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Insulin Signaling Is Necessary for Vitellogenesis in *Drosophila melanogaster* Independent of the Roles of Juvenile Hormone and Ecdysteroids: Female Sterility of the *chico*¹ Insulin Signaling Mutation Is Autonomous to the Ovary

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Abstract

It has been suggested that insulin signaling mutations of *Drosophila melanogaster* are sterile and long-lived because of juvenile hormone (JH) and ecdysteroid deficiency. However, female sterility of an insulin/IGF-like signaling mutant (*chico*¹) of *D. melanogaster* is not mediated by downstream systemic

signaling in terms of major alterations in JH or ecdysteroid levels. *chico*¹ is a null mutation in the insulin substrate protein (CHICO) gene of *D. melanogaster*. Homozygous *chico*¹ females are sterile and their oocytes do not mature beyond the last previtellogenic stage. Homozygous *chico*¹ females exhibit approximately wild-type rates of JH biosynthesis, ovarian release of ecdysteroids and haemolymph ecdysteroid levels, suggesting that these two major hormone systems play no role in producing the sterility. Previtellogenic wild-type ovaries transplanted into homozygous *chico*¹ females underwent vitellogenesis, showing that systemic factors present in mutant females are sufficient to support normal vitellogenesis. *chico*¹ ovaries transplanted into wild-type females did not undergo vitellogenesis indicating that CHICO is necessary in the ovary for vitellogenic maturation. The ovary transplant experiments corroborate the endocrine results and demonstrate that insulin/insulin-like signaling (IIS) is necessary for vitellogenesis even when sufficient levels of JH, ecdysteroids or other factors are present.

Keywords: insulin receptor substrate, Chico, *Drosophila*, ovarian development, receptor mediated endocytosis

Abbreviations: IIS, insulin/insulin-like growth factor-like signaling; IGF, insulin-like growth factor; YP, yolk protein; CA, corpora allata; *DInr*, *Drosophila* insulin receptor; JH, juvenile hormone; JHB3, juvenile hormone bisepoxide; IRS, insulin receptor substrate; INR, insulin receptor

1. Introduction

The *D. melanogaster chico* gene encodes an insulin receptor substrate (IRS)-like protein that is similar to vertebrate IRS1-4 (Bohni et al., 1999). Homozygous *chico*¹ mutant females are sterile and do not develop vitellogenic eggs (Bohni et al., 1999; Drummond-Barbosa and Spradling, 2001). The rate of previtellogenic oocyte production in *chico*¹ females shows a greatly reduced response to a protein-rich diet, indicating a role for insulin/insulin-like growth factor-like signaling (IIS) in regulating the association between reproductive rate and nutrients (Drummond-Barbosa and Spradling, 2001). Females heteroallelic for mutations in the *Drosophila* insulin receptor (*DInr*) are also sterile, and have been reported to have lowered juvenile hormone (JH) biosynthesis to approximately 25% of that of wild type (Tatar et al., 2001). Similarly, *DInr* mutations exhibit lowered ecdysteroid release from the ovary (Tu et al., 2002). Exposure of these mutant *DInr* females to the JH analogue methoprene restored vitellogenesis, leading these workers to conclude that JH was a systemic mediator of the sterility seen in these mutant *DInr* females. JH has been thought to play a predominant role in controlling vitellogenesis (Bownes, 1989; Kelly, 1994; Nijout, 1994), but its direct effect on yolk protein (YP) endocytosis remains uncertain (Raikhel and Dhadialla, 1992). Richard et al. (1998, 2001a, b) implicated a role for ecdysteroids instead. In the present study, we show that IIS is necessary for the onset, and presumably the maintenance, of vitellogenesis in *D. melanogaster* irrespective of the roles of JH and ecdysteroids.

2. Materials and methods

2.1. Genotypes, culture conditions, body weight, and statistical analysis

The *chico*¹ stock used for the present study was backcrossed to flies from the outbred wild-type Dahomey population; the *Cy* balancer chromosome used to maintain the *chico*¹ mutation in stock has been retained (Clancy et al., 2001). *chico*¹ homozygotes were collected from this stock. Dahomey females were collected from an outbreeding population. Unless otherwise described, females heterozygous for *chico*¹ were obtained by crossing homozygous *chico*¹ males to virgin Dahomey females. For all experiments, flies were reared at 24 °C under constant illumination at standardized density (75 eggs per vial) on the *Drosophila* medium described in Carlson and Harshman (1999) and only virgin females of each genotype were used. Ovarian maturity was determined according to King (1970). The mean fresh weight of *chico*¹ females (0.60 mg) was 50% that of Dahomey females (1.21 mg). Consequently, for normalization to body weight, measurements for *chico*¹ females were doubled. Unless otherwise stated, an analysis of variance (ANOVA) was used for the statistics.

