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Do copper tolerant fathead minnows produce copper tolerant adult offspring?

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Abstract

The objective of this study was to determine if the relative Cu tolerance of fathead minnow parents determines the, relative Cu tolerance of their adult offspring. It was hypothesized that the adult offspring of Cu-tolerant minnows would inherit Cu tolerance from their parents. The relative Cu tolerance of 96 adult fish was determined based upon their reduction in swim performance following a sublethal exposure to 150 µg Cu/l. Control, Cu-tolerant and Cu-susceptible lines of fish were produced and fish within each line were allowed to breed. The offspring were raised to adults, then exposed to one of two sublethal Cu concentrations (150 or 225 µg Cu/l) for 8 days. There were no significant differences in relative Cu tolerance, as measured by reduction in swim performance, among the three lines of fish at either dose. However, significant differences in whole body Na⁺ occurred among the fish lines after exposure to 150 µg Cu/l, but not after exposure to 225 µg Cu/l. Significant differences in whole body Cu occurred between Cu-tolerant and Cu-susceptible fish lines after exposure to either Cu dose. The offspring did not inherit the relative Cu tolerance of their parents, however, the selection lines had diverged from each other, particularly with respect to their whole body Cu concentrations after exposure.

Keywords

Copper; Fish; Tolerance; Artificial selection; Whole body Na⁺; Whole body Cu

1. Introduction

The ability of an individual fathead minnow to tolerate an exposure to copper (Cu) is, to some degree, genetic. For example, allozymic variation in polymorphic enzyme loci has been correlated with survival time in fathead minnows exposed to Cu for 96 h (Schlueter et al., 1995, 1997). More recently, relative Cu tolerance of fathead minnows (as determined from reduction in swim performance following a sublethal exposure to Cu) has also been found to correlate with allozymic variation in polymorphic enzyme loci (Kolok et al., 2004).

The results of the correlational studies mentioned above suggest that Cu tolerance is heritable, however, a recent study has found that the relative Cu tolerance of adult fathead minnows does not predict the Cu tolerance of their larval offspring. In that study (Kolok et al., 2004), the relative Cu tolerance of 96 adult fathead minnows was determined following an 8–9 days exposure to Cu. The most Cu-tolerant individuals were bred together, as were the most Cu-susceptible individuals. Some of the resultant larvae were then used in standard larval survivability tests, and median lethal concentrations (LC50s) were produced. The resulting

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LC50 of the larvae produced by Cu-tolerant adults was not significantly different from that of larvae produced by Cu-susceptible adults.

The current study compares the relative Cu tolerance of the Cu-tolerant and Cu-susceptible progeny produced in Kolok et al. (2004), after the progeny have grown to adults. The primary objective of this study was to determine whether the relative Cu tolerance of the adult progeny was influenced by the relative Cu tolerance of their parents. It was hypothesized that the adult offspring of Cu-tolerant minnows would inherit Cu tolerance from their parents.

While the parents of the fish used in this study were chosen for breeding based upon their relative Cu tolerance, other metrics may be more sensitive at elucidating differences among fish from the selection lines. For example, whole body Na^+ has been used as a biomarker of effect in fathead minnows exposed to a variety of ionoregulatory toxicants (Grippio and Dunson, 1991; Croke and McDonald, 2002), including exposure to Cu. Similarly, metal body burden can also demonstrate previous exposure to metals (Birge et al., 2000). Thus, a second objective of this study was to determine whether significant differences existed in whole body Na^+ and whole body Cu concentrations of adult progeny produced by parents with different tolerances for Cu.

2. Materials and methods

2.1. Fish maintenance

Male fathead minnows used in this study were produced by the fathead minnow colony maintained at the University of Nebraska at Omaha (Omaha, NE, USA). Three different lines were used to produce these fish. The parents used to produce the control offspring were randomly obtained from our breeding colony. The offspring of the control parents hatched between 1 and 7 June 2001. The Cu-tolerant and Cu-susceptible groups of offspring were the progeny of parents considered to be tolerant or susceptible based upon the reduction in their swim performance following an 8–9 days exposure to Cu. Details of the breeding program have been presented previously (Kolok et al., 2004). The Cu-tolerant progeny hatched from 8 to 16 July 2001, while the Cu-susceptible progeny hatched from 20 to 24 July.

