

Short Communication B

**ASYNCHRONOUS OVIDUCTAL SEASONAL VARIATION
IN THE UNISEXUAL-BISEXUAL *NACTUS PELAGICUS*
COMPLEX FROM THE VANUATU ARCHIPELAGO
(REPTILIA: SQUAMATA: GEKKONIDAE)**

***Mallory E. Eckstut¹, Alison M. Hamilton^{2,3},
Christopher C. Austin² and David M. Sever¹***

¹Department of Biological Sciences, Southeastern Louisiana University,
Hammond, Louisiana 70402, USA

²Museum of Natural Science and Department of Biological Sciences,
Louisiana State University, 119 Foster Hall, Baton Rouge, Louisiana 70803, USA

³Present Address: Center for Tropical Research, UCLA Institute of the Environment, La
Kretz Hall, Suite 300, Box 951496, Los Angeles, California 90095, USA

ABSTRACT

The South Pacific *Nactus pelagicus* complex (Reptilia: Squamata: Gekkonidae) consists of several genetically distinct but morphologically conserved lineages of *N. multicaerinus*, and a unisexual lineage, *N. pelagicus*, which is a hybrid of two *N. multicaerinus* lineages. A few discrete morphological differences occur between lineages of the *N. pelagicus* complex, but that is all that is known regarding this complex. This complex has been poorly studied and little is known about the general biology and ecology of *N. pelagicus* and *N. multicaerinus*. We analyzed the reproductive morphology using histochemistry in populations of *N. multicaerinus* and *N. pelagicus* from the Vanuatu Archipelago in the South Pacific, the only series of islands where the two species overlap, in order to determine if interspecific differences in reproductive patterns exist and to gather additional knowledge regarding the reproductive ecology of these species. Our findings indicate that both species carry one clutch during the dry season, however there is temporal variation in reproductive activity, with *N. pelagicus*

* Corresponding Author: Email: eckstutm@unlv.nevada.edu, School of Life Sciences, University of Nevada,
Las Vegas, 4505 Maryland Parkway, Las Vegas, Nevada 89154, USA

exhibiting particular histochemical states one and a half to two months prior to *N. multicarinatus*. Furthermore, we found that *N. multicarinatus*, the bisexual lineage, does not store sperm with eggs *in utero*, which has only been observed in one other species of lizard (a skink). These previously unrecognized differences in *Nactus* reproduction provide a foundation for further study of the evolutionary transition to unisexual reproduction and supply additional data on the ecology of this poorly studied gecko complex.

INTRODUCTION

Vertebrate reproduction is highly complex and diverse, and variation in reproductive morphology and the tempo and mode of reproductive cycles are thought to be important contributors to speciation (Lewis 1969; Templeton 1981). Despite their ability to cause reproductive isolation which may, in turn, lead to speciation or to reinforce existing isolation between closely related species, the role of reproductive cycles and morphology in shaping the patterns and processes responsible for the generation and maintenance of unisexual lineages are poorly known. A substantial amount of interspecific variation has been recorded in vertebrate reproductive cycles, clutch sizes, reproductive morphologies, and general morphological traits associated with reproduction (Tinkle et al. 1970; Fox 1977). Reproductive traits and morphologies can range from highly labile to extremely conserved and this variation has strong implications for the evolution of organisms and their life histories (Fox 1977; Sever and Hamlett 2002; Pianka and Vitt 2003).

Phylogenetic constraints may conserve similarities across large taxonomic groups, whereas variation in climate, resource availability, or other selective forces can cause reproductive strategies or morphologies to vary, even among closely related taxa. For example, the large global radiation of gekkonid lizards results from over 200 million years of independent evolution from other squamates (Vidal and Hedges 2005) and includes more than 75 genera and 900 species (Pianka and Vitt 2003). Yet despite this diversity, the majority of geckos have a uniform clutch size of two calcareous-shelled eggs (Bustard 1968; Rose and Barbour 1968). Alternatively, in some non-gekkonid lineages, substantial variation exists in clutch size. Seven of eight lineages of the scincid genus *Emoia* have maintained a fixed clutch size of two eggs, whereas members of a single lineage of *Emoia*, the *E. samoensis* species group, have highly variable clutch sizes both between and within species (Greer 1968; Brown 1991; Cree 1994; Hamilton et al. 2008). Additionally, Walker (1981) also found interspecific variation in clutch size between the closely related teiid lizards *Aspidoscelis sacki* and *A. parvisocius*.

