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INTERACTIONS OF BEHAVIOR AND PHYSIOLOGY DURING THE ANNUAL REPRODUCTIVE CYCLE OF THE RED-SIDED GARTER SNAKE (*THAMNOPHIS SIRTALIS PARIETALIS*)

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ABSTRACT: Canadian populations of the red-sided garter snake (*Thamnophis sirtalis parietalis*) have a severely limited yearly period for growth and reproduction. Breeding occurs immediately on emergence from winter dormancy, before annual gonadal recrudescence occurs. Because this temporal dissociation of gonadal function and reproductive behavior is unlike that occurring in other vertebrates, *T. sirtalis* presents an array of related questions concerning the role that physiology plays in controlling reproductive behavior. Attractivity of females is the result of a pheromone produced in the liver and related chemically to vitellogenin, a precursor of yolk. This pheromone communicates potential fecundity. Male sexual activity requires a period of winter dormancy, and sexual behavior appears to be independent of the presence of the testes and pituitary. Sexual receptivity of females is effected by environmental temperature.

Key words: Reptilia; Serpentes; Colubridae; Dormancy; Pheromone; Reproduction

NATURALISTS have long recognized the existence of defined periods of reproduction in many animal species. Such periods have been called "an artful stratagem" by which temperate species ensure successful reproduction (Reiter, 1980). In a seasonally changing, temperate environment animals cannot breed at just any time. The specific time of seasonal breeding is thought to be determined ultimately to ensure that young are born at the time of the year most favorable for their survival (Baker, 1938). Immediate ecological, internal, and social cues form the proximate factors responsible for coordination and synchrony among breeding animals.

A common aspect of the proximate control of reproductive synchrony is the reciprocal cause and effect relationship between certain physiological events and performance of specific behaviors. We have studied various aspects of the integration of physiology and behavior during the annual reproductive cycle of the red-sided gartersnake (*Thamnophis sirtalis parietalis*) of Manitoba, Canada. This snake was chosen because more is known about the physiology and organismal biology of members of the genus *Thamnophis* than any other reptile group, save the tropical lizard genus *Anolis*. In addition, since the red-sided gartersnake occurs further north than any other reptile in this hemisphere (Conant, 1975), study of this species might provide important insights into the interplay of physiology and behavior in an extreme temperate environment.

NATURAL HISTORY

Populations of *T. sirtalis* extend north in western Canada to the northern edge of limestone soil, which coincides with the beginning of the granitic Precambrian shield (Conant, 1975; F. Cook, pers. comm.). Collapsed caverns or breaks in limestone strata provide suitable overwintering sites where snakes may find refuge from the harsh Canadian winter. These overwintering sites can be many kilometers from suitable summer feeding habitat, and fall and spring migrations over long distances to and from the overwintering dens are required (Gregory and Stewart, 1974). Winters in northwestern Canada can be particularly harsh for an ectotherm. Temperatures are commonly −40 C or lower, and snow cover may be continuous from late September through May. As a result of the Canadian environment, the yearly activity period

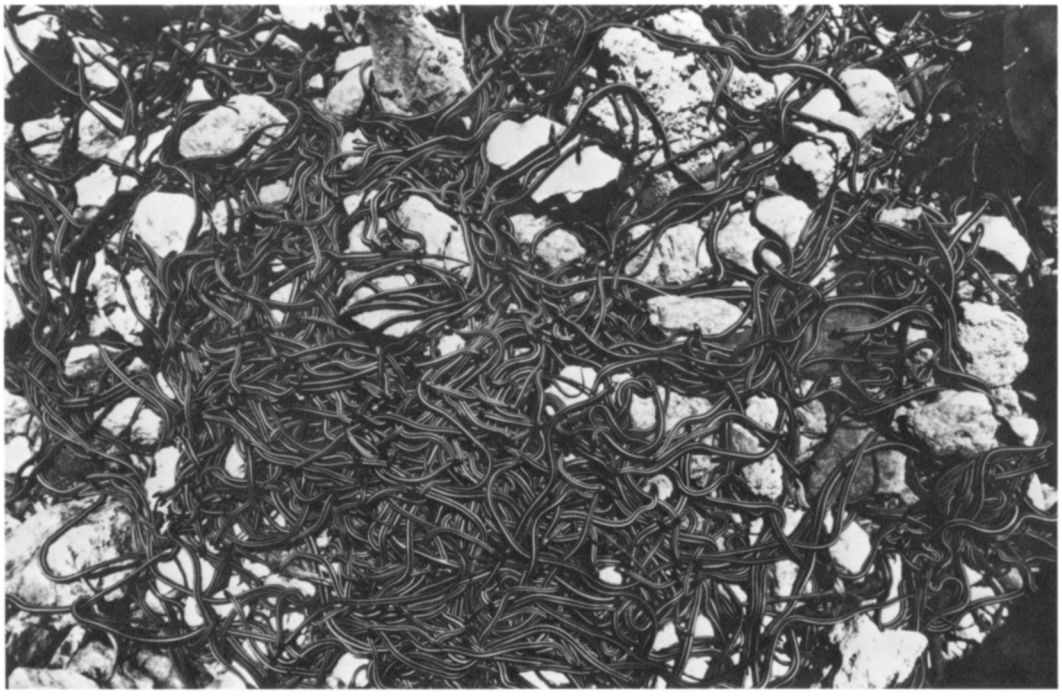

FIG. 1.—Spring at an overwintering den of *Thamnophis sirtalis parietalis* in Manitoba, Canada. In western Canada, snakes overwinter in large aggregations using collapsed caverns as den sites. With the first warm days in spring (usually late April–mid-May) the snakes emerge and breed. (Photo courtesy of Bianca Lavies, National Geographic Society.)

for snakes may be as brief as 3 mo (Gregory, 1976), thereby constraining severely reproduction and growth.

Overwintering aggregations include both male and female snakes, and a single den can contain 10,000 or more animals (Aleksiuk, 1975, 1976; Gregory, 1974, 1976; Fig. 1). Most males emerge from dormancy early in the breeding season and remain in the den area for approximately a month, returning underground nightly. Throughout the breeding period males begin emergence at approximately 0800 h, before the sun has warmed the den floor. Females, on the other hand, emerge singly or in small groups throughout this same period, usually later in the day than the males, but earlier as the season progresses (Fig. 2). Mating occurs upon emergence of females, and females disperse from the den area immediately after mating (Gregory, 1976; Hawley and Aleksiuk, 1975). As a result of this sex difference in spring emergence, males greatly outnumber females at dens. Although sex-ratios in the entire den populations are close to 1:1 (Gregory, 1976), effective sex-ratios during the breeding season strongly favor males. Consequently, each female is vigorously pursued and courted by a number of males, and mating aggregations (or "balls") of up to 100 males courting a single female are often formed (Aleksiuk, 1975; Gardner, 1955, 1957; Hawley and Aleksiuk, 1975; Fig. 3). Male sexual behavior is stimulated by exposure to warm temperatures; at temperatures less than 20 C, less than 50% of males will court (Hawley and Aleksiuk, 1975). Males exposed to 28 ± 4 C in the laboratory court for approximately 3 wk (Camazine et al.,

