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Familial differences in the effects of mercury on reproduction in zebra finches

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ABSTRACT

Ecotoxicologists often implicitly assume that populations are homogenous entities in which all individuals have similar responses to a contaminant. However, genetically variable responses occur within populations. This variation can be visualized using dose–response curves of genetically related groups, similar to the way that evolutionary biologists construct reaction norms. We assessed the variation in reproductive success of full-sibling families of captive zebra finches (*Taeniopygia guttata*) experimentally exposed to methylmercury. We found significant variation among families in the effects of methylmercury on several reproductive parameters. This variation suggests that there may be strong responses to selection for resistant genotypes in contaminated areas. This has important implications for the evolution of tolerance as well as risk assessment and wildlife conservation efforts on sites with legacy contamination.

1. Introduction

Human-induced rapid environmental change (HIREC) is the greatest threat to wildlife populations (Wilcove et al., 1998). HIREC encompasses many environmental disruptions, including habitat loss, species introductions, climate change, and pollution (Sih et al., 2010). Inability of species to respond to these changes increases their probability of extinction (Chevin et al., 2010). Some populations respond to HIREC through evolutionary adaptation, while others do not (Gomulkiewicz and Holt, 1995). Mounting evidence suggests that evolutionary responses to HIREC are becoming increasingly prevalent on a global scale (Smith and Bernatchez, 2008). Evolutionary adaptation requires standing genetic variation in individual resistance to the adverse effects of HIREC (Hendry et al., 2011). Evolutionary responses may be particularly important in the case of pollutants, which can have strong fitness effects (Bickham, 2011). Environmental exposure to pollutants is often chronic and multigenerational, rather than short lived, and can result in an increased probability of extirpation of affected populations if fitness impacts are large and variation in the response to the pollutant is minimal.

Toxicologists generally measure response to a pollutant with a dose–response curve. The response to the toxicant is typically averaged across all individuals in the population, masking within-population variation in resistance to the pollutant. We suggest that we can capture information about heritable variation within a population using dose–response curves made for groups of genetically related individuals, much in the same way as reaction norms are constructed and interpreted (Weltje, 2003). Reaction norms are the set of phenotypes that can be produced by a given genotype when submitted to an environmental gradient (Stearns, 1992), in this case exposure to a pollutant. Reactions norms can be visualized by plotting the phenotypic response of each genotype across the environmental gradient with a separate curve for each genotype. Parallel reaction norms indicate that despite genetic variation, all genotypes react in the same way to the environmental gradient. Conversely, crossed reaction norms indicate a genotype by environment interaction (i.e. genotypes are responding differently to the environmental gradient) and selection will favor different genotypes in different environments (Stearns, 1989). Ideally these curves would be constructed for genetically identical individuals, but in the case of non-clonal species, such as most vertebrates, it is impossible to obtain genetically identical individuals. In these cases reaction norms can be created for full-sibling (i.e. sharing the same father and mother) families. Individuals in full-sibling families are expected to share approximately half of their genes and are more genetically similar to each other than to other members of the population. Dose-response curves are analogous to reaction norms and can be used to assess genetic variation in the response to contaminants (Weltje, 2003). If

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there is a heritable basis to the reaction of related groups to a contaminant, we would expect family-derived dose–response curves to be loosely bundled (i.e., not entirely overlaying each other), and their slopes to be significantly different from one another, perhaps leading to crossing lines (van Noordwijk, 1989). If genetic variation exists, selection could drive adaptation to the pollutant, resulting in a greater proportion of resistant genotypes in populations with multi-generational exposure (Weltje, 2003). If the slopes of family-derived dose–response curves cross it would mean that, potentially, there is selection for different families (and their genotypes) at different levels of contamination. That is, at lower levels of contamination selection may favor a different set of genotypes than at higher levels of contamination. Such a pattern could result in opposing selection for genotypes in contaminated versus uncontaminated areas, potentially constraining the evolution of resistance to toxins if there is gene flow among contaminated and uncontaminated sites. Hence, exploring the patterns of family-derived dose–response curves can elucidate how populations might adapt to contaminant exposure since the genes within families that can resist contaminants can be expected to be over represented in subsequent generations.