While clutch size has gained a significant amount of attention for comparative analysis of reproductive traits, other reproductive characters have also been reported to show interspecific variation. For example, the polychrotid lizard genus *Anolis* has been found to have interspecific variation in oviductal structure and seasonal variation of reproductive activity (Ortiz and Morales 1974; Sever and Hamlett 2002).

Asexually reproducing vertebrate lineages present further complications with regard to reproductive variation and isolation. The majority of unisexual vertebrate lineages arise from interspecific hybridization between two closely related bisexual species (Echelle 1990). Thus, unisexuals are typically morphologically distinct from either parent species. Despite genetic

uniformity of unisexuales, intraspecific variation may also occur because of phenotypic plasticity, a high degree of heterozygosity, multiple hybrid origin events, and the presence of polyploidy in some unisexual lineages associated with hybrid genotypes (Kearney and Shine 2004).

Energy investment in reproduction, egg production, and clutch size differ between unisexual and bisexual species in some unisexual-bisexual complexes, whereas no variation in reproductive traits is seen in other unisexual-bisexual complexes. Congdon et al. (1978) found that some unisexual *Aspidoscelis* (Teiidae) differ from congeneric bisexuals both in the amount of energy invested to each egg and clutch, and in the number of eggs produced per unit of growth in reproductively active females. In addition, Taylor and Caraveo (2003) showed that clutch size differed between two unisexual species of *Aspidoscelis* (*A. exsanguis* and *A. flagellicauda*) that share two of the same parent species (Dessauer and Cole 1989; Lowe et al. 1970; Vrijenhoek et al. 1989). Alternatively, Schall (1978) found no differences in clutch size or reproductive cycles between sympatric populations of unisexual *Aspidoscelis* and hypothesized bisexual parent species, and suggested the lack of variation may result from unisexuales inheriting the reproductive traits of their parent species.

Furthermore, reproductive morphology has previously been shown to be variable within vertebrate lineages and within unisexual-bisexual complexes. Sperm storage characters were shown to have family-level phylogenetic trends in salamanders (Sever and Brizzi 1998), and potential phylogenetic implications were also seen across the subphylum Vertebrata as well as within the sub-order Squamata (Sever and Hamlett 2002). Analysis of the oviductal and ovarian morphologies in the unisexual-bisexual *Heteronotia binoei* complex (Gekkonidae) revealed no reproductive differences between the asexually and sexually reproducing females, but the reproductive morphology seen in all members of the complex differed from what has been reported for other geckos (Whittier et al. 1994). Additionally, Saint Girons and Ineich (1992) found that *Lepidodactylus lugubris* (Gekkonidae) parthenogens were seen to have small ovo-testes and male medullary tissue (indicating some level of hermaphroditism), whereas the rest of the reproductive tract resembled the fertile bisexual females. These unisexuales also exhibited the ability to store sperm in the oviduct (Saint Girons and Ineich 1992), although it is unclear whether or not the sperm are used to fertilize clutches. While it is unnecessary for unisexuales to reproduce with sperm, occasionally syngamy (the inclusion of sperm) producing polyploidy offspring may occur if a unisexual mates with a bisexual parent species (Bogart and Licht 1986).

