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Adenotrophic Viviparity in Tsetse Flies: Potential for Population Control and as an Insect Model for Lactation

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Abstract

Tsetse flies (*Glossina* spp.), vectors of African trypanosomes, are distinguished by their specialized reproductive biology, defined by adenotrophic viviparity (maternal nourishment of progeny by glandular secretions followed by live birth). This trait has evolved infrequently among insects and requires unique reproductive mechanisms. A key event in *Glossina* reproduction involves the transition between periods of lactation and nonlactation (dry periods). Increased lipolysis, nutrient transfer to the milk gland, and milk-specific protein production characterize lactation, which terminates at the birth of the progeny and is followed by a period of involution. The dry stage coincides with embryogenesis of the progeny, during which lipid reserves accumulate in preparation for the next round of lactation. The obligate bacterial symbiont *Wigglesworthia glossinidia* is critical to tsetse reproduction and likely provides B vitamins required for metabolic processes underlying lactation and/or progeny development. Here we describe findings that utilized transcriptomics, physiological assays, and RNA interference–based functional analysis to understand different components of adenotrophic viviparity in tsetse flies.

Keywords

Glossina; tsetse fly; lactation; adenotrophic viviparity; *Wigglesworthia*

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INTRODUCTION

Tsetse (Diptera: Glossinidae)

Tsetse flies (*Glossina* spp.) are members of Hippoboscoidea, a dipteran superfamily containing Glossinidae (tsetse flies), Hippoboscidae (louse flies, sheep keds), and Streblidae and Nycteribiidae (together known as bat flies). Within the Glossinidae, there are 33 extant taxa composed of 22 species, including five subspecies complexes forming 3 subgenera: *Fusca* (Austenina Townsend), *Palpalis* (Nemorhina Robineau-Desvoidy), and *Morsitans* (*Glossina* Wiedemann) (described in 74). *Morsitans* group flies are largely savanna and woodland inhabitants, and *Palpalis* group flies frequent riverine and lacustrine habitats (116). *Fusca* group flies largely inhabit the moist forests of West Africa, although *G. brevipalpis* occurs discontinuously in East Africa, Democratic Republic of the Congo, and Mozambique. The host specificity of the species groups varies; *Palpalis* group flies are strongly anthropophilic, whereas *Morsitans* and *Fusca* are more zoophilic.

Tsetse as Disease Vectors

Male and female adult tsetse flies are obligate blood-feeding vectors of pathogenic trypanosomes. Chief among these are *Trypanosoma* (*Trypanozoon*) *brucei gambiense* and *T. b. rhodesiense*, which cause fatal diseases in humans if untreated. Human African trypanosomiasis (HAT) caused by *T. b. gambiense* represents over 90% of cases and occurs in northwest Uganda, extending into the Central African Republic and southern Chad, along the Congo River north of Brazzaville, and by the Atlantic coast between Gabon and Equatorial Guinea (141). HAT caused by *T. b. rhodesiense* is present east of the Rift Valley, is zoonotic, and causes a more acute disease that is rapidly fatal if untreated. In addition to the impact of HAT, nagana [or, animal African trypanosomiasis (AAT)] caused by *T. b. brucei* and related trypanosomatids, *T. congolense* and *T. vivax*, prevents or limits access to 10 million square kilometers of Africa for cattle farming (124) and has wide implications for land use including constraints on mixed agriculture and lack of animal labor for ploughing (69). Economic losses in cattle production are estimated at US$1–1.2 billion owing to 3 million cattle deaths per year, with total agricultural losses from AAT estimated at US$4.75 billion per year (23). In 2000 the African Union recognized trypanosomiasis as “one of Africa’s greatest constraints to socio-economic development” and began a continent-wide elimination program, Pan African Tsetse Trypanosomiasis Eradication Campaign (PATTEC) (70).

Disease Control Methods

Drugs available for treatment of African trypanosomes are expensive (122). Disease management in late disease stages has a high rate of mortality (66, 99) and a high incidence of failure (22). The ineffectiveness of mammalian vaccines is due to the capacity for antigenic variation by the trypanosomes (59, 65). Active surveillance and patient treatment are essential for disease control but are too expensive to implement at times of low endemicity. Modeling of the different control strategies (whether targeting the animal reservoir, humans, or the vector) shows that vector control is the most efficient method to suppress outbreaks (32, 141). Vector control tools include aerial pesticide sprays (71),

topical insecticide applications for animals, traps/targets to reduce local tsetse populations (68), and sterile insect technique for isolated populations (134).

Unique Aspects of Tsetse Biology

A remarkable adaptation within the Hippoboscoidea, including *Glossina*, is adenotrophic viviparity. The nature of *Glossina* reproduction means that each female produces only 8–10 progeny during the course of her lifetime. Because of this low reproductive capacity, population control methods are highly successful. Glossinidae, and indeed all Hippoboscoidea, are exclusively hematophagous. This highly restricted nutritional ecology has required the establishment of associations with symbiotic bacteria to provide nutritional supplementation (see sidebar, *Wigglesworthia* Symbiosis and Its Role in Host Fecundity and Health of Progeny). Recently, the whole genome sequence (WGS) of *G. morsitans morsitans* has been completed (63), along with the WGS of its obligate endosymbiont, *Wigglesworthia glossinidia* (3, 112). The availability of genomic knowledge coupled with transcriptomic data and downstream functional analyses has expanded our understanding of tsetse's reproductive, nutritive, and symbiotic biologies. In this review, we describe the current state of knowledge on tsetse's reproductive anatomy, processes associated with the *Glossina* gonotrophic cycle (ovulation, oogenesis, larvigenesis, and parturition), milk production, and the role of tsetse's obligate symbionts in fecundity.