Here we constructed family-derived dose–response curves of breeding parameters using a model avian system, the zebra finch (*Taeniopygia guttata*), exposed to methylmercury, which suppresses reproductive success in a number of vertebrate species, including birds (reviewed in Scheuhammer et al, 2007). Anthropogenic sources of inorganic mercury, notably coal-fired power plants, legacy industrial point sources, and artisanal gold mining, have increased the amount of available mercury in the environment at least three-fold (Mason et al., 1994). Once in aquatic systems, microorganisms methylate the inorganic mercury, rendering it bioavailable and allowing it to bioaccumulate and biomagnify (Wiener et al., 2002). Mercury is not restricted to aquatic systems and crosses into terrestrial food webs (Cristol et al., 2008). We compared the effects of methylmercury on breeding success of families of full siblings (i.e. individuals with the same mother and father). If genetic variation exists in the response to mercury, we would expect families to show different dose–response curves. Nearly all studies of genetic variation in response to pollutants have been focused on aquatic invertebrates (e.g. Coutellec et al., 2011; Haap and Köhler, 2009; Pease et al., 2010), with only a few on aquatic vertebrates (e.g. Semlitsch et al, 2000; Athrey et al., 2007; Lind and Grahn, 2011) or terrestrial invertebrates (e.g. Fisker et al., 2011; Fritsch et al, 2011). Despite the fact that many terrestrial vertebrates are affected by pollutants such as mercury (Smith et al., 2007), studies of genetic variation in response to pollutants are lacking in this group, perhaps because of the difficulty of obtaining individuals of known genetic makeup. This is to our knowledge the first study investigating the possibility of a familial genetic basis for resistance to pollutants in a terrestrial vertebrate.

2. Materials and methods

2.1. Study design

We used young, sexually mature (150–400 days old), zebra finches that were captive-bred at the College of William & Mary. All birds used for this study were reproductively inexperienced. None of the birds in this study or their parents had previously been exposed to mercury. All birds were maintained indoors at approximately 20 °C on a 14:10 h light:dark cycle. Food, vitamin-enriched water, and grit were provided *ad libitum*. The birds for this study were bred from an existing population of captive zebra finches and were of known parentage. Because this study was part of a larger reproductive study and we wanted to avoid pseudoreplication caused by siblings, we used a random number generator (random.org) to assign 90 males and 90 females equally to five treatment groups, with the exception that more than four full siblings could not be assigned to any treatment group. Each group was fed a diet containing a constant concentration of methylmercury cysteine (MeHgCys) at 0.0, 0.3, 0.6, 1.2 or 2.4 ppm (fresh weight). The lower mercury doses (0.3 and 0.6 ppm) are at a level similar to the mercury content of common songbird prey items (i.e. spiders) found in the South River watershed, an industrially contaminated site in western Virginia (Cristol et al., 2008). The next higher dose (1.2 ppm) is representative of the highest levels of mercury levels found in prey items on the South River, whereas the highest dose (2.4 ppm) was included to help detect subtle differences at the lower doses. We maintained the birds in single sex cages from the start of dosing until blood mercury levels had plateaued (c. 10 weeks). Because there is wide individual variation in the blood mercury level produced by a given dose of dietary mercury, these discrete mercury doses produced a continuous range of blood mercury levels that we used for analyses (Table 1).

Table 1
Blood mercury concentrations at each dietary treatment level.

Dose	Mean blood mercury	Range
0.0 ppm	0.09 ppm	0.01–0.18 ppm
0.3 ppm	3.95 ppm	2.58–5.99 ppm
0.6 ppm	7.93 ppm	6.21–10.76 ppm
1.2 ppm	16.88 ppm	11.54–22.88 ppm
2.4 ppm	30.61 ppm	23.00–45.93 ppm

Once the blood mercury levels had stabilized, we used a random number generator (random.org) to pair the birds (18 pairs per treatment group) while avoiding pairings of known relatives. Pairs remained on dosed food for the duration of the study. Each pair was housed in a cage (46 × 46 × 76 cm) with a plastic nest box and *ad libitum* nesting material. We allowed the pairs to reproduce for one year, monitoring reproduction each day. Eggs were labeled on the day they were laid, newly hatched chicks were marked on the day of hatching, and nestlings were leg-banded at 10 days to determine offspring fate from laying to independence (50 days). Upon independence, young were removed from parental cages. Hence, we gathered accurate data about the reproductive performance of each pair, including hatching success, fledging success, and total reproductive output. We also sampled blood of adults monthly to monitor their blood total mercury concentrations. Individual mercury levels were determined from the average of all blood samples for each individual throughout the year of breeding ($N = 13$ blood samples per individual). This experiment and all animal handling and care associated with it was approved and overseen by the Institutional Animal Care and Use Committee at the College of William & Mary.

2.2. Food preparation

All food (Zupreem FruitBlend) was dosed with methylmercury cysteine as this is the form of mercury most likely in wild avian diets (e.g. fish and insects, Harris et al., 2003). We added to the pelletized commercial diet an aqueous solution of methylmercury cysteine, representing 10% of the weight of the food. Each batch was assayed to ensure that it fell within 10% of the target mercury concentration. Control (0.0 ppm) food contained only an aqueous solution of cysteine. Food was stored at −20 °C until use to prevent spoilage.

