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RESEARCH ARTICLE

Hormonal control of growth in the wing imaginal disks of *Junonia coenia*: the relative contributions of insulin and ecdysone

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ABSTRACT

The wing imaginal disks of Lepidoptera can be grown in tissue culture, but require both insulin and ecdysone to grow normally. Here, we investigate the contributions the two hormones make to growth. Ecdysone is required to maintain mitoses, whereas in the presence of insulin alone mitoses stop. Both ecdysone and insulin stimulate protein synthesis, but only ecdysone stimulates DNA synthesis. Insulin stimulates primarily cytoplasmic growth and an increase in cell size, whereas ecdysone, by virtue of its stimulation of DNA synthesis and mitosis, stimulates growth by an increase in cell number. Although both hormones stimulate protein synthesis, they do so in different spatial patterns. Both hormones stimulate protein synthesis in the inter-vein regions, but ecdysone stimulates synthesis more strongly in the veins and in the margin of the wing disk. We propose that the balance of insulin and ecdysone signaling must be regulated to maintain normal growth, and when growth appears to be due primarily to an increase in cell number, or an increase in cell size, this may indicate growth occurred under conditions that favored a stronger role for ecdysone, or insulin, respectively.

KEY WORDS: Cell division, Ecdysone, Growth, Imaginal disk, Insulin, Tissue culture

INTRODUCTION

Growth can be due to an increase in cell number, or an increase in cell size, or both. After the discovery that insulin signaling was crucial for normal growth in *Drosophila* (Böhni et al., 1999; Oldham et al., 2000; Chen et al., 1996), there was much interest in determining whether the changes in size that occur due to various genetic and environmental manipulations were due to changes in cell size or in cell number. Much of this research focused on the control of body size and wing size in *Drosophila*. Reduction of the activity of the insulin receptor reduced wing size by a reduction in both cell size and cell number (Brogiolo et al., 2001). Likewise, mutations of the insulin receptor substrate, *chico*, that reduced insulin signaling also reduced body size and wing size. In this case, reduction in cell number accounted for 68% of the total reduction in wing size, and 32% of the reduction was due to a reduction in size of mutant cells (Böhni et al., 1999). Interestingly, the loss of FOXO, a component of the insulin-signaling network, suppressed the reduction in cell number, but not the reduction in cell-size caused by *chico* mutations (Jünger et al., 2003). By contrast, mutations in S6K, another component of the insulin signaling network, severely reduced wing size exclusively by reducing cell size, not cell number (Montagne et al., 1999). In a latitudinal cline of body size in *Drosophila*, differences in wing size among populations were primarily due to differences in cell number (James et al., 1995). A decrease in rearing temperature increased the size of the wings primarily due to an increase in cell size, with no significant change in cell number (Azevedo et al., 2002); at higher temperatures wings were smaller, likewise due to a change in cell size (French et al., 1998; De Moed et al., 1997). The general pattern that seemed to emerge is that environmentally induced changes in tissue size were primarily due to changes in cell size, whereas genetic differences in size were primarily due to changes in cell number.

Because of the extremely successful studies that used manipulations of the insulin signaling network to study growth, there has been a tendency to ascribe the control of growth entirely to variation in insulin signaling, with insulin-like peptides acting as the mediators between nutrition and growth (Britton and Edgar, 1998; Britton et al., 2002; Leopold and Layalle, 2006). Different components of the insulin signaling pathway, such as FOXO and S6K, could perhaps mediate different aspects of growth.

In studies with intact organisms, it is impossible to rule out other factors that might contribute to growth. In insects, insulin is known to interact with other developmental hormones such as ecdysone and the juvenile hormone (Riehle and Brown, 1999; Orme and Leevers, 2005; Gu et al., 2009; Tu et al., 2005; Mirth et al., 2014; Hatem et al., 2015; Nijhout and Callier, 2015). In particular, insulin signaling may play a role in ecdysone secretion by the prothoracic glands (Francis et al., 2010; Boulan et al., 2013). In addition, other growth factors of various molecular types and mechanisms are known to affect growth in insects (Hipfner and Cohen, 1999; Kawamura et al., 1999; Zurovec et al., 2002).

