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Seasonal Testicular Development and Sperm Storage in Tropical and Subtropical Populations of the Brown Tree Snake (*Boiga irregularis*)

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Abstract

The seasonal pattern of testicular development of tropical and subtropical brown tree snakes, *Boiga irregularis*, was investigated. We also examined sperm storage in the reproductive tracts of both sexes. Males in south-eastern Queensland had a testicular cycle that was strongly seasonal, with testicular regression during the wet summer. Males from Papua New Guinea had a continuous testicular cycle, in which sperm was present in some animals every month.

Females from south-eastern Queensland had no stored sperm or sperm storage receptacles in their oviducts. Sperm was found in only one of 28 female reproductive tracts. The sperm was located in the lumen, suggesting recent insemination. In contrast, the presence of sperm in the ductus deferens of most males in both populations indicated that males store sperm. This facilitates the apparent dissociation between the male and female reproductive cycles that has been reported previously in south-eastern Queensland populations. Male *B. irregularis* must store sperm for at least six months, from the time of maximal testicular development in autumn, until ovulation occurs in spring and early summer. This pattern of reproduction in *B. irregularis* fits neither a postnuptial nor a prenuptial pattern, as has been observed in a few other tropical snakes.

Introduction

The influence of the environment on sexual reproduction in reptiles has received a great deal of attention (Duvall *et al.* 1982; Licht 1984; Seigel and Ford 1987; Whittier and Crews 1987; Whittier 1994). However, studies have rarely focused on comparisons between populations of the same species in different regions. Intraspecific geographical variation in the reproductive cycle of males suggests an adaptive response to the local environment (Ballinger 1977). A number of reproductive patterns in reptiles are recognised and these appear to have evolved in response to environmental constraints (Seigel and Ford 1987).

The native range of the brown tree snake, *Boiga irregularis*, extends from the equatorial islands of Papua New Guinea (PNG) and north-western Melanesia to the cool-temperate eastern seaboard of Australia as far as 34°S (Cogger 1992). Given this wide latitudinal distribution, we sought to compare the testicular cycle of this species from specimens collected in subtropical south-eastern Queensland and tropical PNG.

Information about the reproductive cycle of *B. irregularis* in its native range may help us understand the patterns of native species distribution and of invasive species. *Boiga irregularis* represents one of the few colubrids present in Australia. It most likely invaded from Asia relatively recently in geologic terms (Shine 1991). Although there is no common feature of the colubrids that have successfully colonised PNG and Australia, specialised reproductive mechanisms may have contributed to their ability to invade across sea barriers from Asia. For example, the presence of unusual reproductive capabilities explains the distribution of many reptilian taxa on islands in the south Pacific (Gibbons 1985).

Boiga irregularis is an invasive species in the recent historical past as well. It was accidentally introduced onto the island of Guam (13°N) during the 1940s and has since established itself as a serious pest (Fritts 1988); evidence suggests that *B. irregularis* has caused an alarming decline in populations of the native fauna on the island (Savidge 1987; McCoid 1991). As *B. irregularis* presents an immediate threat to biological diversity in regions of high endemism in the Pacific, general information about reproduction in this species is needed.

Study of reproduction in the genus *Boiga* was first conducted in central Java by Kopstein (1938). This work reported that isolated female *B. multimaculata* were capable of producing eggs more than nine months after capture, suggesting a capacity for sperm storage in females. However, no morphological evidence of oviducal sperm storage was presented. Such storage of sperm within the female oviduct for extended periods after mating has been documented in a variety of reptiles (Fox 1956; Saint Girons 1975; Gist and Jones 1987).

We have reported that in subtropical south-eastern Queensland the environment constrains activity and foraging in *B. irregularis* (Whittier and Limpus 1996; Bull and Whittier 1996). This limits the reproductive capacity of females. Moreover, a dissociated pattern of reproduction is observed in *B. irregularis* in this region: when female ovarian development is maximal, males have regressed testes. The consequence of this is that sperm storage in the male or the female is obligatory, at least in the south-eastern portion of the species' range (Whittier and Limpus 1996).