2.3. Mercury analysis

Total mercury levels in food and blood were analyzed using a direct mercury analyzer (Milestone DMA 80), which measures total mercury content. Both food and blood were assayed fresh (i.e. mercury values are not on a dry weight basis). All samples were analyzed using the quality control procedures standardized in our lab (Varian-Ramos et al., 2011). Briefly, standard reference samples (DORM-3, DOLT-4) and machine and sample blanks were run every 20 samples to check calibration and contamination. The machine was recalibrated every two months or as necessary. Duplicate and spiked samples were run throughout the study to verify repeatability (relative percent differences <10%) and recovery rates (>95%).

2.4. Statistical analyses

The 180 birds included in the study belonged to 33 different full-sibling families. Many families contained too few individuals to assess the familial response to mercury accurately, so we only included families with 7 or more individuals. To ensure a good distribution of data from each family across the range of mercury levels, we only included families in which there was at least one individual in at least 4 of the 5 treatment groups. These restrictions resulted in the inclusion of 105 individuals from the 11 most populous families (Table 2). All families had the same male and female parents and shared neither parent with another family (i.e. none were half siblings or had half siblings in any other family). To assess the mercury level for each individual, we used the average concentration of all blood samples for that individual taken over the breeding period; individual blood mercury concentrations remained relatively consistent over time (Buck, 2013). We employed generalized linear models to explain reproductive parameters by average blood mercury, family, and age, as well as the interaction between blood mercury and family. The reproductive parameters we considered were: number of clutches

Table 2
Distribution of individuals in families.

Family	0.0 ppm	0.3 ppm	0.6 ppm	1.2 ppm	2.4 ppm	Total
Magenta	2 ♀	2 ♂, 2 ♀	1 ♂, 1 ♀	1 ♂, 3 ♀	1 ♂, 2 ♀	5 ♂, 10 ♀
Red	1 ♂, 1 ♀	0	2 ♂	2 ♂	1 ♀	5 ♂, 2 ♀
Orange	1 ♂, 1 ♀	1 ♂, 1 ♀	1 ♂, 2 ♀	2 ♂	1 ♂, 2 ♀	6 ♂, 6 ♀
Yellow	1 ♂, 1 ♀	1 ♀	0	1 ♂, 2 ♀	1 ♂	3 ♂, 4 ♀
Green	0	1 ♂, 1 ♀	3 ♂, 1 ♀	1 ♂, 1 ♀	2 ♂	7 ♂, 3 ♀
Cyan	2 ♂, 1 ♀	2 ♂, 2 ♀	2 ♂	1 ♂	1 ♂, 2 ♀	8 ♂, 5 ♀
Blue	0	2 ♂	1 ♂, 1 ♀	2 ♂	1 ♂, 2 ♀	6 ♂, 3 ♀
Purple	1 ♂	2 ♂	2 ♂, 1 ♀	0	1 ♀	5 ♂, 2 ♀
Brown	1 ♂, 2 ♀	0	1 ♀	3 ♀	1 ♂, 2 ♀	2 ♂, 8 ♀
Black	2 ♂	1 ♂, 2 ♀	2 ♀	0	1 ♀	3 ♂, 5♀
Gray	1 ♂, 2 ♀	1 ♀	1 ♀	0	2 ♀	1 ♂, 6 ♀
Total	10 ♂, 10 ♀	11 ♂, 10 ♀	12 ♂, 10 ♀	10 ♂, 9 ♀	8 ♂, 15 ♀	51 ♂, 54 ♀

produced in one year where a clutch was defined as at least 3 eggs laid in succession and separated from other eggs by at least 4 days (number of clutches); median clutch size of all clutches produced in a year (clutch size); proportion of eggs laid that hatched (hatching success); proportion of chicks hatched that survived to fledge from the nestbox (fledging success); and the total number of independent offspring produced in one year (total reproductive success). This final parameter takes into account both rate of reproduction and reproductive success per attempt and is the most comprehensive measure of overall fecundity. In all analyses, family was modeled as a fixed effect because we were looking for specific differences among this set of families. The interaction term between blood mercury and family tested for differences in slope of the family-derived dose–response curves. For count-based reproductive measures (number of clutches, clutch size, total reproductive success) we used a Poisson distribution and a log link function. For reproductive measures that were proportions (hatching success, fledging success) we used a binomial distribution and a logit link function. All statistics were performed in SPSS (version 19, IBM).

