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Proteomics/qPCR protocol to estimate physical ages of wild male oriental fruit flies, *Bactrocera dorsalis* (Hendel)

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ABSTRACT

Laboratory reared fruit flies may have different behavioral phenotypes as compared to the wild in the field. The domesticated lab setting and the absence of factors from the natural environment on lab reared insects may result in different behavioral patterns, such as the length of life cycle. This study was performed to conduct a regression analysis of the ratio of odorant binding protein 99b (OBP99b) expression from 4 to 12-d-old oriental fruit fly males, relative to 1-d-old males to estimate the physical ages of wild male fruit flies. We established two polynomial linear regression equations based on 4–12 days old lab-reared flies using proteomics and qPCR. The equations are $Y = -0.7768 + 0.7205X$, $R^2 = 0.89$ (for proteomics) and $Y = -0.6478 + 0.344X$, $R^2 = 0.64$ (for qPCR). We used these equations to estimate the physical ages of wild-caught male fruit flies. These results indicate that despite multiple behavioral differences between laboratory reared and field-caught flies, the physical ages of both groups are identical. We suggest proteomics and qPCR analysis of selected genes and the proteins they encode may be developed into reliable tools for determining the ages of wild-caught animals, including oriental fruit flies.

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Contents

Introduction

Information on the age of individual field-caught insects is important to research areas such as pest management, and behavioral and

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evolutionary ecology. Knowledge of ages has been crucial to fruit fly control programs, especially for monitoring population structures in Sterile Insect Technique programs (Robson and Crozier, 2009). Data on ages of field-caught male fruit flies in attractant studies is essential. Many techniques to estimate wild-caught insect physical ages have been introduced (Johnston and Ellison, 1982; Camin et al., 1991; Templeton et al., 1993; Robson et al., 2006; Robson and Crozier, 2009; Hugo et al., 2010; and Jang, 2011). A direct method of age determination using counts of growth layers in internal thoracic muscle attachments in *Drosophila* was developed by Johnston and Ellison (1982) and used to age field caught *D. mercatorum* by Templeton et al. (1993). Camin et al. (1991) reported that a simple and accurate way to determine adult age was to measure homogenized head capsules of Mediterranean fruit flies (*Ceratitis capitata* Weidemann) using spectrofluorometry. Adult flies of various ages had significant differences in their level of fluorescence. They also proved that irradiation does not influence the age estimates because no difference in fluorescence was observed between irradiated and nonirradiated flies. Robson et al. (2006) reported that fluorescence spectrophotometry can reliably detect levels of the pteridine 6-biopterin in the heads of individual *Drosophila serrate* Malloch 1927. Robson and Crozier (2009) used spectrofluorometry to confirm the presence of extractable levels of lipofuscins and pteridines based on accumulation of these two chemicals in body tissues through time to estimate the age of the ant *Polyrhachis sexpinosa* Latrielle (Hymenoptera: Formicidae). Hugo et al. (2010) adopted transcriptional assay for the prediction of age of uncaged mosquitoes, *Aedes aegypti* in Northern Australia. Jang (2011) monitored male responses to conspecific advertisement signals in the field cricket *Gryllus rubens* (Orthoptera: Gryllidae) to predict age. All these methods may help achieve some degree of success, but none are ideal.

Odorant binding proteins (OBPs) make up one of the three main types of proteins found in insect olfactory system in addition to odorant receptor and odorant-degrading esterases (Liu et al., 2010). OBPs are water-soluble proteins that present in the chemosensory organs of insects. Zheng et al. (2013) described that these proteins bind various hydrophobic odorant molecules in the environment and transport them through antennal lymph to cell surface odorant receptors of the olfactory neurons. Then the olfactory signal transduction system is induced.

Previously, we performed an 'attract and kill' test in the field using methyl eugenol (ME) and basil oil (Chang et al., 2013). ME, but not basil oil, is supposed to be attractive to reproductively mature male oriental fruit flies. Because insect sensitivity to environmental chemicals may be age-dependent, a tool to identify the male's physical age may help to confirm whether oriental fruit fly males are sensitive to basil oil.

We have identified a large number of proteins expressed during juvenile and adult life stages in oriental fruit flies, *Bactrocera dorsalis* (Hendel), including multiple OBPs. Based on 2 dimensional gel electrophoresis, OBPs are visible only in 9-d-old or older females and 4-d-old or older males. OBP spots increased in size and density with fly age until sexual maturity (Chang et al., 2012a, 2012b). This observation prompted our hypothesis that the age of wild male oriental fruit flies can be determined by the relative expression of OBPs. Here we report on the outcomes of experiments designed to test our hypothesis.

Materials and methods

Insects

Laboratory insects

Male adult fruit flies from liquid diet fed larvae were reared in the laboratory located in the USDA, Agricultural Research Service of Daniel K. Inouya U.S. Pacific Basin Agricultural Research Center in Hilo, Hawaii. Virgin males (4–12-d old) were used for proteomics and qPCR analysis and to establish regression analysis.

Known ages of males with different treatments available in our lab such as virgin and mated males (13, 14, 15-d old), males from irradiated lab pupae with 100 gys and 30 gys (Chang et al., 2015), males from lufenuron (LFN, a chitin synthase inhibitor) diet fed, LFN fed then switched to control diet at 7-d old (recovery), and control diet fed males (24-d old) (Chang et al., 2012a, 2012b) were used for data validation (Table 1). These abovementioned males were randomly selected for data validation purpose only.

Wild insects

Wild males of unknown ages were collected from traps incorporated with methyl eugenol and/or basil oil (1:4) and control (without methyl eugenol) in the field (Chang et al., 2013) and stored at 80 °C freezer for proteomics analysis and estimation of physical age.

Proteomics approach

Sample preparation

Sample preparation followed published procedures (Chang et al., 2015). 1–12-d old male samples (0.2 g per ml buffer, approximately 20–30 adult) from laboratory or field colony 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 were transferred to new vials on ice for immediate use. Three independent biological replicates from the same generation were processed for each treatment.

2D-electrophoresis

Electrophoresis and mass spectrometric protocols followed published procedures (Chang et al., 2015). Five microliters 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). Protein samples were prepared for iso-electric focusing (IEF) as previously described (Chang et al., 2014). A Protean IEF cell system (Bio-Rad, Hercules, CA) was used to perform IEF using the standard protocol and a preset 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/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 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. Entire body of fly was used for protein analysis according to Chang et al. (2015) to establish a baseline information for odorant binding protein (OBP99b). The OBP ratios defined as the ratios of odorant binding protein (OBP99b) presented in each age group verse 1-d-old males lab strain. Mean OBP ratios from each age groups were plotted to regression line using Sigma plot.

MS/MS analysis

Proteins were digested with trypsin gold (Promega, extracted, then lyophilized and reconstituted with water in preparation for MS/MS analysis as described (Chang et al., 2015). A portion of each protein was mixed with alpha-cyano-4-hydroxycinnamic acid matrix and