

LOSS OF EVOLUTIONARY RESISTANCE BY THE OLIGOCHAETE *LIMNODRILUS HOFFMEISTERI* TO A TOXIC SUBSTANCE—COST OR GENE FLOW?

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The oligochaete *Limnodrilus hoffmeisteri* at Foundry Cove (FC), New York evolved genetic resistance to cadmium (Cd) and lost resistance after contaminated sediments were removed by dredging. Selection (on survival time in dissolved Cd) was used to generate tolerance to evaluate fitness cost, the commonplace expectation for evolutionary reversal. The hypothesis that gene flow from neighboring populations could “swamp” resistance was addressed by 16S rDNA sequences. In disagreement with the cost hypothesis, selected-Cd tolerant worms and controls showed no difference in total fecundity or growth rate in environments. Highly-Cd-tolerant worms of the FC-selected population grew rapidly at different temperatures and showed no growth impairment in the presence of Cd, indicating metabolically efficient resistance. Genetic structure at FC was consistent with invasion of genotypes from an adjacent population in the time since dredging. Applying selection to lines from FC and a reference site, demonstrated a more rapid increase in Cd tolerance in FC-origin lines, indicating standing allelic variation for resistance at FC (despite phenotypic erosion). The selection experiment supports the view that resistance is simply controlled—probably by one allele of large effect. Whether such rapid “readaptation” could occur naturally is an important question for understanding broad effects of pollutants.

KEY WORDS: Adaptation, experimental selection, repeatable evolution, 16SrDNA, standing variation.

Rapid evolution is known to occur in a wide range of organisms in response to pollutants, including dioxins, pesticides, and metals (e.g., cadmium, mercury, and copper) (Bradshaw and Hardwick 1989; Klerks and Levinton 1989). Evolutionary reversal is often seen following relaxation of a previously dominating mode of selection (Teotónio and Rose 2001; Sgro and Hoffmann 2004; Lahti

et al. 2009). As Wright (1964) discusses, there are two major hypotheses for a change in gene frequencies, such as would account for resistance reversal—either counter-selection against the original mutation (pleiotropy, commonly referred to as cost), or gene flow from populations lacking genetically based resistance (immigration pressure). Because evidence of the effect of mutation

accumulation alone in eroding traits when they are no longer useful is generally lacking, pleiotropy and immigration pressure stand as major competing or complementary hypotheses in explaining trait loss (Wright 1929, 1964; Maughn et al. 2007).

The steady increase of human disturbance makes it increasingly important to broadly understand potential for adaptation to pollutants. A growing number of examples suggest that fitness costs play an important role in reversing toxicant resistances following relaxation of selection (Lahti et al. 2009), nevertheless, isolating clear counter-selection events in the field is not simple. The fate of resistance alleles may to some extent be conditional on transitory secondary factors, which include environmental extremes and pathogen pressure (Coustau et al. 2000; Sgro and Hoffmann 2004; Bennett and Lenski 2007). These influences, invoking negative fitness correlations, could be short-term and difficult to observe for many reasons. Extrapolating fitness correlations observed in the laboratory (the major source of theoretical understanding) to wild populations carries important caveats. As a filtering process acting in large populations, and over many generations, selection in the field could tend to more readily select for low-cost phenotypes than selection conducted in the laboratory. In cases of insecticide/acaricide resistance, compensatory secondary mutation is often supported (Roush and McKenzie 1987). Culturing of organisms in the laboratory itself imposes selection, altering the genotypic composition of stocks potentially at expense of ecologically relevant genotypes (Hoffmann et al. 2001; Simões et al. 2008). Studies integrating cost and dispersal measurement, supplying independent evidence of each, are clearly required. Persuasive evidence for significant immigration effect is seen in the evolution of insecticide resistances including the practice of using nondosed reservoir populations to prevent widespread fixation of resistance alleles (review by Roush and McKenzie 1987; Raymond et al. 1991, 2001; Carrière et al. 2003).

In this study, we evaluate the occurrence of fitness trade-off and gene flow in an evolutionary loss of cadmium (Cd) resistance observed in the oligochaete *Limnodrilus hoffmeisteri* at Foundry Cove, USA. This population lost resistance rapidly (over nine years, which is equivalent to 9–18 generations) following the removal of localized Cd contamination from across the cove, by sediment dredging in 1994 (US EPA 1998; Levinton et al. 2003; Mackie et al. 2007).

Dumping of cadmium–nickel hydride wastes by a battery factory from 1953 to 1979 brought surface sediment layer Cd concentrations to 10^3 – 10^4 mg/kg over a broad region of Foundry Cove, an unprecedented level in an aquatic system (Occhiogrosso et al. 1979; Knutson et al. 1987). Klerks and Levinton (1989) found that Foundry Cove sediments that were inhabited by *L. hoffmeisteri*, and surprisingly, a diverse and abundant invertebrate community, were toxic to *L. hoffmeisteri* collected from

a neighboring reference population (South Cove, 1.5 km from Foundry Cove); causing 100% or 99% mortality in short exposures. The localized genetic resistance in Foundry Cove *L. hoffmeisteri* involved increased induction of a metallothionein-like Cd-binding protein (MLP) (Klerks 1987; Klerks and Bartholomew 1991) and appeared on the basis of a quantitative genetic analysis to result from a single segregating locus (Martinez and Levinton 1996). By 2002, nine years after dredging, the Foundry Cove and reference populations were statistically indistinguishable in Cd tolerance (Levinton et al. 2003). Our principal question is whether the loss of resistance of Foundry Cove worms could be explained by trade-offs in fitness, or by immigration of nonresistant genotypes from the Hudson River, once the cleanup was done and recruitment was permitted via removal of a strong selective barrier that had isolated Foundry Cove.

In the generation of Cd-tolerant populations (for the major purpose of evaluating costs to Cd tolerance) by selection, we also addressed the potential for rapid evolution at Foundry Cove to occur as a result of retained alleles. Worms were selected on the ability to survive a period of exposure to 1000 ppm dissolved Cd. Selection was conducted using two lines from Foundry Cove and two from South Cove, assessing the null expectation that lines from each area would show equal rates of evolution, consistent with a lack of selection-responsive genetic difference in the field.

A relatively rapid response in selection lines originating from Foundry Cove occurred, suggesting that selection likely elevated the frequency of an original resistance genotype maintained at Foundry Cove. Using selected Cd-tolerant populations, we tested for trade-offs in fecundity, somatic growth, and temperature tolerance in clean-sediment environments. Such trade-offs have been shown following selection for Cd resistance in other organisms (Shirley and Sibly 1999; Xie and Klerks 2004).

The growth rate of highly Cd-tolerant worms originating from Foundry Cove was examined at an abnormal temperature of 35°C. This temperature exceeds the normal local summer maximum temperature encountered by North American and European *L. hoffmeisteri* populations by 1°C or 2°C (Kennedy 1966; Birtwell and Arthur 1980). The high temperature treatment generally widens the window for observing resistance cost. Cost, if present, may be based directly in unfavorable pleiotropy—reduced tolerance of selected-resistant worms to heat stress. If the system acts not as one of stress, but with the higher temperature being favorable generally to rapid growth, metabolic-type “allocation cost”, (Simms and Rausher 1987), may surface—as a smaller unit of increase in the resistant population compared to control.

