Drosophila melanogaster as a model for nutrient regulation of ovarian function

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Abstract

Observed in a wide variety of organism, from invertebrates to mammals, nutritional status modulates the energetically costly effort of producing female gametes. Despite this long-standing link between nutrition and ovarian function, relatively little is known about the cellular and molecular mechanisms that underlie how dietary components modulate egg production. *Drosophila melanogaster*, with its powerful and extensive genetic tools as well as its well-characterized ovarian response to diet, has proven to be instrumental in addressing this issue. This review covers what we currently know about the dietary control of oogenesis in *Drosophila* and the salient features of the fruit fly that make it a model for nutritional control of ovarian function.

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Dynamic organismal macronutrient intake influences ovarian function

Many organisms experience variations in macronutrient intake across small (e.g. meal to meal, day to day, week to week) and large (e.g. infancy, childhood, early adulthood, late adulthood) timescales. These changes can be involuntary, as in the extreme case of famine, or voluntary, as in the case of individuals choosing to go on the low-carbohydrate, high-protein Atkins diet. Variations in macronutrient intake generally result from alterations in all five macronutrients, carbohydrates, proteins, fats, fiber and water, or one macronutrient. For example, under a caloric restriction dietary regimen, there is a 20–40% decrease in total calorie intake without reaching malnutrition (Lee & Longo 2016). Conversely, in cases of over-nutrition, too many nutrients are consumed and too little activity is undertaken, leading to an imbalance between caloric intake and energy expenditure. Dozens of fad diets are often employed for weight loss and/or disease amelioration. For example, a diet low in carbohydrates relative to fats and protein is thought to improve glucose tolerance in patients with type 2 diabetes (Meng *et al.* 2017). Given the inextricable link between nutritional input and whole organismal physiology, alterations in dietary intake can positively or negatively affect metabolism, inflammation, temperature regulation, blood pressure and reproduction (Heymsfield & Wadden 2017).

Nutritional status influences reproduction at the level of gametogenesis, particularly ovarian function, in a wide range of organisms, from invertebrates, like worms and insects, to large domestic animals, like sheep and pigs

(Wheeler 1996, Drummond-Barbosa & Spradling 2001, Terashima & Bownes 2004, Lopez *et al.* 2013, Sohrabi *et al.* 2015, Hohos & Skaznik-Wikiel 2017, Wang *et al.* 2017). For example, high-fat diet leads to poor ovarian function as evidenced by more ovarian follicle death and fewer maturing follicles in rabbits (Cordier *et al.* 2013) or an increase in immature, at the expense of developing, follicles in mice (Solon-Biet *et al.* 2015). Conversely, ovaries from mice fed a calorically restricted diet contained a larger pool of developing follicles and fewer atretic follicles, compared to female mice fed *ad libitum*, ultimately leading to improved fertility and fecundity (Selesniemi *et al.* 2008). The vast majority of research addressing nutritional control of ovarian function in mammalian systems, including humans, has been tied to the framework of obesity (Hohos & Skaznik-Wikiel 2017, McGrice & Porter 2017) and obesity-related reproductive diseases, like polycystic ovarian syndrome (Jarrett & Lujan 2016). This is understandable given that over 50% of reproductive age women are considered to be overweight or obese (Delcore & Lacoursiere 2016) and that most Americans incorporate too few vegetables, fruit and dairy and too much added sugar, saturated fats and sodium, according to the 2015–2020 Dietary Guidelines for Americans. Before we can understand what goes awry under circumstances of aberrant nutrition, it will be important to elucidate the cellular and molecular mechanisms that underlie the ovarian response to an organism's nutritional intake.

Over the last decade, *Drosophila melanogaster*, the fruit fly, has emerged as an important player in metabolism and physiology studies (Baker & Thummel 2007, Rajan & Perrimon 2013, Trinh & Boulianne 2013)

with many focused on the effects of maternal nutrition on reproduction and offspring health (Brookheart & Duncan 2016). The goal of this review is to highlight the utility of *Drosophila* as an *in vivo* model organism to understand the dietary influences on ovarian function. Morphological similarities between fruit fly and human ovaries as well as stages of *Drosophila* oocyte development are described. Readers will find information on the overall influence of diet on ovarian output (i.e. oocyte production) in fruit flies, including the stages of oogenesis particularly sensitive to nutritional input. In addition, the molecular mechanisms known to function within the ovary and other tissues that mediate the effects of diet on ovarian function are discussed. Lastly, established reagents and tools used by *Drosophila* biologists to address this topic are described, including a sampling of *Drosophila* diet recipes, immunocytochemical reagents to visualize oogenesis, and transgenic tools to manipulate ovarian gene expression.

***Drosophila melanogaster* as a model of nutritional physiology**

Several features of *Drosophila melanogaster* make it an ideal model organism to investigate fundamental principles in a variety of biological fields. First, large numbers of flies are easily maintained in a modest amount of space. Adults, which reach 3 mm in size, are housed in vials (for smaller numbers of flies) or bottles (for larger numbers of flies) containing a simple food source of cornmeal, yeast, sugar, water and agar. The 10-day egg-to-adult life cycle, in conjunction with reproductive robustness, allows for the rapid generation of a large number of progeny. Second, *Drosophila* boasts a genomic simplicity while being genetically and biologically complex. The diploid fruit fly has four chromosomes that hold approximately 14,000 protein-coding genes in 143.7 Mbps of DNA compared to the 23 chromosomes that hold approximately 20,000 protein-coding genes in 3 Gbs of human DNA. In addition to making genetic manipulations more straightforward, less genetic redundancy in flies often produces more robust mutant phenotypes due to lack of compensation by related genes. Despite dramatic differences in size, presence of a backbone, appendage morphology, and mode of temperature regulation, *Drosophila* and humans share between 40 and 60% of genes, with up to 75% of human disease-related genes having homologs in flies (Reiter *et al.* 2001). This makes *Drosophila* a well-suited model for the study of human diseases (Millburn *et al.* 2016, Yamaguchi & Yoshida 2018). Moreover, *Drosophila* employs a majority of the organ systems used by humans to regulate physiology including the nervous, musculoskeletal, respiratory, circulatory, digestive, excretory and reproductive systems (drososchools.wordpress.com). Since its early use in elucidating the mechanisms of genetic inheritance, *Drosophila*

melanogaster continues to hold a strong position as one of the premier model organisms used to investigate biological principles in a variety of fields, including cellular biology, development, physiology, behavior/nervous system function, and gene expression.

