AN ANALYSIS OF YOLK PROTEINS FROM THE CARIBBEAN FRUIT FLY, *ANASTREPHA SUSPENSA*

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Abstract

An analysis of the regulation of yolk protein (YP) synthesis in the Caribbean fruit fly, Anastrepha suspensa (Loew), has been initiated. The major polypeptide constituent of oviposited eggs and vitellogenic ovaries, having a molecular mass of approximately 48 kDa, was identified and isolated by gel-filtration and ion-exchange column chromatography, and SDS-polyacrylamide gel electrophoresis. A second less abundant polypeptide having a molecular mass between 47 and 48 kD was detected also. The major site of synthesis of these polypeptides was the ovary with the first appreciable increase in synthesis occurring at 4 to 5 days after adult eclosion. The polypeptides also were produced by adult fat body and found in the hemolymph, but at markedly lower levels compared to ovaries. Minor denatured hemolymph polypeptides in males co-migrated with the YPs, but their identity awaits immunological analysis. Female and male abdomens isolated from 3- to 5-day-old adults were tested for YP synthesis in response to 20-hydroxyecdysone and a juvenile hormone analog. The synthetic rate of fat body and ovarian YP did not differ significantly between untreated and hormone treated abdomens.

Introduction

The yolk proteins (YP) of a variety of oviparous insects have been subject to considerable analysis with regard to their temporal, spatial, hormonal, and sex-specific regulation. These studies not only elucidate an important developmental process, but also have the potential to reveal mechanisms that may be used to manipulate the reproductive capacity of economically important insects.

In particular, isolation and identification of YP gene coding and regulatory regions may allow manipulation of the YP gene product or allow placement of a selectable gene product under YP promoter regulation. Such a YP promoter gene-chimera that offers conditional expression for selection could have potential use in a genetic sexing program. Identification, isolation, and analysis of the YP genes for molecular biological manipulation depends upon first identifying and isolating the YP gene product. Potential uses for the YP regulatory promoter region depend upon defining the physiology of YP synthesis in terms of time and sites of synthesis, sex-specificity, and hormonal control.

For most insects, the adult fat body is a major site of YP synthesis, releasing vitellogenin into the hemolymph for transport to the maturing oocytes where it is taken up and sequestered. Indeed, vitellogenin has been defined as those proteins found in the hemolymph

of reproductively active females that are precursors to the vitellin found in the egg (Telfer, 1954). Within the ovaries, the follicular epithelium also can be a site of YP production in some insects, either sharing the duty of YP synthesis with the fat body, as occurs in several Drosophila species (Brennan et al., 1982; Srdic et al., 1979) or producing unique proteins as occurs in the lepidopterans (Ono et al., 1975; Bast and Telfer, 1976; Shirk et al., 1984). In a variety of insects, 20-hydroxyecdysone (20-HE), juvenile hormone (JH), and neurohormonal factors are required to initiate and maintain the vitellogenic process, although the timing, function, and site of action of these hormones varies considerably, or is not understood clearly (Engelmann, 1983; Girardie, 1983; Hagedorn, 1985).

This report discusses our initial efforts to define the vitellogenic process in the Caribbean fruit fly, Anastrepha suspensa, with the ultimate aim of isolating and characterizing the YP genes. Thus, we placed a primary emphasis on characterizing the major yolk proteins. In contrast to other insects, the major 48 kDa yolk polypeptide found in oviposited eggs is synthesized predominantly by the ovary and not the fat body, and its synthesis is not promoted significantly by either 20-HE or JH, although females do contain high ecdysteroid titers after vitellogenesis has begun.