3. Results

The number of clutches produced per year decreased with increasing mercury level (Wald $\chi^2 = 21.074$, $df = 1$, $P < 0.001$), but there was no significant interaction between family and mercury level (Wald $\chi^2 = 16.084$, $df = 10$, $P = 0.097$). We found no significant response of clutch size to blood mercury level (Wald $\chi^2 = 0.367$, $df = 1$, $P = 0.545$), nor was there a significant interaction between family and mercury level (Wald $\chi^2 = 0.789$, $df = 10$, $P = 1.00$, Fig. 1) indicating a lack of difference in slope of family-derived dose–response curves for clutch size. While there was only a weak negative effect of mercury on the hatching success of eggs at the population level (Wald $\chi^2 = 3.440$, $df = 1$, $P = 0.064$), there was marked variation in how mercury affected the hatching success of eggs among families (Wald $\chi^2 = 95.742$, $df = 10$, $P < 0.001$); some families had decreased hatching success with increasing blood mercury while others had increased hatching success (Fig. 2). Survival of chicks to fledging decreased with increasing blood mercury (Wald $\chi^2 = 12.388$, $df = 1$, $P < 0.001$) and there was a strong interaction between family and blood mercury concentration (Wald $\chi^2 = 58.880$, $df = 10$, $P < 0.001$); some families showed a negative effect of blood mercury on chick survival whereas one family (family 8), surprisingly, showed increased chick survival with higher mercury levels (Fig. 3). The strong effect of mercury on nestling survival to fledging contributed to reduced total reproductive success (as measured by total number of independent offspring per year) with increasing blood mercury (Wald $\chi^2 = 71.127$, $df = 1$, $P < 0.001$), and there was a similarly strong interaction of family with blood mercury (Wald $\chi^2 = 68.310$, $df = 10$, $P < 0.001$). Thus the fitness effects of mercury contamination varied substantially among families and family-derived dose–response curves differed in slope (Fig. 4). Tests for homogeneity of variation for all measures of reproduction revealed that there were no significant differences in the amount of variance across treatment levels (Levene Statistics = 0.721–2.133, $P = 0.082$–0.580) suggesting similar genetic variance regardless of severity of mercury exposure. In a study separate to the one reported here we have already detected significant additive genetic variance (i.e. narrow sense heritability) of blood mercury levels in our captive population (Buck, 2013).

4. Discussion

We found significant variation among families in some but not all of the reproductive responses to methylmercury. Clutch size appeared to be a relatively fixed feature with little plasticity in response to mercury level and little variation in response among families. In contrast, while there was only a weak negative effect of mercury on hatching success when all families were considered together, exploration of family-derived dose–response curves indicated that exposure to mercury increased hatching success in some families and decreased it in others. The unexpected increase in hatching success observed in some families could be the result of increased lethargy in the parents (Evers et al., 2008) resulting in greater time spent incubating, or perhaps mercury at low levels increased egg viability (Heinz et al., 2012). In a typical toxicological study, where effects are averaged across all individuals in the population, these differences would have been obscured, creating an incomplete picture of the effects of mercury on hatching success.

Most families had a strong negative response to mercury exposure both in terms of fledging success and total number of independent offspring produced. However, there were a few families that did not respond to mercury dose or even appeared to have increased reproductive success with increasing mercury levels (e.g. families 4, 8 and 10). Since these differences in response to mercury are associated with full-sibling families, it is likely that there is a genetic component to the response. It is possible that these differences are due to similarities in the natal environment as full siblings were raised by the same parents. However, all sibling families in this experiment were the result of two to four reproductive attempts by the parents, so natal environment is not identical within families.

It is important to note that these differences in the response of families were found in a captive population where birds were held in constant environmental conditions with access to unlimited food and water. It is difficult to predict how the results might differ in wild populations faced with the additional stressors of predation, starvation, and exposure. It is possible that the combination of contamination and natural stressors would result in all families performing equally poorly; however, most wild populations contain much more genetic diversity than our relatively small zebra finch population, therefore, based on the prevalence of genetic diversity in response to contaminants observed across many taxa (e.g. Athrey et al., 2007; Coutellec et al., 2011; Fisker et al., 2011; Lind and Grahn, 2011), it seems likely that this diversity of response to toxins will be found in wild populations as well.

If similar patterns of variation in reproductive success are present in wild birds, adaptation to contaminants could occur rapidly if populations are resident in a contaminated area for multiple generations. In such a situation families with genetic variation endowing increased resistance to the effects of mercury would be under strong positive selection. As a result, highly sedentary populations at sites with legacy contamination may already be adapted to those contaminants. Hence, these populations may be relatively unresponsive to the contaminant despite the presence of substantial pollution. Field studies comparing effects at contaminated sites to reference sites may underestimate the risk of the contaminant if genetic divergence exists between the sites (Morgan et al., 2007). This can have important implications for field-based risk