Worms originating from Foundry Cove and nonselected, nonresistant worms from this source were also subjected to Cd. Growth of worms in the presence of Cd (at either 22°C or 35°C) was used to test for costs associated with active detoxification.

We reason that this could reveal a scenario in which cost is only responsive to facultative genetic induction. In a closed-cell respiration experiment, we compared the ability of the nontolerant and tolerant populations to regulate oxygen consumption rates under different conditions: at 22°C or 35°C, and with and without Cd dosing.

The alternative explanation for loss of resistance by influx of nonresistant genotypes following the cleanup of Foundry Cove was evaluated using genetic data from a mitochondrial gene (16S rDNA). We measured the similarity of the Foundry Cove pre-cleanup genetic sample and the population present one decade after the cleanup, including inspection of the alleles in neighboring areas, to estimate the net influence of immigration to the cove in the time since dredging.

Methods

Foundry Cove (41°24'47.03''N, 73°57'10.86''W) is a 20-hectare tidal freshwater cove within an area of surrounding marshlands, known as Constitution Marsh, in New York State, 90 km north of The Battery (southern tip of Manhattan Island, New York City). We refer subsequently to the eastern areas of Foundry Cove and South Cove as "FC" and "SC." Both coves are separated from the main river by a narrow opening through an embankment that carries a railway trestle (Fig. 1).

In 1994, contaminated sediments were dredged from across FC to 30 cm depth (US EPA 1998), which reduced sediment Cd concentration from approximately 1000 mg/kg dry weight to <100 mg/kg over a broad area (Mackie et al. 2007). This still

represents significant contamination. The background Cd concentration in the Hudson River is circa 10 mg/kg (Bopp et al. 2006). SC experienced minor levels of Cd contamination during the time battery factory wastes were dumped into FC, but is now indistinguishable from the river-wide concentration (Mackie et al. 2007). SC has been used exclusively as the reference in defining Cd tolerance at FC. In preliminary studies, we also assessed Cd tolerance of *L. hoffmeisteri* from two other marsh sites, Stockport Flats ("SP," 42°17'41.92''N, 73°46'25.34''W) and Tivoli Bay ("TB," 42°2'43.45''N, 73°55'25.09''W) (Fig. 1). The median survival time (MST) estimate and 95% confidence interval of the SP population was 23 h, CI = 20–24 h (starting $n = 100$). Tivoli Bay MST was 21 h, CI = 20–23 h ($n = 85$). These data are in agreement with tolerances of samples referenced at SC over multiple years (Levinton et al. 2003).

One thousand six hundred worms were collected at both FC and SC in 2004 to found cultures. Worms were cultured in Tupperware tubs (40 × 25 cm) (Sterilite, Townsend, MA) with 2 cm of sediment overlaid with water. Tubs were maintained in darkness with aeration supplied by an air pump attached to a pipette tip. Individual tubs were seeded with a maximum of 200 adults. Wardley tropical fish flakes (The Hartz Mountain Corporation, Secaucus, NJ) were added after 14 days to new cultures and periodically thereafter when no flakes were remaining. Water was collected from the Hudson River near the opening to FC and filtered through Glass Microfibre Filter paper (a GFC/C, Whatman International Ltd., Kent, UK). The sediment was obtained from FC in 2004–2005 from within the dredged zone; mean dry weight Cd was ~33 mg/kg (Mackie et al.

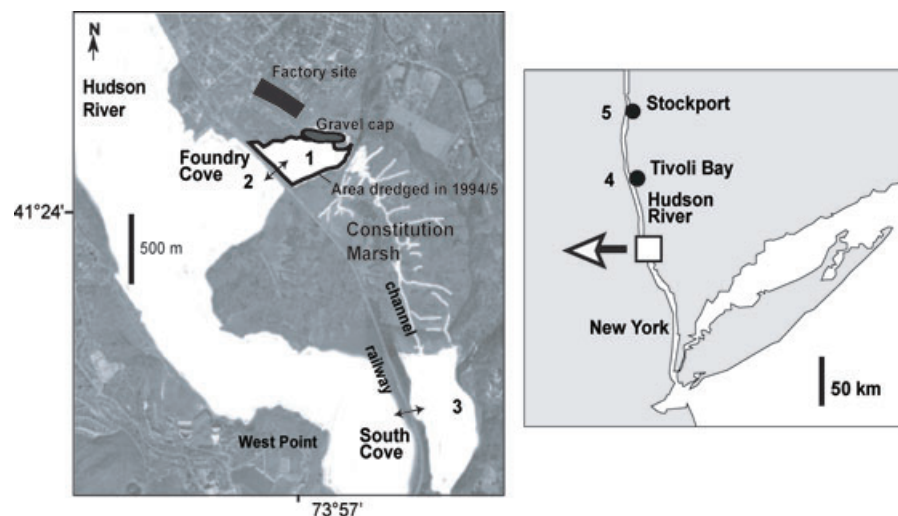


Figure 1. Map of the Constitution Marsh area (left) and general study region (right). The eastern Foundry Cove area was dredged in 1994 to remove Cd-contaminated sediments caused by waste from the Marathon Battery factory from 1953 to 1979. Foundry Cove and South Cove are connected by shallow channels. Two openings (\leftrightarrow , c. 20 m wide) are major points of tidal exchange between Foundry Cove and the main river. 1–5: collection sites of populations used in 16S haplotype analysis. Populations used for cadmium selection were collected within Foundry Cove and South Cove in 2004 in areas 1 and 3.

2007). Sediment was washed through a 1 mm sieve using tap water, then rinsed with distilled H₂O, and boiled or autoclaved to remove live organisms. Trade-off tests were conducted using soft reconstituted water (48 mg/L NaHCO₃, 30 mg/L CaSO₄, 30 mg/L MgSO₄, 2 mg/L KCl) and sediment was collected at SC. Closed-cell respiration analysis of O₂ consumption rates (described below) was conducted in darkness, otherwise fluorescent lighting was present on an 11:13 h light:dark period during assays.

SELECTION EXPERIMENT

Worms were exposed individually to 8.9 μM CdCl₂ (1000 ppm Cd) in 4 mL cell culture wells following the procedure used previously to assay Cd tolerance (Levinton et al. 2003). Survivors (from 20 to 40 worms) were removed to clean sediment where reproduction occurred before the next generation of selection. Exposures were preceded by a 24-h depuration period in soft water to minimize variation in toxicity of the metal due to gut sediments (Gillis et al. 2004). A prodding stimulus was used to confirm death. Survival times were recorded with the aid of a macro in Excel (Microsoft, Redmond, WA) and were rounded to the nearest whole hour in analysis. The Kaplan–Meier (K–M) survival function, MST, and 95% confidence interval (CI) were calculated using the proc lifetest function in SAS 9.1.3 software (SAS Institute Inc., Cary, NC). Survival curves were compared by Peto's log-rank Chi² test (Peto et al. 1977) with survivors as censored observations. At the start of exposures, worms were categorized into length categories: <1 cm (representing 24% of the total used in selection), 1.0–2.4 cm (64%), and >2.4 cm (12%). Size was found to contribute to variance in survival times, with large-bodied worms experiencing reduced risk (J. A. Mackie, unpubl. data). All surviving worms were used to found the subsequent generation in selection, however comparisons of survival data are shown using worms of the middle size range of 1.0–2.4 cm only to reduce heterogeneity. All data are available by request to the lead author.

