

Hormonal and pheromonal correlates of reproductive behavior in garter snakes

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Introduction

In the spirit of this symposium, it is of some heuristic value to speculate on the relationship between hormones, those chemical signals that communicate inside an organism's body, and pheromones, chemical signals that communicate information between two individuals of the same species. Currently, the preponderance of our knowledge of chemical signals concerns mainly internal signalling via the blood-borne, or humoral, factors we know as hormones. However, pheromones or semiochemicals, chemicals with signal function, may well have been the first signals utilized in the evolution of multicellular organisms.

It is especially interesting to speculate, in this venue of a comparative endocrinology proceedings, on the evolutionary relationship of hormones and pheromones. Given the preponderance of current knowledge of hormones and endocrine mechanisms, it is relatively simple to think of hormones as the progenitors of pheromones and that pheromones represent a specialized subset of hormones. However, this may be an overly simplified view of the question. Wilson (1975) hypothesized that whatever communication occurred between the most ancient organisms, it almost certainly was chemical in nature. As organisms evolved from unicellular forms to colonial forms and eventually multicellular organisms, chemical communication must have continued along with them. Indeed, J.B.S. Haldane (as cited in Wilson 1975) speculated in the 1930s that pheromones are the direct lineal ancestors of hormones. Thus, when the metazoan soma was organized in evolution, hormones appeared simply as the intercellular equivalent of the pheromones that mediated behavior among the single-celled organisms (Wilson 1975).

As organisms evolved and became more complex, other forms of communication appeared. As humans, we rely primarily on our visual and auditory senses. Naturally, as the field of animal behavior developed, those investigators studying communication focused on visual and auditory cues, especially among vertebrates. However, more recently, investigators have come to readdress the phenomenon of chemical communication. It is becoming increasingly evident that pheromones remain the fundamental signals for most kinds of organisms (Wilson 1975). Pheromones continue to turn up with great dependability in species whenever a deliberate search is made for them, to an extent that makes it reasonable to conjecture that chemical communication is virtually universal among living organisms (Wilson 1975).

The proceeding hypotheses are based primarily on a myriad of studies with invertebrates, primarily arthropods, and more specifically insects. Indeed, well over 1000 pheromones have been isolated and described in insects (Abelson 1985). However, the situation in vertebrates is very different. Despite some very diligent and careful experiments by investigators of vertebrate chemical communication, the discovery of pheromones in vertebrates is relatively rare, with comparatively few pheromones having been isolated and described. To some degree, this is due in part to the fact that most of the model organisms were mammals. Although most, if not all, mam-

mals utilize chemical signalling to some degree, it is very difficult to study pheromonal communication in mammals. Most investigators of mammalian pheromones have found that responses in the bioassay vanish long before the chemical fractionation procedures reach the level of single compounds. Then the bioassays themselves are frequently complex, are difficult to replicate, and frequently yield nonspecific behaviors such as sniffing.

Taking a clue from Wilson (1975), a small number of investigators have been studying chemical communication in non-mammalian vertebrates and meeting with some degree of success. Pheromonal communication is now well-known in fish. For example, maturational steroids provide pheromonal cues that orchestrate reproductive behaviors in the goldfish (Stacey 1994). It now appears that some of these non-mammalian species may provide excellent models for addressing the original question of how hormones and pheromones have coevolved to mediate behaviors both in an organism and between organisms.

One such vertebrate that has shown great promise as a model for studies of the hormonal and pheromonal mediation of behavior is the Canadian red-sided garter snake, *Thamnophis sirtalis parietalis*. The red-sided garter snake is a good model because there is a great deal of information known about the hormonal mechanisms regulating reproduction as well as the pheromonal cues that are necessary in the mediation of reproductive behavior.

