Biol Trace Elem Res (2014) 160:245–249
DOI 10.1007/s12011-014-0037-1

Decreased Accumulation of Cadmium in *Drosophila* Selected for Resistance Suggests a Mechanism Independent of Metallothionein

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Received: 13 March 2014 / Accepted: 3 June 2014 / Published online: 15 June 2014

Abstract Heavy metals, including cadmium, are common contaminants in environments subject to human activity. Responses to exposure in the fruit fly, *Drosophila melanogaster*, are dosage-dependent and resistance is selectable. While metallothionein-mediated sequestration has been extensively studied as a mechanism of cadmium resistance, a link between selection for resistance and an increased accumulation of cadmium has yet to be demonstrated. To address this need, we have selected wild-type flies for cadmium resistance for 20 generations and tested metal content using mass spectrometry. Resistant flies were observed to contain lower levels of cadmium, arguing for a mechanism of cadmium resistance that is not mediated by increased sequestration. This, coupled with genetic evidence suggesting the involvement of factors located on the X chromosome, suggests a gene other than metallothionein may be involved in resistance in this line.

Keywords *Drosophila melanogaster* · Cadmium · Metallothionein · Heavy metal resistance · Accumulation

Introduction

Heavy metal adaptation is an important biological response to environmental contamination with significant ecological implications (for reviews see [1–3]). Heavy metal toxicity acts in a variety of ways. Organismal responses range from biochemical to physiological. *Drosophila melanogaster* was one of the first terrestrial invertebrates in which adaptation to heavy metals was definitively demonstrated [4]. However, the pathways and the genetics underlying the response and adaptation are not completely understood.

The mechanisms of toxicity are not entirely clear and may depend on multiple mechanisms collectively causing toxicity. In low concentrations, some heavy metals act as essential micronutrients, such as copper, which is an enzymatic cofactor [5, 6]. High levels of exposure to heavy metals, on the other hand, are generally toxic; however, many animals develop resistance to heavy metal exposure (for review see [7]). Cadmium is of particular interest for having highly toxic effects [8] and no known physiological role [9]. Cadmium treatment of *Drosophila* cell lines induces heat shock protein synthesis [10, 11], supporting the hypothesis that toxicity involves DNA damage and protein synthesis interference. Similarly, in exposed *Drosophila* cells, cadmium has been shown to replace zinc in metalloenzymes and also interact with free sulfhydryl groups of other proteins [11]. This may cause a reduction of enzyme functionality [8] and disruption of protein synthesis [11]. Giaginis et al. [8] discuss cadmium inhibition of DNA repair processes, including nucleotide excision repair, base excision repair, and mismatch repair. For example, cadmium has been shown to substitute for zinc in the zinc finger structures contained in the DNA repair protein XPA, causing deformation and subsequent inactivation [12]. In *Drosophila* cells, cadmium toxicity results in a decrease in protein synthesis, followed by cell death [10]. Heavy metals can also be toxic at the physiological level. Cadmium exposure during the larval stage has been shown to reduce viability [13] and extend the developmental period, while reducing overall adult weight, fecundity, and survival [14]. In summary, studies on cadmium in *Drosophila* seem to suggest a variety of different mechanisms that all contribute to toxicity.

Responses to heavy metal exposure seem to vary as much as mechanisms of toxicity and may function independently or in concert. These can generally be classified as avoidance

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behavior, detoxification, compartmentalization, or excretion [2, 14]. Studies on the springtail *Orchesella cincta* (Arthropoda: Collembola, springtails), a soil-living invertebrate, indicate that these organismal responses mediate resistance to environmental toxicity [7, 15–17]. Selection, whether through artificial exposure or natural exposure in a toxic environment, acts on the underlying genetic variation to increase the frequency of resistant phenotypes in the adapted population. In *Drosophila melanogaster*, there are examples of laboratory selected metal resistance and variation in metal response between laboratory and wild lines. Magnusson and Ramel [18] showed significant variation in resistance even among laboratory strains. Heavy metal resistance, specifically cadmium resistance, has become a model system for inbreeding and selection studies (see, for example, [19]). Shirley and Sibly [20] selected cadmium resistant flies for over 20 generations, resulting in a 30 % increase in survival. Resistance of this line was conferred by a single, dominant, sex-linked gene. Christie et al. [21] also found a major factor for cadmium resistance present on the X chromosome of the naturally resistant laboratory line, *v;bw*. The best documented mechanism for heavy metal adaptation in *D. melanogaster* is the duplication of the metallothionein gene [22]. A third chromosomal gene duplication of *MtnA* (formerly named *Mtn*) [22] is correlated with an increase in survival on cadmium of a wild-type strain and also a strain into which a second functional copy of *MtnA* was introduced [23]. Metallothionein-mediated sequestration [13, 24] and subsequent accumulation of cadmium in the fly midgut [25] are widely accepted as a primary response mechanism to heavy metal exposure in both flies and springtails [15, 16, 26, 27].

