1

A brief history of Lepidoptera as model systems

JUDITH H. WILLIS, ADAM S. WILKINS, and
MARIAN R. GOLDSMITH

Introduction

Throughout the twentieth century, the Lepidoptera, especially moths, have played important roles in fundamental studies in physiology, especially endocrinologic phenomena, and in biochemistry. Increasingly, they are also finding use in studies of biological development. Unfortunately, the recent resurgence of *Drosophila melanogaster* as the insect species par excellence for developmental analyses (see Lawrence, 1992) has seemingly eclipsed both past and current work on Lepidoptera. This book is intended, therefore, in part, to act as a counter, even an antidote, to the *Drosophila*-centered view of insect biology. In particular, this introductory chapter is designed both to remind the reader of the special value of the Lepidoptera as model systems and to help preserve our knowledge of key discoveries in this field, discoveries whose general implications extend far beyond the particular organisms under study. The chapters that follow emphasize the streams of contemporary research that have flowed from these beginnings and, we hope, convey the breadth and intrinsic interest of lepidopteran biology today.

In taking stock of the origins of lepidopteran research, one can discern three distinct kinds of initial interest. The first was inspired directly by the beauty and variety of lepidopteran species, in particular, by the variety of wing color patterns. This variety has been and continues to be important in studies of systematics and evolution and in the elaboration of ideas about pattern formation (reviewed in Nijhout, 1991). The detailed analysis of the pigments that decorate Lepidoptera was also important in stimulating research in another direction, in spawning major areas of bio-organic chemistry (reviewed in Kayser, 1985).

The second aspect of Lepidoptera that has favored their use as biological model systems is the large body size of certain species and their amenability to surgical manipulation. Much of what we know about how hormones regulate the lives of all insects had its origins in ligations, transplantations, and parabioses in diverse Lepidoptera. The large size of the caterpillars and adults of certain species has also been significant in many biochemical studies. For instance, most of the insect hemolymph products were first characterized in species of moths with large larvae and pupae that can be bled easily (reviewed in Kanost, Kawooya, Law, Ryan, Van Heusden, and Ziegler, 1990).

The third major impetus to lepidopteran research was economic, and here one can subdivide the kinds of interest into two sorts. On the one hand, several species have direct positive economic value, through their production of silk. The silkworm, *Bombyx mori,* whose cultivation began more than five thousand years ago in China (Kuhn, 1988), is the principal species in this respect. Early work with *Bombyx,* connected to the obviously desirable goals of increasing the quantity and quality of silk, produced some major findings of fundamental import. These included the first identification of microorganisms as a cause of disease, first a fungus in 1835 (Bassi, 1958) and, later, two bacterial species (Pasteur, 1870). In addition, some of the first carefully studied homeotic transformations were reported in *Bombyx* in the late 1930s and early 1940s (Sakata, 1938; Hashimoto, 1941). In fact, the modern genetic analysis of *Bombyx* goes back to the turn of the century (Toyama, 1912; see also Tazima, 1964) and provided some of the first modern accounts of mutations. With respect to fundamental research today, work on silk moths has several major strands: the molecular biology of silk production, whose analysis includes the special transcriptional and translational mechanisms employed by the silk gland; the study of the organization, evolution, and regulation of the large, complex chorion multigene family; the recently begun investigation of homeotic genes and mutant effects at the molecular level; and the combined molecular and genetic analyses of embryogenesis. The development of the genetics of *Bombyx* continues to play a key part in all of this work, and the different facets of gene regulation in the silk glands and the chorion system are described in several of the chapters in this book.

The other, and contrary, economic importance of Lepidoptera concerns their major roles as pests, when they act as consumers of vegetables, orchards, forests, shade trees, and clothing. Our knowledge of pheromone signaling by virgin females arose from the need to identify some vulner-

ability in these remarkably successful pests. One such potential weak spot concerns the sex signaling system by which females attract males (reviewed in Tamaki, 1985), sometimes over considerable distances; the first sex pheromone to be identified chemically was bombykol from *B. mori* (Butenandt, Beckman, Stamm, and Hecker, 1959). This research has led into the detailed characterization of the neural response to pheromones, a subject reviewed by Vogt in this volume. In addition to research strategies based on pheromones, much modern research on lepidopteran control strategies involves recombinant DNA techniques, using baculoviruses, natural insect infectious agents, as vectors. More recently, emphasis in baculovirus research has shifted, in part, toward their employment in basic research on lepidopteran biology. Both of these areas are reviewed by Iatrou in this volume.

