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RESEARCH ARTICLE

Warm and cold temperatures have distinct germline stem cell lineage effects during *Drosophila* oogenesis

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ABSTRACT

Despite their medical and economic relevance, it remains largely unknown how suboptimal temperatures affect adult insect reproduction. Here, we report an in-depth analysis of how chronic adult exposure to suboptimal temperatures affects oogenesis using the model insect *Drosophila melanogaster*. In adult females maintained at 18°C (cold) or 29°C (warm), relative to females at the 25°C control temperature, egg production was reduced through distinct cellular mechanisms. Chronic 18°C exposure improved germline stem cell maintenance, survival of early germline cysts and oocyte quality, but reduced follicle growth with no obvious effect on vitellogenesis. By contrast, in females at 29°C, germline stem cell numbers and follicle growth were similar to those at 25°C, while early germline cyst death and degeneration of vitellogenic follicles were markedly increased and oocyte quality plummeted over time. Finally, we also show that these effects are largely independent of diet, male factors or canonical temperature sensors. These findings are relevant not only to cold-blooded organisms, which have limited thermoregulation, but also potentially to warm-blooded organisms, which are susceptible to hypothermia, heatstroke and fever.

KEY WORDS: *Drosophila*, Temperature, Germline, Stem cell, Cell death, Oogenesis

INTRODUCTION

Reproduction is essential for the survival of species, but it is costly and requires coordination with the physiological and external environments (Diskin and Kenny, 2014; Gaskins and Chavarro, 2018; Albert Hubbard and Schedl, 2019; Drummond-Barbosa, 2019). In light of the current climate crisis, a key and timely question to consider is how temperature impacts reproduction. Several lines of evidence indicate that fertility is negatively impacted by increased environmental temperatures in a wide range of organisms, including plants, corals, insects and vertebrates (Walsh et al., 2019; González-Tokman et al., 2020; Kumar et al., 2021; Mishra, 2021). Insects have limited capacity to thermoregulate and are therefore particularly susceptible to climate change, especially rising temperatures aggravated by loss of refugial habitat (González-Tokman et al., 2020). Insects also have medical, economic and ecological relevance; for example, they represent the most diverse group of animals and include disease vectors, agricultural pests and pollinators (Schowalter et al., 2018). Yet, despite intense research on how temperature is sensed to influence insect behavior using the model insect *Drosophila melanogaster* (Barbagallo and Garrity, 2015; Li and Gong, 2017), much less is known about how chronic exposure of adult females to suboptimal temperatures affects the energy- and resource-intensive process of oogenesis.

Drosophila is a powerful system for investigating fundamental aspects of reproductive biology and it is well suited for physiological studies, given numerous available tools for cell- and tissue-specific manipulation, established methods for analysis of oogenesis and its well-described biology (Hudson and Cooley, 2014; Laws and Drummond-Barbosa, 2015). The *Drosophila* ovary is subdivided into ∼15 ovarioles, each having an anterior germarium followed by developing follicles (Fig. 1A). Each germarium contains two or three germline stem cells (GSCs), and each GSC division yields a GSC and a cystoblast that divides four times to form a two-, four-, eight- and, finally, 16-cell cyst (Fig. 1B). Follicle cells envelop the cyst, producing a follicle that buds off the germarium and develops through 14 stages of oogenesis (including the onset of vitellogenesis at stage 8) to form a mature oocyte ready to be fertilized and laid (Drummond-Barbosa, 2019). GSCs and their progeny grow and divide faster on rich rather than poor diets, and increased GSC loss, death of early germline cysts and degeneration of vitellogenic follicles on a poor diet also help modulate egg production (Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2009). Multiple diet-dependent factors, including insulin-like peptides, the steroid hormone ecdysone, the nutrient sensor Target of Rapamycin (TOR) and adipocyte factors, mediate this response (Drummond-Barbosa, 2019). However, much less is known about the effects of temperature on oogenesis.

Several lines of evidence suggest that increased temperature affects oogenesis in multiple *Drosophila* species. For example, larval development of *D. melanogaster*, *D. pseudoobscura*, *D. simulans*, *D. funebris* and *D. immigrans* at high temperatures (28.5°C to 32.5°C) leads to adult females with reduced fertility (Northrop, 1920; Plough and Strauss, 1923; Alpatov, 1932; Dobzhansky, 1935). *D. suzukii* flies that undergo development at the suboptimal temperature of 30°C (relative to those reared at the optimal 24°C) have reduced survival and fertility, and ovarian size and sperm counts are reduced in females and males, respectively (Kirk Green et al., 2019). Exposure of adult *D. virilis* to acute heat stress of 38°C for 4 h leads to vitellogenic follicle degeneration and accumulation of mature oocytes (Gruntenko et al., 2003). However, how oogenesis is affected in females reared under optimal conditions but chronically exposed to suboptimal temperatures during adulthood has remained largely unexplored.

Here, we report an in-depth analysis of how chronic exposure of adult *Drosophila melanogaster* females to cold or warm temperature influences GSCs and their progeny. We find that, compared with females at the 25°C control temperature, females at 18°C (cold) or 29°C (warm) produce significantly fewer eggs.

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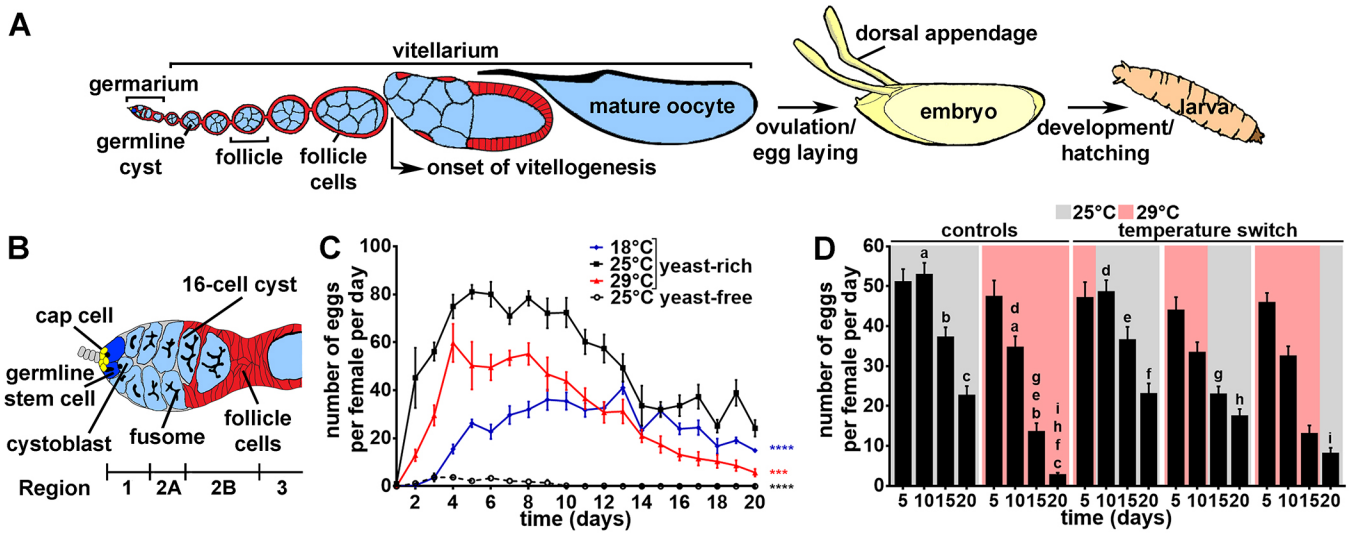

Fig. 1. Egg production decreases in a partially reversible manner upon chronic exposure of adult females to suboptimal temperatures. (A) Diagram of a *Drosophila* ovariole showing an anterior germarium followed by developing follicles (also known as egg chambers). Each follicle consists of a germline cyst (15 nurse cells and one oocyte) surrounded by somatic follicle cells. Follicles develop through 14 stages of oogenesis, with vitellogenesis (i.e. yolk uptake) beginning at stage 8. Once a mature stage 14 oocyte (recognizable by the presence of fully formed dorsal appendages) is ovulated, fertilized and laid as an egg, the resulting embryo develops for ∼24 h, giving rise to a larva that hatches out of the eggshell. (B) Diagram of germarium showing two germline stem cells (GSCs; dark blue) within a niche composed primarily of cap cells (yellow). As each GSC divides, it renews itself and produces a cystoblast that undergoes four rounds of incomplete mitoses to form a 16-cell cyst. The 16-cell cyst is enveloped by follicle cells (red) to make a new follicle that leaves the germarium. GSCs, cystoblasts and germline cysts are identified based on their characteristic fusome morphology (black). Germarium region 1 corresponds to the anterior region containing GSCs, cystoblasts and mitotically dividing cysts. Region 2A corresponds to newly formed 16-cell cysts not yet in contact with follicle cells. Region 2B corresponds to lens-shaped 16-cell cysts being surrounded by follicle cells. Region 3 corresponds to a round 16-cell cyst fully surrounded by a monolayer of follicle cells (also known as a stage 1 follicle) that has not yet completed budding. (C) Average number of eggs laid per *y w* female per day over time upon chronic exposure of adult females to 18°C (cold) or 29°C (warm) compared with 25°C controls. Females on a yeast-free diet at 25°C were used as a negative control (Drummond-Barbosa and Spradling, 2001). Data are mean±s.e.m. from six replicates. ***P<0.001; ****P<0.0001, F-test of third order polynomial fitted curves using 25°C as control. (D) Average number of eggs laid per *y w* female per day in temperature switch experiments. Adult females were initially exposed to 29°C (indicated by red rectangles) for 5, 10 or 15 days prior to being switched to 25°C (indicated by grey rectangles) for remainder of 20-day time course. Females maintained at 25°C or 29°C for entire duration were used as controls. Data are mean±s.e.m. from three independent experiments. Pairwise comparisons, P<0.0001 for a-c,e,f,h; pairwise comparisons, P<0.01 for d,g,i. Multiple t-test with Holm-Sidak method using 29°C as control.