There are three major questions that will be addressed in this short review. The first is, how do hormones affect reproductive behaviors and physiology of garter snakes? Those studies relating to the hormonal control of reproduction in both the male and female garter snake will be examined. The second question is, what role do pheromones play in mediating reproductive behavior in garter snakes? Three specific pheromone systems that mediate reproduction in both the male and female will be examined. Finally, the last question is, how do hormones and pheromones interact to mediate reproductive behavior in garter snakes? To address this question we will examine an interesting case of pheromone mimicry that presents a "natural" experiment which can be exploited to yield interesting insights into the relationship of the hormonal system and the pheromonal system in the mediation of reproduction in garter snakes.

Natural history of the red-sided garter snake

The red-sided garter snake, *T. s. parietalis*, is the most northerly living reptile in North America (Logier and Toner 1961). As such, it is constrained to hibernate for up to 8 months of the year in underground dens or hibernacula. In Manitoba, Canada, male red-sided garter snakes emerge in late April and congregate at the den entrance in aggregations consisting of several thousand animals (Gregory 1975). Shortly thereafter, the females emerge singly or in small groups over the next 3–4 weeks of the mating season. Male garter snakes actively search for sexually attractive females. The males are initially attracted to movement but must actually come into contact with a female before courtship begins.

Upon encountering a sexually attractive, unmated female, a male presses his chin onto her dorsal skin and emits very short, rapid tongue flicks (Noble 1937). At the same time the male traverses up and down the female, turning back at head and tail; the male comes to rest with his head resting behind the female's head. Once stationary, the male's body undulates in caudocephalic waves. Both chin-rubbing and caudocephalic waves are only observed during a mating context and are not associated with any other behaviors (Camazine et al. 1980). Many males will court a female simultaneously, forming a mating ball of 10–100 males (Gregory 1974). However, only one male will mate with the female, while the others disperse to seek other unmated females.

Noble (1937) was the first to demonstrate that the female garter snake produces a pheromone that is sequestered on the skin of her dorsal surface. Sexually active males fail to court females following chemical manipulations of the pheromone on the female's back. Other studies eliminating auditory, visual, and tactile cues led Noble to conclude that the tongue delivers chemical cues to the vomeronasal organ in the roof of the mouth. More recent studies by Halpern and colleagues (Halpern and Kubie 1980; Kubie et al. 1978) confirmed Noble's findings and clearly demonstrated that the vomeronasal system was the sole means by which males recognize females.

Production of the skin-derived pheromone is all that is necessary for males to court females. The fact that dead females are vigorously courted for days illustrates not only the robust response of males to the chemical signal but also precludes behavioral cues from the female from having any important role in male courtship.

Hormonal mediation of reproduction in garter snakes

There are basically three topics that address the role of hormones in the mediation of reproductive physiology in the garter snake. These are (i) effects of hormones on male courtship, (ii) effects of hormones on female attractivity, and (iii) effects of hormones on female receptivity.

In many vertebrates, especially mammals and domesticated species, gamete production, sex steroid hormone secretion, and mating behavior are linked, or occur concurrently. This pattern of reproduction has been termed an "associated" reproductive tactic or sometimes prenuptial gametogenesis (Crews 1984; Volsoe 1944; Lofts 1977; Licht 1984). However, it is now generally accepted that in many vertebrate species gonadal activity and mating are uncoupled or "dissociated" (Crews 1984). The tactics of male garter snakes belong in the dissociated class of reproductive tactics. That is, male garter snakes exhibit sexual behavior when their gonads are completely regressed and circulating levels of sex steroid hormones are at their lowest (Crews et al. 1984). Courtship and mating behavior in the male are not activated by testicular or pituitary hormones but by the male experiencing an increase in the ambient temperature following winter dormancy (Crews 1983).

In male garter snakes, testicular activity is near basal levels at the time of spring emergence. Only after all spring breeding activity has ceased do males eventually recrudescence their testes some 5–10 weeks later (Krohmer et al. 1987). Thus, the sperm used by the male in the spring were produced the previous summer and stored in the epididymides and vasa deferentia over the winter dormancy period.

