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Hormesis and stage specific toxicity induced by cadmium in an insect model, the queen blowfly, *Phormia regina* Meig.

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Abstract

Hormesis is an adaptive response, commonly characterized by a biphasic dose–response that can be either directly induced, or the result of compensatory biological processes following an initial disruption in homeostasis [Calabrese and Baldwin, Hum. Exp. Toxicol., 21 (2002), 91]. Low and environmentally relevant levels of dietary cadmium significantly enhanced the pupation rate of blowfly larvae, while higher doses inhibited pupation success. However, dietary cadmium at all exposure levels adversely affected the emergence of the adult fly from the pupal case. Such findings represent the first report of a heavy metal displaying a hormetic-like biphasic response for pupation success, while at the same time displaying stage-specific toxicity at a later developmental period. These conclusions are based on substantial experimentation of over 1750 blowflies, in

seven replicate experiments, involving 10 concentrations per experiment. These findings indicate the need to assess the impact of environmental stressors over a broad range of potential exposures as well as throughout the entire life cycle.

Abstract

This is the first report of a heavy metal displaying a hormetic-like biphasic response for early developmental success, while at the same time displaying stage-specific toxicity at a later developmental stage.

Author Keywords: Hormesis; Stage-specific; Dose–response; Cadmium; Blowfly

1. Introduction

Hormesis, a dose–response phenomenon characterized as a low dose stimulation, high dose inhibition, is an adaptive dose–response relationship that occurs as the result of a compensatory mechanism following a disruption in homeostasis (Calabrese and Baldwin, 2002). The phenomenon of hormesis is widely reported in the scientific literature and is broadly generalizable according to chemical class, biological model, and endpoint (Calabrese and Baldwin, 1997a). With respect to metazoan model organisms, insects are the most populous class, and serve as valuable models on which to evaluate the physiological effects of terrestrial and aquatic metal contamination (Hare, 1992). Within class Insecta, chemical hormesis has been principally studied with respect to reproductive endpoints. Cadmium specifically induces hormetic responses in a number of organisms (Calabrese and Baldwin, 1999), and is widely recognized as an environmental contaminant (ATSDR, 1999). Given the extensive research on insect models, it is of interest to determine if low-level exposure to this non-essential metal may enhance survivorship, and overall developmental fitness using the queen blowfly (*Phormia regina*), a model on which considerable data exist on developmental and aging processes in multiple physiological systems (Yin et al., 1994).

Although the initial goal of this research was to evaluate the hypothesis that a non-essential metal may induce hormesis, the goal was soon expanded following the unexpected observation of cadmium-induced arrested development in the transition of pupae to adult. This stage specific toxicity was initially observed in flies exposed to high levels (10–160 ppm) of both cadmium chloride and lead acetate during preliminary dose ranging studies. The metal exposed flies appeared to develop normally through the instar stages within the larval diet. The larvae successfully eclosed as pupae and developed a hard, normal appearing (in size and color) pupal case. However, flies did not emerge from the pupal case. Upon evisceration of the pupal case, an apparently fully developed, tanned, adult fly was observed. Similar results were observed in flies exposed to lower doses of cadmium chloride (2 ppm to 2×10^{-2} ppm), and are further discussed in the following sections.

2. Materials and methods

Larvae were selected from a breeding colony of queen blowflies, *Phormia regina*, from the Department of Entomology, at the University of Massachusetts Amherst. This breeding colony was maintained as previously described by Stoffolano (1974).

For all experiments a standard stock laboratory diet was prepared (Stoffolano, 1974). From this stock 50 ml (50 g) of diet per treatment group was removed using a ladle sampling device (Fisher brand cat no. 14-242-20) and placed in plastic petri-dishes (Fisherbrand clear dishes, cat. no. 08-757-12).

Background levels of cadmium in the larval diet were determined by inductively coupled argon plasma–atomic emission spectrophotometer (Spectro-Flame, argon plasma ICP–AES) following an acid digestion. Included in the ICP-analysis were seven total samples, five digested food samples, one sample of 20 ml of H_2O (water control), and one 20 ml sample of 50% nitric acid [H_2O:$HN0_3$, 1/1, v/v] (acid control). The analysis of the water and acid control samples was negative

for cadmium (<0.0002 ppm). The value 0.0002 ppm was therefore used as a background level of cadmium in the larval diet.