Different cellular mechanisms account for the decreases in egg production at 18°C versus 29°C. We found that chronic exposure of females to 18°C improved GSC maintenance and early germline cyst survival but reduced the rate of follicle development, perhaps due to changes in thermodynamics and/or slower aging. By contrast, chronic exposure to 29°C increased early germline cyst death and degeneration of vitellogenic follicles, with little to no effect on GSC numbers or follicle growth rate. Fertility can be affected not only by the rate of oocyte production, but also by the quality of those oocytes (i.e. their ability to support embryo development). We found that oocyte quality is drastically reduced in females at 29°C relative to controls at 25°C. Finally, the negative effects of 29°C on oogenesis are largely independent of diet, male factors or canonical warm temperature sensors. These findings provide a foundation for future research on the mechanisms underlying the distinct effects of temperature on specific steps of oogenesis. This broad question is widely relevant not only to cold-blooded organisms, which have limited ability to regulate their body temperature, but also potentially to warm-blooded organisms, which are susceptible to hypothermia, heatstroke and fever (Cheshire, 2016).

RESULTS

Chronic exposure of adult females to suboptimal temperatures decreases egg production in a partially reversible manner

To determine how chronic exposure of adult females to suboptimal temperatures affects oogenesis, we first measured egg laying rates using the *y w* strain, which is commonly used as a genetic background in *Drosophila* studies. *y w* females maintained with males at a cold (18°C) or warm (29°C) temperature laid fewer eggs relative to those at the 25°C control temperature (Fig. 1C; Fig. S1A,B). Similar temperature effects were observed in Oregon-R-C and Canton-S wild-type strains (Fig. S1C,D). To determine whether warm temperature effects on oogenesis are reversible, females were incubated with males at 29°C for 5, 10 or 15 days prior to switching to 25°C for the remainder of the 20-day experiment. Notably, the effects of 29°C are fully reversible if females are switched to 25°C after the first 5 days, but only partially reversible after 10 or more days at 29°C (Fig. 1D), suggesting that multiple mechanisms account for the effects of temperature on oogenesis.

Chronic exposure of adult females to cold improves GSC maintenance

We and others have previously shown that physiological factors influence oogenesis at multiple steps, including GSC maintenance and proliferation, early germline cyst survival, follicle growth, survival of vitellogenic follicles, and ovulation (Drummond-Barbosa, 2019). We first tested whether chronic exposure to suboptimal temperatures affects GSCs. We counted GSCs over time, and found that GSC numbers were indistinguishable in females at 25°C and 29°C (Fig. 2A,B), showing the normal baseline rate of GSC loss (Xie and Spradling, 2000). By contrast, GSCs were maintained better over time at 18°C compared with the standard 25°C (Fig. 2A,B). Similar results were obtained when we measured

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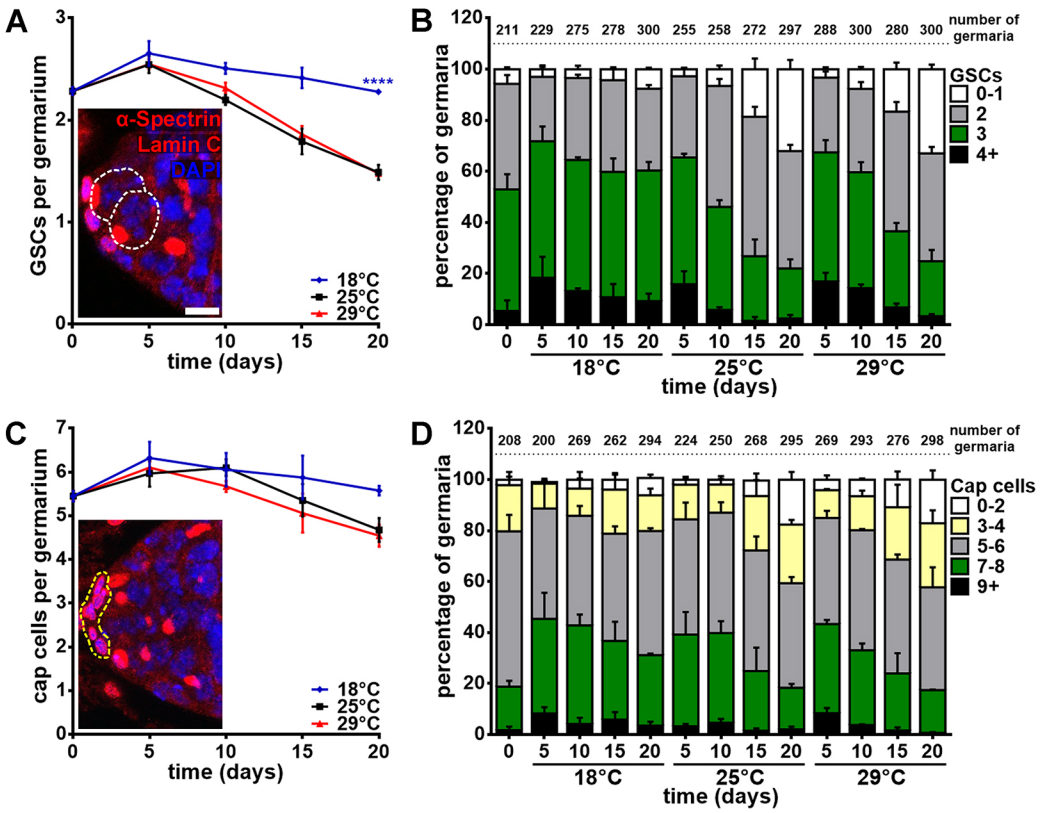

Fig. 2. Chronic exposure of adult females to cold reduces the rate of GSC loss. (A) Average number of GSCs per germarium in adult *y w* females maintained at 18°C, 25°C or 29°C for 0, 5, 10, 15 or 20 days. (B) Frequencies of germaria containing 0-1, 2, 3, or 4 or more GSCs from same raw data as in A. (C) Average number of cap cells per germarium in same females as in A. In A,C, images show examples of germaria from females kept at 25°C for 15 days. α-Spectrin (red), fusome; Lamin C (red), cap cell nuclear lamina; DAPI (blue), nuclei. GSCs (A) and cap cells (C) are outlined. Images in A,C represent projections of three 1 µm confocal sections. Scale bar: 5 µm. (D) Frequencies of germaria containing 0-2, 3 or 4, 5 or 6, 7 or 8, or 9 or more caps cells from same raw data as in C. In B,D, numbers of germaria analyzed are shown above the bars. Data are mean±s.e.m. from three independent experiments. ****P<0.0001, two-way ANOVA with interaction using 25°C as control.

cap cell numbers at different temperatures, although the trend towards increased niche size at 18°C did not reach statistical significance (Fig. 2C,D). These results indicate that GSC loss is not a contributing factor to the reduced egg production at 18°C or 29°C, and that GSC maintenance is actually improved at 18°C, perhaps as a result of a slowdown in aging that occurs at this temperature (Carvalho et al., 2017).

To assess GSC proliferation, we compared the frequencies of GSCs positive for 5-ethynyl-2′-deoxyuridine (EdU), a thymidine analog incorporated into cells in S phase (Chehrehasa et al., 2009), among females maintained at different temperatures (Fig. 3A). Females exposed to 29°C or the control 25°C temperature for 5 days had similar frequencies of EdU-positive GSCs, suggesting that GSCs proliferate at normal rates under warm temperature (Fig. 3B). Intriguingly, the frequency of EdU-positive GSCs in females at 18°C was significantly elevated (Fig. 3B). We speculate that these results reflect prolonged S phase at 18°C slowing down GSC division (perhaps in part due to thermodynamic changes) (Schulte, 2014; Glazier, 2015), based on the lineage-tracing analyses described below.

Temperature modulates the survival of eight-cell germline cysts

We next examined the effect of temperature on early germline cyst survival using the ApopTag TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay to label dying cells (Drummond-Barbosa and Spradling, 2001) (Fig. 4A). The frequency of germaria with early dying germline cysts was significantly increased in females maintained at 29°C for 5 or 15 days, whereas exposure to 18°C reduced early cyst death relative to 25°C at both time points (Fig. 4B). To pinpoint when temperature affects early germline cyst survival, we quantified the number of cystoblasts and the number of two-, four-, eight- and 16-cell cysts (normalized to the number of GSCs) per germarium in females exposed to different temperatures. Although similar numbers of cystoblasts, two- and four-cell cysts are present at 18°C, 25°C and 29°C, there is a marked reduction in the numbers of eight- and 16-cell cysts in germaria at 29°C relative to those at 25°C (Fig. 4C), consistent with our observations that germaria are visibly shorter at 29°C (see Fig. 4A). Conversely, the numbers of eight- and 16-cell germline cysts were significantly higher in females exposed to 18°C relative to 25°C (Fig. 4C), in accordance with the reduced frequency of germaria containing ApopTag-positive cysts at 18°C (Fig. 4B). These results indicate that temperature controls the survival of germline cysts at the eight-cell stage. Specifically, reduced early germline cyst death at 18°C contributes to the increase in eight- and 16-cell cysts, whereas increased death of early cysts at 29°C likely contributes to the reduced number of early cysts and later eggs produced by females at 29°C.

