Hormones, Sex Accessory Structures, and Secondary Sexual Characteristics in Amphibians

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SUMMARY
Gonadal steroid hormones, particularly testosterone (T) and related androgens, are important in the development and seasonal variation of sexually dimorphic organs. Other hormones, such as prolactin, have been found to be necessary in conjunction with gonadal steroids for the full structural development and function of some sex accessory structures (e.g., oviductal and cloacal gland secretions) and secondary sexual characteristics (e.g., genial glands and skin glands of newts). Thyroid hormones work synergistically with prolactin and T in hypertrophy of the tail fin and nuptial pads of American newts (e.g., Notophthalmus viridescens), whereas oxytocin antagonizes the influence of prolactin. In the Japanese newt (Cynops pyrrhogaster), however, estrogens block the action of prolactin on increasing tail height, explaining the sexual differences in tail morphology. Arginine vasotocin (AVT) has been shown to stimulate labor in the viviparous salamandrid Salamandra salamandra, corticosterone influences the development of salamander cloacal glands, and prostaglandin PGF2α causes the release of sperm from the salamander spermatheca by triggering contraction of the myoepithelium.

1. INTRODUCTION
The sex accessory structures of amphibians are the genital ducts and derivatives of these structures. The secondary sexual characteristics are all of the differences between the sexes due to sexual maturation other than those connected with the gonads and their ducts. In this chapter, we review the effects of hormones on development, anatomy, function, and seasonal variation of the sex accessory structures and secondary sexual characteristics of amphibians. We introduce the more general term, secondary characteristics, to refer to non-sexually dimorphic traits that arise at sexual maturity. A list of the structures considered is presented in Table 5.1 and we will report on representative studies related to these organs.

The role of gonadal steroid hormones in causing seasonal changes in sex accessory structures and secondary sexual characteristics has been well-documented (see, for example the reviews by Norris (1987), Fernández and Ramos (2003), Kikuyama, Tanaka, and Moore (2003), and Exbrayat (2006)). In males, the primary testicular steroids are testosterone (T) and 5α-dihydrotestosterone, although others have been detected; i.e., 11-ketotestosterone, 11β-hydroxytestosterone, androstenedione, progesterone (P4), 17β-estradiol (E2), and estrone. In females, the primary steroids are T, androstenedione, E2, estrone, and P4 (Moore & Deviche, 1988). Gonadal steroids are also important in the behaviors associated with these characteristics, but other hormones such as the gonadotropins (GTHs) follicle-stimulating hormone and luteinizing hormone, and the neuropeptide arginine vasotocin (AVT), are also critical in mating behaviors and show changes in concentrations that correlate with gonadal activity and changes in sex accessory structures and secondary sexual characteristics (Deviche, Propper, & Moore, 1990). This chapter, however, is not concerned with behavior, which is covered elsewhere (Chapter 8, this volume). Gonadotropin-releasing hormone (GnRH) stimulates the release of GTHs, and GTHs stimulate development of the gonads and thus production of gonadal steroids. The feedback relationships in these cycles have been examined in a number of amphibians but are not a concern of this chapter (see Chapter 2, this volume).

2. SEX ACCESSORY STRUCTURES
2.1. Wolffian Ducts/Vas Deferens
From each testis, sperm pass through longitudinal tubules into the Wolffian (archinephric, mesonephric) duct; no epididymis exists. The Wolffian duct is both a sperm and
urine duct, but, in plethodontid salamanders, the anterior extension of the Wolffian duct is strictly a sperm duct and the name ‘vas deferens’ is appropriate for this portion (Uribe Aranza´ bal, 2003). Development of the Wolffian ducts in larval and postmetamorphic amphibians is stimulated by androgens (Norris, 1987) and, in Rana japonicus, the effects of T were enhanced by treatment with E2 (Tojio & Iwasawa, 1977).

The caudal portion of the Wolffian duct stores sperm prior to the mating season and becomes secretory in Notophthalmus viridescens, Taricha torosa, Ambystoma tigrinum (Norris, 1987), and Ambystoma dumerillii (Uribe, 2001). Norris, Norman, Pancak, and Duvall (1985) reported that hypertrophy of the vasa deferentia epithelium and secretory activity of the cloacal gland complex in neotenic A. tigrinum were closely tied to high levels of plasma androgens (T and DHT). The diameter of the vasa deferentia was associated with the presence of stored sperm and not with plasma androgen levels.

2.2. Müllerian Ducts

Müllerian ducts develop into functional oviducts under the influence of ovarian estrogens during ontogeny, and this has been documented in many studies (Norris, Carr, Summers, & Featherston, 1997; Greven, 2003). Boisseau (1975) showed that exposure to E2 hastens morphogenesis of ciliated and secretory cells in the salamandrid Pleurodeles waltl. Jego (1977), also in P. waltl, found that E2 had different effects on fucosyltransferase and galactosyltransferase, enzymes involved in the formation of glycoproteins. In the newt Cynops pyrrhogaster, both prolactin (PRL) and sex steroids (E2, T) were needed for production of oviductal mucopolysaccharides (Kikuyama, Seshimo, Shirama, Kato, & Noumura, 1986; Polzonetti-Magni, Carnevali, Yamamoto, & Kikuyama, 1995). Without PRL, E2 could neither induce full structural development nor stimulate egg jelly synthesis in C. pyrrhogaster (Kikuyama et al., 2000).

Clark, Norris, & Jones (1995) studied the interactions of E2 or dihydrotestosterone (DHT) and the pesticides dichlorodiphenyltrichloroethane (technical grade, 80% p,p’-DDT and 20% o,p’-DDT) and dichlordiphenyldichloroethylene (p,p’-DDE) on the development of Wolffian ducts and Müllerian ducts in larval male and female A. tigrinum. 17β-estradiol and DHT stimulated Müllerian duct development in both sexes, whereas the Wolffian duct was stimulated only by DHT. Dichlorodiphenyltrichloroethane had an antiestrogenic effect on gonaduct development, and DDE had an estrogenic effect on the Müllerian ducts of females. The Müllerian ducts of males and the Wolffian ducts of both sexes were unaffected by DDT or DDE alone. Their results contradicted the expected estrogenic actions of DDT and the antiandrogenic actions of DDE.