The potential interactions among these various growth-promoting factors during normal development are manifold and make it difficult if not impossible to isolate a specific role for any one of them. Thus, even though interference with insulin signaling reduces growth and size, it is impossible to tell whether the effect of insulin is direct or indirect, via the stimulation of other factors, or whether it acts in conjunction with, or synergistically with, other essential growth factors.

The wing imaginal disks of Lepidoptera can be cultured *in vitro* and the relative contributions of different hormones to normal growth and differentiation can be studied without potential interference from, or interactions with, other uncontrolled or unknown growth factors that might occur in an intact animal. Culture experiments have shown that wing imaginal disks of *Junonia coenia* and *Manduca sexta* require both 20-hydroxy ecdysone (20E) and insulin for growth (Nijhout and Grunert, 2002; Nijhout et al., 2007). Without hormones, wing disks do not grow *in vitro*. Either hormone by itself supports only a small amount of growth, but with both hormones present, wing disks can be

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stimulated to grow at a rate identical to that seen *in vivo* (Nijhout and Grunert, 2002). In the present study, we attempt to dissect the relative contributions that each of these hormones makes to normal growth.

RESULTS

Control of mitosis

We measured the mitotic index in imaginal disks at various times after culturing with hormones (Fig. 1). Mitoses were counted only on the dorsal epithelium of the disk. At the beginning of the experiment, the average mitotic index of controls was 12±1.3 (s.e.m.). In control disks incubated without hormone, the mitotic index declined rapidly and after 6-10 h no more mitoses were detectable. Disks incubated with insulin had a mitotic profile that was indistinguishable from the controls (Fig. 1), suggesting that insulin cannot maintain or stimulate mitoses. When disks were incubated with 20E, or with 20E and insulin, the mitotic index declined briefly and then rose to threefold the initial value at 8 to 10 h (Fig. 1). The mitotic index then gradually declined and at 20-24 h after the incubation started no more mitoses were detectable.

Overall growth of disks in culture

Wing imaginal disks were cultured for 24 h with hormones. Disks were paired, so that the left disk of a specimen was incubated without hormone and served as a control, and the right disk was incubated with a hormone. The surface areas of the disks, not including that of the tracheal bundles at the base of the disks, were measured and the sizes of experimental and control disks after 24 h in culture are shown in Fig. 2. Disks incubated with insulin or 20E alone grew about 14±4.3% and 26±6.1% larger than controls, respectively, whereas disks incubated with insulin and 20E together grew 100±5.3% larger. Fig. 2 shows the regression of experimental on control sizes of disks of different initial sizes, and suggest that the effects of insulin and 20E are more than just additive. This is in accordance with earlier findings that 20E and insulin act synergistically to stimulate wing imaginal disk growth (Nijhout and Grunert, 2002; Nijhout et al., 2007).

The relative roles of insulin and ecdysone

The data in Fig. 1 show that 20E stimulates mitosis and insulin does not. However, both insulin and 20E are needed for normal growth of disks *in vitro* (Nijhout and Grunert, 2002). In order to determine what aspect of growth was controlled by either hormone, we measured the changes in volume of cells and of nuclei after 24 h of incubation. Fig. 3 shows that insulin and 20E each stimulated about a 20-25% increase in cell volume, and insulin and 20E together caused an increase in average cell volume of about 45%. By contrast, insulin stimulated only a small increase in average nuclear volume (about 20%), whereas 20E stimulated about a 100% increase in nuclear volume. The response to 20E suggest that cells arrested at G2. Incubation with insulin and 20E together stimulated a 50% increase in average nuclear volume.

Growth in cell number or cell size

Growth in size can be due to an increase in cell number, in cell size, or a combination of the two. Our data on increase in cell size and on growth in overall size of the wing disk after incubation with hormones show that insulin induces no increase in cell number, suggesting that this hormone primarily stimulates growth by an increase in cell size (Fig. 4). 20E alone causes a modest increase in cell number (Fig. 4), consistent with the finding that 20E stimulates mitosis (Fig. 1). Growth stimulated by 20E and insulin together was due to a greater (but not statistically significant) increase in both cell number and cell volume (Fig. 4).