Drosophila melanogaster is an excellent model organism to tease apart the intricate relationship between nutritional input and ovarian function. First, individual adult females have the capacity to lay up to 100 eggs per day for several days under optimal conditions (Drummond-Barbosa & Spradling 2001). Secondly, the *Drosophila* ovary responds dramatically to a variety of dietary changes including starvation, dietary restriction, removal of specific macronutrients, and alterations in macronutrient ratios (Sang & King 1961, Chippindale *et al.* 1993, Bradley & Simmons 1997, Drummond-Barbosa & Spradling 2001, Good & Tatar 2001, Mair *et al.* 2004, Terashima & Bownes 2004, Skorupa *et al.* 2008, Lushchak *et al.* 2012, 2014). Lastly, extensive research over several years has uncovered the involvement of multiple nutrient-sensing pathways in the ovary's ability to directly and indirectly sense and respond to nutritional status (Ables *et al.* 2012). Moreover, *Drosophila melanogaster* is a classic and powerful genetic system for which a wealth of reagents and transgenic organisms exist that allows spatial and temporal control of gene expression.

The stem cell-supported ovary powers oocyte production in *Drosophila melanogaster*

In the *Drosophila* ovary, multiple cell types work together to execute 14 developmental stages responsible for the continual production of mature oocytes over a female fly's reproductive lifespan (Spradling 1993, McLaughlin & Bratu 2015). Like mammals, abdomens of *Drosophila* females house a pair of ovaries, each composed of approximately 18 individual units or ovarioles (Fig. 1A and B). The anterior-most tip of an ovariole, called the germarium, contains two stem cell populations: two-three germline stem cells (GSCs) at the anterior and two follicle stem cells (FSCs) just beyond the midpoint of the anterior-posterior axis. Three somatic cell types, including terminal filament, cap and escort cells, make up the stem cell niche that maintains GSCs in an undifferentiated state (Spradling *et al.* 2011). GSCs, which are physically attached to cap cells by cadherins, divide asymmetrically to self-renew (the daughter that remains adhered to cap cells) and generate a cystoblast (the daughter cell that is one cell diameter removed from cap cells). Cystoblasts undergo four synchronous divisions with incomplete cytokinesis, creating two, four, eight and ultimately 16 interconnected germ cells, or germline cysts. In a 16-cell germline cyst, one cell becomes the oocyte proper and the remaining 15 become nurse cells. Before exiting the germarium, individual

Figure 1 The *Drosophila melanogaster* ovary compared to the mammalian ovary. (A) The paired *Drosophila* ovary is composed of several individual ovarioles. (B) A single ovariole contains progressively older follicles composed of germ cells (green) surrounded by a layer of somatic follicle cells (purple). Each follicle contains 16 germ cells, 1 oocyte (oo) and 15 nurse cells (nc). (C) The germarium (g) houses germline stem cells (GSCs, dark green) and their progeny, germline cysts (light green). Cap cells (pink) are a major cellular component of the stem cell niche. Follicle stem cells (FSCs, dark purple) support continued generation of follicle cells (light purple). (D) Similar to fruit flies, the mammalian ovary contains several follicles at various stages of development or maturation (primordial, developing, and mature follicles). Each follicle contains the germ cell or oocyte (green) and a layer(s) of somatic cells (purple).

16-cell cysts acquire a single cell layer of epithelial follicle cells, which originate from FSCs, producing a discrete egg chamber. Each egg chamber undergoes 14 morphologically distinct developmental stages that include endoreplication of nurse cell nuclei, follicle cell proliferation and migration, yolk and lipid uptake by the growing oocyte, deposition of maternal effect products into the oocyte, nurse cell break down and eggshell formation (Spradling 1993, McLaughlin & Bratu 2015). The mature oocyte then traverses the lateral and common oviduct to rest in the uterus where it is fertilized by sperm from a specialized sperm storage organ, the spermatheca, and oviposited on a suitable substrate (Spradling 1993). Under optimal laboratory conditions, a single wild-type adult female can lay approximately 80–100 eggs per day for several days (Drummond-Barbosa and Spradling), with a lifetime fecundity potential of up to 1000 progeny. While many environmental factors affect *Drosophila* egg production, nutrition is one of the most influential.

Drosophila and mammalian ovaries share several characteristics

Despite some obvious differences (e.g. ovarian relative to body size), *Drosophila* and mammalian ovaries have similarities regarding cellular organization and oocyte development (Fig. 1B and D). In both organisms, the ovary produces multiple, self-contained female gametes consisting of a single oocyte and a layer of support cells. In the fruit fly egg chamber, a single layer of epithelial follicle cells encloses nurse cells and the oocyte, while multiple layers of granulosa cells enclose the human ovum. Moreover, each individual reproductive unit in humans and fruit flies undergo a progressive series of coordinated steps to form a mature oocyte. The 14 stages of oocyte development traversed by egg chambers in the *Drosophila* ovariole are akin to human oocytes transitioning from primordial, primary, secondary, early and late antral follicles to germinal vesicle followed by meiotic maturation stages (El-Hayek & Clarke 2016). A major part of this linear development includes support cell proliferation and oocyte growth mediated by support cell–oocyte communication.