Chronic exposure to cold temperature slows down follicle growth

To directly measure the rates of follicle development and growth among females maintained at 18°C, 25°C or 29°C, we took

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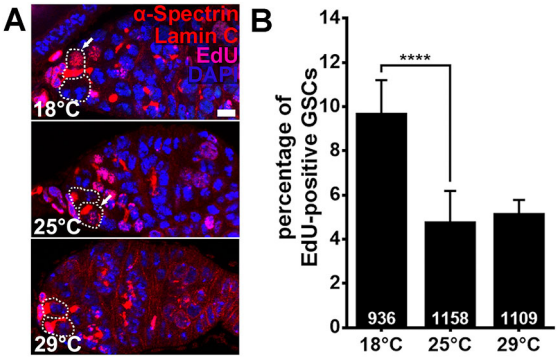

Fig. 3. Germline stem cells have similar proliferation rates at 25°C and 29°C. (A) Examples of germaria from females kept at 18°C, 25°C or 29°C for 5 days. α-Spectrin (red), fusome; Lamin C (red), cap cell nuclear lamina; DAPI (blue), nuclei. EdU (red) can be distinguished from the fusome based on morphology and overlap with nuclei. GSCs are outlined. Arrows indicate EdU-positive GSCs. Images are projections of three 1 µm confocal sections. Scale bar: 5 µm. (B) Frequencies of GSCs in S phase based on EdU incorporation in females described in A. Numbers of GSCs analyzed are shown inside the bars. Data are mean±s.e.m. from five independent experiments. ****P<0.0001, Chi-square test using 25°C as control.

advantage of a well-established lineage-tracing system previously used to measure dietary effects on oogenesis (Drummond-Barbosa and Spradling, 2001). In this system, two defective *tubulin-lacZ* transgenes undergo recombination at 'time zero' (by heat-shock induction of flippase/FRT recombination) to generate a constitutively expressed *tubulin-lacZ* transgene in a small fraction of mitotically dividing cells (Harrison and Perrimon, 1993) (Fig. 5A). Single β-galactosidase (β-gal)-labeled germ cells can only be induced in germarium region 1 (where germ cells undergo mitosis), whereas single follicle cells can become β-gal-positive up until stage 6, after which follicle cells switch from mitotic to endoreplicative cycles (Zielke et al., 2013). As single β-gal-positive cells labeled at 'time zero' divide and grow, their progeny inherit the active *tubulin-lacZ* transgene. This lineage-tracing approach allows us to determine how far germline cysts have progressed through oogenesis since their original labeling in germarium region 1, and how fast follicle cells proliferate (based on the size of clones formed from single labeled follicle cells within the epithelium surrounding germline cysts).

Our lineage-tracing analyses showed distinct effects of 18°C and 29°C on follicle growth. Chronic exposure to 18°C significantly reduces the rate of follicle growth relative to that at 25°C, based on the slowed progression through oogenesis of germ cells labeled during early mitotic divisions (Fig. 5B,C) and on the decreased proliferation rate of follicle cells (Fig. 5D,E). By contrast, we detected a relatively small increase in follicle cell proliferation rate at 29°C (Fig. 5E); however, a corresponding increase in the rate of follicle growth/development was not detected in our germline cyst analysis (Fig. 5C). We also ruled out that incubation of females at 29°C triggers the lineage-tracing system, given that non-heat-shocked females maintained at 29°C have no β-gal-positive cells (Fig. 5F). These results indicate that slowed follicle growth contributes to the lower rates of egg production of females maintained at 18°C but not at 29°C. Furthermore, the slowed follicle growth at 18°C is consistent with our speculation that GSCs divide more slowly due to prolonged S phase (see above and Fig. 3), given that we do not observe accumulation of any intermediate stages of oogenesis between GSCs and developing follicles (Fig. 4C).

Chronic exposure to warm temperature causes death of vitellogenic follicles

Multiple physiological factors affect the survival of follicles in early stages of vitellogenesis in *D. melanogaster* (Drummond-Barbosa, 2019), and acute heat stress (4 h at 38°C) induces follicle degeneration in *D. virillis* (Gruntenko et al., 2003). We therefore compared the frequencies of ovarioles containing degenerating vitellogenic follicles among females maintained at 18°C, 25°C or 29°C temperatures over time (Fig. 6A). No statistically significant differences in vitellogenic follicle survival were observed in females chronically exposed to 18°C relative to 25°C (Fig. 6B). In females exposed to 29°C, vitellogenic follicle survival was not significantly impacted at 5 or 10 days (Fig. 6B); however, the frequency of ovarioles containing degenerating vitellogenic follicles was markedly higher in females exposed to 29°C for 15 or 20 days, relative to 25°C controls (Fig. 6B). These results show that increased

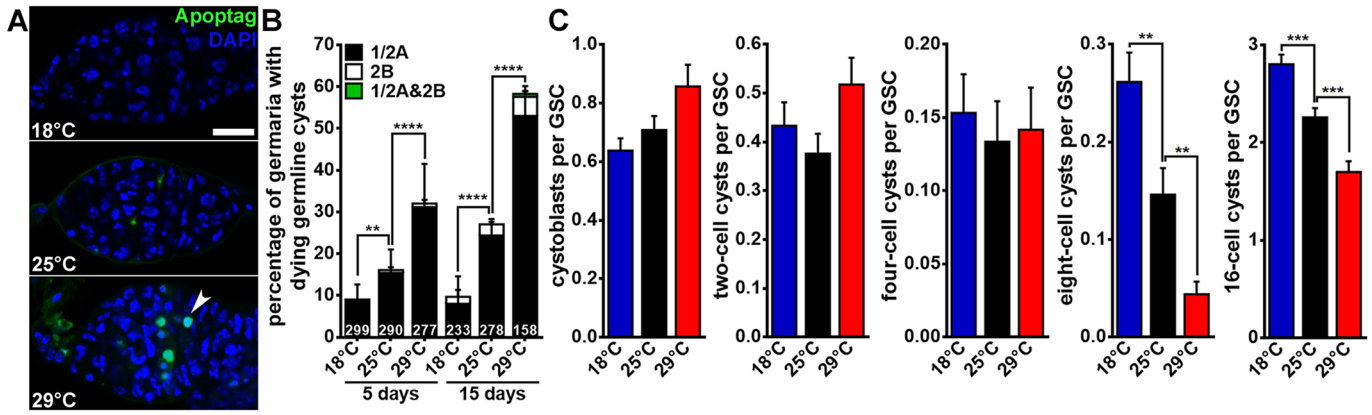

Fig. 4. The survival of eight-cell germline cysts is temperature dependent. (A) Examples of germaria from *y w* females incubated for 15 days at 18°C, 25°C or 29°C. DAPI (blue), nuclei; Apoptag (green), dying cells. Images are projections of three 1 µm confocal sections. Arrowhead indicates dying germline cyst. Scale bar: 10 µm. (B) Percentage of germaria containing Apoptag-positive dying cysts in region 1/2A (meaning late region 1 and/or region 2A; black bar), region 2B (white bar) or in both (green bar) from *y w* females incubated for 5 or 15 days at 18°C, 25°C or 29°C. Data are mean±s.e.m. from three independent experiments. **P<0.01, ****P<0.0001, Chi-square test using 25°C as control. Numbers of germaria analyzed are shown inside the bars. (C) Number of cystoblasts, two-cell cysts, four-cell cysts, eight-cell cysts and 16-cell cysts, normalized to the number of GSCs, in germaria of females incubated for 15 days at 18°C, 25°C or 29°C. The total number of germaria analyzed was 87 (18°C), 87 (25°C) and 78 (29°C). Data are mean±s.e.m. from three independent experiments. **P<0.01; ***P<0.001, two-tailed Mann–Whitney test using 25°C as control.

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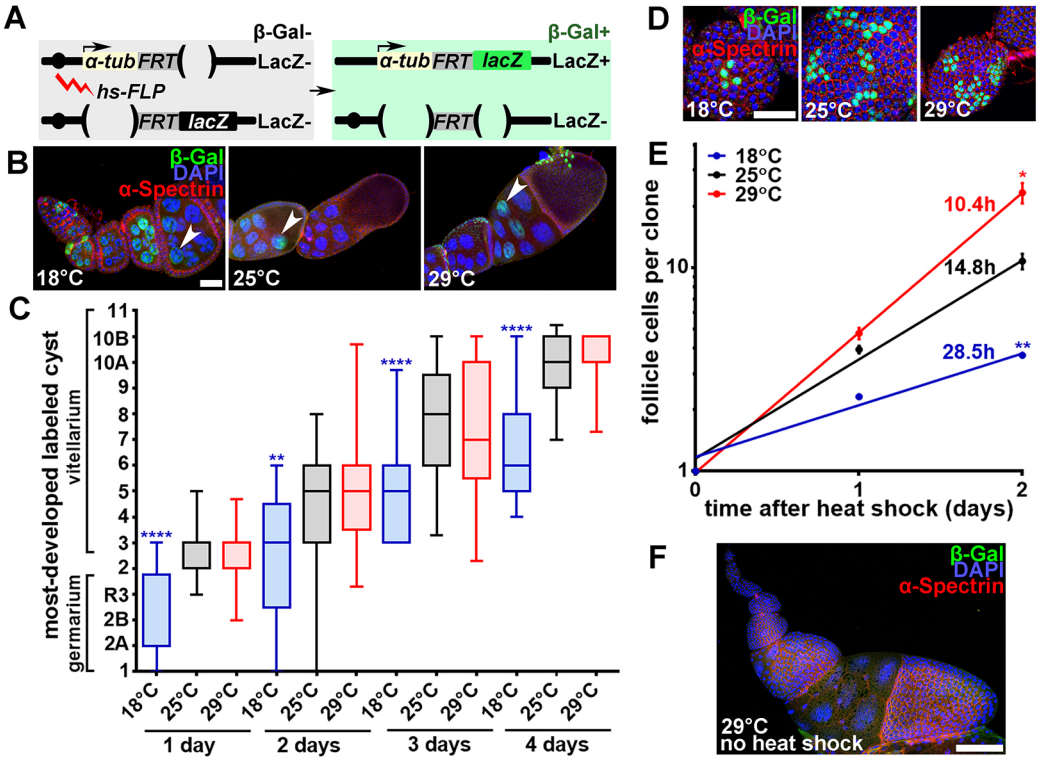