Just as variation exists in the degree to which unisexual lineages share reproductive characteristics with their bisexual parent lineages, the factors responsible for regulating phenotypic diversity in unisexual lineages are variable, and both genetic and environmental factors have been shown to influence unisexual phenotypic diversity (Dawley 1989; Kearney and Shine 2004). Although some research exists on certain aspects of reproduction in a few unisexual-bisexual complexes, for most unisexual-bisexual squamate complexes, it is unknown if the members differ with respect to characteristics such as reproductive morphology, timing of reproduction, and fecundity. Because of this lack of basic knowledge, similarities and differences in reproduction between the parent species and their unisexual lineages are often poorly understood. Differences in reproduction in these complexes have implications for our understanding of the patterns and processes responsible for the generation and maintenance of unisexual lineages.

The *Nactus pelagicus* complex (Reptilia: Squamata: Gekkonidae) of Australia, Melanesia, and Oceania consists of a bisexual, sexually reproducing species (*N. multicaerinus*), within which are at least four genetically distinct lineages (I-IV) based on the number of chromosomes, allozyme data, and variation in mitochondrial restriction fragment length polymorphisms (RFLP) (Donnellan and Moritz 1995; Moritz 1987); and a unisexual, asexually reproducing species (*N. pelagicus*). The *N. multicaerinus* lineages are morphologically similar and are found in New Guinea, the Solomon Islands, northeastern Australia, the northern islands of the Vanuatu Archipelago and two southern islands in Vanuatu (Moritz 1987). However, Zug and Moon (1995) suggested that the species epithet *N. multicaerinus* applies only to the populations found in the southern Solomon Islands and Vanuatu (excluding the islands of Tanna and Erromango), and Zug (1997) designated the Australian bisexuals as *N. cheverti*. Unisexual *N. pelagicus* is found in the Loyalty Islands, New Caledonia, Micronesia, Tonga, Niue, Samoa, Tuvalu, and southern Vanuatu (Moritz, 1987). The only region of overlap between the unisexual species and the lineage IV bisexual species is within the Vanuatu Archipelago (Moritz 1987). Although the unisexuals and a bisexual lineage co-occur on two islands in southern Vanuatu (Aneityum Island and Tanna Island) (Moritz 1987; Eckstut 2006), whether the two species are syntopic is unknown. Based on the number of chromosomes, variation in both allozyme, and mtDNA RFLP data, *N. pelagicus* ($2N = 35$) is hypothesized to have resulted from hybridization between two genetically distinct lineages of the *N. pelagicus* complex; the paternal lineage with a chromosome $2N$ of 28 pattern and the maternal lineage with an as yet undiscovered chromosome $2N$ of 42 pattern (Donnellan and Moritz 1995; Moritz 1987). While the maternal genome ($2N$ of 42) has not yet been identified, the paternal genome ($2N$ of 28) is hypothesized to be the Vanuatu archipelago *N. multicaerinus* lineage (Moritz 1987).

Morphologically, the unisexual *N. pelagicus* is only distinguishable from the hypothesized paternal *N. multicaerinus* by differences in chin shield scalation pattern (Moritz 1987; Zug and Moon 1995; Eckstut 2006), and the two reproductive traits, including placement of eggs in the oviduct and size of reproductively active females (Eckstut et al. *unpubl. data*), and it is currently hypothesized that both species carry one clutch from July through September (the Vanuatu dry season). However, little else is known regarding their reproductive habits throughout the rest of the year, due to limited observations and samples. Given the lack of knowledge regarding the *N. pelagicus* complex (both within and between species), we analyzed reproductive morphology of the Vanuatu members of the *N. pelagicus* complex using light microscopy in order to better understand how the hybrid origin of the unisexual *N. pelagicus* has shaped reproduction in this species. We conducted a comparative analysis of oviductal structure, sperm storage traits (e.g., sperm storage tubule location and structure, and presence of sperm being stored), and seasonal variation of the oviduct during the Vanuatu dry season (July through October).

