Review Article

Vertebrate hormones in insects: the role of estrogen in silkworm – a review

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Abstract: The presence of vertebrate hormones in invertebrates is a well-established fact. Their physiological roles are still far from clear in various invertebrate systems including insects. This review provides some positive evidence toward the establishment of the biological role of vertebrate hormones in silkworm, *Bombyx mori* Linnaeus, 1758, with special emphasis on sex steroids.

Key words: Insect, vertebrate hormones, estrogen, silkworm

1. Introduction
Hormones are signaling molecules that carry messages for various physiological functions and their structures have been conserved during the passage of evolution. In other words, a similar type of signaling molecule is found in different types of organisms like vertebrates and invertebrates. However, this does not mean that all the functions of those molecules remain equally conserved in these two groups of animals. Similar hormones might have different functions in different organisms. The presence of similar hormones in vertebrates and invertebrates has long been questioned. Investigations over the years have suggested that various vertebrate hormones have different functional roles in invertebrates including insects.

This review provides a brief outline of the basic biological roles of vertebrate hormones in insect systems, and especially in silkworm, *Bombyx mori* Linnaeus, 1758 (Lepidoptera: Bombycidae).

2. Vertebrate hormones in insects
A number of invertebrates including insects have been examined for the presence of vertebrate hormone-like materials. Bombyxin is a brain secretory peptide of the silkworm, *B. mori*, which was first identified as an insulin-related peptide of invertebrate origin (Nagasawa et al., 1984). Hemolymph from the tobacco hornworm, *Manduca sexta* Linnaeus, 1763 (Lepidoptera: Sphingidae), was examined for insulin-like, glucagon-like, and gastrin-like peptides. Several immunoreactive components similar in size to vertebrate insulin and glucagon were separated by gel filtration of acid extracts of larval and pupal hemolymph (Kramer et al., 1980). Insulin-like peptide present in *M.*

sexta was found to be very similar to vertebrate insulin in solubility, chromatographic, immunological, and biological properties. A similar peptide obtained from *Apis mellifera* Linnaeus, 1761 (Hymenoptera: Apidae) had similar properties to vertebrate insulin. These results demonstrated that insect insulin-like peptide and vertebrate insulin are structurally similar (Kramer et al., 1982).

Many reports focused on the identification and isolation of vertebrate-type steroids present in invertebrates (Lehoux and Sandor, 1970; Sandor and Mehdi, 1979; Sandor, 1980; De Loof and De Clerck, 1986; Denlinger et al., 1987; Bradbrook et al., 1990). Testosterone- and progesterone-like substances have been detected by RIA in chromatographed extracts of hemolymph from larvae of *Sarcophaga bullata* Parker, 1916 (Diptera: Sarcophagidae). Detections of estrogen- and androgen-like substances in *S. bullata*, *Periplaneta americana* Linnaeus, 1758 (Blattodae: Blattidae), *M. sexta*, *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae), and *Oncopeltus fasciatus* Dallas, 1852 (Hemiptera: Lygaeidae) led to further investigations of this aspect (Mechoulam et al., 1984). The presence of estradiol was demonstrated in the ovary (Ohnishi et al., 1985), in hemolymph (Das and Ray, 2007), in the posterior silk gland (Keshan and Ray, 2001), and in the larval and pupal fat body of *B. mori* (De, 2007; Roy, 2007).