BASIC REPRODUCTIVE PHYSIOLOGY AND ANATOMY

Insect Reproduction

Animal reproduction can be divided into two major strategies: oviparity and viviparity (17, 90). Oviparous females deposit eggs that undergo embryogenesis outside the female. Viviparous females retain their eggs within the reproductive tract until after progeny emerge from the egg and give birth to live offspring. Viviparity is documented for 11 orders of insects (52, 90) and has evolved independently at least 60 times in several fly lineages (90). Among viviparous species, many are facultative (situational oviparity or viviparity) whereas others display obligate viviparity. Ovoviviparity is a third term used to describe reproductive mechanisms whereby embryos develop in the eggs while still in the mother and progeny emerge rapidly after eggs are deposited. Of interest, most, if not all, ovoviviparous insects can give live birth under specific situations, suggesting ovoviviparous insects should be more appropriately classified as facultatively viviparous. Obligate viviparity is further divided into lecithotrophic (nutrients provided only by the egg yolk) and matrotrophic (nutrients provided by the egg yolk and other routes) forms. There are three divisions of matrotrophic viviparity. In hemocoelous viviparity, embryos and larvae develop within the hemocoel and absorb nutrients from the hemolymph (52, 108). In pseudoplacental viviparity, embryos and larvae develop in terminal ovarian follicles and/or a uterus-like structure, such as the brood sac in cockroaches (52, 108, 123). Nutrients are provided by actively secreting, thickened cells that are usually follicular (cells that surrounded developing oocytes in earlier stages) and/or epithelial (52, 108, 123). In adenotrophic viviparity, embryos and larvae develop in the common oviduct (or uterus) and are nourished through glandular secretions from specialized organs (52); this form is employed by *Glossina*.

Adenotrophic Viviparity in Flies: An Unusual Characteristic

In Diptera, adenotrophic viviparity is limited to the superfamily Hippoboscoidea, which includes *Glossina*, the family Sarcophagidae (flesh flies), and the subfamily Mesembrinellinae (Calliphoridae) (49, 90). Within Sarcophagidae, only a single species (*Sarcophaga nigriventris*) may provide nourishment to progeny, whereas all members of Hippoboscoidea and Mesembrinellinae produce nutrients for their intrauterine larvae (90). Flies belonging to Mesembrinellinae provide milk-like products to their progeny via secretions from ectodermal glands in their enlarged spermathecae (49). Other previously identified lecithotrophic flies deposit larval progeny much larger than the eggs or have expanded spermathecae/accessory glands, suggesting that in utero nourishment among dipterans might occur more frequently than has been officially documented (90).

Members of Hippoboscoidea and *S. nigriventris* provide nutrient secretions via the accessory glands (milk gland, or uterine gland). In most flies, accessory glands are small and consist of only a few cells (4). In Hippoboscoidea, accessory glands are expanded into a large, branched organ that contains hundreds to thousands of cells, occupies much of the abdominal space, and is connected to the uterus to provision nutrients directly to feeding larvae (52, 84, 90).

Ovarian, Milk Gland, and Uterine Structure

Three critical adaptations that allow tsetse, along with other members of Hippoboscoidea, to harbor and feed intrauterine larvae are the reduction of ovaries and the expansion of the milk gland and the reproductive tract (Figure 1*a*,*b*). Tsetse ovaries have a reduced ovariole number, two ovarioles per ovary, relative to ovaries from other dipterans, which can contain dozens of ovarioles (Figure 1*a*,*b*). The milk gland that connects to the uterus expands throughout the abdomen as bifurcating tubules and is a permutation of the female accessory glands or paraovaria found in other flies/insects (Figure 1*a*,*b*). This structure consists of the common collecting duct and the proximal and distal tubules (104, 130, 131). The distal milk gland is composed of large columnar and pyramidal cells, which distend as secretory reservoirs accumulate milk secretions. The proximal section contains underdeveloped organelles associated with secretory components (104) (Figure 1*c*). The differential structure of the distal and proximal sections suggests that secretory products are produced in the distal tubules and that the proximal section likely acts as a conduit for milk transfer. The muscular common collecting duct has been suggested as a regulator of milk flow into the uterus and to the larvae (131). The uterus is greatly expanded compared with the reproductive tract of other flies (106). The uterine wall is covered by tracheated muscle lined by a layer of squamous epithelium with cuticular intima (104, 131). A ridged, tongue-like structure with cuticular horns known as the choriothete is distinct from the uterus and causes a thickening of the anteroventral side of the uterine wall due to underlying epithelium with cuboidal cells, considerable connective and muscle tissue, and tight folding of projections toward the uterine lumen (104, 115, 128). The cuboidal cells of the choriothete appear to be secretory (104, 115, 128). Two functions for the choriothete have been suggested. First, the choriothete may facilitate removal of the egg chorion and larval cuticle both mechanically and through the secretion of sticky mucoproteins (104, 115, 128). Second, the choriothete

may serve as an anchor for the larva throughout lactation to ensure correct posture for feeding (115, 128) (Figure 1*d*).

Life Cycle of Tsetse Flies

Females deposit a single larva per gonotrophic cycle (131). The first gonotrophic cycle begins when teneral tsetse females emerge from the puparium. At this time, the first oocyte begins development in the right ovary. Mating typically occurs 3–5 days after adult emergence and sperm are stored in the spermathecae until fertilization during ovulation. The first ovulation occurs ~10 days after adult emergence, followed by intrauterine embryogenesis and larvigenesis (Figure 2). During intrauterine embryonic and first instar larval development, a second oocyte begins development within the left ovary. Embryogenesis takes 3–4 days and is followed by 5–6 days of larval development (120, 131). The second oocyte matures before larval development is completed. Females give birth to their first progeny ~20 days posteclosion. Larvae are deposited on an appropriate substrate and burrow into the ground to pupariate within 1–2 h (131), and adults eclose after 30 days. The fully developed second oocyte in the left ovary is ovulated 20–35 min after larviposition. This development cycle allows deposition of the second larva 9–10 days after the first birth. The duration of progeny development depends on diet and environmental factors, including blood meal availability and host type (120, 131) as well as exposure to abiotic/biotic stressors that interfere with oogenesis or milk production (61).

OOGENESIS AND OVULATION IN TSETSE

Oogenesis

Individual oocyte development in tsetse is similar to that observed in other flies within the brachyceran suborder. The viviparous nature of tsetse's reproductive biology adds a layer of complexity to the system and places significant constraints on the resources utilized for oogenesis in terms of physical space, nutrition, and timing owing to intrauterine larval development (26). Oogenic development in tsetse is conserved relative to that in other related dipteran species and is detailed for a number of fly species (2, 47, 62, 72, 73, 94). Only a single oocyte develops at a time while the remaining three ovarian follicles are held in a state of arrest that is broken upon ovulation of the mature oocyte. Oocyte development alternates between the right and left ovaries during each gonotrophic cycle (118, 119). The mechanism by which tsetse develop only a single oocyte when all ovarioles are exposed to the same hormonal, nutritional, and chemical milieus remains unknown.