MATERIALS AND METHODS

Specimen Collection

Voucher specimens were collected from the Vanuatu Archipelago between early July to late September (the dry season) during the years 2001, 2002, 2004, and 2005. Sampling localities encompassed the geographic distribution of both *N. pelagicus* and *N. multicarinatus* in the Vanuatu Archipelago and included all major islands within the archipelago. Vouchers are deposited in the LSUMZ. Sixteen specimens were for this study. The analyzed specimens were collected from early July to mid October of 2001 - 2005. Two specimens per species were used from four time periods during the Vanuatu dry season (Early to Mid-July, Early to Mid-August, Early September, and Late September/Early October).

To further our understanding of reproduction and sperm storage in the *N. pelagicus* complex, specimens were also obtained from FMNH, and the utilized specimens were collected from December – April between 1929 and 1970. Three total *N. pelagicus* and three *N. multicarinatus* were utilized. However, only the *N. multicarinatus* had been collected from the Vanuatu Archipelago; the *N. pelagicus* specimens were collected from the Mariana Islands and Fiji. Because these specimens were collected from a variety of localities throughout the South Pacific and were collected over an extensive period of time, only sperm storage analysis was conducted on these specimens, and seasonal variation was not analyzed.

Tissue Preparation and Analysis

Tissue was rinsed in water, dehydrated in a graded series of ethanol, cleared in toluene, and embedded in paraffin. Sections (10 μm thick) were cut with a rotary microtome, and then fixed onto slides using albumin. Alternate slides were stained for general cytology using Hematoxylin-Eosin, Alcian Blue at pH 2.8 for carboxylated glycosaminoglycans followed by Periodic Acid-Schiff's procedure for neutral carbohydrates and sialic acids, and Bromophenol Blue for proteins. These procedures were utilized by Sever and Hopkins (2004) for their analysis of sperm storage in *Scincella lateralis*, following Kiernan (1990).

Slides were analyzed using a Leica DM2000 microscope system (Leica Microsystems, Wetzlar, Germany). Photographs were taken using a Leica DF420 digital camera (Leica Microsystems, Wetzlar, Germany). Images were subsequently edited using Adobe Photoshop 7.0 or Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA).

RESULTS

Oviductal Structure

We found no difference between the species regarding oviductal structure. Both species exhibited generic gekkonid oviductal structure as described by Girling (2002) with regard to the infundibular, uterine, and vaginal regions. The uterine tube, isthmus, and uterus were all

considered a single region for this analysis, as all three defined regions in analyzed geckos are morphologically indistinct.

The oviduct consists of three layers: The inner mucosa, the middle muscularis, and the outer visceral pleuroperitoneum. The muscularis of the infundibulum consists only of a thin layer of longitudinal smooth muscle anteriorly and this layer becomes progressively thicker in the posterior infundibulum and throughout the remainder of the oviduct, with the vagina as the most muscular region.

The epithelium of the mucosal layer throughout the oviduct contains ciliated cells alternating with non-ciliated secretory cells. These cells are simple squamous and cuboidal in the anterior half of the infundibulum and are columnar in the remainder of the oviduct.

Simple tubular glands first appear anteriorly in the mid infundibulum and occur throughout the rest of the infundibulum and uterus. The simple tubular glands in the posterior infundibulum are often interspersed with a few branched tubular glands, and resemble sperm storage tubules reported in other squamates. The oviductal epithelium and associated tubular glands share the same staining characteristics (Table 1).

Table 1. Reproductive cycles as characterized by Periodic Acid Sciff's, Alcian Blue, and Bromophenol Blue stains. Unless stated otherwise, histochemical reactions refer to throughout the identified region. Each row represents a specimen. Somewhat positive histochemical reaction is indicated by the symbol (~). Specimens associated with time A indicates specimens from early- to mid-July, B from early- to mid-August, C from early September, and D from late September/early October