Fig. 5. Follicle development is significantly slowed down at 18°C. (A) Diagram of the lineage-tracing system. Heat-shock induction of flippase (*hs-FLP*) leads to the generation of a constitutively expressed *tubulin-lacZ* transgene through FLP/*FRT*-mediated recombination in a fraction of mitotically dividing cells (see text for details). (B) Single confocal sections of ovarioles with β-gal-positive germline clones at 4 days after heat shock, showing how far they have developed (since the 'time zero' initial labeling in germarium region 1) in females maintained at 18°C, 25°C or 29°C. β-gal (green), germline cysts; α-Spectrin (red), fusome and follicle cell membranes; DAPI (blue), nuclei. Arrowheads indicate the most-developed (i.e. most posteriorly located) follicle containing β-gal-positive germ cells in each ovariole. Scale bar: 20 µm. (C) Box and whisker plot showing the stage of the most-developed germline cyst per ovariole at different times after clone induction in females maintained at 18°C, 25°C or 29°C. Forty-five ovarioles were analyzed for each time point. The horizontal line indicates the median, the box indicates the upper and lower quartiles, and the whiskers indicate the maximum and minimum values. Data are from three independent experiments. ***P*<0.01; *****P*<0.0001, Chi-square test using 25°C as control. (D) Single confocal sections of follicles with clones of β-gal-positive follicle cells 2 days after heat shock (when single follicle cells were labeled at 'time zero'). β-Gal (green) labeled follicle cell clones. Scale bar: 20 µm. (E) Log scale plot showing the average number of follicle cells per clone over time. Data are mean±s.e.m. from three independent experiments. Doubling times are shown next to corresponding regression lines. **P*<0.05; ***P*<0.01, two-way ANOVA with interaction using 25°C as control. (F) Ovariole stained as in B and D from a female carrying lineage-tracing transgenes that did not undergo heat shock and were maintained at 29°C for 2 days. Scale bar: 100 µm. This example, illustrating the complete absence of β-gal-positive cells, is representative of all the ovarioles analyzed (*n*=151), indicating that incubation at 29°C is not sufficient to induce the lineage-tracing system.

death of vitellogenic follicles plays a major role in reducing egg production in females exposed to 29°C for 10 or more days, while 18°C has no effect on vitellogenic follicle survival.

Chronic exposure of adult flies to warm temperature lowers oocyte quality and impairs male fertility

Female fertility depends not only on the number of oocytes produced, but also on the quality of those oocytes (i.e. their ability to support development of the future embryo into a larva). We therefore measured the fraction of eggs laid by *y w* females (maintained with males) at 29°C that hatched into larvae (i.e. the hatching rate). Eggs laid by females at 18°C, 25°C and 29°C were allowed to develop at 25°C for 24 h, and the numbers of eggs that hatched were counted. About 90% of eggs from females at the control 25°C temperature hatched at 5 days, and this number steadily decreased over time, such that just under 60% of eggs hatched at 20 days (Fig. 7A). Eggs from females at 18°C maintained an over 80% hatching rate for the entire 20-day period (Fig. 7A), indicating that oocyte quality remains higher over time at the lower temperature compared with at 25°C, possibly as a result of the lower aging rate at 18°C (Carvalho et al., 2017). By contrast, for females at 29°C, only ∼50% of eggs hatched at 5 days, and that number continued to decrease at 10, 15 and 20 days, when almost none of the eggs hatched (Fig. 7A). Daily quantification showed that significant decreases in hatching rates can be detected as early as 3 days after placing females at 29°C relative to 25°C (Fig. 7B). Although we observed some morphological defects in eggs laid at 29°C (Fig. S2A), the low frequency of these defects (Fig. S2B) was not sufficient to explain the large reduction in hatching rates (Fig. 7A). The effects of warm temperature on hatching rates were partially reversible after 5 or 10 days of 29°C exposure (Fig. 7C). Similar effects of temperature on hatching rates were observed in Oregon-R-C and Canton-S strains (Fig. S3).

To determine whether the hatching rate decrease at 29°C results from impaired oocyte quality and/or decreased male fertility, we tested the male and female contributions separately. To test males, we incubated *y w* males and females at 29°C for 17 days, and then substituted 2-day-old virgin *y w* females (previously at 25°C) for the old females before incubating couples at 29°C for 3 additional days (for a total of 20 days for males at 29°C) (Fig. 7D). Virtually none of the eggs laid by these young females (incubated with males kept for 20 days at 29°C) hatched, indicating that males became completely sterile (Fig. 7D). To test females, we either used a similar strategy to the one above and substituted 2-day-old males (previously at 25°C)

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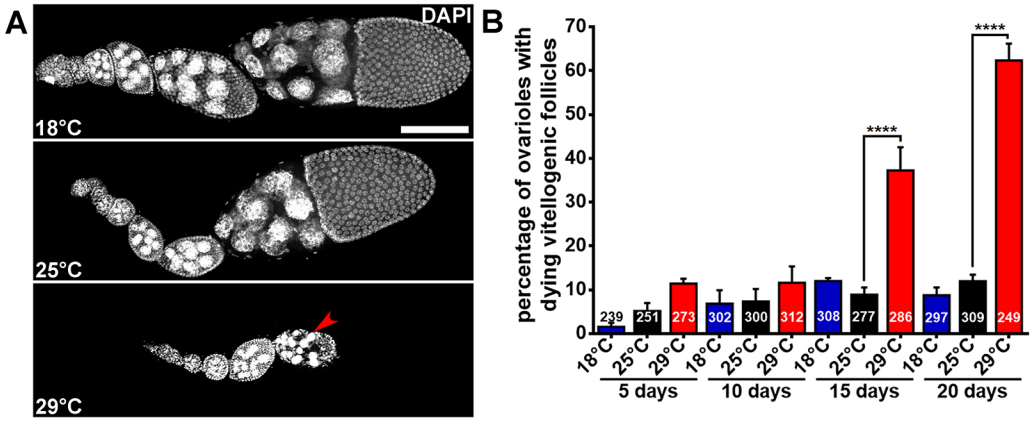

Fig. 6. Chronic exposure of adult females to 29°C leads to increased death of vitellogenic follicles. (A) Examples of ovarioles from *y w* females incubated for 20 days at 18°C, 25°C or 29°C, shown as single confocal slices. DAPI (white) indicates nuclei. Arrowhead indicates a dying vitellogenic follicle with pyknotic nuclei. Scale bar: 65 µm. (B) Frequencies of ovarioles containing dying vitellogenic follicles in *y w* females incubated for five, 10, 15 or 20 days at 18°C, 25°C or 29°C. Numbers of ovarioles analyzed are shown inside or above the bars. Data are mean±s.e.m. from three independent experiments. ****P<0.0001, Chi-square test using 25°C as control. The small trend observed in females exposed to 29°C for 5 or 10 days does not reach statistical significance.

for the old males (Fig. 7E, top graph), or we used virgin females for the 17-day incubation and added the young males for the last 3 days (Fig. 7E, bottom graph), showing that oocyte quality is very low at 29°C. These results show that both oocyte quality and male fertility are markedly reduced over time at 29°C.

The effects of suboptimal temperatures on oogenesis cannot be fully explained by changes in food consumption or by male factors

Nutrient intake and mating are known to affect *Drosophila* oogenesis (Chapman et al., 1996; Soller et al., 1997; Drummond-Barbosa and Spradling, 2001; Bloch Qazi et al., 2003; Barnes et al., 2008). Diet impacts the proliferation and maintenance of GSCs, as well as the proliferation, growth and survival of their progeny (Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2009), while male factors affect vitellogenesis and ovulation (Soller et al., 1997; Bloch Qazi et al., 2003). Although effects of temperature on *Drosophila* mating have been reported (Miquel et al., 1976; Schnebel and Grossfield, 1984; Patton and Krebs, 2001; Miwa et al., 2018; Stazione et al., 2019), the findings are inconsistent owing to experimental differences among studies. For example, chronic exposure (7 days) to 27°C reduced mating, as measured by an 11-min copulation assay (Miquel et al., 1976), while male-female pairings for 48 h in temperatures as high as 30°C had no negative effect on mating, as measured by presence of sperm in sperm storage organs of females (Schnebel and Grossfield, 1984). Other studies showed decreases in mating/mating behavior upon acute exposure (1-4 h) to higher temperatures (33°C-38°C) using different assays (Patton and Krebs, 2001; Miwa et al., 2018; Stazione et al., 2019). We therefore asked whether changes in nutrient consumption or in male factors might account for the effects of temperature on oogenesis.

We determined how incubation of females at 18°C or 29°C affects food consumption using the recently developed consumption-excretion assay (Shell et al., 2018), which allows us to measure the total amount of food medium consumed (internal amount in females plus excretions; see Materials and Methods) by females in a 24 h period. Females chronically exposed to 29°C ate control levels of food at 5 days but significantly less at 15 days (~64% reduction), whereas females at 18°C consumed significantly less food at both 5 and 15 days (69% and 75% reductions, respectively) (Fig. S4). The reduced food consumption by females at 18°C (Fig. S4) could potentially at least in part explain the reduced rate of follicle growth at 18°C (Fig. 5), given the known effects of diet on follicle growth (Drummond-Barbosa and Spradling, 2001). By contrast, the increased germline cyst death at 29°C cannot be fully explained by changes in food consumption, which is not affected at 5 days at 29°C (Fig. S4), when the effect on cyst death is already evident (Fig. 4B). Similarly, the higher levels of vitellogenic follicle degeneration (Fig. 6B) or the reduced oocyte quality (Fig. 7E) at 29°C cannot be explained by the reduced food consumption at 15 days at 29°C because females at 18°C for 15 days have very similar levels of food consumption (Fig. S4) but no changes in vitellogenic follicle survival (Fig. 6B) and improved hatching rates of laid eggs (Fig. 7A) relative to 25°C controls. Altogether, these findings show that changes in food consumption cannot account for most of the effects of temperature on oogenesis.