In fact, this low-temperature overwintering period is critical

to the expression of male sexual behavior in the spring. Hormonal manipulations involving steroids, pituitary and hypothalamic hormones, and neurotransmitters fail to elicit courtship behavior in male garter snakes (see Crews 1987). In addition, castration neither abolishes nor enhances courtship behavior in the male (Crews et al. 1984). Interestingly, courtship behavior can be abolished in the male by radio-frequency lesions to the anterior hypothalamic – preoptic area (AH-POA) (Friedman and Crews 1985; Krohmer and Crews 1987). Crews (1987) has hypothesized that although this area contains sex steroid hormone concentrating neurons (Halpern et al. 1982), it also contains temperature-sensitive neurons (Satinoff 1978). Crews (1987) argues that because there is a lack of evidence of any steroidal induction of sex behavior in male garter snakes, it is reasonable to conclude that the role of the AH-POA in regulating reproductive behavior is not due to the hormone concentrating neurons but rather is due to the presence of the temperature-sensitive neurons (Crews 1987). Thus, for the male red-sided garter snake, activation of reproductive behavior is not due to rising levels of sex steroid hormones but rather is a response to a critical environmental cue, i.e., rising temperatures that signal the onset of spring and the end of winter dormancy.

Female attractivity

Female red-sided garter snakes exhibit a dissociated reproductive tactic as do males. In general, female garter snakes mate when their ovaries are regressed and circulating levels of sex steroid hormones are near basal levels. Temperature appears to be a critical factor mediating female reproductive physiology and behavior as well. Temperature has a stimulating effect on sexual behavior when the ovary is in a regressed condition and levels of sex steroids are low (Whittier and Tokarz 1992; Bona-Gallo and Licht 1983; Garstka et al. 1985; Whittier et al. 1987a). It appears that females must be exposed to at least 7 weeks of 4°C temperature for them to reproduce in the spring (Whittier et al. 1987b).

Female garter snakes attract male courtship and elicit mating by means of a pheromone. Upon emerging from hibernation, female garter snakes possess a skin-derived sexual attractiveness pheromone that is sequestered along the female's entire dorsal surface. Attractivity is dependent on *production* of the sexual attractiveness pheromone by the female and *reception* of the pheromone by the male.

The role of the ovary in the control of sexual behavior in the red-sided garter snake remains unclear, as definitive traditional ovariectomy and hormone replacement have not been attempted (Whittier and Tokarz 1992). A seemingly counter-intuitive phenomenon appears to regulate female attractivity in the red-sided garter snake. Earlier studies showed that injections of exogenous estrogen stimulated female attractivity, as measured by male courtship of the female, in a dose-dependent manner in both intact, sexually inactive, and ovariectomized females (Crews 1976). Further studies indicate that estrogen treatment of intact females induces a pheromone concentrating or releasing phenomenon in which just prior to shedding, the female may be incapable of attracting a male, but following shedding is even more attractive than nonshedding estrogen-treated females (Kubie et al. 1978).

Paradoxically, female garter snakes that emerge from hibernation are virtually all sexually attractive, yet their circulating levels of estrogen are basal (Whittier et al. 1987a). Mating

causes a significant rise in plasma levels of estradiol in the female within 6 h (Garstka et al. 1987; Whittier et al. 1987a). Despite this rapid rise in estradiol, females immediately become sexually unreceptive and within 24 h become unattractive to further male courtship (Whittier et al. 1985; Whittier and Crews 1986a). It now appears that the loss of attractivity in recently mated females does not involve a change in the production or expression of the sexual attractiveness pheromone but rather is due to the presence of a copulatory pheromone deposited by the male during mating (R.T. Mason, unpublished data). The surge in estradiol at the time of mating may facilitate the process of vitellogenesis and initiate ovarian development (Garstka et al. 1982; Whittier and Crews 1986a; Whittier et al. 1987a).