Protein and DNA synthesis

The data presented above suggest that insulin primarily stimulated growth in cell size and 20E simulated cell proliferation and cytoplasmic growth. We next examined the specific contribution of insulin and 20E to protein and DNA synthesis using click technology (Dieterich et al., 2010; Moses and Moorhouse, 2007; Salic and Mitchison, 2008) that allowed us to visualize the incorporation of methionine and uridine derivatives into proteins and DNA, respectively. Paired wing imaginal disks from mid-last instar larva were first incubated for 20-24 h in medium without hormones. The right-hand disk was then incubated with either insulin or 20E for 4 h, and the paired left-hand disk (which acted as the control) was continued without hormones. Both disks were then incubated with either HPG (a methionine derivative) or EdU (a uridine derivative) for 45 min. Fig. 5 illustrates the spatial patterns of DNA and protein synthesis, and shows that insulin stimulated protein synthesis but not DNA synthesis, and 20E stimulated both DNA synthesis and protein synthesis. The spatial patterns of

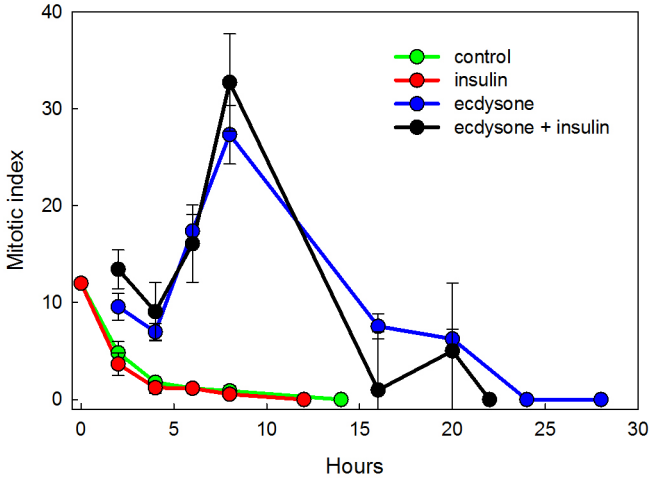

Fig. 1. Control of cell division. Mitotic index (number of mitotic cells, metaphase and anaphase, per 2000 cells) during incubation of wing imaginal disks with different hormones. Each point represents the mean of over 100 counts in three or more replicates. Data are mean±s.e.m. Overlapping error bars indicate data are not significantly different.

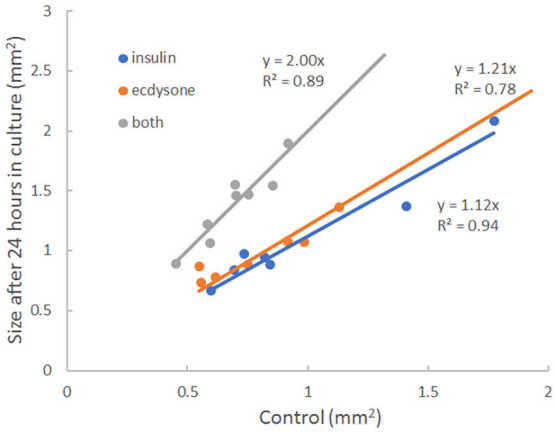

Fig. 2. Growth of cultured wing disks after 24 h in culture with various hormones compared with controls. Size was measured as the area of the wing disk. Control disks were incubated without hormones.

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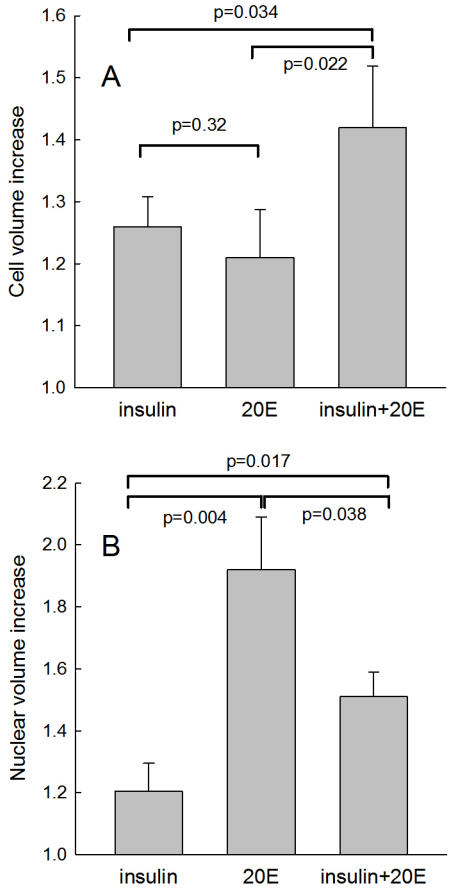