We reasoned that changes in mating (i.e. transfer of male factors) might contribute to the effects of temperature on oogenesis in our experiments, given that both exposure to 29°C (Fig. 6) and lack of mating (Soller et al., 1997) increase death of vitellogenic follicles. Ovulation is controlled by male factors (Bloch Qazi et al., 2003); we therefore reasoned that if 29°C exposure prevented the transfer of male factors to females, ovulation would be significantly impacted. To test this idea, we measured the number of mature stage 14 oocytes present in the ovaries of females at 18°C, 25°C or 29°C (Fig. 8A). Despite a trend towards a slight increase in the number of ovarioles containing two or more mature oocytes at 29°C, this number remained statistically similar at 18°C, 25°C or 29°C (Fig. 8B). These results suggest that mating/transfer of male factors is largely unaffected in our experiments, in stark contrast to certain genetic manipulations that lead to drastic accumulation of mature oocytes as a result of impaired ovulation (Armstrong et al., 2014; Ma et al., 2020). To complement these experiments, we also measured sperm transferred to females at 29°C (Fig. 8C). We incubated *y w* females with *ProtB-eGFP; dj-GFP* males (which produce GFP-labeled sperm) (Yang and Lu, 2011) at 25°C or 29°C for 10 days and quantified the presence of GFP-labeled sperm in the spermatheca, one of the sperm storage organs in females (Mayhew and Merritt, 2013). Nearly all of the spermatheca contained sperm at both temperatures (Fig. 8D), indicating that the increase in early germline cyst death (Fig. 4) and vitellogenic follicle degeneration (Fig. 6), and the reduced hatching rates at 29°C (Fig. 7) are not due to lack of sperm in the females. Nevertheless, to directly ask whether

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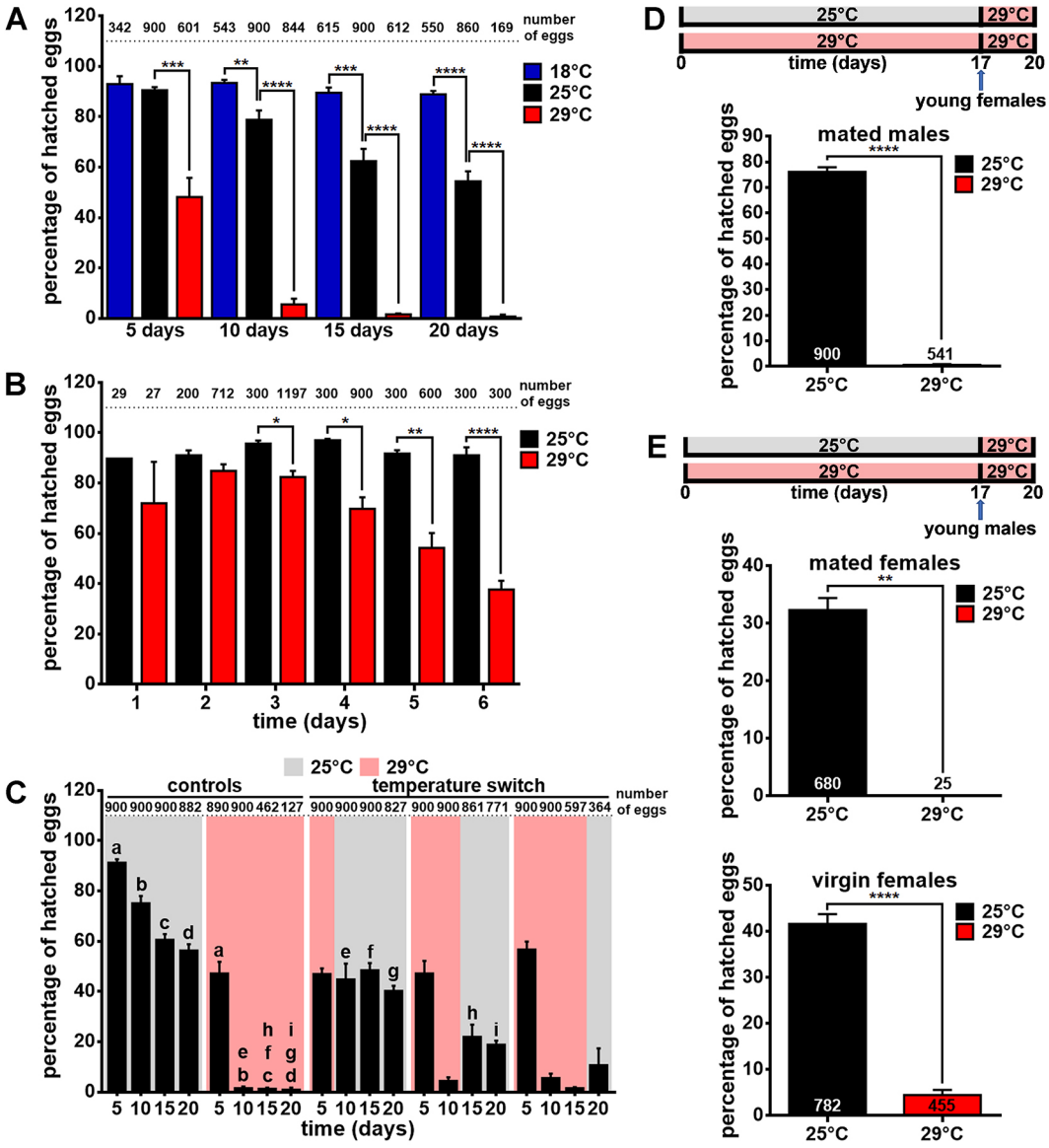

Fig. 7. Chronic exposure to 29°C lowers oocyte quality and impairs male fertility. (A) Percentage of hatched eggs laid by *y w* females incubated with *y w* males for 5, 10, 15 or 20 days at 18°C, 25°C or 29°C. (B) Percentage of hatched eggs laid by *y w* females incubated with *y w* males for 1, 2, 3, 4, 5 or 6 days at 25°C or 29°C. Numbers of eggs analyzed are shown above the bars. Data are mean±s.e.m. from three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, unpaired *t*-test using 25°C as control. (C) Percentage of hatched eggs laid by *y w* females incubated with *y w* males in temperature-switch experiments. Adult females were initially exposed to 29°C (indicated by red rectangles) for 5, 10 or 15 days prior to being switched to 25°C (indicated by gray rectangles) for the remainder of 20-day time course. Females maintained at 25°C or 29°C for the entire experiment were used as controls. Numbers of eggs analyzed are shown above the bars. Data are mean±s.e.m. from three biological replicates. a-g,i are pairwise comparisons, *P*<0.0001; h is a pairwise comparison, *P*<0.01. Multiple *t*-test with Holm-Sidak method, using 29°C as control. (D) Quantification of effect of 29°C on males. *y w* couples were incubated at 25°C or 29°C for 17 days, after which the original females were replaced with 2-day-old virgin females. Original males with new females were incubated for 3 days at 29°C. Eggs laid within the final 24 h were collected to quantify hatching rate. (E) Quantification of effect of 29°C on oocyte quality. *y w* couples (top graph) or *y w* virgins alone (bottom graph) were incubated at 25°C or 29°C for 17 days, then 2-day-old males replaced the original males (top graph) or were added to the virgin females (bottom graph), and couples were incubated for 3 days at 29°C. Eggs laid within the final 24 h were collected to quantify hatching rate. Numbers of eggs analyzed are shown inside or above the bars. Data are mean±s.e.m. from three experiments. ***P*<0.01, *****P*<0.0001, unpaired two-tailed *t*-test using 25°C as control.

the effects of temperature on the ovary depend on any male factor (including pheromones), we tested the effects of 18°C and 29°C on virgin females (i.e. females never exposed to males) (Fig. 8E-G). We found that the increase in early germline cyst death (Fig. 8E) and vitellogenic follicle degeneration (Fig. 8G) at 29°C, and the slowdown of follicle growth at 18°C do not require the presence of males (Fig. 8F). Taken together, these results conclusively show that male factors do not play a major role in how temperature affects the ovary.

The effects of warm temperature on oogenesis are independent of canonical warm temperature receptors

Drosophila has well-studied temperature-sensing mechanisms involving thermosensory proteins in the brain and antenna that control behavior (Barbagallo and Garrity, 2015; Li and Gong, 2017). For example, TrpA1 is expressed in anterior cell neurons in the brain and mediates their activation in response to temperatures above ~25°C (Viswanath et al., 2003; Hamada et al., 2008), while Gr28b is expressed in hot cell neurons in the arista (part of the