In this study we further investigated the capacity for sperm storage in males and females obtained from south-eastern Queensland and compared these patterns to that of males from PNG. We hypothesised that sectioned oviduct material from mature female *B. irregularis* would frequently contain sperm. Moreover, we expected to find this sperm contained in specialised regions of the oviduct wall, and that it would be present from March to October. We also sought to compare the patterns of testicular development in males from the subtropical Queensland environment with that of males from the tropical PNG environment. Finally, we related the pattern of sperm storage and testicular development to long-term records of climate obtained for both areas.

Materials and Methods

Specimen and Sample Collection

Male and female specimens of *B. irregularis* held in the Australian Museum, Queensland Museum and by J. Whittier were examined. The monthly sample of snakes was derived from collections made in different years (1950–1993). Included in the sample were male *B. irregularis* from two areas: (1) south-eastern Queensland, 26–28°S, 152–154°E ($n = 40$), and (2) PNG and associated islands, 2–9°S, 140–150°E ($n = 32$). Preliminary histological analysis of the presence of sperm in the testis and ductus deferens of 22 of the south-eastern Queensland males was previously reported in Whittier and Limpus (1996). In the present study we extended this work in two ways, first by including 18 additional males (12 freshly collected and 6 additional samples from the Queensland Museum). These new animals were selected and collected to fill gaps in the previous sample of males. Second, we extended the investigation of all testicular material to include the observation of spermatogenetic stages. All female specimens ($n = 28$) examined were from south-east Queensland, as few females from PNG were available. Snout to vent length (SVL, ± 0.5 cm) was recorded for each individual; all specimens were selected to be adults of snout–vent lengths greater than 75 cm (Whittier and Limpus 1996).

Female reproductive condition was assessed by observing the gross morphology of the ovaries and reproductive tracts. Females were categorised with respect to follicular size as: non-reproductive (<3 mm); early vitellogenic (3–10 mm); late vitellogenic (>10 mm); or, with oviducal eggs, as gravid.

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Oviducts were serially sectioned in a sagittal plane and the right testis and ductus deferens were representatively sectioned in a transverse plane at 7 μ m. Sections were stained with haematoxylin and eosin.

Reproductive Status of Males

The condition of the seminiferous tubules was classified into three stages of spermatogenesis:

Stage 1. Beginning of spermatogenesis, spermatogonial multiplication, primary and secondary spermatocytes;

Stage 2. Spermatids abundant and transforming, few spermatozoa;

Stage 3. Spermatozoa numerous, spermiation occurring.

The presence or absence of sperm in the ductus deferens was noted. No numerical score of the amount of ductus sperm was made because we found either that sperm was absent or that the ductus was packed with sperm.

Climatology Data

Weather data were obtained from the Bureau of Meteorology, Brisbane, Queensland. Long-term mean values of monthly mean minimum temperature (°C), mean rainfall (mm) and relative humidity (%) at 1500 hours from Brisbane, Queensland, Australia, and Port Moresby, PNG, were used. Low numbers of specimens from regional areas of PNG prohibited a more detailed analysis of potential regional effects of climate on male reproduction. As *B. irregularis* is a nocturnal predator, mean minimum temperature was used because this reflects the night-time temperatures experienced by the snake.

Statistical Analyses

Prior to making comparisons between males collected from south-eastern Queensland and PNG, we evaluated the effect of body length on the stage of testicular development to control for effects of body size on reproductive condition. We compared the mean size of males from the two locations, and then the influence of males' size on the condition of the testicular stage and presence of sperm in the ductus deferens, by ANOVA (SAS 1988). The relationship between testicular development and the presence of ductus sperm was analysed by way of chi-square contingency tables. The monthly level of mean testicular stage and the frequency of ductus sperm of snakes, and the climate of the two areas, were compared using Fisher's Exact tests. Analyses of the effects of climatic factors on reproductive measures were conducted, combining months due to small sample sizes. This was achieved by dividing the year into two seasons on the basis of three climatic conditions, with each climatic condition considered separately, as follows. The wet season in Queensland included all months with a mean total rainfall >75 mm, and the dry season a mean total <75 mm. The warm season in Queensland was defined as all months with a minimum mean >18°C, and the cool season included all months with a minimum mean <18°C. Similarly, the season of high humidity was defined to include all months with a mean humidity >55% at 1500 hours, and the season of low humidity included months with a mean humidity <55% at 1500 hours. Seasons were defined independently of reproductive data.