Time	<i>Nactus multicarinatus</i>			<i>Nactus pelagicus</i>		
	Infundibulum	Uterus	Vagina	Infundibulum	Uterus	Vagina
A	~AB+ (posterior)	PAS++/AB+	AB+	Inactive	Inactive	~AB/PAS+
A	AB++ (posterior) ~AB/PAS+	AB+ (lining)	Inactive	AB++	Inactive	PAS++/AB++
B	(posterior)	AB++	~AB+	AB+++	AB++ ~AB+	Inactive
B	~AB+	~AB+	~AB+ ~AB/	AB++	(lining) AB+/	Inactive Inactive/~BB+
C	Inactive	Inactive	PAS+ ~AB	AB+/~PAS+	~PAS+ AB+/	(lining) Inactive/~BB+
C	Inactive	Inactive	/PAS+	AB+/~PAS+	~PAS+	(lining)
D	AB+++	AB+ (lining)	Inactive	Inactive	Inactive	Inactive
D	AB++	Inactive	Inactive	Inactive	Inactive	Inactive

Sperm Storage Traits

No sperm storage trait differences were observed between the species. Both had what appeared to be sperm storage tubules (Ssts) in the posterior infundibulum (Figure 1). During the dry season, neither bisexual *N. multicarinatus* nor unisexual *N. pelagicus* specimens had sperm in the Ssts during the dry season with eggs *in utero*. During April and February, there were some residual sperm found in the Ssts of gravid *N. multicarinatus*, though no sperm were found in the *N. pelagicus* specimens (Figure 2).

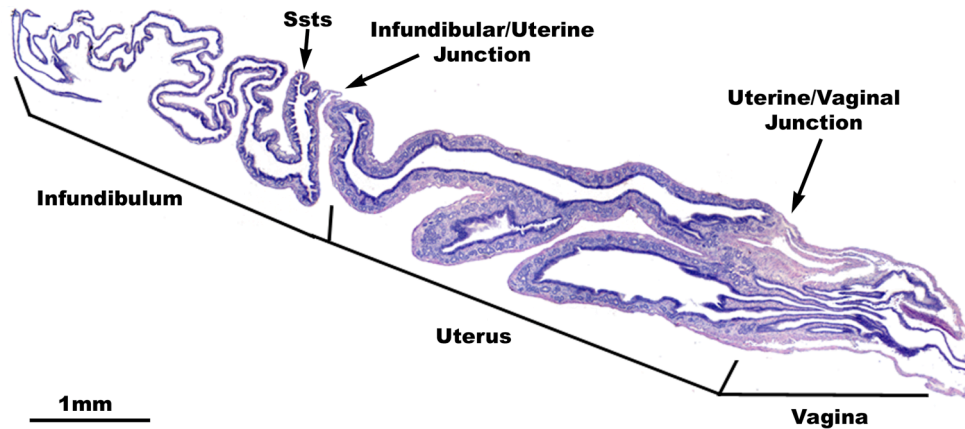


Figure 1. *Nactus multicarinatus* oviductal structure using Hematoxylin-Eosin (H&E) stain for general cytology.

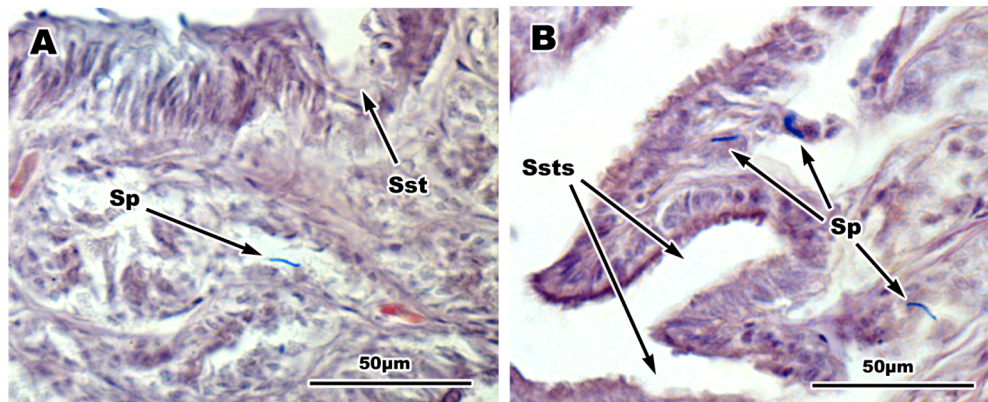


Figure 2. Presence of sperm in the posterior infundibulum of February (A) and April (B) *Nactus multicarinatus* specimens. Stained using H&E, with the sperm H⁺⁺. Sp = Sperm, Ssts = Sperm storage tubules.

