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Proteomic identification of a potential sex biomarker for 2 fruit fly species at pupal stage

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ABSTRACT

Fruit flies are one of the most destructive pests worldwide. The sterile insect technique (SIT) has been one of the most effective fruit fly control techniques. Genetic sexing strains have been developed to facilitate the separation of males and females based on color at the pupal stage for the oriental fruit fly, *Bactrocera dorsalis*, and the medfly, *Ceratitis capitate*, known as dorsalis translocation white pupae (DTWP) and temperature sensitive lethal mutation strain (TSL). In both cases, the females are white and the males are brown. By releasing only sterile males, the control efficiency was increased, and fruit damage due to sterile females reduced. There remains, however, a gap in our understanding of this phenomenon because there is no information on the physiological basis of the sex differences. Here, we report the outcome of a study designed to identify pupal proteins related to the sex differences using these two strains. We collected whole pupae of both species each day for first 10 days for protein analysis. Protein expression was analyzed by 2D electrophoresis, gel densitometry and mass spectrometry. The general odorant binding protein, OBP56d, was differentially expressed in white pupae versus brown pupae for both species. We also confirmed this result with phenotypical characteristics to prove these proteins were not derived from puparia. These proteins had molecular weights between 10 and 15 kDa and a pI of 6.73 for DTWP and 5.71 for TSL.
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Introduction

The sterile insect technique (SIT) is an effective component of several insect pest control programs, including some deployed to manage tephritid fruit fly pests. Conventional SIT programs are based on mass rearing males and females, sterilizing, transporting, and releasing them into the wild. The sterile males compete for and mate with wild females, resulting in reduced fertility at the individual and population levels. These systems have been improved by increasing the field competitiveness and survival of released males. One approach is artificial selection for appropriate phenotypes. For example, McInnis et al. (2002) selected male medfly, *Ceratitis capitata*, for increased survivorship under field conditions. Development of male-only strains led to increased cost effectiveness in rearing and to reduced fruit damage caused by released sterile females.

Genetic sexing strains based on pupal coloration have been developed for the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) known as temperature sensitive lethal [TSL] (Robinson et al., 1999; Fisher, 2000) and the oriental fruit fly, *Bactrocera dorsalis* (Hendel), known as dorsalis white pupal translocation [DWPT] (McCombs and Saul, 1995). In each species, chromosomal translocations link a gene for normal brown pupae to males, and mutant white pupae to females. This translocation-based gender sorting system relies on automated photoelectric scanners to separate female-determined from male-determined pupae (McCombs and Saul, 1995; McInnis et al., 2004). The TSL strain facilitates killing female-determined pupae, by heating to 34 °C for 12 h, thereby creating a male-only colony for release.

The sex-linked pupal coloration opens questions about the underlying mechanisms of the color differences. Some proteins are directly linked to insect color patterns. Insecticyanin, for example, is a blue biliprotein in hemolymph of the tobacco hornworm, *Manduca sexta* (Riley et al., 1984). Two proteins, yellow and ebony, direct pigment patterns in pomace flies, *Drosophila melanogaster* (Wittkopp et al., 2002). Wild-type *D. melanogaster* have a stripe of dark pigment near the trailing edge of the anterior abdominal segments (A2–A6). The stripe is lost in *yellow* mutants and very much darker in *ebony* mutants. The stripe is completely missing in double *yellow*; *ebony* mutants. These two proteins also influence larval colors in silkworms, *Bombyx mori* (Futahashi et al., 2008). Pea aphids, *Acyrthosiphon pisum*, occur in red-green polymorphs. The carotenoid torulene is responsible for the red morph (Moran and Jarvik, 2010). More recently, a single gene, *cortex*, has been identified as regulator of wing pattern changes in many, possibly all, lepidopteran species (Nadeau et al., 2016). More to the point, insertion of a transposable element into *cortex* is responsible for the evolution of industrial melanisms in the peppered moth, *Biston betularia* (Van't Hof et al., 2016). *Cortex* is a member of the fizzy family of cell-cycle regulators and its cognate protein probably acts during early wing disc development. We infer that various proteins and their

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cognate genes are responsible for coloration and sex/color morphs in insects generally, which led us to pose the hypothesis that one or more proteins associated with sex/color formation is differentially expressed between male- and female-determined pupae. We tested our hypothesis by analyzing protein expression in male- and female-determined pupae. Here, we report on the outcomes of our analysis.

Materials and methods

Insects sample collection

Newly laid eggs (<6 h) of the oriental fruit fly, *B. dorsalis* (DTWP) were provided by the DTWP colony rearing group in the Tropical Crop and Commodity Protection Research Unit (USDA Agricultural Research Service, Hilo, Hawaii). Hatched larvae were reared on a liquid diet supplemented with wheat germ oil (WGO) (0.66%, v:v) ad lib (Chang and Vargas, 2007). Upon pupation, pupae were collected at the same time every day for the first 10 days of the pupal stage and sorted by color. Colored pupae of medfly TSL strain, *Ceratitis capitate*, were provided by California Department of Food and Agriculture, Waimanalo, Hawaii. Brown and white pupae (400 each day) were quick-frozen in liquid nitrogen and stored at −80 °C for protein analysis. Protein expression was analyzed by 2D electrophoresis, gel densitometry and mass spectrometry. Identified proteins were confirmed with qPCR as well. We also confirmed our finding with phenotypical characteristics to exclude the hypothesis that these proteins may be derived from color of puparia. All protocols were described below:

Sample preparation

Pupae (0.2 g whole pupae/ml buffer, approximately 20–30 pupae) were homogenized 3× in 1 ml 10 mM Tris-HCl (pH 7.0) containing protease inhibitors (final dilution = 1:100; Sigma, St. Louis, #P8340 for Mammalian Cell and Tissue Extracts) using a Fast Prep-24 instrument (MP Biomedicals, Solon, OH). Homogenates were centrifuged twice at 15,294 ×*g* for 15 min at 4 °C. The resulting infranatants (middle clear layer, just below the supernatant) were transferred to new vials on ice for immediate use. Three independent biological replicates were processed for each age.