The anuran oviduct is divided into three parts (Fernández & Ramos, 2003). The most anterior region is the pars recta, which collects ovulated eggs through the ostium. The pars recta secretes oviductin, which modifies the egg vitelline envelope to make it susceptible for sperm penetration. The middle region of the anuran oviduct is the pars convoluta, which secretes the highly viscous jelly layers onto the eggs. The distal portion of the oviduct is the ovisac, where eggs are held prior to ovulation. In the preovulatory period of the ovarian cycle of Bufo arenarum,
serum T, DHT, and P₄ reach the highest circulating levels whereas E₂ shows the lowest values detected during the cycle (Fernández, Mansilla, & Miceli, 1984). Under these hormonal conditions, the entire oviduct reaches maximal development of secretory cells. A marked decrease in androgen and P₄ circulating levels and a steady increase in E₂ are associated with ovulation (Fernández et al., 1984; Fernández and Ramos, 2003).

The oviduct, in at least some species, may secrete a substance that alerts males to female receptivity. Water that held ovariectomized female newts C. pyrrhogaster treated with PRL and E₂ was significantly more attractive to males than water that held ovariectomized females treated with saline (Toyoda, Tanaka, Matsuda, & Kikuyama, 1994).

In most male anurans and salamanders, the Müllerian duct regresses in the embryo in response to Müllerian-inhibiting hormone (MIH), which is secreted by the Sertoli cells of the developing testis (Akbarsha, Jancy, Smita, & Oommen, 2006). Vestiges of the Müllerian duct persist in some male anurans and salamanders, but they have no function (Duellman & Trueb, 1987). A rudimentary Müllerian duct is retained in male bufonid toads (e.g., Bufo woodhousei) and, if the testes are removed, leaving the ovarian-like Bidder’s organs, the Müllerian ducts may develop into oviducts (Witschi, 1942). The Müllerian duct is retained in male caecilians as a functional glandular structure, the Müllerian gland. The role of MIH in male caecilians is unknown. The caecilian Müllerian gland is involved in the production of seminal fluid and is hypothesized to be homologous to the mammalian prostate gland (Wake, 1981).

Some specializations of the amphibian oviduct are discussed below.

### 2.2.2. Uterus of viviparous anurans

Viviparity is known in several species of African bufonid (including at least Nectophrynoides torquata, Nectophrynoides viviparus, Nimbaphrynoides occidentalis, and Nimbaphrynoides liberiensis (Wake, 1980; 1993)), and in the leptodactylid frog Eleurodactylus jasperi from Puerto Rico (Wake, 1978). After the bufonids exhaust the yolk, they are nourished by ingesting ‘uterine milk,’ a mucoprotein secreted by the oviductal epithelial glands (Vilter & Lugand, 1959). Corpora lutea are present in pregnant N. occidentalis and apparently regulate oviductal nutrient secretion (Lamotte & Xavier, 1972). Intra-oviductal nutrition in E. jasperi is dependent upon yolk throughout uterine development, and no corpora lutea are present in the ovaries (Wake, 1978). Other knowledge about the hormonal influences on gravidity in these species is lacking. For E. jasperi, such information may never be gained as the species may be extinct (Wake & Dickie, 1998).

### 2.2.3. Uterus of Salamandra

Greven (2003) recognized two types of viviparity in salamanders: larviparity, in which the female gives birth to true larvae, and pueriparity, in which the species gives birth to transformed young. The salamanders concerned are all in the European/Asian genus Salamandra (Salamandridae) and, depending upon one’s preference in taxonomy, four to eight species (or more) are involved. Some of these are variably larviparous or pueriparous. Nutritive support after consumption of the initial yolk stores includes oophagy, adelphophagy, and epitelophagy (Greven, 2003). Oophagy and adelphophagy are both forms of embryonic cannibalism, with the former involving ingestion of degrading, unfertilized eggs and the latter the consumption of smaller siblings. Epitelophagy is the ingestion of the uterine epithelial lining from a proliferating zone called the zona trophica (Greven, 2003). Gestation time varies depending upon species and climatic conditions and ranges from five to fourteen months in S. salamandra and up to four years in Salamandra atra and Salamandra lanzai (Greven, 2003).

Knowledge of the hormonal influences on pregnancy in salamanders is limited. Greven and Guex (1994) reported that application of an estrogen increased secretory activity in the oviduct of S. atra. Histology, histochemistry, and ultrastructure have provided evidence for corpora lutea during the gestation period, but these may become reduced late in pregnancy, and embryos remain...
alive and develop after ovariectomy (Greven, 2003). Pregnant females of *S. salamandra* injected intramuscularly with large doses of AVT gave birth to their young but this was difficult to achieve in *S. atra* (Heller, Ferreri, & Leathers, 1970). Birth of young in *S. salamandra*, however, occurs over several weeks whereas two weeks may pass before parturition of the second of two offspring by *S. atra*. Much remains to be learned about viviparity in salamanders.

### 2.2.4. Uterus of caecilians

Wake (1977) estimated that 50% of the 170 known caecilian species are viviparous, and, in two families (Scolecomorphidae (six species) from east Africa and Typhlonectidae (twelve species) from South America), all species are viviparous. All live-bearing caecilians provide maternal nutrition to the developing young via a secretory epithelium that is orally ingested by the fetuses (Wake, 2006). In addition, the highly elaborated gills of embryonic *Typhlonectes compressicauda* form placental-like attachments with the uterine epithelium for nutrient uptake (Exbrayat & Hraoui-Bloquet, 2006). Finally, intra-uterine cannibalism of eggs and/or embryos has been reported for *T. compressicauda* (Exbrayat & Hraoui-Bloquet, 2006). Exbrayat and Delsol (1988) presented morphological evidence that corpora lutea persist through the pregnancy of *T. compressicauda*, and proposed that P₄ was involved in maintaining gestation. Otherwise, little is known about hormonal influences on viviparity in caecilians.

### 2.3. Penis of *Ascaphus*

As indicated in Section 2.3.1, *Ascaphus truei* and its sibling species *A. montanus* are phylogenetically basal frogs that practice internal fertilization. These frogs are the only anurans that truly can be said to engage in copulation while in amplexus (termed ‘copulexus’ by Sever et al. (2001)), and the only anamniotes to possess a ‘true penis;’ i.e., a copulatory organ in which cavernous tissue becomes engorged with blood during erection (Sever et al., 2003). The penis has been traditionally referred to as a ‘tail,’ but it has no relationship to the caudal vertebrae and is a fleshy extension of the cloaca. When engorged, the penis forms a sulcus for passage of sperm and is inserted in the cloaca of the female (Noble, 1925; Noble & Putnam, 1931; Slater, 1931). Nothing is known about hormonal influences on the penis of *Ascaphus*.

