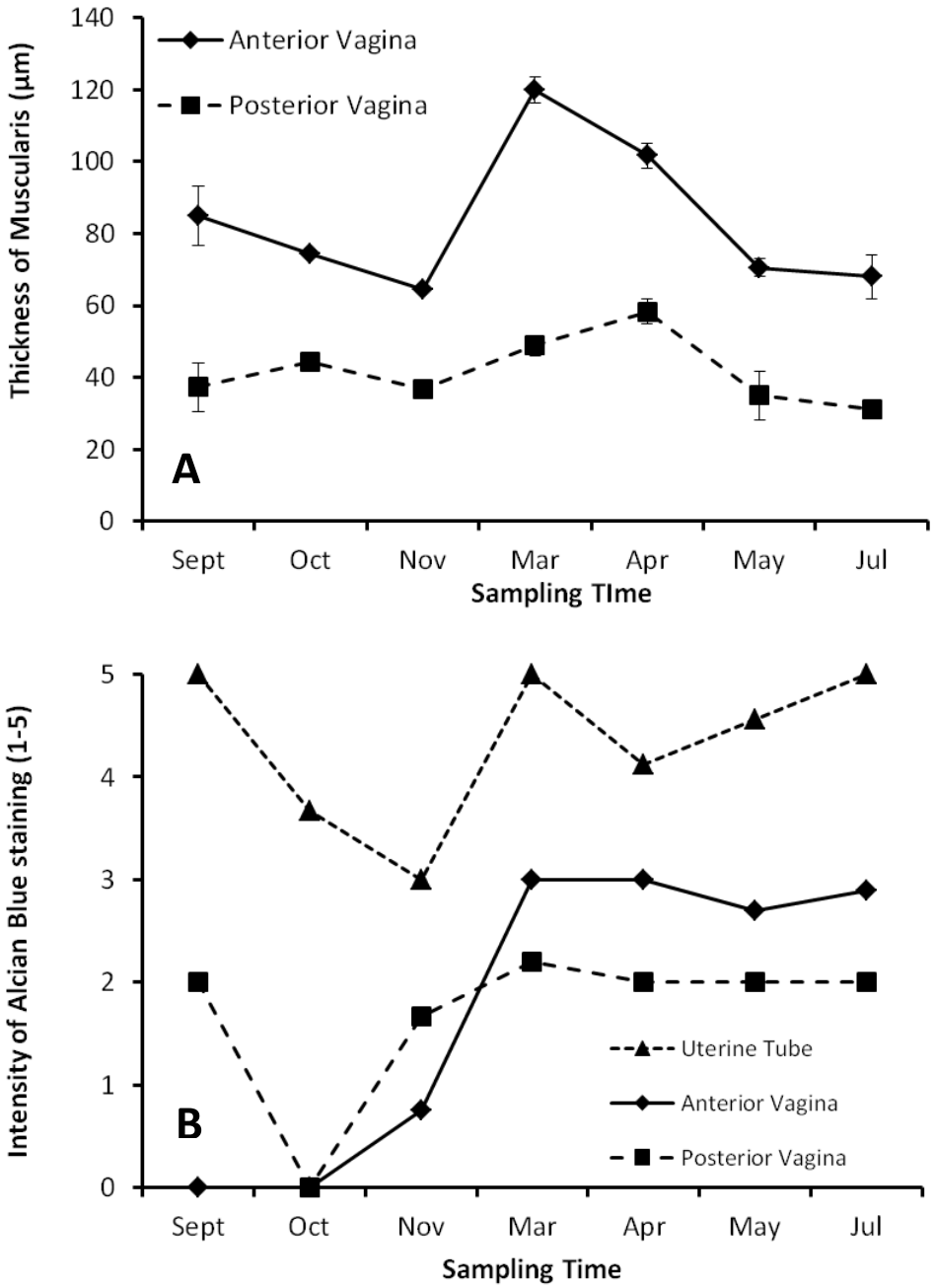


**Figure 3.10.** Seasonal changes in the mean thickness of the muscularis of the anterior and posterior vagina (A) and the staining intensity in the anterior vagina, posterior vagina, and infundibulum (B) of *N. ocellatus* females.