Similarly, for PNG, the year was divided into a wet and a dry season, using the same criteria as for Queensland (75 mm of rainfall). Rainfall data were recorded from Port Moresby, and although annual rainfall there is low compared with that in districts from which some specimens were collected, the annual pattern reflects that of the whole area. Long-term rainfall data from elsewhere in PNG are not available.

The significance of seasonal differences was tested by means of one-way ANOVAs. As temperature and humidity did not vary significantly in PNG, no seasonal definition was inferred for these climatic factors. Differences in testicular development and the presence of ductus sperm in males between these seasons were then compared using chi-square contingency table analysis.

The influence of the female's reproductive cycle on the reproductive activity of males in south-eastern Queensland was tested with a Fisher's Exact test. The female's reproductive status was divided into four categories: (1) vitellogenesis – September and October; (2) ovulation – November and December; (3) egg laying – January to March; and (4) non-reproductive – April to August. These categories were based on observations from museum specimens and basic knowledge of the reproductive biology of *B. irregularis* (Shine 1991; Whittier and Limpus 1996). No data on reproductive activity of females were available for PNG as few females are represented in the museum collections.

Results

The morphology of the oviduct of *B. irregularis* was typical of the structures that have been described in other snakes. We observed furrows in the cranial infundibular region, an area where sperm storage is observed in other snakes, but no sperm storage was evident in our specimens in this, or any other, oviducal region. Sperm were observed in only one of the 28 sexually mature female *B. irregularis* examined. This occurred in the posterior of the vaginal segment near the cloaca of a large female (QM# J28392, registered 4/77, collected in Brisbane, Queensland, SVL = 118 cm) that was in a late vitellogenic stage. In this individual, the sperm did not appear