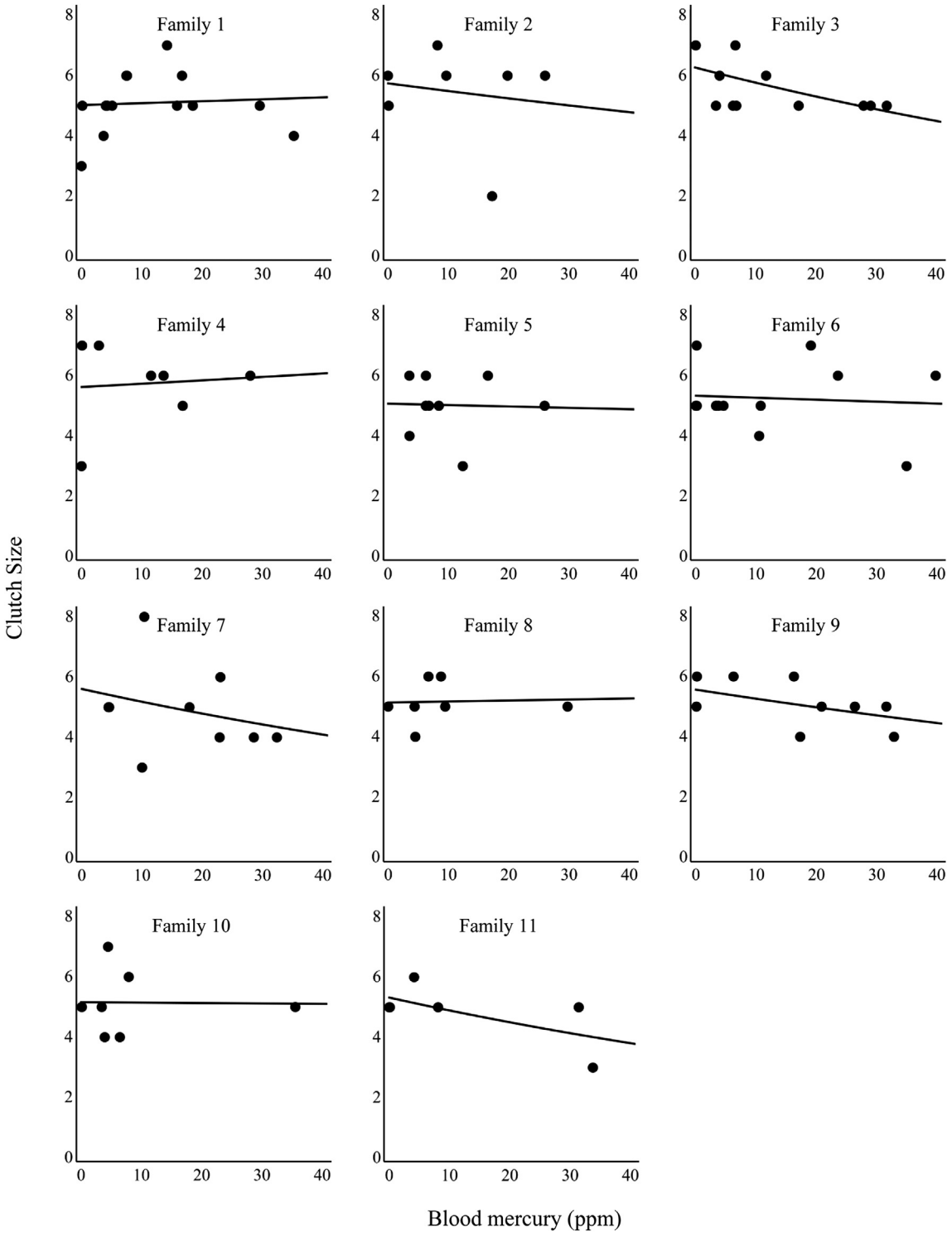

Fig. 1. Response of clutch size to blood mercury level in 11 full-sibling families of zebra finch. Curves in each graph represent the response of that family from the generalized linear model.

assessments because risk will be dependent on the contaminant history and genetic history of the studied population (Medina et al., 2007). It may be necessary in some cases to perform common garden, relocation (or cross-fostering), or dosing experiments in order to examine whether any adaptation has occurred. If strong selection for resistant genotypes occurs at polluted sites, this may have other consequences for the population as a whole. Strong selection for a limited number of families or genotypes can reduce genetic diversity in a population, which in turn can limit that population's ability to adapt to additional stressors.

