Endocrine network essential for reproductive success in *Drosophila melanogaster*

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Ecdysis-triggering hormone (ETH) was originally discovered and characterized as a molt termination signal in insects through its regulation of the ecdysis sequence. Here we report that ETH persists in adult *Drosophila melanogaster*, where it functions as an obligatory allatotropin to promote juvenile hormone (JH) production and reproduction. ETH signaling deficits lead to sharply reduced JH levels and consequent reductions of ovary size, egg production, and yolk deposition in mature oocytes. Expression of ETH and ETH receptor genes is in turn dependent on ecdysone (20E). Furthermore, 20E receptor knockdown specifically in Inka cells reduces fecundity. Our findings indicate that the canonical developmental roles of 20E, ETH, and JH during juvenile stages are repurposed to function as an endocrine network essential for reproductive success.

*Significance*

Endocrine networks are the foundation of estrous cycles in most vertebrates. However, hormones regulating reproduction in invertebrates often are examined in isolation rather than as part of an emergent endocrine context. Here we show that a highly conserved endocrine network consisting of ecdysone, ecdysis triggering hormone, and juvenile hormone interact in *Drosophila melanogaster* to promote reproductive success. These findings provide a foundation for future studies on the endocrine regulation of reproduction in invertebrates.


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transcripts fluctuate in phase during the first 3 wk of adulthood, increasing in intensity through day 5, dropping on days 8 and 10, and increasing again up to day 20. In contrast, the male expression pattern shows a steady increase during the first 2 wk of adulthood and remains strong through day 20. In general, expression of ETH and ETHR is in phase, suggesting a common upstream regulator.

Inka cells of Drosophila larvae are the sole source of ETH (4). We asked whether the same is true during adulthood by conditionally ablating Inka cells in posteclosion flies using an Inka cell-specific Gal4 driver to express the apoptosis gene reaper (ETH-Gal4 > tubulin-Gal80ts/UAS-rpr). Flies were raised at 18 °C, at which temperature Gal80 inhibits Gal80, over 95% lethal eclosion deficiency was observed. Escapers were deficient in tanning, likely related to insufficient release of bur- sicon, known to be regulated by ETH (15, 16). When the temperature shift was postponed until after eclosion, all flies survived but were completely devoid of ETH transcript (Fig. S2). These data confirm that Inka cells are the sole source of ETH in adult Drosophila.

ETH Induces Calcium Mobilization in the CA. ETHR transcripts have been detected in the CA of Bombyx and Aedes, suggesting these glands are targets of ETH (5, 6). We found that exposure of CA excised from day 3 adults to ETH induces robust calcium mobilization (Fig. 2). Using two different genotypes [Aug21-Gal4 > UAS-GCaMP3 (Fig. 2 B and C) or JHAMT-Gal4 > UAS-GCaMP5 (Fig. 2 A, D, and E)], we observed calcium mobilization in both males and females for more than 45 min.

Latency to ETH-induced calcium mobilization, defined as the time elapsed between ETH treatment and the first peak with amplitude greater than background activity (established during the 4 min of recording before treatment), was concentration-dependent and sexually dimorphic. CA responses occurred within 1,000 s in 58 of 60 experiments; the two nonresponders were females at the lowest concentrations of ETH tested (200 nM). When latencies for each sex and dose were compared using a 2 × 3 factorial ANOVA, both factors were found to be significant effectors of latency at P < 0.001. Latency to calcium mobilization was significantly shorter for male CA compared with those of females and latency was inversely proportional to concentration (Fig. 2E).

Calcium Mobilization in the CA Following ETH Exposure Depends on Level of ETHR Expression. Upon RNAi silencing of ETHR using the genotype JHAMT-Gal4 > UAS-GCaMP5/UAS-ETHR-Sym, the percentage of female responders exposed to 5 μM ETH decreased from 100% to 66% in controls and from 100% to 83% in males. Among the responders, latency and variance were decreased from 100% to 66% in controls and from 100% to 83% in males. Among the responders, latency and variance were significantly increased in both sexes after RNA silencing (P < 0.05) (Fig. 2F). Thus, calcium mobilization in the CA in response to ETH exposure depends upon relative abundance of ETHR transcripts.