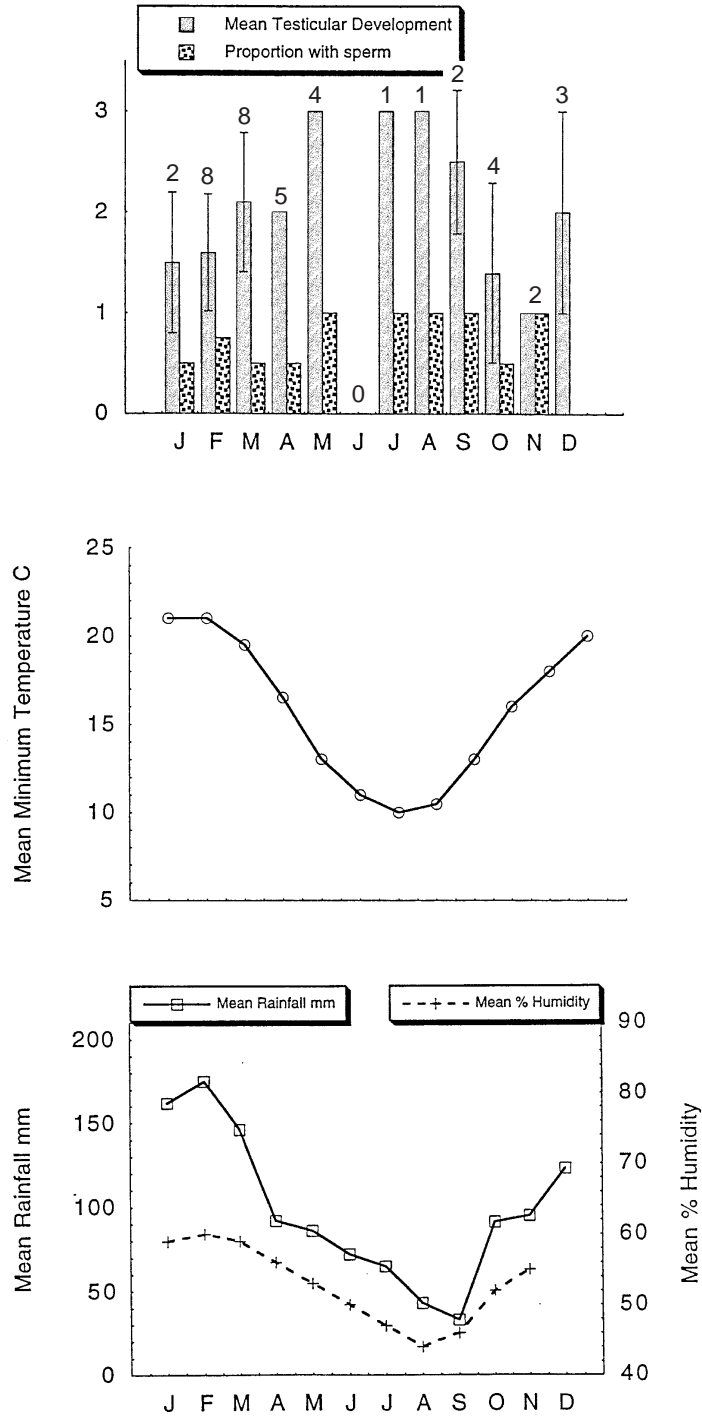


Fig. 1. Seasonal variation in the testicular cycle of *B. irregularis* and climatic factors in south-eastern Queensland. A, Mean testicular development and proportion of males with sperm in the ductus deferens (sample sizes above each bar); B, mean minimum monthly temperature (°C); C, mean monthly rainfall (mm) and mean monthly % humidity at 1500 hours.