The progeny from the three lines were raised for their first month at 26 °C and fed live *Anemia nauplii* twice per day. After the fish were 1 month old, the water temperature was gradually lowered to room temperature (20 °C) and the fish were switched to a diet of high-protein flake food (Aquatic Ecosystems, Apopka, FL, USA). Fish were maintained in dechlorinated Omaha tap water: alkalinity = 107 mg/l as CaCO_3 ; hardness = 215 mg/l as CaCO_3 ; pH = 8.22; conductivity = 730 μS .

2.2. Pre-exposure and post-exposure U_{cri} and the Cu exposures

In experiment 1, 27 male fish from the three lines (10 resistant, 9 susceptible and 8 unselected) were subjected to a swim performance, exposed to 150 $\mu\text{g/l}$ Cu for 8 days, then immediately challenged with a second bout of swimming. None of these fish died during the experimental regime.

In experiment 2, 28 male fish from the three lines (10 resistant, 10 susceptible and 8 unselected) were subjected to a swim performance, exposed to 225 $\mu\text{g/l}$ Cu for 8 days, then immediately challenged with a second bout of swimming. Two of the males from the susceptible line died during the 8 day exposure.

Immediately prior to the first battery of swim performances conducted during experiment 1, six unexposed fish were sacrificed and stored at – 80 °C. The whole body Na^+ and Cu

concentrations ($\mu\text{mol/g}$) from these fish provided a baseline for comparison with the Cu-exposed fish.

The swim performances were determined using two open system swim chambers, according to previously published protocols (Kolok et al., 1998, 2002, 2004). Critical swimming speeds (U_{crit}) were determined using two open system swim chambers maintained in a large 4001 trough. To initiate the swim performance, a fish was placed into the chamber and given approximately 1.5 h to habituate to a water velocity of 15 cm/s. After the habituation period, the fish was forced to swim against a water velocity that increased 5 cm/s every 20min until the fish fatigued. Fatigue was determined when the fish could no longer be persuaded to swim off the flow straighteners located at the end of the chamber.

Two swim performances were conducted on each fish, one prior to Cu exposure (pre-exposure U_{crit}) and the second immediately after the Cu exposure (post-exposure U_{crit}). The pre-exposure U_{crit} was conducted over a 5-day period, with six fish generally processed per day. Immediately, following the pre-exposure U_{crit} , the fish were lightly anesthetized by immersion in a buffered (pH = 7.2), sublethal solution of 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222). The fish were then weighed and fin clipped for identification of individuals. Previous studies have found that the paired pelvic fins and the single anal fin could be clipped from the fish without influencing U_{crit} (Kolok et al., 1998). The fish resumed their normal behaviors once returned to the recovery aquaria.

Fish were given at least 5 days to recover from the pre-exposure U_{crit} before they were exposed to Cu in a 1001 aquarium, according to a previously designed protocol (Kolok et al., 2002, 2004). Static renewal of one-third of the exposure water was conducted daily with laboratory stock water, and the water was appropriately respiked to maintain the desired Cu concentration. The exposure aquarium was subdivided into five compartments, with the use of standard plastic aquarium dividers. Water could freely move from one compartment to the next, but the fish could not.

The fish were sequentially introduced into the exposure aquaria (over a 5-day period) so that each group of fish was exposed for 8 days before being removed. On the morning of the eighth day of exposure the post-exposure U_{crit} of the fish was determined. Percent reduction in U_{crit} was calculated as: $100(\text{pre-exposure } U_{\text{crit}} - \text{post-exposure } U_{\text{crit}})/\text{pre-exposure } U_{\text{crit}}$.