Methods

YP identification and isolation

One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to O'Farrell (1975) with modifications and sample preparation described in Belote et al. (1985). Gels were either straight 10% acrylamide or 9-12% gradients as noted in the text. YP purification was achieved as described by Shirk et al. (1984). Crude egg extract proteins were precipitated in 75% ammonium sulfate and resolved by S-300 Sephacryl gel permeation chromatography. YP containing fractions were detected by UV absorbence (280 nm) and verified by SDS-PAGE. S-300 peak fractions were pooled, concentrated by dialysis, and applied to a DEAE Sepharose Cl-6B ion-exchange column and eluted with increasing concentrations of KCl (80 ml steps of 50, 100, 180, and 500 mM KCl). YP containing fractions were detected by UV absorbence and SDS-PAGE. Immunoblots were performed according to Towbin et al. (1979) using antisera raised against Drosophila melanogaster egg extract (Dm anti-yolk; J. H. Postlethwait, unpublished). Relative measures of protein synthesis were determined by densitometry of autoradiograms.

Sample preparation

Samples were prepared from the hemolymph or tissues diluted or dissected, respectively, in insect Ringer's solution. Isolated abdomens were prepared by tying nylon monofilament between the abdomen and thorax, cutting away the thorax, and incubated as described by Handler and Postlethwait (1977, 1978). Animals or abdomens were injected with 0.5 to 1.0 µl of 20-HE or ^{35}S-methionine dissolved in Ringer's. JH analog methoprene; (ZR515) was dissolved in acetone and topically applied.

Radioimmunoassay for ecdysteroids

The radioimmunoassay for ecdysteroids was performed according to Borst and O'Connor (1974) as described by Handler (1982), except that samples of approximately 100 mg/ml homogenized tissue in 70% methanol were prepared, with 0.15 ml duplicate aliquots assayed. DHS I-15 antiserum was utilized (Soumoff et al., 1981).

Results and Discussion

Yolk polypeptide identification and isolation

The major yolk polypeptide constituents of A. suspensa ovaries and oviposited eggs were identified by SDS-PAGE. Figures 1 and 3 show that the predominant polypeptide in both oviposited eggs and vitellogenic ovaries had an electrophoretic mobility corresponding to a molecular mass of approximately 48,000 Daltons. Several other polypeptides found in eggs, ovaries, female hemolymph and male hemolymph, also co-migrated on SDS-PAGE. While some of these may be minor yolk constituents, our present analysis focused on the major 48 kDa YP. Identification of vitellogenin in the Caribfly was confused by the finding that a

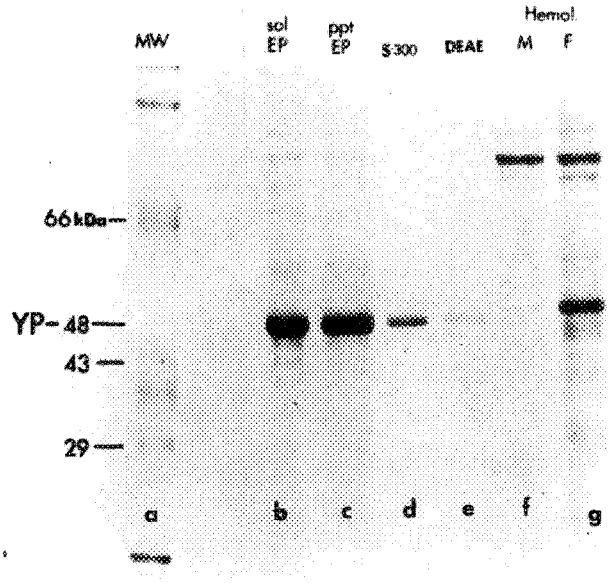

Figure 1. Identification of YP on 10% SDS-PAGE stained for protein. Molecular weight standards (lane a), water soluble proteins from homogenized oviposited eggs (lane b), ammonium sulfate precipitated egg proteins (lane c), UV absorbence peak fraction (fraction 24) of precipitated egg proteins run on an S-300 gel filtration column (lane d), UV absorbence peak fraction of S-300 fraction 24 run on a DEAE ion-exchange column (lane e), hemolymph proteins from 3-4d males (lane f), hemolymph proteins from 3-4d females (lane g).

female-specific 48 kDa polypeptide was not apparent in the hemolymph. A 48 kDa hemolymph polypeptide was observed in females and males (Fig. 2), but only as a minor constituent and there was no apparent immunocross-reactivity with the Dm YP antiserum (Fig. 3, see below). If the female hemolymph polypeptide is indeed vitellogenin, then either it is a minor contribution to the yolk, or it fails to accumulate due to rapid uptake.