2.2. Ovary transplantation

Single immature ovaries were taken from *chico*¹ homozygotes and transferred into a reproductively capable female. Heterozygote and Dahomey females were the reproductively capable females used as hosts for ovaries from *chico*¹ homozygotes. The reciprocal experiment was to surgically implant a single immature ovary from the heterozygote, a genotype that actively produced eggs, into a *chico*¹ homozygote female. Ovaries from newly eclosed females are relatively small and contain only previtellogenic oocytes even in reproductively capable genotypes. Single ovaries taken from 1–4 h post-eclosion adults were transplanted into host females of the same age. The hosts were dissected 4 days later; both host ovaries and the transplanted ovary were examined (Wilson, 1982). The criterion for vitellogenic oocyte maturation throughout the study was the presence of stage 8, or later, vitellogenic oocytes.

2.3. Methoprene exposure

*chico*¹ females were exposed to ZR-2008 (s-methoprene), the active enantiomer of methoprene (Wilson and Fabian, 1986) which is a JH analog. Exposure was either by topical administration in 0.25–0.5 µl of acetone or by continuous exposure to methoprene in vapor. For exposure by vapor, a cotton plug is permeated with the methoprene which is volatile and the plug is used to cap a vial with flies (Wilson and Chaykin, 1985, Tatar et al., 2001). For the former, doses of 20–200 ng per fly were applied at day 2–3 post-eclosion and females dissected four days later. *chico*¹ females were continuously exposed to methoprene vapor while on fresh food. The efficacy of the methoprene used in these experiments was regularly verified using reproductively capable females in which it stimulated vitellogenesis and egg production.

2.4. JH biosynthesis

The radiochemical assay for JH production in vitro, based on that developed by Tobe and Pratt (1974), was carried out in minimal essential medium (MEM) supplemented with L-

[³H-methyl]-methionine (Amersham, specific activity 81 Ci/mmol) as the methyl-group donor, and with the JH-esterase inhibitor octyl-1, 1,1-trifluoropropanone (OTFP) to a concentration of 0.5 μ M as described previously (Richard et al., 1989; Altartz et al., 1991). Single adult female CA or isolated 3rd instar larval brain-ventral ganglion-ring gland complexes (as positive controls) were incubated for 2 h at 25°C in the presence or absence of 20 μ M farnesoic acid (FA a JH precursor kindly provided by Dr. Stephen S. Tobe, University of Toronto). Scintillation counts of hexane extracts of the incubation medium were used to assess JH biosynthesis by each CA (each data point). Pooled hexane fractions were subjected to silica gel thin layer chromatography with a JH III standard visualized under UV light as described previously (Richard et al., 1989).

2.5. Ecdysteroid assays

Ecdysteroids were quantified by radioimmunoassay (RIA), using the SHO3 antibody (Kiriishi et al., 1990), as described by Warren et al. (1984) with modifications. Two ovaries from each female were incubated in 10 μ l of Grace's medium at 25°C for 5 h. Each replicate determination of ecdysteroids released from ovaries was based on 40–60 separately incubated pairs of ovaries. At 24 h post-eclosion, two replicate determinations were made for the homozygous *chico*¹ mutant, the *chico*¹/Dahomey heterozygote and Dahomey; at 96 h seven replicate determinations were made for the mutant, two for the heterozygote and sixteen for Dahomey. Ecdysteroids released into incubation solution were extracted in 70% methanol for 4 days at 4°C. The extraction time and temperature were designed to match those in a previous study (Harshman et al., 1999).

Haemolymph ecdysteroids were quantified as described in Harshman et al. (1999) except that cells were removed from the haemolymph samples by centrifugation at 13,000 \times g for 10 min before extraction of ecdysteroids from the supernatant. For each replicate determination, haemolymph was drawn from 150–200 females of each genotype. There were two replicate determinations of haemolymph ecdysteroids per genotype on day 1 posteclosion and three replicate determinations for each genotype on day 4 post-eclosion.

2.6. Ovariole number and nurse cell counts

Ovariole numbers were determined as described by Coyne et al. (1991) by counting the ovarioles from both ovaries from 20 females per genotype. Nurse cells were counted in stage 7 egg chambers from 10–15 females per genotype (1–4 chambers/female). The number of nurse cells was determined by three independent counts of each egg chamber using a confocal microscope and an optical Z-series of Systox-green-stained ovaries.

2.7. Yolk protein receptor mRNA abundance measurement by semi-quantitative RT-PCR

For reverse transcriptase (RT) PCR of the YP-receptor gene (*yolkless*), the following primers were used to amplify a predicted 409 bp fragment: 5'-CAACATCCAGAACGAAGCGG-3' forward primer; 5'-CAACTTGGCATCCACCCAGTAG-3' backward primer. The PCR reaction was conducted with 1 μ g total RNA using a Stratagene ProStar RT-PCR kit (52°C annealing temperature, denaturation at 95°C, extension at 68°C). RNA was extracted from ovaries using the Qiagen RNeasy kit.

2.8. Western blots for yolk protein

Western blots for YP (1 µg protein per lane) were conducted as described by Richard et al. (2001a) using a YP antibody kindly provided by Mary Bownes (University of Edinburgh).

