Published in final edited form as:

Aquat Toxicol. 2005 August 30; 74(2): 97–109. doi:10.1016/j.aquatox.2005.05.005.

Acclimation to Cu in fathead minnows: Does age influence the response?

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Abstract

This study had two primary objectives. The first was to determine if the length of exposure necessary for acclimation to Cu to develop in larval fathead minnows (*Pimephales promelas*) is different than that for juveniles. The second objective was to determine whether the acclimatory response, as determined by organism survival, is consistent with acclimation as determined by whole-body Na^+ . Six experiments were conducted: four using larval (<20-d-old) and two using juvenile (<60-d-old) fathead minnows. Within each experiment, fish were allocated to one of four groups: unexposed, continuously exposed, episodically exposed or naïvely exposed. The continuous group was exposed to a sublethal Cu exposure (125 $\mu\text{g/L}$) for 8, 12, 16 or 20 d and then subjected to a survival test at a lethal dose (375 $\mu\text{g/L}$). Fish in the episodic group were exposed to the sublethal dose for either 4 or 8 d, given a depuration period of varying lengths (4–16 d) then subjected to a survival test. Naïve minnows were maintained in clean water then given the survival challenge. Results from survival tests show that the larvae acclimate after only a 4-d sublethal exposure to Cu. In contrast, juveniles require a 16-d exposure to acclimate. Once acclimation had developed, there was a strong relationship between larval survival and whole-body Na^+ . Acclimated larvae maintained whole-body Na^+ relative to unexposed fish, while unacclimated larvae did not. Interestingly, this was not the case for juveniles, as acclimated and unacclimated groups did not differ with respect to whole-body Na^+ concentrations. The results of this study show that age influences the time course and possibly the mechanisms of acclimation in fathead minnows exposed to Cu.

Keywords

Acclimation; Copper; Fathead minnow; Organism age; Whole-body sodium

1. Introduction

Several toxicological studies have shown that fish exposed to sublethal levels of Cu are capable of acclimation (Dixon and Sprague, 1981; Buckley et al., 1982; Benson and Birge, 1985; Laurén and McDonald, 1987; Wilson and Taylor, 1992). Acclimation to Cu has been defined as the ability to tolerate typically lethal Cu exposures while maintaining normal physiological function (McDonald and Wood, 1993). Several factors influence the ability of a fish to acclimate to Cu including exposure duration and dose (Dixon and Sprague, 1981), as well as interspecies differences (McDonald and Wood, 1993). A potential modifying factor that is often overlooked in studies investigating acclimation is organism age or developmental stage. For example, in adult fathead minnow exposed to metals, acclimation occurs relatively slowly

(14 d) and attenuates rapidly (7 d) (Benson and Birge, 1985). This is also true for juvenile fathead minnows (Hobson and Birge, 1989). However, little is known about the acclimatory response of larval fathead minnows.

Whole-organism endpoints (i.e., survival) are typically used to determine if acclimation occurs; however, more reductionistic physiological endpoints may also be used as an indicator of acclimatory response. Whole-body Na^+ serves as one such indicator, as acclimation to Cu is typically accompanied by recovery of Na^+ ionoregulation and subsequently whole-body Na^+ levels (Laurén and McDonald, 1987; McGeer et al., 2000). Several studies have utilized whole-body Na^+ concentrations to determine the mechanisms underlying Cu tolerance and toxicity (Laurén and McDonald, 1987; Grippo and Dunson, 1991; Croke and McDonald, 2002; Kolok et al., 2002). Generally, it would be expected that if one biological indicator (e.g., survival) indicated tolerance, then other biological indicators (e.g., whole-body Na^+) would also be indicative of tolerance (Croke and McDonald, 2002). This expectation has only recently begun to be tested.

This study has two primary objectives. The first is to determine if the length of exposure necessary for acclimation to develop in larval fathead minnows is different than that for juveniles. The second objective is to determine whether the acclimatory response, as determined by organism survival, is consistent with acclimation as determined by whole-body Na^+ .

2. Materials and methods

2.1. Fish maintenance

All of the fish used in these experiments were produced by the University of Nebraska at Omaha fathead minnow colony according to the protocols outlined in Peake et al. (2004). Once the larvae hatched, they were transferred via pipette from 1-L hatching chambers to 2-L beakers containing 1.8 L of heated (26 °C) water. Each beaker contained 75 larvae to maintain a density of approximately 41 larvae/L. This density was justified based on Environmental Protection Agency guidelines suggesting a maximum density of 150 larvae/L for larvae up to 28-d-old (US EPA, 1994). Larvae in these beakers were either directly used in experiments or were raised to juveniles. After 3 weeks, fish raised to juveniles were transferred to 40-L aquaria. After 5 weeks the fish were gradually switched from their larval diet (1-d-old *Artemia* nauplii twice daily) to high-protein flake food (Aquatic Ecosystems, Apopka, FL, USA). One week prior to the initiation of the juvenile experiment, the water temperature was reduced from 26 °C to room temperature (approximately 23 °C).

