Chapter 15

Juvenile and Steroid Hormones in *Drosophila melanogaster* Longevity

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I. Introduction

Hormones coordinate diverse developmental and physiological processes and regulate the allocation of metabolic resources to different organs and life-history stages (Finch & Rose, 1995). Many insects display an amazing amount of phenotypic variation in their life histories, which is mediated by the effects of hormones (Dingle & Winchell, 1997; Nijhout, 1994). Juvenile hormone (JH)[1] and the steroid hormone ecdysone (active form: 20-hydroxy-ecdysone, 20E) have fascinating physiological effects on various aspects of development and the adult phenotype. Consequently, the endocrine effects of a single hormone on multiple traits ("hormonal pleiotropy") may offer promising insights into the mechanisms regulating complex phenotypes (Dingle & Winchell, 1997; Ketterson & Nolan, 1992; Zera & Harshman, 2001), including the aging phenotype (Bartke & Lane, 2001; Finch & Rose, 1995; Kenyon, 2001; Tatar, 2004; Tatar *et al.*, 2003).

The study of endocrine aspects of aging is not new. In 1889, Charles-Edouard Brown-Séquard, the "father" of endocrinology, was one of the first to suggest that alterations of endocrine glands or hormone metabolism may be determinants of human aging (Hayflick, 1994). For the case of insects, Meir Paul Pener showed more than 30 years ago that removal of JH extends life span in three grasshopper species (Pener, 1972). While JH and 20E are best known for their major roles in pre-adult development and adult reproduction (Kozlova & Thummel, 2000; Riddiford, 1993, 1994; Truman & Riddiford, 2002;

[1]Abbreviations: aa,. abnormal abdomen; CA, corpus allatum, corpora allata; CC, corpus cardiacum, corpora cardiaca; CNS, central nervous system; DAF, dauer formation; dFOXO, *Drosophila* forkhead transcription factor FOXO; DILPs, Drosophila insulin-like peptides; EcR, ecdysone receptor; 20E, 20-hydroxy-ecdysone; IGF, insulin-like growth factor; InR, insulin-like receptor; IPCs, insulin-producing cells; JH, juvenile hormone; JHA, juvenile hormone analog; JHB_3, juvenile hormone 3 bisepoxide; MET, methoprene-tolerant; MNCs, median neurosecretory cells; USP, ultraspiracle.

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Wyatt & Davey, 1996), these hormones also show intriguing effects on insect longevity. Recent studies have shown that downregulation of JH or 20E can slow senescence (Herman & Tatar, 2001; Simon *et al.*, 2003; Tatar *et al.*, 2001a,b). Here we review hormonal effects on aging in *Drosophila melanogaster* (for a review of the genetics of aging in *Drosophila*, see Stearns & Partridge, 2001; also see Ford & Tower, Chapter 15, this volume). Although we will focus our discussion on *Drosophila,* a model system with both ample genetic and endocrinological data, we will also draw parallels to the endocrine control of aging in other systems (other insects, the nematode *Caenorhabditis elegans*, and vertebrates). We shall argue that studying endocrine regulation can offer promising insights into the mechanisms and the evolution of senescence.

II. JH and 20E: Two Major Insect Hormones

A. JH

The insect hormone JH is a sesquiterpenoid compound produced by the corpora allata (CA), a pair of endocrine glands with nervous connections to the brain (Nijhout, 1994; Tobe & Stay, 1985). In larval *Drosophila*, the single corpus allatum (CA) makes part of the so-called ring gland, a compound structure consisting of the CA and two other endocrine tissues, the prothoracic glands and the corpora cardiaca (CC); in adult flies, the CA is closer to the CC, whereas the prothoracic cells of the ring gland have degenerated (see Bodenstein 1950; Richard *et al.*, 1989; Siegmund & Korge, 2001).

JH is a major insect hormone but may also exist in other arthropods and can be produced by some plant species (Tobe & Bendena, 1999). In most insects, JH regulates critical physiological processes, including metamorphosis and reproduction (Dubrobsky, 2005; Gilbert *et al.*, 2000; Riddiford, 1993). Insects produce at least eight different types of juvenoids (0, I, II, III, 4-Methyl-JH, JH III-bis-epoxide [JHB_3], and the two hydroxy-JH's 8′-OH-JH III and 12′-OH-JH III), the most common type being JH III (Darrouzet *et al.*, 1997; Richard *et al.*, 1989; Riddiford, 1994). *D. melanogaster* produces both JH III and JHB_3, both of which have endocrine function in dipterans (Riddiford, 1993; Yin *et al.*, 1995). Whereas the effects of JH III are better known, JHB_3 appears to be the major product of the CA in higher dipterans (so-called cyclorrhaphan flies, including *Drosophila* and the housefly *Musca domestica*), yet its function awaits further study (Richard *et al.*, 1989; Teal & Gomez-Simuta, 2002; Yin *et al.*, 1995).

In pre-adult development and metamorphosis, JH functions as a "status quo" hormone, allowing continued growth after ecdysteroid-induced molting (Riddiford, 1996). Metamorphosis can only take place when the ecdysteroids act in the absence of JH. During the final larval instar, the JH titer declines due to a cessation of synthesis and increased degradation in the hemolymph and target tissues. Absence of JH triggers the release of prothoracicotropic hormone (PTTH), which in turn induces the secretion of 20E and the onset of metamorphosis (Nijhout, 1994). Although an experimental withdrawal of JH during development can lead to premature metamorphosis, an excess of JH prior to pupation results in delayed metamorphosis (Hammock *et al.*, 1990; Nijhout, 2003; Riddiford, 1985). Unlike in some other insects, application of exogenous JH does not prevent the larval-pupal transformation in *Drosophila* (Riddiford & Ashburner, 1991). However, JH can disrupt the metamorphosis of the nervous and muscular system and disturb the normal differentiation of the abdomen in the fly (Restifo & Wilson, 1998; Riddiford &

Ashburner, 1991). Furthermore, high concentrations of exogenous JH can prolong developmental time or even inhibit eclosion without affecting the larval-pupal transformation (Riddiford & Ashburner, 1991).

In the adult fly, JH is crucial for the coordination of reproductive maturation in both sexes. In females, JH acts on oocyte maturation, including stimulation of vitellogenin synthesis and uptake of vitellogenin by the ovary (Bownes, 1982, 1989; Dubrovsky *et al.*, 2002; Gavin & Williamson, 1976; Postlethwait & Weiser, 1973; Shemshedini & Wilson, 1993), and sexual receptivity (Manning, 1966; Ringo *et al.*, 1991). In contrast, much less is known about the effects of JH on male reproduction. By analogy with other insects, JH may affect protein synthesis in the male accessory glands, sexual maturation, courtship behavior, and pheromone production (Bownes, 1982; Cook, 1973; Manning, 1967; Nijhout, 1994; Teal *et al.*, 2000; Wilson *et al.*, 2003). In other insects, JH can also affect diapause regulation, migratory behavior, wing length polyphenism, horn development in scarab beetles, seasonal form development, locomotory behavior, immune function, caste determination and division of labor in social hymenopterans and isopterans, and learning and memory (Belgacem & Martin, 2002; Hartfelder, 2000; Nijhout, 1994; Riddiford, 1994; Rolff & Siva-Jothy, 2002; Teal *et al.*, 2000; Wyatt & Davey, 1996). Thus, JH is truly a "master" hormone in insects (Hartfelder, 2000; Wheeler & Nijhout, 2003). As we shall discuss in this review, JH also has intriguing effects on insect aging.

B. 20E

Steroid hormones, such as ecdysteroids (including ecdysone and its active form 20-hydroxyecdysone, 20E), are another class of vital hormones in insects. In pre-adult flies, 20E is produced in the larval prothoracic gland, which (together with the larval CA and the CC) makes part of the larval ring gland in dipterans (Bodenstein, 1950). In adult female flies, the ovary is the major 20E producing tissue (Chavez *et al.*, 2000; Gäde *et al.*, 1997; Gilbert *et al.*, 2002; Hagedorn, 1985; Kozlova & Thummel, 2000), and 20E appears to be produced in both ovarian follicle and nurse cells (Chavez *et al.*, 2000, Gilbert *et al.*, 2002; Schwartz *et al.*, 1985, 1989). Unfortunately, we know almost nothing about the production and metabolism of 20E in adult male *Drosophila* and other insects (Nijhout, 1994; Riddiford, 1993); by analogy with other insects, *Drosophila* males may produce 20E in their testes (Gäde *et al.*, 1997; Hagedorn, 1985). Together with JH, 20E is an important regulator of developmental transitions and metamorphosis (Dubrovsky, 2005; Kozlova & Thummel, 2000). In adults, 20E is well known for its effects on oogenesis, much like JH, yet other adult functions have remained elusive (Dubrovsky, 2005; Kozlova & Thummel, 2000). Similarly, the adult function of 20E in male *Drosophila* is poorly understood (Riddiford, 1993); 20E may affect *Drosophila* spermatogenesis, as it does in other insects (Nijhout, 1994).

C. Interaction Between JH and 20E

Both JH and 20E play major antagonistic or synergistic roles in regulating *Drosophila* development (Dubrovsky, 2005; Kozlova & Thummel, 2000, 2003; [AU1] Riddiford, 1993; Truman & Riddiford, 2002; Zhou & Riddiford, 2002). The interaction between JH and 20E seems to take place in target tissues such as the fat body (adipose tissue), epithelium, and the ovary. For example, 20E circulating in the hemolymph appears to inhibit JH-induced production of vitellogenin in the fat body (Engelmann, 2002; Soller *et al.*, 1999; Stay *et al.*, 1980). However, in some insects, such as the silkworm

Bombyx mori, 20E can stimulate JH synthesis (Gu & Chow, 2003), and JH itself can stimulate 20E production in certain immature lepidopterans and possibly other insect species (Hiruma *et al.*, 1978). Thus, while 20E and JH appear to co-regulate reproduction, there is an intricate yet not well understood hormonal feedback between these key hormones (Dubrovsky, 2005).