Water samples (125 ml) were collected once per day during the Cu exposure. Immediately, after being collected, the samples were fixed with nitric acid to a pH of <2.0, and stored below 4 °C prior to analysis, Cu analysis was performed by flame atomic absorption spectrometry according to previously established methods (US Environmental Protection Agency, 1983).

2.3. Whole body Na^+ and Cu

Immediately, following the post-exposure U_{crit} , the fish were sacrificed, measured for fork length (± 1 mm) and body mass (± 1 mg), then frozen whole at -80 °C for subsequent analysis. Whole body Na^+ and whole body Cu was determined for all of the exposed fish that survived the two experiments ($n = 53$) and for the six unexposed fish. Fathead minnows were digested in a mixture of concentrated nitric acid, sulfuric acid and hydrochloric acid (Grippo and Dunson, 1991). These solutions were then diluted with deionized water to a standard volume, then analyzed for whole body Na^+ and Cu with a flame atomic absorption spectrometer.

2.4. Statistical analysis

Data throughout this study are reported as means ± 1 S.E.M., and the minimum significance level set at $P < 0.05$. Body mass and whole body ion data were compared among the three fish lines with ANOVA followed by Neuman-Keuls multiple comparison tests. Percent reduction

in U_{crit} was compared among the fish lines with parametric (ANOVA) and non-parametric tests (Kruskal–Wallis ANOVA). The results for these comparisons were the same regardless of the statistical method employed.

2.5. Size adjustment

At the beginning of experiment 1 (22 January 2002) the fish were between six months and eight and a half months old, with the control fish being the oldest and largest. Males from the control line (mean body mass, $4.3\text{g} \pm 0.2$) were significantly larger ($P < 0.05$) than the males from the Cu-tolerant (mean body mass, $3.6\text{g} \pm 0.1$) and Cu-susceptible ($3.0\text{g} \pm 0.1$) lines. Body mass, however, was not significantly correlated with any of the other traits measured in this study (linear regression, $P > 0.05$ in all cases), therefore no attempts were made to size adjust the measured traits.

At the beginning of experiment 2 (26 February 2002), the males selected for analysis from the control line (mean body mass, $5.6\text{g} \pm 0.3$) were significantly larger ($P < 0.05$) than the males from the Cu-tolerant (mean body mass, $3.3\text{g} \pm 0.1$) and Cu-susceptible ($3.9\text{g} \pm 0.1$) lines. Variation in body mass was significantly correlated with variation in whole body Na^+ ($r^2 = 0.37$, $P = 0.024$), but not whole body Cu ($r^2 = 0.04$, $P = 0.55$) nor percent reduction in U_{crit} ($r^2 = 0.0025$, $P = 0.79$). Whole body Na^+ was size adjusted by using the slope of the body mass/whole body Na^+ relationship, as per Packard and Boardman (1987) then adjusting all of the measurements to the mean body mass of all 28 fish (i.e. 4.2 g). Size adjusted values for whole body Na^+ of the fish exposed to 225 μg Cu/l are featured throughout the rest of the paper.

3. Results

In experiment 1, the fish were exposed to a mean Cu concentration of $151.1\text{ }\mu\text{g Cu/l} \pm 1.7$. In experiment 2, the fish were exposed to a mean Cu concentration of $224.5\text{ }\mu\text{g Cu/l} \pm 1.9$. Concentrations of Cu were consistent throughout each exposure.

After being exposed to 150 μg Cu/l in experiment 1, percent reduction in U_{crit} was not significantly different among the fish lines ($P > 0.05$, Fig. 1A). Similarly, after being exposed to 225 μg Cu/l in experiment 2, percent reduction in U_{crit} was not significantly different among the fish lines ($P > 0.05$, Fig. 2A).