The question remains: if estrogen induces the production and expression of the sexual attractiveness pheromone, yet the circulating levels of estrogens are basal at the time when the females are most attractive, how does estrogen production regulate female attractivity at spring emergence? The answer may be found by looking at the male's annual cycle for clues. Males produce their sperm in the summer and store it over the winter for immediate use on spring emergence. Perhaps females produce their sexual attractiveness pheromone in the fall and store it in and on their skin through hibernation. Support for this hypothesis comes from a review of earlier studies. Whittier and Tokarz (1992) describe years in which two-thirds of females returning to hibernacula in the autumn had recently deposited sperm in their oviducts (Halpert et al. 1982; Whittier and Crews 1986a). Blood samples from females collected in the fall showed elevated levels of estradiol (Whittier et al. 1987a). Thus, female red-sided garter snakes may be producing their sexual attractiveness pheromone in the late summer and fall and sequestering it in their skin throughout hibernation. This would account for the attractivity of females upon entering hibernation and immediately upon emergence from hibernation.

Female receptivity

It seems clear that sexual attractivity and receptivity are regulated differentially in the female garter snake because some attractive females are unreceptive to male courtship (Whittier and Tokarz 1992). Receptivity in the garter snake is assumed only when actual mating occurs (Whittier and Tokarz 1992; Ross and Crews 1977, 1978; Whittier et al. 1985).

As discussed earlier, females become refractory to continued courtship after mating. Usually they become immediately sexually unreceptive and unattractive. Although there is a rapid and pronounced estrogen surge within 4–6 h after mating in the female, it appears that this phenomenon may facilitate vitellogenesis and ovarian recrudescence (Garstka et al. 1982). Ovarian development can occur without a postmating surge, as females that mate in the previous fall but not on spring emergence can still go on to ovulate and produce young (Whittier and Crews 1986a; Whittier et al. 1987a; Mendonca and Crews 1989). Finally, this estrogen surge after mating in female garter snakes appears to be linked to a neuroendocrine reflex, as spinal transection or anesthetization of the female's cloaca before mating will prevent the postmating estrogen surge and subsequent ovarian development (Mendonca and Crews 1990).

The loss of sexual receptivity in the female garter snake is probably not directly correlated to the postmating estrogen surge. Female garter snakes have a rapid and pronounced loss

of receptivity immediately following mating. Because the estrogen surge postmating occurs on the order of hours, there must be other putative factors responsible for this behavior.

It appears that prostaglandins may be mediators of sexual receptivity in female red-sided garter snakes (Whittier and Crews 1986b, 1989; Whittier and O'Connor 1991). Females injected with PGF_{2α} were significantly less receptive than untreated controls (Whittier and Crews 1986b). Lower doses of the same prostaglandin did not significantly inhibit mating but did increase the latency to mating. In field studies, recently mated females had significantly higher plasma concentrations of PGF_{2α} than courted, but unmated, controls (Whittier and Crews 1989). Levels of prostaglandins are significantly elevated within minutes of copulation and remain elevated for up to 24 h (Whittier and Crews 1989). Although some of the endogenous prostaglandins may be produced by the female herself, further evidence demonstrates that a significant amount of the prostaglandin measured in the female postmating is derived from the material expressed into the female's cloaca by the male during copulation (Whittier and O'Connor 1991). Finally, these prostaglandins directly stimulate the ovaries to secrete estrogens and are in part responsible for the postmating estrogen surge in females (Whittier and O'Connor 1991).

Sex pheromone systems in garter snakes

Investigators have hypothesized for over 50 years now that sex pheromones play an important role in orchestrating sexual behavior in snakes (Noble 1937). It is now widely accepted that many, if not all, snakes utilize pheromones to one degree or another (Mason 1992). The Canadian red-sided garter snake has proven to be an excellent model for investigating the mechanisms by which pheromones mediate reproductive behaviors.