Fig. 3. Growth of cell and nuclear volume. (A) Fold-increase in cell volume after culturing wing disks with hormones for 24 h. (B) Fold-increase in nuclear volume after 24 h. Data are mean±s.e.m. Each value is based on 10-12 determinations. Significance of differences are indicated (*t*-test, unequal variances).

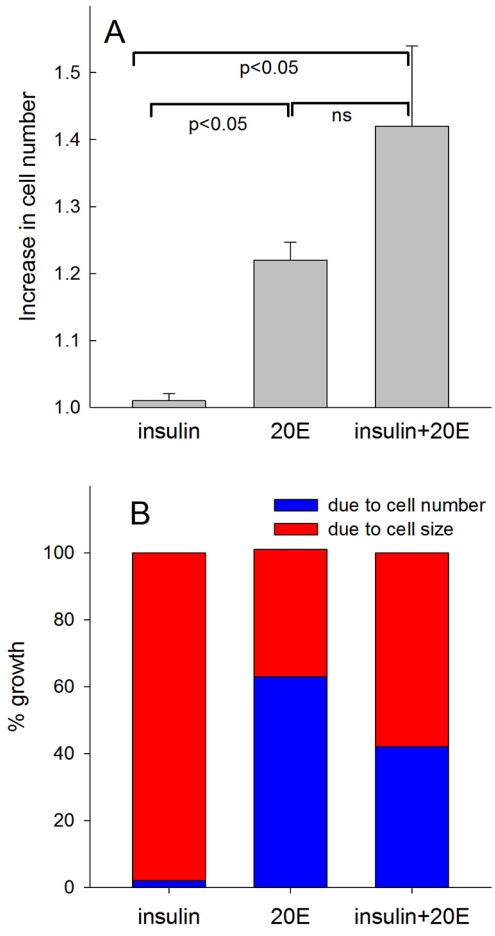

Fig. 4. Growth in cell number. (A) Average fold-increase in cell number after a 24 h incubation with insulin and/or 20E. Counts were made as described in the Materials and Methods. Each value is based on three replicates. Data are mean±s.e.m. Differences are significant at $P<0.05$ (*t*-test, unequal variances), except where indicated (ns). (B) Relative contribution of an increase in cell number or cell size to the growth of the wing imaginal disk when stimulated with insulin and/or 20E.

insulin- and 20E-stimulated protein synthesis were distinctly different (Fig. 6). Insulin stimulated protein synthesis only in the inter-vein regions, whereas 20E also stimulated protein synthesis in the peripheral region of the wing disk, distal to the bordering lacuna, and in the tracheal building cells of the wing veins (Figs 6 and 7). 20E stimulated a more widespread and spatially homogeneous pattern of protein synthesis than did insulin. In the inter-vein region, 20E and insulin appear to stimulate about the same amount of protein synthesis in the 30 min window of this experiment, and almost two times as much protein synthesis in the wing margin and the tracheal building cells in the wing veins (Fig. 7).

DISCUSSION

Lepidopteran wing imaginal disks, taken from feeding larvae in the middle of the last larval instar, can be grown in tissue culture provided they are supplied with two endocrine factors: 20-hydroxyecdysone (20E) and an insulin-like growth factor. The 20E requirement is rather precise. Optimal growth occurs at low levels of ecdysone (0.1 µg/ml=200 nM). Ecdysone concentrations above or below this optimum support substantially less growth (Nijhout and Grunert, 2002). The growth-promoting effect of the insulin-like growth factor bombyxin saturates at about 0.1 mg/ml (in the presence of optimal 20E), and higher concentrations do not inhibit growth. Either hormone by itself supports little growth *in vitro*, but together they support growth at the same rate observed *in situ* (Nijhout and Grunert, 2002; Nijhout et al., 2007). In the present study, we examined the relative contributions of the two hormones to normal growth and how they contribute to growth by cell enlargement and cell division.