Materials immunologically related to luteinizing hormone (LH), follicle-stimulating hormone (FSH), and gonadotropin-releasing hormone (GnRH) were identified in neural tissues of *Locusta migratoria* Linnaeus, 1758 (Orthoptera: Acrididae) and *S. bullata* by means of

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the peroxidase-antiperoxidase method, where several polyclonal antisera and a monoclonal antiserum were used in such studies (Theunis et al., 1989). Pimentel et al. (1996) showed that insulin is a growth factor for embryonic *Drosophila* neural cells and remains present in the embryo after neurogenesis. They also suggested its potential hormonal role in *Drosophila* and other insects. Different kinds of vertebrate hormone-like substances, including insulin- and glucagon-like peptides, adipokinetic hormone, somatostatin-like peptides, gastrin, cholecystokinin-like peptides, vasopressin, endorphin, and encephalin, have been isolated from various insect species and also from other invertebrates (Duve et al., 1979; Remy et al., 1979). Van Noorden and Falkmer (1980) described the presence of somatostatin in five species of insects and seven species of other invertebrates as a phylogenetically old hormone since it was detected in animals belonging to the protostomian evolutionary line (Arthropoda, Mollusca, and Annelida). Their findings indicate that the vertebrate hormones might have originated in neural tissue before the development of the vertebrate line of evolution.

3. Role of vertebrate hormones in insects

In insects, evidence for the involvement of vertebrate steroids in the control of reproduction is scanty. In oviparous vertebrates estrogen induces synthesis of vitellogenin protein (oocyte-maturing egg yolk precursor protein) in the liver. Experimental data obtained from invertebrates mostly favor the idea that estrogen might play a role in vitellogenesis (Novak and Lambert, 1989). The first sets of reports involving vertebrate steroids in an insect were made about 80 years ago using extracts of moth from Java (*Attacus atlas* Linnaeus, 1758; Lepidoptera: Saturniidae); it caused estrus in castrated mice (Steidle, 1930; Loewe, 1931; Loewe et al., 1932). Choudhury (1986) demonstrated the role of thyroxine as a stimulating influence on various physiological processes in silkworm, *B. mori*. Reddy et al. (1996) demonstrated that the fat body ATPases were influenced by thyroxine in all the stages of silkworm in a dose-dependent manner. Kim et al. (1999) described that a vertebrate hormone, L-3,5,3′-triiodothyronine (T_3), induces volume reduction in the follicle cells of *L. migratoria* and *Rhodnius prolixus* Stål, 1859 (Hemiptera: Reduviidae), and their findings strongly suggest that T_3 and JH act via the same receptor in follicle cells. Estrogen-induced effect on NAD^+ and $NADP^+$-linked enzyme systems and protein content of fat body cells strongly supported the steroid hormone effect on early moth eclosion in *Antheraea mylitta* Drury, 1773 (Lepidoptera: Saturniidae) (wild tasar silkworm) by triggering tissue metabolism in advance for pupal–adult transformation, thereby proving the controlling of the diapause regulatory mechanism in this insect (Chaudhuri

et al., 2000). In addition, a dose-dependent enhancement in malic enzyme (malate dehydrogenase) activity by E_2 treatment indicates possible receptor-mediated hormonal action associated with receptor saturation and physiologic response in the fat body cells of this insect (Chaudhuri et al., 2000).

Bhakthan and Gilbert (1968) demonstrated several vertebrate adipokinetic hormones and their effects on the release of lipids from the insect fat body. In their experiment, ACTH was found to be without effect. They also showed inhibition of the release of diglycerides and free fatty acids by insulin from the pupal fat body of *Hyalophora cecropia* Linnaeus, 1758 (Lepidoptera: Saturniidae), an effect analogous to that exerted on vertebrate adipose tissue. Growth hormone, gonadotropin (HCG), and thyroxine exerted adipokinetic effects on the release of diglycerides and free fatty acids. Epinephrine greatly stimulated the release of free fatty acids from the fat body of four species of cockroaches. In the case of the fat body of pupal or adult *H. cecropia*, epinephrine also stimulated the rate of free fatty acid release, but it had a dual effect in that it inhibited the release of diglycerides. Thus, increase of lipolysis in the cockroach fat body in the presence of epinephrine may be mediated by cyclic 3′,5′-AMP (Bhakthan and Gilbert, 1968).