Currently, there are four pheromone systems in garter snakes that interact to orchestrate sexual behavior. Female sexual attractiveness pheromones are estrogen-induced pheromones that arise from the skin lipids on the female's dorsal surface. These pheromones serve to attract males and advertise the female's species and the fact that she is reproductively competent. Male sex recognition pheromones are also part of the skin lipids, although they are limited to males. These pheromones advertise the male's gender and indicate that he is an inappropriate individual to court. Copulatory pheromones are a recent discovery that shed light on previous studies of copulatory plugs. These pheromones advertise the female's mated status and render her sexually unattractive for 24 to 36 h. During this time she may disperse from the hibernaculum relatively unfettered by further male courtship.

Finally, pheromone mimicry is an interesting natural experiment. A small percentage of males in the population produce the female sexual attractiveness pheromones and very little of the male sex recognition pheromones. They present a model to investigate the hormonal interaction with pheromone production. By studying the hormonal differences between these female mimics and comparing them with the situation in males and females, we have been able to exploit these differences to gather knowledge about the mechanism of hormone action on pheromone production.

Female sexual attractiveness pheromones

Upon emergence from hibernation in late April, Canadian red-sided garter snakes commence breeding activity. In some of the large communal dens or hibernacula, populations in

excess of 10 000 animals are known. As with most vertebrates, males arrive on the breeding grounds first and await the emergence of females, which emerge singly or a few at a time over the course of the 4-week breeding season. This presents a problem for the males: how does one find and recognize the females? Although females are larger than males in terms of snout-vent length and body mass, there are no differences in markings or coloration. Thus, it is a problem for males to recognize a slightly larger female snake among thousands of ardent male suitors. In a similar fashion, snakes do not vocalize and they do not have ears, so auditory cues can be eliminated.

Garter snakes convey a great deal of information by chemical communication via semiochemicals or pheromones. Female garter snakes have been known for some 50 years to produce a sex attractant pheromone on their dorsal skin (Noble 1937). We were able to isolate, characterize, and synthesize this pheromone and test its activity in bioassays performed in the field (Mason et al. 1989, 1990; Mason 1993). The sexual attractiveness pheromone of the female red-sided garter snake is a series of long-chain saturated and monounsaturated methyl ketones ranging in mass from 394 to 532 Da. These contact pheromones are relatively nonvolatile, thus indicating the necessity of the male's tongue to pick up these odorant molecules.

Male garter snakes also possess a set of semiochemicals. During the breeding season male garter snakes will tongue-flick any individual with which they come into contact. Females are immediately courted whereas males are rapidly abandoned and ignored. It now appears that males possess another unique set of skin-derived semiochemicals that advertise their sex (Mason et al. 1989). Squalene is one component of a multi-component mixture that identifies males and serves as a male sex recognition pheromone.

These two pheromone systems may be exaptations of a pre-existing lipid system in these snakes. All terrestrial life forms possess lipids on their skin or cuticle that retard transcutaneous water loss (Hadley 1985). Reptiles in general, and snakes in particular, are known to possess complex mixtures of skin lipids that serve, among other things, to reduce transcutaneous water loss (Burken et al. 1985). Natural selection may have selected for those males that could detect and perceive endocrine-associated changes in females that were reflected in the skin lipid profiles. Estrogens and other steroids are known to affect skin lipid components (Hasselquist et al. 1980; Ozasa and Gould 1982; Onouchi and Kato 1983).

Copulatory pheromones

Males can discriminate between mated and unmated females and rarely are seen to court mated females. In the field, many males often court a single female. Whenever one male eventually mates, the other males disperse before the rival male even finishes mating. Field studies of *T. s. parietalis* show that females become unattractive for 24–36 h after mating (Ross and Crews 1977, 1978; Whittier et al. 1985). Males leave a gelatinous copulatory plug in the cloaca of mated females and it has been suggested that the copulatory plug possesses an inhibitory pheromone that renders mated females unattractive (Ross and Crews 1977, 1978; Devine 1975, 1977). However, other work has demonstrated no such pheromonal properties associated with the copulatory plug (Whittier et al. 1985).