Cadmium chloride (Fisher, lot no. 733629) was added to each food sample from serial dilutions contained in glass test tubes, using double de-ionized water as a solvent. The $CdCl_2$ dilutions were transferred to each food sample by means of a calibrated automatic pipette in 1 ml amounts. This dilution scheme, combined with the 50 g diet, produced 10 dietary treatments, ranging in nominal concentration from 200.002 ppm to 2×10^{-5} ppm, of $CdCl_2$. Concurrent vehicle controls received 1 ml of double de-ionized water (Sham). The treated diet was then allowed to cool and solidify at room temperature. All diets were stored for 48 h in a growth chamber (in a controlled environment of 28±2 °C, and 70% r.h. on a 16:8 light:dark photoregime) before the introduction of larvae.

The larvae used in each experiment were 48-h-old larvae reared on non-contaminated diet. The larvae were originally selected from beef liver that was placed in the breeding colony for a period of 1 h. Sexually mature female flies were allowed to oviposit on the liver. At the end of 1 h, clusters of eggs (approximately 500) were collected from the beef liver with a small metal spatula, then washed and separated with de-ionized water in a glass petri-dish. Groups of the separated eggs were then transferred with a disposable glass pipette onto non-contaminated larval diet contained in a 250-ml container. After 48 h in the non-contaminated diet, larvae were removed from the media, washed with de-ionized water, and placed into petri-dishes containing 50 g of diet and their respective treatment scheme (cadmium chloride treatment or control). The petri-dish was then either covered (experiments 4–7) or not (experiments 1–3) and placed in individual 22 oz containers (Sage Products, inc. Reorder no. 2400). The cover was removed on day 7 (in experiments 4–7) and mature larvae were allowed to crawl out of the dish into a thin layer of sand (“play sand”) under the petri-dish. In experiments 1–3, all groups were maintained in the previously described fly rearing room (Stoffolano, 1974). In experiments 4–7, all groups

were maintained in a Pervical Growth Chamber (28±2 °C, and 70% r.h. on a 16:8 light:dark photo regime).

On day 14, each group's mature pupae were sifted from the sand bed and the number of pupae recorded. The pupae were then placed back in the 22 oz containers and returned to their respective rearing location until emergence. Following emergence the number of adult flies was recorded.

3. Statistical evaluation

Statistical tests to determine if a model system displays a hormetic response are not well defined (Callahan et al., 2001 and Deng et al., 2001). Therefore, several statistical tests and a previously described algorithm were integrated and used in an evaluation of the consistency of the hormetic hypothesis to explain the experimental observations.

The data were initially evaluated using a chi-square analysis to determine if the observed stimulation of survival parameters of larvae receiving a cadmium treatment differed significantly from the non-exposed control.

Deng et al. (2001) have proposed that the experimental data should be modeled to confirm the existence of hormesis. Using the procedure outlined by Deng et al. (2001), data on the combined pupation success of cadmium-fed flies were modeled. The data were fit to a regression model and the adequacy of a linear regression line, as well as a polynomial regression line, was evaluated.

The individual experiments were then evaluated to determine if the biphasic dose–response curves were consistent with the hormesis hypothesis as defined by the Hormesis Scoring System (Calabrese et al., 1999).

To evaluate if the differential mortality of developmental stages within the same organism was consistent with the stage specific susceptibility of blowfly larvae to cadmium, pupae–adult mortality data were compared to larvae–pupae mortality

data. Using a chi-square analysis, the odds of an adult to emerge (relative to the number of pupae that failed to emerge) was compared to the odds of larvae to pupate (relative to the number of larvae that failed to pupate).

4. Results

In general, there was an enhancement in pupation success at low concentrations of cadmium and a reduction of success at higher concentrations (Fig. 1). This conclusion is supported by a three-tiered evaluation consisting of (1) chi-square statistical analysis of the pupae survival data, (2) statistical modeling of the dose–response curves (Deng et al., 2001) and (3) the application of an algorithm and ranking scheme to assess hormesis (Calabrese et al., 1999).