In this chapter, we look more closely at the origins of some of these lines of research under, admittedly, somewhat arbitrary headings. As we hope will become apparent, the current molecular studies are not only adding much of direct and central interest to our understanding of particular phenomena but are serving to bring together previously disparate areas, in particular those of hormonal physiology and gene action in development.

Endocrinology

Early endocrinologic studies

One of the pioneers of modern endocrinologic research in Lepidoptera, Carroll Williams, once remarked at the start of his graduate course in insect development that some scientists are problem-driven and select the species most suitable for analysis of their specific problems, whereas others become enamored of a particular species and analyze diverse problems as they are revealed in their favorite model system. Williams himself was probably in the second group. His Ph.D. thesis was on *Drosophila* flight, but most of his research was directed toward solving interesting problems that arose as he studied *Hyalophora cecropia,* though later he also worked on *Manduca sexta.*

Williams's prime research organism, *H. cecropia,* in fact, has had strong attractions for both problem- and organism-centered biologists and was important in some of the earliest grafting experiments to investigate de-

velopmental and physiological problems. Thus, a pioneer series of grafting protocols was carried out on the large pupae of this species by the Harvard biologist H. E. Crampton (1900) to test the suggestions of his colleague, A. G. Mayer (1896), that hemolymph contributed to wing pigmentation. Crampton found that in most instances of interspecies grafts, wing pigmentation was independent of hemolymph composition. Mayer's work, it should be noted, had involved a histological analysis of wing development in several Lepidoptera, including *H. cecropia,* and was focused on the steps involved in producing a patterned wing from an imaginal disc. (Mayer's paper, incidentally, contains a concluding section entitled "Summary of Conclusions Believed to Be New to Science." Would that such conclusions were a requirement of today's journals!)

Though Crampton's procedure was, in principal, a crucial breakthrough in methodology, there is no evidence indicating that his experiments were known to the next worker to employ them. This was the Polish biologist S. Kopec (1922), whose surgical manipulations were conducted on a far smaller species, *Lymantria dispar,* the gypsy moth. Kopec's papers established the field of insect endocrinology and, indeed, also that of invertebrate endocrinology. Kopec described the motivation for his research as a "wish to investigate the relation which probably exists between the nervous system and the processes of metamorphosis in insects." Kopec took the progeny of a single gypsy moth female, ablated their brains or ganglia, placed ligations, transplanted tissues, and studied the resulting effects on molting. Reading his paper (published in English) in the *Biological Bulletin* is a humbling experience. Berta Scharrer (1987) has summarized the impact of these experiments as "not only the first demonstration of an endocrine activity in any invertebrate, but . . . also the first indication anywhere in the animal kingdom that the nervous tissue is capable of producing hormones."

All students of insect endocrinology appreciate that these discoveries were confirmed and elaborated by V. B. Wigglesworth in the bug *Rhodnius prolixus* (Wigglesworth, 1934, 1940). Nevertheless, lepidopterans continued to play a key role in permitting generalizations of Wigglesworth's schemes and in expanding and clarifying key issues. Wigglesworth's discovery of a diffusible factor (i.e., juvenile hormone) that inhibited metamorphosis (Wigglesworth, 1936) was followed quickly by two sets of experiments in *B. mori.* In the first, the French worker J. J. Bounhiol (1938) confirmed Wigglesworth's hypothesis that the corpora allata (CA) are the source of juvenile hormone. He showed that ablation of the CA

from early instar *Bombyx* larvae resulted in premature metamorphosis, an event signaled by the premature spinning of a diminutive cocoon. Inside the cocoon, the tiny pupa metamorphosed into a tiny adult, the first evidence that metamorphosis in holometabolous insects is also influenced by the CA. Ablation, however, provides only half of the proof needed to establish an endocrine source definitively.

The second half was produced in a set of experiments by a Japanese scientist, S. Fukuda (1944), who implanted CA from early instars into a final instar *Bombyx* larva; the host molted to a supernumerary giant larva that subsequently yielded a giant cocoon, giant pupa, and giant moth. (A drawing immortalizing these experiments was subsequently published in *Scientific American* [Williams, 1958], and slides with this figure have since been projected on countless lecture room screens.)