Oviductal Seasonal Variation

Seasonal variation of the oviducts was observed between *N. pelagicus* and *N. multicarinatus*. The state of the oviduct of *N. pelagicus* appears to be one and a half to two months ahead of the oviductal state of *N. multicarinatus* (Table 1). The July *N. pelagicus* more closely resembled the September *N. multicarinatus* specimens (Figure 3), with both samples exhibiting little activity in the oviduct. Alternatively, the August *N. pelagicus* specimens more closely resembled the early October *N. multicarinatus* specimens (Figure 4), with heavily AB⁺ posterior infundibular and uterine regions. Furthermore, the July and August *N. multicarinatus* samples resembled none of the *N. pelagicus* samples, whereas the September and late September *N. pelagicus* samples resembled none of the *N. multicarinatus* samples.

CONCLUSION

Oviductal Asynchrony Between *N. pelagicus* and *N. multicolorinatus*

Analysis of oviductal seasonal variation shows greater insight into previously unrecognized, cryptic differences between the hypothesized paternal *N. multicolorinatus* lineage located in northern Vanuatu and the unisexual *N. pelagicus*. How long these species retain one clutch is unknown. Our findings indicate that there are at least two clutches in *N. multicolorinatus* per year: At least one in the wet season, and one in the dry. However, while we have no evidence at present to suggest that *N. pelagicus* has multiple clutches per year, though we suggest that multiple clutches is probable due to overall reproductive similarity to the paternal *N. multicolorinatus* lineage. Furthermore, Eckstut et al. (*unpubl. data*) identified a likely scenario of both *N. pelagicus* and *N. multicolorinatus* developing only one clutch during the dry season in Vanuatu (July through October). Our finding of asynchrony in the oviducts does not refute the possibility of only one clutch in the dry season. However, these findings do indicate a potential temporal variation in reproductive activity, with *N. pelagicus* exhibiting particular histochemical states roughly one and a half to two months prior to *N. multicolorinatus* (Table 1). Eckstut et al. (*unpubl. data*) found that the egg size did not differ between *N. pelagicus* and *N. multicolorinatus*. Our results, however, depicting asynchrony may indicate that growth rate and/or overall size of eggs may differ between species.