The effect of feeding mulberry leaves treated with thyroid powder on larval growth and cocoon formation was analyzed in *B. mori* and it was suggested that thyroxine affects protein metabolism in this insect (Thyagaraja et al., 1991). The studies of Reddy et al. (1996) indicated that thyroxine has a controlling influence on the ATPase system in the silkworm fat body. Thyagaraja et al. (1993) showed that feeding thyroxine to larvae of the bivoltine silkworm *B. mori* resulted in increased pupal hemolymph protein and ecdysteroid levels. It also increased yolk deposition and accelerated oocyte maturation. Due to early yolk deposition, mature oocytes were more numerous than vitellogenic oocytes in the ovarian tubules of thyroxine-treated insects. They also showed that oocyte size became significantly larger following the thyroxine treatment. Pupal–adult eclosion was accelerated in this situation. Thyagaraja et al. (1993) suggested that the vertebrate hormone thyroxine affects insect development and maturation through alterations in ecdysteroid patterns. Davey (2000) demonstrated that thyroid hormones ingested by locusts have the potential to be used as hormonal signals in the control of egg production.

Exogenous steroids have been implicated in the control of reproduction in the rabbit flea, *Spilopsyllus cuniculi* Dale, 1878 (Siphonoptera: Pulicidae) (Rothschild and Ford, 1964, 1966). Reproduction in this insect takes place in synchrony with that of its mammalian host and it seems to be controlled by the steroid hormones

available from the host. Estrogen and corticoids from pregnant mammalian blood triggers vitellogenesis in the flea, whereas progesterone-rich postpartum blood induces ovary regression. It is noteworthy once again that 'heterophyllic' effects of steroid hormones are better demonstrated in a host–parasite interrelationship in a number of animals including in echinoderms, where an estrogen/progesterone antagonism is observed (Lafont, 1991). Allelochemical functions have been described in dytiscid beetles. Amounts in milligram ranges of testosterone, deoxy-corticosterone, and a wide array of C21 steroids are produced and secreted by the prothoracic glands of these beetles. These compounds are thought to protect these insects against various predators (Lafont, 1991). The natural existence of 'nonecdysteroid' steroids was reported in many insects (Lafont, 1991). One group of workers represented the steroids found in the defensive systems of beetles as mostly of the nonvertebrate type, and another group suggested that steroids are of the 'vertebrate-type' and are usually found in the hemolymph, gonads, or whole-body extracts in insects. If such molecules play a hormonal role, one can expect a binding factor for these compounds. In this respect Paesen et al. (1988) searched the binding factors for several steroid classes and they were able to demonstrate the presence of a pregnenolone binding factor in the copulatory organ of the locust. An induction of vitellogenesis in insects by ecdysone was demonstrated for *D. melanogaster* and for milkweed bug, *O. fasciatus* (Rankin and Jäckle, 1980; Jowett and Postlethwait, 1981). After topical treatment or injections of steroids (progesterone, testosterone, pregnenolone, estrone estradiol) to insects the effect on vitellogenesis could not be demonstrated in males or females. There only exists a report concerning possible other functions of vertebrate steroids in insects. It has also been demonstrated that there might be a defensive role of steroids in dytiscid water beetles and in the carrion beetle, *Silpha americana* Linnaeus, 1758 (Coleoptera: Silphidae) (Meinwald et al., 1985). Gawienowski et al. (1987) showed an increase in the growth rate of *O. fasciatus* after application of cortisol. Induction of Vg protein in males by transplanted oocytes was described much earlier in *Diploptera punctata* Eschscholtz, 1822 (Blattodea: Blaberidae); *Pieris brassicae* Linnaeus, 1758 (Lepidoptera: Pieridae); and *Aedes aegypti* Linnaeus, 1762 (Diptera: Culicidae) (Hagedorn and Fallon, 1973; Karlinsky and Lamy, 1976; Mundall et al., 1979). Paesen et al. (1988) demonstrated cytosolic binding proteins for pregnenolone in spermathecal cells of *L. migratoria*. Yang et al. (2010) confirmed that female silkworm powder extracts have significant estrogenic effects in mice.