III. Effects of JH and 20E on *Drosophila* Aging

A. JH and Agings

As we will discuss below, there is now increasing evidence showing that JH is a key regulator of aging in several insect species, including *Drosophila* (see also Tatar, 2004; Tatar *et al.*, 2003).

1. JH and the Abnormal Abdomen Syndrome

One of the first indications of an effect of JH on dipteran life history was found in Hawaiian *Drosophila mercatorum* (DeSalle & Templeton, 1986; Templeton, 1982, 1983; Templeton & Rankin, 1978; Thomas, 1991). In this species, the *abnormal abdomen* (*aa*) genotype has a decreased JH esterase (JHE) activity, which may lead to a high JH titer in the hemolymph (Templeton *et al.*, 1993; Thomas, 1991). The *aa* phenotype has increased developmental time, early sexual maturation, increased fecundity, and decreased longevity among females (Hollocher & Templeton, 1994; Templeton, 1982, 1983; Templeton & Rankin, 1978; but see Thomas, 1991). In males, developmental time is not affected, whereas sexual maturation is delayed, mating success decreased, and longevity increased (Hollocher & Templeton, 1994). Thus, the *aa* geno-type affects male and female life history differently.

Although *aa* females seem to be long-lived (Templeton, 1982, 1983), the effects of *aa* on life span and other life-history traits may depend on nutrient conditions (Thomas, 1991). Contrary to the findings of Templeton (1982, 1983), both males and females of *aa* genotypes generally exhibited greater longevity than non-*aa* genotypes when reared on various concentrations of dry yeast in the food medium (Thomas, 1991). This life-span extension was observed for all yeast concentrations, except for the concentration used by Templeton (1982, 1983), which reduced life span. Thus, nutrition may affect *D. melanogaster* life span and other life-history traits through changes in JH signaling (see section V).

In addition, reproduction appears not to trade off with survival in these long-lived *aa* genotypes because females showed both greater fecundity and longevity than non-*aa* females (Thomas, 1991). This contradicts many experiments in *Drosophila* that show that life-span extension is typically accompanied by reduced reproduction (for a review, see Stearns & Partridge, 2001). Thus, the study by Thomas (1991) adds to a growing number of examples suggesting that the tradeoff between reproduction and life span can be uncoupled under some circumstances (Barnes & Partridge, 2003; Good & Tatar, 2001; Hwangbo *et al.*, 2004; Leroi, 2001; Marden *et al.*, 2003; Tu & Tatar, 2003).

While the various life-history effects observed in *D. mercatorum* may indeed be proximally controlled by JH, a direct proof for such an effect is lacking. Whether the *aa* genotype has an increased JH titer remains to be determined using a direct JH titer assay rather than measuring the turnover rate of degradation enzymes (Thomas, 1991). Yet, despite the uncertainty surrounding the pleiotropy of the *aa* genotype and the role of JH in the *aa* syndrome, it is interesting to note that *aa* genotypes differ remarkably from wildtype flies in *both* life span and JH metabolism,

suggesting that JH may be a proximate determinant of life span.

2. JH, Reproductive Diapause, and Senescence Plasticity

Many adult insects use token cues to initiate diapause in response to seasonably predictable stressful or harsh environmental conditions. Diapause is a hormonally mediated state of reduced metabolism, developmental arrest, increased stress resistance, and altered behavior (Nijhout 1994; Tatar & Yin, 2001); the developmental arrest associated with diapause is reflected in an arrest of oogenesis, male accessory gland synthesis, and mating ("reproductive diapause"). In many insects, JH is proximately involved in regulating diapause (Nijhout, 1994; Tatar, 2004; Tatar & Yin, 2001).

JH controls reproductive diapause in insects as variable as butterflies (*Danaus plexippus*; Herman & Tatar, 2001; Tatar, 2004; Tatar & Yin, 2001), several grasshopper species (Pener, 1972; Tatar & Yin, 2001), and several species of *Drosophila* (*D. macroptera* and *D. grisea*: Kambysellis & Heed, 1974; *D. melanogaster*: Saunders *et al.*, 1989; Tatar & Yin, 2001; Tatar *et al.*, 2001a).

For example, several temperate-zone species of *Drosophila*, including *D. melanogaster*, *D. triauraria, D. littoralis*, and the cave-dwelling species *D. grisea* and *D. macroptera*, are known to over-winter as diapausing adults (Kambysellis & Heed, 1974; Saunders *et al.*, 1989; Tatar, 2004; Tatar & Yin, 2001). As shown by Tatar and colleagues (2001a), traits specific for diapause in *D. melanogaster* (arrest of oogenesis, resistance to exogenous stress, negligible senescence during diapause) are controlled by JH (Tatar & Yin, 2001). JH may thus be a key mediator of senescence plasticity and the tradeoff between reproduction and longevity.

Diapausing females downregulate JH synthesis in response to shorter day length and cool temperatures. Consequently, females enter ovarian arrest and show reduced age-specific mortality as compared to non-diapausing female cohorts that were started synchronously with the diapausing cohort. The diapause phenotype can be rescued by application of the synthetic JH methoprene; this treatment terminates ovarian arrest, makes flies more sensitive to oxidative stress, and reduces post-diapause longevity (Tatar *et al.*, 2001b). Methoprene is a JH analog (JHA) that is chemically more stable and much more potent than JH itself (see Wilson, 2004, and references therein). Although high doses of methoprene, a commonly used insecticide, can be toxic to insects, insect physiologists have confirmed in numerous reports that methoprene behaves as a faithful mimic of JH action in insects in general and in *Drosophila* in particular, both *in vivo* and *in vitro* (e.g., Riddiford & Ashburner, 1991; Wilson, 2004, and references therein; but also see Zera, 2004).

In *C. elegans*, larval diapause (formation of so-called dauer larvae) is under the control of the insulin signaling pathway, which signals through an insulin-like receptor encoded by the *dauerformation 2* gene (*daf-2*), the homolog of the *Drosophila insulin-like receptor* (*InR*) locus. Mutations in *daf-2* cause dramatic life-span extension (Dorman *et al.*, 1995; Kenyon *et al.*, 1993; Kenyon, 2001). Interestingly, in *D. melanogaster*, mutant *InR* genotypes live longer and exhibit small and immature ovaries, very similar to those observed in diapausing wildtype *Drosophila* (Tatar *et al.*, 2001b). This suggests that flies in reproductive diapause "phenocopy" the phenotype of *InR* mutants. Thus, there may exist an analogy between diapause and insulin signaling in *C. elegans* and *D. melanogaster*.

3. JH in Mutants of the Insulin Signaling Pathway

Several *D. melanogaster* mutant genotypes of *InR* and *chico* (encoding the insulin-receptor substrate) are long-lived (Clancy *et al.*, 2001; Tatar *et al.*, 2001b; Tu *et al.*, 2002a). For example, a heteroallelic *InR* mutant (InR^{p5545}/InR^{E19}) produces dwarf females with extended life span up to 85 percent (Tatar *et al.*, 2001b). Similarly, homozygous mutants of *chico* are sterile and very long-lived dwarfs, whereas heterozygous mutants of *chico* exhibit normal body size, reduced fecundity, and extended life span (Clancy *et al.*, 2001; Tu *et al.*, 2002a).

However, not all *InR* mutant alleles increase longevity: since *InR* gene is a highly pleiotropic locus, some alleles may have deleterious developmental effects carrying over to adults and counterbalancing the positive effects on aging (Tatar *et al.*, 2001b). Furthermore, unlike in *C. elegans*, hypomorphic insulin signaling mutants in *Drosophila* may have different effects on life span in males and females (Tu *et al.*, 2002a). While *Drosophila* mutants of *InR* and *chico* extend female longevity by 36 to 85 percent, the same alleles do not seem to produce an extension of mean longevity in males (Clancy *et al.*, 2001; Tatar *et al.*, 2001b). However, males of *InR* heteroallelic mutants have an increased life expectancy measured at age 20 days (Tatar *et al.*, 2001b), and the $chico^1$ mutation extends male longevity but has age-independent effects on adult mortality that counteract the strong impact of slow aging on life expectancy seen in *chico* mutant females (Tu *et al.*, 2002a). Similarly, reducing insulin signaling by experimental ablation of insulin-producing cells (IPCs) reduces age-dependent mortality, yet this effect is masked at young ages due to a high age-independent risk of death (Wessells *et al.*, 2004; but see Broughton *et al.*, 2005).

Interestingly, the extended life-span phenotype of some insulin signaling mutants is likely to be caused by JH deficiency. JH synthesis is negligible in *InR* dwarfs (Tatar *et al.*, 2001b), and homozygous *chico* mutants are also JH deficient (Tu *et al.*, 2005). Although *InR* mutant females are infertile with non-vitellogenic ovaries, egg development can be restored by application of methoprene. Furthermore, treatment with methoprene restores wildtype longevity to the long-lived *InR* mutants. Thus, JH deficiency, resulting from mutation in the insulin signaling pathway, may retard senescence, possibly through hormone-mediated effects on adult reproduction, physiology, and somatic maintenance. Consequently, Tatar and colleagues (2001b) suggest that infertility may not be the direct cause of slowed aging but that JH may simply control both fertility and longevity; JH may therefore be a key regulator for both traits. However, JH synthesis is also known to be reduced in a homozygous *InR* mutant genotype with normal life span; thus, the lack of JH may not be sufficient to extend life span under all circumstances (Tatar *et al.*, 2001b). Similarly, whether short-lived insulin signaling mutants have upregulated JH is currently unknown.