Yet, more than two decades were to pass before the hormonal activity now known as juvenile hormone (JH) was first partially purified, this preparation being described as a "golden oil" (Williams, 1956a). It is a remarkable coincidence that adult males of *H. cecropia,* Williams's favorite experimental insect, have the most abundant source of juvenoids yet discovered. That the first extracts were produced when Williams was visiting Wigglesworth might not appear to be so remarkable, except that Williams was studying longevity in adults at that time, not the control of metamorphosis. His finding set off a fierce competition to purify JH and identify its chemical nature, turning former collaborators into competitors. It was H. Röller, a scientist previously unknown to the American "moth club," who won the race by purifying the active principle from Cecropia and elucidating the structure of what is now designated JH I (Röller, Dahm, Sweely, and Trost, 1967). Röller had been a student of Piepho and had worked on the influence of the CA in adult insects, so he was well qualified to enter the competition. He was also fortunate in his collaboration with the chemist K. H. Dahm, another German émigré. Their publications were followed by the discovery of JH II from *H. cecropia* (Meyer, Schneiderman, Hanzmann, and Ko, 1968); this form of JH was missed by Röller because he had relied on a beetle, *Tenebrio,* for his bioassay (Röller and Bjerke, 1965). *Tenebrio,* as it happens, is not as sensitive to JH II as the lepidopteran (*Galleria*) that Meyer et al. used for their bioassays.

The first suggestion that juvenoids might have a polyisoprenoid structure with an epoxide component was based on theoretical considerations by Bowers, Thompson, and Uebel, (1965). Only a few years later, the

hypothesized compound was isolated from a lepidopteran, *M. sexta,* and christened JH III (Judy, Schooley, Dunham, Hall, Bergot, and Siddall, 1973). It is only within the lepidopterans that a single species appears to synthesize more than one form of juvenoid; indeed, five are now recognized in *Manduca* (Schooley, Baker, Tsai, Miller, and Jamieson, 1984). All other orders of insect tested to date use only JH III, with the exception of flies, which make a *bis*-epoxide of JH III (Richard et al., 1989), and hemipterans, which have JH I (Numata et al., 1992).

The elaborate steps that were first needed to purify JH would not have been necessary had Williams done one additional experiment: to pinpoint the location of JH in *H. cecropia* males. The original isolation was from the abdomens of males, and it took 11 years to separate active juvenoids from abdominal lipids. In 1976, two decades after JH had first been isolated from *H. cecropia* abdomens, Röller's group discovered that all of the juvenoids in the abdomen of the *H. cecropia* male are found in the tiny accessory gland, nicely sequestered from the lipid-rich fat body (Shirk, Dahm, and Röller, 1976). Indeed, we now know that adult male CA secrete JH acid, and that the methyl transferase that converts it to JH is in the accessory gland (Weirich and Culver, 1979). The physiological function of this storage, however, remains obscure; males that are allatectomized as pupae mate normally, and their progeny develop unimpaired (Williams, 1959).

In the 1940s, the ideas of Kopec, who was executed by the Nazis in 1941, were continued by others, resulting in the discovery of ecdysone, the "molting hormone." Fukuda (1940) and Williams (1947) independently showed that molting was initiated by the prothoracic gland. Williams, using isolated abdomens of *H. cecropia* as his assay material, showed that the brain, acting as an endocrine organ, stimulated the prothoracic glands to produce molting hormone. Thus, by the 1950s, the fundamentals of insect endocrinology were established – a brain hormone drives prothoracic glands, and their secretion, in turn, causes molting. The CA control the nature of the molt: When juvenoids are abundant, the molt is larval-to-larval; when their titer is low or absent, the molt is larval-to-pupal or pupal-to-adult.

As with JH, Lepidoptera were the first source of the ecdysteroids, produced by the prothoracic gland. Ecdysone was isolated from 500 kg of male *B. mori* pupae, using a fly bioassay discovered by Frankel, perfected by Baker and Plagg, and exploited with brilliant success by Butenandt and Karlson (1954). (The females from the same shipment of *Bombyx* yielded

the adults that provided the pheromone for Butenandt; see Horn, 1989.) Concern that ecdysone might be no more than a tanning agent (given that is what the assay measured) was dispelled by Williams, who demonstrated that it causes adult development and molting in abdomens isolated from diapausing *H. cecropia* pupae (see Butenandt and Karlson, 1954). It took a further 1,000 kg of *Bombyx* pupae to yield 250 mg of ecdysone, sufficient to provide analytic and crystallographic evidence that at least some invertebrates use steroids as hormones (Huber and Hoppe, 1965; Karlson, Hoffmeister, Hummel, Hocks, and Spiteller, 1965).