3. Results

3.1. Ovary transplants

We tested the importance of systemic factors in the sterility phenotype of *chico*¹ homozygote females by reciprocal ovary transplantations (table 1). The characteristics of all egg stages is well established (King, 1970); stages 1–7 are previtellogenic and 8–14 are vitellogenic. The stages are easily visualized and scored under a dissecting microscope at 200X. In particular, previtellogenic stage 7 oocytes and vitellogenic stage 8 oocytes are readily and unequivocally distinguishable. In the case of reproductively capable ovary transplants into *chico*¹, a robust vitellogenic response was seen in five implants, including 15–20 vitellogenic oocytes spanning the entire range of stage 8 to mature stage 14 eggs in each ovary. In every instance, the oocytes of host *chico*¹ ovaries terminated development at previtellogenic stage-7, typical of the mutant phenotype. In the reciprocal experiment, transplanted *chico*¹ ovaries failed to develop oocytes beyond stage 7 even though the *chico*/Dahomey (*N* = 7) or Dahomey (*N* = 2) host ovaries became fully vitellogenic in every recipient female. Therefore, *chico*¹ ovaries failed to develop vitellogenic oocytes even in a host environment that supports normal levels of vitellogenesis and egg production, and *chico*¹ females provided systemic factors necessary for vitellogenic oocyte maturation.

Table 1. Vitellogenic oocyte development following ovary transplantation between *chico*¹ homozygotes and *chico*¹/Dahomey heterozygote females

Host	Implant	Post-implant hosts	Implant ovaries	
			Vitellogenic	Non-vitellogenic
<i>chico</i> ¹ /Dahomey	<i>chico</i> ¹	Ovaries vitellogenic	7	0
Dahomey	<i>chico</i> ¹	Ovaries vitellogenic	0	2
<i>chico</i> ¹	<i>chico</i> ¹ /Dahomey	No vitellogenesis	5	1
<i>chico</i> ¹ /Dahomey	<i>chico</i> ¹ /Dahomey	Ovaries vitellogenic	9	0

3.2. Methoprene administration

The failure of *chico*¹ ovaries to undergo vitellogenesis when transplanted into wild-type hosts suggested that exogenous JH analog administration would fail to stimulate vitellogenesis of *chico*¹ females. We tested this hypothesis by exposure of *chico*¹ to methoprene. The two modes of exposure were topical administration using a range of methoprene concentrations (20–200 ng per fly) and by exposure to methoprene vapor. Topical application of methoprene, including the highest dose, which is considerably more than required for a biological response, did not stimulate vitellogenesis or egg production in *chico*¹ homozygotes at 1 week after application (*N* = 15). Methoprene vapor also failed to initiate vitellogenesis or egg production in newly eclosed *chico*¹ females exposed for 4 days beginning at

1–6 h post-eclosion ($N = 17$), or older females after 6 days of exposure beginning at 3–4 days post-eclosion ($N = 23$).

3.3. JH biosynthesis

Previous research reported that insulin receptor mutation synthesized relatively low levels of JHs (Tatar et al., 2001) which share a range of phenotypes with *chico* including sterility. We measured the in vitro rate of production of JH. For all genotypes at both ages posteclosion, almost all of the JH produced in vitro was JHB₃. Specifically, the maximum amount of JHIII produced from a single corpora allata in any assay was less than 5% and the remainder was JHB₃. In the first experiment (fig. 1A), we assayed homozygous *chico*¹ at 24 and 96 h, a heterozygote genotype consisting of *chico*¹ over a balancer chromosome at 24 h, and Dahomey females at 24 and 96 h. The analysis of variance on the raw data did not indicate an effect of age ($P = 0.6219$), genotype ($P = 0.7627$), nor interaction between age and genotype ($P = 0.3074$). However, when JH synthesis was normalized for body size (fig. 3A), there was an effect of genotype ($P < 0.0001$), but not of age ($P = 0.2503$) nor of interaction between age and genotype ($P = 0.2424$). Per unit weight, *chico*¹ females synthesized JH at almost two times the rate of the other genotypes. There was no significant difference between *chico*¹ homozygotes and wildtype at 24 or 96 h posteclosion ($P < 0.2500$). However, the normalized data indicated greater JH biosynthesis by *chico*¹ females at 24 h ($P = 0.0006$) and 96 h ($P = 0.0162$) post-eclosion. JH biosynthesis by heterozygote females at 24 and 96 h was compared with that by Dahomey females at the same ages, using a second batch of labeled precursor (fig. 1B). Statistical analysis of the data indicated that there was a significant interaction between genotype and age for the heterozygous females ($P = 0.0028$) and a statistically significant difference between genotypes at 96 h ($P = 0.0003$), when heterozygotes produced JH at a rate 33% greater than Dahomey. The rates of JH synthesis were lower in this experiment, and in that reported in figure 1C, because a different batch of radiolabeled methionine was used (fig. 1B and C). In a third experiment (fig. 1C), JH biosynthesis by CA from 24 h *chico*¹ homozygotes and Dahomey was compared. The positive control third larval instar brain/ring gland complexes also produced substantially less JH when the second lot of radiolabel was used (data not shown). There was no significant difference between genotypes when the raw data were analyzed (*t*-test for unequal variances, $P = 0.1618$; Wilcoxon two-sample, $P = 0.6269$). Normalization of the data to body weight again showed that mutant females produce JH at a greater rate on a per weight basis (*t*-test for unequal variances, $P = 0.0266$; Wilcoxon two-sample test, $P = 0.0422$).