4. Effects of Manipulating JH on Life Span

Using mutants to examine the effects of JH on life span may be problematic because mutants can exhibit unspecific pleiotropic effects that are unrelated to changes in JH signaling. This problem may be overcome by examining the effects of applying exogenous JH or JHAs such as methoprene. Treating wildtype flies with methropene increases early fecundity but decreases longevity and stress resistance (Flatt & Kawecki, in preparation; Salmon *et al.*, 2001, Tatar *et al.*, 2001a,b). For example, methoprene treatment of diapausing *D. melanogaster*

restores vitellogenesis and egg production yet increases demographic senescence (Tatar *et al.*, 2001a). Similarly, as discussed above (section III.A.3), Tatar and colleagues (2001b) found that the sterility phenotype of *InR* mutants can be rescued by methoprene treatment, which restores egg development and reduces life expectancy to that of wildtype flies, whereas methoprene treatment of *InR* wildtype controls did not increase adult mortality.

Application of commonly used JH inhibitors, such as precocene (Wilson *et al.*, 1983) or fluvastatin (Debernard *et al.*, 1994), can reduce or inhibit JH synthesis in the CA and may thus be used to study the effects of JH deficiency upon life span. However, these inhibitors also appear to have unspecific and toxic effects (e.g., Debernard *et al.*, 1994; Zera, 2004, and references therein). For example, high doses of fluvastatin kill locusts, whereas surgical removal of CA is not lethal (Debernard *et al.*, 1994). Thus, the usefulness of JH inhibitors for manipulating life span through changes in JH signaling is questionable. A different approach is to select wildtype flies for resistance to toxic doses of methoprene (T. Flatt & T. J. Kawecki, unpublished results). Flies that evolve specific insensitivity to JH, either by constitutive upregulation of JH esterases or by reduced JH binding, may exhibit increased longevity because JH deficiency is known to slow aging. Interestingly, flies selected for methoprene resistance rapidly evolved both methoprene- and JH III-resistance and showed extended life span (T. Flatt & T. J. Kawecki, unpublished results). However, the underlying mechanisms for this lifespan extension are unknown and may not be JH-related.

B. 20E and Aging

Although we know far less about the effects of steroid hormones on life span than about JH effects on aging, it now seems clear that 20E is a second candidate regulator of *Drosophila* aging (also see Simon *et al.*, 2003; Tatar, 2004; Tatar *et al.*, 2003).

1. *Long-Lived Insulin Signaling Mutants Have Reduced 20E Titers*

In adult insects, ecdysteroids are made in the ovaries and the testes (Chavez *et al.*, 2000; Gäde *et al.*, 1997; Gilbert *et al.*, 2002; Hagedorn, 1985). Tu and colleagues (2002b) measured ecdysteroid synthesis in isolated ovaries of *InR* mutants *in vitro* and found that ovarian ecdysteroid synthesis of mutant females was reduced as compared to wildtype. How 20E deficiency affects aging in *InR* mutants is currently not understood; 20E may affect life span by serving as a pro-aging hormonal signal or by regulating the relationship between reproduction and aging (Tatar, 2004; Tatar et al., 2004; Tu *et al.*, 2002b). Clearly, it would be interesting to see whether and how treatment of 20E-deficient *InR* mutants with 20E affects aging. In addition, because 20E is a major product of the *Drosophila* ovary and plays a pervasive role in female reproduction, we may speculate that the sex-specific effects of insulin signaling on aging seen in the fly may be related to differences in 20E signaling between males and females.

2. *Mutations in the Ecdysone Receptor Extend Life Span*

Simon and colleagues (2003) demonstrate that flies heterozygous for mutations of the *ecdysone receptor* (*EcR*) gene exhibit increased life span and stress resistance without decreases in reproduction or activity. Although almost nothing is known about the production, metabolism, and role of 20E in adult male *Drosophila*, it is interesting to note that mutations in *EcR* extended life span in both males and

females, suggesting that 20E signaling affects aging in both sexes. Furthermore, a mutant involved in the 20E biosynthesis pathway (*DTS-3*) displays the same phenotype; this phenotype can be rescued by the application of 20E (Simon *et al.*, 2003). These results are consistent with reduced post-eclosion levels of ecdysteroids in long-lived females from a selection experiment for life-span extension (Harshman, 1999). Thus, the few examples at hand clearly suggest that 20E deficiency slows aging.

C. Interaction Effects of JH and 20E on Aging

Interactions between JH and 20E may not be restricted to pre-adult development, metamorphosis, and reproduction (Dubrovsky, 2005), but may also extend to the aging phenotype. In mosquitoes, application of bovine insulin acts directly in the ovary to regulate ecdysteroid synthesis (Riehle & Brown, 1999); insulin is also known to regulate germ-line stem cell proliferation in *D. melanogaster* ovaries (Drummond-Barbosa & Spradling, 2001). As discussed above (sections II.C and III.B.1), the ovaries of the JH-deficient *InR* mutants produce little ecdysteroids (Tu *et al.*, 2002b), and 20E can, depending on the species, inhibit or stimulate JH synthesis (Gu & Chow, 2003; Soller *et al.*, 1999). Thus, 20E may be a gonad-derived signal through which insulin and JH affect insect aging (Tatar, 2004; Tatar et al., 2003). In addition, reproductive diapause and diapause senescence may not be exclusively controlled by JH. Application of JH III or JHB_3 to abdomens of diapausing female flies can restore vitellogenesis (Saunders *et al.*, 1989). However, terminating diapause by warming flies from 11 °C to 25 °C results in a significant increase in the synthesis of ecdysteroids, but not JH (Saunders *et al.*, 1989). Furthermore, the injection of 20E can also elicit vitellogenesis and terminate diapause (Richard *et al.*, 1998, 2001), as has been observed with JH. This suggests that JH and 20E may interact in affecting diapause and levels of age-specific mortality during diapause. Interestingly, although JH and 20E often have antagonistic effects on the same trait or process, both hormones seem to have positive effects on female reproduction and negative effects on female life span. However, whether and how 20E interacts with JH to affect male life history remains unknown. Clearly, the interactive effects of these hormones on aging await further study.

IV. Candidate Genes Affecting Life Span Through JH and 20E Signaling

A. Insulin Signaling Affects Both JH and 20E

The insulin/IGF (insulin-like growth factor) signaling pathway has profound effects on aging in a variety of organisms, such as the nematode *C. elegans*, *Drosophila*, and rodents, and is suspected to have similar effects in humans (Kenyon, 2001; Partridge & Gems, 2002; Tatar, 2004; Tatar et al., 2003). Studies of mutants of *InR*, *chico*, and *EcR* suggest that JH and 20E may be secondary pro-aging signals downstream of insulin/IGF (see section III). As discussed above, long-lived mutants of *InR* are both JH and 20E-deficient, suggesting that insulin signaling is a major regulator of these secondary hormones. Indeed, the stimulation of ecdysteroid synthesis by insulin signaling is well known in many insects (Graf *et al.*, 1997; Hagedorn, 1985; Nagasawa, 1992; Riehle & Brown, 1999). For example, mosquitoes synthesize ovarian ecdysteroids after a blood meal, and this synthesis depends on insulin signaling. Sugar-fed mosquito females do not produce ecdysteroids, but application of exogenous bovine insulin can stimulate ovarian ecdysteroid

synthesis (Riehle & Brown, 1999). But how does insulin signaling regulate JH and 20E synthesis?

In response to environmental or internal stimuli such as nutrition, insulin-producing cells (IPCs, belonging to the class of median neurosecretory cells, MNCs) located in the *pars intercerebralis* of the brain produce seven different *Drosophila* insulin-like peptides (DILPs; Brogiolo *et al.*, 2001; Broughton *et al.*, 2005). These DILPs are then released into the protocerebrum, at the CC, and into the hemolymph (Ikeya *et al.*, 2002; Rulifson *et al.*, 2002). Little is known about the effects of individual DILPs, but the expression of the genes *dilp3* and *dilp5* seems to be regulated by nutrition, and overexpression of *dilp1–7* promotes growth (Hwangbo *et al.*, 2004; Ikeya *et al.*, 2002; Rulifson *et al.*, 2002). Insulin signaling may regulate JH and 20E synthesis in at least two not mutually exclusive ways (Tatar, 2004). First, circulating DILPs in the hemolymph activate insulin signaling by binding to the *InR* receptors in the target tissues and hence stimulate growth in these tissues. For example, *InR* mutants are dwarfs with both reduced JH synthesis and corpus allatum (CA) size; insulin may thus affect JH synthesis by affecting CA development and growth (Tatar *et al.*, 2001b; Tu *et al.*, 2005). Similarly, *InR* mutants have an approximately 50 percent reduction of wildtype ovariole number; mutants may therefore produce less 20E because of a reduced number of ovarian follicle cells synthesizing 20E (Tu & Tatar, 2003; Tu *et al.*, 2002b;). Second, JH and 20E synthesis may be indirectly modulated by insulin signaling. For example, because JH synthesis is regulated by neuropeptides (Tobe & Bendena, 1999), DILPs may indirectly regulate the production of JH by affecting these neuropeptides. In *chico* mutants, JH synthesis relative to CA size is disproportionately reduced, suggesting that insulin signaling can regulate adult JH synthesis independent of affecting CA development and growth (Tu *et al.*, 2005). The insulin signaling–dependent regulation of JH and 20E may also be supported by the observation that ablation of IPCs can extend life span in *Drosophila* (Broughton *et al.*, 2005; Wessells *et al.*, 2004). However, it is currently unknown whether this effect occurs through the downregulation of JH or 20E. Clearly, future work needs to test whether ablation of IPCs or downregulation of DILP expression reduces JH and/or 20E. Similarly, reducing insulin signaling by transgenically overexpressing *dPTEN* (encoding a phosphatase and tensin homolog protein that antagonizes insulin signaling) and *dFOXO* (a forkhead transcription factor downstream of insulin signaling whose activity is inhibited by insulin signaling) in the head fat body of the fly can slow aging (Hwangbo *et al.*, 2004), yet it remains to be tested whether these genes regulate longevity through effects on JH or 20E production. Figure 15.1 shows an integrated model of the endocrine regulation of *Drosophila* aging.