### 2.4. Cloacal Glands of Salamanders

The seven families of salamander comprising the traditional suborder Salamandroidea are unique among vertebrates in the possession of a distinct set of male cloacal glands that make spermatophores as well as female cloacal glands that store sperm (Sever, 1991a; 2003). The result is internal fertilization either in the oviduct (some salamandrids) or as eggs pass through the cloaca (rest of the species). The remaining three families of salamander reproduce by external fertilization and either lack cloacal glands (Sirenidae (Sever et al., 1996)) or generally possess just one type of cloacal gland that is similar in both males and females (Hynobiidae, Cryptobranchidae). These are the three basal families of urodeles (Larson, Weisrock, & Kozak, 2003; Wiens, Bonett, & Chippindale, 2005). Sever (1991b) proposed that cloacal glands of hynobiids and cryptobranchids function in pheromone production and that this is the ancestral function of cloacal glands.

Development of cloacal glands is controlled by sex hormones associated with maturation and sexual activity, and the glands are most hypertrophied during the breeding season (Norris & Moore, 1975; Norris, 1987; Iwata, Toyoda, Yamamoto, & Kikuyama, 2000; Kikuyama et al., 2000). Individual gland clusters are virtually unrecognizable prior to sexual maturity or in individuals collected outside the breeding season (Wild, 1925; Williams, Martan, & Brandon, 1985; Sever 1994). Mature neotenic salamanders have cloacal gland clusters similar to those of metamorphosed salamanders (Licht & Sever 1991; Trauth, Sever, & Semlitsch, 1994; Krenz & Sever, 1995), except for neotenic hemidactyliines in which, the pheromone-producing glands are absent or reduced (Sever, 1985). Some relevant papers on the hormonal control of cloacal glands are presented below.

Benson (1965) in an unpublished dissertation studied the effects of various hormone treatments on the cloacal glands of *N. viridescens*. Evidence from castrates injected with androgens (T and dehydroepiandrosterone) and GTHs (chorionic gonadotropin (CG) and luteinizing hormone (LH)) indicates that the cloacal glands are stimulated directly by male hormones and only indirectly by GTHs. In addition, cloacal glands of castrates were found to be directly stimulated by three other steroidal hormones (estriol, P₄, and corticosterone), whereas a fourth, deoxycorticosterone acetate (DOCA), had no effect. In intact animals, the corticosteroid DOCA induced massive involution of the cloacal glands whereas estril, P₄, and corticosterone caused only mild inhibition.

Norris and Moore (1975) conducted a study in which they injected immature male and female *A. tigrinum* larvae with T, E₂, P₄, T + E₂, T + P₄, or E₂ + P₄ on alternate days for 40 days. Only T treatment induced development of male-type cloacal glands. 17β-estradiol significantly reduced the response to T, and P₄ had no effect.
Sever (1980) injected the paedomorphic plethodontid *Eurycea tynerensis* with l-thyroxin pentahydrate to induce metamorphosis to study the effects on male cloacal glands. Individuals were also injected with T because they were collected while in a sexually inactive condition. Paedomorphic *E. tynerensis* lack one type of cloacal gland, called the vent gland, which arises from the cornified epidermis lining the posterior angle of the cloacal orifice. Larval salamanders lack the cornified epidermis found in metamorphosed salamanders. Although the epidermis changed to the metamorphosed condition in the *E. tynerensis* induced to metamorphose, vent glands did not appear, suggesting that *E. tynerensis* has lost the genetic ability for the development of vent glands. Other cloacal glands were similar in both paedomorphic and metamorphosed *E. tynerensis*, and similar to those of other species of *Eurycea*.

Sodelfrin is a pheromone produced by the dorsal gland of the newt *C. pyrrhogaster* (Kikuyama et al., 1995; Toyoda et al., 1995). Sodelfrin synthesis appears to be under the control of PRL and androgens because the amount of the pheromone increases following treatment with both and less markedly with androgens alone (Yamamoto, Toyoda, Tanaka, Hayashi, & Kikuyama, 1996). These hormones also affect the structural development of the dorsal gland and ventral gland. In hypophysectomized and/or castrated animals treated with prolactin and androgens and those injected with saline, the dorsal glands were similar to those of breeding and non-breeding males, respectively (Kikuyama et al., 1995). The same conditions probably apply to production of a similar pheromone, silefrin, from the dorsal gland of *Cynops ensicauda* (Yamamoto et al., 2000).

Little work has been done on hormonal influences on sperm storage in the female spermatheca, which can occur through periods when the ovary is quiescent and gonadal steroids are presumably at low levels. Hardy and Dent (1987) studied the effects of some neurotransmitters and hormones on the release of sperm from the spermatheca. They injected into the spermathecal region of females with either saline, saline plus acetylcholine, norepinephrine, AVT, or the prostaglandin PGF2α. The number of sperm present in the cloaca was highest in those females injected with PGF2α. Hardy and Dent proposed an active role for the spermathecal myoepithelium in the discharge of stored sperm and a role for PGF2α in triggering that discharge.

2.5. Phalldodeum of Caecilians

The phalldodeum comprises the posterior wall of the male cloaca and is contained in a connective tissue capsule from which it is separated by a periphalldodeal space (Exbyrat & Estabel, 2006). Extrusion of the phalldodeum during sexual activity involves eversion of the tubular structure so that the inner spinous epithelium becomes situated on the external face of the phalldodeum (Tonutti, 1931). The linings of the phalldodeum vary with the sexual cycle, but the hormonal influences on these changes have not been described.

3. SECONDARY SEXUAL CHARACTERISTICS

Secondary sexual characteristics are common in amphibians. Darwin reviewed secondary sexual characteristics in amphibians and noted that many develop during the breeding season (Darwin, 1871), thus implicating the role of sex steroids in their development. Shine (1979) summarized the patterns of sexual dimorphism in amphibians, particularly those associated with sexual selection. In this section we review the hormonal basis of these traits, which represent a small subset of all dimorphic characteristics.