To assess whether population source (FC or SC) affected selection response, lines from each locality were exposed together (and all other culturing steps handled simultaneously). Two synchronously selected FC/SC line pairs (referred to as selection groups A and B) were analyzed. Assays of selection Group A and Group B were usually carried out on separate days, which facilitated monitoring of a large number of worms. Furthermore, a third line from each locality was used assess tolerance deviation related to population sampling and handling steps, but not Cd exposure. These lines are referred to as random controls (Falconer 1981; Muir 1986). Survival under Cd exposure in the random control lines was generally monitored in tandem with the selective assay of the group B FC/SC selected line pair. At the completion of each selection cycle, nonexposed parents from random

lines were selected haphazardly and moved to a fresh culture tub, at a number equal to the lowest number of survivors extracted from any of the four selected lines in that generation. We make a distinction between random controls and base cultures, large (1000+) populations that were maintained over the course of the selection experiment (which lasted 12 months). In the final phenotype assessment (F3 assay round, Fig. 2), Cd tolerance of populations sampled from the FC base culture and SC culture was indistinguishable. From the combined base culture data, a Weibull function was derived (by least squares regression), and used to visually compare survival variation in Cd-selected lines and the sub-sampled 'random' control from each source.

TESTS FOR COSTS

Grand-progeny of F3-exposed survivors were used in tests for deleterious effects arising from Cd-tolerance selection. Following three generations of selection, survivors from selection groups A and B were pooled in cultures (maintaining FC or SC origin, 200 founders in each case). Culturing in pristine (SC) sediment followed to exclude the possibility of confounding epigenetic effects of parental Cd exposure in the life-history assays. Nonselected (control) populations used in life-history assays were reared with 200 grandparents from either FC or SC base culture.

Reproductive output was measured by placing mature worms (showing clitellar development) in groups of 10 (FC-Sel, SC-Sel, FC-Non Sel, SC-Non Sel; 10 replicates per treatment) in clean-sediment microcosms. To standardize initial mating opportunity, groups were held in sediment (20% volume) for 14 days. The adults were then recovered and transferred (minus cocoons and juveniles) to fresh microcosms (20% volume). This sediment was augmented with 0.04 g of ground fish flakes. At days 15 and 30, clean sediment was added to 40% and then 60% of container volume. The experiment was stopped after 55 days. Sediment, adults, juveniles, and cocoons were recovered and preserved in 70% ethanol containing Rose Bengal stain. Cocoons were ruptured using forceps to release embryos. Juveniles, cocoons, and number of embryos per cocoon were counted under a dissecting light microscope at 20×.

Average worm growth rate was measured at the ambient laboratory temperature (~22°C) in clean sediment. Worms were held singly in microcosms, consisting of a circular plastic container (base diameter: 7.4 cm, opening: 9 cm diameter, and height: 7 cm) and 2.0 mm of sediment and soft reconstituted water. Worms were normally prereproductive. No food was added to containers. Worms, which typically burrowed below the sediment–water interface, were recovered after 28 days using a 63 μm sieve. Photographs were taken with a standard length scale at the start and end of the experiment using a Nikon D1 digital camera (Nikon, Tokyo, Japan) equipped with a macro lens and lengths determined using ImageJ software (<http://rsb.info.nih.gov/ij/>).

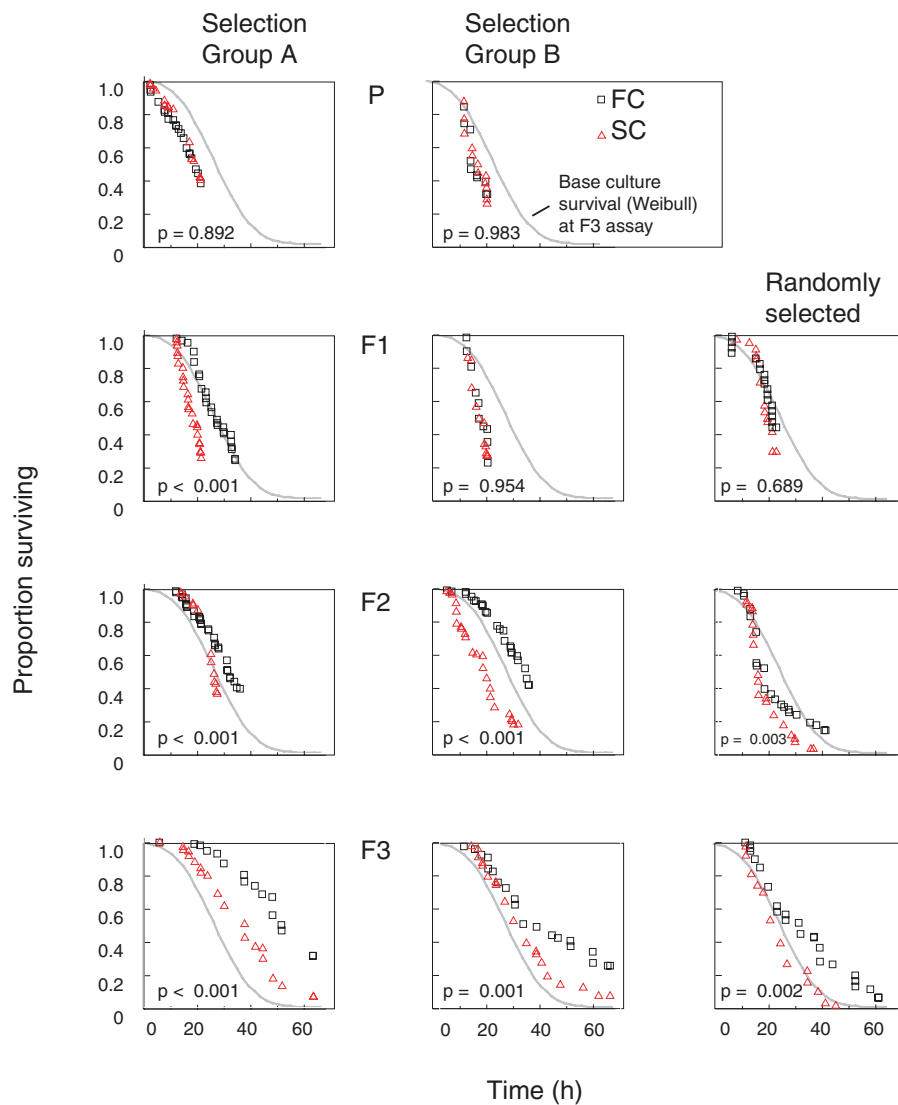


Figure 2. Survival-curve response to cadmium selection. *P*-values are Peto's log-rank test of the assumption of no-difference between co-assayed lines from Foundry Cove (FC) and South Cove (SC), (selection groups A and B and random control lines). Survival time distributions are imposed on the Weibull function (shape parameter, $k = 2.983$, and scale parameter, $\lambda = 30.48$) that was fit to nonselected FC and SC base culture populations assessed in the F3 tolerance assay. FC and SC base culture survival functions were not statistically distinguishable.