In humans, granulosa cell number increases creating multiple layers from a single cell layer (Gougeon 2010), while *Drosophila* follicle cells undergo several rounds of division to produce 650–1000 from approximately 80 cells (Horne-Badovinac & Bilder 2005). Concomitantly, the human oocyte grows in size from 40 to 140 μm (Gougeon 2010), while *Drosophila* oocytes grow from 10 to approximately 500 μm (Spradling 1993). Transport of cellular constituents from support cells to the oocyte mediates this extensive increase in size, thus preparing oocytes for early embryonic development following fertilization. In *Drosophila*, the 15 nurse cells and oocyte are all interconnected by actin and microtubule rich intercellular bridges or ring canals (Lu et al. 2017). Given that the *Drosophila* oocyte is transcriptionally silent, nurse cells provide the oocyte with mRNAs, organelles, and proteins important for oocyte specification, embryonic patterning, and energy reserve (Mahowald & Strassheim 1970, Spradling 1993, Clark et al. 2007, Nicolas et al. 2009, Lu et al. 2017). For example, mitochondria redistribute in a stage-specific manner from nurse cells to the oocyte through ring canals (Cox & Spradling 2003). In fetal mouse ovaries, transport of cellular material through transient, interconnected germ cell cysts is thought to play a role in oocyte determination (Lei & Spradling 2016, Ikami et al. 2017). Similar to *Drosophila* ring canals, proper mammalian follicular maturation relies on transzonal projections, actin- and connexin-rich cytoplasmic extensions that connect granulosa cells to the underlying oocyte (El-Hayek & Clarke 2016). Transzonal projections allow granulosa cells to provide growth factors, metabolites, and amino acids to the growing oocyte (El-Hayek & Clarke 2016). While it is not thought that transzonal projections

mediate the transport of organelles, mammalian ovaries do experience modifications of cellular components. For example, mitochondria in human oocytes move from the cellular periphery to the central zone (Sundström *et al.* 1985). Lipid droplets also redistribute during oocyte maturation and growth in mammals in addition to an increase in size and number (Gu *et al.* 2015). In *Drosophila*, lipid accumulation is a hallmark of the process of vitellogenesis during oocyte development (Buszczak *et al.* 2002, Parra-Peralbo & Culi 2011).

Arguably, the major difference between human and *Drosophila* ovaries is the existence of a stem cell population. *Drosophila* ovarian germline stem cells have been extensively characterized (Spradling *et al.* 2011), while the presence of stem cells in the mammalian/human ovary remains controversial (De Felici & Barrios 2013, Grieve *et al.* 2015, Hummitzsch *et al.* 2015). Nonetheless, conserved biological phenomena, like cellular proliferation, survival and intercellular signaling, must properly occur in ovaries of both organisms to generate functional female gametes.

Dietary macronutrient composition determines ovarian function in *Drosophila melanogaster*

Various types of standard fruit fly laboratory diets contain ingredients that provide carbohydrates, protein, and fats. An extensive body of *Drosophila melanogaster* literature exists examining how dietary macronutrients influence egg production, the ultimate output of ovarian function. Female flies fed a carbohydrate-only diet, in the form of sucrose or molasses, display a severe reduction in egg-laying rates (Bownes & Blair 1986, Drummond-Barbosa & Spradling 2001) and lifetime fecundity (Good & Tatar 2001). For example, a standard diet supplemented with yeast paste results in an average of 100 eggs in 24 h per female, while a diet of only 10% sucrose results in 10 eggs per female in the same amount of time (Drummond-Barbosa & Spradling 2001). Varying the total amount of food available, by diluting standard fly food media, shows that egg-laying rate correlates positively with increasing total food level (Chapman & Partridge 1996, Bross *et al.* 2005). The vast majority of studies, however, manipulate the protein-to-carbohydrate ratio (P:C) by changing the amount of sucrose or yeast added to standard food media.

Despite differences in feeding paradigms (i.e. exact P:C ratios, yeast vs a defined protein source, solid vs liquid diets, and length of feeding), there is an overall consensus that dietary protein supersedes carbohydrates in promoting egg production. For example, increasing protein concentration by adding yeast to diets containing a constant carbohydrate concentration, leads to increased egg laying (Bradley & Simmons 1997, Zajitschek *et al.* 2013). More recently, nutritional geometry has been employed to analyze the independent and interactive effects of protein

and carbohydrates on life-history traits influenced by nutrition, like reproduction (Archer *et al.* 2009, Simpson *et al.* 2015, Jang & Lee 2018). In studies testing over 20 different diets, it was revealed that the maximal rate of egg production is achieved when female flies consume a diet high in protein but with moderate-to-low levels of carbohydrates (Lee *et al.* 2008, Skorupa *et al.* 2008, Lushchak *et al.* 2012, Jensen *et al.* 2015). Moreover, the dietary condition of low overall food amount, that is low protein and low carbohydrate, built into these nutritional geometry studies show the expected detrimental effect on egg production (Chapman & Partridge 1996, Lee *et al.* 2008, Skorupa *et al.* 2008). Interestingly, diets high in carbohydrates negate the positive effects of protein. Female fruit flies fed diets containing high-protein and high-carbohydrate levels have smaller ovaries with less active oogenesis (Brookheart *et al.* 2017) and lay very few eggs (Skorupa *et al.* 2008). Likely because of the small amount of lipids supplied in the diet relative to proteins and carbohydrates, the role that dietary fats play in modulating *Drosophila* egg production has received little, if any attention. Thus, in *Drosophila melanogaster*, dietary protein acts as the most prominent influencer of ovarian function with some modulation by dietary carbohydrates.