Initial efforts to purify the YP are represented as SDS-PAGE samples from crude soluble protein egg extract, ammonium sulfate precipitation, and fractions from S-300 Sephacryl and DEAE ion-exchange column chromatography (Figs. 1 and 3). A single band was resolved in DEAE fractions 51 (Fig. 3f) and 52 (not shown) although when combined and concentrated, a doublet was observed (Fig. 3e). These fractions eluted between 180 and 500 mM KCl on a step gradient. Resolution should be increased by repeating the purification with a continuous linear KCl gradient.

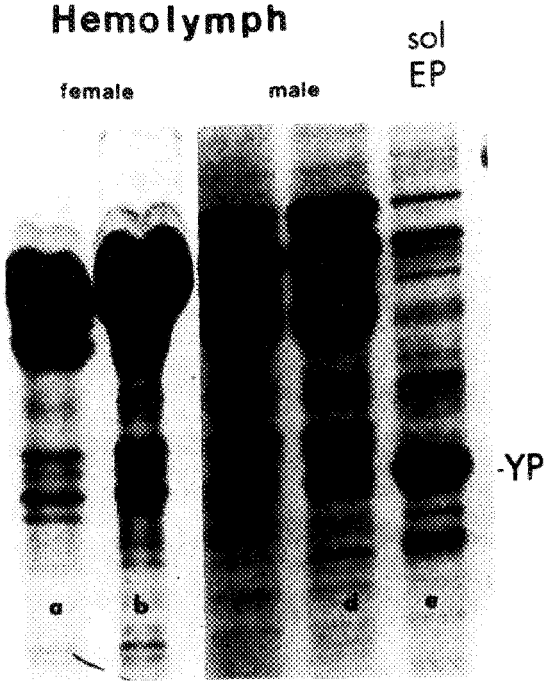

Figure 2. Hemolymph polypeptides identified on 9-12% gradient SDS-PAGE stained for protein. Hemolymph from 5-6d females (lanes a-b), hemolymph from 4-5d adult males (lane c-d), soluble proteins from oviposited eggs (lane e).

In an effort to further define the YPs and determine if they share antigenic homology with D. melanogaster YPs, which have similar molecular weights (Warren et al., 1979), immunoblots were performed using Dm anti-yolk antisera against A. suspensa YP from SDS-PAGE. Figure 3B is an immunoblot of identical samples stained for protein in

Figure 3A. The Drosophila antibody bound with an array of polypeptides extracted from Drosophila ovaries, with greatest cross-reaction with the three YPs. Cross-reactivity was also significant with the A. suspensa YPs, including those from ovaries, egg extracts and purified protein fractions. Both members of the polypeptide doublet from the combined DEAE fractions cross-reacted. Interestingly, the minor 48 kDa polypeptide from female hemolymph did not show cross-reactivity, nor did any polypeptides of the same molecular mass from male hemolymph. We observed significant binding of the Dm anti-yolk antiserum to Caribfly YP, yet the Dm anti-yolk antisera had higher binding affinity for the Drosophila YPs. This suggests some, though not total, homology between the YPs of the two species. We are encouraged to attempt the use of cloned genomic YP DNA from Drosophila (Barnett et al. 1980) to isolate the YP gene(s) from A. suspensa since the antigenic homology may indicate sufficient structural homology to allow for specific hybridization between the heterologous genes.