A key stage of oocyte development is vitellogenesis, which is the synthesis, secretion, and uptake of yolk proteins. Brachyceran fly species produce lipase-derived yolk proteins (57, 117). Most species have multiple yolk protein genes, two to five depending on the species (19, 57, 87). However, tsetse is unique in that it carries only a single yolk protein gene orthologous to *Drosophila yp2* (6). In addition to the reduction in yolk protein genes, the tissue specificity of tsetse yolk protein genes differs from that of most brachyceran flies such as *Drosophila*, in which yolk proteins are produced by both the fat body and ovarian follicle cells (20, 46, 64, 67, 142). In tsetse, yolk protein is synthesized only by the follicle cells of the developing oocyte (6, 58, 62), as in *Stomoxys calcitrans* (29, 60). The *Drosophila* yolk

protein gene (*yp2*) is expressed in a follicle-cell-specific manner (48), suggesting that follicle-specific expression for *yp2* orthologs likely occurs in most brachyceran flies.

Regulation of oogenesis in Diptera is a complex process and integrates signaling from multiple sources. The regulatory mechanisms associated with oogenesis in the Nematocera (primarily mosquitoes) are well characterized owing both to their significance as vectors and to the phenomenon of anautogeny (the requirement of a blood meal to initiate oogenesis) (reviewed in 7, 107). This process is regulated via signals from ecdysteroids (E), juvenile hormones (JH), insulin/insulin-like growth factors, and target of rapamycin (TOR), as well as peptide hormones such as ovarian ecdysiotropic hormone (OEH) and oostatic hormone (18, 21, 43, 50, 53–55, 84).

Comparative analysis of the literature on regulation of oogenesis in brachyceran species is less conclusive. All the aforementioned signaling pathways associated with oogenesis in Nematocera are also necessary for brachyceran oogenesis (1, 28, 33, 109, 110, 126, 127). However, these pathways function differently within the context of Brachycera. In most brachycerans (under optimal conditions), oocyte development is a constitutive process rather than an “on” or “off” state as observed in anautogenous mosquitoes. This is not to say that the process is unconditional, as nutritional status influences brachyceran oogenesis (125). Little is known regarding hormonal regulation of oogenesis in tsetse. As observed in other related flies, JH appears to play a significant role in oogenesis in tsetse. Flies subject to the removal of the biosynthetic source of JH (the corpora allata) soon after eclosion are unable to provide viable oocytes. This effect is reversible by the ectopic application of a JH analog (42). Further analysis is needed to understand the mechanisms by which oogenesis in tsetse is regulated.

Ovulation

Ovulation in tsetse is coordinated by the mating and pregnancy status of the fly. Females carrying an intrauterine embryo or larva will not ovulate and will hold the next developed oocyte until parturition or abortion. The presence of a developing or developed oocyte within the ovaries inhibits oogenesis in the other three follicles. This stasis is broken upon parturition or abortion of the primary offspring and ovulation of the penultimate oocyte, which occurs within 24 h of parturition. This cycle implies that the presence of an intrauterine offspring directly or indirectly inhibits ovulation and oogenesis beyond the penultimate oocyte. Detailed physiological analyses have determined the mechanisms regulating ovulation in tsetse. The two key inputs regulating ovulation are mating status and oocyte development. Mating status is determined by mechanical stimuli resulting from copulation, which must occur for at least 1.5–2 h (27, 120).

The mating status of a female does not trigger ovulation, as most females mate approximately 2–3 days posteclosion and do not ovulate until their first oocyte is ready at 8–10 days posteclosion (131). Injection of hemolymph or ovary/oviduct extracts from mated ovulating females into mature virgin females stimulates ovulation, which indicates the presence of a hemolymph or reproductive tissue-borne regulatory factor (28, 113).The presence of a mature oocyte in mated females appears to stimulate the release of an ovulation-stimulating factor into the hemolymph. This substance may be synthesized within

the median neurosecretory cells (MNCs) and transported to a neurohemal organ, and it appears to require cyclic AMP as a second messenger (36). A study in *G. austeni* showed that ablation of the brain MNCs before or after mating in teneral females disrupts ovulation (45). The development of new proteomic and metabolomic analyses will advance these classic physiological studies and will facilitate the identification of factors regulating ovulation in tsetse.

TSETSE LACTATION AND UNDERLYING MECHANISMS

Structural Changes in the Milk Gland Associated with Lactation

Milk production is a complex process requiring massive physiological changes to support the growth of a larva that increases over 100-fold in dry mass over a 6-day period (37, 80). These changes occur in two major organs: the milk gland and the fat body. The fat body undergoes a two- to threefold increase in lipid content before the first lactation cycle and again following each parturition in subsequent dry phases. These lipid reserves are broken down and mobilized to the milk gland during lactation (4, 5, 11, 81). Structural changes in the milk gland are inverse to those of the fat body (37). Distal milk gland tubules are 30–40 μm wide prior to lactation and increase to 80–100 μm at the midpoint of lactation (37). Following parturition, the tubules revert to their prelactation width (37). The increased size of the milk gland corresponds to increased levels of rough endoplasmic reticulum and Golgi apparatuses (37, 56), which function in the production of milk proteins (111, 129, 131), and enlarged secretory reservoirs filled with milk products (8, 37).

Milk products are added to the secretory reservoirs by merocrine secretion (87, 131). A porous plug termed the rete retains the reservoir contents (56, 86). The volume of the secretory reservoirs increases nearly 100-fold between parturition and day 6 of the subsequent pregnancy cycle (8, 37, 86). A net loss of materials from the reservoir is observed during the remainder of the second pregnancy cycle (days 6–8) (56, 86). Histochemical analyses indicate that the primary contents of the secretory reservoir are proteins and phospholipids (86). These contents are comparable to the gut contents of third instar larvae with the exception that there are high levels of triacylglycerides (TAGs) in larvae, suggesting a conversion of phospholipids to TAGs, which are easily stored in the larval gut (31).