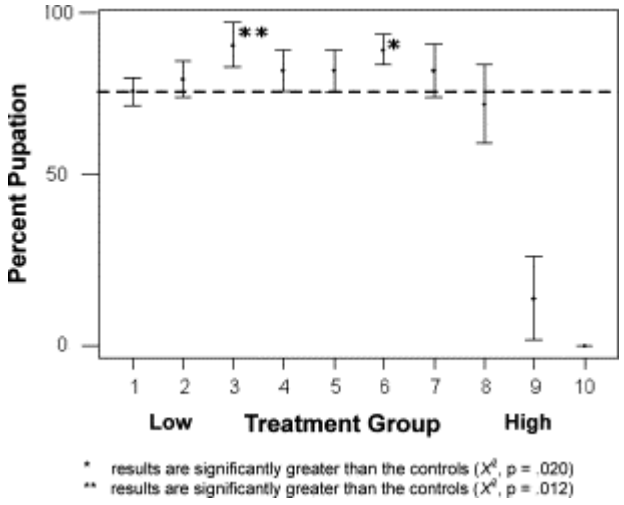

Fig. 1. Mean pupation success of cadmium-fed larvae from all 7 experiments. Results from rearing 1750 two-day old *P. regina* larvae on a cadmium contaminated diet. The dashed line indicates the mean value of the control (No. 1 on the *x*-axis). The *x*-axis is the treatment group corresponding to the concentration of cadmium in the larval diet (see Table 1). The *y*-axis corresponds to the mean percentage of originally introduced 2-day old larvae to pupate. The interval bars shown represent the standard error of the mean.

When the data from seven experiments were combined, six treatments containing cadmium-fed larvae had a mean pupation value greater than that of the mean pupation of the controls (Table 1). Two of the treatment groups (3 and 6) yielded a significant increase (P <0.05) in pupation success of cadmium-fed larvae compared with the concurrent vehicle controls.

Table 1. Developmental success of cadmium-fed larvae

Treatment group	Cadmium conc. of larval diet (ppm)	(a) mean% pupation (S.E.M.)	(b) mean% emergence (S.E.M.)	(c) Pupae deaths (% of total larvae)	(d) Stage specific deaths (% of total pupae)
10	200.0002	0.0 (0)	0 (0)	100	0
9	20.0002	13.9 (12.3)	0.0 (0)	86	100
8	2.0002	70.9 (11.4)	20.8 (14.9)	29	69†
7	0.2002	80.7 (7.8)*	54.5 (12.2)	19	44
6	0.0202	86.7 (4.4)**	57.8 (17.0)	13	45†
5	0.0022	80.5 (6.2)*	77.0 (9.5)	19	17
4	0.0004	80.7 (6.0)*	55.3 (15.2)	19	45†
3	0.00022	88.1 (6.5)***	67.7 (11.2)	12	25†
2	0.0002	78.1 (5.3)*	64.7 (14.4)	22	36†
1 (Control)	> 0.0002	74.4 (4.2)	79.2 (9.5)	26	16

Column (a) is the mean percent pupation of seven experiments, with the standard error of the mean percent pupation data in parenthesis. Column (b) is the mean percent of adults to emerge from pupae from a combination of six experiments, with the standard error of the mean emergence data in parenthesis. Column (c) is the percent of larvae that failed to successfully reach the pupa stage. Shown in column (d) is the percent of pupae whose development was arrested as a result of cadmium and did not emerge as adults.*Greater than the control. **Significantly greater than the controls (×2, P=0.020). ***Significantly greater than the controls (×2, P=0.012). †Statistically significant (P <0.001, ×2).

When the pupae survival data were fit to a regression model, the combined data was best explained by a quadratic model (P <0.0001), which is indicative of a hormetic dose–response relationship (Deng et al., 2001).

Each of the seven experiments, as well as the combined results, were determined to be consistent with the hormesis hypothesis when evaluated

relative to parameters described by Calabrese et al (1999). The individual scores ranged from Low–Moderate, to High evidence of hormesis using this established ranking system.

The adult emergence from the pupal stage was generally lower among cadmium-fed larvae when compared to the rate of transition from larvae to pupae (Table 1). This stage specific toxicity was concentration-dependent and more evident between treatments groups 6–9 (0.02 – 20.0 ppm) (Fig. 2 and Fig. 3). The lower concentrations used in this study reveal a generally linear response of 35% of the pupae failing to eclose as adults, while the higher concentrations appear to be associated with a sharp increase in mortality.