In addition to exploring whether sedentary populations have adapted to the effects of multi-generational exposure to contaminants, our study also underscores the importance of quantifying the extent of gene flow between populations at contaminated and

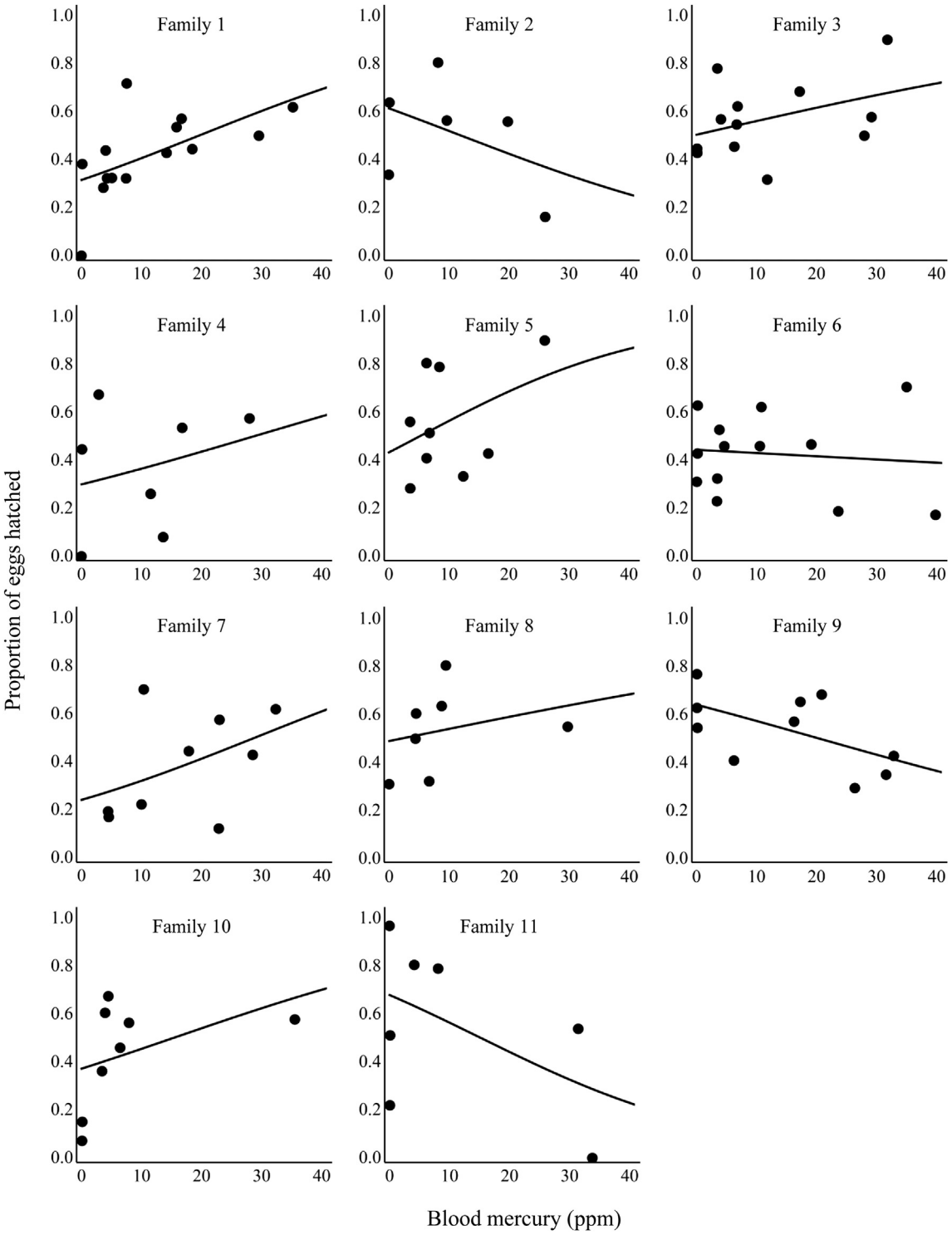

Fig. 2. Response of hatching success to blood mercury level in 11 full-sibling families of zebra finch. Curves in each graph represent the response of that family from the generalized linear model.

uncontaminated sites (Theodorakis, 2001). Because the family-derived dose–response curves differed substantially in slope, and even intersected one another, it is possible that families under the strongest negative selection at a contaminated site may be under the strongest positive selection at an uncontaminated site. Within our study, the families of zebra finches with the highest reproductive success at high mercury levels had relatively low reproductive success at control levels. This suggests that there may be a cost associated with resistance to mercury (Wilson, 1988). If this is true in wild populations, then gene flow will constrain rates of local adaptation as resistant families would be expected to be uncommon in uncontaminated populations. Additionally, the negative effects of contaminants on populations might be greater than we currently think as families who breed successfully on