Metabolic Aspects Underlying Tsetse Lactation

During larvigenesis, a combination of amino acids, free fatty acids (FFAs), and diacylglycerols (DAGs) is transported to the milk gland via the hemolymph to support milk synthesis (5, 81, 97, 131). These nutrients are generated either directly from blood meals ingested during larvigenesis or indirectly from the breakdown of lipid reserves (75, 96, 106, 134). Lipids are the primary nutritional component of milk during the early phases of pregnancy, and proteins are critical in late lactation as lipid reserves decline (82, 96, 132).DAG, which is transported by lipophorin from the fat body to the milk gland, is the main lipid moiety provided for milk fat synthesis (15, 76, 101, 105). FFAs in the hemolymph are directly absorbed by the milk gland through passive diffusion and/or an uncharacterized protein-mediated mechanism (75, 121, 133). Lipids are likely directly

incorporated into the milk to act as the milk fat source. Free amino acids are transferred into the milk gland through uncharacterized amino acid transporters (76, 79, 95, 96, 105). A key aspect of tsetse's metabolic physiology is the reliance on proline, rather than sugars, as a hemolymph-borne energy source (24, 25, 88). Proline is obtained primarily from digested blood and by conversion of alanine to proline via the breakdown of fat body lipids (24, 25, 88, 89). Thus, amino acids fulfill dual roles by providing energy to support milk production and functioning as building blocks for milk protein synthesis.

Proteinaceous Components of Tsetse Milk

We have provided in Figure 3 a synopsis of the roles of the major proteins found in tsetse milk. Twelve major milk proteins in *G. morsitans* have been identified. These proteins represent 47% of all female gene transcripts during lactation and less than 4% during the dry period (12). Osir et al. (102) discovered the first milk protein, Milk Gland Protein 1 (MGP1), and Attardo et al. (6, 8) determined its full sequence. Following the identification of MGP1, a family of unrelated tsetse-specific milk proteins, MGP2–10, was identified (12, 144). Proteomic analysis of larval gut contents validated their role as milk components (12). In addition, analyses of the milk transcriptome/proteome identified an acid sphingomyelinase (aSMase1) and a transferrin protein as major constituents of tsetse milk. The aSMase1 protein is activated by the low pH of the larval gut and aids in lipid digestion (13). Little is known about the physiological role of transferrin in the milk (51, 126). In addition to the major milk proteins, proteomic analysis identified multiple minor constituents (12). One of these is peptidoglycan recognition protein-LB, PGRP-LB (135), which suppresses immune function to facilitate successful transfer of bacterial symbionts between mother and offspring.

Transcriptional Regulation of Milk Proteins

Milk protein gene expression is tightly regulated and shows a rapid increase in transcript abundance during lactation, followed by an abrupt decline within 24 h of parturition (4, 12). The completion of the WGS from *G. morsitans* has aided analysis of gene regulatory sequences. However, the inability to generate transgenic tsetse flies because of their reproductive physiology required the analysis of tsetse regulatory elements in the heterologous *Drosophila* system (4). Transformation of *Drosophila* with the tsetse *mgp1* regulatory sequence fused to a reporter gene showed conservation in tissue-specific expression between species. *Drosophila* bearing this construct show reporter gene expression restricted to the female accessory glands. Comparative bioinformatic analysis of the minimal *mgp1* promoter sequence and putative regulatory sequences from the other MGP genes identified a single conserved transcription factor binding site for the homeodomain transcription factors. Differential RNA-seq analysis and tissue-specific qPCR analyses revealed a candidate homeodomain factor, *ladybird late* (*lbl*), whose knockdown yielded a reduction in fecundity owing to reduced milk protein levels (4). LBL therefore likely represents at least one of the factors regulating the expression of tsetse milk protein genes.

Water Transfer into the Milk Gland

In addition to proteins and lipids, water represents the third and most abundant component of the milk (31). Ten genes coding for aquaporins (AQPs), transmembrane proteins that act as water channels, were identified in the tsetse genome—two more than are known from any other insect (14). Two AQP genes, *Drosophila integral protein A* (*DripA*) and *DripB*, are highly expressed in the milk gland (14). Suppression of expression of either *DripA* or *DripB* had only a minor impact on progeny production. However, combined suppression of their expression drastically reduced progeny output (14). Progeny from AQP-deficient mothers showed a significant reduction in water content (14). These results indicate that AQPs play an important role in milk hydration.

Hormonal Regulation of Milk Production, Larvigenesis, and Birth

The tsetse lactation/larvigenesis cycle is hormonally regulated. Cross talk among JH, E, IIS, the poorly defined parturition hormone (PH), and various neurosecretory factors is necessary to orchestrate lactation and parturition (11, 34, 41, 42, 77, 78, 83). Topical application of the developmental hormones JH or E induces larval abortion in *G. morsitans* (34). These larvae abort as second or third instars, lack complete cuticle melanization, and are unable to pupariate. The mechanism behind this phenomenon remains poorly characterized. However, larvae can survive for several days in utero in a starved female, arguing against starvation as a mechanism for the rapid abortion induced by E or JH analogs (34).

Like *Drosophila*, tsetse express two JH receptor paralogs, the bHLH PAS family transcription factors *Methoprene tolerant* (*Met*) and *germ-cell expressed* (*gce*) (10). The presence of duplicate JH receptors is thus conserved within Muscomorpha (137), and these proteins regulate divergent target genes as in *Drosophila* (40, 93). Investigators have examined JH action during tsetse reproduction by manipulating hormone titer (11, 34, 41, 42, 78, 83). Allatectomy (CAX), or removal of the corpus allatum, has been a popular approach for removing circulating JH in adults. As shown by Ejezie & Davey (42), allatectomized *G. austeni* did not produce offspring unless given exogenous JH. The timing of CAX was crucial—early allatectomized flies failed even to produce vitellogenic oocytes and late CAX occasionally allowed flies to produce some offspring. In contrast, Langley& Pimley (83) observed no effect of CAX on *G. morsitans* reproduction, perhaps reflecting inherent differences among tsetse species or surgical technique.

Lipid homeostasis between stored TAGs and milk-borne lipids is critical for tsetse lactation (5). The release dynamics of adipokinetic hormone (AKH), a lipid-synthesis-stimulating factor from the corpus cardiaca, closely follows the pregnancy cycle, showing a dramatic increase to promote lipid breakdown during mid-pregnancy (11, 105, 106). Both the AKH system and Brummer lipase, a downstream target of the IIS pathway, are required for tsetse lactation; knockdown of either inhibits the breakdown and utilization of stored lipids (5). Additionally, JH can affect milk production. The average volume of the corpora allata in *G. austeni* fluctuates in accordance with the milk gland, reaching maximum size 2 days before both maximal milk gland size and synthetic activity during each cycle (41). CAX induces fat body hypertrophy and suppresses milk production; JH replacement therapy restores the capacity for lactation (42). A recent molecular analysis demonstrated that lipogenic genes,

lipid levels, and JH-/IIS-associated gene transcripts show maximal levels at the onset of lactation, followed by minimal levels at larviposition (11).These results suggest that JH plays an important role in maintaining lipid stores and lipogenesis by the fat body that precedes lactation. Thus, lack of JH likely interferes with the transition between dry and lactating periods, particularly by affecting lipid homeostasis, impairing milk gland function (11, 42).