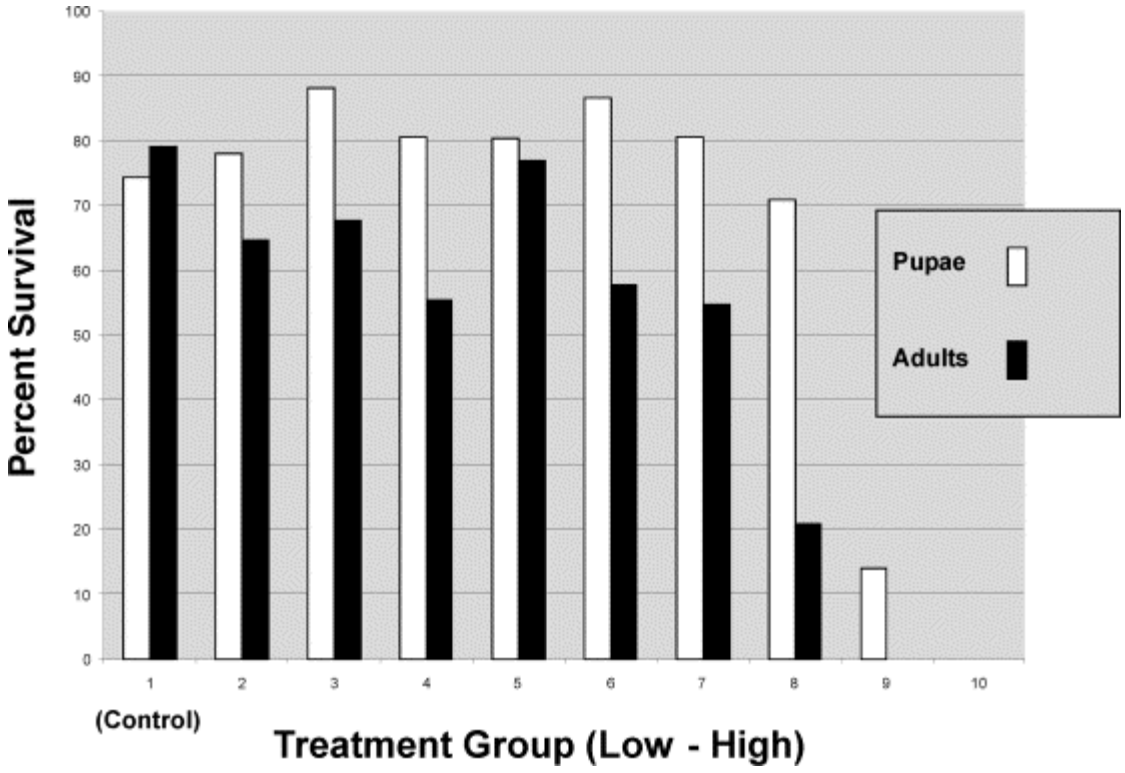

Fig. 2. The effect of cadmium on pupae–adult development. Shown is the number of pupae (white column) and adults (black column) vs. their respective treatment group (corresponding to the concentration of cadmium in the larval

diet). The region of the white column that extends beyond the black column is where the immature development of the cadmium-fed larvae has been arrested.

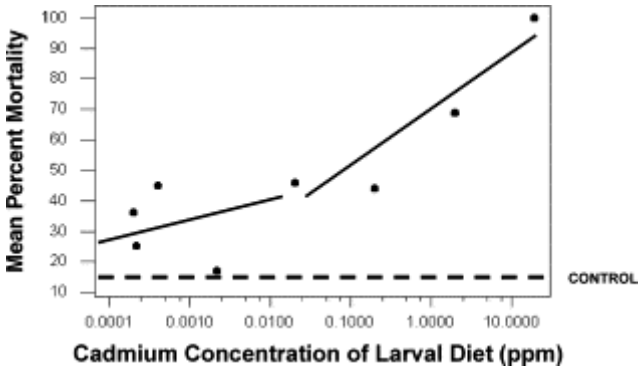

Fig. 3. Dose–response trends in the pupae stage specific mortality data. The data from column (c) on Table 1 has been plotted; it yields two unique vectors within the dose–response continuum.

5. Discussion

The present findings represent the first report in an animal model of a hormetic response in one developmental stage (pupation), while the subsequent developmental transition (adult emergence) displays a stage specific toxicity. In addition, cadmium-induced pupation success and the associated dose–response relationship being consistent with the hormetic hypothesis are novel findings for any heavy metal.