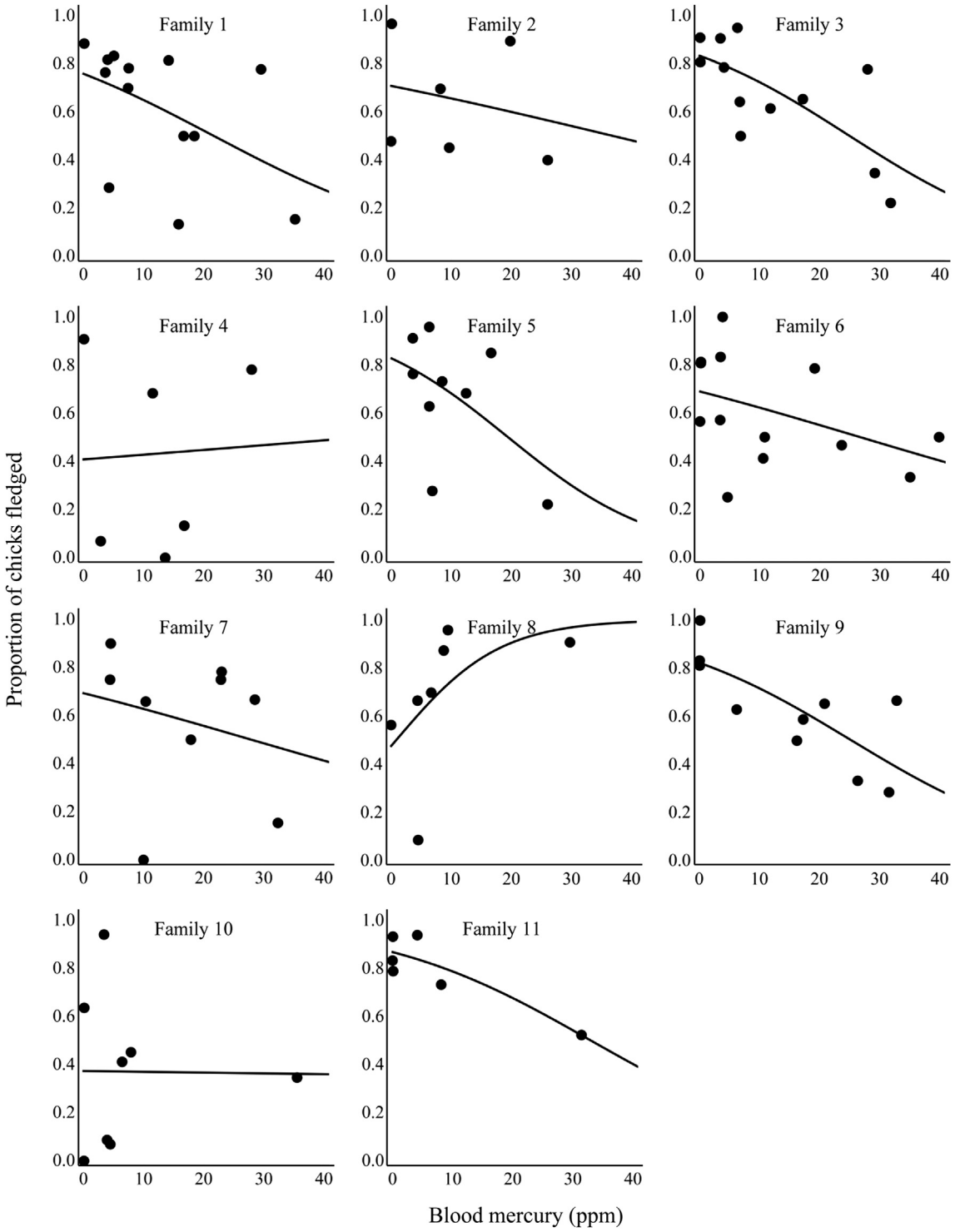

Fig. 3. Response of fledging success to blood mercury level in 11 full-sibling families of zebra finch. Curves in each graph represent the response of that family from the generalized linear model.

contaminated sites could produce offspring that are less competitive when they disperse to breed in uncontaminated areas. Trade-offs between pollutant resistance and fitness in uncontaminated areas have been demonstrated in several groups (e.g. Agra et al., 2010; Semlitsch et al, 2000; Xie and Klerks, 2003) but are not universal (e.g. Arnaud and Haubruge, 2002; Saro et al., 2012). Therefore, it is crucial to understand the basic life history of species living in contaminated areas. For an accurate assessment of risk, it will be important to quantify patterns of movement and dispersal to estimate gene flow in and out of contaminated sites. The patterns of intersecting family-derived dose–response curves in our zebra finches suggest the possibility that the families under strongest selection at contaminated sites may not be the best adapted to environmental conditions at less contaminated sites.