2D-electrophoresis

Electrophoresis and mass spectrometric protocols followed published procedures (Stanley et al., 2008; Chang et al., 2012). Five μl of 2D gel protein standards (Bio-Rad, Hercules, CA, #161-0320) were added to each sample tube. Protein concentration (5 μg/μl) was determined using the Pierce Micro BCA Protein Assay Kit, using BSA as a quantitative standard (Rockford, IL). A Protean isoelectrical focusing (IEF) cell system (Bio-Rad, Hercules, CA) was used to perform IEF using the standard protocol and a pre-set linear volt ramp program (8000 V and 50 μA/strip max, 35,000 vH).

For the second dimension, the IPG strips were equilibrated (15 min in buffer: 6 M urea, 2% SDS, 20% glycerol, 130 mM DTT, 0.375 M Tris-HCl, pH 8.7 [Buffer I] followed by 6 M urea, 2% SDS, 20% glycerol, 135 mM iodoacetamide, 0.375 M Tris-HCl, pH 8.7 [Buffer II]). Prior to running, molecular weight standards (10 μl/lane, Bio-Rad #161-0363) were applied to each gel (precast gels, 8–16% Tris-HCl, Bio-Rad #345-0105), and proteins were separated on SDS-PAGE using the Criterion Cell system (Bio-Rad, Hercules, CA, #165-6001). Gels were stained with Coomassie Blue G-250 (BioSafe Stain, Bio-Rad) and analyzed using Delta 2D software (Decodon GmbH, Greifswald, Germany). Protein spots with densities significantly different between treatments (Students' *t*-test, $p < 0.05$) were removed from the gels using a 1.5 mm spot picker (The Gel Company, San Francisco, CA) and stored at −80 °C. One gel was run for each independent biological replicate and three independent biological replicates were performed.

MS/MS analysis

Mass spectrometric protocols followed published procedures (Stanley et al., 2008; Chang et al., 2012). 4700 MALDI TOF-TOF mass spectrometer was used in this study.

Selected protein spots were prepared for mass spectrometry by transferring them to clean tubes, digesting with trypsin, extracting peptides from the gels, and then lyophilizing and reconstituting the peptides with water for MS/MS analysis as described (Stanley et al., 2008). A portion of each protein was mixed with alpha-cyano-4-hydroxycinnamic acid matrix and applied to the MALDI target for analysis. The resulting sequence data, combined with observed MW and pI values, were used to establish protein identities. MS/MS ion searches were performed for each spot using the Mascot search engine v. 2.4 (www.matrixscience.com) on an in-house server against a custom *B. dorsalis* protein database derived from mRNA sequence and the NCBInr Metazoa protein database. E-values and frequency of matches to a specific protein were the primary criteria for these determinations. The lowest E-values and highest frequency were usually selected. All reported matches in the species specific *B. dorsalis* database had higher significance levels compared to the Metazoa database. The custom *B. dorsalis* peptide database, generated from a draft annotation set of the *B. dorsalis* genome is available as supplemental information (Supplementary file 3).

Quantitative PCR (qPCR)

Ten odorant binding proteins (OBPs) primers from *Drosophila* genes used in Zheng et al. (2013) for *B. dorsalis* genes along with chitin binding proteins and chitin synthases listed in Table 1 were used in this study for qPCR analysis to confirm our finding.

The qPCR protocol followed published procedures (Chang et al., 2012). Total RNA was isolated from separated brown and white DTWP pupae using a Macherey-Nagel NucleoSpin RNA II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Four pupae of each color per age group were homogenized in 600 μl of lysis buffer (RA1) and 6 μl of β-mercaptoethanol using a RNase-free pestle and 1.5 ml microcentrifuge tube, following the manufacturer's protocol. Total elution volume was 60 μl. Total RNA concentrations were measured using a Qubit Fluorometer using Qubit RNA BR reagents (Invitrogen, Carlsbad, CA). The first-strand cDNA was synthesized using Moloney Murine

Table 1
Identification of OBP56d expression ratio between white and brown *Bactrocera dorsalis* pupae.

Spot ID	W/B ratio + SE	p values
1-1	2.23 ± 0.68	0.0036*
1-2	2.61 ± 1.45	0.0015*
2-1	4.20 ± 0.02	0.0001*
2-2	3.33 ± 0.49	0.00005*
3-1	3.17 ± 0.23	0.0015*
3-2	3.03 ± 0.59	0.0003*
4-1	2.94 ± 0.55	0.0102*
4-2	3.22 ± 1.00	0.0063*
5-1	2.76 ± 0.53	0.0658**
5-2	5.43 ± 0.78	0.0336*
6-1	3.25 ± 0.81	0.0474*
6-2	4.16 ± 0.96	0.0316*
7-1	2.57 ± 0.66	0.0807**
7-2	3.06 ± 0.61	0.0031*
8-1	3.10 ± 0.29	0.0678**
8-2	0.99 ± 2.29	0.9399
9-1	1.97 ± 1.39	0.1364
9-2 + 3	1.09 ± 1.54	0.6274
10-1	0.77 ± 0.34	0.4193
10-2 + 3	1.21 ± 0.22	0.1504

SE: standard errors.
* 95% significance.
** 90% significance.