Parturition

The identity of PH, a factor that induces larval expulsion by pregnant females, remains enigmatic. Some evidence suggests that both the mother and the larva play active roles in orchestrating the timing of parturition (35, 38).With regard to humoral factors, a uterine-derived substance with PH activity elicits abortion when it is injected during early pregnancy and parturition when injected during late pregnancy, but only in neck-ligated flies, suggesting the requirement of additional input from the central nervous system (39). PH activity was also observed in uterine extracts from six *Glossina* species and even in extracts from the common oviduct of the flesh fly *Sarcophaga bullata* (20). The final E peak during pregnancy has been postulated to initiate the release of PH (114), and E application alone increases both the frequency and the amplitude of uterine muscular contractions in vitro (3). Thus, PH activity perhaps results from the integration of several distinct molecules or pathways rather than a single, elusive bioactive compound.

Antioxidant Production During Lactation Protects Milk Gland Function and Maintains Fecundity

Tsetse females are fertile throughout their entire life span and undergo multiple pregnancies without showing significant reproductive senescence (77, 91). This is surprising because the tsetse milk gland generates a substantial amount of milk in a short period (12), which likely yields oxidative stress and damage. Tsetse females appear to manage reproduction-associated oxidative stress by upregulating antioxidant enzymes (Figure 3). Suppression of this response increased accumulation of oxidative damage and reduced reproductive output because of a diminished ability to produce milk proteins during subsequent reproductive cycles (91). When this response is suppressed in early reproductive cycles, cumulative damage and reproductive senescence occur (91). Thus, the antioxidant response is a critical mechanism that facilitates maximum reproductive output and prevents premature reproductive senescence due to oxidative stress during tsetse lactation (91).

COMPARATIVE ASPECTS OF MATROTROPHIC VIVIPARITY AND LACTATION

Matrotrophic Viviparity and Lactation-Like Systems Among Insects

Research on the adenotrophic viviparous biology outside of *Glossina* for other closely related Hippoboscoidea is sparse (85). Structurally, the milk glands from sheep keds and tsetse flies are quite similar; the only difference is that the secretory reservoir of sheep keds is bilobed (85). Histochemical analyses of the secretory reservoir suggest that sheep ked milk is similar to tsetse milk (85). Variation in milk gland cell size and the abundance of

endoplasmic reticulum from sheep ked glands is similar to that seen in tsetse during lactation (85). In contrast to tsetse, sheep keds have higher milk lipid content and a reduced dependence on stored nutrients derived from early blood meals, as female sheep keds are wingless and live in constant, direct contact with the host, allowing more frequent feeding by the mother.

Diptera outside the Hippoboscoidea, specifically *S. nigriventris* and members of Mesembrinellinae, likely produce milk products via accessory glands and the spermathecae, respectively (49, 91). Other nondipteran insects provide nourishment to developing embryos and larvae through hemocoelous or pseudoplacental viviparous mechanisms. Select dermapterans (52); the viviparous cockroach, *Diploptera punctata* (124, 143); aphids (16); and certain psocopterans (52, 108) undergo pseudoplacental viviparity (52). Hemocoelous viviparity in strepsipterans and dipterans has been documented (52, 108). Recent studies on the dermapteran *Arixenia esau* revealed that it uses a two-phase reproductive strategy termed pseudoplacento-uterotrophic viviparity, in which nutrients are provided to progeny in both the terminal ovarian follicle and the uterus (132). Two separate cell types associated with these tissues are responsible for nutrient production in *Arixenia* lactation (132). In *D. punctata*, developing progeny remain in the mother's brood sac and are provided milk-like substances via secretory cells in the brood sac walls (124, 143). For aphids, nutrients are provided through the amnio-serosal membrane that immediately surrounds the developing embryo (16). Outside these examples, little is known about nutrient transfer in other viviparous insect systems.

Similarities in Lactation Among Vertebrates and Invertebrates

Tsetse lactation shares many analogous features with mammalian lactation. First, tsetse flies undergo punctuated periods of milk synthesis and related metabolic activities, followed by rapid involution preceding an extended dry period (37, 131). Second, lipocalins are a conserved class of proteins found in tsetse and mammalian milk and likely transport specific hydrophobic moieties to the developing offspring (6). Third, sphingomyelinase activity in both tsetse and mammalian milk has been documented (13, 100). The tsetse MGP2–10 proteins appear analogous to mammalian caseins; they act as a major source of amino acids and phosphate and are critical to milk homeostasis (12). Unlike the caseins, the MGP2–10 family likely does not transport calcium, as only trace amounts of calcium are detected in tsetse milk (31). This finding is unsurprising because insects synthesize a chitin-based exoskeleton rather than a calcium-based endoskeleton (12). Another distinguishing feature of tsetse milk is that it lacks sugar, which is usually abundant in the milk of most mammals (31). This difference likely reflects tsetse's dependence on amino acids rather than glucose as a nutrient source within the blood. Finally, both mammalian milk and tsetse milk provide bacterial symbionts to juvenile progeny (see sidebar, *Wigglesworthia* Symbiosis and Its Role in Host Fecundity and Health of Progeny, above). These parallels are likely due to the fact that although tsetse and mammals are invertebrates and vertebrates, respectively, both are eukaryotic organisms that must provide nursing offspring similar milk-borne nutrients when no other food is available or utilized.