While most studies focus on how dietary changes in protein and/or carbohydrates in general modulate egg production, each class of macronutrients encompasses a variety of molecules: 20 amino acids from protein, simple and complex sugars from carbohydrates, and unsaturated and polyunsaturated fatty acids from fat, which could individually influence ovarian function. For example, adding back only essential amino acids rescued the reduced fecundity in *Drosophila* females fed a restricted diet (Grandison *et al.* 2009). Moreover, elimination of a single amino acid, methionine, from a full diet resulted in females laying fewer eggs while adding back methionine alone showed the same rescue of egg production as adding back essential amino acids (Grandison *et al.* 2009). Recently, it was shown that the effect of dietary methionine on egg production is concentration dependent and influenced by the presence of other amino acids (Lee *et al.* 2014). Arginine has also been shown to influence *Drosophila* ovarian function. Increasing or decreasing the concentration of arginine alone in the fly diet resulted in increased or decreased egg laying, respectively (Piper *et al.* 2017). While carbohydrates do not support *Drosophila* ovarian function as robustly as protein, specific carbohydrates differentially affect egg production when provided in a diet containing a constant amount of protein. Female flies fed the monosaccharides glucose or fructose, individually or in combination, lay significantly more eggs than their counterparts fed the disaccharide sucrose (Lushchak *et al.* 2014). Taken together, these studies highlight the specificity of individual macronutrients in controlling ovarian function.

Nutrition controls *Drosophila* egg production at multiple points during oogenesis

The cellular responses that occur during oocyte development in response to a protein-poor diet have been well characterized for *Drosophila* females. In ovaries of adult female flies fed a protein-poor diet, germline and follicle stem cells show reduced proliferation rates (Drummond-Barbosa & Spradling 2001) and GSCs are lost more rapidly from the stem cell niche (Hsu & Drummond-Barbosa 2009). Germline cysts present in the germarium exhibit an increase in programmed cell death as well as autophagy in females fed a protein-poor diet (Drummond-Barbosa & Spradling 2001, Hou *et al.* 2008, Barth *et al.* 2011). Increased cell death rates during mid-oogenesis, egg chamber stages 7–9, are also observed under protein-poor dietary conditions (Drummond-Barbosa & Spradling 2001, Pritchett *et al.* 2009). Upon exit from the germarium, discrete egg chambers are developmentally delayed due to reduced proliferation of somatic follicle cells and defects in the mitotic-to-endocycle switch of nurse cells (Drummond-Barbosa & Spradling 2001, Jouandin *et al.* 2014). Protein-poor dietary conditions also induce microtubule reorganization, leading to perturbed nurse cell transport to the developing oocyte (Shimada *et al.* 2011). Moreover, protein-deficient diets result in ovaries in which vitellogenesis, yolk protein and lipid uptake by the oocyte, is blocked (Drummond-Barbosa & Spradling 2001, Terashima & Bownes 2004, Mazzalupo & Cooley 2006, Pritchett & McCall 2012). Lastly, mature oocytes are retained within the ovary, failing to be ovulated (Drummond-Barbosa & Spradling 2001). Not surprisingly, many of these effects of protein-poor diet are reversible, allowing female flies that are re-fed a normal diet to increase egg production (Drummond-Barbosa & Spradling 2001). For example, feeding a protein-rich diet to previously starved female flies reverses the microtubule reorganization induced by protein-poor diet, thus restoring intercellular transport from nurse cells to the oocyte (Shimada *et al.* 2011). Thus, nutritional input influences multiple stages of oogenesis at the cellular level to ultimately affect the final readout of ovarian function and egg production.

Molecular mechanisms involved in the nutritional control of ovarian function in *Drosophila*

A complex interplay between diet-dependent hormones, nutrient sensing, and inter-organ communication mediate the effects of diet on ovarian function. *Drosophila melanogaster's* extensive genetic toolkit in combination with reliable cell biological methods has allowed investigators to uncover the mechanism of action of several highly conserved signaling pathways, including insulin/insulin-like growth factor signaling (IIS), steroid hormone signaling, as well as target of rapamycin (TOR)

and AMP-activated protein kinase (AMPK)-mediated signaling. For more detailed discussion on the material described below, readers are directed to several excellent reviews on *Drosophila* ovarian response to diet (Ables *et al.* 2012, Ables & Drummond-Barbosa 2017, Laws & Drummond-Barbosa 2017, Templeman & Murphy 2018, Mirth *et al.* 2019).

In *Drosophila*, insulin-like peptide (ILP) binding to a single insulin receptor (InR) activates two main downstream signaling cascades, the PI3K/Akt and Ras/MAPK axes (Boucher *et al.* 2014). Of the seven ILPs, ILPs 2,3, and 5 are secreted from brain median neurosecretory cells in response to feeding to control oocyte development (Ikeya *et al.* 2002, LaFever & Drummond-Barbosa 2005). These brain-derived ILPs act directly on the ovarian germline, promoting GSC proliferation, egg chamber growth and progression through vitellogenesis (LaFever & Drummond-Barbosa 2005). They also act on GSC niche cap cells to indirectly maintain GSCs by regulating niche size, that is, cap cell number, and cadherin-dependent adhesion of GSCs to cap cells (Hsu & Drummond-Barbosa 2009, Hsu & Drummond-Barbosa 2011, Yang *et al.* 2013). Follicle cells also employ IIS to mediate starvation-induced microtubule reorganization and P-body aggregation in underlying previtellogenic egg chambers that serves as a protective mechanism against nutrient stress (Burn *et al.* 2015). Moreover, cell-autonomous IIS controls the mitotic-to-endocycle cell switch in follicle cells, which correlates with vitellogenic entry of mid-stage egg chambers (Jouandin *et al.* 2014).

The diet-dependent steroid hormone ecdysone, known for its role in timing transitions during larval development (Yamanaka *et al.* 2013), functions in the nutrient response of the *Drosophila* ovary throughout oogenesis. Ecdysone binds to its heterodimeric nuclear hormone ecdysone receptor and ultraspiracle (EcR/USP) to act on targets like E74, E75 and broad, early response genes expressed in the ovary (Riddiford *et al.* 2000, Belles & Piulachs 2015). In the *Drosophila* ovary, ecdysone signaling functions cell-autonomously to promote GSC maintenance and proliferation (Ables & Drummond-Barbosa 2010) and non-cell-autonomously in escort cells to regulate germline differentiation, including the formation of multicellular cysts, oocyte meiotic entry and egg chamber formation (König *et al.* 2011, Morris & Spradling 2012). Furthermore, survival of previtellogenic and vitellogenic egg chambers requires ecdysone signaling (Buszczak *et al.* 1999, Carney & Bender 2000, Ables *et al.* 2015). Recently, a host of ecdysone-responsive genes have been identified that function in the control of GSC and FSC maintenance, germline cyst and ovarian somatic cell development, as well as encapsulation (Ables *et al.* 2016). An intricate crosstalk mechanism exists between IIS and ecdysone signaling whereby each diet-dependent pathway influences activity of the other and modulate similar aspects of oogenesis.