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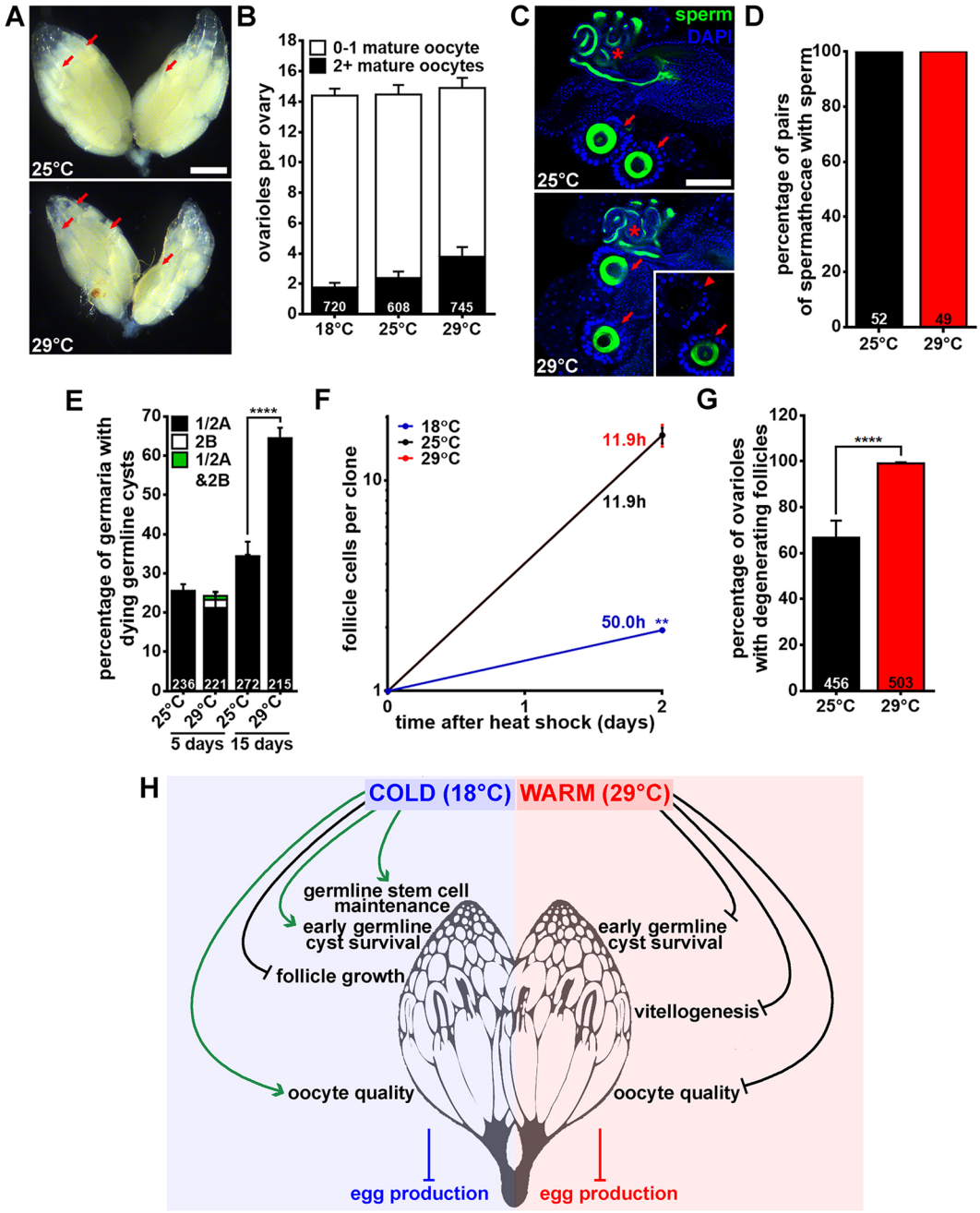

Fig. 8. The effects of temperature on oogenesis are male independent. (A) Pairs of ovaries from *y w* females incubated for 15 days at 25°C or 29°C. Mature oocytes are recognizable by the presence of dorsal appendages (indicated by arrows; see Fig. 1A). Scale bar: 200 µm. (B) Number of ovarioles per ovary with 0-1, or 2 or more mature oocytes from females maintained at 18°C, 25°C or 29°C for 15 days. Numbers of ovarioles analyzed are shown inside the bars. Data are mean±s.e.m. from four independent experiments. No statistically significant differences (Mann–Whitney test using 25°C as control). (C) Spermatheca pairs from *y w* females incubated with *ProtB-eGFP;dj-GFP* males for 10 days at 25°C or 29°C. Sperm (green) are labeled with ProtB-eGFP/dj-GFP. DAPI (blue) indicates nuclei. Arrows indicate spermathecae containing sperm. The inset shows a rare example of 29°C female with one empty spermatheca (arrowhead). Asterisks indicate seminal receptacles. Scale bar: 100 µm. (D) Percentage of spermatheca pairs containing *ProtB*-GFP;*dj*-GFP sperm at 25°C or 29°C. Numbers of spermatheca pairs analyzed are shown inside the bars. No statistically significant differences (Chi-square test using 25°C as control). (E) Percentage of germaria containing Apoptag-positive dying cysts in region 1/2A (black bar), region 2B (white bar) or in both (green bar) from *y w* virgin females incubated for 5 or 15 days at 25°C or 29°C. Numbers of germaria analyzed are shown inside bars. Data are mean±s.e.m. from three independent experiments. ****P<0.0001, Chi-square test. (F) Log scale plot showing the average number of follicle cells per clone. The theoretical 'time zero' value is based on the fact that single labeled follicle cells are generated at the time of flippase induction by heat shock (see Fig. 5A). Data at 2 days are mean±s.e.m. from three independent experiments. Doubling times are shown next to corresponding lines. **P<0.01, two-way ANOVA with interaction using 25°C as control. (G) Frequencies of ovarioles containing degenerating follicles in *y w* virgin females incubated for 15 days at 25°C or 29°C. Numbers of ovarioles analyzed are shown inside bars. Data are mean±s.e.m. from two independent experiments. ****P<0.0001, Chi-square test using 25°C as control. (H) Model for how cold and warm temperatures affect rates of egg production and oocyte quality. Chronic exposure of adult females to 18°C or 29°C reduce egg production rates through entirely distinct cellular effects. At 18°C, slowed follicle growth leads to a lower rate of oogenesis, whereas at 29°C, the increase in both early germline cyst death and vitellogenic follicle degeneration reduces egg production. Notably, 18°C (relative to 25°C control temperature) appears to have beneficial effects to the ovary, including improved GSC maintenance, early cyst survival and oocyte quality. By contrast, oocyte quality is severely impaired at 29°C, further contributing to the drastic drop in fertility at 29°C.

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antenna) and also senses warm temperature (Ni et al., 2013). Despite sensing similar temperatures, these neurons project to different brain regions that control distinct behaviors (Frank et al., 2015; Liu et al., 2015). Although mechanisms of temperature sensing have been studied in the context of *Drosophila* behavior, we reasoned that they might also mediate other responses to temperature in the body, including the temperature effects on oogenesis.

We therefore tested whether warm temperature sensors are involved in the 29°C effects on the ovary. We analyzed early germline cyst death, vitellogenic follicle degeneration and oocyte quality in females homozygous for null *TrpA1* and hypomorphic *Gr28b* alleles ($TrpA1^{1}$ and $Gr28b^{c01884}$, respectively; Fig. S5A,B) in the same genetic background as the *y w* controls at 29°C. There were no significant differences in early germline cyst death among control and experimental females exposed to 29°C for 10 days (Fig. S5C). Homozygous $TrpA1^{1}$ or $Gr28b^{c01884}$ had statistically similar levels of dying vitellogenic follicles to *y w* females, while double mutant $Gr28b^{c01884}$; $TrpA1^{1}$ females showed an increase in the percentage of ovarioles with degenerating vitellogenic follicles (Fig. S5D). However, the biological relevance of these results is unclear owing to their high variability and small statistical significance. Intriguingly, eggs laid by homozygous $TrpA1^{1}$ females, but not by $Gr28b^{c01884}$ homozygotes, had markedly improved oocyte quality relative to those laid by *y w* control females incubated at 29°C (but not at 25°C) for 10 days (with young *y w* males substituted for original males prior to egg collection and hatching rate measurement) (Fig. S5E,F). However, this phenotype was not reversed by a previously published genomic rescue construct (Hamada et al., 2008), indicating that the $TrpA1^{1}$ mutation is not responsible for it (Fig. S5F). Although we cannot rule out the possibility that the remaining *Gr28b* mRNA expression in $Gr28b^{c01884}$ females (Fig. S5B) might have obfuscated additional roles of *Gr28b*, these results suggest that the effects of 29°C on oogenesis are largely independent of canonical warm temperature receptors and that as yet unknown mechanisms are at play instead.

DISCUSSION

According to the United Nations, 'climate change is the defining crisis of our time, and it is happening even more quickly than we feared'. As temperatures steadily rise on Earth, we all notice its many effects, including natural disasters, weather extremes, environmental degradation, higher disease spread and negative economic impact (Masson-Delmotte et al., 2021). Insects are particularly vulnerable to suboptimal environmental temperatures owing to their limited capacity for thermoregulation (Colinet et al., 2015; Walsh et al., 2019; González-Tokman et al., 2020), while their reproductive fitness is essential for their long-term survival as species. Yet, the cellular effects of chronic adult exposure on specific stages of insect oogenesis have remained largely unexplored. Our data delineate how chronic exposure of adult *D. melanogaster* to warm or cold temperatures leads to significant reduction in rates of egg production through remarkably distinct mechanisms (Fig. 8H). Cold temperature improves GSC maintenance, survival of their progeny and oocyte quality, but slows down follicle growth to decrease egg output. Conversely, warm temperature increases death of eight-cell germline cysts and degeneration of vitellogenic follicles, and markedly impairs oocyte quality, with minor (if any) effects on follicle growth. Remarkably, GSCs, which are often lost faster in response to physiological changes (Drummond-Barbosa, 2019), remain unaffected at warm temperatures, suggesting the existence of protective mechanisms. This study provides a conceptual framework for investigating the effects of temperature on the oogenesis of insects in general, which have enormous ecological, agricultural, medical and economic relevance (Schowalter et al., 2018; Walsh et al., 2019; González-Tokman et al., 2020; Masson-Delmotte et al., 2021). It also highlights the crucial importance of research efforts towards gaining a deeper knowledge of how temperature impacts the reproduction of cold-blooded organisms around the globe. Our findings are also potentially relevant to warm-blooded animals [including humans, which are susceptible to fever, heat stroke, hypothermia and thermoregulatory disorders (Cheshire, 2016)], as suggested by studies describing negative effects of heat stress on the reproduction of mammals (Roth and Wolfenson, 2016; Jensen et al., 2021).