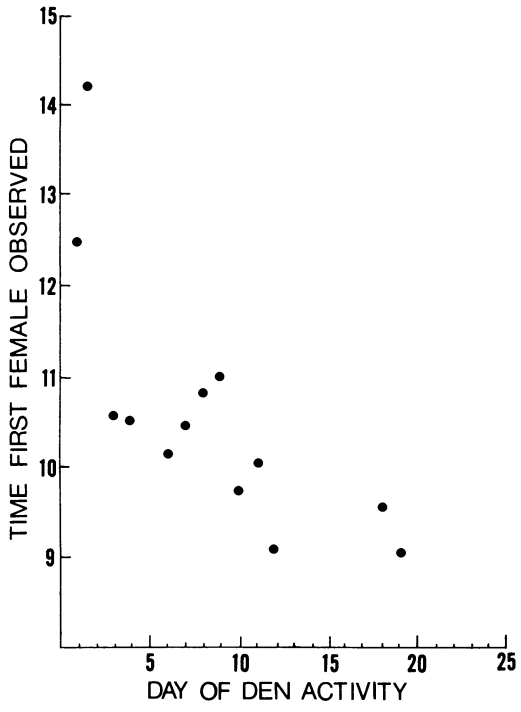

FIG. 2.—Pattern of emergence of female *Thamnophis sirtalis* at a Manitoba den. Observations began on 24 April 1981 and continued for 19 d. Females emerged earlier in the day as the breeding season progressed and hence were present longer during the day.

FIG. 3.—A mating aggregation of *Thamnophis sirtalis*. Males are attracted to females by a species-specific pheromone. Because few females are present at any given time, many males will simultaneously court a single female, and a "mating ball" is formed.

1980). Males actively courting in the field have cloacal temperatures of 25–29 C ($\bar{x}$ = 27.5; Garstka, unpubl. data).

It is advantageous for a male to identify suitable mates rapidly because of the male bias in effective sex ratio. Further, males can fertilize more than one female, even on the same day (Blanchard and Blanchard, 1941*a*; Devine, 1977*a*). Although there is a high degree of competition among males for mates, territoriality and male aggressive combat are absent (Devine, 1977*a*; Gregory, 1976). Pair bonding is not known to exist in *Thamnophis* (Devine, 1977*a*).

Courtship behavior of male *T. sirtalis* is similar to that of other species of *Thamnophis* (Aleksiuk and Gregory, 1974; Camazine et al., 1981; Carpenter, 1977; Crews, 1976; Fitch, 1965; Hawley and Aleksiuk, 1975; List, 1950; Munro, 1948; Noble, 1937; Pisani, 1976). After visual contact is made, the male investigates the female with the vomeronasal sense by tongue-flicking (Halpern and Kubie, 1980; Meredith and Burghardt, 1978). Females are recognized by pheromonal cues and are then courted by males (Burghardt, 1970; Crews, 1976; Kubie, 1977; Kubie et al., 1978*b*; Noble, 1937). Larger females are preferred by males (Aleksiuk and Gregory, 1974; Hawley and Aleksiuk, 1976*a*). However, ovariectomized females of any size are unattractive (Crews, 1976; Kubie, 1977; Kubie et al., 1978*a*), suggesting that an ovarian product may be responsible for female attractivity. Because larger females are preferred, the amount of this

product may vary with female size. If a male finds a female sexually attractive, his tongue-flicking increases in frequency, and he initiates "chin-rubbing." In this behavior the male presses his labial–mental area against the dorsolateral skin of the female and repeatedly traverses her length rapidly. Next, he aligns his body with the body of the female, chinning along her back and sides. These traverses along the female continue until the male comes to rest with his body alongside or over the body of the female. The male next aligns his cloaca with the cloaca of the female and begins caudocephalic and cephalocaudal contractile waves along the length of his body (Noble; 1937; Pisani, 1976). These undulations continue until intromission.

The male attempts intromission by rolling the tail region of the female upward with his tail. During courtship females increase their ventilatory volume but not rate. Sexually receptive females remain stationary while being courted. The female may play an active role during intromission; she must not only remain stationary, but may lift her tail and gape her cloaca in response to the male's attempts at intromission (Carpenter, 1977; Crews, 1976; Hawley and Aleksiuk, 1976*a*; Noble, 1937). A nonreceptive female retreats from the courtship advances of the male and may vibrate her tail. After intromission of a single hemipenis, the male ceases moving and the female may drag him as she moves.

Following intromission by the successful male, other males cease courting the female and disperse from the mating pair (Devine, 1975, 1977*a*; Fitch, 1965; Ross and Crews, 1977, 1978). Copulation usually lasts for ca. 17 min in *T. sirtalis* (Crews, 1976; Devine, 1977*a*; Fitch, 1965) and 90 min in *T. radix* (Ross and Crews, 1978). Recently mated female *T. sirtalis* and *T. butleri* have a translucent gelatinous plug in the cloaca (Devine, 1977*a*; Garstka and Crews, unpubl. data). Mated females continue to be unattractive, become intolerant of further courtship, and depart quickly from the den area (Devine, 1977*a*; Gregory, 1974, 1976). Ross and Crews (1977, 1978) reported that a pheromone contained in the copulatory plug inhibits sexual activity of males.

After dispersal to summer feeding grounds, mated females give birth to young sometime in August (Gregory, 1976). Brood size in *Thamnophis* is positively associated with female body length (Fitch, 1965, 1970; Ford and Ball, 1977; Gregory, 1976). Small females may not produce young every year (Gregory, 1976; Hawley and Aleksiuk, 1976*a*).

Snakes remain on summer feeding grounds until fall. Adult body size in Canadian *T. sirtalis* (male >20 cm snout–vent length; female >40 cm SVL) is usually reached after the first entire summer of growth. Thus, reproductive individuals are normally in at least their second year. Adults migrate back to dens in September, and adults of both sexes return to the same dens year after year (Gregory, 1976). Neonates may spend the first winter at or near the feeding grounds, and it is not known how they first locate a den (Gregory, 1976).

Reproductive Physiology

Vertebrates can exhibit either of two patterns of annual gonadal development in relation to periods of breeding. In most seasonally breeding species, gamete maturation occurs as a single discrete event during or immediately before the breeding season. This has been termed the "prenuptial" pattern (Licht, 1981; Lofts, 1969, 1977; Volsøe, 1944). Alternatively, gametes can be produced in an interrupted manner, with growth arrested during a period of dormancy. This latter pattern has been termed "postnuptial." Both sexes in *Thamnophis* follow the interrupted, or postnuptial, pattern. Final maturation of one season's gametes occurs after the breeding season ends (Cieslak, 1945; Fox, 1952, 1954; Gregory, 1976; Hawley and Aleksiuk, 1976*b*; Fig. 4).