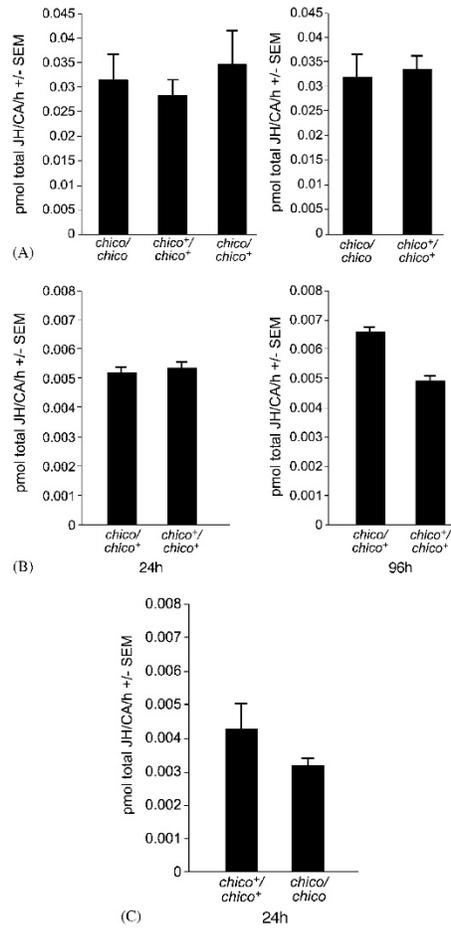


Figure 1. JH production by *corpora allata* isolated from adult female *chico* homozygotes, *chico*/*+* heterozygotes and Dahomey, measured by radiochemical assay. (A) *chico/chico* at 24 ($n = 12$) and 96 h ($n = 12$), *chico*/*+* at 24 h ($n = 13$), *+/+* at 24 ($n = 12$) and 96 h ($n = 16$) post eclosion. (B) *chico*/*+* at 24 ($n = 11$) and 96 h ($n = 13$), *+/+* at 24 ($n = 14$) and 96 h ($n = 14$) post eclosion. (C) *chico/chico* ($n = 9$) and *+/+* ($n = 11$) at 24 h post eclosion. In each experiment, incubation was for 2 h at 25°C. Data shown in A were obtained using one batch of tritiated methionine, whereas data in B and C were obtained using a second batch of label.

3.4. Ecdysteroids

Ecdysteroids could be responsible for the control of YP uptake (Richard et al., 1998) and a deficiency could cause the sterility phenotype of *chico*. The amount of ecdysteroids released from in vitro incubated ovaries removed from the mutant homozygotes, the heterozygote (*chico*¹/Dahomey), and wildtype Dahomey were determined at 24 and 96 h (fig. 2A). There were statistically significant differences in the amount of ecdysteroids released by the three genotypes ($P = 0.0021$). *chico*¹ ovaries released less ecdysteroids than the heterozygote ovaries ($P = 0.0068$) and Dahomey ($P = 0.0032$), especially at 24 h post-eclosion

(fig. 2A). When normalized by body weight, there was no overall difference among genotypes ($P = 0.2849$), but there was an interaction between day and genotype ($P = 0.0501$) reflecting relatively low release of ecdysteroids on day 1 by *chico*¹ females and relatively high levels compared to the other genotypes on day 4 (fig. 2A).

The abundance of haemolymph ecdysteroids was measured for all three genotypes because the circulating level of systemic hormones is more relevant to physiological activity than the rate of biosynthesis or release (fig. 2B). There was an overall effect of age ($P = 0.0124$), but not of genotype ($P = 0.4947$). There was no statistically significant genotype-by-age interaction although the ecdysteroids present in day 1 in *chico*¹ females were low compared to those in the other genotypes. The effect of age may be due to the greater amount of haemolymph that can be removed from immature versus mature adult females (Handler, 1982). When haemolymph ecdysteroid abundance was normalized by size (fig. 3B), there was an indication of statistically significant differences between genotypes ($P = 0.0568$) due largely to the relatively high level in *chico*¹ females, compared to the other genotypes, at day 4 post-eclosion (fig. 2B).