CONCLUSION

Tsetse flies are one of the most unique insect vectors owing to their adenotrophic viviparous reproduction. This peculiar reproductive strategy yields very few progeny and is an excellent target for population control. Recent studies, particularly the completed tsetse genome project and its associated functional genomics projects (4, 12, 14, 63, 91), along with previous biochemical and physiological studies, have helped elucidate the underpinnings of tsetse reproduction. The *Wigglesworthia* symbionts are also critical to reproduction and progeny development. The mechanisms by which this bacterial association is critical to tsetse physiology have begun to be established (3, 92, 103, 112, 136, 138, 139). The processes of tsetse reproduction and reliance on its symbionts provide targets for methods to suppress tsetse fecundity, including: (*a*) interfering with digestion and nutrient mobilization to disrupt milk production, (*b*) suppressing milk protein expression, (*c*) inhibiting the tsetse stress response during reproduction to disrupt subsequent gonotrophic cycles, and (*d*) disrupting the synthesis of vitamins and cofactors by *Wigglesworthia*. These targets could be exploited to generate tsetse-specific population control tools, thus reducing the prevalence of African trypanosomiasis.

Many aspects of tsetse reproductive physiology are analogous to those of mammalian reproduction. This begs the question of whether tsetse can be used as a model system to study lactation and maternal-derived nutrition. Availability of the sequenced tsetse genome (63) in combination with transcriptomic and physiological studies establishes the foundation for tsetse as a model organism. Initial studies of the role of the microbiome in immune development indicate that tsetse will find utility as a model to address complex questions associated with maternally derived nutrient provisioning (138, 139). Further studies regarding multigenerational effects in relation to changes in milk content and transfer of environmental toxicants in milk will benefit from the adoption of tsetse as a model species for lactation biology.

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Glossary

Adenotrophic viviparity	a form of matrotrophic viviparity in which progeny are provided nutrients within a uterus through glandular secretions until live birth
Matrotrophic viviparity	nutrients during gestation are provided by mechanisms beyond that of the egg yolk
Milk gland	common term used to describe the tsetse fly accessory gland; also known as the uterine gland
E	ecdysteroids

JH	juvenile hormone

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WIGGLESWORTHIA SYMBIOSIS AND ITS ROLE IN HOST FECUNDITY AND HEALTH OF PROGENY

Tsetse's vertebrate blood diet is rich in protein and lipids but low in specific micronutrients such as vitamins. To supplement their diet, all tsetse species and individuals harbor the enteric symbiont *Wigglesworthia*, a member of *Gammaproteobacteria*, intracellularly in bacteriocytes that form the bacteriome organ in the anterior midgut (3, 136). The symbiont is also present extracellularly in the lumen of the milk gland (9) and is transferred in milk to colonize the milk gland and gut bacteriome organs of the progeny. The *Wigglesworthia*-tsetse association is ancient and, as a consequence of its strict vertical transmission, displays concordant evolution with host species phylogeny (30). The WGS of *Wigglesworthia* from two tsetse species, *G. brevipalpis* and *G. morsitans*, have been determined, and both were found to be reduced to near 700 kb, approximately 10 times smaller than in most other Proteobacteria (3, 112). Despite this size reduction, the *Wigglesworthia* genome has retained the ability to synthesize B vitamins, including B_1 (thiamine), B_2 (riboflavin), B_3 (nicotinamide), B_5 (pantothenic acid), B_6 (pyridoxine), and B_9 (folic acid) (3, 112). Females that receive tetracycline-supplemented blood meals to clear *Wigglesworthia* cannot support larval development and abort their progeny (98, 99, 104). Loss of fecundity can be partially recovered by dietary supplementations with micronutrients from yeast extracts or B vitamins (92, 98, 103). Beyond the nutritional role *Wigglesworthia* plays in host fecundity, *Wigglesworthia* presence during larval development is essential for immune system maturation (138–140). Adult progeny that lacked *Wigglesworthia* during larval development were immunocompromised, characterized by deficient hematopoiesis (139) and gut immune integrity (138, 140), and were highly susceptible to trypanosome infections.

SUMMARY POINTS

1. Viviparity occurs in many insect systems, but adenotrophic viviparity is relatively rare, only definitively documented among members of the dipteran superfamily Hippoboscoidea and the subfamily Mesembrinellinae. The organ that produces the milk-like secretions differs between the two independent occurrences of adenotrophic viviparity evolution.
2. Adenotrophic viviparity in Hippoboscoidea features three major morphological changes: an expanded uterus to support the development of the intrauterine larva, an expanded accessory gland that secretes milk, and reduced ovariole development.
3. Tsetse larvae require substantial maternal transfer for development, resulting in a 50% reduction in maternal lipid reserves. Disruption of nutrient transfer results in delayed parturition or abortion.
4. Milk secretions contain 12 major proteins, which are expressed in accordance with the lactation cycle. During lactation, the major milk proteins represent 47% of the mother's transcriptional output.
5. Tsetse's obligate symbiont, *Wigglesworthia glossinidia*, is transmitted to the intrauterine larva via milk. In the absence of *Wigglesworthia*, emerging adult progeny are immunocompromised and infertile. Sterility resulting from the absence of *Wigglesworthia* can be rescued through dietary supplementation with yeast extract and B complex vitamins.
6. Tsetse lactation is functionally analogous to mammalian lactation, as are the major proteins involved. This is unsurprising because the basic nutritional requirements for most eukaryotes are equivalent.

FUTURE ISSUES

1. Regulation of oogenesis: What are the roles of hormonal and nutritional inputs (e.g., JH, E, insulin-like peptides, oostatic hormone, amino acid signaling) in the regulation of oogenesis? How does the tsetse fly restrict oocyte development to a single oocyte per gonotrophic cycle?
2. Regulation of ovulation and parturition: Which hemolymph-borne factors appear to regulate ovulation and parturition in tsetse? From what tissue do these factors originate? How is their activity regulated during oogenesis and larvigenesis?
3. Regulation of lactation-specific products: How is homeodomain factor activity regulated? What factors beyond a single homeodomain factor regulate milk production?
4. Prevention of larval movement until birth: What mechanisms are responsible for suppressing the movement of a larva (other than feeding) within the uterus, as an active, motile larva could damage the uterus? This reduced movement may be due to delayed neural development (44).
5. Role of obligate symbiosis in fecundity and host immunity: Which B vitamins and other symbiont-produced factors are required for milk production and maturation of larval immunity? For which host pathways and/or metabolites are these vitamins essential for maintaining functionality?
6. Convergent evolution: What are the common mechanisms of milk generation among insects, and what situations facilitated the transition from oviparity to matrotrophic viviparity? How is this transition similar to that which occurred in vertebrates?
7. Harnessing adenotrophic viviparity for vector control: Can vulnerabilities in the reproductive cycle be exploited to manage tsetse populations? Can compounds that interfere with milk protein regulation or *Wigglesworthia* fitness be used as tsetse-specific insecticides?