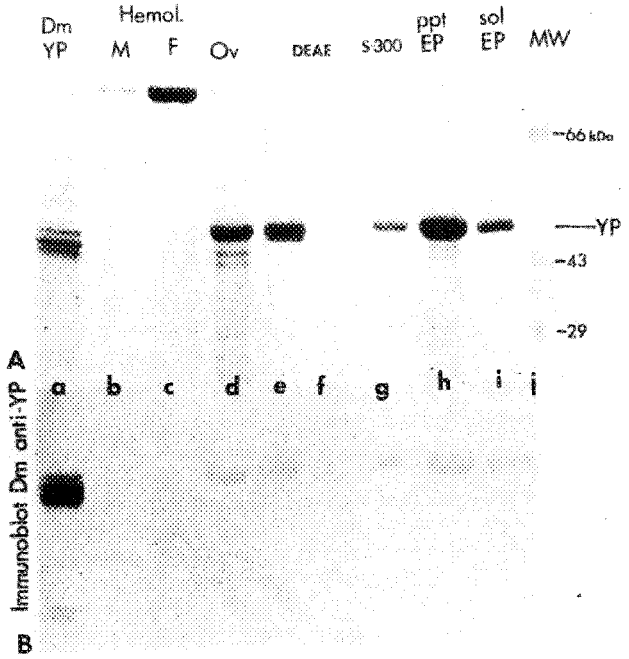

Figure 3. Identification of YP on 9-12% gradient SDS-PAGE stained for protein (A), or electroblotted onto nitrocellulose and cross-reacted with Dm anti-yolk antisera (B). D. melanogaster ovarian proteins (lane a), 4 d adult male hemolymph (lane b), 4 d adult female hemolymph (lane c), A. suspensa ovarian proteins (lane d), DEAE fractions 51 and 52 (lane e), DEAE fraction 51 (lane f), S-300 fraction 24 (lane g), ammonium sulfate precipitated egg proteins (lane h), soluble egg proteins (lane i), molecular weight standards (lane j).

Site of synthesis

The majority of insects studied thus far contain a hemolymph-borne YP or vitellogenin in adult females, that presumably is synthesized by the fat body (c.f. Kunkel and Nordin, 1985). For some Drosophila species, the ovaries also contribute YP, as was most conclusively demonstrated by in situ hybridization of cloned YP gene DNA to follicular epithelium RNA in D. melanogaster (Brennan et al., 1982).

Our SDS-PAGE analysis of female hemolymph proteins did not reveal a major YP constituent. To determine the site(s) of YP synthesis, this analysis was continued by measuring protein synthesis in vivo and in in vitro tissue culture by incorporation of radiolabeled methionine. Figure 4 shows an autoradiogram of radiolabeled polypeptides resolved by SDS-PAGE. Lanes a-c shows in vivo radiolabeled proteins contained in pre-vitellogenic, early, and late vitellogenic ovaries taken from females that were injected with ^{35}S-methionine. YP synthesis was first detected in early vitellogenic ovaries where the 48 kDa doublet was resolved (7% of total synthesis); extremely high levels of synthesis were observed 2 days later in late-vitellogenic ovaries where the doublet was obscured due to the massive amounts of YPs produced (24% of total synthesis). Hemolymph taken from the same animals showed synthesis of the 48 kDa polypeptide only in late-vitellogenic females (Fig. 4d-f), although at a rate considerably less than a higher molecular weight hemolymph protein (7% vs. 18% of total synthesis).