Using selected and nonselected populations of FC-origin only, individuals were grown in clean or Cd-spiked microcosms. This experiment was conducted at a low temperature (LT, 22°C) or high temperature (HT, 35°C) using a chamber set to 36°C. Water temperature was 34–35°C due to evaporative cooling. Cd-treated microcosms were dosed with 10 µg of Cd/g (dry weight) prepared in sediment plus 2.2 µM Cd water used to overlay sediment. This dosage was found in preliminary trials to be nonlethal to control worms. The Cd was added (as CdCl₂) to SC sediment using slurry spiking (Simpson et al. 2005), allowing 14 days for equilibration. Nondosed sediments were treated in parallel with water. Fish flakes (0.02 g) were added to sediment prior to the commencement. After 28 days, worms were measured. Sacrificed worms were frozen (–70°C) until measurement of Cd body burden.

CADMIUM ACCUMULATION

To obtain enough tissue for measurement of Cd whole-body concentration by atomic absorption (AA), worms from within treatments were pooled into sets of five to seven individuals (~0.001–0.004 g dry weight, three replicates per treatment) Worms were placed into preweighed acid washed vials, dried at 65°C. Dry weights were obtained using a Mettler Toledo AX205 DeltaRange balance (Mettler Toledo, Columbus, OH). Concentrated HNO₃ (5 mL) was then added to each vial and samples were refluxed (3–4 days) on medium heat until tissues dissolved. Samples were then evaporated to dryness, re-suspended in 3 mL of 2% ultragrade HNO₃, and filtered using 0.45 µm filters (Millipore, Billerica, MA). Filtered samples were then analyzed for Cd using a 3100 Graphite Furnace Atomic

Absorption Spectrometer (PerkinElmer, Waltham, MA) calibrated with standards (0, 1, 2, 5 ppb Cd) prepared from 2% ultra-grade HNO₃ and a Cd atomic absorption standard (supplied by VWR, West Chester, PA). Quality control and quality assurance samples included (1) standard tissue samples (~95% recovery), (2) standards run as unknowns (yielding an error of less ~5%), (3) digestion and filter blanks (all blanks were at or below background and were consistently 10-fold (or more) lower than sample concentrations).

RESPIRATION RATE MEASUREMENT

Oxygen consumption rate was measured using groups of three adults from FC-selected and FC base culture descendent lines. Worms were placed in a 15-mL Falcon tube that was filled with water and sealed. Solutions were air-saturated by an aquarium pump. In half, water was dosed with Cd (2.2 μM). Dosed and nondosed groups were held at 22°C or 35°C. The experiment was conducted without sediment and in darkness to minimize effects of microbial respiration or photosynthesis. Dissolved oxygen was measured with a polarographic electrode (Strathkelvin 928). Oxygen concentrations were recorded in eight blank (worm-free) vessels held under each condition. Oxygen consumption was assessed after 24 h. The total dry weight of worms in each tube was recorded at the end of the assay. Oxygen consumption (mg O₂/mg dry weight) was determined by subtracting final oxygen quantities of experimental vessels from blanks. Mortality during the experimental assay was limited to the deaths of one or two worms from the nonselected population. This occurred in five of the eight microcosms in the HT +Cd treatment. Analyses were run including these tubes, unbalanced without these tubes, and with the averages of the remaining three tubes to balance the design. Results were not different across these analyses.

STATISTICAL ANALYSES

Experiments examining the effects of selection history, temperature, and Cd exposure on growth or respiration rate were analyzed by three-factor analysis of variance (ANOVA). Nested ANOVAs were tested using the restricted maximum likelihood (REML) technique on number of embryos per cocoon, with microcosm as a nested random factor. Tukey's HSD tests were conducted to identify significant pairwise relationships. Statistical tests were conducted using JMP, version 4.0.4 (SAS Institute Inc., Cary, NC) and the R statistical software package, version 2.7.2 (R Foundation for Statistical Computing, Vienna, Austria). A log transformation was used to correct for heterogeneous variances in respiration data. Significance implies that $P < 0.05$, unless otherwise stated.

GENETIC ANALYSIS

We compared 16SrDNA nucleotide sequences of populations collected earlier at FC in the period of 1993–1994 at FC and 1994 at

SC (collector: C. Sturmbauer), and collections made in the period of 2004–2005 at FC and SC, and 2004 at TB and SP. Worms were collected from the main Hudson River channel in an arc 150 m from the entrance to FC in 2006 to test the hypothesis of a direct dispersal occurring through the narrow opening to FC. Collection sites are shown in Figure 1. Worms were sieved in the laboratory from sediment and preserved in ethanol (70–85%). DNA was isolated by Chelex (Biorad, Hercules, CA) or phenol–chloroform extraction and PCR-amplified and sequenced using 16SAR and 16SBR primers (Kocher et al. 1989). Sequences of FC and SC worms collected in 1993 were obtained previously (C. Sturmbauer, unpubl. data). Sequences of FC and SC worms collected in 1994 (stored in EtOH at 4°C for 13 years) and recently collected samples were obtained on an automatic sequencer (ABI 3130xl; PE Applied Biosystems, Foster City, CA) using BigDye dideoxy-terminator chemistry (v 3.1, Applied Biosystems, Foster City, CA). A list of unique haplotypes was generated using DNACol-lapser version 1.0 (<http://www.birc.au.dk/fabox/>). Sequences of all haplotypes were submitted to GenBank (acquisition numbers: EU160464–160491).

A median branching network of haplotype sequences was constructed using TCS software (Clement et al. 2000). Genetic relatedness of population samples was computed as Slatkin's linearized F_{ST} (or Φ_{ST}) (Slatkin 1991, 1995). Substitution of Nm for Φ_{ST} , in

$$Nm = 1/2(1/\Phi_{ST} - 1).$$

Wright's (1951) Island equilibrium model estimator of the number of migrants per generation (Nm) moving between subpopulations was used to define a migration rate among population pairs (Hudson et al. 1992; Slatkin 1995). Analysis of molecular variance (AMOVA) inferred from metric distances (Excoffier et al. 1992), population diversity indices, and minimum spanning network distances (Rohlf 1978) were calculated using Arlequin (Excoffier et al. 2005). The Unweighted pair group method with arithmetic mean (UPGMA) was used to join populations according to the Φ_{ST} matrix. The similarity profile, (or SIMPROF), test (Clarke et al. 2008), a nonparametric permutation test, was used to cluster populations that were not distinguished statistically, at a P -value of 0.05. The SIMPROF was conducted using Bray–Curtis similarity matrices of square-root transformed haplotype frequencies in Primer, version 6.1 (PRIMER-E Ltd., Plymouth, UK).