Drosophila contains an insulin receptor homologue, encoded by the *inr* gene located at position 93E4-5 on the third chromosome. The receptor protein is strikingly homologous to the human insulin receptor, suggesting a conserved function for this growth factor family in the regulation of growth and body size (Chen et al., 1996).

4. Biological role of vertebrate hormones in silkworms

The physiological function of estrogens of egg-laying vertebrates in the induction of vitellogenin synthesis led to the recently introduced concept that the endocrine systems of vertebrates and insects might have more elements in common than generally assumed. Ogiso and Ohnishi (1986) failed to demonstrate a biological function of estradiol in *B. mori*, but they predicted such function of estradiol in the silkworm system. In the insects so far investigated, there is no clear-cut relationship found between sex and steroid distribution, as males may contain higher amounts of estrogens and lower amounts of androgens than females (Mechoulam et al., 1984). Those amounts indeed undergo large changes during development, but hardly any conclusion could be drawn from the available data. It has been demonstrated that the *Bombyx* ovary might have the capacity to synthesize and accumulate vertebrate steroid hormones. It has been recently suggested that steroid hormones in both vertebrates and invertebrates may play a role in calcium homeostasis because calcium has a role in all cell types; the effect of E_2 could be cell-specific and not necessarily linked to the classical concept of reproduction in female system (De Loof et al., 2014; De Loof, 2015). The presence of sex steroids in invertebrates has been studied for many years and other findings can demonstrate the role of estrogen in the alteration of female-specific protein in the hemolymph and ovary in *B. mori* (Das and Ray, 2014a). Several other estradiol-17β-induced changes in the metabolism in the posterior silk gland (Keshan and Ray, 1998, 2001), pupal fat body, and ovary (Das and Ray, 1996; De, 2007; Roy, 2007) have been demonstrated. Only one type of specific [³H]estradiol-17β binding site in the posterior silk gland, pupal ovary, and fat body of *B. mori* has been identified and the magnitude of 3H binding could be correlated with the changes in some metabolic enzyme activities in these organs (Keshan and Ray, 2001; De, 2007; Roy, 2007).

The high activities of steroid-metabolizing enzymes like 17β-hydroxysteroid dehydrogenase (17β-HSD) and Δ^5,3β-hydroxysteroid dehydrogenase (Δ^5,3β-HSD) have been detected in pupal ovaries with appreciable levels of estradiol-17β (Das and Ray, 2007). Such an occurrence indicates the endogenous origin of estrogen in the ovary (Das and Ray, 2007). Unless 17α-hydroxylase and aromatase enzyme activities are detected in the pupal ovary, the biosynthesis of estradiol-17β cannot be confirmed in *B. mori*. Findings of Keshan and Ray (2001) demonstrated that E_2 action on the silk gland might be a

receptor-mediated phenomenon. They also described the specific effects of estradiol on silk gland function, fat body, and ovary as nuclear-mediated phenomena (Keshan and Ray, 2001; De, 2007; Roy, 2007).