Cold and warm temperatures slow down egg production through distinct cellular mechanisms

In contrast to previous studies in the *Drosophila* genus that described the negative effects on oogenesis of subjecting adults to acute thermal stress (Gruntenko et al., 2003) or of rearing flies under suboptimal temperatures (Northrop, 1920; Plough and Strauss, 1923; Alpatov, 1932; Dobzhansky, 1935; Kaliss and Graubard, 1936; Cohet and David, 1978; Kirk Green et al., 2019; Klepsatel et al., 2019), our study teased out adult-specific effects of suboptimal temperatures (18°C and 29°C). Notably, we revealed how the decreases in egg production of adult females exposed to 18°C versus 29°C stem from negative effects on different processes during oogenesis. Exposure to cold temperature slows down follicle growth, whereas incubation of females in warm temperature increases early germline cyst death and degeneration of vitellogenic follicles. These findings indicate that entirely dissimilar (as yet unknown) underlying molecular mechanisms are responsible for decreasing the number of eggs produced at different suboptimal temperatures. Similar to 18°C exposure, a yeast-free diet leads to reduced follicle growth; however, females on a yeast-free diet also have increased GSC loss and death of early cysts and vitellogenic follicles (Drummond-Barbosa and Spradling, 2001; Hsu and Drummond-Barbosa, 2009). Nevertheless, it is possible that the reduced food consumption at 18°C contributes to the slowed follicle growth but is not sufficiently severe to cause the additional responses observed on a yeast-free diet. By contrast, as explained in the Results section, the early germline cyst death and vitellogenic follicle degeneration induced at 29°C are not explainable by changes in food consumption, and might, for example, involve stress signaling pathways instead (Krebs and Loeschcke, 1994; Jevtov et al., 2015; Huelgas-Morales et al., 2016). Intriguingly, although many types of physiological stress can lead to GSC loss (Drummond-Barbosa, 2019), GSC numbers in females maintained at warm temperatures remain unaffected over time, suggesting the existence of as yet unknown protective mechanisms for GSCs.

Potentially beneficial effects on oogenesis and stem cells of chronic exposure to cold temperature

In contrast to studies on reproductive diapause, which is triggered by colder temperatures (e.g. 10°C to 12°C) combined with a short photoperiod, and involves impairment of vitellogenesis (Saunders et al., 1989), we focused on how the suboptimal temperature of 18°C (routinely used in *Drosophila* laboratories) impacts oogenesis. Other than slowed follicle growth, the effects of 18°C on oogenesis were apparently beneficial, including improved GSC maintenance, early germline cyst survival and oocyte quality. It is conceivable that these effects are a secondary consequence of the extended lifespan of *Drosophila* maintained at lower temperatures, such that they remain 'physiologically younger' than their 25°C counterparts (Smith, 1958; Yarger and King, 1971; David et al., 1975; Carvalho

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et al., 2017), although we cannot rule out additional mechanisms. Interestingly, it was recently shown in humans that ambient temperatures have effects on total fertility rates that persist into the following generation (Jensen et al., 2021). Future studies using additional insects and mammalian models should explore the extent of evolutionary conservation and the relevant mechanisms of how cold temperatures affect oogenesis (especially germ cell survival and oocyte quality).

Our work showed that GSC maintenance is improved at 18°C, representing one of the few examples of studies exploring the effects of low temperature on stem cells. Effects of cold temperatures on mammalian stem cells based on *in vitro* studies have been reported. For example, bone marrow-derived mesenchymal stem cells differentiate into beige-like adipocytes at 32°C as opposed to white adipocytes at 37°C (Velickovic et al., 2018). Processing of human cord blood at 4°C within minutes of birth appears to improve the numbers of hematopoietic stem cells (Broxmeyer et al., 2021). How cold temperatures impact the self-renewal and other properties of various types of tissue stem cells throughout the body is a fascinating question for further investigation.

Warm temperature drastically decreases oocyte quality and leads to male infertility over time

Many processes during oogenesis contribute towards making a high-quality oocyte, including the production/localization of maternal polarity determinants, specialized cell cycles of follicle cells and germ cells (including oocyte meiosis), quality control of organelles, and the transport and accumulation of RNA molecules, proteins, lipids, carbohydrates and other biomolecules (for future embryonic development) (McKim et al., 2002; Swanhart et al., 2005; Mishra and Chan, 2014; Conti and Franciosi, 2018; Merkle et al., 2020). Defects in any of these processes can lead to impaired oocyte quality. For example, prolonged retention of mature *Drosophila* oocytes in ovarioles prior to egg laying increases meiotic spindle instability and leads to embryo aneuploidy (Greenblatt et al., 2019), while loss of the translational regulator encoded by *Fmr1* impairs the translation of large mRNAs in mature oocytes and causes neural defects in the resulting embryos (Greenblatt and Spradling, 2018). Therefore, the drastic reduction in the quality of oocytes produced by females chronically exposed to 29°C that we observed might result from defects in any of the oogenesis processes mentioned above and/or from temperature-induced metabolic alterations or damage to crucial biomolecules (Sokolova, 2013). Finally, although mild heat stress during development is known to affect male fertility in *Drosophila* species (Young and Plough, 1926; Frankel et al., 1971; Cohet and David, 1978; Chakir et al., 2002; Vollmer et al., 2004; Kirk Green et al., 2019), we showed that males that develop at room temperature (23°C) and are placed at 29°C as adults drastically lose fertility. The mechanisms underlying low oocyte quality and male infertility at warm temperature are important topics for continued investigation.

MATERIALS AND METHODS

Drosophila strains and culture conditions

Fly stocks were maintained at 21-23°C on standard medium containing 4.64% w/v cornmeal, 5.8% v/v molasses, 1.74% w/v yeast and 0.93% w/v agar, with added potato flakes. *y w*, Oregon-R-C and Canton-S were used as wild-type strains. *X-15-29*, *X-15-33* and *MKRS, hs-FLP* strains used for lineage tracing have been described previously (Harrison and Perrimon, 1993). The *w**; *ProtB*-eGFP; *dj*-GFP transgenic line has been described previously (Santel et al., 1997; Jayaramaiah Raja and Renkawitz-Pohl, 2006; Yang and Lu, 2011). The *TrpA1* genomic rescue transgene (a gift from Paul Garrity, Brandeis University, Boston, MA, USA) has been previously described (Hamada et al., 2008). The $TrpA1^{1}$ null (Kwon et al., 2008) and $Gr28b^{c01884}$ hypomorphic (Thorne and Amrein, 2008) alleles were verified using genomic PCR and RT-PCR, respectively (see below). $TrpA1^{1}$, $Gr28b^{c01884}$ and the *TrpA1* genomic rescue were backcrossed into an isogenized *y w* background for six generations.

For most experiments, 0- to 1-day-old females (with *y w* males, except for experiments using virgin females) raised at room temperature (23°C) were incubated at 18°C, 25°C or 29°C for up to 20 days at ≥70% humidity in standard medium supplemented with dry yeast, except where noted. Food was changed daily, and temperature and humidity remained stable, based on daily monitoring during experiments (Fig. S1A,B). For temperature reversion experiments, flies were incubated at 29°C for five, 10, 15 or 20 days prior to being switched to 25°C for the remainder of the 20-day time course. Control flies were incubated either at 25°C or 29°C for the entire duration of temperature reversion experiments.

Measurement of food consumption

To measure relative food media consumption among females on different temperatures, we used the consumption-excretion dye-based method as described previously (Shell et al., 2018), with minor modifications. Briefly, ∼0.5 ml of standard medium with 0.25% FD&C Blue No. 1 (Spectrum Chemicals) were poured into the center of vial foam plugs (avoiding borders) and allowed to cool and solidify at room temperature for same-day use. Ten 0- to 1-day-old *y w* couples were incubated at 18°C, 25°C or 29°C for 4 days on standard medium, and females were subsequently transferred to empty vials closed with the blue food-containing plugs and maintained at their respective temperatures for 24 h prior to sacrifice. (To control for background absorbance, *y w* females were transferred to empty vials closed with plugs containing standard medium without the dye.) To harvest the dye inside the females (internal dye), females were homogenized in 1.5 ml of water, samples were centrifuged at 10,000 ***g*** for 1 min to pellet debris, and supernatant was collected. To harvest the dye excreted by females (excreted dye), 3 ml of water were used to rinse the vial walls and water extracts were vortexed for 10 s. Absorbance at 630 nm was measured using a Synergy H1 spectrophotometer (Biotek) and converted to amount of medium consumed (µg/fly) based on a standard curve of pure FD&C Blue No. 1 in water and known concentration of dye in the food. Statistical analysis was performed using an unpaired *t*-test (GraphPad Prism) with 25°C as control.

Egg counts and hatching rate measurements

To measure the number of eggs laid, five couples were maintained in inverted perforated plastic bottles closed with molasses/agar plates covered by a thin layer of wet yeast paste, in six replicates, at 18°C, 25°C or 29°C. Plates were changed twice a day, and eggs laid within a period of 24 h were counted every day to calculate the average number of eggs produced per female per day. For statistical analysis, we used *F*-test of third order polynomial (GraphPad Prism) fitted curves, with 25°C as control.

To measure hatching rates, eggs laid overnight prior to the 5-, 10-, 15- and 20-day time points of egg-laying experiments at 18°C, 25°C or 29°C were collected. For each genotype/condition/time point, 10 groups of 10 eggs were placed in a molasses plate around a small quantity of yeast paste and incubated at 25°C for 24 h in a humid chamber (covered Pyrex dish with wet paper towels) in triplicate. The unhatched eggs were counted and subtracted from the total to calculate the number of hatched eggs. To analyze the male contribution to hatching rates at 29°C, *y w* couples were incubated at 25°C or 29°C for 17 days, at which point females were replaced by 2-day-old virgin *y w* females and all couples were incubated for 3 additional days (with original males) at 29°C prior to egg collection and quantification of hatching rates as described above. To analyze the female contribution (i.e. oocyte quality), *y w* couples or *y w* virgin females were incubated at 25°C or 29°C for 17 days, then 2-day-old males either replaced original males or were added to the virgin females, respectively, and all couples were incubated for 3 days at 29°C prior to egg collection and hatching rate quantification. Data from three independent experiments were subjected to an unpaired *t*-test (GraphPad Prism) with 25°C as control.