Given the limited magnitude of the stimulatory response (i.e. usually 30–60% greater than controls at maximum), a stringent study design and replication requirement is necessary to ensure reliable judgments on causality (Calabrese and Baldwin, 2002). These experiments were replicated seven times to assess whether the low-dose stimulatory response was due to random variation, and not necessarily an example of hormesis. Although the overall experimental conditions evolved improvements in the precision of temperature/relative humidity regulation, in addition to slight changes in sample size, the collective findings provide consistent support for the hormesis hypothesis. These findings have an additional advantage of providing an estimate of variability for the two major developmental processes (pupation and adult emergence) studied, as well as the variability with respect to hormetic dose–responses.

The composite (i.e. combination of the seven individual experiments) dose–response displays a clear example of a hormetic response (Fig. 1). However, when the seven experimentally derived dose–responses were initially subjected to the Calabrese & Baldwin, 1997a and Calabrese & Baldwin, 1997b methodology for consistency to the hormetic/β-curve, one scored “High” (No. 7), one scored “Moderate” (No. 3) and the remainder scored “Low–Moderate” (Nos. 1–2, and Nos. 4–6). These differences in scoring were not the result of any differences in experimental design, but rather in the critical nature of the low dose stimulation. While the overall response was clearly supportive of the cadmium-induced hormetic response for pupation success, the inherent variability in the larval developmental success reflects the formidable challenge that replication of an hormetic response within an animal system represents.

The causes of widespread variability within hormetic responses are unknown. In an effort to estimate the background variability within this model system and to address how it may be affected by the larvae to food ratio in the absence of cadmium, a range of food was offered to the developing larvae. A similar variability in pupation was also observed under highly controlled environmental conditions in the absence of cadmium (data not shown), as was observed during

the cadmium exposure experiments. While additional research under strictly regulated experimental conditions is warranted, the present data suggest relatively high background variability within this model system.

The mechanisms by which low doses of cadmium may enhance pupation success are unknown. A possible interpretation such as enhanced food consumption by sub-optimal dietary conditions remains to be explored (Simkiss et al., 1993). However, this phenomenon is an unlikely explanation for the experiments described herein, given that sub-optimal dietary conditions are reported to accelerate the rate of development, and not the overall success of pupation as reported here. Experimental investigations revealing mechanisms involved in cadmium detoxification within terrestrial invertebrates have been previously reviewed (Dallinger, 1996). Although data were not collected on the cellular and molecular fate of metals within the insect model used in this study, it is reasonable to expect similar conserved detoxification mechanisms upon exposure to cadmium. Perhaps, the most widely reported metal detoxification mechanism within insect populations is the production of a cadmium binding protein, with properties characteristic to that of metallothionein (Akoi et al., 1984). While the induction of this protein is common to a number of insects, its induction and role appears to differ widely within dipteran insect species (Akoi et al., 1984). Laboratory results suggest that gene expression of this iso-metallothionein, which confers resistance to the toxic effects of cadmium, is dependent upon the stage of larval development (Akoi and Suzuki, 1984). This may be a profitable area of follow-up research to explore the mechanistic background of the stage specific effects observed in the experiments presented here.

Stage specific toxicity observed in these experiments are consistent with reports in the mosquito (*Aedes aegypti*) (Rayms-Keller et al., 1998) (*Aedes albopictus*) (Bellini et al., 1988), fruit fly (*Drosophila melonogaster*) (Lynch et al., 1991), housefly (*Musca domestica*) (Raina et al., 2001), and chironomids (Timmermans et al., 1992 and Wentsel et al., 1978). The stage-specific effects in these blowfly experiments are in general agreement with the previously reported studies.

However, the aforementioned studies failed to explore as broad of a dose range as the one employed herein. This broad dose range, coupled with multiple endpoints (pupation and emergence) may account for the convergence of two unique phenomena within the same experimental population.

These results have the potential for broad based implications in both toxicology and risk assessment. Classic studies in these disciplines often explore only single static endpoints, and not a continuum of multiple developmental endpoints as seen here. This study is novel in that it demonstrates that an early developmental hormetic response (in pupation) may be associated with an increased risk of death (adult emergence).

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