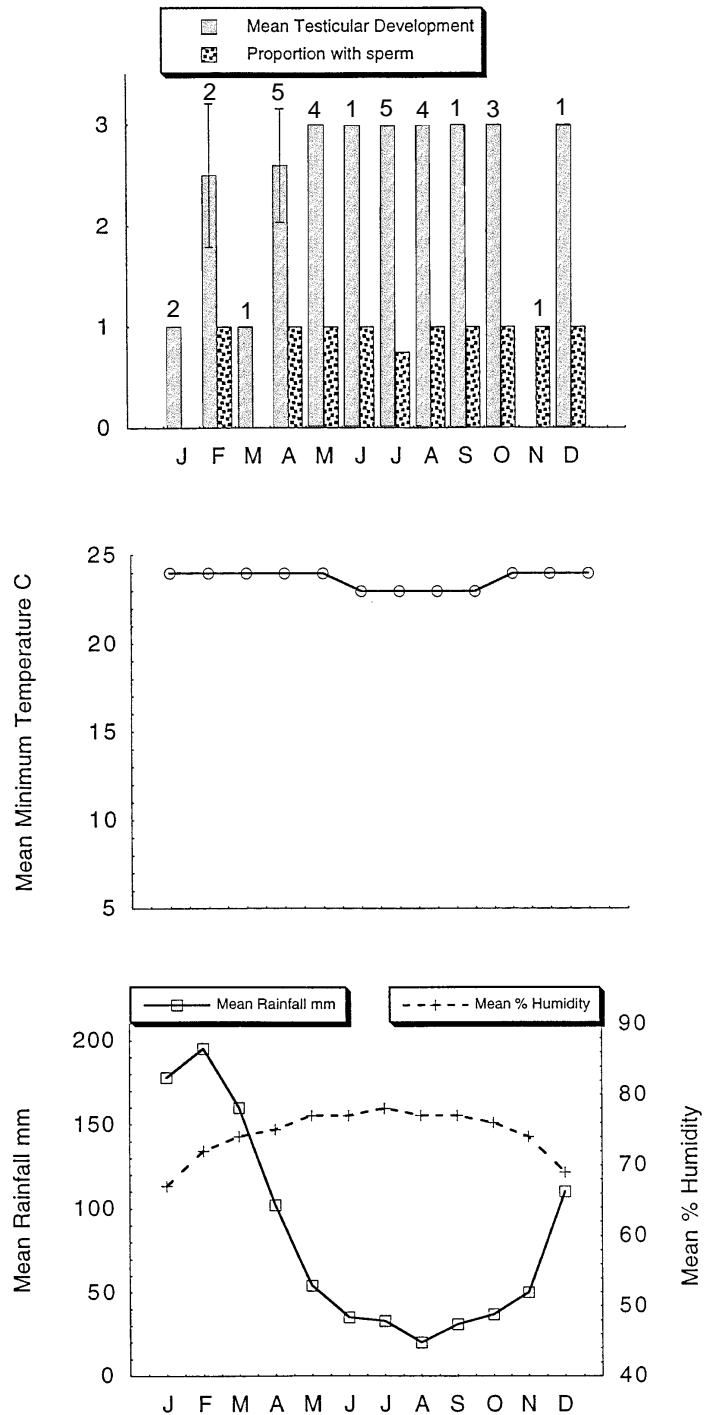


Fig. 2. Seasonal variation in the testicular cycle of *B. irregularis* and climatic factors in Papua New Guinea. A, Mean testicular development and proportion of males with sperm (sample sizes above each bar); B, mean minimum monthly temperature (°C); C, mean monthly rainfall (mm) and mean monthly % humidity at 1500 hours.

to be associated with the epithelium in a manner indicative of sperm storage. Instead, the spermatozoa were free in the lumen, surrounded by the carrier matrix that transports the sperm mass. Sperm storage receptacles, as previously described in other snakes, were not found in any of the females examined.

The males examined were all above the minimum size at sexual maturity (Whittier and Limpus 1996) and ranged from 80 to 150 cm in snout–vent length (SVL) with a mean of 114.5 ± 6.1 cm. There was no significant difference between the mean SVL of Queensland and PNG specimens ($F = 0.1495$, $P < 0.05$, d.f. = 1). Male size did not significantly influence the condition of the gonads or the presence of sperm in the ductus deferens ($F = 0.1256$, $P < 0.05$, d.f. = 2).

Testicular development of *B. irregularis* in south-eastern Queensland varied significantly throughout the year (Fig. 1A). In winter, all males collected in May, July and August (none were obtained in June) had mature sperm in the testis and were classed as Stage 3. In spring (September–November), mean testicular development declined, with 30% of males in Stage 3, and 67% regressed or in early recrudescence phases, in Stage 1. In summer (December–February) and continuing into early autumn in March and April, mean testicular development reflected the majority of males that were in a late recrudescence Stage 2. No males were found with sperm in the testes in the hottest months of the year, January and February.

When we examined the ductus deferens of *B. irregularis* from south-eastern Queensland, we found males with the capacity to inseminate females present throughout the year (Fig. 1A). Sperm were found in the duct of 70% of all snakes examined, including individuals from every season. All males collected in the months of May, July, August and September had sperm in their ductus deferens, coinciding with the months in which testicular sperm were being produced. During the rest of the testicular cycle, the presence of sperm in the ductus was variable. Males with no sperm in their ductus deferens (30%) were found in October, and from January to April.

Testicular development of *B. irregularis* from PNG differed from that observed in males from south-eastern Queensland (Fig. 2A; $P = 0.0256$, Fisher's Exact test). First, the period of maximal testicular development (Stage 3) was extended from February to December in PNG. Thus a larger proportion of males examined from PNG (80%) had mature sperm in the testis. Mean testicular development was lower from January to April, with most males either with regressed or early recrudescence testes (Stage 1) or with late recrudescence testes (Stage 2).