To verify the presence of ETHR transcript in the CA, we performed qPCR on isolated glands of male and female 4-d-old adult flies of genotype JHAMT-Gal4 > UAS-CD4-tgGFP/UAS-ETHR-Sym. We observed GFP labeling solely in the CA, which were carefully extirpated under fluorescence optics. Analysis by qPCR revealed the presence of ETHR transcript in both the male and female CA; relative transcript abundance was significantly higher in males (P < 0.05). Expression of UAS-ETHR-Sym resulted in significant knockdown of ETHR in CA of both sexes (P < 0.05) (Fig. 2F).

ETH Is an Obligatory Allatotropin in both Males and Females. We tested whether ETH is required for maintenance of JH levels using two genetic approaches to interrupt signaling: (i) RNAi silencing of ETHR in the CA using the JHAMT-Gal4 driver, and (ii) conditional ablation of Inka cells using the apoptosis gene reaper (rpr). RNA knockdown of ETHR expression in the CA
(genotype JHAMT-Gal4 > UAS-ETHR-Sym) led to a ~70% reduction of JH levels in males and ~85% reduction in females (Fig. 3A). We observed no defects in body size, head eversion, or time to eclosion in ETHR-silenced flies.

Conditional ablation of Inka cells was accomplished using ETH-Gal4 > tubulin-Gal80ts/UAS-rpr. Because ablation of Inka cells during larval or pupal stages leads to lethal ecdysis defects, flies were moved to Gal80-inactivating warmer temperatures only after eclosion to the adult stage (within 8 h posteclosion).

Both males and females subjected to Inka cell ablation exhibited markedly depressed levels of JH, 94% in males and over 99% in females (Fig. 3B).

**Disruption of ETH Signaling Reduces Fecundity and Impairs Vitellogenesis.**

We observed clear reproductive phenotypes associated with impaired ETH signaling. ETHR silencing using the JHAMT-Gal4 driver and two double-stranded RNA constructs directed toward mutually exclusive sequences in the ETHR gene (UAS-ETHR-Sym and UAS-ETHR-IR2) (see Materials and Methods for details) resulted in a 35% decrease in egg production in mated female flies (Fig. 3C). Fecundity was restored to normal levels following topical application of 3.4 pg of the JH analog methoprene, a known agonist of the Drosophila JH receptors Met and Gce, on the day of eclosion (17). Similarly, ablation of Inka cells, the source of ETH, led to a ~30% drop in egg production; methoprene treatment again rescued egg production to normal levels (Fig. 3D). ETHR silencing in the CA (JHAMT-Gal4 > UAS-ETHR-Sym) or conditional Inka cell ablation (ETH-Gal4 > tubulin-Gal80ts/UAS-rpr) reduced ovary size in day 5 virgin females (Fig. 4A and B). Interestingly, despite their smaller size, ovaries from Inka cell-ablated flies retained more mature (stage 14) eggs than controls (Fig. S3). Reduction in ovary size resulting from either treatment was rescued by topical treatment with methoprene, whereas mature egg number was unaffected.

To investigate this seemingly conflicting dichotomy, we examined oocytes and ovarioles of affected flies. First, we scored numbers of eggs at successive stages of oogenesis (staging according to ref. 18) and found that, although development through stage 7 was normal, a significantly greater number of oocytes from ETH-deficient flies degenerated during stages 8–9, suggesting that the balance between 20E and JH was perturbed (19) (Fig. 4C and D and Fig. S4). Furthermore, progressing oocytes in stages 9–13 were present in much lower numbers.
following Inka cell ablation (Fig. 4D and Fig. S4). A balance between JH and 20E determines whether oogenesis will progress beyond the midoogenesis checkpoint stage (7, 8). JH deficiency results in activation of caspases and apoptosis, marked by DNA fragmentation and obvious with DAPI and TUNEL staining (19, 20). We observed increased degeneration of oocytes in ETHR-deficient flies compared with controls (Fig. 4C and D).

We also found that stage 14 eggs were thinner, relatively translucent, and often did not activate (incomplete inflation) in PBS (21). Eggs were depleted or devoid of yolk (Fig. 4E). Protein (Bradford) assays showed marked reduction of soluble protein in eggs from ETHR-deficient or Inka cell-ablated flies (Fig. 4F).

Yolk protein gene expression is directly related to JH levels (22). To determine whether yolk protein transcription is diminished following disruption of ETH signaling, we performed qPCR for yolk protein genes in 4-d-old virgin females (Fig. 4G). Expression of both Yp1 and Yp2 was significantly reduced following either Inka cell ablation or ETHR silencing in the CA.