There are other traits, however, that arise at sexual maturity and are not primary or secondary sexual characteristics. We introduce the term ‘secondary characteristics’ to refer to traits that arise at sexual maturity, yet that are not necessarily sexually dimorphic. Secondary characteristics include, as a subset, secondary sexual characteristics, which are traits that arise at sexual maturity and are sexually dimorphic. An example of a monomorphic secondary characteristic is the adult coloration of males and females of the African frog, *Hyperolius viridiflavus*. The adult phenotype is different from the juvenile color pattern, yet is not sexually dimorphic. In one of the few studies to address the hormonal basis of a monomorphic secondary characteristic, Hayes (1997) showed that both T and E2 induce the adult color pattern in this sexually monochromatic frog. The Harderian gland in frogs is another example of a secondary characteristic; it is sexually dimorphic in mammals but is not dimorphic in frogs. The presence of androgen receptors in both males and females explains the lack of dimorphism in frogs (D’Istria et al., 1991; Chieffi-Baccari et al., 1993). Some secondary characteristics are behavioral; in some species of ranid frog, females as well as males produce mating vocalizations (Emerson & Boyd, 1999).

Although the examples above are not traditional secondary sexual characteristics because they are not sexually dimorphic, they do develop as a consequence of sexual maturation. By identifying these traits with a specific term, we acknowledge their derived status from the juvenile condition. Because of sex steroid differences in males and females, dimorphism or secondary sexual traits are predicted to develop at sexual maturity. Thus, the intriguing question with secondary characteristics is, ‘Why are they *not* dimorphic?’ One potential hormonal mechanism for monomorphic secondary characteristics is high levels of androgens in females (Staub & De Beer, 1997).
3.1. Amphibian Skin

Because sex steroid receptors are present in the integument, D’Istria, Delrio, and Chieffi (1975) suggested that the entire integument of amphibians be considered a secondary characteristic. They collected adult males of *Rana esculenta* and *Triturus cristatus* in breeding condition, castrated them, and treated them with [3H]-T, [3H] E2, [3H]-estrone, or [3H]-P4. In *R. esculenta*, they found receptors for [3H]-T whereas, in *T. cristatus*, only receptor sites for [3H]-E2 were observed. No morphological effects were described. In a later study, the thickness of the epidermis, height of granular cells, and diameter of mucous glands were strongly correlated with androgen levels in both males and females (D’Istria, Picilli, Basile, Delrio, & Chieffi, 1982).

The presence of multicellular mucous and serous (granular) exocrine glands in the dermis of metamorphosed skin is a synapomorphy for extant Amphibia (Sever & Houck, 1985). In addition, many amphibians have lipid glands and mixed mucous-serous glands in the dermis, plus specialized glands representing modified mucous or serous glands (Staub & Paladin, 1997; Brizzi, Delfino, & Jantra, 2003). These specialized glands include poison glands used in defense and breeding glands used in social communication and reproduction (Thomas, Tsang, & Licht, 1993; Brizzi et al., 2003; Sever, 2003).

3.2. Anuran Breeding Glands

Thomas et al. (1993) found sexually dimorphic skin glands in 14 frog species representing six families, and described the histology and histochemistry of these glands. The glands were apparently found only in males (although only female *Rana pipiens* were examined). In 12 species these breeding glands were multicellular alveolar glands that stained for neutral mucoproteins. In the other two species, the glands were similar to the protein-secreting granular glands. Specialized mucous glands on the dorsal surface of the glands were similar to the protein-secreting granular stained for neutral mucoproteins. In the other two species, breeding glands were multicellular alveolar glands that cause the venter of the male to adhere to the skin of the female during amplexus. Such glands were first reported in the Microhylidae for *Kaloula conjuncta* from the Philippines by Taylor (1920), who stated that males adhere to females by virtue of a slimy secretion from the belly. Inger (1954) later reported ‘belly glands’ from *Kaloula picta* and *Kaloula rigida*, and noted that they were absent in other members of the genus. Fitch (1956) described adhesion during amplexus of the North American microhylid *Gastrophryne olivacea* and, subsequently, Conaway and Metter (1967) described adhesive glands in *Gastrophryne carolinensis*. Adhesive glands are also known from male *Brevicips* in the south African family Brevicipitidae (Poynton, 1964; Visser, Cei, & Gutierrez, 1982). Because *Brevicips* is not closely related to microhylids, the adhesive glands in that taxon must have evolved independently (Siegel, Sever, Schriever, & Chabarria, 2008). Ultrastructurally, the adhesive glands of *Gastrophryne* appear to be modified mucous glands.

3.2.1. Adhesive glands

Among the more unique types of breeding glands are adhesive glands in the pectoral region of certain male frogs that cause the venter of the male to adhere to the skin of the dorsal of the female during amplexus. Such glands were first reported in the Microhylidae for *Kaloula conjuncta* from the Philippines by Taylor (1920), who stated that males adhere to females by virtue of a slimy secretion from the belly. Inger (1954) later reported ‘belly glands’ from *Kaloula picta* and *Kaloula rigida*, and noted that they were absent in other members of the genus. Fitch (1956) described adhesion during amplexus of the North American microhylid *Gastrophryne olivacea* and, subsequently, Conaway and Metter (1967) described adhesive glands in *Gastrophryne carolinensis*. Adhesive glands are also known from male *Brevicips* in the south African family Brevicipitidae (Poynton, 1964; Visser, Cei, & Gutierrez, 1982). Because *Brevicips* is not closely related to microhylids, the adhesive glands in that taxon must have evolved independently (Siegel, Sever, Schriever, & Chabarria, 2008). Ultrastructurally, the adhesive glands of *Gastrophryne* appear to be modified mucous glands.

The adhesion of bisexual pairs during amplexus in these species has been proposed as an adaptation for protecting a female’s backside from a rival male (Fitch, 1956), keeping pairs together in case of a mating disturbance (Fitch, 1956), helping a male with short arms to stay amplexed to his potential mate (Wager, 1965; Conaway &
Metter, 1967), or causing strong adherence for burrowing into a nesting chamber (Visser et al., 1982).