Most sexual activity in *T. sirtalis* oc-

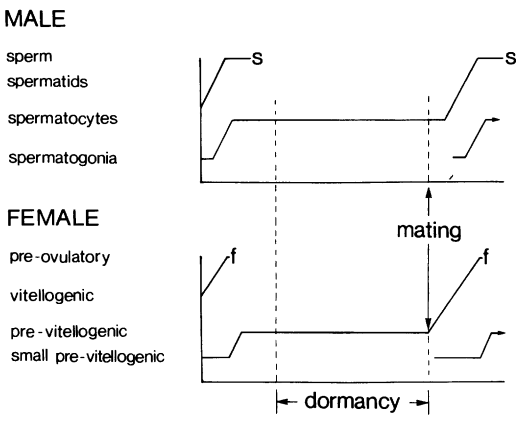

FIG. 4.—The annual cycle of gametogenesis in *Thamnophis sirtalis*. Both sexes of the red-sided garter snake exhibit an interrupted, or "postnuptial," pattern of gametogenesis. Mating occurs before the gonads are fully recrudesced and before maturation of the gametes. Mature gametes are shed at "s" (spermatozoa into the accessory sex structures) and "f" (follicles into the oviduct). Males store spermatozoa through the dormancy period until mating the following spring.

curs immediately following emergence from winter dormancy (Aleksiuk and Gregory, 1974). Testicular recrudescence begins after sexual activity has ceased in late spring. Spermatozoa are evacuated from testicular tubules into the epididymides and ductus deferentia in late summer, and are stored in the ductus deferentia until the following year. Testes are reduced in size and regressed when snakes enter winter dormancy. During winter dormancy, testes contain only spermatogonia and spermatocytes (Camazine et al., 1980; Gregory, 1976; Hawley and Aleksiuk, 1976*b*; Thatcher, 1922; Tsui and Licht, 1974). Thus, spring courtship activity occurs before testes undergo recrudescence (Aleksiuk and Gregory, 1974; Gregory, 1976; Hawley and Aleksiuk, 1976*b*).

Similarly, ovarian follicular growth in *T. sirtalis* is of the interrupted, or postnuptial, type (Gregory, 1976). After ovulation, some small (<1 mm long) follicles undergo an initial "hydration" phase (Dessauer and Fox, 1959) when water accumulates and the follicle growth to 4–6 mm in length. Follicles remain at this size during winter dormancy and are therefore small when females emerge in spring. Following mating, follicles undergo a period of rapid growth. Follicles first lengthen to 8–10 mm, and then begin to accumulate yolk (Dessauer and Fox, 1959). Pre-ovulatory follicles are 13–17 mm long or longer, ovoid, and yellow; ovulation occurs 6–8 wk after mating (Gregory, 1976). Ovarian follicles of unmated females, however, do not undergo the second phase of follicular growth (Blanchard and Blanchard, 1941*a*,*b*). Further, follicles of unmated females may decrease in size (Bona-Gallo and Licht, pers. comm.; Garstka and Crews, unpubl. data). Ovulation, which occurs 6–8 wk after copulation (Gregory, 1976), is not directly induced by mating.

In animals exhibiting prenuptial gametogenesis, steroid production coincides with the display of reproductive behavior. Dependence of sexual behavior on gonadal steroids is well documented in many animal species having prenuptial gametogenesis (Bermant and Davidson, 1974); injection of gonadal extracts or synthetic androgens into males immediately following castration, or after the decline of sexual behavior, will either maintain or reinstate sexual activity to its preoperative levels. Similarly, treatment of ovariectomized females with gonadal steroids present during estrus will elicit receptive behavior.

However, an inverse relationship between sex steroid levels and sexual activity has been reported for several species exhibiting postnuptial gametogenesis. Male green turtles (*Chelonia mydas*), noctule bats (*Nyctalus noctula*), and brown bats (*Myotis lucifugus*) have low androgen titers during the breeding season (Gustafson and Shemesh, 1976; Licht et al., 1979; Racey, 1974). In both captive and wild *T. sirtalis*, androgen levels are variable during winter dormancy and fall

during the spring breeding period (Camazine et al., 1980; Hawley and Aleksiuk, 1976). Estrogen levels in females are lowest during dormancy, rise rapidly after mating and are again low during the yolking phase of follicular growth (Garstka et al., unpubl. data).

Statement of Questions

The information presented thus far suggests that the physiological factors regulating some aspects of reproductive function and behavior of *Thamnophis* may differ considerably from those in other vertebrates. In particular, the dissociation of reproductive behavior and gonadal recrudescence suggests that the factors responsible for sexual behavior may differ from those responsible for gonadal activity. We next will discuss in detail several of these problem areas in a sequence corresponding to the temporal sequence of events during a natural breeding period.

Specific questions are: (1) How do females become attractive immediately upon spring emergence, before gonadal recrudescence has occurred and when there seems to be a connection between an ovarian product and attractivity? (2) Because males are courting before testicular recrudescence occurs, while circulating androgen levels are variable or low, what is regulating male sexual behavior? (3) How is female sexual receptivity, which is usually connected with hormonal changes related to follicular maturation and ovulation, occurring before follicular maturation?

Pheromonal Basis of Female Attractivity

Female vertebrates play an active part in many aspects of breeding synchrony. Beach (1976) formalized descriptive terms for these roles. Those actions initiated by the female to establish a sexual interaction he termed "proceptive behaviors" in contrast to "receptive behaviors," which are the actions of a female demonstrating a willingness to mate. The stimulus value of a female in evoking male sexual interest he termed "attractivity."

Previous investigators, beginning with Noble (1937), showed that perception of a pheromone present on or in the skin of attractive females is necessary to elicit male courtship (Gillingham and Dickinson, 1980). Many of these same investigators searched unsuccessfully for structures in the skin of female snakes which might serve as sites of synthesis, storage, or release of the attractiveness pheromone. Cloacal glands, which are the only exocrine glands in *Thamnophis*, seem not to be involved in sexual attractivity (Munro, 1948; Noble, 1937).

Our investigation (Garstka and Crews, 1981) sought first to identify the female attractiveness pheromone and its source, and then to determine what the pheromone communicates and why it is attractive. Although there are no exocrine structures in the skin which could be responsible for the presence of the pheromone, other vertebrates possess pheromones in the urine or feces which are not necessarily of glandular origin (Muller-Schwartze and Mozzell, 1977). Upon reading the description of a dermal vascular bed (Rauch, 1978) in *Thamnophis*, we reasoned that the pheromone could be carried in the circulation of the female and transferred via that route to the skin. It could then be sequestered from the circulation into an active site and dispersed during courtship.

Because chin-rubbing is displayed exculsively in a sexual context, it formed the basis of a behavioral bioassay for the attractiveness pheromone. Chin-rubbing by at least one of two sexually active males tested constituted a positive response. To test the hypothesis that the attractiveness pheromone is in the circulation, sera from estrogen-treated females and untreated males were applied to the backs of males, and male courtship of these serum-coated males was record-

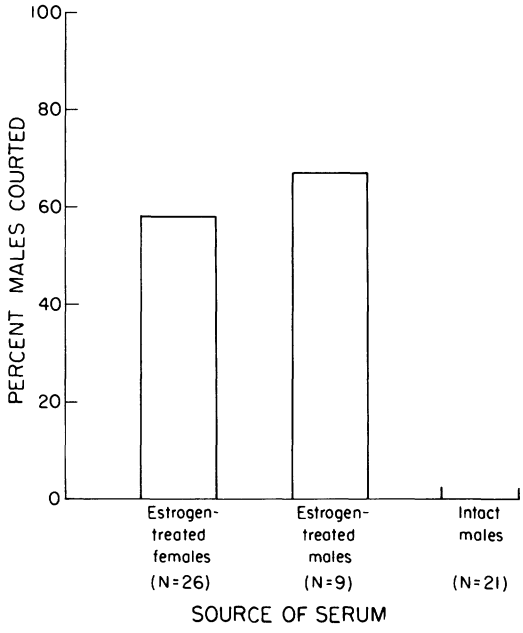

FIG. 5.—Serum contains the active attractiveness pheromone of female *Thamnophis sirtalis*. Conspecific males with 500 μl of serum from estrogen-treated females applied to their backs were courted by sexually active males; sera of males did not elicit courtship ($X^2 = 17.8$, $P < 0.005$). Males with sera of estrogen-treated males applied were also courted.

ed. Males receiving an application of sera from estrogen-treated females were courted, while the sera of untreated males did not elicit courtship. Males with an application of sera of estrogen-treated males were also courted (Fig. 5).