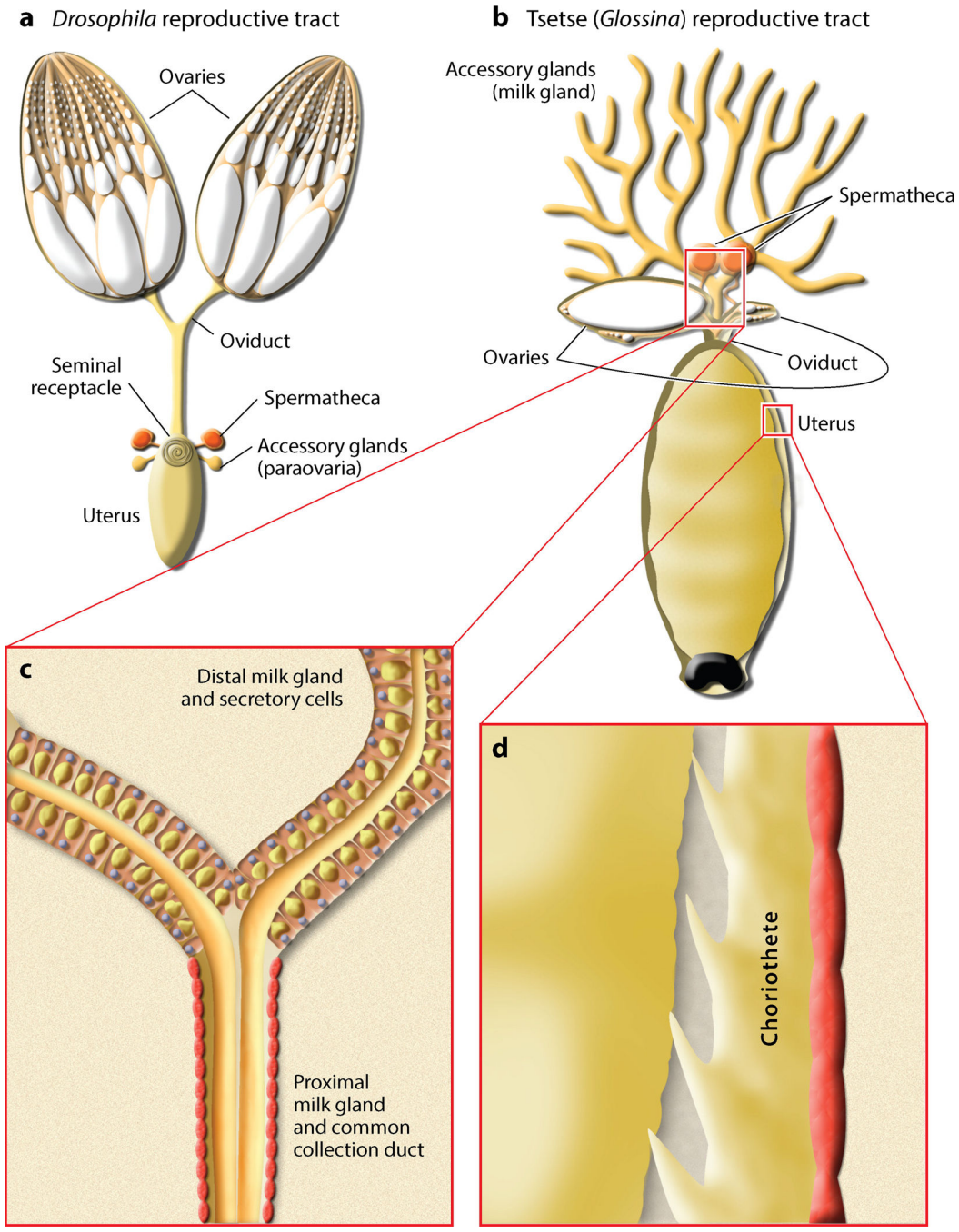

Figure 1.

Comparative morphology of dipteran reproductive organs. Illustrations of reproductive tract morphology from (*a*) *Drosophila* and (*b*) *Glossina*. (*c*) Magnified view of the *Glossina* milk gland tubules highlights the change in tubule and secretory cell physiology in the transition between the distal and proximal milk gland. (*d*) Magnified representation of the intrauterine wall and choriothete structure.