Later endocrinologic studies

Williams (1967) speculated that hormones could be third-generation pesticides (following the first generation – kerosene, arsenates, nicotine, and rotenone – and the second – organics such as DDT). He predicted that insects could not mount resistance to hormonally active materials. Even though this prediction proved naive, industry and governments were not deterred from becoming interested in insect biochemistry. Research teams were established to identify hormone analogues, especially those that might have specificity for particular pests. Plants turned out to be repositories of phytoecdysteroids, juvenoids, and antijuvenoids (Bowers, 1985; Horn, 1989). Not surprisingly, much of this work relied on lepidopteran assay systems. Lepidopterans also provided the material and assay system that led to the amino acid sequence for the prothoracicotropic hormone (PTTH), the brain factor that initiates insect endocrinology and triggers each molt (Kawakami et al., 1990).

One of the most useful lepidopteran model systems for endocrinologic studies has proven to be *M. sexta,* although fruitful results have been primarily in basic research rather than insect control. It was Lawrence Gilbert who first recognized its potential to be the "white rat" of insect physiology (see Gilbert, 1989, for review). He learned that *Manduca,* unlike native American silk moths, can be raised in the laboratory on an artificial diet with ease, speed, and considerable synchrony. It has a facultative, photoperiodically controlled pupal diapause.

An instance of the usefulness of *Manduca* for bioassays involved the fortuitous discovery of a few black larvae in the colony that Lynn Riddiford and Carroll Williams were using at Harvard. A stock of these larvae was established and subsequent analyses revealed that this abnormal pigmentation could be prevented if fourth instar larvae were treated with

juvenoids at the time of head capsule slippage. This led to a rapid bioassay for juvenoids (Truman, Riddiford, and Safranek, 1973).

The special feature of *Manduca,* however, which is responsible for its prominence, is that key events in larval development can be timed with great precision. Using classic techniques of ligation, Truman and Riddiford (1974; Truman, Riddiford, and Safranek, 1974; Fain and Riddiford, 1976, and references therein) established the critical periods for release of the major hormones. Their work established that if close attention were paid to rearing conditions, one could predict precisely when PTTH, juvenoids, and ecdysteroids would be released in the final instar.

As techniques became available, hormone titers were measured. Small blips of ecdysteroids found early in the last instar had been seen in radioimmunoassays of many species. It was the synchrony of *Manduca* development, and Riddiford's development and use of protocols to monitor epidermal development with tissue culture and transplantation, that revealed that these blips constituted a "commitment peak." Larval cells become committed to pupal development as judged by their becoming insensitive to juvenoids (Mitsui and Riddiford, 1978; see also Riddiford, this volume).

The programming of metamorphosis has also been studied in *Galleria mellonella,* where it has been shown that allatectomized larvae can bypass the pupal stage and form adult cuticle (Sehnal, 1972). In terms of the now better understood role of ecdysteroids in triggering the transcriptional activation of particular genes (see the section "Molecular studies"), this finding is now less mysterious than it seemed at first. A provocative account of other studies on *Galleria* has been provided by Kumaran (1991).

An important aspect of current endocrinologic studies involves the characterization of particular cellular effects with hormonal changes. One of these phenomena is programmed cell death. Finlayson's (1956) precise account of which muscles break down at adult eclosion in several moths was followed by Lockshin's extensive studies (see Lockshin, 1985, for review) and those of the Truman group (Schwartz and Truman, 1982; see also Truman, this volume). Studies of programmed cell death have culminated in the discovery that baculoviruses produce proteins that curtail apoptosis (a particular scenario of cell death) in infected lepidopteran cells (Clem, Fechheimer, and Miller, 1991).

Diapause and eclosion

Two other hormonally triggered phenomena that have been investigated intensively in Lepidoptera are diapause and eclosion. Lepidoptera, indeed,

were the key subjects for studies that defined diapause in insects, elucidated the basis for entry and exit, and characterized its metabolism. Duclaux, in 1869 (cited in Lees, 1955), recognized that diapausing *Bombyx* embryos would not develop at room temperature unless they had been subjected to a period of chilling. Diapause, as a term, was first applied to grasshopper embryonic movements, but by 1904, was restricted to describe arrested development (Lees, 1955).