A recent publication of Das and Ray (2014b) suggested that E_2 treatment significantly augmented the 5th instar larval life span or, more technically, the initiation of mean spinning time. The maximum reduction of the initiation of spinning time was found to be 2 days after E_2 treatment. The effect was very similar to that found with application of ecdysone or 20-hydroxyecdysone as reported earlier by other workers (Chou and Lu, 1980). Ecdysone, when present in high concentrations, might function as an androgenic sex steroid. It is also the precursor of 20-OH-ecdysone, which is the molting hormone of insects, and the counterpart of estrogens in vitellogenic females of many species, as well (De Loof and Huybrechts, 1998). Whether estrogen acts through its conversion to ecdysone or alone as gonadotropin is a matter for further investigation. It might also be possible that treatment of E_2 stimulates the brain, which also regulates the synthesis and release of prothoracicotropic hormone (PTTH) and thus stimulates the activity of the prothoracic gland for secretion of ecdysone for the shortening of the larval period. Chawna et al. (2007) demonstrated the shortening of larval duration after the application of phytoestrogen in *Pueraria mirifica* Airy Shaw & Suvat. (Fabales: Fabaceae) in their study; however, the spinning of larvae has not been synchronized and that was not an economic character of silk production in silkworm in their experiment. Whatever the mechanism of the action of E_2 may be on larval life, the effect of the hormone (E_2) as demonstrated on the larval life of silkworm is a beneficial one (Das and Ray, 2014b). Keshan and Ray (2000) suggested that silkworm larvae have been found to be highly sensitive to exogenous hormones, and the effects of hormone applications are very much dependent on the age of the larvae and the particular dose of the hormone. The unchanged cocoon shell weight (without pupa) with the reduction of the 5th instar larval life span by 2 days clearly guarantee the possibilities of net increase in silk production after E_2 treatment. The unaltered number of eggs laid and unchanged hatchability after various doses of E_2 treatment also ruled out any ill effect of E_2 treatment (Das and Ray, 2014b).

The indication of estradiol-17β-induced alteration of female-specific protein (at 170 and 43 kDa) signifies the possible involvement of this vertebrate sex steroid in the biological processes of silkworm, *B. mori* (Das and Ray, 2014a). A single injection of 2 μg of E_2 per gram of body weight on the 3rd day of the 5th instar larvae female silkworm (*B. mori*, race Nistari) led to more accumulation of both 170- and 43-kDa proteins in the hemolymph from day 3 to 7 of pupal life with a peak on day 7, in comparison to the untreated (control) hemolymph. On the contrary, the ovarian extracts revealed the highest accumulation of both the proteins in female silkworm pupae on day 4, which thereafter declined gradually to match the control level on day 8. The fertilized egg extracts, irrespective of being from control or E_2-treated silkworms, failed to establish quantitative differences of the proteins in question. Recent findings of Shen et al. (2015) confirmed that estradiol treatment by injection or by feeding to male larvae in the final instar stage induced and stimulated male BmVg protein accumulation in hemolymph. They also showed that vertebrate E_2 induced Vg synthesis in a dose-dependent fashion in males. Therefore, it may be concluded that estradiol has a sex hormone-like function, which can induce and maintain female-typical BmVg protein levels in male silkworms. Transplantation of ovaries in male silkworms from 5th instar female silkworms showed further development and egg production in fewer numbers in the male, and also the ovaries contained BmVn (vitellin; Shen et al., 2015). Induction and maintenance of female-typical protein has been shown to be stimulated by exogenous estradiol (Das and Ray, 2014a). The pathway of E_2 action in males might be different from that of female silkworms. These pathways have to be explored in the future.

Shen et al. (2015) also demonstrated that removal of female silkworm ovaries resulted in an increase of BmVg protein in fat bodies and its accumulation in hemolymph. They also confirmed after ovary transplantation from female to male silkworms that E_2 may be synthesized in the ovaries of silkworm. Earlier it was demonstrated that female silkworm pupal powder extracts have significant estrogenic effects in mice (Yang et al., 2010). The presence of E_2 receptors in pupal ovaries is already known (De, 2007).

Hormones play major roles in vertebrate and invertebrate systems. Heterologous hormonal actions have also been investigated for some years. In particular, the actions of vertebrate hormones in invertebrates have created much sensation in regards to the topic of "reverse endocrinology" (Lafont, 1991). Perhaps cholesterol is a very primitive organic material related to life processes. Estradiol, being a cholesterol derivative, may claim its presence across the phylogeny. The vertebrate signaling molecules that are present in invertebrates show an unorganized nature of action and become more and more precise in their functions during the evolutionary ascent to vertebrate nature.

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