When exposed to 150 μg Cu/l, significant differences occurred in whole body Na^+ ($\mu\text{mol/g}$) among the fish from the three selection lines ($P < 0.05$, Fig. 1B). The mean whole body Na^+ concentration of the minnows produced by Cu-tolerant parents was significantly greater than the mean whole body Na^+ concentration of the fish produced by the control fish, but not the Cu-susceptible fish ($P < 0.05$). If the unexposed group (dashed line in Fig. 2B) is added to the analysis, the results are essentially the same. Significant differences occurred in whole body Na^+ among the fish from the three selection lines and the unexposed fish ($P < 0.05$). The mean whole body Na^+ concentrations of the unexposed minnows and the minnows produced by the Cu-tolerant parents were significantly greater than the mean whole body Na^+ concentration of the fish produced by the control (unselected) fish, but not different from that of minnows produced by the Cu-susceptible fish ($P < 0.05$).

Whole body Cu was also affected by the relative Cu tolerance of the minnows parents ($P < 0.05$, Fig. 1C). The mean whole body Cu concentration of the minnows produced by Cu-tolerant parents was significantly greater than the minnows produced by either the controls or of the Cu-susceptible parents ($P < 0.05$).

When exposed to 225 $\mu\text{g/l}$ Cu, there were no significant differences in whole body Na^+ among the fish from the three selection lines ($P>0.05$, Fig. 2B). If the unexposed group (dashed line in Fig. 2B) is added to the analysis, the results are slightly different. Significant differences occurred in whole body Na^+ among the fish from the three selection lines and the unexposed fish ($P<0.05$). In this case, the mean whole body Na^+ concentration of the minnows produced by the unexposed fish was significantly greater than the mean whole body Na^+ concentrations of the fish in all of the other lines ($P<0.05$).

There were no significant differences in whole body Cu among the fish from the three selection lines ($P>0.05$), although these data may have been inappropriately biased by data from one individual. One control fish had a whole body Cu concentration (0.232 $\mu\text{mol/g}$) that was more than three times higher than the next highest whole body Cu concentration (0.07 $\mu\text{mol/g}$) of any other fish in experiment 2. If data from this fish is excluded from analysis, there is a significant difference among the three groups of fish in whole body Cu concentration ($P<0.05$, Fig. 2C). The whole body Cu concentration of the Cu-tolerant fish was significantly greater than that of the Cu-susceptible fish ($P<0.05$). A significant difference between the Cu-tolerant fish and the Cu-susceptible fish is also evident if these two lines of fish are compared directly to each other (t -test, $P<0.05$), without considering the fish from the control line.

4. Discussion

The objective of this study was to determine whether the relative Cu tolerance of parent fathead minnows influenced the relative Cu tolerance of their adult offspring (as determined by post-exposure reductions in swim performance). Results from this experiment do not convincingly support this hypothesis. After one generation of selection, there were no significant differences in the relative Cu tolerance of the fish.

While the selection regime did not generate lines of fathead minnows with different levels of Cu tolerance, there were significant differences in whole body Na^+ between the Cu-tolerant and control fish exposed to 150 $\mu\text{g Cu/l}$. Cu is known to be toxic to fish by causing impaired branchial ionoregulation (Lauren and McDonald, 1985), specifically by impairing Na^+/K^+ activated adenosine triphosphatase (ATPase) activity (Stagg and Shuttleworth, 1982; Lauren and McDonald, 1987; Pelgrom et al., 1995; Li et al., 1998). If the exposure to Cu is chronic, impaired Na^+ ionoregulation can lead to significant reductions in whole body Na^+ (Grippio and Dunson, 1991; Croke and McDonald, 2002). For male fathead minnows, it has also been previously demonstrated (Kolok et al., 2002) that significant correlations exist between whole body Na^+ levels and relative Cu tolerance (as determined from post-exposure reduction in U_{crit}). In this study, selection for relative Cu tolerance produced adult offspring that were significantly better at maintaining whole body Na^+ when exposed to 150 $\mu\text{g/l}$ Cu than were fish from the control line.