Metallothionein (MT) production and regulation as a general response to heavy metal exposure has been well studied and recently reviewed [5, 28]. This gene family is comprised of small, cysteine-rich proteins that facilitate binding of heavy metals [29] and mediate sequestration [19, 30]. Five genes of the MT family have been characterized in *D. melanogaster* and are found in an array on the third chromosome [22, 31, 32]. Genes in the MT family are found across the organismal spectrum, from microorganisms, including yeast and algae, to higher vertebrates, including birds and mammals [11]. Metallothioneins are conserved during evolution, as evidenced by both promoter [24] and protein [33] sequence homology.

The primary location of MT production, in the fly midgut, creates high cadmium localization in the abdominal region, as seen in the housefly, *Musca domestica* [34], the midgut and intestines of the springtail, *Orchesella cincta* [35], and midgut of *D. melanogaster* [13]. Lauverjat et al. [25] observed a greater number of midgut lysosomes, likely to contain metallothionein-bound metals, in resistant *Drosophila* strains exposed to cadmium. These data suggest metallothionein is a large and effective contributor to cadmium resistance through lysosomal sequestration in the midgut.

Metallothionein is not the only adaptation to heavy metal exposure in flies. Gill et al. [36] studied MT production and its correlation to resistance in two strains of flies: the sensitive Austin strain and the resistant *v;bw* strain. Cadmium exposure induced increased MT production in both of these strains; however, MT production increased markedly more in the sensitive Austin strain. In comparing survival with these MT levels, the resistant *v;bw* line exhibited small increases in MT corresponding with high resistance, whereas the large MT increases observed in the Austin strain had comparatively little effect on increasing survival. Even with MT levels surpassing that of the resistant *v;bw* strain at higher levels of cadmium exposure, Austin was consistently two to three times more sensitive than the *v;bw* flies. This indicates resistance does not depend exclusively or in a straightforward way on metallothionein-mediated sequestration. In studying cadmium-adapted midge larvae *(Chironomus riparius)*, Postma et al. [37] has alternatively suggested a form of resistance mediated by increased metal excretion efficiency.

One prediction of metallothionein-mediated resistance is an increase in cadmium concentrations through increased sequestration. For example, Berger and Dallinger [35] used flame atomic absorption spectrophotometry to follow both cadmium and copper in the terrestrial gastropod, *Arianta arbustorum,* after laboratory exposure. They found that cadmium levels increased in the midgut and intestines over the first ten days of exposure and then plateaued as the rates of uptake and loss reached equilibrium. No studies in *Drosophila* have suggested a specific alternative mechanism to metallothionein-mediated sequestration. One strong prediction of a metallothionein-mediated resistance is an increase in cadmium sequestration as observed by Berger and Dallinger [35] in *A. arbustorum*. In this report, we investigate *Drosophila* resistance to cadmium exposure by quantifying the heavy metal content of a wild-type strain raised on cadmium and selected for resistance over 20 generations. We suggest that an alternative mechanism, analogous to the increased excretion in the midge larvae, exists in the *Drosophila* response to cadmium exposure.

Materials and Methods

Selection for Resistance

The Berlin wild-type stock (Bloomington *Drosophila* Stock Center, IN) was used in this experiment. Magnusson and Ramel [18] found this strain to be relatively sensitive to heavy metal exposure. Flies were allowed to lay on grape juice agar plates (20 % agar and 6 % sugar in grape juice (*w*/*v*)) for 15–20 h. The eggs were then immediately counted under a light microscope, transferred onto 1 cm×2 cm filter paper, wetted with water, and placed into vials containing 5 ml instant *Drosophila* media (Carolina Biological) made with 4.5 ml of

$CdCl_2$ solution (0–0.2 mg/ml). $CdCl_2$ solutions were prepared at concentrations of 0 (control), 0.002, 0.02, 0.08, 0.1, and 0.2 mg/ml and used in the dose-response assay. At 0.08 mg/ml concentration of $CdCl_2$, 8.2 % of exposed flies survived. This provided sufficient survival and good selective pressure. This concentration was used in the selection for cadmium resistance.