**Impaired ETH Signaling Reduces Male Reproductive Potential.** Previous studies demonstrated that JH is necessary for normal male accessory gland functions in a variety of insects (23–25), and that JH induces accessory gland protein synthesis in *D. melanogaster* (26, 27). However, reproductive impairment associated with JH deficiency in male *Drosophila* has not been reported. We...
disrupted ETH signaling in posteclosion males via ETHR knockdown in the CA or Inka cell ablation. Day 4 males were paired with wild-type females of the same age, placed in a 1-cm-diameter courtship chamber, and observed for 30 min to confirm copulation. Immediately after mating, inseminated females were isolated and allowed to lay eggs for 3 d. After 72 h, we removed the female and counted larvae and unhatched eggs over the next 24 h to assess both egg production and viability. Egg production in females mated with JH-deficient males was reduced (Fig. S5A and B), and egg viability did not differ significantly from controls (Fig. S5E). Egg production was rescued by topical treatment of JH-deficient males with methoprene (1.7 pg). Females (day 4)
20E Regulates ETH Signaling During the Adult Stage. During juvenile stages, expression of genes encoding ETH and ETHR is induced by ecdysteroids (10, 11, 13). We examined whether ETH and ETHR transcript levels are influenced by 20E in adult flies. Injection of 20E (150 pg) into male and female flies led to significant and sustained ~twofold elevation of the ETH precursor transcript in both males and females (Fig. 5 A and B). With regard to ETHR expression, 20E-injection elicited much stronger elevation of ETHR transcript in females compared with males. At 1 h postinjection, we observed a 6-fold increase in females, but only a 1.5-fold increase in males. However, at 4 h postinjection, ETHR transcripts increased nearly 100-fold, whereas male transcript levels returned to baseline, if not slightly below control levels.

We then asked whether steroid signaling in Inka cells affects fecundity. We tested this by suppressing EcR expression in Inka cells specifically, either through RNAi silencing or expression of an EcR dominant-negative allele. Indeed, both of these treatments led to significant reductions in both female fecundity and male reproductive potential (Fig. 5C and Fig. S5 C). Both of these phenotypes were rescued by topical application of methoprene, suggesting that reduced fecundity resulting from elimination of EcR in Inka cells is the result of JH deficiency, and that 20E acts through ETH from Inka cells, which in turn targets the CA to maintain normal JH levels.

Discussion

We have shown that Inka cells and expression of genes encoding ETH signaling molecules persist well into the adult stage of male and female Drosophila. Our findings indicate a vital functional role for ETH as an obligatory allatotropin for maintenance of JH levels required for normal vitellogenesis and fecundity in females and reproductive potential in males. A critical upstream signal for regulation of ETH gene expression is 20E. Thus, 20E, ETH, and JH comprise a hormonal network essential for normal reproductive physiology in both male and female flies.

ETH and ETHR transcripts and ETH-like immunoreactivity in Inka cells persist for at least 3 wk posteclosion in both males and females. ETH and ETHR transcripts appear to be in phase with one another and also following an infradian rhythmicity, with band intensity levels similar to reported peaks of fecundity (28). A total of nine Inka cell pairs are present, two of which are located in the thorax and seven in the abdomen. The pattern of Inka cell distribution in adults is particularly interesting. Unlike larval Inka cells, which are evenly distributed throughout the animal (4), adult cells are more strategically located. In the thorax, an anterior pair is situated in close proximity to the CA, particularly with the allatotropic action of ETH described here. Abdominal Inka cells are more concentrated posteriorly, particularly, in the female, where four of the seven pairs are clustered in terminal segments closely associated with reproductive organs, thought to be the most prominent source of 20E (29).

We present evidence that ETHR is expressed in the CA of Drosophila, in agreement with previous reports documenting ETHR expression in CA of the silkworm, B. mori, and yellow fever mosquito, A. aegypti (5, 6). In Aedes, ETH was reported to stimulate activity of the rate-limiting enzyme in JH biosynthesis, JH acid methyl-transferase via calcium release from stores, while not affecting JHAMT gene expression. RNAi knockdown of ETHR using the CA driver JHAMT-Gal4 causes marked reduction of JH levels and reproductive loss-of-function phenotypes, including reductions in ovary size, fecundity, yolk deposition, yolk protein expression, and lower male reproductive potential; ovary size and fecundity loss-of-function phenotypes are restored to normal levels by methoprene rescue. Indeed, the magnitude of reduced egg production in response to disrupted ETH signaling is comparable to that resulting from total ablation of the CA (Fig. S6) (22, 30).