In addition to controlling growth in a variety of cell and tissue types across organisms, the amino acid sensor, TOR, and glucose and energy sensor, AMPK, function in *Drosophila* to control ovarian response to nutritional status (Grewal 2009, Bland *et al.* 2010, Ben-Sahra & Manning 2017, Lin & Hardie 2018). Cell-autonomous TOR-mediated signaling regulates GSC maintenance and proliferation as well as early germline cyst survival (LaFever *et al.* 2010, Sun *et al.* 2010). Egg chamber growth relies on TOR signaling within the germline (LaFever *et al.* 2010). Moreover, follicle cell size, controlled intrinsically by TOR, indirectly modulates growth of the underlying germline cyst to match growth of somatic and germ cells in the developing egg chamber (LaFever *et al.* 2010). Similar to IIS, TOR-mediated signaling modulates the mitotic-to-endocycle switch and progression through vitellogenesis (LaFever *et al.* 2010, Pritchett & McCall 2012). Interestingly, regulators of TOR activity control oocyte meiotic entry, but seem to do so independent of dietary input (Wei *et al.* 2014). Like TOR signaling, AMPK has a diet-independent function in follicle cell encapsulation of germline cysts (Laws & Drummond-Barbosa 2016). Importantly, however, AMPK is responsible for mediating several effects on oogenesis elicited by a protein-poor diet, including the decrease in GSC and follicle cell proliferation (Laws & Drummond-Barbosa 2016). Similar to the non-cell-autonomous role of TOR-mediated follicle cell signaling in controlling germline growth, AMPK functions in follicle cells to restrict germline cyst growth in female flies fed a poor diet (Laws & Drummond-Barbosa 2016).

Given that multiple organs sense and respond to changes in nutritional input, the role that inter-organ communication plays in coordinating whole organism physiological responses has received increasing attention (Droujinine & Perrimon 2013).

Specifically, *Drosophila* adipose tissue, or the fat body, has recently arisen as a nutrient-sensing depot that refines the ovarian response to diet at multiple stages of oogenesis. Adipocytes, the primary cell type of *Drosophila* adipose tissue, regulate GSCs and their progeny using a variety of nutrient-sensing and metabolic pathways. First, GSCs are maintained by adipocyte sensing of amino acids via the amino acid response pathway (Armstrong *et al.* 2014) and by the IIS/Akt/GSK3 β axis (Armstrong & Drummond-Barbosa 2018). Furthermore, adipocyte IIS promotes germline cyst survival via InR/Akt signaling and as yet to be identified Akt targets (Armstrong & Drummond-Barbosa 2018). Intriguingly, progression through vitellogenesis is also influenced by adipocyte InR activity, but not through the canonical PI3K/Akt axis (Armstrong & Drummond-Barbosa 2018). Lastly, TOR-dependent amino acid sensing in adult adipocytes promotes ovulation of mature oocytes (Armstrong *et al.* 2014). In *Drosophila* adipose tissue, metabolic enzymes and nutrient transport proteins expressed in a diet-dependent manner have been shown to control ovarian

function. For example, several enzymes involved in fatty acid oxidation are down regulated in fat bodies from females fed a protein-poor diet and are important for GSC maintenance under fed conditions (Matsuoka *et al.* 2017).

Taken together, findings from the described studies underscore the multifaceted regulation of *Drosophila* ovarian function. Not only do different cell types within the ovary use a variety of nutrient-sensing pathways to directly respond to dietary changes, somatic and germ cells within the ovary communicate this information to each other. As an added level of regulation, IIS and TOR-mediated signaling are used again in the adipose tissue to control similar aspects of oogenesis. It remains to be determined if AMPK also functions in adipose tissue to communicate information about nutritional status to the ovary. In multicellular organisms, humans and *Drosophila* alike, whole organism physiological responses to nutrition depend on inter-organ communication (Droujinine & Perrimon 2013). Therefore, it is important to identify the factors, and their modes of action, which relay nutritional information in a complex network of tissue crosstalk. The ability to manipulate gene expression tissue specifically firmly positions *Drosophila melanogaster* as an *in vivo* model system to address this challenge. In fact, several studies in adult flies have shown inter-organ communication from muscle-to-fat, fat-to-brain, and fat-to-ovary (Rajan & Perrimon 2013, Demontis *et al.* 2014, studies described in this review). In future studies, it will be interesting to determine if other nutrient-responsive tissues, such as the gut and muscle, also act to refine the ovarian response to diet.

Dietary manipulations for *Drosophila melanogaster*

Drosophila melanogaster is incredibly amenable to dietary manipulations, including alterations of food composition and quantity, as well as feeding schedule. While a variety of feeding media exist, nearly all contain ingredients that provide the three main macronutrients, protein, carbohydrates and lipids. For general fly maintenance, *Drosophila* biologists use a standard, or undefined, medium of yeast, sugar, cornmeal/corn flour, agar, and water with or without anti-fungal or preservation agents (Table 1). The source of each ingredient varies depending on the fly food recipe being used; for example, Baker's versus Brewer's yeast or corn syrup versus sucrose, for the protein or carbohydrate source, respectively. Despite these slight differences, standard diets are composed of 1.5–10% yeast, 5–10% cornmeal, 5–10% sugar, and 0.5–3% agar (percentages are w/v). To achieve broad manipulations, like dietary restriction, high-fat or high-sugar diets, standard medium can be modified by removing or adding ingredients. For dietary restriction, the quantity of yeast is usually reduced by half (Bass *et al.* 2007), while the

Table 1 Ingredients for example standard, semi-defined, and chemically defined *Drosophila* diets.