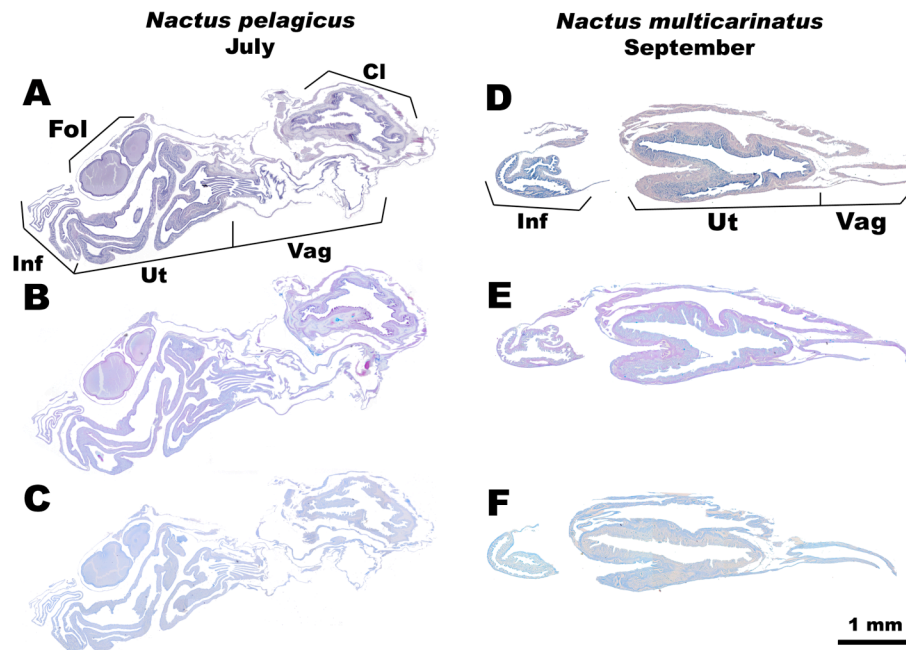


Figure 3. Oviducts of July *Nactus pelagicus* and September *N. multicolorinatus*. A-C represent a July *N. pelagicus* stained using H&E, PAS/AB, and BB, respectively. D-F represent a September *N. multicolorinatus* stained using H&E, PAS/AB, and BB, respectively. None of the oviducts exhibit strong histochemical reactions. Inf = Infundibulum, Ut = Uterus, Vag = Vagina, Cl = Cloaca, and Fol = Vitellogenic Follicles.

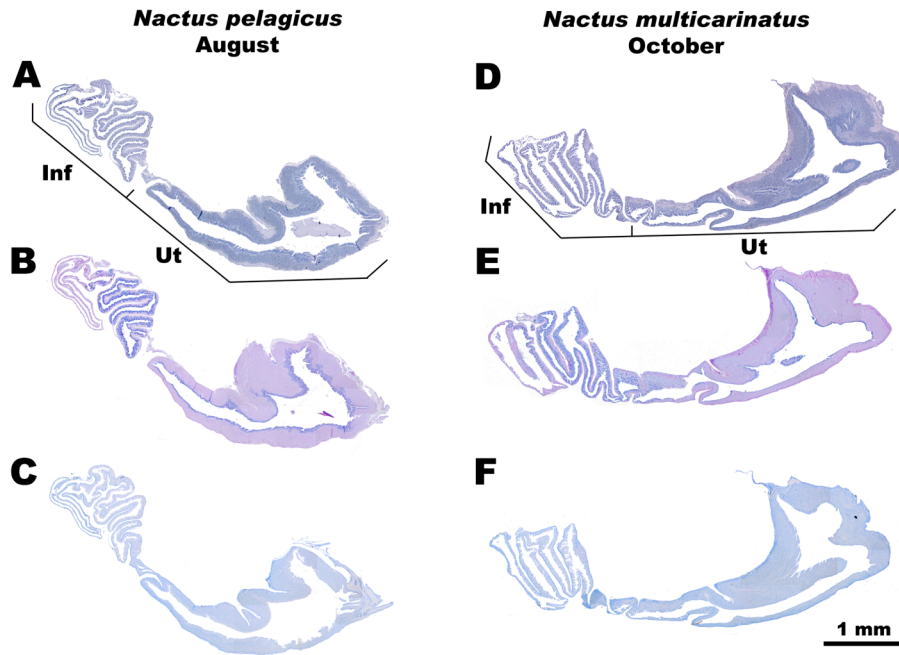


Figure 4. Oviducts of August *Nactus pelagicus* and October *N. multcarinatus*. A-C represent an August *N. pelagicus* stained using H&E, PAS/AB, and BB, respectively. D-F represent an October *N. multcarinatus* stained using H&E, PAS/AB, and BB, respectively. Both stained heavily AB+ in the posterior infundibular and uterine regions. Inf = Infundibulum and Ut = Uterus.

The differences we found between a presumed parent species and its hybrid offspring could have arisen for a variety of reasons, perhaps as a result of the unisexuals, inheriting a mixture of traits from each parent. These differences in seasonal variation may be the result of maternal inheritance, in which *N. pelagicus* inherited its reproductive cycle from the undiscovered maternal lineage of *N. multcarinatus*. Alternatively, the *N. pelagicus* seasonal variation may represent an intermediate state between the maternal and paternal traits. However, these hypotheses can only be resolved by discovery of the maternal lineage and analysis of the maternal lineage's reproductive biology. These questions may only be thoroughly addressed by more complete yearly sampling of *N. pelagicus* and the paternal *N. multcarinatus* lineage.