It has been proposed that oogenesis in Drosophila depends upon balanced levels of JH and 20E (19). Under normal conditions, JH stimulates yolk protein synthesis in the fat body. In the ovary, JH in combination with other factors promotes endocytosis of yolk proteins into developing oocytes (22, 31). The combinatorial effect of synthesis and uptake leads to adequate yolk deposition in mature oocytes and normal progression of oogenesis. However, during situations of stress, ecdysteroid levels rise, causing nurse cell apoptosis and follicle degeneration. We show marked follicle degeneration and a decrease in late-stage oocytes following Inka cell ablation (Fig. 4 E and F and Fig. S4). A previous study on the role of EcR in
oogenesis using a temperature-sensitive EcR mutant reported disruption in progression to late-stage oogenesis, as well as an increase in the number of stage 14 oocytes (32). Our findings suggest that loss of EcR expression and consequent disruption of oogenesis could be attributable to reduced expression of the ETH gene in the Inka cell. Furthermore, we suggest that ETH could be important for balancing 20E and JH levels. Unlike steroids and JH, hemolymph ETH concentration can change rapidly, as it does over a matter of minutes during ecidyse (4). Thus, ETH may contribute increased plasticity to the stress response system, which is known to work over a span of hours or even days; further experiments are necessary to test such a prediction.

Ramifications of low JH in male flies have been described as “enigmatic” (22). Although a variety of insects show reduced accessory gland production and a reproductive cost stemming from reduced JH, such events have not been associated with changes in reproductive potential of adult males (33). Following disruption of ETH signaling, we subjected JH-deficient males to a single mating to same-aged wild-type females on day 4 and observed a significant reduction in reproductive potential. This effect was not seen when males were raised in groups with females (7), nor was the effect obvious when males were mated on day 10. Although the mechanism of this impairment is currently unclear, investigations in other species provide a clear link between JH and accessory gland protein synthesis. Our experimental evidence suggests partner fecundity impairment could be because of a reduced rate of accessory gland protein synthesis in JH-deficient males. Accessory gland proteins have an indispensable role in stimulating female fecundity; sex peptide transfer can enhance reproductive output in females by stimulating JH synthesis, intestinal remodeling, and germ-line stem cell proliferation in females; and ovulin stimulates growth of octopaminergic neurons that regulate ovulation (34–38). By day 10, JH-deficient flies may have “caught up” to controls through accumulation of accessory gland proteins, providing sufficient protein ejaculate for normal egg production in the mated females. In groups, multiple matings can occur, and more matings may compensate for a reduced accessory gland protein dose on the initial mating. Low JH males show no reproductive potential deficit (7), which could be explained by lower sex-peptide transfer from the male, resulting in a shorter time to remating (28). Therefore a fresh dose of oogenesis-stimulating accessory gland protein is released into the female.

Based on the work presented herein, it appears that normal levels of JH in Drosophila adults depend upon ETH signaling. Furthermore, our findings indicate that 20E regulates ETH in Inka cells via EcR activation, thus regulating JH levels indirectly. This interpretation is supported by the fact that reduced fecundity and male reproductive potential following RNAi knockdown of EcR in Inka cells was rescued by the JH analog, methoprene.

On the other hand, injection of 20E results in a sustained, approximately twofold increase in ETH in both sexes. Whereas 20E treatment increased ETHR transcript dramatically in females, males exhibited only a small, transient increase. Nonetheless, although ETH transcript number in males may increase only slightly in response to 20E, its dual regulation of ETH and ETHR transcripts could have multiplicative effects on ETH target tissues.

ETH, previously known only for its regulation of ecidyse, now acquires a critical role in the adult stage as a promoter of reproduction. Three hormones interrelated by their canonical roles in morphogenesis are shown here to maintain their relationship despite dramatic reorganization of the body plan following metamorphosis. The diversity of allato-regulators in Drosophila, including insulin-like peptides and biogenic amines (40–43), also influence JH production; each may influence this network in a context-specific manner to coordinate and optimize reproductive behaviors. The ability of the CA to integrate a variety of inputs, including nutrition and steroid levels, into a proreproductive signal bears a striking similarity to the mammalian GnRH neuron, which integrates complex hormonal information regarding stress, nutrition, and circadian rhythm into its activity, the emergent hormonal state determining whether reproduction is appropriate (44).