	Standard (BDSC)	Meridic (Reis 2016)	Holidic (Piper et al. 2017)
Protein	Baker's yeast (16); Soy flour (10)	Casein (73.3)	Alanine (1.1); Arginine (1.63); Asparagine (1.03); Aspartate (1.17); Cysteine (0.34); Glutamate (1.52); Glutamine (1.12); Glycine (0.77); Histidine (0.65); Isoleucine (1.12); Leucine (2.03); Lysine (1.37); Methionine (0.6); Phenylalanine (1.01); Proline (0.98); Serine (1.38); Threonine (1.11); Tryptophan (0.32); Tyrosine (0.93); Valine (1.2)
Carbohydrates	Light corn syrup (7% v/v)	Sucrose (13.3)	Sucrose (17.12)
Lipids	Yellow cornmeal (67)	Cholesterol (0.4)	Cholesterol (0.1)
Other	Agar (5)	Agar (5); Choline (0.32); Inosine (0.85); Uridine (0.76)	Agar (20); Inositol (0.005); Choline (0.05); Inosine (0.65); Uridine (0.06); Thiamine/B1 (0.0014); Riboflavin/B2 (0.0007); Nicotinate/B3 (0.0084); Pantothenate/B5 (0.0108); Pyridoxine/B6 (0.0017); Biotin/B7 (0.0001); Folic acid/B9 (0.0005)

Concentrations in g/L for all except where indicated.

amount of sugar added is 2–3X more for a high-sugar diet (Musselman et al. 2011) and coconut oil is added for a high-fat diet (Birse et al. 2010). Importantly, flies and mammals fed these diets share many phenotypes regarding lifespan, reproduction and metabolism (Trinh & Boulianne 2013).

In efforts to standardize diets for studies that aim to better understand the nutritional control of *Drosophila* physiology, several semi-defined (meridic) and chemically defined (holidic) diets have been developed (Table 1; Piper 2017). Unlike standard diets, meridic and holidic media use defined macronutrient sources. While live yeast serves as the protein source in standard diets, meridic diets contain casein and holidic diets use explicit concentrations of individual amino acids (reviewed in Piper 2017). In addition, meridic and holidic diets can contain single or specific combinations of glucose, fructose, sucrose, lactose, and trehalose as the carbohydrate source and lecithin, cholesterol, ergosterol, or inositol as the lipid source (Table 1, Piper 2017). Additional ingredients not added to standard diets include nucleosides, trace metals, and vitamins. As a testament to the inextricable link between nutritional input and organismal physiology, complete diets that only differ in their macronutrient source and concentration exert their own influence on developmental timing, egg production and survival. For example, female flies fed a meridic diet containing sucrose and casein at a high P:C survive longer than female flies fed a holidic diet containing glucose, sucrose, lactose, trehalose and purified amino acids at a low P:C (Lee & Micchelli 2013, Reis 2016). Thus, 'control' diets should elicit a response from which deviations in either direction for a given physiological response are readily apparent. Regarding egg production, female flies fed a sucrose-based diet supplemented with wet yeast paste (i.e. protein-rich) lay an average of 90 eggs/day while those fed only sucrose (i.e. protein-poor) lay an average of 1.5 eggs/day (Drummond-Barbosa & Spradling 2001). Therefore, a meridic diet like that used by Sang and King (1961) or a holidic diet like that used by Lee and Micchelli (2013) which result in females that lay approximately 50 eggs per day provide a baseline to

which dietary manipulations can be compared. The dramatic response of the *Drosophila* ovary to dietary changes underscores the need to pay special attention to nutrient composition for studies in which ovarian function is being assessed.

Tools to analyze the *Drosophila* ovary

The overall cellular organization and stages required for proper oocyte development (Spradling 1993) have been well described owing to the extensive *Drosophila melanogaster* toolkit. Established cell biological methods in combination with genetic techniques allow visualization and quantification of ovarian cellular composition (stem cells, germline, somatic cells, cyst types, etc.). A core set of antibodies and transgenic reporter lines are routinely used to mark ovarian cell types (Lie-Jensen & Haglund 2016). Antibodies that recognize VASA, a DEAD-box RNA helicase required for oogenesis in *Drosophila* (reviewed in Lasko 2013) label germ cell cytoplasm (Lasko & Ashburner 1990) (Fig. 2A, C and F), while an anti-orb antibody is enriched in the oocyte (Lantz et al. 1994). Labeling ovaries with antibodies that target alpha-spectrin, phosphorylated-MAPK (pMAPK) or fasciclin III identifies somatic cell populations like escort cells and follicle cells (Fig. 2D and H). A transgenic fly line in which the gene encoding Fax, a membrane protein involved in axon formation, has an in-frame GFP can be used to highlight all somatic cells (Buszczak et al. 2007). For analyses focused on stem cell niche and germline stem cells, antibodies against LaminC (labels terminal filament and cap cell nuclear lamina) and phosphorylated-Mad as well as Nanos (labels germline stem cells) are generally used (Lie-Jensen & Haglund 2016). Transgenic lines in which genes important for maintenance of stemness are fused to reporter genes, like dad-lacZ and bam::GFP, are often used to highlight germline stem cells (Song et al. 2004). Antibodies that recognize Hts-F and alpha-spectrin label the fusome, a specialized structure in germline cysts whose branching morphology correlates with cyst cell number/stage in the germarium (de Cuevas & Spradling 1998) (Fig. 2E, F and G).

Figure 2 Commonly used antibodies in the *Drosophila* ovary. (A, B, C, D and E) 20× magnification confocal images of whole ovarioles labeled with DAPI to visualize nuclei (blue in A and B), VASA to visualize germ cells (red in A and C), pMAPK to visualize somatic cells (green in A and D), LaminC and alpha-spectrin to label cap cell nuclei, fusomes and cell membranes (grayscale in A and E). (F, G and H) 63× magnification images of germaria. (F) Germ cells (green), fusomes and cell membranes (red). (G) Single channel, grayscale image of LaminC and alpha-spectrin. (H) Escort cells (red) and follicle cells (FasIII, green).