Although JH and 20E are not produced by *C. elegans* and rodents, the existence of secondary pro-aging hormones in these organisms has been postulated (Tatar *et al.*, 2003; also see Gill *et al.*, 2004). For example, in rodents, insulin signaling in the hypothalamus regulates the pituitary gland, which secretes secondary hormones such as thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), growth hormone (GH), and luteinizing hormone (LH); TSH in turn regulates the thyroid gland to produce the thyroxin hormones T3 and T4. The pituitary may be seen as the mammalian equivalent of the CC and CA, and thyroxin has been tentatively suggested to share cellular functions with JH (Davey, 2000). Remarkably, a new study supports the idea that thyroxin may be a pro-aging hormone like JH (Vergara *et al.*, 2004). Long-lived Snell dwarf mice (mice homozygous for the *Pit1* mutation)

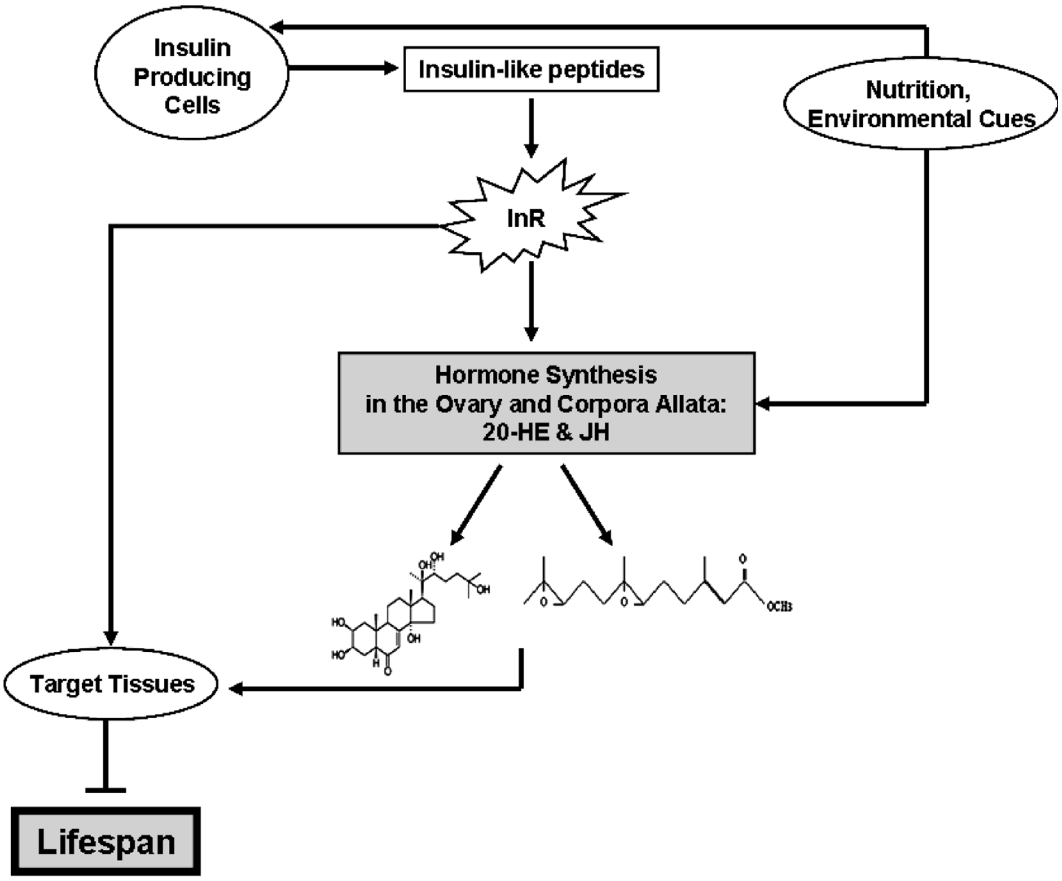

Figure 15.1 Integrated model for the endocrine regulation of aging, based on studies in *D. melanogaster* (see Hwangbo *et al.*, 2004; Tatar, 2004; Tatar *et al.*, 2003) External cues like nutrition stimulate insulin-producing cells (IPCs) to secrete insulin-like peptides (DILPs), which bind and activate the insulin-like receptor at the target tissues (InR in *D. melanogaster*, DAF-2 in *C. elegans*). Ligand binding at InR in turn induces the insulin/IGF-1 signaling cascade in cells of the CNS and other tissues, such as the fat body (primary insulin signaling). Induction of insulin/IGF-1 signaling suppresses a forkhead transcription factor downstream of insulin signaling (dFOXO in *D. melanogaster*, DAF-16 in *C. elegans*) required for life-span extension by slowed insulin signaling. Activation of this transcription factor (or inactivation of InR or ablation of IPCs) extends life span (Broughton *et al.*, 2005; Hwangbo *et al.*, 2004; Kenyon *et al.*, 1993; Tatar *et al.*, 2001b; Wessells *et al.*, 2004). Insulin signaling also has secondary effects: (1) insulin signaling affects insulin production by participating in endocrine and paracrine regulatory feedback circuits to regulate DILP transcription (Hwangbo *et al.*, 2004) and (2) insulin signaling affects the production of secondary aging regulatory signals such as JH from the CA or 20E from the gonads (Simon *et al.*, 2003; Tatar, 2004; Tatar *et al.*, 2001b, 2003; Tu *et al.*, 2002b;). In *Drosophila* and other insects, these secondary endocrine signals (unknown hormones in *C. elegans*, but see Gill *et al.*, 2004) suppress life-span extension (as well as stress resistance, immunity, and somatic maintenance) and upregulate gonad activity and reproduction (Tatar, 2004; Tatar *et al.*, 2003). Note that external cues may also directly affect the production of JH and 20E. Thus, insulin signaling may either directly affect aging (through dFOXO) or indirectly (through secondary pro-aging hormones). See text for further details.

have multiple hormonal defects, but whether these deficiencies are causally responsible for the slow aging phenotype has remained unclear (Tatar *et al.*, 2003). Vergara and colleagues (2004) show that treatment with the thyroxine hormone T4 [AU2] restores the reduced senescence phenotype in the long-lived Snell mice. Although it

cannot be ruled out that the T4 treatment results in a toxic effect reducing life span (Vergara *et al.*, 2004), the restoration of the anti-aging effects by T4 is highly reminiscent of the life-span reduction seen in long-lived JH-deficient flies when treated with methoprene.

B. Genes Involved in JH Signaling

The genetics of JH signaling are currently not well understood (Dubrovsky, 2005). Moreover, the life-span effects of most *Drosophila* genes involved in JH signaling are unknown. Yet, despite our limited understanding, there exist several interesting candidate genes implicated in JH signaling that may affect the aging phenotype.

A major reason limiting our understanding of JH signaling is the unknown nature of the JH receptor (Dubrovsky, 2005; Gilbert *et al.*, 2000; Henrich & Brown, 1995; Jones & Sharp, 1997; Truman & Riddiford, 2002). Recent evidence suggests that *ultraspiracle (usp)*, encoding a retinoid X receptor, may be a JH receptor because JH is closely related to retinoic acid (RA) and USP protein can bind JH (Gilbert *et al.*, 2000; Jones & Sharp, 1997; Truman & Riddford, 2002). Although USP does not show high-affinity binding to JH (Jones & Sharp, 1997), it forms a heterodimer with the ecdysone receptor (EcR) (Gilbert *et al.*, 2002; Truman & Riddiford, 2002), which is itself known to have effects on *Drosophila* life span (Simon *et al.*, 2003). Thus, given the common interactions between JH and 20E and given that mutations in *EcR* or JH deficiency extend life span, *ultraspiracle* appears to be a promising candidate gene affecting aging. Yet, while *usp* probably plays a functional role in JH signaling, the low binding affinity for JH is not consistent with *usp* encoding a JH receptor (Dubrovsky, 2005).

Another candidate gene for the JH receptor is the X-linked gene *Methoprene-tolerant* (*Met*), encoding a 85 kD high-affinity JH binding protein essential for transducing JH signals (Ashok *et al.*, 1998; Pursley *et al.*, 2000; Restifo & Wilson, 1998; Shemshedini & Wilson, 1990; Wilson *et al.*, 2003). *Met* mutant flies produce JH in normal amounts but are up to 100 times less sensitive to JH III and methoprene than Met^{+} flies (Pursley *et al.*, 2000; Shemshedini & Wilson, 1990; Shemshedini *et al.*, 1990). In adults, the *Met* gene has important effects on life-history traits, such as developmental time, onset of reproduction, and age-specific fecundity (Flatt & Kawecki, 2004; Minkoff & Wilson, 1992; Wilson & Ashok, 1998; Wilson *et al.*, 2003). Given these pleiotropic life-history effects of *Met*, presumably mediated by JH signaling, one may expect that this locus also affects life span. However, inconsistent with the notion of a JH receptor, *Met* null mutants are completely viable. Thus, the exact role of *Met* in JH signaling remains unclear (Dubrovsky, 2005).