Developmental signaling roles for ecdysteroids, ETH, and JH have been characterized in a number of holometabolous insect species (45–47). In particular, periodic molting and ecidyse occur through bouts of steroid (20E) surge and ebb. The 20E surge promotes synthesis of ETH in Inka cells via transcription factors cryptocephal and EcR-B2 (48). Meanwhile, 20E represses transcription of βFTZ-F1, an orphan nuclear receptor necessary for secretory competence (49). Subsequent decline of steroid levels de-represses βFTZ-F1, leading to acquisition of secretory competence and release of ETH.

Although ETH is known to be under control of 20E during developmental stages (9, 10), we show here that this relationship persists into the adult stage. It will be interesting to ascertain whether fluctuation of 20E levels during adulthood functions in a similar manner to trigger synthesis and release of ETH for reproductive functions. A reasonable prediction might be that ETH functions as a link between 20E and JH signaling to promote successive, nonoverlapping surges of these hormones, similar to cyclic hormonal fluctuations in mammals (44).

We propose a model depicting chemical signaling among members of the 20E-ETH-JH network (Fig. 6). A 20E surge stimulates production of ETH in Inka cells and ETHR in target tissues, such as the CA. If the CA is primed by insulin and other cues, when ETH release occurs upon steroid ebb, active JHAMT stimulates JH biosynthesis and release from the CA. Normal JH levels elevate, promoting normal rates of egg production in females and reproductive potential in males. According to previous reports, circulating JH levels can inhibit 20E production during the adult stage (50–52). Indeed, mutually exclusive fluctuations of E75A and E75B are observed throughout the lifespan of Drosophila, and it has been proposed that these are indicators of hemolymph concentrations of JH and 20E, respectively (53). Coordinated fluctuations of 20E and JH could facilitate oogenesis through sequential steps of development in the ovariole (29, 54). Real-time hormone measurements are required to validate this model.

Materials and Methods

Fly Stocks. Flies used for immunohistochemistry, calcium imaging, and CA ETH silencing were raised at 23 °C on standard cornmeal-agar media under a 12:12-h light:dark regimen. Inka cell-ablated flies were raised at the Gal80° permissive temperature (18 °C). Following eclosion, they were moved to the nonpermissive temperature (29 °C) for 24 h, then moved to 23 °C until day 4. CA-ablated flies were reared as described previously (22) at 29 °C, isolated before eclosion, and transferred to isolated chambers held at 23 °C before mating and fecundity analysis. The JHAMT-Gal4 fly line has been described recently (55). Use of double-stranded RNA constructs for silencing of ETHR [UAS-ETHR-Sym; UAS-ETHR-IR2 line, Vienna Drosophila Resource Center (VDCR) transgenic stock ID dna697] were described recently (15). Aug12-Gal4 flies were obtained from S. Korge, Freie Universität, Berlin, Germany. All other fly lines were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN): UAS-Red Stinger (BS no. 8574), UAS-mCD8-GFP (BS no. 5137), UAS-CD4-tDdGFP (BS no. 35838), UAS-rpr (BS no. 8524), UAS-NIpr1.HA (BS no. 2371; referred to as UAS-NIpr1 henceforth), UAS-GCaMP3 (BS no. 32235), UAS-GCaMP5 (BS no. 42037), TubbGal80° (BS no. 7017), ETH-Gal4 (BS no. 51982), UAS-ecr-RNAi (BS no. 37059), UAS-EcR.B1 (BS no. 6869). All flies used for behavior experiments were backcrossed for at least five generations into the Canton-S background.

Visualization of Inka Cells. We crossed ETH-Gal4 transgenic flies with UAS-Red Stinger flies to produce progeny expressing GFP + RFP for double immunohistochemical staining. Day 4 adults were dissected in PBS and fixed in 4% paraformaldehyde in PBS overnight at 4 °C. After washing with PBS-0.5% Triton X-100 (PBST) five times and blocking in 3% normal goat serum in PBST for 30 min at room temperature, samples were in-
cubated with anti-Eth antiserum (1:1,000 dilution in PBS; previously described in ref. 15) for 2 d at 4°C. Tissues were washed with PBS three times, incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) overnight, and washed four times for 10 min each in PBS before imaging.