Sperm Storage Variability in Geckos

Our analysis of *N. multcarinatus* shows more variation in sperm storage traits than previously observed within both sub-order Squamata and family Gekkonidae. Sperm storage studies of other lizard species have shown that the majority of lizards retain oviductal sperm while eggs were *in utero*. We found no evidence for oviductal sperm storage with eggs *in utero*, and the scarce number of sperm we observed in the Ssts are likely remnants of a previous mating and not actually sperm storage. Examination of male reproductive cycles is necessary to see when males are producing and storing sperm, and thus capable of mating throughout the year, when females are laying multiple clutches.

The lizards that have been observed to store sperm with eggs *in utero* range across several unrelated lizard taxa, including *Anolis sagrei* (family Polychrotidae; Sever and Hamlett, 2002), *Holbrookia propinqua* (Phrynosomatidae; Adams and Cooper 1988), *Acanthodactylus scutellatus* (Lacertidae; Bou-Resli et al. 1981), *Hemidactylus frenatus* (Gekkonidae; Murphy-Walker and Haley 1996), and *H. turcicus* (Gekkonidae; Eckstut et al. 2009). Previous to this study, the one known exception was a species of skink, *Scincella lateralis* (Sever and Hopkins 2004). All of the species mentioned above have infundibular sperm storage except for *A. sagrei* (utero-vaginal) and *S. lateralis* (vaginal), and the sperm are more sequestered in special glands in *A. sagrei* than in *S. lateralis*.

This analysis of the Vanuatu *N. pelagicus* complex provides knowledge of a second lizard species that does not store sperm with eggs *in utero*. We found no evidence of sperm being stored in *N. multicoloratus*, the bisexual species, during the Vanuatu dry season when all analyzed specimens were carrying eggs *in utero*. Geckos are often thought of as reproductively conservative, especially since the majority of geckos have a uniform clutch size of two calcareous-shelled eggs (Bustard 1968; Rose and Barbour 1968). However, this may be due to the length of time it takes for the clutch to develop *in utero* before oviposition, which may influence whether or not sperm can be utilized to fertilize several clutches. *Hemidactylus turcicus* (Gekkonidae) produce between 3 and 4 clutches between May and August of the southeastern United States, indicating rapid development of the eggs within the uterus and quick deposition, and a strong potential for fertilization of multiple clutches by stored sperm from matings preceding the first clutch (Eckstut et al. 2009). However, *N. multicoloratus* likely only develops one clutch over the four months that were sampled (July through October). Storing sperm during that time is potentially both inefficient and unnecessary, especially if the sperm gradually degrade over time. *Scincella lateralis*, the other known species of lizard that does not store sperm with eggs *in utero*, was seen to have epithelial cells in the sperm storage area that contained lysosomes, which may be involved in spermophagy (Sever and Hopkins 2004). Degradation of sperm may be related to the concept that certain vertebrate lineages do not use sperm that become embedded in the oviductal epithelium for fertilization (Sever and Brizzi 1998).

Despite the amount of informative data gathered from this analysis, many of our hypotheses are purely speculative at this time due to a lack of readily available specimens from the Vanuatu archipelago from throughout the year. Further examination of annual reproductive cycles are needed in both species, both to determine if *N. multicoloratus* is able to store sperm at any point throughout the year, and to fully address how variable these species are with regard to one another, as well as in comparison to other gekkonid and squamate lineages.

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Reviewed by

Brian I. Crother,

Southeastern Louisiana University, Hammond,
Louisiana 70402 (Email: bcrother@selu.edu);

Roldán Valverde,

Southeastern Louisiana University, Hammond,
Louisiana 70402 (Email: rvalverde@selu.edu).