In very recent work (R.T. Mason, unpublished data), it was found that the transient loss of females' attractiveness is not due

to a change in the females' attractiveness pheromones. Pheromone extracts from the dorsal surfaces of recently mated females were just as effective in eliciting male courtship as were pheromone extracts from unmated females. Copulatory plugs from recently mated females when applied to the backs of unmated females also failed to diminish the test females' attractiveness. It now appears that the source of the copulatory pheromones that render females transiently unattractive after mating is a secretion associated with the act of mating that is expressed by the male or female or both. This secretion is smeared around the entire cloacal region of both the male and female during copulation. After mating, this secretion can be clearly seen as a shiny coating of the entire cloacal area and up onto the lateral body surface. In similar transfer experiments, this material, when transferred to the backs of unmated females, rendered them as unattractive as mated females (R.T. Mason, unpublished data). Further studies are proceeding in the analysis of the putative semiochemicals responsible for this phenomenon.

Pheromone mimicry

The final pheromone system found in garter snakes is an unusual example of pheromone mimicry. In field studies in Manitoba, a small proportion of male red-sided garter snakes consistently elicits courtship from courting males in the same fashion as do females (Mason and Crews 1985). These female mimics, or she-males (Mason and Crews 1985), are anatomically and morphologically male and they court and mate with females. However, they can be discerned from other courting males on the basis of their attractiveness. The she-males seem to be a source of confusion to the other males in the mating ball. In competitive mating trials, she-males mate more than twice as often as do normal males, indicating a strong selective advantage to those males possessing this trait. Chemical analyses of the skin lipids of she-males reveal that the lipids possess methyl ketones that are intermediate to those of both males and females (Mason 1993). Further, she-males have very low levels of squalene in their skin, essentially the same as females (Mason et al. 1989).

These she-males present an interesting natural experiment that helps to shed new light on the role hormones play in regulating pheromone production and expression. As discussed earlier, estrogen is known to stimulate the production and expression of the sex attractiveness pheromones in females. Perhaps she-males possess high levels of estrogen that in turn cause the production of sexual attractiveness pheromones. Examination of circulating levels of sex steroid hormones in these snakes revealed that she-males had levels of estradiol similar to those of males and significantly less than those of females (Mason and Crews 1985). However, she-males had significantly higher plasma concentrations of testosterone than did normal males (Mason and Crews 1985). It was hypothesized that these high levels of testosterone in the plasma may be converted by aromatase enzymes to high levels of estrogen at the level of the target organ (Mason 1993). In this case, the target organ for pheromone production is the skin. Preliminary evidence indicates that female's skin has the highest aromatase levels followed by that of she-males and finally males (R.T. Mason and R.W. Krohmer, unpublished data).

Finding significant amounts of aromatase in the skin of the she-males provides an opportunity to exploit this interesting phenomenon to gain a better understanding of the hormonal

control of pheromone production in the garter snake. These she-males will hopefully provide a useful tool for elucidating the physiological mechanisms by which the sexual attractiveness and male sex recognition pheromones are produced. In the future, studies will examine the biosynthesis of pheromones, including their site of production and the biochemical pathways involved. In addition, the integration of the pheromone system and the endocrine system will continue to be examined. For instance, much is known of the reproductive endocrinology and reproductive behavior of garter snakes. Similarly, there is a growing body of knowledge of the pheromones involved in reproduction. However, how are the two functions linked? Once the male perceives the female sexual attractiveness pheromones, what physiological processing occurs in the brain that initiates the expression of sexual behavior? These kinds of integrative questions are currently being examined and the garter snake continues to provide a challenging model in this regard.

Acknowledgements

I thank David Crews and Jeremy McNeil for inviting me to participate in this symposium. This work was supported by the National Science Foundation (NSF) through INT-9114567, an NSF Young Investigator Award, and by an Oregon State University Public Health Service institutional grant.

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