When we examined the ductus deferens of *B. irregularis* from PNG, we found only three males (12%) without sperm; these were collected in January, March and July. All of the other males, collected throughout the year, had ductus sperm present. Although a larger proportion of males from PNG (88%) had sperm in the ductus when compared with those from south-eastern Queensland (70%), this difference was not statistically significant ($\chi^2 = 0.116$, $P < 0.05$).

In males sampled from both regions, there was no relationship between testicular development and presence of sperm in the ductus deferens ($P = 1.0$, Fisher's Exact test). Sperm were absent from the ductus in some fully developed Stage 3 males (17%), but present in most others (83%). Similarly, sperm were absent from the ductus in some testicular Stage 1 males (20%), yet present in most Stage 1 males (80%). Overall, 80% had sperm in the ductus deferens throughout the year.

Significant differences were found in the mean minimum temperatures ($P = 0.00067$, Fisher's Exact test) and the relative humidity at 1500 hours ($P = 0.0$, Fisher's Exact test) at the two sites in every month of the year (Figs 1B, 1C; 2B, 2C). Although the mean annual rainfall in Port Moresby (1125 mm) and south-eastern Queensland (1190 mm) are similar, rainfall from other areas of PNG from which specimens were collected is known to be greater, ranging from 3000 to 5000 mm. The pattern of seasonal rainfall was significantly different between south-eastern Queensland and PNG, with south-eastern Queensland having a shorter wet season from December to April, compared to the longer wet season in PNG, lasting from October to May (Figs 1C, 2C).

The influence of climatic factors and the differences between the two regions in testicular development were investigated. In south-eastern Queensland there was no significant difference in the distribution of males from the three testicular stages, nor of males with sperm in the ductus deferens, between the wet season and the dry season ($P = 0.819$, Fisher's Exact test; and $\chi^2 = 0.406$, $P > 0.05$; d.f. = 4). Similarly, there were no significant effects of temperature or humidity on the incidence of males in the three testicular stages or on the proportion of males with sperm in the ductus deferens, respectively ($\chi^2 = 0.406$, $P > 0.05$, d.f. = 4 for temperature; $\chi^2 = 0.3062$, $P > 0.05$, d.f. = 3 for humidity; $P = 0.313$, Fisher's Exact test for both temperature and humidity). In PNG the wet season/dry season dichotomy also did not significantly affect the distribution of males of different reproductive condition ($\chi^2 = 0.116$, $P > 0.05$, d.f. = 3).

In the south-eastern Queensland specimens, testicular development varied significantly with respect to the reproductive condition of females (Fig. 3; $P = 0.013$, Fisher's Exact test). There was an inverse relationship between the gonadal maturation of males and the stages of the ovarian cycle of females. When females were regressed (from April to August), 90% of males were in the maximal Stage 3 of testicular development, but when females were undergoing vitellogenesis (during spring months, September and October), 43% were in Stage 3 while 57% of males were regressed in Stage 1. During ovulation (during November and December), 60% of males were regressed in Stage 1. While females were laying eggs (from January to March), 35% of males were in Stage 2 and 41% of males were in Stage 3. Although the testicular stage of the males was significantly related to the ovarian cycle of the females, the presence of sperm in the ductus, which exhibits a less defined pattern throughout the year, was not ($P = 0.808$, Fisher's Exact test).