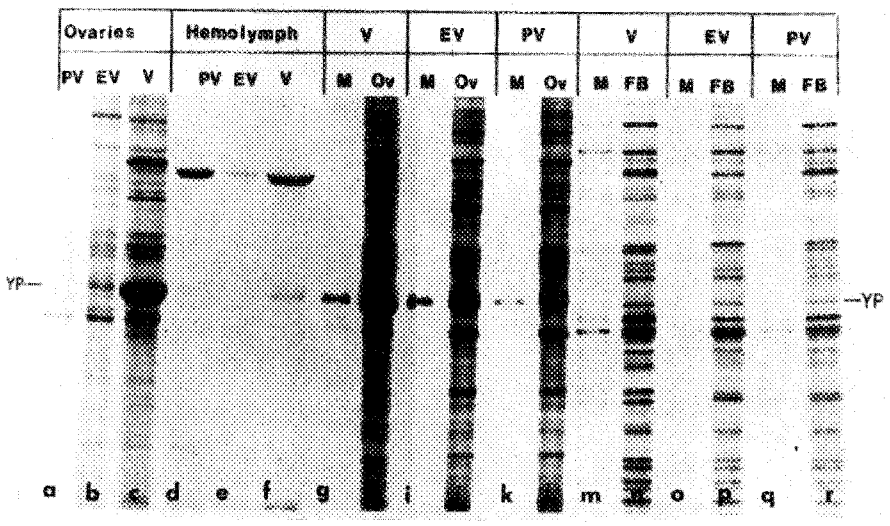

Figure 4. YP synthesis in vivo and in vitro indicated on a 9-12% gradient SDS-PAGE autoradiogram. PV, pre-vitellogenic 3 d adult females; EV, early vitellogenic 4-5 d adult females; V, vitellogenic 6 d adult females; M, culture media; Ov, cultured ovaries; FB, cultured abdominal walls containing adult fat body. Adult females were injected with 3 µCi ^{35}S-methionine, incubated for 3 hr and dissected. In vivo cultured ovaries (lanes a-c) and hemolymph (lanes d-f). In vitro culture of ovaries (lanes g-l) and abdominal walls (lanes m-r) with 15 µCi ^{35}S-methionine.

Ovaries dissected from the same age females and radiolabeled in vitro exhibited high overall total protein synthesis with significantly high YP synthesis levels at all three stages (Fig. 4h,j,l). Interestingly, the culture media from all three ages contained almost exclusively the 48 kDa YP doublet (Fig. 4g,i,k). Abdominal walls, which had large amounts of fat body attached in addition to muscle and cuticle, were also incubated in vitro. Total protein synthesis from these tissues was extensive, although only a single polypeptide (co-migrating with the lower ovarian polypeptide) was observed, whose synthesis was moderate and equivalent at all three ages (approx. 2-3% of total synthesis; Fig. 4m-r)). The cultured fat body secreted an array of proteins, of which the 48 kDa polypeptide was a minor contribution (3-5% of total synthesis).

These experiments strongly suggest that the ovary is the major, if not the exclusive site of YP synthesis in A. suspensa. The 48 kDa polypeptide found in the hemolymph, presumably secreted by the fat body, may indeed be a YP constituent, but its contribution appears to be minor. Vitellogenic and pre-vitellogenic ovaries cultured in vitro are competent to support a high level of YP synthesis which can be secreted. While leakage cannot be excluded, the pre- and early-vitellogenic ovaries are not disrupted easily, and certainly other polypeptides are conserved within the ovary. The selective secretion of ovarian YP in vitro suggests that either this is an artifact of in vitro conditions (e.g. a lack of feedback inhibition) or re-uptake of YP in vivo occurs too rapidly for appreciable accumulation in the hemolymph.

Hormonal control of YP synthesis

In a wide variety of insect species, as well as oviparous animals in general, hormones have been found to have a regulatory influence on YP synthesis, as well as other aspects of the vitellogenic process. In D. melanogaster both 20-HE and JH apparently have equivalent regulatory influences on YP synthesis, although 20-HE only affects fat body synthesis (Postlethwait and Handler, 1979; Jowett and Postlethwait, 1980). On the other hand JH affects both fat body and ovarian YP synthesis, as well as promoting YP uptake by the oocytes (Postlethwait and Handler, 1978; 1979). In another dipteran, Aedes aegypti (L.), both 20-HE and JH influence YP synthesis but in this species JH does not stimulate synthesis directly, rather it is a prerequisite for the more direct action of 20-HE (Hagedorn, 1983). In contrast to the positive hormonal influence in these species, in the lepidopteran Plodia interpunctella JH has no effect on YP synthesis, while 20-HE inhibits YP synthesis at concentrations above 10^{-8} M (Shirk and Brookes, 1986).

Analysis of hormonal regulation of YP synthesis in A. suspensa generally followed the protocol we established for a similar analysis in D. melanogaster. Abdomens were isolated from females previous to or at the initial appearance of vitellogenic oocytes (approximately 3, 4, and 5 days after eclosion), incubated for 24 hr, and then treated with either 10^{-4} M 20-HE or 10^{-3} M juvenile hormone analog (methoprene). At 6 to 8 hr after hormone treatment, the ovaries and body walls were dissected and incubated separately in vitro with 15 µCi ^{35}S-met for 3 hr. Autoradiography of the proteins resolved by SDS-PAGE indicate relatively low levels of 48 kDa polypeptide synthesis in both untreated and hormone treated abdominal tissues, yet total protein synthesis in these samples was extensive (Fig. 5). This test fails to demonstrate substantial stimulation of YP synthesis by either 20-HE or JH.