Mutations of the *apterous* (*ap*) gene result in sterile females due to the development of non-vitellogenic ovaries (Ringo *et al.*, 1991). Mutant males are behaviorally sterile, spend less time courting, and are less likely to perform some elements of courtship behavior than age-matched wildtype males, yet they have fertile gametes. Adult mutant flies are JH deficient (Altatraz *et al.* [AU3] 1991), and application of JH or methoprene to newly eclosed mutant females results in vitellogenic oocytes (Postlethwait & Weiser 1973), suggesting that the development of oocytes is profoundly affected by JH. A recent study clearly supports the notion that *apterous* is involved in JH signaling; in *apterous* mutants, the levels of two JH-inducible genes (Dubrovsky *et al.* 2002), *JhI-21* and *minidiscs* (*mdn*), are strongly reduced, and methoprene treatment can rescue this defect. Thus, since *apterous* mutants are JH-deficient, like long-lived mutants of *InR* and *chico*, *apterous* may be an

interesting candidate gene affecting aging.

Flies with a mutation in the *cricklet* (*clt*) gene have reduced yolk protein synthesis, larval fat bodies persisting into the adult stage, and an arrested oocyte development in the pre-vitellogenic stage (Shirras & Bownes, 1989). Methoprene has no effect on the fat body synthesis of yolk and vitellogenesis in the mutants; ovarian transplant experiments suggest that females have sufficient JH concentrations to promote oogenesis (Shirras & Bownes, 1989). This indicates that the gene may encode a protein downstream of JH synthesis, such as a receptor or transcription factor, which is nonfunctional in the mutants (Shirras & Bownes, 1989). However, as for many other genes affecting JH signaling, the potential effects of *clt* on aging await further study.

In female *Drosophila*, the post-mating response consists of increased egg deposition and reduced receptivity to males and is regulated by sex peptide (SP), contained in the male seminal fluid and transferred to the female upon mating. SP is known to stimulate JH synthesis in the mature CA (Moshitzky *et al.*, 1996). Consequently, JH appears to be a downstream component in the SP response cascade, causing the progression of vitellogenic oocytes after mating or SP application (Moshitzky *et al.*, 1996; Soller *et al.*, 1999). Remarkably, work by Geiger-Thornsberry & Mackay (2004) shows that the *sex peptide* locus (*Acp70*, *accessory protein 70*) harbors genetic variation for life span. Thus, the *sex peptide* gene is another promising candidate gene for aging studies, in particular since it may affect life span through its effects on female JH signaling (Flatt, 2004).

Biogenic amines, such as dopamine, octopamine, and serotonin, are known to affect JH synthesis in insects (Lafon-Cazal & Baehr, 1988; Rachinsky, 1994; Roeder, 1999). For example, octopamine stimulates JH production in locusts (Lafon-Cazal & Baehr, 1988), and both octopamine and serotonin promote JH synthesis in honeybees (Rachinsky, 1994). In *D. melanogaster* and *D. virilis*, dopamine seems to stimulate synthesis of JH in immature females but inhibits its synthesis in mature, intensely reproducing females; similarly, octopamine appears to block JH production (Chentsova *et al.* 2002; Grutenko *et al.*, 2001; Rauschenbach *et al.*, 2002). Two recent experiments suggest that biogenic amines are important determinants of life span. Sze and colleagues (2000) found that a serotonin-synthesis mutant in *C. elegans* exhibits extended reproductive life span, and De Luca and colleagues (2003) report that the gene coding for the enzyme dopa decarboxylase (DDC), required for the final catalytic step in the synthesis of dopamine and serotonin, harbors significant amounts of variation for *Drosophila* longevity. Thus, although *C. elegans* is not known to produce JH, these results suggest that biogenic amine metabolism may affect the levels of secondary endocrine hormone with effects on aging. However, it remains to be determined whether *ddc* mutants in *Drosophila* exhibit variation in JH signaling that may, at least partially, account for the variation in life span attributed to this locus.

Table 15.1 summarizes information on some *Drosophila* genes involved in JH signaling; these genes may be promising candidate genes affecting aging.

C. Genes Involved in 20E Signaling

In contrast to JH, we have a fairly good understanding of the molecular mechanisms of both ecdysteroid synthesis and 20E action, particularly at the onset of metamorphosis (Dubrovsky, 2005). For example, 20E binds to the ecdysone receptor (EcR), which forms a heterodimer receptor complex with USP (Gilbert *et al.*, 2002; Truman & Riddiford, 2002), and recent work

Table 15.1

Examples of *Drosophila* Genes Involved in JH Signaling. Because JH is known to have major effects on insect life span, these genes represent candidate genes for aging. Further information on each of these and further candidate genes, including references, can be found on the flybase Website at: http://flybase.bio.indiana.edu; this database is searchable using gene names, flybase accession numbers, or keywords.

Gene	Function	Mutant Phenotype and Biological Processes	Flybase Accession
Acp70 (sex peptide; accessory gland peptide 70A)	Male product contained in seminal fluid; has hormone activity; negatively regulates female receptivity and post-mating response	Female mating defective; injection of *Acp70A* stimulates JH synthesis in the female, increases egg production and mimics the effects of mating; genetic variation for life span detected as a function of this gene	FBgn0003034
adp (adipose)	Involved in carbohydrate and lipid metabolism	Mutations result in hypertrophied adult fat body with enlarged lipid vesicles and hypertrophied female corpora allata; mutants are viable, starvation resistant, male and female fertile, yet egg hatchability is reduced and eclosion delayed	FBgn0000057
ap (apterous)	Product exhibits zinc ion binding and is involved in neurogenesis	Mutations affect halteres, muscle development, neuroanatomy, ovarian development, oogenesis, and female receptivity; some mutants are JH-deficient; JH application can restore vitellogenesis and positively affects ovarian maturation	FBgn0000099
clt (cricklet)	Carboxylesterase activity	Defective in yolk protein synthesis, histolysis of the larval fat body, vitellogenesis, and synthesis of larval serum protein 2; gene may encode a protein essential for mediating JH signaling in target tissues	FBgn0000326
e (ebony)	Product exhibits beta-alanyl-dopamine synthase activity, involved in cuticle pigmentation	Mutations affect wing and adult cuticle; mutants are locomotor rhythm and body color defective; 1-day-old mutant females show a significantly lower JH-hydrolyzing activity as compared to wildtype	FBgn0000527

(*continues*)

Table 15.1 (*Cont'd*)

Gene	Function	Mutant Phenotype and Biological Processes	Flybase Accession
Fas2 (fasciclin 2)	Involved in neuronal cell recognition and organ looping and symmetry	Mutations affect the embryonic neurons and 13 other tissues; mutants are larval recessive lethal and have embryonic neuroanatomical defects; the *spin* mutant allele affects neurosecretory cells innervating the corpus allatum, male genitalia rotation, and interacts with the *Met* locus	FBgn0000635
fs(2)B (female sterile (2) Bridges)	Female reproduction; also affects endocrine function	Recessive female sterile; cells of the corpus-allatum-corpus-cardiacum (CA/CC) complex of homozygous mutant females can probably not release hormone product and undergo degenerative changes; enlarged abdominal fat cells	FBgn0000949
ibx (icebox)	Encodes a product involved in female courtship behavior	Various mutant phenotypes, including female mating defective, female courtship defective, yet fertile and viable; treatment with JH analog methoprene results in more mating in homozygous females	FBgn0041750
InR (insulin-like receptor)	Insulin-like receptor; binds *Drosophila* insulin-like peptides	Heterozygous mutants are dwarf, female sterile, and long-lived; some mutants have lowered JH and 20E production	FBgn0013984
jhamt (JH acid methyltransferase)	JH acid methyltransferase activity involved in JH biosynthesis	Unknown	FBgn0028841
Jhbp-30, 63, and 80 (JH binding proteins – 30kD, 63kD, and 80kD)	JH binding activity in larval fat body cell nuclei	Unknown	FBgn0013282; FBgn0013283; FBgn0013284
Jhe (JH esterase)	JH esterase activity involved in JH catabolism	Unknown	FBgn0010052
Jheh1, Jheh2, and Jheh3 (JH epoxide hydrolases 1, 2, 3)	JH epoxide hydrolase activity involved in JH catabolism; maybe involved in defense response	Unknown	FBgn0010053; FBgn0034405; FBgn0034406

(*continues*)

Table 15.1 (*Cont'd*)

Gene	Function	Mutant Phenotype and Biological Processes	Flybase Accession
JhI-1and JhI-26 (JH-inducible proteins 1 and 26)	Unknown; gene expression induced by JH	Unknown	FBgn0028426; FBgn0028424
JhI-21 (JH-inducible protein 21)	L-amino acid transporter activity; amino acid metabolism?	Unknown	FBgn0028425
l(2)gl (lethal (2) giant larvae)	Product with myosin binding, involved in neurotransmitter secretion	Mutations affect the dorsal mesothoracic disc, the larval brain, and 21 other tissues; recessive lethal and tumorigenic; wildtype allele important for onset of vitellogenesis and oocyte growth, follicle cell migration and organization, and germline cell viability; some mutants have reduced size of the larval ring gland (consisting of the corpora cardiaca, the corpora allata, and the prothoracic gland), presumably resulting in endocrine deficiency	FBgn0002121
Methoprene-tolerant (Met 5 Rst(1)JH, Resistance to JH)	Encodes a product with JH binding, a putative JH receptor; involved in regulation of transcription	Loss-of-function alleles have reduced numbers of stage S8 to 9 and stage S10 to 14 oocytes, are methoprene and JH III resistant, viable, but have reduced female fertility; *Met* interacts with *Br* and *Fas2*	FBgn0002723
mama (maternal metaphase arrest)	?	Mutants show lipid accumulation, hypertrophied corpora allata, are viable, recessive female sterile, maternal effect recessive lethal, and reduced male fertile	FBgn0000988
Mdh1 (malate dehydrogenase 1)	L-malate dehydrogenase activity	Unknown; response of Mdh1 to JH depends on ecdysteroids; during interecdysial period of the last instar *Mdh1* rapidly responds to JH by increasing activity	FBgn0002699