In order to determine what kind of chemical the pheromone is, extracts of skin were made and tested similarly. We reasonsed that since the pheromone is active on contact, and not volatile, it might be a protein. Certain classes of lipids, including steroids, are also not volatile. Devine (1977*b*) reported aggregative properties associated with skin lipids, and several classes of lipid are known to be present in snake skin (Ahern and Downing, 1973). Lipid (chloroform : methanol, 2:1), soluble protein (phosphate-buffered saline [PBS]), and bound protein (1 M NaCl in PBS) were

TABLE 1.—Lipid content ($\bar{x} \pm$ SE) of sera used to elicit courtship from males.

Source of sera	*n*	Concentration (mg/100 cm³)
Estrogen-treated females		
Sera eliciting courtship	15	2990 ± 270
Sera not eliciting courtship	11	2890 ± 300
Intact males		
Sera not eliciting courtship	19	670 ± 40
Estrogen-treated males		
Sera eliciting courtship	6	1579 ± 274
Sera not eliciting courtship	3	1109 ± 394

extracted from the body skin of four estrogen-treated females whose sera had elicited courtship. Chloroform : methanol extracts were applied directly to males after removing the solvent. Aqueous extracts were dialyzed (spectrapor, MW cutoff 3500) against five changes of distilled water at 5 C and lyophilized; lyophilisates were dissolved in 1 cm³ distilled water prior to behavioral testing. Courtship was elicited by all lipid extracts, but by none of the protein extracts. This indicated that the pheromone is a lipid.

On the basis of these results, behaviorally tested sera were analyzed for lipid. Lipid concentration was significantly lower in sera from males than in sera from estrogen-treated females ($t = 9.81$, df = 43, $P < 0.001$; Table 1). Estrogen treatment significantly increased serum lipid in males ($t = 5.04$, df = 26, $P < 0.001$; Table 1). Because neither lard (1 g) nor estradiol (500 μl of 1 mg/cm³ estradiol benzoate in vehicle) elicited male courtship, the behavioral response in these tests was due to a specific lipid, and not solely to the quantity of lipid or the presence of estrogen.

Finally, to determine the source of the pheromone, extracts of other tissues were tested for pheromonal activity. We reasoned that a lipid pheromone could be either released from fat-body storage sites or directly synthesized in the liver. To determine if either organ contained

the attractiveness pheromone, homogenates of livers and fat bodies of intact females which were perfused extensively with saline were tested. None of the six fat-body preparations elicited courtship, but both liver homogenates tested were effective. Two homogenates of livers from estrogen-treated males also were effective. This indicates that the liver is the source of the pheromone.

Because there is an annual cycle in liver size in female *T. sirtalis* related to the cycle of yolk synthesis and deposition (Aleksiuk and Stewart, 1971; Dessauer et al., 1956), and because sera and livers from estrogen-treated males were positive for pheromonal activity, we investigated the possibility of a relationship between the active pheromone and vitellogenin, the circulating precursor of yolk. Estrogen treatment is known to induce vitellogenin synthesis in males as well as females, although males do not normally synthesize this lipoprotein (Wallace, 1979; Wallace and Bergink, 1974). In an initial test of the hypothesis that the pheromone is related to vitellogenin, yolk from yolking ovarian follicles (500 μl) of *T. sirtalis*, *T. melanogaster*, *T. eques*, *Anolis carolinensis*, and the chicken, *Gallus*, was applied to the backs of males. Males with an application of yolk from conspecific females were courted, but yolk from other species did not elicit courtship.

Vitellogenin was then isolated by the method of Ansari (Ansari et al., 1971; Gapp et al., 1979). The vitellogenin purified by this method was tested and found to be effective at 50 mg lyophilizate/cm³ Ringer's saline. All four males tested courted the test male.

To determine the mechanism of release of the female attractiveness pheromone, frozen sections of skin were prepared for histological examination and stained for lipid with Sudan Black B (Humason, 1979). Two lipid-containing areas of the skin were identified.

Numerous lipid-positive vesicles were observed in the striated muscle and connective tissue deep in the dermis of estrogen-treated females (Fig. 6). Lipid-positive cells were seen in the dermis, regardless of the phase of the shedding cycle (Jackson and Sharawy, 1978; Landmann, 1979, 1980; Landmann et al., 1981; Maderson, 1964; Maderson et al., 1970*a*,*b*). Untreated males lacked this intense lipid-staining. However, estrogen-treated males developed the dense dermal staining pattern of treated females.

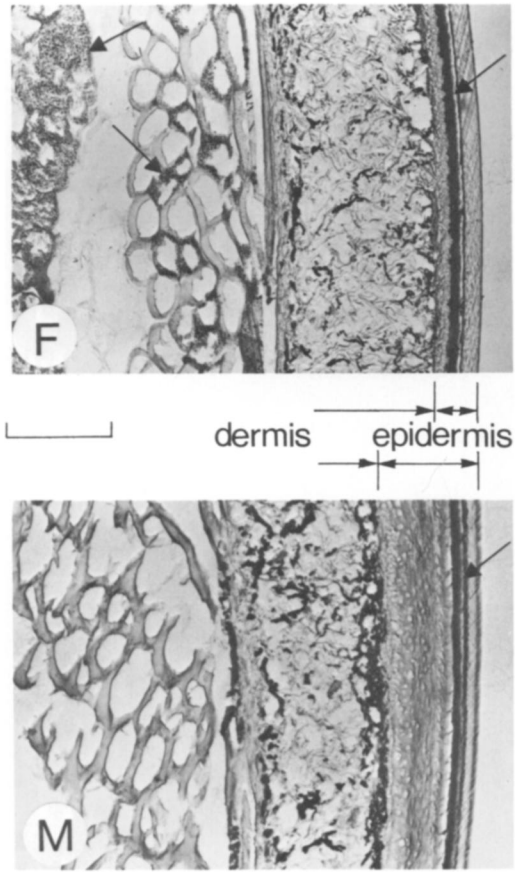

FIG. 6.—Section through skin of estrogen-treated female (F) and untreated male (M) *Thamnophis sirtalis* stained with Sudan Black B. Lipid is present in two areas of the skin: the mesos and α keratin of the epidermis in both sexes, and connective tissue and muscle of the dermis in the treated female. The scale bar is 200 μm.

Lipid staining also was apparent in the epidermis of both males and females. Lipid is present in both inner and outer epidermal generations but is trapped in these tissues between highly keratinized layers. This trapped lipid is sequestered in epidermal cells during skin generation (Jackson and Sharawy, 1978; Landmann, 1979, 1980; Landmann et al., 1981) and forms a barrier to water loss (Landmann, 1980; Landmann et al., 1981; Roberts and Lillywhite, 1980).

Some lipid material, however, is present outside the skin of females under the edges of scales on the dorsal and especially lateral areas. This suggests that a mechanism is present in these snakes which allows lipid to pass through the skin. Further, both *T. sirtalis* and *T. melanogaster* quickly become sexually attractive with estrogen treatment, irrespective of the shedding cycle stage (Garstka and Crews, unpubl. data).