While preparation of ovarian tissue for immunocytochemical analysis varies depending on the structures to be visualized, most protocols include a fixation step, followed by washing in PBS with or without detergent, a blocking step, incubation in primary antibodies followed by incubation in the appropriate fluorescently tagged secondary antibodies (Lie-Jensen & Haglund 2016). Protocols can vary at each step including (1) fixative type (aldehyde- vs alcohol-based), time and temperature, (2) detergent type (Triton-X-100 vs Tween-20) and concentration, and (3) antibody incubation time and temperature. Therefore, immunocytochemical protocol optimization should be a first step when using new/unfamiliar antibodies.

Like the mammalian ovary, oocyte formation in *Drosophila* requires proper regulation of cellular proliferation, survival, and changes in gene expression. A variety of antibodies and reagents are used to evaluate these fundamental cellular processes. Proliferation of germline stem cells, germline cyst cells and follicle cells supports robust egg production in well-fed *Drosophila* females and division of these cell types is

decreased under nutrient-poor conditions (Drummond-Barbosa & Spradling 2001, Ables *et al.* 2012). Using a combination of cell labeling (described above) and Click iT chemistry, proliferation rate in the *Drosophila* ovary is often measured by quantifying the percentage of cells that incorporate 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analog. In addition, a snapshot of cell division can be obtained by phospho-histone H3 (PH3) antibody labeling that identifies cells undergoing mitotic divisions. Apoptotic and autophagic forms of programmed cell death function in the *Drosophila* ovary to support normal oocyte formation or respond to environmental stressors (Jenkins *et al.* 2013, Peterson *et al.* 2015). For example, nutrient-poor conditions lead to increased apoptosis and autophagy in the germline as well as somatic cells in early and middle stages of oogenesis (Drummond-Barbosa & Spradling 2001, Hou *et al.* 2008, Nezis *et al.* 2009, Barth *et al.* 2011). To assess apoptotic cell death in the *Drosophila* ovary, immunoreactivity to an effector caspase, cleaved Dcp-1, and/or TUNEL labeling are often used (Meehan *et al.* 2015) along with the presence of pyknotic nuclei highlighted by DAPI staining. To assess autophagic cell death, a dye that labels acidic organelles (LysoTracker) is used as a proxy for the presence of autophagosomes (DeVorkin & Gorski 2014) in addition to transgenic fly lines in which proteins required for autophagy are fluorescently tagged (mCherry-Atg8 and LC3-GFP) (Hou *et al.* 2008, Nezis *et al.* 2009). Changes in gene expression drive the highly coordinated processes required for proper development of mature oocytes (Spradling 1993, Baker & Russell 2009, Adrian & Comeron 2013). As described, dietary manipulations often negatively influence formation of mature oocytes. GFP protein-trap lines, in which proteins are tagged with green fluorescent protein at endogenous loci (Morin *et al.* 2001, Kelso *et al.* 2004, Buszczak *et al.* 2007), have been used to visualize factors expressed in the ovary that are modulated by diet (Hsu & Drummond-Barbosa 2017).

Manipulation of gene expression in the *Drosophila* ovary

The exquisite ability to alter gene expression is a major strength of using *Drosophila melanogaster* as a model system. The FLP/FRT system uses flippase (FLP)-mediated recombination of genomic elements flanked by FLP recognition target (FRT) sites to generate clones of mutant cells juxtaposed to cells with wild-type gene function (Xu & Rubin 1993). Genetic mosaic analysis in the *Drosophila* ovary using fly lines carrying mutant alleles for nutrient-sensing pathway components (Table 2) (Laws *et al.* 2015) has uncovered roles for IIS, ecdysone, TOR- and AMPK-mediated signaling in GSCs,

Table 2 Fly lines carrying mutant alleles for nutrient-sensing pathway components.

Pathway	Mutant alleles	References
IIS	<i>chico</i> ¹ , <i>dinr</i> ^{E19} , <i>dinr</i> ³³⁹ , <i>dinr</i> ³⁵³ , <i>dFOXO</i> ²¹ , <i>dFOXO</i> ²⁵	Fernandez <i>et al.</i> (1995), Chen <i>et al.</i> (1996), (Böhni <i>et al.</i> 1999), Brogiolo <i>et al.</i> (2001), Fernandez <i>et al.</i> (1995); (Jünger <i>et al.</i> 2003)
TOR	<i>Tor</i> ^{R248X} , <i>Tor</i> ^{P2293L} , <i>Tor</i> ^{W1251X} , <i>Tsc1</i> ^{Q87X}	Tapon <i>et al.</i> (2001), Zhang <i>et al.</i> (2006)
AMPK	<i>AMPKα</i> ^{D2} , <i>AMPKα</i> ¹ , <i>AMPKα</i> ^A	Lee <i>et al.</i> (2007), Haack <i>et al.</i> (2013), Haelterman <i>et al.</i> (2014)

FSCs, and their progeny in response to dietary changes (Ables *et al.* 2012, Laws & Drummond-Barbosa 2017).