Mitosis in the wing imaginal disks of *Junonia coenia* is stimulated by 20E, whereas insulin can neither stimulate nor maintain mitoses (Fig. 1). This suggests that 20E acts as a mitogen, which is in accordance with the findings of Kato and Riddiford (1987), who showed that 20E alone is sufficient to induce mitoses in cultured epidermis of *Manduca sexta*. The frequency of mitoses first declined for about 4 h after initiation of culture before rising to a peak at about 8 h. A similar initial decline in mitoses was found by Koyama et al. (2004), who also documented a brief decline in S-phase cells after exposure to 20E. Continuous exposure to 20E stimulated a round of mitoses (Fig. 1), but did not appear to be capable of maintaining continuous mitoses. In cultured *Drosophila* wing disks, insulin is also incapable of stimulating or maintaining mitoses (Dye et al., 2017). The nuclear volume of the wing disk cells approximately doubled during a 24 h incubation with 20E (Fig. 3B), suggesting that cells arrested at G2. This may be related to the finding that insect cell lines stimulated by very high concentrations (1 µM or about 0.5 µg/ml) of 20E arrest at G2

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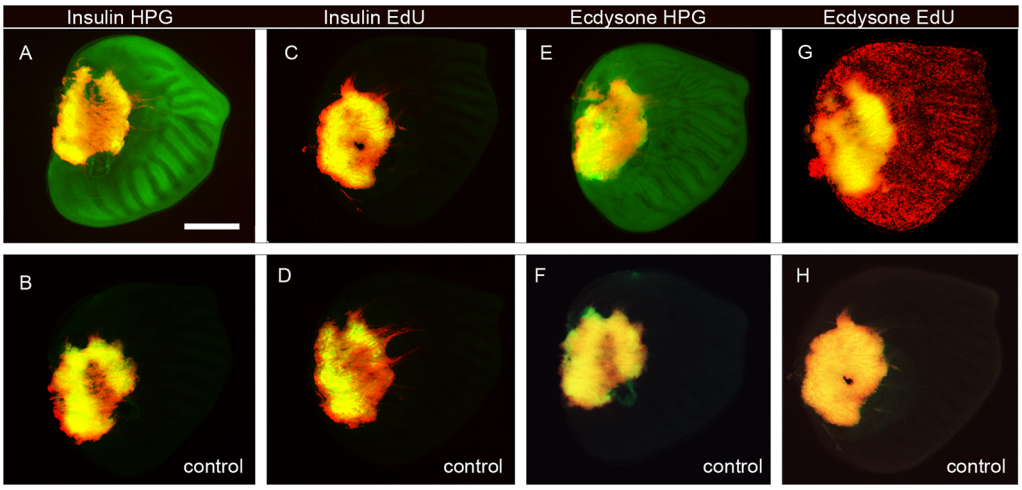

Fig. 5. Patterns of protein and DNA synthesis. (A,B) Patterns of protein synthesis (as HPG fluorescence; green channel, ex/em 493/519 nm) stimulated by insulin (A). (C,D) Patterns of DNA synthesis (as EdU fluorescence; red channel) stimulated by insulin (C). (E,F) Patterns of protein synthesis (as HPG fluorescence) stimulated by 20E (ecdysone) (F). (G,H) Patterns of DNA synthesis (as EdU fluorescence) stimulated by 20E (H). Controls were incubated without hormone. Experimental and control disks are paired left and right wings from the same individual, but both are printed here as right-hand disks. In all cases, the protein or DNA signals were recorded at ex/em 492/520 nm, and are depicted in the green channel for protein synthesis (A,B,E,F) and the red channel for DNA synthesis (C,D,G,H). Background autofluorescence was recorded at ex/em 550/570 nm. The large amorphous area on the right side of each disk is autofluorescence (in both the ex/em 492/520 nm and ex/em 550/570 nm channels) of a dense tracheal bundle that supplies the tracheae in the wing veins. Scale bar: 300 µm.