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Ovulation analyses and quantification of sperm transfer to females

For ovulation analyses, ovaries dissected in Grace's Insect Medium (Caisson Labs) were fixed for 15 min in fixing solution [5.3% formaldehyde (Ted Pella) in Grace's medium]. Ovaries were rinsed and washed twice for 5 min each in PBS [10 mM NaH_2PO_4/$NaHPO_4$ and 175 mM NaCl (pH 7.4)] and kept at 4°C prior to analysis under a stereomicroscope. Mature oocytes are recognizable by their fully developed dorsal appendages (Spradling, 1993). To quantify the number of stage 14 mature oocytes per ovariole, we teased the ovarioles individually under the stereoscope and counted the number of mature oocytes per ovariole. Data from four independent experiments were subjected to the Mann–Whitney test (GraphPad Prism), with 25°C as control. Images of whole ovaries and laid eggs were captured using a Zeiss Axiocam ERc 5s attached to a Zeiss Stemi 2000-CS stereomicroscope.

To quantify the transfer of sperm from *ProtB*-eGFP; *dj*-GFP males to *y w* females, spermathecae were dissected in Grace's insect medium and fixed for 15 min in fixing solution (5.3% formaldehyde in Grace's medium). Spermathecae were rinsed and washed for 15 min in PBST (PBS plus 0.1% Triton X-100) before mounting in Vectashield containing DAPI (Vector Labs). Images were collected using a Zeiss LSM700 confocal microscope. The presence of sperm in spermatheca was determined based on intense green fluorescence. Spermathecae with fewer than 10 spermatozoa were considered absent of sperm. Data from two independent experiments were subjected to a Chi-square test, using 25°C as control.

Lineage-tracing analysis

β-Galactosidase (β-gal)-positive clones from single mitotically dividing cells were produced as previously described (Drummond-Barbosa and Spradling, 2001). Newly eclosed *y w; X-15-29/X-15-33; MKRS, hs-FLP* females (with zero-to-one-day-old *y w* males, except for virgin experiments) were maintained at 18°C, 25°C or 29°C on standard food with dry yeast for 3 days. Flies were then transferred to vials containing wet Kimwipes and immersed in a 37°C water bath for 1 h to induce flippase expression (and the generation of single β-gal-positive cells at day zero). Flies were subsequently transferred to standard food with dry yeast and maintained at their respective temperatures for 1-4 days prior to dissection. As a control, flies were maintained at 29°C throughout the experiment without a 37°C heat shock to ensure that no clones are induced due to incubation at 29°C. Flies were transferred to fresh food vials daily throughout the experiments.

As follicles develop and progress through oogenesis, the rate of follicle cell proliferation is proportional to the growth rate of the underlying germline cysts. Therefore, we analyzed β-gal-positive germline and follicle cell clones at different time points after heat shock to assess the rate of follicle growth/development. For germline clones (which were all originally labeled in region 1 of the germarium, where early germ cells are mitotically dividing), we identified for each time point which oogenesis stage the most developed β-gal labeled cyst had reached in each ovariole analyzed. Data from three independent experiments were subjected to a Chi-square test (Microsoft Excel), with 25°C as control. For follicle cells (which were labeled within the mitotically dividing population of follicle cells), the number of cells per clone was counted in stages 4-6 follicles at different time points, and doubling times were calculated using regression line equations (GraphPad Prism). Statistical analysis of data from three independent experiments was performed using two-way ANOVA with interaction (GraphPad Prism).

Ovary immunostaining and fluorescence microscopy

Ovaries were dissected and ovarioles teased apart in Grace's medium, fixed for 15 min at room temperature in fixing solution, and then rinsed and washed three times for 15 min each in PBST (PBS plus 0.1% Triton X-100). After overnight incubation at 4°C in blocking solution [5% normal goat serum (MP Biochemicals) plus 5% bovine serum albumin (Sigma) in PBST], ovaries were incubated overnight at 4°C in the following primary antibodies diluted in blocking solution: mouse monoclonal anti-α-Spectrin (3A9) [Developmental Studies Hybridoma Bank (DSHB), 1:20]; mouse monoclonal anti-Lamin C (LC28.26) (DSHB, 1:20); and chicken β-gal antibody (ab9361) (Abcam, 1:200). Ovaries were washed as above and incubated with Alexa Fluor 488- or 568-conjugated secondary antibodies (Molecular Probes, 1:400; A11034 and A11004, respectively) in blocking solution for 2 h at room temperature. Samples were washed and mounted in Vectashield containing DAPI. Data were collected using a Zeiss AxioImager-A2 fluorescence microscope or a Zeiss LSM700 confocal microscope.

GSC, cap cell and cystoblast/early germline cyst quantification

Cap cells were identified based on their ovoid shape and Lamin C-positive staining, whereas GSCs were identified based on their juxtaposition to cap cells and typical fusome morphology and position (de Cuevas and Spradling, 1998). Data from three independent experiments were subjected to two-way ANOVA with interaction (GraphPad Prism), using 25°C as control. For cyst distribution analysis, the number of cystoblasts and two-, four-, eight- and 16-cell germline cysts present in germaria were identified based on fusome morphology (de Cuevas and Spradling, 1998), and normalized to the number of GSCs. Statistical analysis of data from three independent experiments was performed using the Mann–Whitney test (GraphPad Prism), with 25°C as control.

Analysis of GSC proliferation

For EdU incorporation analysis, intact dissected ovaries were incubated for 1 h at room temperature in 100 µM EdU (Molecular Probes) diluted in Grace's insect medium (Bio Whittaker), washed and fixed as described above. Following primary antibody incubation, samples were subjected to the Click-iT reaction according to the manufacturer's instructions (Life Technologies) for 30 min at room temperature. Ovaries were washed, incubated with secondary antibodies and washed again prior to mounting and microscopy, as described above. As a measure of GSC proliferation, we calculated the fraction of EdU-positive GSCs as a percentage of the total number of GSCs analyzed per condition. Data from five independent experiments were subjected to Chi-square analysis, using 25°C as control.

Analysis of early dying cysts and degenerating vitellogenic follicles

To determine the percentage of germaria containing dying germline cysts, the ApopTag Fluorescein Direct *In Situ* Apoptosis Detection Kit (S7160, Millipore Sigma) was used as described previously (Drummond-Barbosa and Spradling, 2001). Progression through vitellogenesis was assessed using DAPI staining. The percentage of ovarioles containing one or more dying vitellogenic follicles (recognized by the presence of pyknotic nuclei), as opposed to exclusively healthy vitellogenic follicles (Spradling, 1993), were analyzed on Zeiss Imager.A2 X-Cite Series 120. Data from three independent experiments were subjected to Chi-square analysis, using 25°C as control.

Genomic polymerase chain reaction and RT-PCR

Genomic polymerase chain reaction (PCR) was used to verify the *TrpA1*^{1} allele, originally generated by ends-out homologous recombination (Kwon et al., 2008) (see Fig. S5A). For genomic DNA extraction, seven to 10 flies were kept in a 1.5 ml tube at −20°C for at least 30 min. Samples were homogenized in 100 µl of lysis buffer [0.5% SDS, 100 mM Tris (pH 7.4), 100 mM NaCl and 100 mM EDTA (pH 8.0)] using a motorized pestle. An additional 900 µl of lysis buffer was added, and samples were vortexed well before incubation at 65°C for 30 min. 400 µl of homogenate was transferred to a new tube, 800 ml of ice-cold 1.43 M LiCl/4.3 M KAc solution was added, and samples were placed on ice for a minimum of 10 min. Samples were centrifuged at 10,000 ***g*** for 15 min and 800 µl of supernatant were transferred into a new tube. 600 µl of isopropanol was added and samples were mixed well and centrifuged for 15 min. Pellets were washed once with ice-cold 70% ethanol and centrifuged again for 15 min. Dry pellets were resuspended in 50 µl DNase/RNase-free water. PCR was performed using previously described *TrpA1* P1 primers, 5′-GCTTGGCTGCAGGTCGA-CTCTAGAG-3′ and 5′-GCAGGAGGTACACGCCAAGGATGCTC-3′ (Kwon et al., 2008), and the Phusion Plus PCR Master Mix F631S

(ThermoScientific) in an MJ Research PTC-200 Thermal Cycler. PCR using *y w* flies or without fly DNA served as negative controls.

The insertional *Gr28b*^{c01884} allele was verified using RT-PCR as previously described (Thorne and Amrein, 2008). Ten adult female heads were incubated in RNA*later* Stabilization Solution (ThermoFisher Scientific) for 10 min. After RNALater removal, 250 µl of lysis buffer from the RNAqueous-4PCR Total RNA Isolation Kit (ThermoFisher Scientific) were added and samples were homogenized using a motorized pestle for RNA extraction according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 500 ng total RNA using oligo (dT) primers and SuperScript II Reverse Transcriptase (ThermoFisher Scientific) according to the manufacturer's instructions. cDNA was amplified through a 35-cycle reaction (94°C for 30 s, 61°C for 30 s and 72°C for 1 min for *Gr28b*; 94°C for 30 s, 64°C for 30 s and 72°C for 30 s for *α-tubulin*) using previously described primers for the *Gr28b* common exon, 5′-CCCATCAATGGGACACCCGAAGCCT-3′ and 5′-GAGATAAGTGGTCAAGGCCCCGCTG-3′ (Thorne and Amrein, 2008), and for *α-tubulin* (used as normalization control), 5′-GCTGTTCCACCCC-GAGCAGCTGATC-3′ and 5′-GGCGAACTCCAGCTTGGACTTCTTGC-3′ (Ponton et al., 2011). Reactions using cDNA from *y w* female heads or without cDNA served as positive and negative controls, respectively.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.C.P.G., D.D.-B.; Methodology: A.C.P.G., D.D.-B.; Validation: A.C.P.G.; Formal analysis: A.C.P.G.; Investigation: A.C.P.G.; Writing - original draft: A.C.P.G., D.D.-B.; Writing - review & editing: A.C.P.G., D.D.-B.; Visualization: A.C.P.G.; Supervision: D.D.-B.; Project administration: D.D.-B.; Funding acquisition: D.D.-B.

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