Immunofluorescence was recorded using a confocal microscope (Leica model SP2) with FITC filter in the Institute of Integrative Genome Biology core facility at the University of California, Riverside.

RT-PCR. Fifteen Canton-s wild-type flies were collected on the days relative to eclosion: −2, −1, 0, 3, 5, 8, 10, 13, 16, and 20 d after eclosion. Following homogenization of whole flies, cDNA was prepared using a SuperScript III kit (Invitrogen). cDNA was normalized and incubated for 20 cycles with actin, ETHR, or ETHR-B primers and Invitrogen Taq polymerase. InKA cell ablation was confirmed by processing 15 day 4 females of InKA cell-ablated facility at the University of California, Riverside.

Fig. 6. Model for gonadotropic coregulation by the hormonal network consisting of 20E, ETH, and JH in Drosophila adults. (A) Ecdysone (20E) induces expression of ETH in InKA cells and ETHR in target tissues. The CA integrates ETH and other cues to determine JH level; high JH exerts negative feedback inhibition on 20E production. (B) Timing of 20E and JH release into the hemolymph. Ecdysone induces ETH synthesis, but inhibits its release, possibly through inhibition of the secretory competence factor jFTZ-F1. ETH release occurs as 20E levels decline.

Calcium Imaging. The CA and esophagus of 4-d males and females (n = 24) were extirpated and placed in a Petri dish. We used an imaging set-up consisting of a Polychrome V monochromator (TILL Photonics/FEI) as light sources and a TiLL Imago CCD camera. The microscope (Olympus Model BX51W) was equipped with a 40x NA 0.8 objective. Binning on the chip (8 × 8) was set to give a sampling spatial rate of 1 μm per pixel (image size 172 × 130 pixels, corresponding to 172 μm and 130 μm). Images were taken at a rate of 1 Hz. The excitation wavelength was 488 nm, and exposure time was 25 ms. Fluorescent light passing an excitation filter (370–510 nm) was directed onto a 500-nm DLP mirror followed by a 515 LP emission filter for EGFP. One-hour-long continuous images were acquired from each CNS preparation and ETH was applied into a bathing media −5 min after imaging onset. The volume of applied ETH was 3.6 μL. We used a mixture of ETH1 and ETH2 for all experiments; 300 nM ETH (300 nM ETH1 plus 300 nM ETH2) and 600 nM ETH (600 nM ETH1 plus 600 nM ETH2) was added to a stagnant bathing bath with a micropipette. Fluorescence intensity was calculated as ΔF/F0; mean fluorescence over the entire 100 frames was taken, for each pixel, as an estimate for F.

Laity Experiments. For dose–response curves, the CA from InKA-Gal4 > UAS-GCaMP5; UAS-ETHR-Sym and InKA-Gal4 > UAS-GCaMP5 were dissected as above using similar imaging settings, using ETH1 only. As considerable constitutive activity was observed using the more-sensitive GCaMP5, latency was defined as time to first atypical ΔF/F0 peak, as recorded by the software. After 240 s of recording, establishing baseline activity, 20 nL of 10 times the noted concentrations was added to a bath with 180 μL fly saline and the CA were recorded for 1,000 s. Each data point contains latencies from 8 to 12 CA. ETHR-silenced imaging was performed with InKA-Gal4 > UAS-GCaMP5; UAS-ETHR-Sym and InKA-Gal4 > UAS-GCaMP5 flies.

Methoprene Treatment. Within 24 h of eclosion, adult males or females were cold-anesthetized and treated topically on the dorsal side of the abdomen with 72 (females) or 36 (males) nL of either acetone, or 0.01% methoprene
Ovary Size Measurement. Four days after eclosion, ovaries were dissected from females of the genotypes JAAM-Gal4/+; JHAM-Gal4/+; UAS-ETH-Sym/++; UAS-Gal80> UAS-Gal80/+ > UAS-ops. Ovarioles were then scored in single blind manner with an ocular micrometer. In cases where ovaries were not symmetrical, ovaries were not used for size determination.