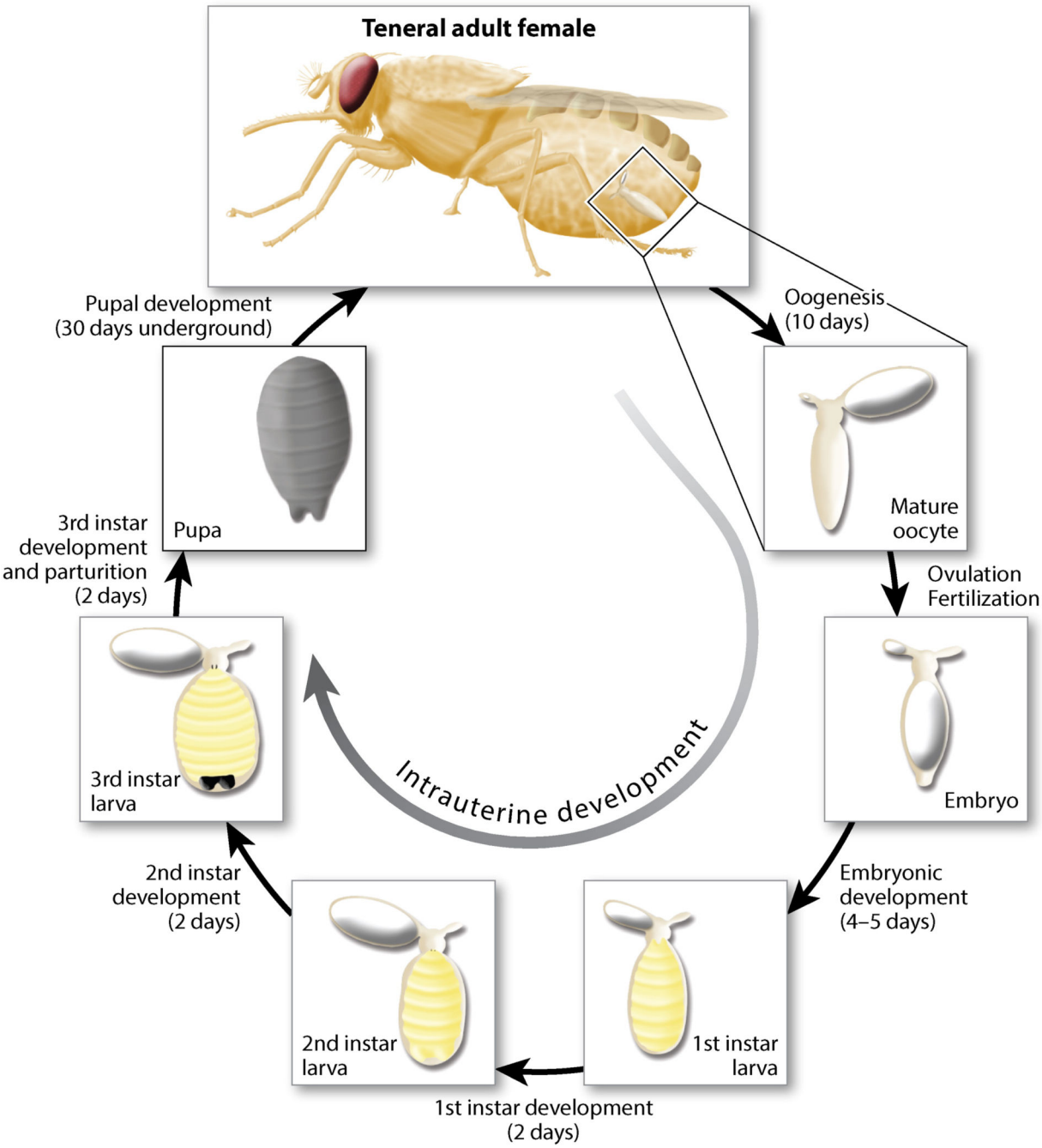

Figure 2.

Schematic of the first gonotrophic cycle of a *Glossina morsitans* female under optimal environmental and nutritional conditions. The different stages of oogenesis, embryogenesis, and larvigenesis within the *Glossina* reproductive tract (ovaries and uterus) are shown.

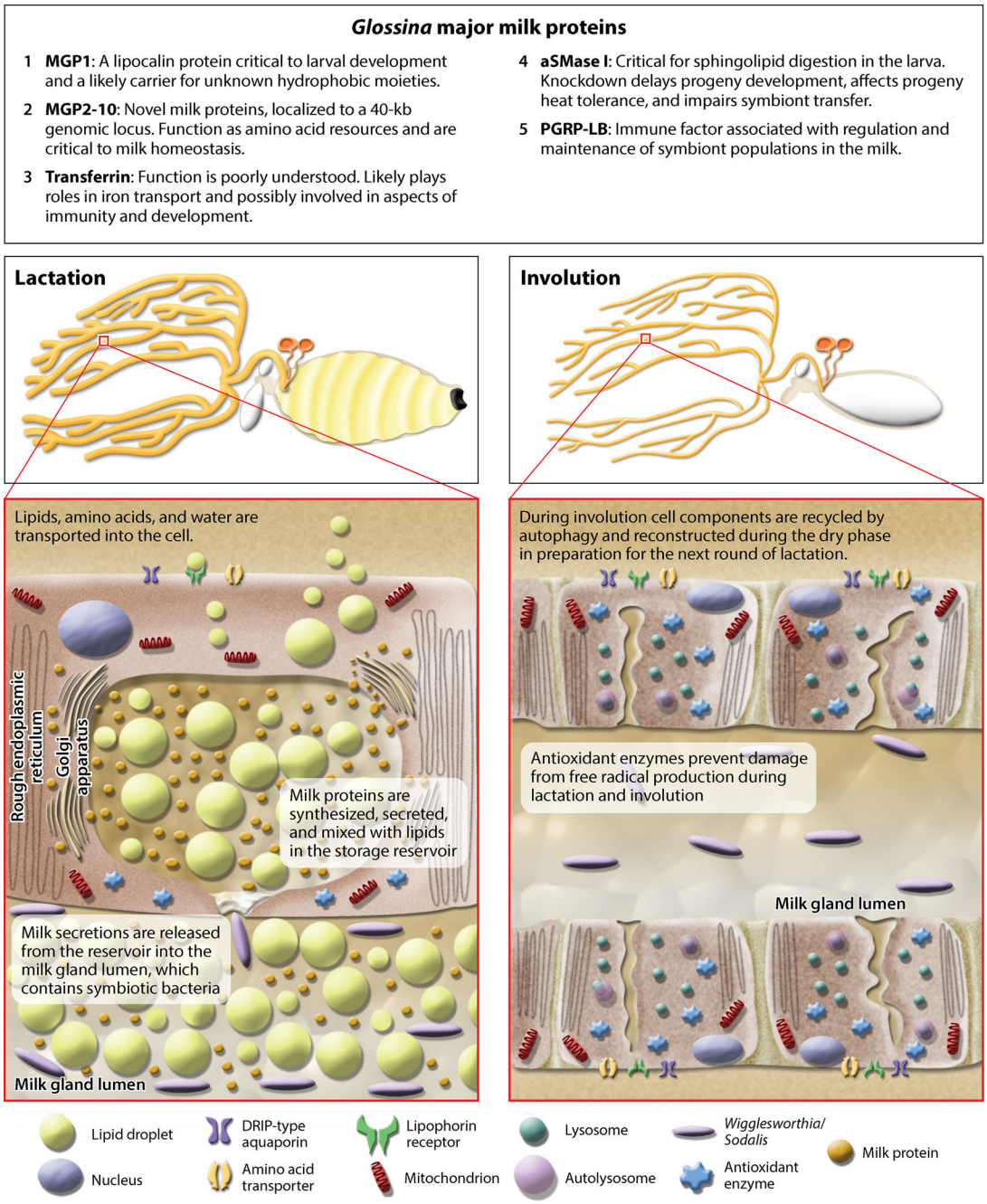

Figure 3.

Overview of *Glossina* lactation. Top: Description of major milk proteins. Bottom left: Diagram of a reproductive tract during lactation, featuring milk gland secretory cells undergoing milk generation and secretion (*inset*). Bottom right: Diagram of a reproductive tract during the dry period after birth, featuring milk gland secretory cells undergoing involution (*inset*).