The vials containing the transferred eggs were incubated at 25 °C. Emergent adults were collected and counted every 2–3 days for the duration of emergence (9 days from the first emergence) to keep maximum adult cadmium exposure at 72 h. Percent survival was calculated as the (number of adult flies)/(number of eggs transferred). Emergent adults were kept on standard molasses agar medium with no cadmium until the end of that generation's emergence (9 days). These adults were used to establish the next generation (see above) and then stored at −80 °C for mass spectrometry analysis. Flies were selected for 20 generations using this protocol. Data from generations five to six were not included in the analysis due to inconsistencies in the ways the flies were handled.

Cadmium Levels in Fly Tissue

Frozen fly samples were dehydrated and digested in an Anton Paar Multiwave 3000 Microwave Reaction System. Dehydrations were performed using the Anton Paar Drying Rotor 1DRY accessory for the Multiwave 3000 system. Digestions were run in an 8xf100 digestion rotor using 4 ml HNO_3 (TraceMetal Grade, Fisher Scientific) and 2 ml H_2O_2 (30 %, Optima Grade, Fisher Scientific) per sample as per manufacturer muscle digestion recipe. Metal content was obtained using a Perkin Elmer ELAN DRC-e inductively coupled plasma-mass spectrometer (ICP-MS) as per manufacturer instructions.

Data Analysis

The survival rates and mass spectrometry data were compared using two-tailed *t* tests between groups.

Results

The dose-response assay for $CdCl_2$ suggested that 0.08 mg/ml $CdCl_2$ gave the best balance between survival and selection (data not shown). A control line was selected on water at each generation in parallel with the cadmium-exposed line. The survival of the control line had no general increasing or decreasing trend through the 20 generations, showing a nearly horizontal trend-line slope of 0.0899. The average survival of the controls was 78.7 %. After exposure to 0.08 mg/ml $CdCl_2$, only 8.2 % of Berlin flies emerged in the first generation. The line selected for survival on $CdCl_2$ increased to 68.7 % survival at generation 20, demonstrating a significant 8.6-fold increase in survival ($p<0.01$) (Fig. 1).

Heavy metal content of the cadmium-selected flies in generations 1–20 was determined using ICP-MS. Cadmium levels in the controls raised on water remained minimal, averaging 1722.111 ppb (standard deviation=1006.894). This was distinctly lower than the cadmium resistant line, in which cadmium content was consistently over 20,000 ppb of cadmium. During 20 generations of selection on 0.08 mg/ml $CdCl_2$, the cadmium content in flies varied, but generally decreased at a rate of 1069 ppb of cadmium per generation (see trend line, Fig. 2). Flies in generation 20 exhibited a significant 1.5-fold decrease in cadmium content from the first generation of exposure ($p<0.01$).

Discussion

Mechanisms of Cadmium Resistance

Wild-type flies exposed to cadmium responded with a dosage-dependent decrease in survivorship. Previous experiments [14] had suggested that survival can be selected to establish a resistant line that can be used to investigate the heavy metal response. After 20 generations of selection, resistant flies

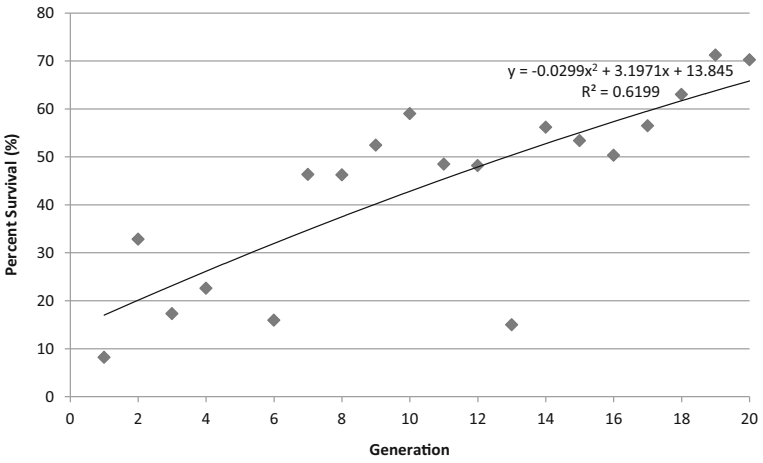

Fig. 1 Survival of flies selected for cadmium resistance on media containing 0.08 mg/ml $CdCl_2$ solution. Twenty generations of selection resulted in survival 8.6 times higher than the first-generation exposed to cadmium ($p<0.01$)