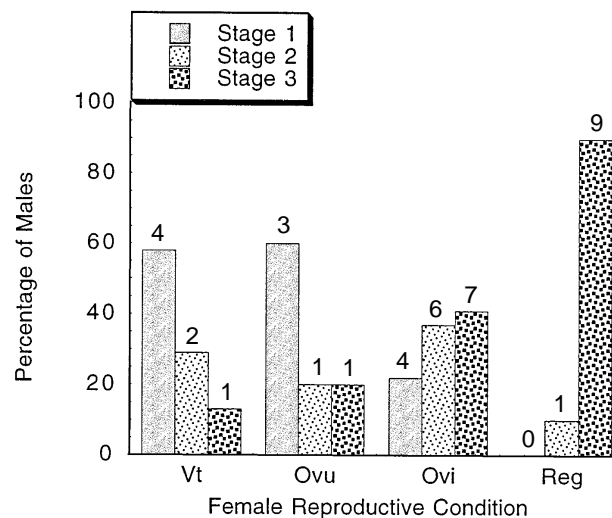


Fig. 3. Percentages of males in each of three testicular developmental stages (stage 1 = regressed; stage 2 = developing; stage 3 = maximal) in south-eastern Queensland with respect to the stage of the seasonal female reproductive cycle (Vt = vitellogenesis; Ovu = ovulation; Ovi = oviposition; Reg = regressed). Sample sizes are noted above each bar.

Discussion

The oviduct of *B. irregularis* appeared similar to that of other oviparous reptilian species. However, histological examination of serial sections of the reproductive tracts of 28 adult females found no seminal receptacles for the storage of sperm in either the vaginal (Ludwig and Rahn 1943; Halpert *et al.* 1982; Gist and Jones 1987) or the infundibular (Fox 1956; Fawcett *et al.* 1972; Hoffman and Wimsatt 1972) segments of the oviduct. In the single individual in which

sperm was recorded, the sperm was believed to be from a recent insemination and was free in the lumen. Because the female had large vitellogenic follicles we inferred that the sperm mass was in the process of migrating towards the site of fertilisation in the infundibulum.

Sperm can be stored for short periods in snakes within the lumen of the vaginal segment, without the use of specialised crypts (Saint Girons 1975). Although it is possible that female *B. irregularis* store sperm on a short-term basis, the absence of seminal receptacles in the oviduct suggests that longer-term storage may not commonly occur. We conclude that females in eastern Australia do not have obligatory oviducal sperm storage.

Our results suggest that male *B. irregularis* store sperm. Although sperm storage has been documented in only a few species of male snakes (Johnson *et al.* 1982; Saint Girons 1982; Mitchell and Zug 1984), it is accepted as an obligate component of postnuptial spermatogenesis (Saint Girons 1982; Schuett 1992).

The pattern of testicular development of *B. irregularis* in tropical PNG resembled reproductive patterns found in reptiles from tropical areas, particularly those with a distinct wet/dry season (Fitch 1982; Saint Girons 1982; Seigel and Ford 1987). There was an extended period of maximal testicular development from May to December and evidence of a short period of regression from January to March. This pattern is comparable to those of the tropical colubrids *Dendrelaphis pictus*, *Ptyas mucosus* and *Rhabdophis subminiatus* in south-east Asia (Saint Girons 1982). It probably represents a phylogenetically conservative pattern for *B. irregularis*, which is thought to have originated in tropical Asia (Shine 1991).

The pattern of spermatogenetic activity of *B. irregularis* in subtropical south-eastern Queensland was defined by a more pronounced cycle than was observed in males from tropical PNG. However, it was not a typical postnuptial cycle such as has been reported in North American colubrids from temperate climates. In the typical postnuptial cycle, spermatogenesis is maximal during the spring and early summer, often after mating has occurred (Seigel and Ford 1987). Sperm used during mating in these postnuptial species is from previously stored ductus sperm. In contrast, in south-eastern Queensland *B. irregularis* begins testicular recrudescence in autumn and maximal testicular development is observed during the winter, when animals are inactive. Testicular regression begins in spring and continues through summer, the testes being at a minimal stage in late summer.

Boiga irregularis in south-eastern Queensland also departs from the typical temperate-zone postnuptial pattern with regard to the presence of sperm in the ductus deferens. Rather than having sperm in the ductus during only a portion of the season, ductus sperm was present in some males collected in every month of the year. This suggests that a portion of the male population of *B. irregularis* in subtropical Queensland may be capable of inseminating females, if they are vitellogenic, in any month of the year. Timing of mating is not known in these areas; a single instance of mating in *B. irregularis* has been recorded in south-eastern Queensland in March (Whittier and Limpus 1996). At this time of year a male could use either previously stored ductus sperm (and thus be classed as postnuptial) or newly developed testicular sperm (prenuptial) or, possibly, both. The relationship between evacuation of previously stored ductus sperm and the production of new sperm is not known in this species.