The phenomenology of diapause has been studied extensively, with Williams (1956b) defining critical periods for chilling and Danilevskii (1965) providing detailed life histories that reveal the genetic plasticity of photoperiodic response. The cause of arrest in the pupae of *H. cecropia* was pinpointed by Williams (1952) as a quiescence of the brain/prothoracic gland axis, while at the same time the basis of diapause in the embryos of *Bombyx,* which involves the brain and subesophageal gland, was being clarified by Fukuda (1951) and Hasegawa (1951). The first indications, however, that the phenomenon involves an endocrinologic signal came from a much earlier study of Umeya (1926, cited in Denlinger, 1985), who showed that hemolymph transfusions alter the incidence of diapause. The *Bombyx* diapause factor, which leads to embryologic arrest, has recently been purified and sequenced (Imai et al., 1991). The active agent in some larval diapauses was identified as JH when Chippendale and Yin (1973) used ligations and hormone injections with the corn borer, *Diatraea grandiosella,* to show that juvenoids are the principal regulatory agents for entry and maintenance of the diapause state.

Eclosion, the emergence of an insect from the cuticle of the previous stage, is also under hormonal control, but in this instance, the control involves a novel peptide hormone; research on eclosion mechanisms is, in contrast to the other studies, fairly recent. Truman first established that eclosion of silk moths occurs at the appropriate day in development and at a certain photophase when an eclosion hormone is released (Truman and Riddiford, 1970; Truman, 1985). His laboratory shifted to *Manduca* and went on to purify eclosion hormone, obtain its sequence (Marti, Takio, Walsh, Terzi, and Truman, 1987), and isolate and characterize its gene (Horodyski, Riddiford, and Truman, 1989). Other researchers have also begun to work on eclosion hormone, simultaneously obtaining its sequence from *Manduca* (Kataoka, Troetschler, Kramer, Cesarin, and Schooley, 1987) and *Bombyx* (Kono, Nagasawa, Isogai, Fugo, and Suzuki, 1987).

One of the targets of eclosion hormone is the insect's central nervous system (CNS); the molecular events that accompany acquisition of de-

velopmental competence in the CNS to respond to the hormone have also been described (Morton and Truman, 1986). In silk moths that lack valves in their cocoons, eclosion is facilitated by the secretion of a trypsinlike enzyme termed, simply, "cocoonase" (Kafatos and Williams, 1964), which served as one of the early insect models for studying the regulation of gene activity.

Nonendocrinologic physiology: biochemistry of the hemolymph

Although a major function of the hemolymph is the carrying of endocrinologic agents (in addition, of course, to basic nutrients for cell growth and metabolism), it has also proven to be a rich source of other substances. In particular, the characterization of various protein families of biological interest was often first possible in the Lepidoptera because of their large size. Products first isolated in the Lepidoptera were then subsequently sought and found in other insects, including *Drosophila.* One group of note are the vitellogenins, the yolk proteins. Telfer (1954), studying *H. cecropia,* was the first to recognize female-specific proteins in the hemolymph of insects and subsequently showed that the vitellogenins are taken up by the ovaries (Telfer, 1960) by passing between follicle cells (Telfer, 1961).

In addition to the yolk proteins, there are numerous insect storage and transport proteins of significance that were first isolated and characterized in the Lepidoptera. These include the first insect hemolymph protein of known function, apart from hemoglobin, namely, lipoprotein I (Chino, Murakami, and Harashima, 1969); the first methionine-rich storage proteins (Tojo, Betachaku, Ziccardi, and Wyatt, 1978); the recognition of arylophorins as a class of proteins with high content of aromatic amino acids (Telfer, Keim, and Law, 1983); first recognition of flavoproteins with high histidine, associated copper, and no cystine (Telfer and Massey, 1987); the first insect iron-binding proteins (Huebers et al., 1988); and the first insect serine protease inhibitors (serpins) (Kanost, Prasad, and Wells, 1989). For hemolymph biochemistry, the reader should consult reviews by Kanost et al. (1990) and Telfer and Kunkel (1991). The utility of lepidopterans as models for understanding lipid transport is summarized by Law and Wells (1989). Diverse tissues synthesize hemolymph proteins (Palli and Locke, 1987, 1988; Sass, Kiss, and Locke, 1993, 1994).