(Mottier et al., 2004; Dinan et al., 1990). These same concentrations also inhibit growth of intact wing imaginal disks *in vitro* (Nijhout and Grunert, 2002). Insulin by itself caused about a 20% increase in nuclear volume, and 20E in combination with insulin caused an ~50% increase of nuclear volume (Fig. 3B). As insulin does not stimulate DNA synthesis (Fig. 5), this increase in nuclear volume may be due to the import of metabolites, proteins and nucleotides into the nucleus in preparation for DNA synthesis. It is not clear why mitoses stop after 20-25 h of incubation with 20E. Preliminary experiments indicate that mitoses do not initiate again with more prolonged culture, so it is possible that we are missing a required signal in this *in vitro* system. In *Manduca sexta*, 20E levels appear to fluctuate with an approximately daily rhythm (Wolfgang and Riddiford, 1986), so it is possible that fluctuations of 20E are required to maintain cell divisions.

Incubation of wing disks with insulin alone causes about a 14% increase in size of the disk after 24 h of incubation. With 20E alone,

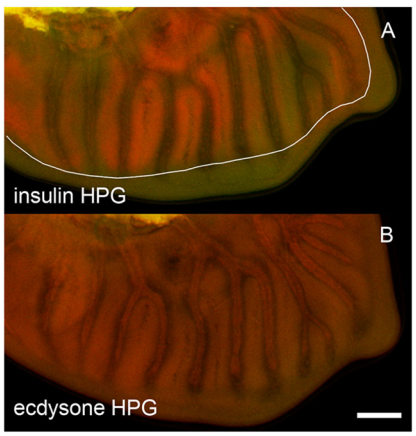

Fig. 6. Spatial patterns of protein synthesis, visualized as HPG incorporation (red channel), after stimulation with insulin or 20E. (A) Insulin primarily stimulates protein synthesis in the inter-vein regions and is not homogeneous across the wing surface. (B) 20E stimulated protein synthesis more homogeneously across the entire wing surface, including the wing veins and the area distal to the bordering lacuna. The thin line in A shows the path of the bordering lacuna. Green channel is background autofluorescence. Scale bar: 100 µm.

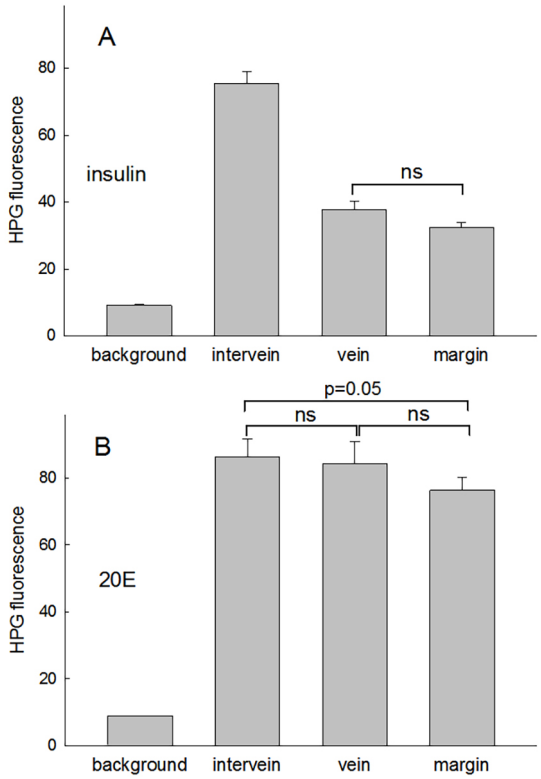

Fig. 7. Spatial patterns of protein synthesis. (A,B) Relative amounts of protein synthesis, based on HPG fluorescence, induced by insulin (A) and 20E (B) in different regions of the wing imaginal disk. Data are mean±s.e.m. Differences are significant at $P<0.05$ (*t*-test, unequal variances), except where indicated (ns).

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the increase is about 26%; with both hormones it is about 100% (Fig. 2). Insulin and 20E both produced an ~20-25% increase in cell volume, and both hormones together increased cell volume by about 45% (Fig. 3A). This suggests that both hormones can independently stimulate protein synthesis and cytoplasmic growth, and that together they act to increase cytoplasmic volume.

Incubation with hormones also caused an increase in cell number under some conditions. Insulin by itself did not increase the number of cells (Fig. 4A), so the increase in wing size stimulated by insulin must be entirely due to an increase in cell size. 20E by itself stimulated about a 25% increase in cell number and, together, insulin and 20E stimulated an increase of nearly 50% in the number of cells (Fig. 4A).