Egg Production. Newly eclosed males and females of noted genotype were kept in incubators at 50% humidity in isolation vials until day 4, at which point they were paired with a wild-type Canton-S male of the opposite sex in confiscent chambers. Following mating, females were isolated in 23 °C until day 4, at which point they were mated to Canton-S males and moved to incubators maintained at indicated temperatures.

Egg Staging and Protein Content. Ovaries dissected from day 4 virgin females were immediately fixed for 1 h in 4% paraformaldehyde, washed 5× in 0.1% PFA and incubated overnight in 0.5 mg/ml DIAP and 2% NGS in PBST. Samples were washed 5× and TUNEL-stained using the Roche In Situ Cell Death Detection Kit, TMR Red according to the manufacturer’s protocol. Egg staging was performed as described by Wijesekera et al. (56). Ovaries of virgin day 5 females of indicated genotype were raised individually in isolation vials, dissected in PBS, and 10, mature stage 14 oocytes were collected from 10 sample flies. Eggs were removed and placed in tubes containing MilliQ water, homogenized, and centrifuged (15,000 × g for 5 min. The supernatant containing soluble protein was recovered and subjected to Bradford assay according to the protocol described by Thermo Scientific (https://www.thermoscientific.com/order/catalog/product/23236) using a Nanodrop 2000 at the UCR Genomics Core Facility.

JH Determination. JH III was extracted from flies, labeled with a fluorescent tag and analyzed by reversed phase high-performance liquid chromatography coupled to a fluorescent detector, as previously described (57), with 100 flies for each sex/genotype divided into two groups for statistical comparison (one-way ANOVA).


Fig. S1. Images of Inka cells in vivo in ETH-Gal4 > UAS-RedStinger adults, Inka cell nuclei (red, indicated by white arrowheads) visible through the cuticle after 4 h of H₂O₂ treatment in an adult female, locations of thoracic (A and C) and abdominal (B and D) cells in color (A and B) and black and white (C and D). (Scale bar, 100 μm.) Higher-magnification images of Inka cells on trachea (E and F). (Scale bar, 50 μm.)

Fig. S2. RT-PCR of ETH transcript deficiency in ETH-Gal4 > UAS-rpr flies. Inka cell-ablated flies showed no detectable levels of ETH mRNA on day 4 post-eclosion.

Fig. S3. ETH-Gal4 > UAS-rpr (Inka cell-ablated flies) (A) but not CA-ETHR-silenced (B) flies showed an increase in mature eggs (stage 14) retained. Methoprene treatment had no effect on egg retention. Error bars represent SEM; NS, P > 0.05; ****P < 0.0001.
Fig. S4. Ovarioles of day 5 females of Inka cell-ablated and control flies were separated from ovaries and DAPI stained. Stage 8–13 eggs including eggs degenerating at stage 8 and 9 (8D, 9D), from each ovary were totaled, defined as progressing (P) or degenerating (D) and compared (n = 15). Error bars represent SEM. NS, P > 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Fig. S5. Impaired ETH signaling reduces reproductive potential of males. Reduced egg production following mating of Canton-S females with males following Inka cell ablation (A) (ETH-Gal4; tubulin-Gal80°/UAS-rpr) or ETHR-knockdown in the CA (B) (JHAMT-Gal4 > UAS-ETHR-Sym or UAS-ETHR-IR2) and rescue following topical treatment with methoprene (n = 15–25). (C) Impairment of male reproductive potential following suppression of EcR expression in Inka cells (n = 20–25). (D) Reproductive potential of Inka cell-ablated males returns to normal by day 10 (n = 10–15). (E) Hatch rate of females mated to low JH males (n = 20–25). Error bars represent SEM. NS, **P < 0.01; ****P < 0.0001.
Fig. S6. CA-ablated flies show reproductive potentials reduced to levels comparable to those of ETH-JH interrupted flies (see Fig. 3). Number of eggs produced by Canton-S females mated to males of the indicated genotype in the 72 h following mating (left side) for CA-ablated flies (Aug21-Gal4 > UAS-NIPP1) as well as genetic controls (n = 15–25). Number of eggs produced in the 72 h following mating to a Canton-S male (right side) by CA-ablated flies (Aug21-Gal4 > UAS-NIPP1) and genetic controls (n = 15–25). Error bars represent SEM. P > 0.05; **P < 0.01; ****P < 0.0001.