(*continues*)

Table 15.1 (*Cont'd*)

Gene	Function	Mutant Phenotype and Biological Processes	Flybase Accession
Rbp9 (RNA binding protein 9)	RNA binding, involved in egg chamber formation	Loss-of-function mutations affect the ovariole, the cystocyte, and the egg chamber and are female sterile; cells of the corpus-allatum corpus-cardiacum complex of homozygous mutant females can probably not release their hormone products and undergo degenerative changes	FBgn0010263
Tbh (tyramine ? hydroxylase)	Tyramine-beta hydroxylase activity involved in behavioral response to ethanol	Loss-of-function mutations are viable, male fertile and female sterile; under normal conditions, young mutant females have a higher JH-hydrolyzing activity than wildtype	FBgn0010329
usp (ultrapiracle)	Product with ligand-dependent nuclear receptor activity; forms heterodimer with ecdysone receptor; binds JH with low affinity; has cell-autonomous role in controlling neuronal remodeling	Mutations affect the embryonic/larval anterior spiracle, the imaginal discs, the embryonic larval midgut, and are recessive lethal, hypoactive and touch sensitivity defective; mutations show a range of imaginal disc phenotypes	FBgn0003964
Vha44 (vacuolar H[+] ATPase)	Hydrogen-exporting ATPase activity, phosphorylative mechanism, involved in JH biosynthesis	Unknown	FBgn0020611
Yp 1, 2, and 3 (yolk proteins 1, 2, and 3)	Structural molecule activity involved in vitellogenesis	Mutations conditionally affect egg production and the adult fat body and are dominant female sterile; the JH analog methoprene upregulates yolk proteins	FBgn0004045; FBgn0005391; FBgn0004047

confirms the importance of this heterodimer complex for *Drosophila* development (Hall & Thummel, 1998; Hodin & Riddiford, 1998; Dubrovsky, 2005). When bound to the receptor, 20E induces a number of early response genesm such as *Broad* (*Br*), a gene essential for transducing 20E signals

during metamorphic development of larval and imaginal tissues (Restifo & Wilson, 1998; Zhou & Riddiford, 2002), as well as *E74* and *E75,* which seem to be required for oogenesis (Dubrovsky, 2005; Kozlova & Thummel, 2000). In contrast to the genetic control of JH synthesis, the field has recently witnessed major progress in identifying critical genes required for 20E synthesis, involving key enzymes encoded by genes such as *defective in the avoidance of repellents* (*dare*), *disembodied* (*dib*), *ghost* (*gho*), *phantom* (*phm*), *shade* (*shd*), *shadow* (*sad*), s*pook* (*spo*), and *Start1* (Buszczak *et al.*, 1999; Chavez *et al.*, 2000; Gilbert *et al.*, 2002; Petryk *et al.*, 2003; Roth *et al.*, 2004; Warren *et al.*, 2004). Because these [AU6] genes affect 20E synthesis and because 20E deficiency is known to slow aging, some of these genes may be interesting candidate genes affecting aging.

Table 15.2 summarizes information on some fly genes known to be involved in 20E signaling; our future understanding of the endocrine regulation of aging may be improved by studying the effects of these genes on the aging phenotype.

Table 15.2
Examples of *Drosophila* Genes Involved in 20E Signaling. Because 20E affects insect aging, these genes may represent candidate genes for aging. Further information on each of these and other candidate genes can be found at: http://flybase.bio.indiana.edu ; this data base is searchable using gene names, flybase accession numbers, or keywords.

Gene	Function	Mutant Phenotype and Biological Processes	Flybase Accession
EcR (ecdysone receptor)	20E receptor	Constitutive heterozygous mutants extend life span; follicle cell expression of dominant negative for EcR results in female sterility; germline clones have arrested mid-oogenesis	FBgn0000546
E74 and E75 (ecdysone-induced proteins 74 and 75)	Orphan receptors; transcription factors required for mediating 20E response	Germline clones have arrested mid-oogenesis, degenerate egg chambers; some mutants have low 20E titers	FBgn0000567; FBgn0000568
Br (broad)	Transcription factor; a major 20E inducible gene; also interacts with the *Met* locus	Various mutant phenotypes, including developmental arrest, metathoracic tarsal segment formation, optic lobe formation, effects on adult brain, and reduced transcription rate or stability of the small heat shock protein mRNAs	FBgn0000210

(*continues*)

Table 15.2 (*Cont'd*)

	Gene	Function	Mutant Phenotype and Biological Processes	Flybase Accession
	dare (defective in the avoidance of repellents)	Adrenodoxin reductase required for ecdysteroid synthesis	Various mutant phenotypes; blocked ecdysteroid synthesis; abnormal response to olfactory stimuli, degenerate nervous system; germ-line clones arrest oogenesis at stage 8/9	FBgn0015582
	dib (disembodied)	Mitochondrial cytochrome P450 required for hydroxylation in ecdysteroid biosynthesis	Low ecdysteroid synthesis and various other mutant phenotypes, including no differentiation of the cuticle or the head skeleton	FBgn0000449
[AU7]	gho (ghost)	?; involved in ecdysteroid biosynthesis pathway	Mutants have undifferentiated cuticle due to defect in 20E signaling but normal embryonic ecdysteroid titers	FBgn0001106
	InR (insulin-like receptor)	Insulin-like receptor, Ibinding *Drosophila* insulin-like peptides	Heterozygous mutants are dwarf, female sterile, and long-lived; some mutants have lowered JH and 20E production	FBgn0013984
[AU8]	phm (phantom)	?	Mutations affect the embryonic cuticle, the embryonic head, and oogenesis; embryonic recessive lethal; mutants display a posterior contraction and poorly differentiated cuticle	FBgn0004959
	sad (shadow)	Mitochondrial cytochrome P450 required for hydroxylation in ecdysteroid synthesis	Mutants have low larval ecdysteroid titers; no differentiation of the cuticle or the head skeleton	FBgn0003312
	shd (shade)	P450 enzyme, converting ecdysone to 20E	Mutants have no differentiation of cuticle or head skeleton; ovarian enzyme activity required for female fertility; low embryonic 20E production	FBgn0003388
[AU9]	spo (spook)	?, transporter for shuttling	Mutants with low embryonic ecdysteroid titers; no differentiation of the cuticle or the head skeleton	FBgn0003486

D. Interactions between JH and 20E Signaling

As discussed above (section IV.B), the interactive effects of JH and 20E are supported by the finding that the ecdysone receptor (EcR), affecting fly life span, dimerizes with the USP, which is a candidate receptor for JH (Dubrovsky, 2005; Truman & Riddiford, 2002). The potential importance of this receptor complex for aging is underscored by the suggestion that the molecular chaperones Hsp70 and Hsp90 and the histone deacetylases Sin3A/Rpd3 interact with EcR/USP (Arbeitmann & Hogness, 2000; Tsai *et al.*, 1999). These genes are known to affect life span: overexpression of chaperones extends longevity in *D. melanogaster* and *C. elegans* (Tatar *et al.*, 1997; Yokoyama *et al.*, 2002), and mutation of the gene encoding Rpd3 increases the life span of yeast and *D. melanogaster* (Kim *et al.*, 1999; Rogina *et al.*, 2003). It is thus interesting to speculate that the aging effects of EcR, and potentially those of the EcR/USP complex, may be mediated by these genes.

Interestingly, two 20E-induced transcription factors, *Br* and *E75*, seem to be intimately involved in the cross-talk between JH and 20E signaling (Dubrovsky, 2005). JH and 20E are known to regulate the expression of *Br* (Dubrovsky, 2005, and references therein), and preliminary data suggest that *Br* interacts epistatically with the putative JH receptor gene *Met* (Restifo & Wilson, 1998). In view of this interaction, it would be interesting to study the effects of *Br* on aging. The *E75* gene can be activated by both JH and 20E, and the isoform *E75A* is the first transcription factor whose expression is known to be directly induced by JH (Dubrovsky, 2005, and references therein). Thus, E75 may be an important mediator of the interaction between JH and 20E (Dubrovsky, 2005), and it would be important to examine whether this locus affects aging.

V. Hormones, Nutrition, and Life Span

A. Nutrition Regulates JH and 20E Synthesis

In insects, nutritional status is well known to affect both development and reproduction. Proper nutrition provides an organism with the energy required for development, growth, reproduction, and somatic maintenance. In some insects, insufficient nutrition suppresses egg development by inhibiting CA or ovarian function, as seen in mosquitoes and some higher dipterans (Wheeler, 1996). For example, vitellogenesis in the mosquito *Aedes aegypti* depends on interactions among JH, 20E, and other endocrine factors. These hormones, however, are released only after a blood meal (Dhadialla & Raikhel, 1994). A protein-rich diet is also necessary to initiate JH and 20E synthesis in other higher flies, such as the house fly *M. domestica* (Adams & Gerst, 1991, 1992) and the black blow fly *Phormia regina* (Liu *et al.*, 1988; Yin & Stoffolano, 1990; Yin *et al.*, 1990; Zou *et al.*, 1989).

In *D. melanogaster*, yeast appears to be a major stimulus in activating the endocrine system. For example, DILP production by the IPCs is activated upon yeast feeding in adult flies that were yeast-deprived as third instar larvae (Tu & Tatar, 2003). DILPs may be required for JH and 20E synthesis because reduced insulin signaling, as observed in *InR* and *chico* mutants, is known to result in JH and 20E deficiency. This hypothesis may be supported by the observation that JH synthesis is elevated upon adult yeast feeding in flies that were yeast-deprived as third instar larvae as compared to control flies yeast-starved both during third larval instar and adulthood (Tu & Tatar, 2003). Thus, these results suggest that JH production is directly regulated by adult feeding, not larval feeding. 20E also responds to nutrition in the fruit fly. For instance,

flies fed on a diet consisting only of sugar and water produce much less ecdysteroids at both 24 hours and 48 hours of adulthood than yeast-fed control flies, whereas re-feeding female yeast after a 24-hour yeast starvation period induces a high ovarian ecdysteroid production (Schwartz *et al.*, 1985). The above results therefore suggest that the effects of dietary manipulation on life span may be mediated by nutrition-induced changes of pro-aging hormones such as JH and 20E.