Results

RESPONSE TO SELECTION

Cultures collected at FC and SC in 2004, were subdivided, using 171–195 founders per line to commence selection (Table 1). In 1994, prior to dredging, the wild FC *L. hoffmeisteri* population had an MST of 30 h in the Cd exposure assay (Fig. S1 in Levinton

Table 1. Parameters of the selection experiment to increase Foundry Cove (FC) and South Cove (SC) cadmium tolerance. Pairs of lines in selection groups A and B were exposed to cadmium synchronously.

Population	Generation	Source	Starting number	Exposure stopping time (h)	Proportion surviving	Median survival time and 95% CI (h)
Selection group A						
P		FC	180	21	0.43	19, 17–19
		SC	182	21	0.42	19, 16–20
F1		FC	341	34	0.23	29, 29–32
		SC	410	21	0.29	19, 17–19
F2		FC	339	36	0.37	33, 30–35
		SC	275	22	0.38	25, 25–26
F3		FC	96	64	0.39	52, 48–63
		SC	96	64	0.07	38, 38–42
Selection group B						
P		FC	171	26	0.40	18, 17–19
		SC	195	27	0.40	22, 21–22
F1		FC	93	18	0.24	14, 14–18
		SC	96	18	0.25	14, 13–16
F2		FC	465	40	0.48	39, 38–39
		SC	256	36	0.40	25, 22–25
F3		FC	60	64	0.27	39, 31–57
		SC	120	64	0.04	33, 28–33

et al 2003); in summary, three generations of selection resulted in greater tolerance in the two independently selected lines originating at FC (MST of 52 or 39 h, Table 1).

Initial, survival functions (and corresponding MST 95 percentile ranges, Table 1) of FC- and SC-origin lines did not differ. FC- and SC-source base populations maintained over the period required to carry out selection did not differ in tolerance according to a log-rank test at the end of the experiment: FC_{Base(n=60)} – MST = 24 h, CI = 21–28 h; SC_{Base(n=60)} – MST = 25 h, CI = 22–28 h; $P = 0.681$; (combined-data Weibull function shown in Fig. 2).

In an early response to selection, Cd tolerance increased in the FC source lines relative to SC source lines. In selection group A, survival functions diverged following one generation of selection ($P < 0.0001$); in group B, divergence in favor of FC occurred following two generations ($P < 0.0001$) (Fig. 2).

After two generations of selection, the SC randomly selected control was similar to a base culture function, whereas tolerance of the FC-origin random line was slightly increased. Tolerance of the FC-random line exceeded SC-random line in F2 and F3 generations according to log-rank comparisons (Fig. 2). Both of the FC-origin selection lines were significantly more Cd tolerant than the FC random line in the F2 and final F3 assay (four comparisons, P values < 0.01 ; not shown), which was indicative of a significant response to experimental selection.

LIFE-HISTORY ASSAYS

Selection did not significantly affect the number of free-living juveniles ($F = 1.4984$, $P = 0.2315$, Fig. 3A) or unhatched cocoons ($F = 1.1167$, $P = 0.3551$, Fig. 3B) that were present in microcosms after 55 days of reproduction. The number of embryos per cocoon, which ranged from 1 to 27 (mean = 6.3, SD = 3.2), varied in response to selection ($F = 4.9113$, $P = 0.0031$). This, however, occurred with a significant selection history \times source interaction ($F = 12.2500$, $P = 0.0013$). The FC-origin selected population had fewer embryos per cocoon than the FC-origin control line (but did not differ significantly from SC control line). Conversely, the SC-origin selected line had more embryos per cocoon than the nonselected control from that location (Fig. 3C). Total reproduction, inferred as number of free-living juveniles and embryos, although nominally lower in selected populations, was not significantly affected by selection ($F = 3.041$, $P = 0.090$, Fig. 3D).

Offspring of selected and nonselected populations, originating from either FC or SC, showed no significant difference in growth rate in clean sediment (ANOVA for $N = 142$, $df = 3$, $P = 0.62$, data not shown). The three-factor ANOVA model (Table 2A) supported a significant relationship between selection history and effect of Cd ($S \times Cd$, $F = 6.6668$, $P = 0.0107$), and temperature on growth ($F = 4.6752$, $P = 0.0320$). Selected and nonselected populations had indistinguishable growth rates at 22°C and 35°C in the absence of Cd. Worms of each population

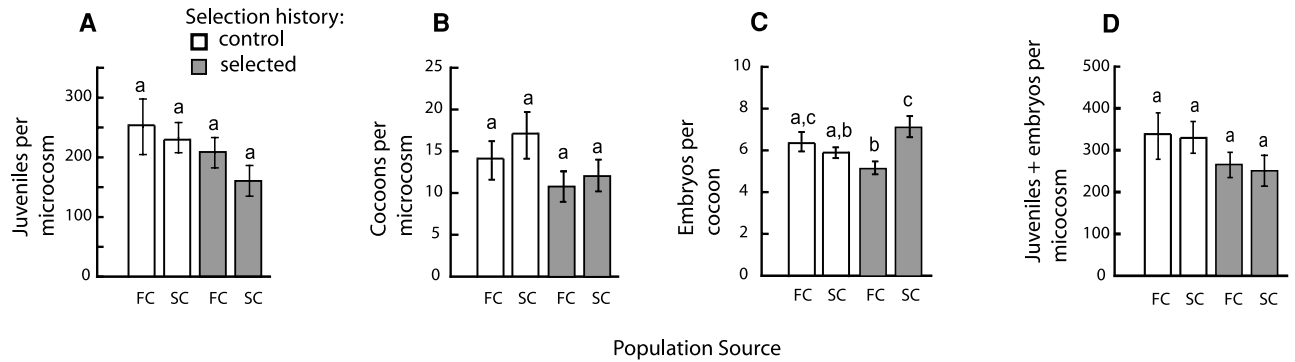


Figure 3. (A–D) Measures of mean reproduction in microcosms (each holding 10 adults for 55 days, $n = 10$) of selected and nonselected *L. hoffmeisteri* populations originating from FC or SC. Error bars are standard error of mean. Shared letters indicate relationships that are not significantly different as confirmed by Tukey’s HSD tests ($\alpha = 0.10$).

grew more rapidly at 35°C. The presence of Cd in microcosms inhibited growth of the nonselected worms; selected worms had unchanged growth rate in the presence of Cd (Fig. 4A).

A two-factor ANOVA examining FC-selected and control worms held in Cd-treated microcosms indicated a highly significant effect of temperature on total body burden of Cd ($P < 0.000$). Cd accumulation was approximately twofold greater at 35°C than 22°C (Fig. 4B). Although selected worms had nominally greater Cd accumulation at both temperatures, this difference was not statistically significant ($P = 0.0908$, Fig. 4B). As only three tissue

pools in each of four treatments were compared, this assessment has limited sensitivity.

RESPIRATION RESPONSE

Control worms analyzed at 22°C, in the absence of Cd consumed oxygen at a rate of $160 \pm 31 \mu\text{g O}_2/\text{mg}/24 \text{ h}$, which is in the upper range of respiration rate measures made for other tubificids (Johnson and Brinkhurst 1971). Selection state was very significant ($P < 0.0001$) (Table 2B), with worms selected for Cd tolerance having lower respiration than nonselected worms (Fig. 4C). Temperature was also significant (Table 2B), positively affecting respiration rate. Cd exposure had no effect on respiration rate, although there was a significant interaction between Cd exposure and temperature. This is partially explained by the very high respiration experienced by nonselected worms at combined high-Cd and high temperature, but may signal other complex interaction effects that are difficult to explain with our design.