# CHAPTER FOUR

## General Discussion:

This thesis provides detailed anatomical descriptions of seasonal changes in the testes, epididymides, and oviducts of *N. ocellatus*, an analysis of the viability of sperm whilst stored in the epididymides, and a description of male mate preferences and mating patterns over the course of an entire mating season. In doing so, this thesis provides support for previous work describing the reproductive cycle of *N. ocellatus*, expands on our previous knowledge of their mating patterns, and offers the opportunity for future work to enhance our understanding of the causes and consequences of sperm storage for male and female reproductive success within temperate-climate reptiles.

My observations confirmed that matings took place in autumn and spring. However, females were approximately three times more likely to receive copulations in autumn than in spring, suggesting there was facultative sperm storage over winter by females. Since mating strategies are commonly influenced by sperm viability and its consequences on fertilization success (Galvani and Johnstone, 1998; Cornwallis and O'Connor, 2009), the higher number of copulations in autumn compared to spring and the resulting differences in the duration of male and female sperm storage, suggest that the oviduct is better suited for maintaining sperm viability than the epididymis in *N. ocellatus*. Indeed, my sperm viability analyses revealed that sperm viability decreased significantly between autumn and winter in the epididymis. Although I was unable to investigate sperm viability and survival in the female reproductive tract, the presence of acid muccopolysaccharides in areas of sperm storage, and their increased

concentration during periods of oviductal sperm, further suggests that the oviduct is well adapted for long-term sperm storage. These secretions are hypothesized to be involved in the nourishment and/or maintenance of sperm during storage in a variety of taxa (Hamlett et al., 2002; Storrie et al., 2008). *Ex situ* experiments provide the opportunity to control access to mates, and as a result, the timing of copulation and the duration of male and female sperm storage. The paternity of resulting offspring can subsequently be assessed to determine the consequence of variation in the mating patterns of *N. ocellatus* on reproductive success.

In addition to male and female sperm storage duration, the timing of copulations could also influence the order in which males mate with the same female, and by consequence, their likelihood of fertilizing a female's ova. Mating order has been reported to influence a male's reproductive success in a variety of polygamous species and taxa (see Lessells and Birkhead, 1990). The occurrence of copulations in spring in *N. ocellatus* suggests that benefits may exist from mating later than competing males with the same female. This type of last-male sperm precedence commonly occurs in a variety of taxa (Lessells and Birkman, 1990; Briskie, 1996; Birkhead, 1998; Kraaijeveld-Smit et al., 2002; Eady and Tubman, 2011). In some species, sperm has been described to become stratified or layered, giving the last male to copulate with a female precedence in fertilizing ova, and thus, an advantage over earlier-mating competitors (see Lessells and Birkhead, 1990). The predominance of autumn copulations in *N. ocellatus* suggests that their mating patterns are influenced by sperm viability; however, the occurrence of spring copulations suggests that their mating patterns may be influenced by multiple factors including sperm competition resulting

from mating order. Although the consequences of male mating order are still unknown in this species, similarly to above, *ex-situ* mating experiments can manipulate male mating order by controlling access to females and later assess the paternity of resulting offspring to investigate the consequences of male mating order on reproductive success.

This thesis also described further mating patterns that provide insight into the factors which may drive mate choices in *N. ocellatus*. Promiscuous females with multiple copulation scars received fewer new copulations than those that only had a few scars, suggesting that male mate preferences were driven by female phenotype, and the likelihood of fertilizing ova. Females with multiple copulation scars, and thus, sperm from multiple males within their reproductive tracts, potentially indicated a high degree of sperm competition, and as a result a lower chance of fertilization (Parker, 1970; Trivers, 1972; Engqvist and Reinhold, 2006; Ball and Parker, 2007). Females with fewer copulation scars, by comparison, carried sperm from fewer competitors and potentially offered a higher probability for each suitor to fertilize her ova. Indeed, the driving of male mate choices by the probability of fertilizing ova, and ultimately reproductive success, is widespread (Trivers, 1972; Bondurianski, 2001; Olsson, 2001; Hardling et al., 2008). Interestingly, however, the overall number of copulations females received was low considering the length of the protracted mating season, suggesting that copulation frequency may be controlled by females for example. Indeed, since copulations are commonly associated with costs to females (e.g. exposure to predators, Rowe, 1994; disease, Thrall et al., 2000, and physical harassment from males, Chapman et al., 1995), females should only mate enough