Several species of the related natricine genera *Natrix* (= *Rhabdophis*) and *Macropisthodon* (Nakamura, 1935; Smith, 1938), as well as the gekkonid lizard *Diplodactylus* (Rosenberg and Russell, 1980), possess ductless, dermal poison glands. In these species, muscular contractions force glandular exudate through ruptures in the skin in the hinge region between scales where the keratinization is only one cell thick. Dermal lipid-staining in *Thamnophis* females was concentrated in the hinge regions adjacent to the dermal vascular bed. Further, examination of the inner surface of fresh skin revealed thinnings of the skin in the hinge regions of anterior dorsal scales. These thin areas were visible because the lighter pigment of the dorsal stripes showed through.

The lack of a sex or treatment difference in lipid staining within the epidermis, the fact that epidermal lipid is trapped between keratinized layers, and the presence of the lipid on the outside of the skin suggest that the presence of the pheromone on the skin of female *Thamnophis* is a consequence of an active process which may be functionally similar to ejection of poison in some other reptiles. One possibility is that the increase in size due to increased ventilatory volume exhibited by females during courtship acts to disperse the pheromone. During this hyperventilation, skin of the anterior body is stretched and adjacent scales are moved apart. This may serve to expose trapped lipid and, we hypothesize, to eject pheromonal material.

It is clear that a relationship between vitellogenin and attractivity means that males can, in effect, assess the reproductive potential of a female. Vitellogenin content reflects the size or number of ovarian follicles which can be yolked. Female *T. sirtalis* are larger than males (Carpenter, 1952*a*,*b*, 1955; Fitch, 1965; Gregory, 1976), and males prefer larger females (Aleksiuk and Gregory, 1974; Hawley and Aleksiuk, 1976*a*). Offspring number is positively correlated with increasing body length of females in *Thamnophis* (Aldridge, 1979; Fitch, 1965; Ford and Ball, 1977; Gregory, 1976). Therefore, one would predict that male choice would be reflected in females of generally larger body size, and with more or larger ovarian follicles.

To test this prediction, intact (untreated) females were chosen at random from a group of females in low temperature dormancy in the laboratory. Each of 27 females were offered to two males in courtship tests, and then sacrificed. The males had been out of artificial dormancy for 12–14 d. A comparison between those females courted and those not courted (Table 2) revealed that, as in previous field and laboratory studies, males preferred larger females. However, if body size is removed as a variable by analysis of covariance, it is apparent that the groups differ significantly in follicle number ($F_{1,23} = 4.86, P < 0.01$). This suggests that large females are courted preferentially not because of their size per se, but as a consequence of the quantity of pheromone, which reflects their higher potential fecundity.

Previous investigators reported that female *T. sirtalis* are unattractive when

TABLE 2.—Stimulus value of intact female *Thamnophis sirtalis* after 6 mo dormancy. Data are presented as means ± SE.

Condition of female	Sexual attractivity		
	Courted (n = 11)	Not courted (n = 16)	Significance level
Body size			
Snout–vent length (cm)	62.9 ± 3.1	52.8 ± 1.8	< 0.005
Body mass (g)	86.5 ± 10.4	46.1 ± 5.8	< 0.005
Nutritional state			
Fat-body mass/body mass (× 100)	4.61 ± 0.80	4.17 ± 0.62	NS
Serum protein (g/100 cm^3)	4.7 ± 2.8	4.3 ± 0.3	NS
Serum lipid (g/100 cm^3)	1.1 ± 0.1	1.3 ± 0.1	NS
Serum glucose (mg/100 cm^3)	88 ± 9	148 ± 26	NS
Ovarian state			
Ovary mass/body mass (× 100)	1.85 ± 0.19	1.36 ± 0.16	NS
Number of follicles >0.5 mm	74 ± 7	45 ± 4	< 0.001
Length of largest follicle (mm)	7.3 ± 0.6	5.6 ± 0.5	< 0.05
Oviduct mass/body mass (× 100)	1.13 ± 0.11	0.96 ± 0.12	NS
Serum estradiol (pg/cm^3)	255 ± 81	144 ± 30	NS

ovariectomized (Crews, 1976; Kubie, 1977; Kubie et al., 1978*a*), further demonstrating that the quantity of attractiveness pheromone is independent of body size. In order to verify this observation, two large female *T. sirtalis* were ovariectomized, allowed to go through two shedding cycles, and offered to sexually active males 2 d out of dormancy, in alternation with intact, estrogen-treated females. Males courted both groups of females equally. A second, similar test with new males 10 d later produced equivalent results. However, when males were retested 12 d out of dormancy, all preferred intact, estrogen-treated females, and none courted ovariectomized females.

To determine if sensitivity of males to the attractiveness pheromone was changing, two ovariectomized females were offered, in alternation with two intact, estrogen-treated females, to males in daily tests. In addition, sexually active males previously naive to ovariectomized females first were offered ovariectomized females in alternation tests on day 10 and 13. Male *T. sirtalis* are initially indiscriminate of female stimulus quality and will even court ovariectomized females for the first few days after emergence. After 7–10 d, males show a preference for intact, estrogen-treated females when offered ovariectomized females as the other choice (Fig. 7). This effect is not experiential, as 10 and 13 d naive males exhibited a preference for estrogen-treated females.

We believe that this change in sensitivity to the pheromone, at the ultimate level, reflects a change in availability of mates. Because of the sex difference in emergence behavior, there is an extreme male bias in the effective sex ratio at the onset of the breeding season. Under these conditions it is advantageous for a male to court any female he can locate. However, females begin emerging earlier in the day as the breeding period progresses and some males have dispersed, so the effective sex ratio changes. Although the effective sex ratio probably never becomes biased toward females, it may approach 1:1. Thus, with more females to choose from, it is advantageous for a male to choose the potentially most fecund females for mating.

REGULATION OF MALE SEXUAL BEHAVIOR

The dependence of reproduction on all levels of the hypothalamic–pituitary–gonadal (HPG) axis of the endocrine system is well documented in tetrapods following the prenuptial pattern of gametogenesis

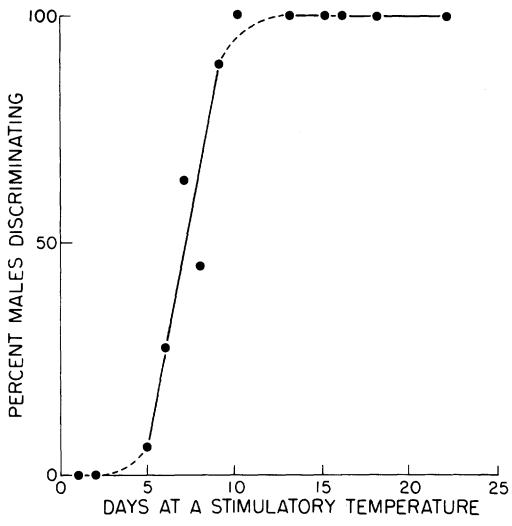

FIG. 7.—Mate discrimination by male *Thamnophis sirtalis*. Males were offered two ovariectomized females in alternation with two intact, estrogen-treated females in 2 min tests each day. Males initially showed no preference for females in either group, but after 5–10 d at 28 ± 4 C all males showed a strong preference for estrogen-treated females.