Table 2. Three-factor ANOVA tests of effects of selection, temperature, and cadmium on (A) growth rate ($n=22$), and (B) mass-corrected respiration rate of *Limnodrilus hoffmeisteri* ($n=8$). For respiration rate, arcsine transformation was applied to correct for heterogeneous variances, and missing values were replaced with average of remaining values in treatment (see text). P -values < 0.05 are shown in bold.

Source	df	SS	F	P
(A) Growth rate				
Selection (S)	1	0.2099	1.9024	0.1696
Temperature (T)	1	0.5159	4.6752	0.0320
Cadmium (Cd)	1	0.0763	0.6917	0.4068
S×T	1	0.0595	0.5396	0.4636
S×Cd	1	0.7356	6.6668	0.0107
Cd×T	1	0.0505	0.4575	0.4997
S×T×Cd	1	0.0943	0.8549	0.3565
Residual	168	18.5374		
(B) Respiration Rate				
Selection (S)	1	1.0033	39.4673	<0.0001
Temperature (T)	1	0.7140	28.0879	<0.0001
Cadmium (Cd)	1	0.0329	1.2927	0.2604
S×T	1	0.0318	1.2506	0.2682
S×Cd	1	0.1616	6.3549	0.0146
Cd×T	1	1.7642	69.399	<0.0001
S×T×Cd	1	0.2437	9.5847	0.0031
Residuals	56	1.4236		

16S POPULATION COMPARISON

Sequencing of a 422 base pair segment of 16S in 224 individuals resulted in 28 haplotypes (Appendix S1). The sequences formed three clades (Fig. 5). Values of Tajima’s D (Tajima’s 1996) and Fu’s F_s (Fu 1997) statistics did not differ from neutral model expectations in any of the populations (data not shown). The FC precleanup population genetic diversity ($h = 0.8089$) and nucleotide diversity ($\pi = 0.0174$) were within the ranges of other Constitution Marsh populations (Table 3). Interestingly, there was a higher proportion of private alleles (haplotypes occurring only in that sample) in the precleanup population compared to other Constitution Marsh collections (23% vs. 0–10%, Table 3).

The northern populations TB and SP contained haplotypes of clade 1 and 3 only (Appendix S1) and had relatively high average nucleotide diversity ($\pi > 0.04$, Table 3). At least one haplotype from each of the clades (1–3) occurred in the different areas sampled within Constitution Marsh. The AMOVA and SIMPROF tests supported genetic similarity of TB and SP (Table 4, Fig. 6).

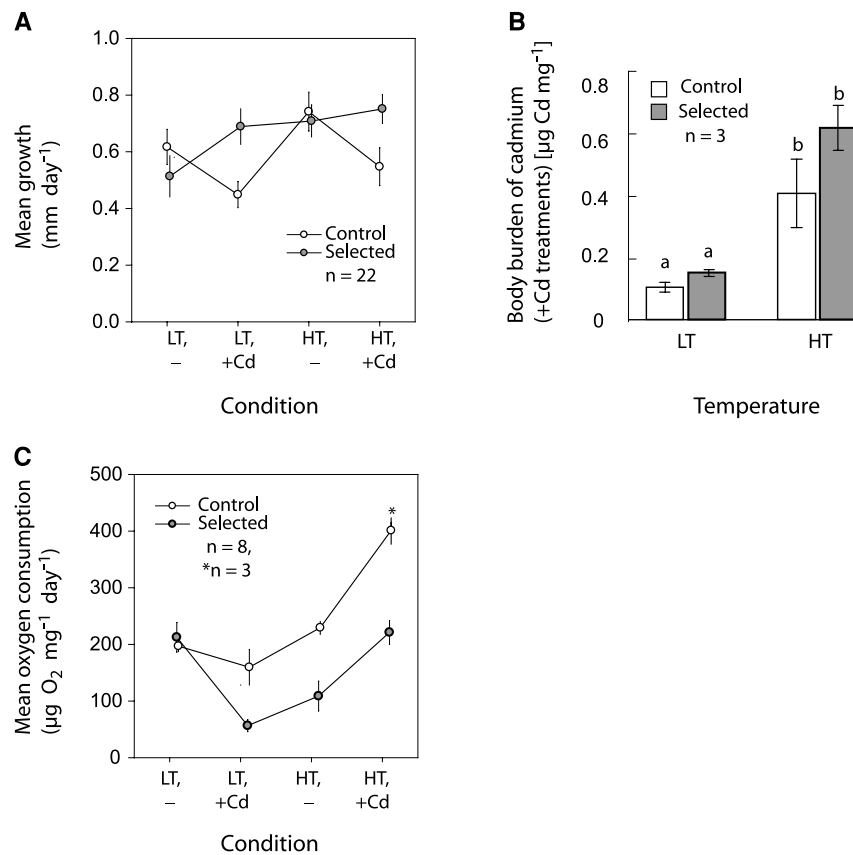


Figure 4. (A) Profiles of growth of selected/nonselected FC-origin worms over different conditions (LT = 22°C, HT = 35°C; ± Cd in microcosms). (B) Final whole body concentrations of Cd in worms from dosed microcosms. Shared letters indicate relationships that are not significantly different (P -value < 0.05) in Tukey's tests. Body burdens of Cd in worms from nondosed microcosms were <0.03 $\mu\text{g Cd mg}^{-1}$ (data not shown). (C) Profile of mass-corrected O₂ consumption rates of worms held for 24 h in respiration chambers under different conditions. (*Sample size was lower in the case of the nonselected worms in the HT, +Cd treatment as five respiration chambers were excluded from measurement because they contained one or two dead worms.) Error bars are standard error of means. Statistically significant effects and interactions within A and C are shown in Table 2.

The precleanup FC population differed significantly from other Constitution Marsh populations, including the sample collected at FC ten years after dredging. The postcleanup FC sample and the main Hudson River had the highest similarity of any two populations, supporting recent exchange. The genetic pairwise correlation, $\Phi_{ST} = 0.0011$, corresponds to an estimated migration rate (Nm) of 454 breeding individuals/generation, which was one order of magnitude higher than Nm between any other two subpopulations (Table 4). The next lowest population-pair differentiation was between recently collected samples from FC and neighboring SC ($\Phi_{ST} = 0.0169$), corresponding to Nm of 29 individuals/generation.

Discussion

Our selection protocol mirrors an earlier selection experiment. Klerks and Levinton (1989) were able to increase the resistance of populations taken from the reference site, SC by exposure to dissolved Cd after only three generations of selection. Under

the current selection regime, lines from FC, (collected after reversal in Cd tolerance) responded to selection by developing greater resistance than lines from the reference site. This demonstrates that even after the cleanup, a significant genetic factor underlying Cd resistance remained in greater frequency within the FC population and was recruited in response to selection. This has occurred despite the fact that standard Cd exposure of the nonselected population was not able to detect significant differences in median survival of FC and SC, nine years after Cd was removed from FC by dredging (Levinton et al. 2003).