The *Gal4/Gal80^{ts}/UAS* system, a mainstay in *Drosophila* genetic manipulation, allows tissue/cell-type specificity as well as temporal control of gene expression (Brand & Perrimon 1993, del Valle Rodríguez *et al.* 2011). In this bipartite genetic tool, one transgenic fly line harbors a tissue-specific promoter that controls the expression of the transcription factor Gal4, while a second transgenic fly line harbors a transgene of interest downstream of upstream activating sequences (UAS), binding sites for Gal4. Following standard genetic crosses, the gene of interest will be expressed in a specific tissue in progeny containing *Gal4* and *UAS* genetic elements. Incorporating a temperature-sensitive inhibitor of *Gal4*, *Gal80^{ts}*, provides temporal control of gene expression as switching flies to the permissive or restrictive temperature for *Gal80^{ts}* represses or inhibits transgene expression, respectively. A variety of transgenic *Gal4* fly lines are available that drive gene expression in defined cell populations in the *Drosophila* ovary (Table 3) (Hudson & Cooley 2014). With appropriate UAS transgenes, gene expression can be modified in several ways. Incorporation of open reading frames downstream of UAS allows overexpression (Bischof *et al.* 2013), while incorporation of short inverted repeats complementary to a gene of interest downstream of UAS allows RNA interference-mediated knockdown (Kaya-Çopur & Schnorrrer 2016). In addition to fly stocks available at the Bloomington Drosophila Stock Center (BDSC; flystocks.bio.indiana.edu), the Zurich ORFeome Project (FlyORF; flyorf.ch) contains over 3000 overexpression lines covering approximately 2850 genes. The Vienna *Drosophila* Resource Center (VRDC; stockcenter.vdrc.

at) has created and maintains an RNAi library of over 25,000 fly lines, covering 91% of the genome (Dietzel *et al.* 2007). The Transgenic RNAi Project (TRiP; flyrnai.org), whose collection is housed at the Bloomington Drosophila Stock Center, has created over 12,000 fly lines and additional tools for the fly community to generate their own RNAi lines (Ni *et al.* 2011). Many transgenic lines exist that allow inhibition or activation of IIS-, TOR-, or AMPK-mediated signaling by targeting various components of each pathway (Table 4). More recently, the CRISPR-Cas9 system has been adapted in *Drosophila*, including tissue specificity, and will likely play a major role in assessing gene function as the tools are refined (Bassett & Liu 2014, Port *et al.* 2019).

Concluding remarks

Under optimal nutritional conditions, *Drosophila melanogaster* females are supremely poised for producing a large number of eggs over a significant portion of their lifespan. Many observations over several decades have shown that a wide range of dietary changes manipulating macronutrient levels impinges on ovarian output. Because of the incredible experimental tools available in *Drosophila*, a host of studies have deciphered numerous cellular and molecular mechanisms that mediate the ovarian response to diet. Importantly, what we have learned from *Drosophila* is broadly applicable to other organisms, including mammals and humans. Despite some morphological and cell biological differences, human and *Drosophila* ovaries must both regulate cellular proliferation, survival and differentiation to produce a functional gamete. Likely a result of the multifactorial nature of human

Table 3 Transgenic *Gal4* lines driving expression in the *Drosophila* ovary.

	<i>Gal4</i> driver name	Ovarian expression	Available Stocks		References
			BDSC	Kyoto	
Germ cells	<i>bam-Gal4:VP16</i>	Germline stem cells and early germ line	80579	n/a	Chen and McKearin (2003)
	<i>GreenEye.nos-Gal4</i>	Germline stem cells and germ line	32180, 32179	n/a	Holtzman <i>et al.</i> (2010)
	<i>MTD-Gal4</i>	Germline stem cells and germ line	31777	n/a	Grieder <i>et al.</i> (2000)
	<i>nanos-Gal4</i>	Germ line cells	4442, 32563	107748	Tracey <i>et al.</i> (2000)
	<i>nanos-Gal4-VP16</i>	Germline stem cells and germ line	4937, 7253, 7303, 64277, 77923	107955	VanDoren <i>et al.</i> (1998)
	<i>otu-Gal4::VP16</i>	Germline stem cells and germ line	58424	n/a	Rorth (1998)
Somatic cells	<i>bab1-Gal4</i>	Cap and terminal filament cells	6802, 6803	n/a	Cabrera <i>et al.</i> (2002)
	<i>c587-Gal4</i>	Escort cells and germarium follicle cells	67747	n/a	Song and Xie (2003)
	<i>en2.4-Gal4</i>	Follicle stem and follicle cells	1973	106609	Harrison <i>et al.</i> (1995)
	<i>GR1-Gal4</i>	Follicle stem and follicle cells	36287	n/a	Tran and Berg (2003)
	<i>tj-Gal4</i>	Somatic cells	n/a	104055	Olivieri <i>et al.</i> (2012)

Table 4 UAS-controlled transgenes for nutrient-sensing pathway manipulation.

Pathway	Transgene	Reference
IIS	<i>UAS-InR</i> , <i>UAS-InR^{GL00139}</i> , <i>UAS-Akt1^{GD1361}</i> , <i>UAS-Akt1^{HMS00007}</i> , <i>UAS-foxo^{KK108590}</i> , <i>UAS-foxo</i> , <i>UAS-Pten</i>	Huang <i>et al.</i> (1999), Hwangbo <i>et al.</i> (2004), Tang <i>et al.</i> (2011), Willecke <i>et al.</i> (2011), Sieber <i>et al.</i> (2016), Armstrong and Drummond-Barbosa (2018)
TOR	<i>UAS-Tsc1/2</i> , <i>UAS-RagA^{T16N}</i>	Tapon <i>et al.</i> (2001), Kim <i>et al.</i> (2008), Armstrong <i>et al.</i> (2014)
AMPK	<i>UAS-AMPKα^{WT}</i> , <i>UAS-AMPK^{DN}</i> , <i>AMPK^{KK102684}</i>	Johnson <i>et al.</i> (2010), Stenesen <i>et al.</i> (2013), Laws and Drummond-Barbosa (2016)

diet, that is, regional differences, socioeconomic status, psychological and behavioral control, little is known about the underlying biology that links mammalian ovarian function to nutritional status, particularly obesity/overnutrition. Given the high degree of conservation between organ systems, physiology, nutrient-sensing pathways, and metabolism, *Drosophila* has developed in to a model for obesity and obesity-related diseases, including metabolic syndrome and type 2 diabetes (Baker & Thummel 2007, Trinh & Boulianne 2013, Smith *et al.* 2014, Álvarez-Rendón, Salceda & Riesgo-Escovar 2018).

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