B. Nutrient Sensing Pathways Affect Aging Through Hormones

An adequate physiological response to nutrient levels is a key determinant of survival and somatic maintenance. In *Drosophila*, responses to nutrition are mediated by highly conserved nutrient sensing pathways, such as the insulin/IGF signaling pathway and the target of rapamycin (TOR) pathway, both of which are major growth regulators (Chen *et al.*, 1996; Ikeya *et al.*, 2002; Oldham *et al.*, 2000).

For the case of insulin signaling, the gene expression of *dilp3* and *dilp3*, but not *dilp2*, is regulated by nutrient availability, and overexpressing *dilp1–7* promotes growth (Ikeya *et al.*, 2002). Interestingly, recent work shows intimate connections between DILP expression, insulin signaling, and the fat body, which presumably represents a major nutrient sensing tissue in the fly. The fat body is an important target for both JH and 20E, a major organ for the synthesis of vitellogenins and antibacterial peptides, and also has essential nutrient storage functions.

In *C. elegans,* activation of the gene *daf-16*, a forkhead transcription factor downstream of insulin signaling, is required for the life-span extension induced by reduced insulin signaling (Kenyon *et al.*, 1993). Interestingly, in adult *Drosophila*, limited activation of *dFOXO*, the homolog of *C. elegans daf-16*, in the head (pericerebral) fat body uniquely reduces expression of neuronally synthesized DILP2 (but not that of DILP3 and DILP5), represses endogenous insulin-dependent signaling in the abdominal fat body, and extends life span (Hwangbo *et al.*, 2004). These nonautonomous and systemic effects suggest that the adult head fat body is a major endocrine site (Hwangbo *et al.*, 2004). Furthermore, these studies indicate that DILPs 2, 3, and 5 may have different physiological functions (Hwangbo *et al.*, 2004; Ikeya *et al.*, 2002). DILP2 responds to long-term downregulation of insulin signaling, whereas DILP3 and DILP5 do not. In contrast, DILP3 and DILP5 respond to acute starvation, whereas DILP2 does not. Thus, DILP3 and DILP5 may mediate the short-term response to changes in nutrient levels, whereas DILP2 may adjust a fly's life history in response to sustained periods of reduced insulin signaling. Clearly, it would be very informative to examine the effects of individual DILPs on aging and on the production of JH and 20E in different developmental stages and under different nutritional conditions (also see Figure 15.1).

In addition to insulin signaling, the *TOR* and the *slimfast* pathways are major nutrition sensing pathways regulating growth through the fat body (Colombani *et al.*, 2003). The involvement of the fat body in insulin, *slimfast,* and *TOR* signaling and its response to nutrition challenge suggest that this tissue is important for both development and the regulation of aging. It is thus conceivable that downregulating the *slimfast* and *TOR* pathways in the fat body may increase longevity. Indeed, recent work demonstrates that constitutive suppression of the *dTOR* pathway, either ubiquitously or in the fat body only, can extend life span (Kapahi *et al.*, 2004). This life-span extension depends on nutritional conditions, suggesting a possible link between the *TOR* pathway and dietary restriction (Kapahi *et al.*, 2004). For example, in yeast

(*Saccharomyces cerevisiae*), the *TOR* pathway mediates cell growth in response to nutrient availability, in part by inducing ribosomal protein gene expression through histone acetylation (Rohde & Cardenas, 2003). Thus, although the longevity extension by downregulation of *TOR* may relate to caloric restriction, the underlying mechanisms remain unclear. Interestingly, the *dTOR* and *slimfast* pathways are known to interact with the insulin signaling pathway (Colombani *et al.*, 2003; Hafen, 2004; Oldham & Hafen, 2003), but whether and how *dTOR* and *slimfast* affect hormones such as JH and 20E remains unknown.

C. Connection Between Caloric Restriction, Hormones, and Life Span

To date, caloric restriction has been one of the most effective interventions extending life span in model organisms, including yeast, nematodes, flies, and rodents (Kenyon, 2001; Masoro, 2000; but see Carey *et al.*, 2002). Similarly, as discussed above, nutrition has major effects on the production of hormones (DILPs, JH, and 20E) that are intimately involved in the regulation of aging. It is thus interesting to speculate that the longevity effects of caloric restriction may be mediated by hormones.

In *Drosophila*, dietary restriction affects both reproduction and age-specific mortality (Good & Tatar, 2001). For instance, low adult nutrition induces an arrest in early stem-cell proliferation and alters the frequency of cell death at two pre-vitellogenic checkpoints; this response requires intact insulin signaling (Drummond-Barbosa & Spradling, 2001). Similarly, caloric (or dietary) restriction can dramatically extend fly life span (Chapman & Partridge, 1996; Chippindale *et al.*, 1993). In contrast, complete yeast starvation of adult flies shortens life span (Chippindale *et al.*, 1993; Good & Tatar, 2001; Tu & Tatar, 2003), suggesting that yeast is a crucial dietary component for survival and somatic maintenance.

Yeast restriction during late development may silence insulin signaling throughout metamorphosis into adulthood, and thereby extend life span. To test this hypothesis, Tu and Tatar (2003) studied aging in adult flies that were yeast-deprived as third instar larvae. As expected, adult flies from yeast-deprived larvae phenocopied insulin signaling mutants by exhibiting prolonged developmental time, small body size, reduced ovariole number, and reduced fecundity (Tu & Tatar, 2003). Furthermore, yeast deprivation reduced insulin signaling: adult flies from yeast deprived larvae had reduced numbers of insulin-positive vesicles. However, unlike constitutive insulin signaling mutants of *InR* or *chico*, adults from yeast-deprived larvae did not exhibit decreased age-specific mortality. Interestingly, yeast feeding increased both insulin-like peptide and JH levels in adult flies from yeast-deprived larvae as compared to flies that were yeast-deprived throughout both their larval and adult life (Tu & Tatar, 2003). This suggests that adult insulin and JH are regulated by adult nutritional state and that slowed aging specifically requires reduced insulin signaling or JH deficiency in the adult.

A better understanding of the interplay between nutrition and hormones in affecting aging is likely to come from genetic analysis. For example, recent findings show that mutations in the gene encoding the Rpd3 histone deacetylase, likely to be involved in caloric restriction, promote life span (Rogina *et al.*, 2003). Interestingly, the histone deacetylases Sin3A/Rpd3 interact with the EcR/USP complex (Tsai *et al.*, 1999), suggesting that they may respond to JH and 20E signaling. Furthermore, in yeast, caloric restriction extends life span by activating Sir2, a member of the sirtuin family of NAD^+-dependent protein deacetylases.

In *C. elegans*, a homolog of Sir2 appears to act in the insulin signaling pathway upstream of DAF-16; overexpression of the *Sir2* gene extends worm life span in a *daf-16* dependent manner (Tissenbaum & Guarente, 2001). Sir2 can also be activated by several sirtuin-activating compounds (STACs) found in plants. For example, the natural compound resveratrol, found in red wine, activates sirtuins in both *C. elegans* and *Drosophila* and extends both worm and fly life span (Wood *et al.*, 2004). The life-span extension induced by resveratrol seems to be independent of caloric restriction: resveratrol does not increase life span in calorically restricted long-lived worms and flies, suggesting that resveratrol affects life span through a mechanism related to caloric restriction (Wood *et al.*, 2004). It is noteworthy that the structure of resveratrol is somewhat similar to that of JH or 20E, with multiple six-carbon rings and long carbon chain branches, and it will be interesting to determine whether and how JH and 20E signal through a sirtuin pathway to regulate aging under starvation conditions. However, in summary, our current understanding of how nutrition and hormonal signaling interact in affecting life span remains very limited.

VI. Hormonal Effects on Stress Resistance and Immunity

A. Hormones and Stress Resistance

Upregulation of stress resistance is thought to be one of the major ways for organisms to regulate senescence (Jazwinski, 1996; Johnson *et al.*, 1996; Lithgow, 1996). During the aging process, molecular chaperones such as heat-shock proteins are thought to combat stress-related senescent dysfunction. For example, transgenic *Drosophila* with extra copies of the heat shock protein gene *hsp70* show increased life span upon heat shock induction (Tatar *et al.*, 1997). Similarly, long-lived *InR* mutants or transgenic flies overexpressing *dFOXO* have elevated resistance to paraquat, a commonly used free-radical reagent (Hwangbo *et al.*, 2004; Tatar *et al.*, 2001b). Since *InR* mutants have low JH and 20E synthesis, it is possible that JH and 20E negatively regulate stress response in flies. For example, high JH and 20E levels may increase reproduction at the cost of decreased stress resistance and shortened life span. This model is indeed supported by recent experiments. For example, Salmon and colleagues (2001) found that methoprene application increased reproduction in female fruit flies yet decreased stress resistance, measured as the susceptibility to starvation and oxidative stress. Another good example comes from burying beetles (*Nicrophorus spp.*), in which starvation stress decreases both the JH titer and fecundity, whereas treatment with JH or a JH analog reduces starvation resistance (Trumbo & Robinson, 2004).