Metter and Conaway (1969) studied the effects of sex hormones on the development of adhesive glands in both G. carolinensis and G. olivacea. Pellets of 95% T propionate and 5% petroleum jelly and pellets of estradiol benzoate (E2-B) were inserted into dorsal lymph sacs of adult females and juveniles of both sexes, and injections of human CG (hCG) were given to adult females and juvenile males. In addition, adult males were castrated, and their breeding glands underwent full regression in 28 days, whereas mucous and granular glands were unaffected. Adhesive glands developed to an apparently functional state in females and juveniles treated with T after 28 days. 17β-estradiol-B caused no change in glands in any animal tested. Human CG caused some development of adhesive glands in juvenile males, but no treated juvenile males lived longer than 14 days. Human CG produced no effects on females. Metter and Conaway (1969) proposed that the adhesive glands are derived from mucous rather than granular glands, based upon the similarity of regressed and undeveloped adhesive glands to immature mucous glands.

3.3. Nuptial Excrescences of Anurans

A good review of hormonal influences on the glandular nuptial excrescences (‘pads’) occurring in many anurans is presented by Brizzi et al. (2003). These pads are modified patches of skin occurring on the fingers (‘thumb pads’), hands, and/or forearms (‘nuptial pads’) of males in breeding condition. The pads are keratinizations of the skin superficial to large dermal mucous glands. Pads have been implicated in aiding the male to clasp the female during mating (Duellman & Trueb, 1986). Hypertrophy of the pads is associated with annual variation in testicular hormones, and the pads can be induced by treatment with androgens in adult males, adult females, and even tadpoles. For example, testosterone cypionate implants in R. pipiens stimulated thickening of the epidermis and dermis, formation of keratinized papillae, hypertrophy of gland epithelium, and accumulation of secretory product (Lynch & Blackburn, 1995). Androgen receptors in the nuptial pad of Rana chensinensis are positively correlated with development of these secondary sexual characteristics (Yang, Zhang, & Cui, 2005). Androgen receptor mRNA increases in the thumb pad (R. esculenta) following treatment with T (Varriale & Serino, 1994). An extensive literature exists on the effect of androgens on anuran nuptial pads, and Brizzi et al. (2003) give references for 15 species from six families. Duellman and Trueb (1987) state that nuptial excrescences are ‘nearly universal’ in frogs that have amplexus in water and are best developed in stream breeders. The normal role of estrogens in the development and maintenance of nuptial pads and other secondary sexual characteristics is less studied. One study does show that the combination of E2 and T results in greater development of the thumb pad in Rana nigromaculata than treatment with T alone; injection with only E2 had no effect on thumb pad development (Iwasawa & Kobayashi, 1974).

3.4. Tusks/Spines

Duellman and Trueb (1986) provide a review of spines and tusks present in males, and in some females, of many species of anuran. Spines can be prepollical or humeral and are used in male–male combat, although, in some species with spines, no male–male combat has been observed. Tusks on the lower jaw are also a secondary sexual characteristic and are believed to be used for defense of foam nests, for example. Relative to studies on nuptial excrescences, the hormonal basis of these traits is largely unknown.

3.5. Tail Fin and Nuptial Pads of Newts

During the breeding season, males of N. viridescens have increased tail fin height and possess black, keratinized nuptial pads on the hind legs. Females lack these characteristics, and a decline in tail fin height and loss of nuptial pads occurs in males at the end of the mating season or in breeding males conditioned to a laboratory environment of room temperature and long day lengths (Singhas & Dent, 1975).

Singhas and Dent (1975) performed a number of experiments on hormonal control of these characteristics. For tail height, decline was unaffected by thyroidectomy or administration of T on alternate days, but decline was slowed by autografting the pituitary gland and was halted by administration of PRL on alternate days. Tail height of laboratory-conditioned males was restored by administration of PRL over a period of 2–3 weeks; this response was reduced in hypophysectomized males but subsequently restored by additional treatment with thyroid hormone or thyroid-stimulating hormone (TSH). For nuptial pads, decline as in the tail fin was delayed by autografting of the pituitary and administration of prolactin, but in addition by thyroidectomy. T alone did not affect the loss of nuptial pads, but loss was prevented by the combination of T and PRL, and this combination caused restoration of pads in laboratory-conditioned males. In hypophysectomized, laboratory-conditioned males, thyroxine (T4) or TSH was needed in addition to T and PRL to restore nuptial pads. Luteinizing hormone but not P4 effectively substituted for T.

Thus, thyroid hormones play an important role in both tail height and nuptial pad presence, and seem to work synergistically with PRL and T. Singhas and Dent (1975) proposed that the function of thyroid hormone is a passive one and that it acts in a general way to promote the
wellbeing of the animal, perhaps through stimulating sloughing of the skin.

In a later study, Dent (1982) studied the interactions between T4 and oxytocin (OXY) with PRL on the growth of tail fins in N. viridescens. Again, he found that physiological levels of T4 did not antagonize the stimulatory effect of PRL on growth of the adult tail fin. Oxytocin, however, in doses of 100 µU antagonized the stimulatory action of 0.3 U of prolactin. Dent (1982) proposed that the antagonistic interaction between PRL and T4 seen in larvae persists in adult urodeles when omo regulatory action predominates and is lacking in situations in which the primary action of PRL is the stimulation of growth.

Adult male Cynops also possess a broader tail with a well-developed fin as compared to the female. Kikuyama et al. (1986) found that PRL increased tail height and that estrogens blocked the action of PRL in C. pyrrhogaster and C. ensicauda. The antagonism by estrogens may explain the sex difference in the tail morphology of Cynops.

### 3.6. Teeth of Plethodontids

Noble and Pope (1929) noted that the males of ‘most’ plethodontids have premaxillary teeth that are elongate, monocuspid, and directed more or less forward. These teeth are used in the ‘vaccination’ mode of pheromone delivery during courtship (Houck & Arnold, 2003). The male abrades the female’s skin with his premaxillary teeth and rubs secretions from his mental gland into the scraped site. Because this mode of pheromone delivery exists in some members in all lineages of plethodontids, vaccination is considered the ancestral condition in plethodontids. In Desmognathus spp., such teeth can develop in an adult female if a testis is transplanted into the body (Noble & Pope, 1929). Sever (unpublished) induced development of enlarged premaxillary teeth, mental glands, and cirri in females of Euryccea quadrigitidigita injected with T enanthate.

Molecular techniques allow a more mechanicistic description of how these derived monocuspid premaxillary teeth in plethodontids arise. Immunohistological studies on the plethodontid salamander Bolitoglossa schizodactyla show that different dental lamina have differential expression of androgen receptors; androgen receptor expression is limited to the premaxillary lamina (Ehmcke, Wistuba, Clemen, & Schlatt, 2003). The distribution of androgen receptors explains the variation in tooth shape and size among the different tooth-bearing bones as well as explaining the sexual dimorphism in tooth type (Ehmcke et al., 2003).