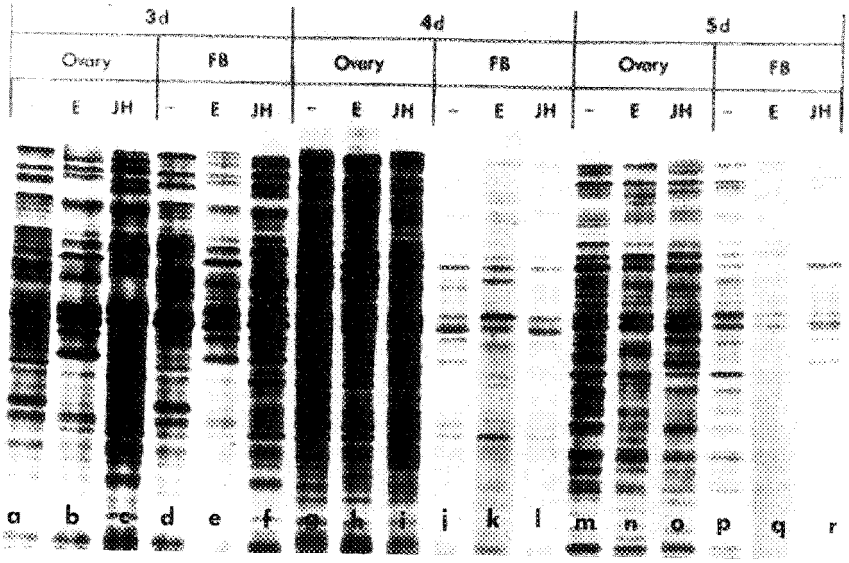

Figure 5. Influence of 20-HE (E) and JH on ovarian or fat body (FB) synthesis of YP. Abdomens isolated from 3, 4, and 5 d adult females were treated with hormone for 6-8 hr, tissues were dissected and incubated for 3 hr in Ringer's solution with 15 μCi ^{35}S-methionine. Tissues and media were dissolved in sample buffer, and run on 9-12% gradient SDS-PAGE which was autoradiographed. Tissues from abdomens untreated with hormone (lanes a,d,g,j,m,p); tissues from abdomens treated with 10^{-4} M 20-HE (lanes b,e,h,k,n,q); tissues from abdomens treated with 10^{-3} M JH analog methoprene (lanes c,f,i,l,o,r).

Specificity in terms of hormone concentration or times of hormone sensitivity were not tested; thus this negative result does not rule out hormonal control of YP synthesis in Anastrepha. However, it is apparent that the endocrine physiology of vitellogenesis in this species differs from that in D. melanogaster.

Ecdysteroid titers in adult animals

Ecdysteroids have been found to promote YP synthesis experimentally in various insects, and upon RIA analysis physiologically significant amounts of hormone have been found in adults systemically or in homogenates (Hagedorn, 1983; 1985). In some species females have higher ecdysteroid titers compared to males, which might be related to vitellogenesis. While ovarian secretion of hormone has been detected in some instances (Hagedorn et al., 1975), for other species ovaries accumulate ecdysteroids for use by the zygote during embryogenesis (Lagueux et al., 1981). In Drosophila, ecdysteroids have been found in ovaries, though their ability to secrete hormone is subject of debate (Handler, 1982; Bownes et al., 1984; Schwartz et al., 1985). The ovaries are clearly not necessary for YP synthesis by the fat body

(Postlethwait et al., 1980; Belote et al., 1985), and hemolymph and whole body ecdysteroid titers do not differ significantly between D. melanogaster males and females (Handler, 1982; Bownes et al., 1984).