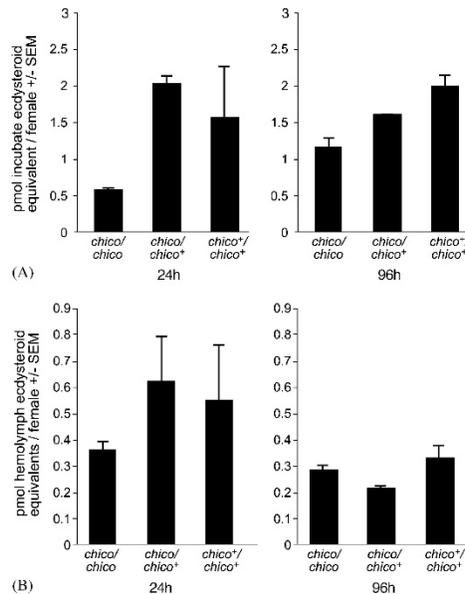


Figure 2. (A) Ecdysteroids released by isolated ovaries: *chico/chico* ($n = 2$), *chico*^{+/} ($n = 2$), *+/+* ($n = 2$) at 24-post eclosion; ($n = 7$), *chico*^{+/} ($n = 2$), and *+/+* ($n = 16$) at 96 h post eclosion where n is the number of replicate determinations. (B) Haemolymph ecdysteroid titers: *chico/chico*, *chico*^{+/} and *+/+* at 24 h post eclosion ($n = 2$ per genotype); *chico/chico*, *chico*^{+/}, and *+/+* at 96 h post eclosion ($n = 3$ per genotype). Haemolymph was drawn from 150 to 200 females of each genotype for each replicate determination (n).

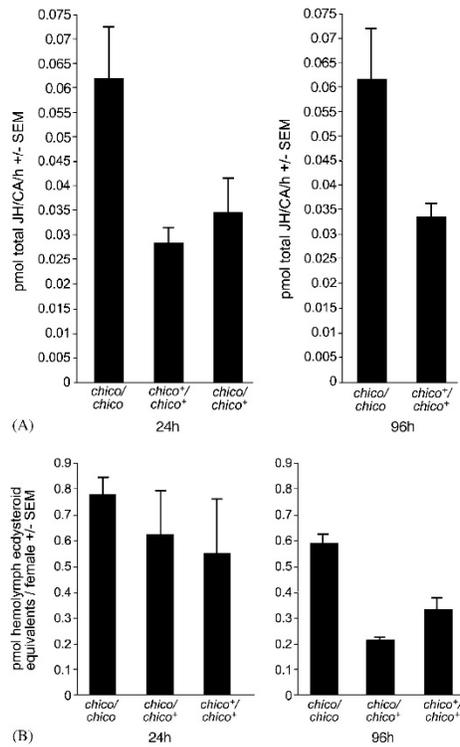


Figure 3. (A) *chico/chico* females are half the size of the heterozygote and wild-type females. JH biosynthesis data (fig. 1A) normalized by body size. (B) Hemolymph ecdysteroid data (fig. 2B) normalized by body size.

3.5. Ovariole and nurse cell numbers

Ecdysteroids are reported to be synthesized by nurse cells (Freeman et al., 1999) and thus we determined the number of nurse cells as a possible explanation for low ecdysteroids on day 1 post-eclosion. The number of ovarioles and number of nurse cells in stage seven egg chambers were counted to characterize the rate and state of ovarian maturation of homozygous *chico*¹ females (table 2). On day 1 post-eclosion, *chico*¹ females had an average of 11.2 nurse cells per egg chamber and on day 4 they had 14.8 nurse cells per egg chamber, a statistically significant difference ($t = 5.8277$; $P < 0.025$). The heterozygote and Dahomey had 15 nurse cells per egg chamber. The low number of nurse cells observed on day 1 in the present study corroborates an earlier observation that *DInr* mutations exhibit delayed egg chamber maturation (Chen et al., 1996). By day 4, posteclosion *chico*¹ egg chambers had approximately the standard number (15) of nurse cells. When the mean number of ovarioles per ovary was compared for *chico*¹, heterozygote and wild-type genotypes (table 2), the statistical analysis revealed an interaction between genotype and age ($P < 0.0001$) partially resulting from a relatively low number of ovarioles in *chico*¹ females on day 4.

Table 2. Number of ovarioles and nurse cells in *chico*¹ homozygotes, *chico*¹/Dahomey heterozygotes, and wild-type Dahomey females(1) Mean (SEM) nurse cell number per egg chamber in stage 7 oocytes in *chico*¹ homozygotes (the heterozygote and Dahomey had 15 nurse cells per egg chamber)

	Day 1	Day 4
<i>chico</i> ¹ / <i>chico</i> ¹	11.2 (0.54)	14.8 (0.30)

(2) Mean (SEM) number of ovarioles per ovary

Genotype	Day 1	Day 4
<i>chico</i> ¹ / <i>chico</i> ¹	17.2 (0.78)	14.7 (0.44)
<i>chico</i> ¹ /Dahomey	20.0 (0.45)	19.9 (0.42)
Dahomey	19.7 (0.43)	19.7 (0.38)

*SEM, standard error of the mean

3.6. Yolk protein and yolk protein receptor mRNA

An absence of YP or yolk protein receptor could have been responsible for the sterility phenotype of *chico*. The level of YP (47 kDa) in the ovaries of *chico*¹ females was lower than in the other genotypes (fig. 4A). YP-receptor mRNA was present at low levels in homozygous *chico*¹ ovaries compared to heterozygote and wild-type Dahomey ovaries as indicated by the relative band intensity of the amplicons based on normalization to 1 µg polyA mRNA in each amplification (fig. 4B).