times to guarantee the fertilization of their ova (see Simmons et al., 2005). Although this thesis provides evidence for the likelihood of a female receiving a copulation being influenced by the number of copulations she had previously received, the factors driving the total number of copulations received by females were not determined. The consequences of variation in copulation frequency, however, could be better understood by *ex situ* manipulative experiments controlling for the number of mates a female receives, and assessing female and offspring health, to give us a better insight into the factors driving copulation frequency in *N. ocellatus*.

Furthermore, this thesis also offers support for previous descriptions of the reproductive cycles of male and female *N. ocellatus*, reveals interesting previously undescribed aspects of these cycles, and provides detailed histological descriptions of the mechanisms underlying sperm storage. My analyses confirmed that male *N. ocellatus* sperm production occurs once per year (Jones et al., 1997; Wapstra et al., 1999) and that once sperm were produced, they travelled into the epididymis, and were stored in its posterior segment until ejaculated, similarly to that of a number of lizard species (Marion, 1982; Flemming, 1993; Van Wyk, 1995; Ibargüengoytía, 2004). Interestingly, my work also indicated that spermatogenesis followed a different progression to what was previously described for this species. Specifically, I showed that early- and late spermatogenesis were temporally separated by a long refractory period which began in late autumn, and that the late-summer peaks in testes size and androgen levels previously reported to be associated with spermatogenesis (Jones, et al., 1997) were in fact only associated with the final step of sperm production (late spermatogenesis). Similar spermatogenic refractory periods are common in many

birds and reptiles (Marion, 1982; Licht, 1984; Bharti et al., 2011), but controversies exist as to whether they are triggered by temperature effects or hormonal stimulation, specifically FSH (Follicle Stimulating Hormone) (Licht, 1984; also see Gribbins, 2011). Although FSH fluctuations have yet to be studied in this species, the refractory period takes place just prior to winter, suggesting it may be driven by environmental temperatures. Further examinations of the effects of temperature and FSH on spermatogenic progression and regression, however, would provide a better understanding of the factors driving the reproductive cycle of male *N. ocellatus*.

My analyses revealed that sperm viability decreased with time, being highest in early autumn and lowest in winter. However, live sperm proportions were also higher in spring than in winter. This result was unexpected because no new sperm were produced in spring. The spring increase in sperm viability occurred concurrently with a significant decrease in epididymal sperm abundance, suggesting the presence of a mechanism to release dead sperm from the epididymides, decreasing the volume of sperm, but raising the overall proportion of live sperm present. Indeed such mechanisms for epididymal cleansing exist in many taxa, whereby dead and defective sperm are evacuated from, or leak out of the epididymides to increase overall sperm viability prior to a secondary mating period (Millar, 1972; Lincoln, 1974; Jones, 2004), but none had previously been noted in *N. ocellatus*. Such mechanism are known to commonly occur in conjunction with a decrease in androgen levels (Jones, 2004), and indeed, such a decrease was previously recorded between August and September in this species (Jones et al., 1997). Although this work revealed the existence of a mechanism to maximize sperm viability in conjunction with male emergence from

hibernation and previously measured androgen fluctuations, detailed accounts of the cellular processes underlying this mechanism in *N. ocellatus* do not exist. Closer observations using SEM may better describe the interactions between sperm and the epididymis, and elucidate how dead and/or defective sperm are removed.