The balance of how much growth was due to an increase in cell size or cell number depended on the hormonal treatment. Insulin stimulated only an increase in cell size. Under stimulation by 20E alone, about 65% of growth was due to an increase in cell number and 35% due to an increase in cell size. Under stimulation by both hormones, about 40% of growth was due to an increase in cell number and 60% was due to an increase in cell size (Fig. 4B). This suggests that differences in the concentration or the relative timing of 20E and insulin secretion can bias growth to appear to be largely due to cell size, if insulin predominates, or to cell number, if 20E predominates.

Label-incorporation experiments show that insulin stimulates protein synthesis but not DNA synthesis, whereas 20E stimulates both (Fig. 6). The patterns of protein synthesis induced by 20E and insulin are different, however. Insulin induced protein synthesis primarily in regions between the wing veins, but in a pattern that was quite variable across the wing surface. Protein synthesis stimulation by 20E, by contrast, was more homogeneous across the wing surface, and 20E also stimulated protein synthesis in the wing veins and in the peripheral margin of the wing, distal to the bordering lacuna (Figs 6 and 7). The bordering lacuna defines the margin of the adult wing. Cells distal to the bordering lacuna undergo programmed cell death during the pupal stage.

These findings support those of Fujinaga et al. (Fujinaga et al., 2017) who worked with cultured male genital imaginal disks of *Bombyx mori* during pupal and early adult development. *Bombyx* has a unique insulin-like protein, called BIGFLP, that rises to extremely high levels in the pupal stage (Okamoto et al., 2009). Its secretion is controlled by 20E, which also rises to extremely high levels during the pupal-adult stage (Okamoto et al., 2011, 2009). Both 20E and insulin signaling are required for growth, with 20E primarily stimulating protein synthesis and elongation of the disk, and insulin signaling stimulating protein synthesis and overall growth. Fujinaga et al. (2017) showed that BIGFLP acts via the insulin signaling pathway and 20E acts via the mitogen-activated protein kinase pathway.

The hormonal control of wing disk growth in *Drosophila* is somewhat different. *Drosophila* wing disks, grown in culture, grow for about 7 h with insulin alone, for about 24 h with 20E alone, and for about 7 h with both hormones present (Dye et al., 2017). 20E thus appears to be sufficient to maintain normal growth and cell proliferation, as it does in *Junonia*, but insulin somehow inhibits growth after a period of time in culture. In this regard the *Drosophila* wing disks resemble the *Manduca* epidermis, where 20E alone is sufficient to maintain growth *in vitro* (Kato and Riddiford, 1987), although a potential role of insulin signaling in epidermal growth has not yet been investigated.

Both insulin and 20E signaling stimulate protein synthesis in cultured genital imaginal disks of *Bombyx mori* pupae. However, there are several interesting differences from the control of growth in the wing disks of *Junonia* larvae. First, in *Bombyx*, insulin signaling stimulates DNA synthesis (Okamoto et al., 2009) and in *Junonia* it does not. Second, in *Junonia* there is an optimal concentration of 20E above which growth is inhibited, and in *Bombyx* the concentration of 20E that supports growth is well above this optimum: a concentration at which growth would be almost completely inhibited in *Junonia*. Finally, in *Bombyx*, 20E induces an elongation of the genital disk, perhaps resembling wing disk eversion in *Drosophila*, which is also provoked by higher concentrations of ecdysone (Dye et al., 2017). In *Junonia*, neither hormone induced a change in shape of the disk.