B. Hormonal Effects on Immunity

The optimal function of the immune system is of crucial importance for survival and somatic maintenance (Arlt & Hewison, 2004). For example, there are many well-known links between longevity and immune-response genes in mammals (Flurkey *et al.*, 2001), such as genes of the major histocompatibility complex (MHC, see review by Ginaldi & Sternberg, 2003). In contrast to mammals, insects such as *Drosophila* do not possess adaptive immunity but exhibit innate immunity to combat microbial infections (Hoffmann, 2003; Tzou *et al.*, 2002).

How hormones in general modulate immune function in insects is not well understood, but recent studies suggest that JH regulates immunity (Rantala *et al.* 2003; Rolff & Siva-Jothy, 2002). In the mealworm beetle (*Tenebrio molitor*) immunity (phenoloxidase levels) is reduced by mating activity, and this trade-off seems to be regulated by JH (Rolff &

Siva-Jothy, 2002). Application of the JH inhibitor fluvastatin increases immune activity; thus, JH specifically downregulates immune function (Rolff & Siva-Jothy, 2002). Similarly, Rantala and colleagues (2003), using the same species, have shown that the tradeoff between immune function and sexual advertisement (i.e., pheromone production) is mediated by JH. Thus, JH, a major gonadotropic hormone, has negative effects on immune function. This observation is interesting in view of the fact that reproductive hormones in vertebrates can often have negative effects on the immune system, as is the case for testosterone (e.g., Casto *et al.*, 2001).

[AU11]

To initiate an improved understanding of hormonal effects on immunity, our laboratory has recently begun to explore the effects of JH on primary immune response genes in *Drosophila* (M. Tatar, unpublished). In this preliminary experiment, flies were yeast-starved for 5 days to lower their endogenous JH titer and to synchronize their physiology. Subsequently, the JH analog methoprene was topically applied to individual flies, using ethanol-treated flies as control. RNA transcript levels from these two groups were then analyzed using Affymetrix gene chips (two replicate chips per group). From these data, with the FatiGO software (Al-Shahrour *et al.*, 2004), we find that genes with functions for response to biotic stimuli are relatively enriched by JH treatment. This gene ontology category includes genes involved in response to microbial infection, starvation, and oxidative stress. Our set of JH responsive genes (criterion: at least two-fold change in gene expression as compared to the untreated control) consisted of 270 probe sets (160 downregulated and 110 upregulated, of a total 14,009 sets with 6,142 annotated). Noticeably, for this set, 12.9 percent of the genes showing changes in gene expression belong to the category "response to biotic stimuli."

To test whether different categorical responses represent the biological effect of JH as distinct from a chance observation, we compared the observed representation within gene ontology categories to the expected representation assuming a process of random sampling. Genes involved in "monosaccharide metabolism" were significantly over-represented (3.91 percent observed relative to 1.21 percent expected from the annotated genome; Fisher's Exact Test, $P = 0.0032$). As well, the categories "response to pest/pathogen/parasite" and "response to biotic stimuli" were enriched relative to chance expectation (respectively: 3.91 percent versus 1.25 percent, $P = 0.0040$; 13.04 percent versus 7.55 percent, $P = 0.0051$). Genes involved in response to biotic stimuli (including parasites and pathogens) are thus significantly over-represented, suggesting that JH influences the state of adult defense response, including immune function.

Among the genes that show a response to biotic stimuli, is there any bias toward upregulation versus downregulation? For instance, among "cell organization and biogenesis" genes, 16.84 percent were upregulated relative to 4.41 percent downregulated (Fisher's Exact Test, $P = 0.0024$). In contrast, among genes in the "biotic response" category, 17.65 percent were downregulated compared to only 6.32 percent in the upregulated set (Fisher's Exact Test, $P = 0.0159$). Thus, although JH stimulates the expression of some of these genes, these data overall suggest that JH functions to suppress many aspects of cellular and systemic stress response (see Table 15.3).

VII. Conclusions

Here we have reviewed the effects of hormones on fly aging. The hormonal mechanisms affecting aging are manifest both as genetic polymorphisms (as seen in endocrine deficient mutants) and as phenotypic/physiological plasticity (as seen

Table 15.3

JH-Induced *Drosophila* Genes Involved in Defense or Immune Response. The table provides information on genes involved in defense, stress, or immune response whose expression is either upregulated or downregulated by application of the JH analog methoprene (M. Tatar, unpublished data). Further information on each of these genes can be found at: http:/ /flybase.bio.indiana.edu.

Gene Name	Function in Reaction to Biological Stimulus	Downregulated or Upregulated	Flybase Accession
Dorsal-related immunity factor	Defense and immune response, response to fungi	Up	FBgn0011274
Turandot M	Humoral defense mechanism	Up	FBgn0031701
Tetraspanin 96F	B-cell mediated immunity	Up	FBgn0027865
Traf3	Defense response	Up	FBgn0030748
CG6662	Defense response	Up	FBgn0035907
Tetraspanin 74F	Defense response	Up	FBgn0036769
CG6435	Defense response, defense response to bacteria	Down	FBgn0034165
CG7627	Defense response, response to toxin	Down	FBgn0032026
JH expoxide hydrolase 2	Defense response, response to toxin	Down	FBgn0034405
Drosocin	Defense response to gram-positive and -negative bacteria	Down	FBgn0010388
Immune induced molecule 23	Defense response	Down	FBgn0034328
PHGPx	Defense response, response to toxin	Down	FBgn0035438
Ejaculatory bulb protein III	Response to virus	Down	FBgn0011695
CG12780	Gram-negative bacterial binding, defense response	Down	FBgn0033301
Metchnikowin	Antibacterial and antifungal humoral response	Down	FBgn0014865
CG1702	Defense response, response to toxin	Down	FBgn0031117
CG6426	Defense response to bacteria	Down	FBgn0034162
CG5397	Defense response	Down	FBgn0031327
CG13422	Defense response to gram-negative bacteria	Down	FBgn0034511
CG10307	Defense response	Down	FBgn0034655
Transferrin 1	Defense response	Down	FBgn0022355
18 wheeler	Antibacterial humoral response, immune response	Down	FBgn0004364
Diptericin B	Antibacterial humoral response	Down	FBgn0034407
Hemolectin	Defense response	Down	FBgn0029167
Diptericin	Defense response to gram-negative bacteria	Down	FBgn0004240

(*continues*)

Table 15.3 (*Cont'd*)

Gene Name	Function in Reaction to Biological Stimulus	Downregulated or Upregulated	Flybase Accession
CG1681	Defense response	Down	FBgn0030484
CG2736	Defense response	Down	FBgn0035090
CG8336	Defense response	Down	FBgn0036020
takeout	Behavioral response to starvation	Down	FBgn0039298
CG18522	Defense response; reactive oxygen species metabolism	Down	FBgn0038347

in reproductive diapause and senescence plasticity). These sources of variation are likely to involve common endocrine regulatory mechanisms.

As in worms and rodents, reduced insulin signaling can slow aging in the fly (see Figure 15.1). Downstream of insulin signaling, fly aging seems to be regulated by secondary hormones, such as the sesquiterpenoid JH and the steroid 20E. Several lines of evidence indicate that both JH and 20E deficiency can dramatically slow aging while increasing stress resistance or immune function. Insects such as *Drosophila* may use JH and 20E to adaptively coordinate (and, if necessary, trade off) the expression of the "reproductive function" versus the "survival function" in response to environmental cues such as temperature or nutrition. Yet, although in some cases JH and 20E co-affect life span and reproduction, endocrine interventions typically slow aging without causing costs in reproduction. The conditional uncoupling of reproduction and survival will thus require us to rethink classical models for the evolution of senescence, including Williams' (1957) antagonistic pleiotropy hypothesis and the concept of senescence tradeoffs caused by costs of reproduction. Recent work on the *Drosophila* fat body has also advanced our understanding of the tissue specificity of endocrine effects on life span. The fat body appears to regulate aging both autonomously and non-autonomously by affecting insulin signaling and probably secondary hormones such as JH and 20E.

Despite these insights into the endocrine control of aging in *Drosophila* and other insects, there remain many unresolved and difficult questions. For instance, we need to know how general the effects of JH and 20E upon aging are: Does JH or 20E deficiency invariably extend life span, or is this a conditional effect, for instance depending on species, nutrition, temperature, and sex? Do both JH and 20E have identical effects on life span, or do they differ and how? Answering these questions will depend on suitable tools for manipulating JH and 20E signaling. For example, do synthetic JH and 20E inhibitors or inhibitory neuropeptides such as allatostatins extend life span? Does overexpression of JH- or 20E-degrading enzymes in transgenically engineered insects slow aging? Similarly, the molecular details of JH and, to a lesser extent, 20E signaling remain relatively poorly understood. This raises many difficult questions: What is the molecular nature of the JH receptor, and how does it modulate aging? Do most, if not all, JH and 20E signaling genes affect aging? Through which downstream genes do the JH and 20E signaling pathways regulate aging? How exactly does insulin signaling affect JH and 20E signaling? What is the nature of the secondary hormones mediating the effects of fat body insulin signaling on whole-animal

life span? How do caloric restriction and hormones interact at the molecular level to affect longevity? We can make further observations and ask related questions. For example, JH and 20E seem to modulate evolutionarily relevant tradeoff relationships between fitness components. How, mechanistically, do JH and 20E modulate these tradeoffs between reproduction and survival, stress resistance, and somatic maintenance? Are the endocrine loci affecting these tradeoffs genetically variable and subject to natural selection in wild populations? Finally, we may ask: Do other insect hormones than JH and 20E have major effects on the aging phenotype? What are the functional equivalents of JH and 20E in other organisms and how do they affect aging?

These are but a few of the most pressing questions to be addressed in the near future. To address them, we will need to combine molecular and evolutionary genetics, endocrinology, nutritional physiology, and biodemography. We have many hypotheses, patterns, and models, but little solid data. Understanding the genetic and endocrine regulation of aging remains a fundamental, yet promising, challenge for modern molecular biogerontology.

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