Clearly, *B. irregularis* in the subtropical portion of its range maintains a range of reproductive options that are more diverse than is seen in colubrids in subtropical and temperate zones elsewhere. The testicular cycle and the reliance on stored ductus sperm in this species appears to be a pattern intermediate between those of colubrids from temperate and tropical areas. The reproductive patterns exhibited by male *B. irregularis* in both the tropical and subtropical portions of their range do not fit into the classic prenuptial/postnuptial patterns described in temperate-zone snakes and these terms, as previously suggested by Saint Girons (1982), may not be useful in tropically derived species.

Although the postnuptial/prenuptial dichotomy is not particularly useful in describing the testicular cycle of male *B. irregularis*, we confirm that the cycle of males and females is dissociated in south-eastern Queensland (Whittier and Limpus 1996). We found a significant negative relationship between the timing of testicular development and the reproductive

condition of female *B. irregularis*. Males have regressed or, at most, early recrudescing testes during the ovulation and egg-laying phase, and have maximal testicular development when females are non-reproductive. This dissociation is facilitated by males both producing and storing sperm in their ductus deferens over winter. Thus, although males appear capable of inseminating females nearly all year, we found no evidence that females store sperm for long periods in their oviducts prior to ovulation.

Ultimately, any interpretation of these intersexual differences in reproductive patterns requires an understanding of the selective forces determining the times of onset of recrudescence and gonadal regression. Climatic factors are often cited as proximate cues for controlling the timing of reproduction in reptiles (Duvall *et al.* 1982; Seigel and Ford 1987; Whittier 1994). For female *B. irregularis* in south-eastern Queensland initial follicular development commences in winter, a process that is most likely supported by lipids stored in abdominal fat bodies from the previous summer. Increases in temperature and/or rainfall may act as proximate cues for favourable conditions that ensure reproductive success.

In male *B. irregularis*, a proportion of the population from subtropical south-eastern Queensland have testes that are quiescent in the warm summer months of high rainfall and humidity. In tropical Papua New Guinea, fewer animals undergo regression; those that were regressed were collected in the wettest months of the year (January–March). The significantly different pattern of testicular development between the populations and the significant climatic differences between the two locations suggests that environmental factors may be permissive cues that regulate testicular development. Future study of both male and female *B. irregularis* in more narrowly defined regions of PNG would contribute to our understanding of the factors that regulate reproduction in this species.

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Appendix. Material Examined

Females:

Queensland Museum Specimens: J3244, J4794, J8597, J10263, J11181, J11269, J26074, J28392, J32238, J32266, J36753, J37174, J47313, J48104.

J. M. Whittier's specimens: JMW004, JMW017, JMW031, JMW033, JMW035, JMW036, JMW038, JMW040, JMW041, JMW042, JMW044, JMW045, JMW046, JMW048.

Males – South-eastern Queensland Specimens:

Queensland Museum Specimens: J5406, J8005, J8186, J8401, J8454, J9089, J9820, J9907, J20295, J28066, J33457, J33461, J33464, J33465, J33468, J33498, J40293, J40936, J40949, J47015, J47396, J48096, J48129, J48582, J48599, J49797, J49798, J51763, J54584.

J. M. Whittier's specimens: JMW001, JMW002, JMW003, JMW005, JMW007, JMW008, JMW009, JMW020, JMW021, JMW028.

Males – PNG Specimens:

Australian Museum: S28706, S40712, S40347, S86923, S93217, S115330, S120876, S121440, S121187, S122370, S122372, S124365, S124799, S124816, S124835, S124836, S127469, S129052, S130424, S134940, S134944, S134994, S135312, S135580, S136278, S137231, S137234, S142882.