In addressing the major aims, we found that there was little or no cost to tolerance produced by selection. Second, immigration was found to have strongly influenced the genotypes present at FC in the time since dredging.

LACK OF EVIDENCE OF COST

The only measurement consistent with a possible deleterious effect of selected Cd tolerance in the mean number of embryos per

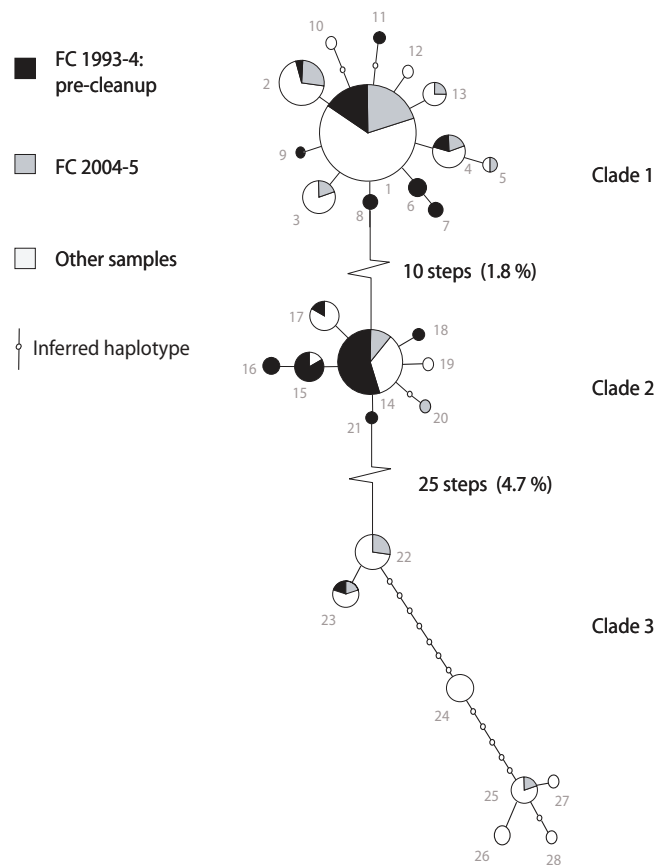


Figure 5. Parsimony network of 16S haplotypes from 224 sampled individuals; small, unnumbered circles are nonsampled intervening haplotypes inferred within three subnetworks calculated in TCS (Clement et al. 2000). Shading contrasts haplotype occurrence within Foundry Cove before, and one decade after dredging. Circles of sampled haplotypes (numbered, Appendix S1) are sized in proportion to frequency, including samples from the main Hudson River, South Cove, Stockport and Tivoli Bay (nonshaded areas). Minimum spanning (stepwise) distances among these clades (1–3) are shown along with average (Kimura 1980) percentage divergence.

cocoon. This was positive in the SC-selected (low Cd tolerance) population and negative in the FC-selected (high Cd tolerance) population relative to the averages in one of the two nonselected population sources. Total reproduction (the number of free-living juveniles plus the total number of embryos encased in cocoons per microcosm) was not affected by selection. Growth of the worms (which is primarily by production of segments) clearly did not differ among selected and nonselected populations in sediments lacking Cd. The dosage of Cd used in our second growth experiment hindered growth of nonselected control worms from FC, as occurs when a related species is exposed to Cd (Gillis et al. 2002). But growth of Cd-selected *L. hoffmeisteri* was not adversely affected by Cd, at either 22°C or 34°C, a finding that indicates a surprising metabolic efficiency of the selected tolerant genotype.

The lack of evidence of cost associated with Cd tolerance of *L. hoffmeisteri* contrasts with two previous studies using Cd selection to elevate tolerance in unrelated organisms. *Drosophila* lines grown on Cd-containing medium for 20 generations exhibited a 44% reduction in fecundity, and reductions in male and female emergence weights in Cd-free environments (Shirley and Sibly 1999). Killifish lines exhibiting increased Cd tolerance after six generations of selection using a similar Cd exposure protocol to the current experiment, showed 18% lower fecundity, slowed rate of maturation, and reduced brood size, life span, and heat tolerance compared to controls (Xie and Klerks 2003, 2004).

The high temperature treatment used in our experiment was stimulatory to somatic growth rate generally, and resulted in more uptake of Cd by worms in dosed microcosms. Increased temperature promotes accumulation of heavy metal in a wide range of organisms (Philips 1976; Tessier et al. 1994; Whittaker et al. 2006). In *L. hoffmeisteri*, Cd sequestration is rooted in the binding of the metal to high molecular weight proteins in chloragoc (digestive tissue); the sequestering was more rapid FC worms compared to SC, when populations were examined prior to FC dredging (Wallace et al. 1998).

One cannot determine yet how stress will effect respiration in an oligochaete, and much less the adaptive significance of changes in respiration rate. At the core of this argument, the same stressor—exposure to heavy metals, organic contaminants, temperature, and pH difference—may strongly perturb respiration rate, upward or downward, in different species (Brinkhurst et al. 1982). Selection for Cd tolerance in the FC-origin population affected respiration rate response significantly. The pattern was complex. At 22°C in the absence of Cd, selected and nonselected worms had similar mass-corrected respiration rate. Overall variance in the mean respiration rate was increased in comparisons at 35°C. Tolerant worms showed lowered levels of respiration in the presence of dissolved Cd at 22°C, and in the absence of Cd at 35°C. This may be the result of either a reduced cellular aerobic demand because of decreased activity (such as movement or digestion), or increased efficiency of anaerobic metabolism.

IMMIGRATION PRESSURE

The population present at FC a decade after dredging and the nearby main Hudson River had high 16S genetic similarity, with estimated Nm being ten times greater than between other pairs of populations. Dispersal of worms across the narrow bridge opening to FC appears certainly have influenced population structure in the time since dredging. Local movements or worms within this marsh system could also have contributed to genetic change, requiring investigation. Two years after dredging, the density of *L. hoffmeisteri* at FC was one-third that of SC as a result of the impact of dredging (Kelaher et al. 2003). Thus, a smaller than predicted number of immigrants

Table 3. Diversities of *L. hoffmeisteri* collections characterized by 16S sequence haplotype. "Before" and "after" refer to dredging of FC that occurred in 1994.

Population	Period of sampling (Year)	Sample size (<i>n</i>)	Distinct haplotypes (<i>k</i>)	<i>k/n</i>	Private alleles	Polymorphic sites (<i>S</i>)	<i>S/n</i>	Gene diversity (<i>h</i> ±SE)	Nucleotide diversity (<i>π</i> ±SE)
FC before	1993–4	57	15	0.2632	0.23	39	0.68	0.8089±0.0392	0.0174±0.0090
FC after	2004–5	40	13	0.3250	0.10	40	1.00	0.7987±0.0574	0.0216±0.0113
outside FC	2006	34	9	0.2647	0.00	36	1.06	0.8039±0.0432	0.0184±0.0100
SC before	1993	37	8	0.2162	0.03	16	0.43	0.6607±0.0806	0.0086±0.0050
SC after	2004–5	19	9	0.5000	0.00	35	1.84	0.8538±0.0680	0.0332±0.0175
SP	2004	19	6	0.3158	0.26	36	1.89	0.8596±0.0393	0.0423±0.0220
TB	2004	18	7	0.3889	0.11	36	2.00	0.6928±0.1143	0.0406±0.0213

(than under the Island model that assumes a negligible influence of immigration on population size) could have been effective in changing genotypic composition within the former area of resistance.