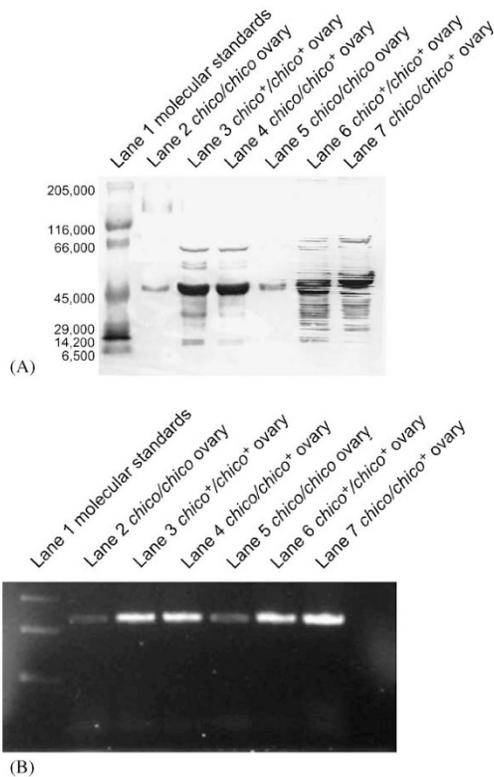


Figure 4. (A) Western blot of YP in *chico/chico*, *chico/+*, and *+/+* ovaries at 24 and 96 h post-eclosion. The YP band, prominent (1 μ g total protein per lane for all genotypes) in the reproductively capable heterozygotes and wild-type ovaries, was observed at 47 kDa. (B) YP receptor gene (*yolkless*) mRNA abundance in *chico/chico*, *chico/+*, and *+/+* ovaries at 24 and 96 h post-eclosion. Amplicons from the *yolkless* gene (shown on the gel) were derived from reverse transcriptase PCR using 1 μ g mRNA per amplification from each genotype.

4. Discussion

The ovary transplant experiments indicate that CHICO in the ovary is necessary for vitellogenic maturation. From these experiments we conclude that IIS (functional CHICO) is necessary for vitellogenesis even when sufficient levels of JH, ecdysteroids, or other factors are present.

4.1. Sterility of *chico¹* females was autonomous to the ovary

Ovaries from immature, reproductively capable females became vitellogenic after transplantation into *chico¹* females (table 1). The endogenous milieu in *chico¹* females was therefore capable of supporting the vitellogenic maturation of normal ovaries. In contrast, ovaries transplanted from *chico¹* homozygous females into reproductively capable females failed to mature to the first vitellogenic stage. The effect of the *chico¹* mutation on vitellogenesis in homozygous females is therefore endogenous to the ovary and the sterility of

*chico*¹ females is attributable to the absence of CHICO in the ovary itself. IIS may therefore play a role in regulation of YP uptake into oocytes.

Lowered levels of JH biosynthesis in vitro by *DInr* heteroallelic mutant females compared to wild-type females has been reported (Tatar et al., 2001). These authors argued that lowered JH production in the *DInr* mutant females, approximately 23% of wild type, was responsible for their sterility. This view was supported by the finding that addition of the JH analog methoprene, stimulated a low level of egg production. The results of the present study demonstrate that the sterility of *chico*¹ females is autonomous to the ovary itself. Our results therefore do not support the argument that IIS in *D. melanogaster* nonautonomously affects reproduction as inferred from work on insulin receptor mutations (Tatar et al., 2001). However, the insulin receptor mutation is not the same as the insulin receptor mutation used in the present study, and there could be different effects associated with each. Nevertheless, the effect of the *chico*¹ mutation on female reproduction was entirely ovary-autonomous.

4.2. JH biosynthesis or circulating ecdysteroids in mature *chico*¹ females was approximately at wild-type levels

The absolute rate of JH biosynthesis in *chico*¹ females was similar to that in other genotypes (fig. 1). When normalized for body size, the dwarf *chico*¹ females were actually found to produce more JH per body mass than the other genotypes (fig. 3A). Our data therefore do not support the finding of Tatar et al. (2001) of lowered JH biosynthesis in females mutant for IIS and hence provide no evidence that lowered production of JH plays any role in the sterility of *chico*¹ females. In any case, JH biosynthesis rate may not reflect the circulating titer of this hormone since at least two JH-degrading enzymes exist in *D. melanogaster*. The circulating level of hormones is a more meaningful physiological measure, but to date there have been no measures of JH titers in *D. melanogaster* haemolymph. A stronger indication that JH signaling plays no role in the sterility of *chico*¹ females came from the results of application of the JH agonist methoprene, which did not rescue sterility or vitellogenic egg production in the present study. In general, the present study provides no evidence for a systemic JH effect on reproduction as described earlier (Tatar et al., 2001). Furthermore, *chico*¹ females were found to be longer-lived than wildtype controls (Clancy et al., 2001), but the mutant does not produce JH at a significantly lower rate than wild-type females, thus suggesting that JH biosynthesis does not play a role in the extension of lifespan by *chico*¹ (the longevity phenotype was observed under similar conditions to those used in the present study).