In females, my histological analyses confirmed that *N. ocellatus* stored sperm from early autumn, through to ovulation in late spring, and further identified that they did so in different areas to those previously described for skinks (Saint Girons, 1973; also see Sever and Hopkins, 2004). This supports recent work suggesting that sperm storage locations and mechanisms vary interspecifically (Birkhead and Møller, 1993; Girling, 2002; Murphy et al., 2006; Holt and Lloyd, 2009). I revealed that female *N. ocellatus* stored sperm in the epithelial folds and lumen of the anterior and posterior vagina, rather than the infundibulum, as was once suggested for all skinks (Saint Girons, 1973; Sever and Hamlett, 2002). Additionally, sperm were also found more anteriorly in the vagina in May than in April, suggesting that sperm migrated from the posterior vagina to the anterior vagina in early autumn. Indeed, anteriorward sperm migrations have previously been noted in other squamate reptiles as well, and have been suggested to result from ciliary action in the vagina (Halpert et al., 1982; Perkins and Palmer, 1996). Although the manner by which sperm migrated up the oviduct of female *N. ocellatus* could not be ascertained in this study, the vagina was indeed heavily ciliated, suggesting that sperm may travel anteriorly by the same mechanism as has been described in other species (Halpert et al., 1983; Perkins and Palmer, 1996). In addition to storage-related morphological variations, the oviduct also underwent several structural changes in the uterus and vagina in conjunction with vitellogenesis.

Specifically, the posterior vagina's muscularis and epithelium, the anterior vagina's serosa and epithelium, and the uterus' muscularis, serosa and epithelium were all at their thickest mid-vitellogenesis. Indeed, peaks in thickness of muscularis and epithelial cell heights have previously been described in many other reptiles in conjunction with vitellogenesis (Palmer and Guillette Jr., 1990; Girling, 1997; Corso et al., 2000; Girling, 2002). Lastly, the tubular glands present in the uterus also underwent seasonal changes, growing in size as the mating season progressed, and degrading after females ovulated. Although similar glands have been reported as sperm storage locations in some reptile species (Palmer and Guillette Jr., 1992; Sarkar et al., 1995; Girling, 2002), they are typically associated with eggshell production (Palmer et al., 1993; Girling, 1998; Girling, 2002). Indeed, any viviparous species retain eggshell production glands and the yolk masses produced by female *N. ocellatus* are covered by a thin shell membrane (Stewart and Thompson, 2004). Therefore the changes in the tubular glands of the uterus are likely better explained by vitellogenesis and the shelling of the yolk, rather than sperm storage. Such seasonal changes in oviductal structure are known to be triggered by hormonal changes in a variety of taxa (Callard et al., 1978; Sarkar et al., 1996; also see Girling, 2002), and indeed, changes in estradiol and progesterone have been noted over the course of the mating season of *N. ocellatus* as well, notably during vitellogenesis, when most structural changes occurred (Jones et al., 1997). Furthermore, the hormone assays previously undertaken showed that progesterone and estradiol levels were lower in autumn, when females received few copulations, and were elevated in spring, when copulations were more sparse (Jones et al., 1997). The conjunction of the seasonal changes described herein with the mating patterns I discussed above and previously

undertaken hormone assays, suggests that the oviduct of female *N. ocellatus* is a hormonally-controlled complex environment with significant structural changes in relation to vitellogenesis, as well as secretions perhaps dedicated to sperm storage. Although the interactions between sperm and the female reproductive tract were not discernible in this study, the relationship between sperm and the epithelium, as well as the acid muccopolysaccharide secretions discussed above, may be more closely investigated using SEM observations to better explain the mechanisms underlying long term sperm storage in female *Niveoscincus ocellatus*.

In summary, this thesis provides an insight into the mechanisms underlying seasonal changes in the reproductive and sperm storage organs of male and female *Niveoscincus ocellatus*as well as their effects on male sperm viability and female mating patterns in a wild population. Such a combination of morphology, physiology and behaviour is essential for creating an understanding of the proximate mechanisms underlying long term sperm storage. This thesis therefore provides a valuable basis from which future work can explore the consequences of long term sperm storage for both male and female reproductive success, and ultimately the strength and direction of sexual selection in this species.

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