What might explain these differences? It could be that the control of imaginal disk growth is simply different in different species. For example, in *Manduca* there is no optimal concentration of 20E (Nijhout et al., 2007), much as is seen in *Bombyx*. It is also possible that control is different at different stages of development. Our work in *Junonia*, and that in *Drosophila*, was carried out during the middle of the last larval stage, when the larva is still feeding, whereas the work on *Bombyx* was carried out during pupal and adult development when there is no nutritive input. When insects are feeding, insulin secretion is stimulated by nutrition, whereas after feeding stops, the secretion of insulin is stimulated by ecdysone (Nijhout and Callier, 2015). It is therefore possible that during stages when there is no nutritive input, the control of tissue growth changes. In *Manduca*, for example, there is a switch in the control of wing disk growth during late larval life, when the growth of disks becomes uncoupled from nutrition when larvae pass the critical weight. Before that time, starvation stops disk growth and after reaching the critical weight, disk growth continues even when larvae are starved (Tobler and Nijhout, 2010). Finally, it could be that different imaginal disks are controlled differently by the two hormones. It is clear that even different locations in the same wing imaginal disk respond differently to ecdysone and insulin. The response of a tissue to a hormone is not a property of the hormone but of the tissue, and is due to differences in the signaling pathway downstream of the hormone receptor. It is not unreasonable to assume that during the evolution of metamorphic growth and morphogenesis, different imaginal disks in different lineages and species evolved different responses to hormones during the adaptive evolution of those disks and the adult structures they generate. Elucidation of the intracellular pathways that generate tissue-specific responses to the same hormone should help resolve how and why those differences evolved.

MATERIALS AND METHODS

Larvae of *Junonia coenia* were derived from an established laboratory colony reared on an artificial diet and kept at 25°C under a 18:6 light:dark photoperiod. All experiments were carried out on wing imaginal disks from larvae on day 3 or 4 of the 5th instar. Wing imaginal disks were cultured individually in wells of 24-well plates (Argos, catalog number P1024) in 1 ml of supplemented Grace's medium (Gibco, 11605-094) or of methionine-free Grace's medium (Gibco, 11595-030), with 10% fetal calf serum (Atlanta Biological, S11150) and 10% antibiotic/antimicotic (Sigma, 15240-062) on a rotating platform at 90 rpm. Hormones used were 20-hydroxy ecdysone (Sigma, H5142) used at 0.1 µg/ml and insulin (Sigma, I9278) used at 10 µl/ml. Nuclei were stained with Hoechst-33258 (Invitrogen, H1398). To detect cell outlines, actin was stained with Alexa Fluor 488 phalloidin (Invitrogen, A12379). Light and fluorescence microscopy were carried out using a Leica DMRBE microscope fitted with a Hamamatsu ORCA-RE camera. Confocal microscopy was carried out with a Zeiss 880 inverted confocal microscope.

Mitotic indices were determined in the dorsal wing disk epithelium by counting mitotic metaphase and anaphase figures, and total number of nuclei in fields of 90×90 µm taken at three locations in each disk, on at least six disks for each data point. Cell sizes and nuclear volumes were determined from confocal *z*-stacks using the segmentation and measurement tools of Amira 6.3 (FEI Houston).

For growth experiments, disks were incubated with either 20E or insulin or both for 24 h, after which they were fixed in 4% paraformaldehyde in PBS for 1 h and stained with Hoechst (100 µg/ml stock in PBS used 4 µl/ml) for 30 min and phalloidin (80 U/ml in PBS) for 12 h. Protein and DNA synthesis were detected by incorporation of HPG (homopropargylglycine), a methionine analog, or EdU (5-ethynyl-2′-deoxyuridine), respectively, using click technology (Dieterich et al., 2010; Moses and Moorhouse, 2007; Salic and Mitchison, 2008). For DNA synthesis, we used the Click-iT Plus EdU Alexa Fluor 488 imaging kit (Invitrogen, C10637). For protein synthesis, we used the Click-iT HPG Alexa Fluor 488 protein synthesis assay kit (Invitrogen, C10428). For DNA or protein synthesis, disks were incubated for 20-24 h without hormone, then for 4 h with either insulin or 20E, followed by 45 min with either EdU or HPG, for DNA or protein synthesis, respectively, by adding 10 mM EdU at 5 µl/ml, or 50 µM HPG at 4 µl/ml to the culture medium. All incubations for protein synthesis were carried out in methionine-free medium. Controls were assayed for HPG or EdU incorporation after culture without hormones. Wing disk areas were measured on calibrated photographic images using the Fiji distribution of ImageJ (Schindelin et al., 2012).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: H.F.N.; Methodology: H.F.N., L.W.G.; Validation: H.F.N.; Investigation: E.L., L.W.G.; Writing - original draft: H.F.N.; Visualization: H.F.N.; Supervision: H.F.N.; Project administration: H.F.N., L.W.G.; Funding acquisition: H.F.N.

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