(Bermant and Davidson, 1974). In these species, gonadal recrudescence coincides with sexual activity, and the hormones involved in gonadal recrudescence have been shown to influence male reproductive physiology and behavior profoundly. Castration results in a decline of reproductive behavior, while implantation of testes or injection of gonadal extracts or exogenous sex steroids reinstates behavior to preoperative levels. Pituitary gonadotropins indirectly control sexual behavior by regulating gonadal activity (both gametogenesis and steroidogenesis). Hypothalamic releasing hormones not only regulate synthesis and release of pituitary tropic hormones, but can play a direct role in the control of sexual behavior (Foreman and Moss, 1977; Moss and McCann, 1973).

Our studies of sexual behavior of male *T. sirtalis* (Camazine, 1981; Camazine et al., 1980) first explored the relationship between various levels of the HPG axis and male sexual activity. Because we have thus far been unable to document a cause and effect relationship between the components of the HPG axis and male sexual activity, we have begun to study the relationship between neural activation and male sexual behavior. The concept of male arousal is involved intimately with the communicative aspects of behaviors facilitating reproduction. We have developed a model for how such neural activation might affect behavior directly, and have tested certain hypotheses derived from this model.

In male rats, arousal is seen as an increase in theta activity of the hippocampus beginning after a male first senses a female visually or olfactorily, and this activity continues until intromission (Adler, 1978; Komisaruk, 1977). The archipallium of reptiles is homologous to the mammalian hippocampus and also receives neurons from the vomeronasal system via the olfactory bulb. In addition, the archipallium has connections with the hypothalamus through a structure homologous or identical with the fornix (Halpern, 1974; Romer, 1967). The vomeronasal system is required for courtship behavior in male *Thamnophis*; either removing the tongue, destroying Jacobson's organs, or damaging the vomeronasal nerve will end male sexual behavior (Kubie, 1977; Kubie et al., 1978*b*; Noble, 1937). Sensitivity to the female attractiveness pheromone changes through the breeding season. Males become highly discriminatory, preferring intact, estrogen-treated females. We hypothesized that changes in hypothalamic activity could directly mediate the vomeronasal system. This would not only offer a more direct route for hypothalamic mediation of sexual behavior, but also account for the observed close relationship between vomeronasal function and behavior. In order to assess direct neural control of behavior, we tested effects of catecholamine neurotransmitters and substances known to affect nerve conduction on male sexual activity.

The few studies with lizards, the only reptiles studied to date, have revealed the same overall pattern of hormonal control of reproductive behavior as seen in other tetrapods. Castration is followed by a rapid decrease or cessation of sexual behavior, and a marked decrease in height and secretory activity of epithelial cells of the renal sex segment (analogous to the mammalian prostate and seminal vesicles) and epididymal tubules (see Crews, 1979). Hypophysectomy causes testicular atrophy—specifically, cessation of spermatogenesis and a reduction in size of the interstitial cells (site of androgen production). This in turn causes a decline in height and activity of the androgen-sensitive renal sex segment (Crews, 1979). Studies involving steroid binding, lesions, and hormone implants have verified the role of the hypothalamus in sexual behavior (Crews, 1980). The direct effect of hypothalamic releasing factors on behavior has been examined in only one reptile, *A. carolinensis* (Alderete et al., 1980). Luteinizing hormone-releasing hormone (LH-RH) was capable of inducing sexual receptivity in ovariectomized, estrogen-primed *Anolis*.

Subcutaneous pellets of crystalline testosterone propionate (TP) or silastic capsules of free testosterone (T) have been used to stimulate courtship activity in intact, sexually inactive male *T. sirtalis* (Crews, 1976; Ross and Crews, 1977, 1978) and *T. radix* (Kubie et al., 1978*a*,*b*). The effects were variable, however, and stimulation of sexual behavior usually occurred in only 30–50% of males. Other investigators have encountered similar difficulties in inducing sexual activity in male *Thamnophis* (M. Devine and N. Ford, pers. comm.).

In an effort to reconcile inconsistent published results using androgen implants in *Thamnophis* males with field data showing low circulating levels of androgen during the breeding season (Hawley and Aleksiuk, 1976*b*), we began our investigations with an experiment to determine if exogenous androgens can stimulate sexual behavior in intact, sexually inactive males (Camazine and Crews, unpubl. data). Animals were maintained under stimulatory environmental conditions simulating summer (28 ± 4 C; 14:10 LD). Eighteen males were pretested for 7 consecutive days, and seven low courting males were selected and then given silastic capsules of TP subcutaneously. The males were subsequently tested for 11 d. Implantation of TP failed to stimulate sexual behavior in any of the experimental males. In studies with *T. radix* (Camazine and Crews, unpubl. data), sexually inactive castrated males were treated with T, dihydrotesterone (DHT), or DHT in combination with estradiol. None of these treatments restored sexual activity.

We next examined the effects of castration and androgen therapy in male *T. sirtalis* (Camazine, 1981; Camazine et al., 1980; Garstka et al., unpubl. data). Twenty males were placed in a stimulatory environment following 7 wk of low temperature dormancy (7.5 ± 2.5 C; 0:24 LD). These males then were tested for courtship activity for 5 d, and 15 sexually active males were selected. Males were then either castrated, castrated and given T-replacement (a subcutaneous silastic implant of T) therapy, or sham-operated. Animals in all three experimental groups exhibited a similar period of intense sexual activity lasting approximately 10 d, followed by a gradual decline in sexual behavior (Fig. 8; Table 3). Serum androgen levels were highest in the T-implanted group and lowest in the castrated group. Testicular recrudescence occurred in the sham-operated group following the decline in courtship behavior. The most conservative conclusion to draw from these unusual results is that the neural systems subserving sexual behavior had been activated previously, either before, during, or immediately following winter dormancy.

The fact that courtship activity becomes more intense with increasing time in low temperature dormancy (Fig. 9)

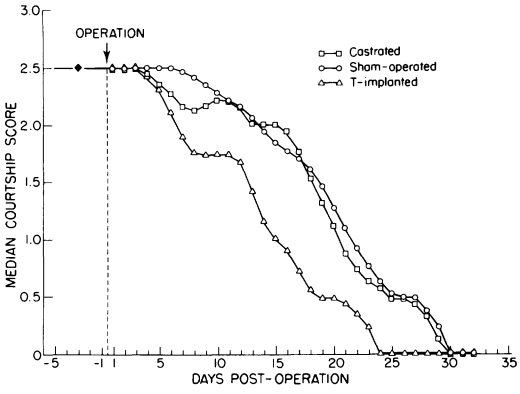

FIG. 8.—Castration of sexually active male *Thamnophis sirtalis* fails to influence courtship behavior. Median daily courtship scores (a quantitative ordinal measure of chin-rubbing behavior; see Camazine et al., 1980) are presented for males before and after surgery. Each group contained five males, each of which was tested daily for sexual activity with two intact, estrogen-treated females, from Camazine et al., 1980).

adds support to the activation hypothesis. To test this hypothesis, sexually inactive males were either castrated, castrated and given T-replacement therapy, or sham-operated in the fall, immediately prior to 7 wk of low temperature dormancy. As in the previous experiment, males in all three groups exhibited a period of intense sexual activity following dormancy. After several days, snakes in all three groups began similar declines in behavior (Table 3). As in the previous study, attendant changes in testicular activity and serum androgens were observed. Further, males castrated either during or before the previous summer's period of testicular recrudescence displayed courtship behavior after 7 wk of low temperature dormancy (Table 3).