The amount of circulating ecdysteroid did differ significantly between *chico*¹ females and the other genotypes on day 4 (fig. 2B). If the amount of haemolymph was normalized by body size, then again, *chico*¹ females had more ecdysteroids in the haemolymph (fig. 3B). Our results therefore provide no support for the suggestion that defective IIS results in a diapause-like state characterized by low levels of JH biosynthesis and ecdysteroid abundance (Tatar et al., 2001). Furthermore, the levels of JH and ecdysteroids in *chico*¹ females were sufficient to support vitellogenesis of ovaries transplanted into homozygous mutant females.

The transition from the previtellogenic to the vitellogenic state of oocytes occurs either as a developmental event in young adult females, or as a reversal of reproductive diapause (Saunders et al., 1989). Diapause in *D. melanogaster* is associated with decreased in vitro ovarian ecdysteroid and corpora allata JH production (Saunders et al., 1990) and application of ecdysteroids to diapausing females, or temperature elevation, stimulates vitellogenesis and results in the appearance of the protein components of YP endocytosis (Richard et al., 1998, 2001a, b). However, incubation of diapausing ovaries in the presence of 20-hydroxyecdysone does not initiate vitellogenesis implying that something else may be required (Richard et al., 2001b). We suggest that IIS may play this role.

4.3. The homozygous mutant ovary was structurally similar to wild type

The reproductive system of *chico*¹ homozygotes was normal in terms of the numbers of ovarioles and nurse cells per egg chamber on day 4 post-eclosion (table 2). An unusually low number of nurse cells were present in *chico*¹ on day 1 perhaps reflecting the slow development typical of this mutation (Bohni et al., 1999). However, the number of nurse cells was approximately normal in *chico*¹ ovaries by day 4 suggesting that in this regard the ovaries attained normal maturation. A lag in nurse cell maturation could have contributed to low levels of ecdysteroid released by the *chico*¹ homozygote ovary on day one (fig. 2A) as nurse cells are a possible site of ecdysteroid biosynthesis (Freeman et al., 1999). The number of ovarioles in *chico*¹ was within the normal range of 15–20 per ovary (Bate and Arias, 1993) as were the numbers for the heterozygote and wild-type Dahomey (table 2). If the ovariole number reflects the number of units in an ovariole that can synthesize hormone, then there was no evidence that the amount of ecdysteroids in the haemolymph of *chico*¹ homozygotes was a result of, or even compensation by, an unusually high number of ovarioles. The relatively low level of ovariole numbers in *chico*¹ females on day 4 post-eclosion, compared to day 1 in the homozygous mutants, is surprisingly given that ovariole number is determined before eclosion and the relevant adult tissue is non-mitotic.

D. melanogaster is a model system for investigation of the control of vitellogenesis by hormones and other factors. The hormonal control of vitellogenesis in *D. melanogaster* has been associated with JH, 20-hydroxyecdysone, the ecdysone receptor, the ecdysone early response genes and the sex peptide (Freeman et al., 1999; Bownes et al., 1996; Soller et al., 1997; Buszczak et al., 1999; Carney and Bender, 2000). JH has been implicated in YP synthesis by the ovary and YP uptake into the oocyte (Postlethwait et al., 1980), and 20-hydroxyecdysone in fat body and ovarian follicle cell YP synthesis (Bownes, 1989). JH can also stimulate ecdysteroid production (Kelly, 1994; Richard et al., 1998). JH has been thought to play a predominant role in controlling vitellogenesis (Kelly, 1994; Nijout, 1994), but its direct effect on YP endocytosis remains uncertain (Raikhel and Dhadialla, 1992). Richard et al. (1998, 2001a, b) hypothesized that ecdysteroids and not JHs may play the major role in controlling vitellogenesis; this is based on observations that both *apterous*^{56f} mutant females (Altaratz et al., 1991) and females recovering from diapause (Richard et al., 1998) are JH-deficient yet produce ovarian ecdysteroids and are fertile. Based on the arrest of ovarian maturation in IIS mutations prior to the onset of YP uptake by oocytes, previous studies have suggested that the IIS pathway may play a role in vitellogenesis (Chen et al.,

1996), perhaps in conjunction with control of ecdysteroid biosynthesis (Drummond-Barbosa and Spradling, 2001). Alternatively, insulin could control YP uptake by oocytes in *D. melanogaster* via receptor-mediated endocytosis in a manner analogous to that in vertebrates (Schonbaument al., 2000). From the transplant experiments, we surmised that levels of JH and ecdysteroids, and any other requisite factors, were sufficient in *chico*¹ homozygotes for vitellogenesis. The transplant experiments demonstrated that IIS was necessary for the transition from the final previtellogenic stage to stage 8 vitellogenic oocytes.