Little is known about movement patterns. Limited sampling (two surveys, totaling 2.5 h, unpubl. data) using plankton nets revealed no *L. hoffmeisteri* dispersing in the water column during tidal influx to FC. Among the captured material were some specimens of oligochaete species with a recognized distribution in the water column (J. S. Levinton and J. A. Mackie, unpubl. data). *Limnodrilus hoffmeisteri* resides in sediments and thus it seems likely to move between sites in this way, however subterranean dispersal potential has not been directly investigated. Movements with floating vegetation or the sediment surface layer (bedload transport) also present potential dispersal opportunities requiring investigation.

RAPID RESPONSE TO SELECTION AS EVIDENCE OF STANDING VARIATION

The importance of standing variation, that is the concept of selection altering preexisting variation rather than de novo mutation

is conceptually simple and used in allelic models, and but has only become viewed as a potentially useful/predictive ecological tool (Barrett and Schluter 2008; Kitano et al. 2008; Simões et al. 2008). In pesticide cases, the presence of one or two genes of large effect is a leading hypothesis for repeated resistance outbreak (e.g., a single gene conferring resistance to different *Bacillus* toxins, Tabashnik et al. 1997; and esterase gene amplification in mosquito, causing repeated outbreaks of organophosphate resistance, Raymond et al. 1991).

A significant question presented by the current study is why mean phenotypic differences were not apparent in postcleanup populations sampled at FC from 2004 onward. A single dominant gene is on theoretical grounds supported as the most likely means of housing an adaptive variation that can be "grabbed" rapidly in response to selection; second, rapid response to selection is favored when deleterious effect of the mutation is minimal (Orr and Betancourt 2001; Hermisson and Pennings 2005). Suggestive of the single-gene principle, crosses among precleanup FC and SC *L. hoffmeisteri* indicated the original tolerance mutation was a single genetic element, with probable-dominant effect (Martinez and Levinton 1996). In selection, we observed a lesser increase in

Table 4. Population differentiation based on 16S rDNA. Lower left matrix: pairwise Φ_{ST} . Asterisks indicate samples that were distinguished in AMOVA comparison: **P*-value < 0.05, ***P*-value < 0.001. Migration (*Nm*) is shown next to pairs with low, nonsignificant Φ_{ST} . Upper right matrix: Bray–Curtis similarities calculated using square root-transformed haplotype frequencies. Both Φ_{ST} and B–C are used to assess population-group level relatedness (Fig. 6).

	Population sample						
	FC before	FC after	Outside FC	SC before	SC after	SP	TB
FC before	—	0.4033	0.4510	0.4562	0.4343	0.1132	0.2392
FC after	0.1938**	—	0.7388	0.4033	0.5881	0.4037	0.4397
outside FC	0.0848*	0.0011 (<i>Nm</i> =454)	—	0.7177	0.7512	0.3381	0.3255
SC before	0.2729**	0.0321 (<i>Nm</i> =15)	0.0277 (<i>Nm</i> =18)	—	0.5963	0.2704	0.3368
SC after	0.1181**	0.0169 (<i>Nm</i> =29)	0.0241*	0.1902**	—	0.3867	0.3642
SP	0.9193**	0.5051**	0.6531**	1.1305**	0.2441**	—	0.5681
TB	0.4322**	0.1163**	0.2143**	0.4143**	0.0453	0.0661	—
					(<i>Nm</i> =11)	(<i>Nm</i> =7)	

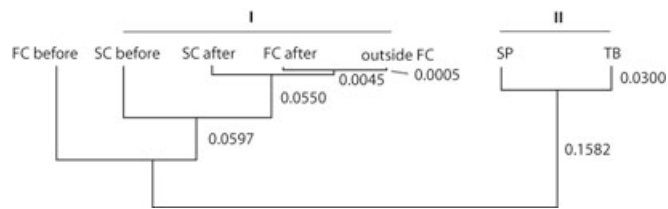


Figure 6. UPGMA cladogram of Φ_{ST} distances among populations. Branch lengths are shown at nodes. I and II indicate groups of populations that were not distinguished from one another according to the similarity profile (SIMPROF) test, at P -value of 0.05.

Cd tolerance in the FC-origin random (non-Cd selected) line. This response was not seen in the random line from SC. This result could indicate correlation between a trait responding to culturing and genetic basis of Cd tolerance, but different possibilities (gene linkage, or sampling effect) were not investigated.

Knowledge of the genomic basis of Cd tolerance in *L. hoffmeisteri* is lacking. Studies of the precleanup FC worms suggest tolerance can be related to an efficient upregulating mutation on metallothionein-like protein expression (Klerks 1987; Klerks and Bartholomew 1991). A gene duplication, or mutation affecting promoter-level expression of a metallothionein-like gene, are important hypotheses to address in examining Cd resistance. Maroni et al. (1987) found that wild *Drosophila* lines with more copies of a metallothionein gene had greater tolerance to copper. With such a mechanism, one might expect little cost or trade-off, because there is not likely to be a great cost in maintaining copies of a gene.

COST ASSUMPTION AND IMPORTANCE OF SELECTION MILEAU OF THE STUDY

Although unseen environmental circumstances in the field and the possibility of complex cost scenarios mean that it is never possible to renounce the possible occurrence of historical trade-offs with complete certainty, our experiments failed to show reduced performance of cd-resistant worms that would be symptomatic of an adverse pleiotropic effect of genetic Cd tolerance. The 16S data on the other hand support the occurrence of immigration in “balancing” haplotype patterns at FC and surrounding populations following the removal of strongly selective Cd concentrations from the field site. This provides reason to question whether cost was involved in the resistance reversal. Analogously, some insecticide resistance mutations are confirmed to not be deleterious in environments lacking the insecticide (french-Constant 1994; Daborn et al. 2001).

Just how “unusual” is the observation of metabolically efficient resistance? The two studies cited above (Shirley and Sibly 1999; Xie and Klerks 2004) reveal costs related to selection for Cd tolerance through selection. The intense selection for Cd resistance in these studies (*Drosophila* and Killifish), imposed in

relatively small laboratory populations, might in itself have contributed adverse life-history effects, and hence trade-offs. The FC scenario suggests the importance of considering effects of selection history. The accelerated selection response of FC lines to experimental selection by Cd suggests that a repeated pollution event in the field could hypothetically cause resistance to return rapidly from a localized standing source of low-cost variation. The strength of selection and type of event that could cause this to happen in the field require evaluation.

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Correction added after online publication August 3, 2009: On page 10, column 2, lines 38–45 were corrected.

Supporting Information

The following supporting information is available for this article:

Appendix S1: Frequency distribution of 16S haplotypes, showing site and collection period (years).

Supporting Information may be found in the online version of this article.

(This link will take you to the article abstract).

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