Because our findings suggested that the testes are not necessary for sexual behavior in male *T. sirtalis*, we then hypothesized that pituitary secretions might control sexual behavior by acting directly on the brain. Three experiments were performed to test this hypothesis. First, males were hypophysectomized or sham-operated while still in low temperature

TABLE 3.—Response of male *Thamnophis sirtalis* to surgical removal of endocrine glands. A plus means that males exhibited courtship behavior.

Treatment group	*n*	Courtship behavior
Castration		
While sexually active:		
control	5	+
castrated	5	+
castrated with subcutaneous capsules of testosterone	5	+
Before winter dormancy:		
control	10	+
castrated	10	+
castrated with subcutaneous capsules of testosterone	10	+
During previous recrudescence:		
control	5	+
castrated	5	+
castrated with subcutaneous capsules of testosterone	5	+
Before previous recrudescence:		
control	10	+
castrated	10	+
castrated with subcutaneous capsules of testosterone	10	+
Hypophysectomy		
During winter dormancy:		
control	5	+
hypophysectomized	5	+
Before winter dormancy:		
control	6	+
hypophysectomized	6	+

dormancy. The males were then placed in a stimulatory environment. Both groups showed initial high levels of sexual activity followed by a decline in courtship behavior (Table 3). Histological examination of the urogenital system revealed that unlike the sham-operated controls and all previous groups, the hypophysectomized males still retained spermatozoa in their ductus deferentia. Thus, there may be pituitary-mediated autolysis of spermatozoa.

Secondly, groups of six males each were either hypophysectomized or sham-operated prior to being placed in low temperature dormancy. Males in both groups displayed characteristic sexual

behavior when removed from dormancy and tested for sexual activity 7 wk later (Table 3).

Finally, sexually inactive males were treated with arginine-8-vasotocin, which has been shown to be involved with induction of male sexual behavior in amphibians (Moore and Zoeller, 1979). Although this neurohormone is released from the pars nervosa, it is hypothalamic in origin and thus its release might not be blocked by hypophysectomy. Sexually inactive males were treated, and tested at 1, 6, and 24 h. No sexual activity was observed (Table 4). Sexually inactive males were then treated with LH-RH. They were tested at 1, 6, and 24 h after treatment, and again no sexual activity was noted (Table 4).

In those animals where changes in photoperiod have been shown to be involved in regulation of seasonal breeding, the pineal and its active hormone, melatonin, mediate the behavioral effects of changes in photoperiod (see Turek and Campbell, 1979). Although photoperiod is believed to be of minor importance in regulating sexual behavior of male *T. sirtalis*, exposure to cold temperatures for at least 7 wk is necessary for male sexual behavior. Sexually inactive males were treated with melatonin in both oil and ethanolic saline vehicles, and tested for sexual activity at 1, 2, 6, and 24 h. No sexual activity was observed (Table 4).

Evidence is accumulating that the catecholamine neurotransmitters are important in modulating sexual behavior (see Meyerson et al., 1979). These chemicals can act as mediators of the sex steroids (Carter and Davis, 1977) or act independently. For example, 3-OH-tyramine (dopamine) is known to increase the initiation of mounts by castrated male rats (Malmnas, 1977). Consequently, we treated sexually inactive male *Thamnophis* with either dopamine, epinephrine, norepinephrine, or serotonin. There was no behavioral change in tests at 1, 6, and 24 h (Table 4).

Additionally, we attempted to initiate

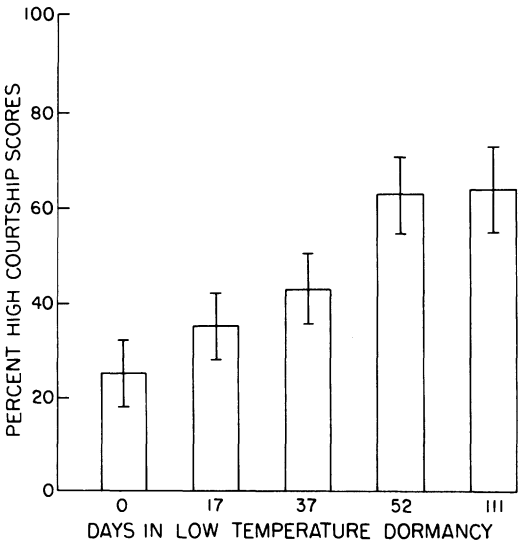

FIG. 9.—Effect of time in artificial winter dormancy on sexual behavior of male *Thamnophis sirtalis*. Males were placed in cotton bags in a modified refrigerator (see Camazine et al., 1980). Groups of 20 males were removed at 3, 5, 7, and 15 wk to a stimulatory environment and tested daily for 4 d with two intact, estrogen-treated females. Courtship was ranked on a quantitative scale and was found to be maximal after 7 wk (52 d) of dormancy.

a state of arousal by direct electrical stimulation (Crowley et al., 1973), because electrical stimulation can release neurotransmitters. Electrodes were placed subcutaneously between the ventral scutes approximately 8 cm apart or in the rear of the head near the quadrate bones. Monophasic pulses of 10 v and 100 ms in duration were given for 2 s at a rate of 5/s. The males became quite agitated and locomotory behavior increased, but no sexual behavior was observed.

There is an annual cycle of thyroid activity coincident with the reproductive cycle of the cobra *Naja naja* (Bona-Gallo et al., 1980) and the European adder *Vipera berus* (L. Kelleway, pers. comm.). In male *Vipera*, circulating levels of thyroxine are greatest during the period of sexual activity. Thyrotropin releasing hormone (TRH) has been shown to induce female sexual receptivity in ovariectomized, estrogen-primed *A. carolinensis*

TABLE 4.—Response of sexually inactive male *Thamnophis sirtalis* to injections of hormones, neurotransmitters, metabolic effectors, and electrolytes. A plus means that males exhibited courtship behavior; a minus means that such behavior was not elicited.

Treatment	Dosage	*n*	Effect
Environmental regimen			
7 wk at 5 ± 3 C; 0L:24D		> 100	+
Steroid hormones			
Testosterone (proprionate)	Implant	9	–
Neurohormones			
Luteinizing hormone-releasing hormone	500 ng/g	4	–
Arginine-8-vasotocin	5 μg/g	5	–
	50 μg/g	2	–
Melatonin	1 μg/g	5	–
	10 μ/g	2	–
	100 μg/g	2	–
Neurotransmitters			
Dopamine	5 μg/g	2	–
	50 μg/g	2	–
Epinephrine	0.5 μg/g	1	–
	5.0 μg/g	1	–
Norepinephrine	5 μg/g	2	–
	50 μg/g	2	–
Serotonin	5 μg/g	2	–
	50 μg/g	2	–
Metabolic effectors			
D-glucose	200 μg/g	2	–
L-thyroxine	100 μg/g	2	–
L-triiodothyronine	100 μg/g	2	–
Electrolytes			
Ca^{2+} (as chloride)	0.1 meq/g	2	–
K^+ (as chloride)	0.1 meq/g	2	–
Na^+ (as chloride)	2.0 meq/g	2	–

(Alderete et al., 1980). Further, courtship activity in male *T. sirtalis* is induced by changes in temperature, and there are metabolic changes attendent with the change from sexual activity to inactivity (Garstka et al., unpubl. data). Thus, certain metabolic effectors were used to examine the role of metabolic change in inducing male sexual behavior. Thyroxine (T_4) and triiodothyronine (T_3) were administered to sexually inactive males, and sexual activity was tested at 1, 6, and 24 h. No behavioral change was observed (Table 4).