Two non-exclusive hypotheses could explain the failure of YP uptake in *chico*¹ homozygotes: [1] defective IIS results in relatively low levels of YP and YP-receptor that constrains YP uptake, and/or [2] defective IIS affects the dynamics of assembly of the protein components of receptor mediated endocytosis at the cell surface. In wild-type flies, YP-receptor mRNA and the receptor itself are synthesized at previtellogenic stages considerably before YP uptake (Schonbaum et al., 2000). This indicates that previtellogenic stages do not fail to take up yolk because of an absence of the receptor and supports the idea that YP-receptor relocalization from within the oocyte to the cortex marks the transition to vitellogenesis (Schonbaum et al., 2000). IIS can play a role in regulation of membrane trafficking including recycling of proteins occurring in a number of mammalian transmembrane receptor complexes such as the InR (Corvera and Czech, 1998). A similar mechanism may underlie *Drosophila* (insect) YP endocytosis.

5. Longevity

IIS evidently plays an evolutionarily conserved role in the determination of fecundity and lifespan and it is pertinent to discuss both phenotypes in the context of our study. The effect of reduced IIS signaling was first discovered to extend lifespan in the nematode worm *Caenorhabditis elegans* (Klass and Hirsh, 1976; Friedman and Johnson, 1988; Kenyon et al., 1993; Kimura et al., 1997), and reduced IIS has since been demonstrated to increase longevity and to induce female sterility in *Drosophila* (Clancy et al., 2001; Tatar et al., 2001). In the mouse, heterozygosity for the insulin-like growth factor 1 receptor (Holzenberger et al., 2003) and ablation of the insulin receptor in white adipose tissue (Bluher et al., 2003) both extend lifespan. In the mouse, mutations in insulin/IGF signaling can also cause female sterility (Brüning et al., 2000; Burks et al., 2000). The pathway evidently plays an evolutionarily conserved role in the determination of fecundity and lifespan.

The pathways and mechanisms through which lowered IIS can extend lifespan and reduce fecundity remain to be elucidated. In *C. elegans*, extension of lifespan by reduced IIS is mediated, at least in part, by dephosphorylation and relocation to the nucleus of a winged fork head transcription factor *daf-16* (Lin et al., 1997; Ogg et al., 1997). The fork head transcription factor *daf-16* transduces insulin-like metabolic and longevity signals in *C. elegans*. Expression of *daf-16* in neuronal tissue appears to be crucial for the extension of lifespan (Wolkow et al., 2000). Although homologues of this gene are present in both *Drosophila* and the mouse, their role in extension of lifespan by reduced IIS in these organisms has not yet been determined. In *C. elegans*, modulations of lifespan and fecundity by IIS are thought to be mediated by a systemic signaling event downstream of *daf-16*, possibly involving steroid hormones (Gerisch et al., 2001).

In *Drosophila*, mutations in genes encoding IIS components that have so far been shown to increase lifespan are also associated with sterility or reduced fecundity (Bohni et al., 1999; Clancy et al., 2001; Tatar et al., 2001; Drummond-Barbosa and Spradling, 2001). It has been suggested that both the increased longevity and the sterility associated with reduced IIS in *Drosophila* are mediated by reduced production of the sesquiterpenoid JH from the *corpora allata* (Tatar et al., 2001; Tatar and Yin, 2001). Both vitellogenesis of eggs, which is absent in long-lived IIS mutant female flies, and normal lifespan have been reported to be restored in females mutant for *Inr* that are treated with the JH analogue methoprene (Tatar et al., 2001). Methoprene did not restore vitellogenesis in *chico*¹ mutant females in the present study.

Flies mutant for the nuclear hormone receptor for ecdysone, the other major systemic hormone in insects, have been recently reported to be long-lived (Simon et al., 2003), and females mutant for this receptor have previously been demonstrated to be sub-fertile or sterile (Carney and Bender, 2000). Production of ecdysone by the ovary, the main site of synthesis, has been reported to be reduced in flies mutant for the IIS receptor (Tu et al., 2002). It remains to be determined if reduced IIS and ecdysone signaling affect fecundity and lifespan by overlapping or separate mechanisms. Hence in *Drosophila* as well as *C. elegans*, there is evidence implicating a second systemic signaling event in mediating the effect of reduced IIS on lifespan and fecundity.

In the present study, hemolymph ecdysteroid levels in *chico* females were also similar to those in wild-type females. We could therefore find no evidence that these two hormones (JH, ecdysteroids) play a role in producing either the sterility or the increased lifespan of *chico* females. Our results imply either that reduction of IIS modulate fecundity by different mechanisms, both within *Drosophila* and between *Drosophila* and *C. elegans*, or that the systemic environment is in fact irrelevant to the fecundity defects associated with mutant IIS in both organisms.

6. Conclusions

Our study indicates that defective insulin/insulin-like growth factor-like signaling (IIS) in *chico*¹ mutant females blocks vitellogenesis irrespective of sufficient levels of juvenile hormone and ecdysteroids to support vitellogenic egg maturation. Regardless of whether IIS acts directly on the yolk protein uptake system or through some intermediate, such as the ecdysone receptor, it is clear that IIS plays an ovarian autonomous role in vitellogenesis.

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