### 3.7. Muscles

A wide body of literature demonstrates the androgenic basis of sexually dimorphic forelimb (e.g., Regnier & Herrera, 1993) and laryngeal muscles of anurans (e.g., Tobias et al., 1993). Kelley’s pioneering work on the larynx of African clawed frogs (X. laevis) (see Kelley (1986) for a review of early work) has led to an understanding of the androgenic basis of dimorphism in behavior (e.g., Fischer & Kelley, 1991), axon number (e.g., Kelley & Dennison, 1990), and muscle tension, twitch type, and fiber recruitment (e.g., Sassoon et al., 1987; Tobias et al., 1993). Recent work on this model system shows that T can also explain sexual dimorphism in neuron size. When female X. laevis are treated with T, masculinization of laryngeal muscle and motoneuron size is complete after four weeks (Potter et al., 2005).

Another sexually dimorphic characteristic in X. laevis is the synapse strength at laryngeal muscles. Juveniles of both sexes have weak synapses and the strong synapse in laryngeal muscles of mature females is controlled by 17β-estradiol (Tobias & Kelley, 1995). Weak synapses are those in which the pre-synaptic neuron does not typically activate the post-synaptic neuron, and strong synapses are those in which the pre-synaptic neuron typically activates the post-synaptic neuron. 17β-estradiol exerts this effect via receptors in the laryngeal muscles (Wu, Tobias, & Kelley, 2003).

Forelimb muscles are often used by anurans during the breeding season to defend a territory and to grasp a female during amplexus, and some urodeles grasp females during amplexus as well. Sexual dimorphism in forelimb muscle mass is expected with these behaviors. For example, studies on the explosive breeder Rana temporaria show that many aspects of strength and stamina of the extensor carpi radialis muscle (used in clamping) show sexual dimorphism. Muscle length and mass were larger, muscle force was greater, and relaxation times slower in males than in females (Navas & James, 2007). With T injections, forelimb muscles hypertrophied independently of muscle innervation (Thibert, 1986). In R. pipiens, T increased muscle mass in the 22 different muscles of the frog forelimb but did not change the water or protein content (Sidor & Blackburn, 1998). Immunocytochemistry on the flexor carpi radialis muscle in male X. laevis revealed that androgen receptors are present in all forelimb muscles and particularly in those muscles innervated by spinal nerve 2 (Dorlochter et al., 1994). These muscle fibers hypertrophied in response to T administration, whereas muscles innervated by different spinal nerves did not (Dorlochter et al., 1994). Further, studies of the effects of T on synapse efficiency found that T enhances characteristics critical for successful amplexic behavior, such as fatigue resistance (Nagaya & Herrera, 1995).

T can also affect the contractile properties of muscle. When trunk muscles (used for sound production in males) of post-breeding season male Hyla chrysoscelis were treated with T, muscle mass and contractile speed increased to levels found during the breeding season. In females, T
administration had the same effect, increasing the mass and contractile speed of the trunk muscles (Girgenrath & Marsh, 2003).

### 3.8. Vomeronasal Organ

A growing body of literature exists on the vomeronasal organ (VNO) in plethodontid salamanders, which is sexually dimorphic (e.g., Dawley, 1992). The VNO lies in the nasal capsule and detects chemical signals such as pheromones. In male *Plethodon shermani*, the VNO is 1.7 times larger in volume than that of females, despite males being smaller than females (Woodley, 2007). The hormonal basis of this sexual dimorphism is not yet understood. Androgen production at sexual maturity does not explain the sexual dimorphism because subadults show dimorphism as well (Woodley, 2007). In contrast, no dimorphism occurs in the volume of the main olfactory epithelium or in the muscles associated with the naris in *P. shermani* (Woodley, 2007). Little is known about the VNO of frogs and caecilians.

### 3.9. Color

Thirty-two species of anuran are known to be dimorphic in color and relatively little is known about the hormonal basis of color changes (Hoffman & Blouin, 2000). The handful of studies that do address this question show that coloration can be controlled by steroids. In the cricket frog, *Acris gryllus*, T implants caused male secondary sexual characteristics, such as yellow coloration and darkening of the vocal sac, to develop (Greenberg, 1942). T and P₄ administration to *R. pipiens* darkened the skin by dispersing melanosomes within melanophores (Himes & Hadley, 1971). In the reedfrog *Hyperolius argus*, mature males retain the solid green color of juveniles and develop a vocal sac, whereas mature females develop a reddish brown dorsum with white spots (Hayes & Menendez, 1999). In a study examining the effects of steroids in both primary and secondary sexual differentiation, T induced vocal sac development and E₂-induced dorsal color change in this species (Hayes & Menendez, 1999). In contrast, both T and E₂ induced the adult color pattern in the sexually monochromatic frog *H. viridiflavus* (Hayes, 1997).

#### 3.10.1. Caudal glands in the middorsal tail base

Hypertrophy of granular and/or mucous glands in the middorsal tail base has been reported in a number of salamanders. In *Ambystoma gracile* (Ambystomatidae), enlarged granular glands along the tail ridge are not sexually dimorphic and their toxic secretions are used in defense (Brodie & Gibson, 1969). In *Ambystoma maculatum*, however, glands in this area become hypertrophied in both males and females when used for nutrient storage (Williams & Larsen, 1986).

Hypertrophied granular glands in the middorsal tail base have been reported in both males and females of European plethodontid *Hydromantes* spp. (Brizzi, Calloni, & Delfino, 1991). These caudal glands in *Hydromantes* may be defensive because they do not vary seasonally (Brizzi et al., 1991). In some other plethodontids, glands in this area that hypertrophy during the breeding season have been reported only in males. These caudal courtship glands have been reported in species of *Desmognathus, Eurycea*, and *Plethodon* (Noble, 1929; 1931; Newman, 1954; Sever, 1989; Truth, Smith, Cheng, & Daniel, 1993; Houck & Sever, 1994; Mary & Truth, 2006) and probably occur widely in the Plethodontidae. Histochemically, the staining characteristics of caudal courtship glands are similar to mental glands, indicating the presence of a mucoprotein or glycoprotein (Sever, 1989; Truth et al., 1993). However, caudal courtship glands cytologically resemble granular glands whereas mental glands are modified mucous glands (Sever, 1976b; 1989).