When exposed to 225 $\mu\text{g Cu/l}$, differences in the whole body Na^+ of the fish from the three lines were no longer evident. This suggests that none of the fish were able to compensate for the effects of Cu on their ability to maintain whole body Na^+ at this concentration. McDonald and Wood (1993) have hypothesized that acclimation to metals occurs in an exposure 'window for acclimation'. Concentrations in excess of the upper boundary of the window would be expected to cause a decline in metal tolerance, or ultimately in death. Our results on whole body Na^+ are consistent with this model. The upper boundary of the window for acclimation was probably less than 150 $\mu\text{g/l}$ Cu for the fish produced by the control and Cu-susceptible adults, since these fish were not able to maintain normal levels of whole body sodium at either exposure. In contrast, the upper boundary of this window was greater than 150 $\mu\text{g/l}$, but less than 225 $\mu\text{g/l}$, for the Cu-tolerant fish, since they maintained near-normal levels of whole body Na^+ at 150, but not 225 $\mu\text{g/l}$ Cu (Figs. 1B and 2B). Artificial selection appears to have changed

the upper limit of the window for acclimation in the Cu-tolerant fish. Additional generations of selection will be necessary to determine the extent to which this upper limit can be further elevated.

The minnows produced by Cu-tolerant parents also accumulated significantly greater whole body Cu concentrations than fish from the Cu-susceptible line (Figs. 1C and 2C). While greater body burdens of metals are often associated with more pronounced toxic effects, this does not appear to be the case for fathead minnows and Cu. In fact, the highest whole body Cu concentration was found in fish produced by Cu-tolerant parents. Previous findings support the hypothesis that the most Cu-tolerant fathead minnows are those that experience the greatest whole body Cu concentrations. Whole body Cu concentration was found to be positively and significantly correlated with Cu tolerance (as determined from post-exposure reduction in U_{crit}) in sublethally exposed male fathead minnows (Kolok et al, 2002).

Recent research efforts have tested the sensitivity of different metrics that can be used as bioindicators of metal exposure. In general, the more sensitive bioindicators are those that are either related to Na^+ ionoregulation (whole body Na^+ , net Na^+ flux, Na^+ uptake), or to gill Cu-binding characteristics (Croke and McDonald, 2002; Taylor et al., 2000). Less sensitive bioindicators are whole body performances such as growth and swim performance (Taylor et al., 2000). Results from this selection experiment are consistent with the published literature in that significant differences among the selected lines occur in whole body Na^+ and Cu, but do not occur in the swim performance metric. Thus, the selection regime has been successful in altering the physiology of the Cu-tolerant fish relative to the other two lines. The lack of any significant difference in Cu tolerance between the lines may be primarily due to the relative insensitivity of the swim performance metric that is being used to ascertain relative Cu tolerance. Additional generations of selection may lead to significant differences in relative Cu tolerance.

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Fig. 1. The mean (±S.E.) percent reduction in swim speed (A), whole body Na⁺ (B) and whole body Cu (C) concentrations for adult fathead minnows from three different breeding lines exposed to 150 μg Cu/l for 8 days. Sample sizes were: Cu-tolerant 10, unselected 8, Cu-susceptible 9. Different numbers denote significant differences (ANOVA, Neuman-Keuls multiple comparison tests) in the metrics among the three groups. The dashed lines represent the mean whole body Na⁺ or whole body Cu concentration in unexposed minnows.

Fig. 2.

The mean ($\pm$ S.E.) percent reduction in swim speed (A), whole body Na^+ (B) and whole body Cu (C) concentrations for adult fathead minnows from three different breeding lines exposed to 225 μg Cu/l for 8 days. Sample sizes were: Cu-tolerant 10, unselected 8, Cu-susceptible 8. Different numbers denote significant differences (ANOVA, Neuman Keuls multiple comparison tests, $P < 0.05$) in the metrics among the three groups. The dashed lines represent the mean whole body Na^+ or whole body Cu concentration in unexposed minnows.