Because there is exchange between blood serum and the fluid bathing neurons, the ionic composition of the blood influences the transmission of neural impulses (Smith, 1966). In mammals, infusion of hypertonic saline into the circulation results in drinking behavior, and a deficiency of glucose may induce seizures or other severe behaviors (Smith, 1966). Guthrie (1980) presented evidence that seasonal changes in neural activity in the snail *Helix* are related to changes in electrolyte concentration of axons and circulation. Seasonal changes in body composition are well documented in *T. sirtalis* (Aleskiuk and Stewart, 1971; Hirth, 1966) which may relate to survival overwinter. In nature, *T. sirtalis*

neither eat nor drink during the courtship period. This, in addition to the changes in body composition related to the dehydration experience during winter dormancy (Aleksiuk and Stewart, 1971), may influence neural excitibility and behavior. Further, temperature can influence other behaviors of *T. sirtalis*. Cold is known to effect aggregative behavior in these snakes (Aleksiuk, 1976; Gregory, 1975).

Two experiments tested the effect of circulating electrolytes on male sexual behavior. In the first experiment, Na^+, K^+, and Ca^{2+} were administered to sexually inactive males. Tests at 2, 6, and 24 h showed no increase in sexual behavior (Table 4). However, the dosage of Na^+ slowed locomotory activity and eventually killed the snakes, and Ca^{2+} increased motor activity. In the second experiment, glucose was administered to sexually inactive males with similar negative results (Table 4). Thus, sexual behavior in male *T. sirtalis* occurs before testicular recrudescence and is apparently independent of the endrocrine events known to cause sexual behavior in other species.

Regulation of Female Sexual Receptivity

Sexual receptivity in vertebrates is characterized by unambiguous behaviors such as lordosis in the rat and the neck-bend in *A. carolinensis*. Such behaviors typically can be induced in ovariectomized females with exogenous estradiol or estrogen followed by progesterone (Tokarz and Crews, unpubl. data). Sexual behaviors of female *Thamnophis* are not as well defined. Further, because there is no close connection of cyclic gonadal development, receptivity, and mating as in most species, applying the concept of sexual receptivity to *Thamnophis* may be difficult.

Receptive behavior of female *Thamnophis* is usually described as the female remaining stationary, raising her tail, and gaping her cloaca (Carpenter, 1977; Crews, 1976). Other authors, however, have argued that while females have an active part in copulation, no specific behavior is involved in receptivity (Blanchard and Blanchard, 1941*a*). Rather, it is only the cessation of resistance by the female to the male which is required for copulation to occur. Thus, a female only accepts or refuses copulation.

TABLE 5.—Relationship between body temperature and time to copulation of female *Thamnophis sirtalis*.

Cloacal temperature (C)	Time to copulation (min)	Body size	
		Snout-vent length (cm)	Body mass (g)
10.5	14.7	69.0	127.1
12.5	13.2	60.5	74.0
14.8	6.7	51.0	64.1
14.8	12.4	54.0	75.8
15.2	1.7	59.5	70.7
22.5	not mated	59.0	90.7
25.2	not mated	56.0	59.3
25.5	not mated	61.5	106.9

Our observations in the field (Garstka and Crews, unpubl. data) indicate that female *T. sirtalis* are mated while still cold from recent emergence. Such females typically have cloacal temperatures below 15 C, while courting males have cloacal temperatures of 25–28 C. Intromissions may also occur while females are moving and actively struggling on their way out of the den. Males mating with tailless females have been observed, and copulations with dead females and into wounds of females are also known to occur. Two males can intromit at the same time into the same female (Brennan, 1924; Crews and Garstka, pers. observ.).

Male *Thamnophis* possess sensory knobbed supra-anal scales that are used to locate the cloaca of the female (Noble, 1937; Pisani, 1976). In addition, there is an enlarged, hooked, proximal spine on each hemipenis which is everted before the rest of the hemipenis. When the male is attempting to copulate, with his tail under that of the female, this spine is oriented anteriorly and may serve to lift the anal scale of the female and open the

cloaca (Pisani, 1976). Thus, by choosing cool, sluggish females, males may be able to force a copulation.

In order to test whether forced copulations occur, females of varying body temperatures were introduced into an arena containing 20 sexually active males, and time to copulation was noted. Females whose body temperatures were low (<15 C) were mated quickly, while those whose body temperatures were warm (20 C) never mated (Table 5). Thus, warming may terminate female sexual receptivity.

To test the hypothesis further that exposure to warm temperature terminates female sexual receptivity, two females which had been tested at warm body temperatures were cooled down the next night and retested at body temperatures of 7.0 C and 8.5 C. These females resisted mating for 40 min. Blanchard and Blanchard (1941*a*) reported that females fled from males during the warmer, later part of the breeding season and avoided mating.

These data seem to contradict previous reports (Aleksiuk and Gregory, 1974; Hawley and Aleksiuk, 1975). In those studies matings increased with environmental temperatures. We have found that male courtship activity in the field is related directly to body temperature (Garstka and Crews, unpubl. data). As in the study by Hawley and Aleksiuk (1975), there was a thermal threshold of 20–25 C for sexual activity; males with body temperatures above 25 C were all sexually active. However, females are mated immediately on emergence. Thus, the environmental temperature necessary to evoke male sexual activity is not related necessarily to the body temperature of mating females.

Because female *T. sirtalis* can avoid mating, even while cold, it is clear that female receptivity must exist. Further, observations that courtship times can be quite variable and that copulation may not occur even after hours of courtship also indicate that there is an active role of females in initiation of copulation (Blanchard and Blanchard, 1941*a*; Hawley and Aleksiuk, 1976*a*; Noble, 1937). What behavioral change occurs, however, is not known.

Summary

We have shown through an analysis of the natural history of the red-sided gartersnake (*T. sirtalis*) that a temporal dissociation of gonadal function and reproductive behavior occurs in both sexes. Because of this, certain questions arise concerning the role that physiology plays in controlling reproductive behavior.

We first showed how female attractivity is communicated to males by a pheromone present on the skin of the female. This pheromone is present in an active form in the circulation and liver of females, and can be induced in sera and livers of males with estrogen treatment. The pheromone is related chemically to vitellogenin, the circulating precursor of yolk, and may be a lipid-rich portion of the vitellogenin molecule. Because of the relationship between quantity of yolk and reproductive potential, the pheromone communicates potential fecundity.

Male sexual behavior is stimulated by temperature; a "priming" period in dormancy followed by higher temperatures elicits courtship behavior from males. The hormonal or neural basis of sexual behavior is not yet known. The acute presence of the testes or pituitary is not required, and no hormonal or neurotransmitter treatment as yet used has stimulated sexual behavior in sexually inactive males.

Although no specific behavior seems to be associated with sexual receptivity of females, the females do take an active role in initiation of copulation. Females must accept copulation before intromission can occur. Receptivity seems to be related to body temperature; females with cloacal temperatures above 20 C do not accept copulation in the field.

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