The caudal courtship glands apparently function during courtship in the tail straddle walk, a synapomorphy for the Plethodontidae (Houck & Sever, 1994). During the tail straddle walk, the female’s chin is placed directly over the caudal courtship glands, so gland secretions are received by the distal ends of the female’s nasolabial grooves (Noble, 1929; Arnold, 1977). Caudal courtship pheromones presumably increase the likelihood that the female will remain with the male during tail straddling, and therefore increase the chances for insemination (Houck & Sever, 1994).

Males of the west Asian salamandrids *Mertensiella caucasica* and *Salamandra luschani* possess a dorsal tail tubercle that may be 3.5 mm long and 2.3 mm high in an animal of 65–70 mm snout-to-vent length (Sever, Sparreboom, & Schultschik, 1997). Thus, the dorsal tubercle is a grossly distinct structure and remains so independently of breeding condition (Klewen, 1988). Histological and molecular evidence suggest that the dorsal tail tubercles are not homologous in *M. caucasica* and *S. luschani* and result from convergent evolution. In *M. caucasica*, the tubercle consists primarily of elongate mucous glands, with granular glands occurring only at the base. In *S. luschani*, mucous glands and granular glands occur
throughout the tubercle. Female *S. luschani* have a slight tubercle as well (Staub, Palmer, Carnes, Quitiquit, & Susantio, 2005).

The role of the dorsal tubercle in courtship in the two species is similar. The dorsal tubercle acts as a pseudospermatophore that is inserted and then withdrawn when the female is about ready to pick up the real spermatophore (Rehberg, 1981; Klewen, 1988; Schultschik, 1994). Apart from a mechanical stimulus and maneuvering device, the caudal tubercle may additionally provide a specific chemical stimulus, since it contains glands with specific types of secretion (Sever et al., 1997).

Work on the hormonal control of the diverse caudal glands described above is sorely lacking.

3.10.2. Caudal glands in the midventral tail base

In the plethodontid *Aneides lugubris*, modified granular glands in this area are more hypertrophied in males than females (Staub & Paladin, 1997). The enlarged glands of males seem similar in appearance and histochemistry (e.g., positive with the periodic acid and Schiff’s reagent procedure, PAS+) to middorsal caudal courtship glands, but their function is unknown (Staub & Paladin, 1997). In *Plethodon cinereus*, enlarged granular glands (PAS−) in this area are used in scent-marking and are not sexually dimorphic (Simons & Felgenhauer, 1992; Jaeger & Gabor, 1993). Once again, hormonal studies on these glands are needed.

3.10.3. Cloacal glands that secrete onto epidermal areas

The cloacal gland complexes were discussed in Section 2 but need to be mentioned here because the dorsal glands and vent glands of males of many taxa secrete onto the epidermis surrounding the posterior end of the cloaca rather than into the cloaca itself or in addition to sites within the cloaca (Sever, 1991a). In the Salamandridae, secretion onto the epidermis external to the cloaca is restricted to terrestrial breeders (Brizzi, Delfino, & Jiantra, 1996). In the Plethodontidae, these glands are rather reduced compared to those in other families (Sever, 1994). Males of *Rhyacotriton olympicus* (Rhyacotritonidae) have greatly enlarged vent glands that secrete through pores lateral to the cloacal orifice (Sever, 1988). Specialized male dorsal or vent glands are secondarily lacking in some taxa, and this loss is often associated with neoteny or derived courtship patterns (Sever, 1991a; Brizzi, Calloni, Delfino, & Tanteri, 1995; Brizzi, Delfino, Rebelo, & Sever, 1999). As with other cloacal glands, these glands are controlled by gonadal steroid hormones.

3.10.4. Genial glands

Genial glands are an autapomorphy for the salamandrid genus *Notophthalmus* (Salamandridae). Genial glands are found at the base of three or four shallow invaginations (‘pits’) posterior to the eye (Hilton, 1902; Rogoff, 1927). The term ‘genial’ is from the Latin root *gena*, meaning the cheek or chin. At the base of the pits are numerous acinar glands consisting in the active condition of cuboidal epithelial cells that contain a colloidal eosinophilic substance.

Pool and Dent (1977) studied the hormonal control of the genial glands of *N. viridescens*. Both males and females have genial glands, which are similar in structure and secretory activity, but females only have about a third as many glands as males. In other species in the genus (*Notophthalmus meridionalis* and *Notophthalmus perstriatus*), genial glands have only been reported in males (Mecham, 1967; 1968). When newts are in breeding condition, the genial glands produce large quantities of a glycoproteinaceous product, and secretory cells become quiescent during the non-breeding season (summer).

In gonadectomized male newts with quiescent glands, the glands remain unchanged after treatment with saline, prolactin, or T, but are transformed to the breeding state by treatment with PRL and T in combination (Pool & Dent, 1977). Genial glands from ovariectomized females respond similarly to PRL in combination with T, but fail to respond to PRL with E2. Thus, females also require a steroid plus PRL to induce hypertrophy of the genial glands, but that steroid is not E2. Pool and Dent (1977) proposed that the T used in their experiments served as a substrate for formation of a steroid that is active in the female newt. Perhaps estrogens in a form other than E2 could be as effective as T in stimulating the activity of female genial glands.

3.10.5. Mental glands

Sexually dimorphic chin glands have been reported in males of one species in the Salamandridae and occur widely in the Plethodontidae. During courtship in the salamandrid *T. torosa*, the male restrains the female by dorsal amplexus, during which he clasps the female with both his forelimbs and hindlimbs. The male slides forward so that his submandibular area can be rubbed across the female’s snout (Davis & Twitty, 1964; Arnold, 1972). According to Smith (1941), the male is applying substances from specialized skin glands that can be distinguished from mucous and granular glands by the nature of their secretions. These specialized submandibular glands are absent in females of *T. torosa*. Smith (1941) speculated that males of *Taricha rivularis* would also have specialized chin glands because of similarities in amplexical behavior with *T. torosa*. 
Outside of the breeding season, the specialized courtship glands are difficult to distinguish from mucous or granular glands (Smith, 1941). Although Smith’s (1941) observations of unique submandibular glands in male Taricha are interesting, they have not subsequently been verified. If such glands exist, they certainly have been independently derived within the genus.