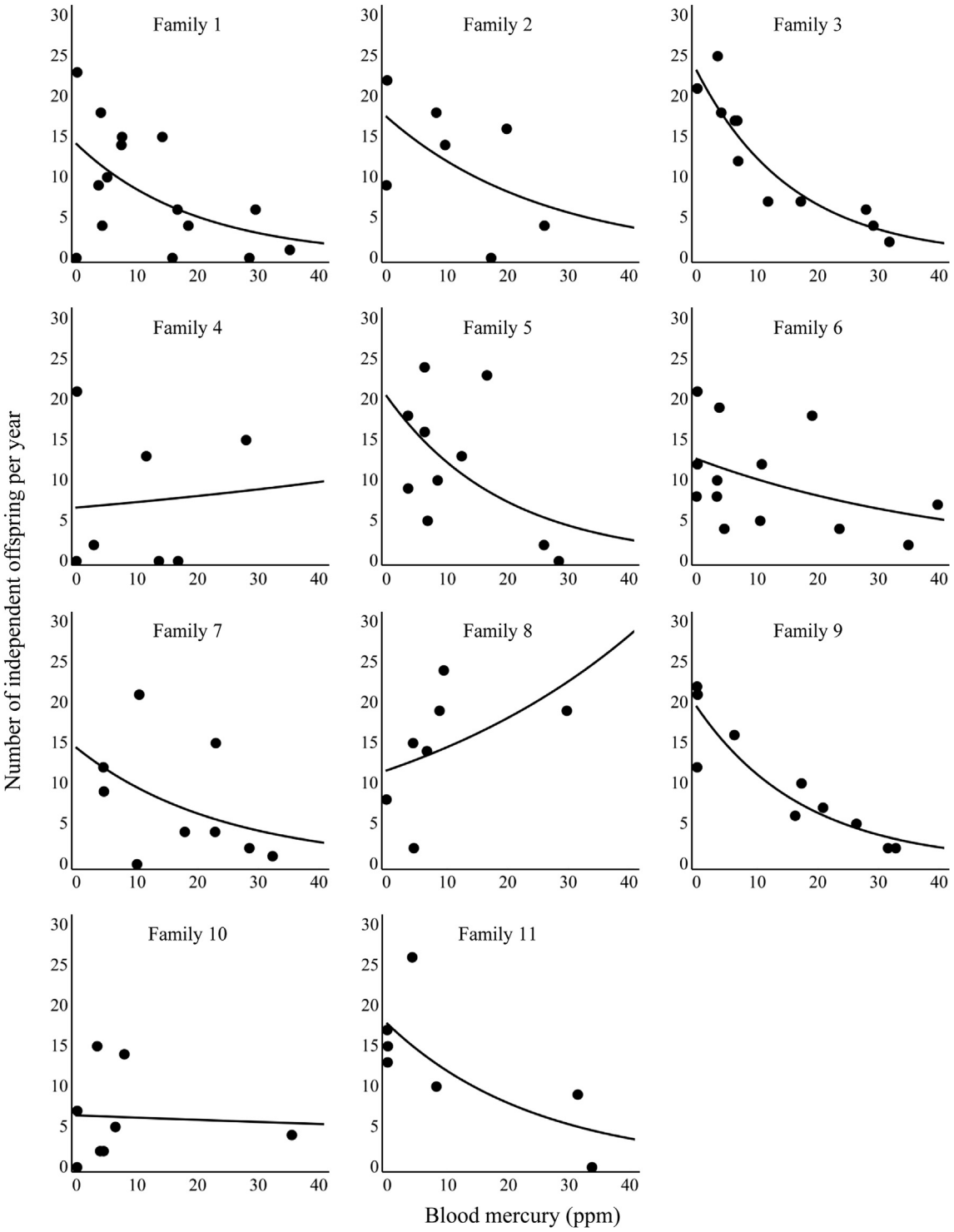

Fig. 4. Response of total independent offspring to blood mercury level in 11 full-sibling families of zebra finch. Curves in each graph represent the response of that family from the generalized linear model.

5. Conclusions

Genetic variation in response to environmental contaminants appears to be widespread across invertebrate (e.g. Barata et al., 2002a; Barata et al., 2002b; Coutellec et al., 2011; Fisker et al., 2011; Fritsch et al, 2011; Haap and Köhler, 2009; Pease et al., 2010) and vertebrate taxa (e.g. Athrey et al., 2007; Lind and Grahn, 2011), yet this phenomenon is still generally underappreciated by environmental toxicologists. Such genetic variation has important consequences that should be considered in future studies. For example, dosing studies that use inbred lines of model organisms may limit our knowledge to the response of a single genotype. Therefore results from such studies may not be representative of the response in a diverse wild population. Careful

choice of test species and consideration of genetic effects are important for improving environmental risk assessments (Breitholtz et al., 2006). Furthermore, the degree of genetic similarity of populations on contaminated and reference sites needs to be carefully assessed, taking into account the consequences of gene flow and the possibility that genotypes resistant to contaminant exposure may have relatively low fitness in uncontaminated areas. Variation in response to contaminants may ultimately allow some populations to adapt and persist in our increasingly contaminated global environment but we stress that these selected populations may end up with a different genetic make-up than that required to thrive in uncontaminated areas.

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