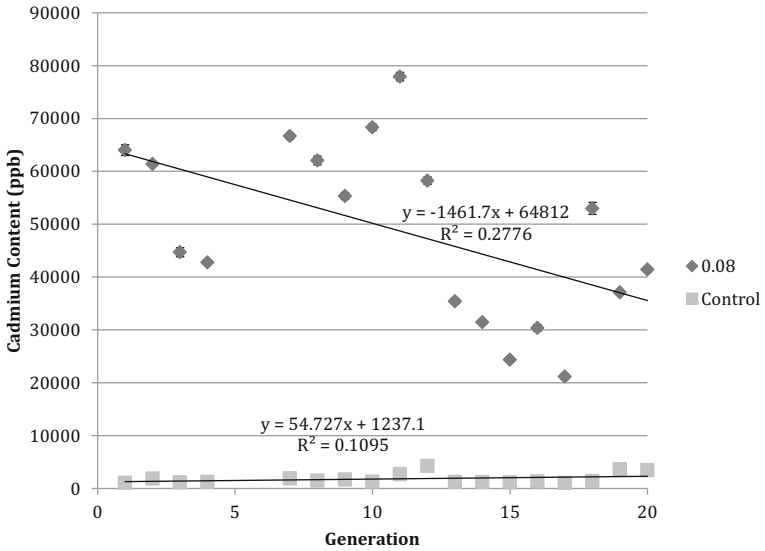

Fig. 2 Cadmium content (ppb) of flies selected for cadmium resistance on media containing 0.08 mg/ml $CdCl_2$ solution. Cadmium content generally decreased as flies were selected for resistance to cadmium. Cadmium content of flies raised on 0.08 mg/ml $CdCl_2$ decreased 1.5-fold in twenty generations ($p<0.01$)

survived 8.6 times better than unselected flies. This suggests that the wild-type population contained underlying genetic diversity that was selected by exposure and exploited to increase the genetic factors contributing to heavy metal response. This is consistent with the increase in survivorship following selection for cadmium resistance observed by Shirley and Sibly [20].

Previous work has suggested that the response to heavy metals in flies is mediated by metallothionein [13, 24] and results in increased sequestration of the heavy metal in the lysosomes of the gut [25, 34]. In this case, resistant flies should have had increased levels of heavy metals. The present experiment sought to test this prediction. Surprisingly, the cadmium content in flies raised on 0.08 mg/ml $CdCl_2$ decreased as selection continued and by generation 20 was a significant 1.5-fold lower than in the first generation ($p<0.01$). Decreasing cadmium concentration suggests an excretory mechanism, rather than an increased rate of sequestration, in response to toxicity. This would suggest cadmium resistance in this line is mediated by a factor other than metallothionein. Preliminary data from this selected line indicate that an important component of the selected cadmium resistance may be conferred by the X chromosome (data not shown). A number of authors have reported an X chromosome contributor [20, 21, 36] to cadmium resistance suggested to be either an additional metallothionein gene or a gene coding for a regulator of metallothionein, based largely on the pervasiveness of the metallothionein model for heavy metal mediation. However, the entire metallothionein gene family is now known to reside on the third chromosome in *Drosophila* [22, 31, 32], and no evidence links metallothionein regulation to the X chromosome. Other pathways mediating cadmium resistance have been described including both catalase and glutathione in *C. riparius* [38]. Next to metallothionein, glutathione is the best characterized pathway mediating heavy metal toxicity and most of the glutathione work has been done in plants (for review see [39]). In mouse heavy metal mediation is split between metallothionein and glutathione [40]. Less work has been done on the glutathione pathway in mediating heavy metals in *Drosophila*, although the X-linked glutathione synthetase gene in *Drosophila* has been shown to mediate arsenic susceptibility [41]. Work is currently underway to characterize the X-linked factor in our cadmium resistant line.

Acknowledgments Special thanks to Dr. Yong-Jun Li for his extensive help with mass spectrometry runs and Ellen Zirkelbach. This project was partially funded by Tri-Beta National Biological Honor Society, Loyola Marymount University (LMU) Molecular Analytical Nano Energy (MANE) grant and Air Force Office of Scientific Research (AFOSR) internal funding (award #0011), LMU Undergraduate Research Opportunity Program, LMU Honors Program, and LMU Department of Biology.

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