Specialized chin glands occur in males of many of the Plethodontidae but are lacking in some representatives of each clade (Houck & Sever, 1994). These glands are often referred to in the older literature as ‘mental hedonic glands’ (see Sever (1976a) for a review). Because the designation ‘hedonic’ refers to pleasure-giving, Arnold (1977) suggested avoidance of the judgmental term, and these chin glands are now referred to as ‘mental courtship glands’ or simply ‘mental glands’ (Houck & Sever, 1994), or sometimes as ‘submandibular glands’ (Rollmann, Houck, & Feldhoff, 1999). The term ‘mental’ is from the Latin mentum, meaning the chin. The first good anatomical study of these glands was by Noble (1927), and notable subsequent studies include those by Weichert (1945), Truffelli (1954), Lanza (1959), and Sever (1976a).

Generally, the glands are either in a pad of short, dorsoventrally oriented tubules that secrete over a small to large area of the skin of the lower jaw, or the glands form a fan-shaped cluster, antero-posteriorly oriented, of short (desmognathines) or long (many Eurycea and Oedipina) tubules that secrete at the apex of the lower jaw. In those species in which the nature of the secretions has been analyzed, the secretions have been shown to contain glycoproteins (Houck & Sever 1994; Feldhoff, Rollmann, & Houck, 1999). In P. shermani, a 22-kd protein called plethodontid receptive factor (PRF) has been isolated and shown to increase female receptivity during courtship (Rollman et al., 1999). Variation in PRF among populations of P. shermani has been demonstrated (Rollmann, Houck, & Feldhoff, 2000).

Sever (1976b) reported that cirri and mental glands can develop in female E. quadridigitata injected 2–4 times with 0.1 ml T enanthate over 28 days. The individual glands of the mental glands that formed on the females were larger in size but fewer in number than those found in males of similar size. The induced mental glands were derived from dermal mucous glands, the only glands otherwise found in the dermis of the lower jaw of females.

3.10.6. Nasolabial glands (cirri)

Members of the Plethodontidae possess clusters of glands in the lateral nasal region that are distinct from the more medial intermaxillary glands (Whipple 1906; Seifert 1937). These glands are called the nasolabial glands and they secrete alongside a groove that passes from the external naris to the edge of the upper lip. The nasolabial glands and the nasolabial groove are autapomorphies for the Plethodontidae. When a plethodontid taps its snout to the substrate, capillary action transfers aqueous odor solutions from the tip of the labia into the nasal cavity, where the solutions are delivered to the VNO (Brown 1968; Dawley 1998).

During the breeding season in males of many plethodontid species, hypertrophy of the nasolabial glands results in protrusions below the upper lip called cirri. A cirrus, therefore, is composed of hypertrophied nasolabial glands and incorporates the nasolabial groove (Sever, 1975; 1980). In some Eurycea, the growth into elongate cirri is especially prominent. The differential hypertrophy of nasolabial glands in males is thought to aid in the detection of pheromones used to locate potential mates (Sever 1980; Dawley 1992; Dawley & Crowder, 1995). For hormonal control, see Section 3.10.5: Sever (1976) induced growth of mental glands and cirri in females of E. quadridigitata by 2–4 injections of 0.1 ml T enanthate over 28 days. As in males, the nasolabial groove continued onto the naris. The only difference in external appearance of the cirri of males and the induced cirri of females was that the cirri of females were usually wider than those of males.

3.10.7. Skin glands of Taricha

In male Taricha (Salamandridae), an overall cutaneous hypertrophy occurs during the aquatic breeding stage (Halliday, 1998). Whether just one type of skin gland or all types are involved has not been determined. In Hynobius nigrescens (Hynobiidae), cutaneous hypertrophy associated with aquatic breeding occurs in both males and females and apparently involves only the serous glands (Hasumi & Iwasawa, 1990). The structural changes from the terrestrial type skin to the aquatic type skin are induced by PRL (Dent, 1975).

4. FUTURE WORK

As indicated in our preceding review, we know little or nothing about hormonal involvement in the development and function of various sex accessory structures (i.e., oviductal sperm storage tubules of Ascaphus, the uterus of viviparous amphibians, the penis of Ascaphus, etc.) and secondary sexual characteristics (i.e., tusks and spines, the VNO, various salamander courtship glands, etc.). Thus, much basic research is still needed, and, indeed, we cannot claim that hormonal control of a sex accessory structure or secondary characteristic of any amphibian has been completely elucidated.

Conducting studies in a phylogenetic context is important to fully understand the role of hormonal
mechanisms in the evolution of sex accessory structures and secondary characteristics. For example, a group of southeast Asian frogs (e.g., the Rana blythi species group) have evolutionarily lost many of the typical anuran secondary sexual characteristics; they have no vocal sacs, no advertisement call, no enlarged forelimbs, and no nuptial pads (Emerson, Rowsomitt, & Hess, 1993; Emerson, 1996). These changes are associated with low androgen levels that seem to have allowed the evolution of derived male parental care. As another example, Hayes (1997) argues that the difference in color pattern between two species of Hyperolius (one being sexually dimorphic and the other not) is a result of constraints due to the underlying hormonal basis of color change in these two species. We encourage the use of phylogenies for interpreting the evolutionary context of variation in hormone action among species.

Mechanistic studies examining the role of steroids on nervous tissue during sexual maturation will help to explain the development of dimorphism and monomorphism in secondary characteristics. The rapid (non-genomic) effects of steroids and their potential influence on secondary characteristics have not been studied. For example, E2 affects ion transport in frog skin (Harvey, Alzamora, Healy, Renard, & Doolan, 2002) and this could potentially have dimorphic consequences.

Especially missing from this review are studies on the hormonal control of the sex accessory structures and secondary characteristics of caecilians. Head size dimorphism (Teodecki, Brodie, Formanowicz, & Nussbaum, 2008) and sexual differences in growth rates (Kupfer, Kramer, & Himstedt, 2004) have been reported, but the hormonal bases of these traits are not known.

Finally, more studies are needed that investigate the role of estrogens in males and androgens in females, and the synergistic effects of these hormones on secondary characteristics in both sexes. Exploring the hormonal basis of secondary sexual characteristics and more generally of secondary characteristics will prove to be a rich and rewarding enterprise.

**REFERENCES**


Chapter 5  Hormones, Sex Accessory Structures, and Secondary Sexual Characteristics in Amphibians