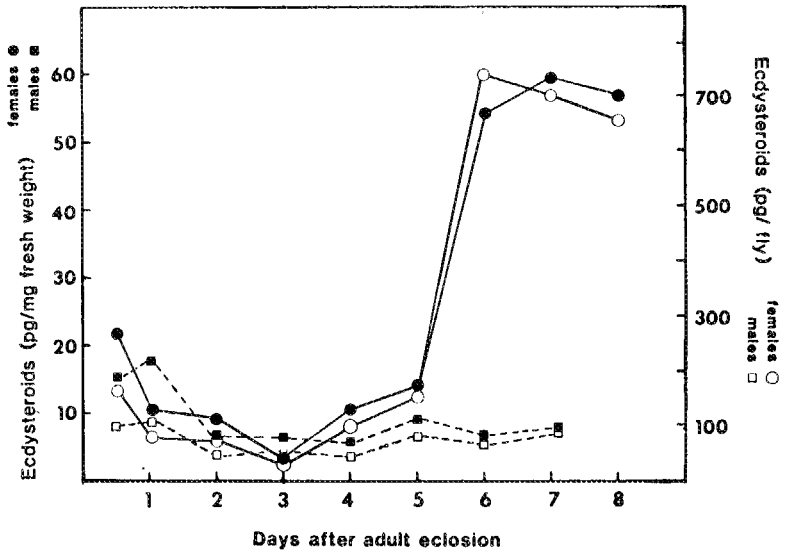

Figure 6. Ecdysteroid radioimmunoassay of whole animal homogenates extracted in 70% methanol. Values represent the mean of two separate assays (each using replicate samples) performed on the same extraction samples. Ecdysteroids are 20-HE equivalents.

As reported above, 20-HE did not stimulate YP synthesis in the Caribfly under our experimental conditions, though it remains of interest whether sex-specific levels of ecdysteroids exist in adult flies. We performed two RIA analyses on a single series of adult male and female whole animal homogenates extracted in 70% methanol. Significant titers were detected in both males and females, with relatively low levels until 5 days after eclosion in both sexes (Fig. 6). At day 6, titers in the females increased to a level of approximately 54 pg/mg fresh weight, compared to 7 pg/mg fresh weight in males. Titers remained at this level for at least 2 succeeding days. The increase in female titer correlates with the time of increasing YP synthesis and the initial appearance of vitellogenic oocytes. While a relationship between YP synthesis and ecdysteroid titer might be deduced, it should be noted that YP synthesis and secretion occured in 3-day-old ovaries cultured in vitro (Fig. 4), at a time when 20-HE titers were low and comparable to titers in males.

Conclusions

In summary, the YP of Anastrepha suspensa is limited to a single major 48 kDa polypeptide, and possibly another minor polypeptide with a slightly lower molecular weight. Synthesis of the polypeptides is almost exclusively limited to the ovaries, and is first detected at an early stage of yolk deposition. YP derived from the fat body is

minimal, and further immunological studies are necessary to determine if the fat body and hemolymph 48 kDa polypeptide is indeed female-specific and/or deposited into the oocyte. Stimulation of YP synthesis by either 20-HE or a JH analog was not observed in our studies, although females late in vitellogenesis have ecdysteroid titers 6- to 7-fold higher than sibling males. Elucidation of the hormonal regulation of Anastrepha vitellogenesis awaits a more comprehensive analysis.

The prodigious ovarian synthesis of YP has allowed us to isolate mRNA sufficient to produce cDNA libraries from which YP cDNA clones may be isolated (Handler, unpublished). In addition, antigenic homology between Drosophila and Anastrepha YP suggests that Anastrepha YP genomic DNA may be isolated by hybridization to D. melanogaster YP DNA. With cloned YP DNA in hand, we may then be able to define the YP promoter regulatory region by gene-fusion and transformation into Drosophila techniques. Of particular interest will be the ability of the YP promoter region to confer sex-specific expression upon a selectable gene-product such as alcohol dehydrogenase, which has potential use in a genetic-sexing program. In addition, YP gene expression itself may be used as a marker to identify and characterize sex-determining genes in A. suspensa as has been done in Drosophila (Belote et al., 1985). The eventual isolation and manipulation of sex-determining genes will add a new dimension to our ability to genetically sex tephritid populations, and perhaps control their population size.

Acknowledgment

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