Throughout all of the experiments, minnows were maintained in aerated, dechlorinated tap water and were kept under a photoperiod of 16 h light:8 h dark. Static renewal of water in the beakers or aquaria occurred daily. Renewal water had the following water quality—(mean $\pm$ S.E.): pH, 8.15 ± 0.01 ; conductivity, 789 ± 3 μS ; hardness (expressed as mg/L CaCO_3), 159 ± 3 ; alkalinity (expressed as mg/L CaCO_3), 73 ± 5 ; Ca, 38.8 ± 1.2 mg/L; Mg, 17.9 ± 0.8 mg/L; Na, 62.6 ± 5.5 mg/L; K, 7.1 ± 1.0 mg/L; Cl, 21.8 ± 1.9 mg/L and SO_4 , 181 ± 11 mg/L.

2.2. Exposure regime

2.2.1. Larvae—Four larval experiments were conducted. At the beginning of each experiment, minnows were allocated to one of four groups: unexposed, continuously, episodically or naïvely exposed (Fig. 1). Minnows in the unexposed group were not exposed to Cu throughout the duration of each experiment. Minnows in the continuous group served as a positive control and were exposed to a sublethal Cu dose (125 $\mu\text{g/L}$) followed by a 96-h lethal Cu dose (375 $\mu\text{g/L}$). The episodic group was exposed to the sublethal dose, given a

depuration period of varying length, and was then exposed to the lethal dose. Naïve minnows served as a negative control and were maintained in clean water, then subjected to the lethal dose. The only difference between the experiments was in the length of the depuration period, and consequently in the age of the larvae at the beginning of the lethal exposure. In experiment 1, the depuration period was 4 d, and the fish were 8-d-old at the initiation of the lethal exposure. Experiments 2–4 featured depuration periods of 8, 12, and 16 d, with the fish being 12-, 16- and 20-d-old at the beginning of the lethal exposure.

Each larval experiment used 280 larvae equally divided into one of the four experimental groups discussed above. Each 2-L beaker contained 70 newly hatched larvae (≤ 24 -h old) that were produced by a minimum of eight parental pairs. Larvae from each parental pair were evenly distributed among the experimental groups. The first two experiments were run concurrently, as were the second two experiments. During lethal exposures, dead larvae were counted and removed 3, 6, 12, 24, 48 and 96 h after initiation of the lethal exposure.

2.2.2. Juveniles—In the juvenile experiments, minnows were allocated to the same four groups as previously discussed for the larvae. The first juvenile experiment mirrored larval experiment 2 with the fish in the episodic group having a 4-d sublethal exposure, 8-d depuration and 4-d survival test (Fig. 1). The depuration period remained the same in the second juvenile experiment, but both the sublethal exposure duration and survival challenge were extended to 8 d. The sublethal exposure period was extended due to the lack of acclimation observed in the first experiment. The duration of the survival challenge was lengthened because in the first experiment there was very little mortality prior to 48 h.

The juvenile experiments used 200 fish equally divided into one of the four experimental groups. Each experimental group contained 50 juveniles produced by a minimum of nine parental pairs. Juveniles from each parental pair were evenly distributed among the experimental groups. Three 60-L aquaria were used for each experiment: clean (no Cu), sublethal (125 $\mu\text{g/L}$ Cu), or lethal (375 $\mu\text{g/L}$ Cu), which were further subdivided into compartments using plastic screens. Fish were transferred to treatment compartments once they reached between 46- and 52-d-old and had an average mass of 0.476 ± 0.005 g (S.E.). At initiation of the experiment, fish were placed into each of the unexposed and naïve compartments within the clean tank, and 50 fish were placed into each of the episodic and continuous compartments within the sublethal tank. At the end of the acclimation period, the episodically exposed fish were transferred into an additional compartment within the clean tank for a depuration period. Following the depuration period, the naïvely, episodically, and continuously exposed fish were transferred into the lethal tank for the survival trial as described above for the larval experiments. On days when aquarium transfer occurred, all of the fish were transferred from their home aquaria into the appropriate treatment, while fish that did not change aquaria were transferred into an empty adjacent compartment. In both experiments, dead fish were counted and removed 3, 6, 12, 24, 48 and 96 h after initiation of the lethal exposure. In experiment 2, dead fish were also counted and removed at 192 h.

2.3. Cu analysis

During the experiments, a water exchange ($>80\%$ of total volume in the larval exposures and $>30\%$ in the juvenile exposures) was conducted daily. A stock solution of CuSO_4 was used to manually dose all of the experimental tanks. Water samples (125 ml) were collected daily from each experimental aquarium or beaker. Each water sample was acidified with nitric acid to a pH of <2.0 and stored at 4°C . Actual Cu concentrations were determined by flame atomic absorption spectrometry according to previously established methods (US EPA, 1983). The actual Cu concentrations in the larval beakers averaged 113.1 $\mu\text{g/L}$ (90.5% of nominal) during sublethal exposures and 290.3 $\mu\text{g/L}$ (77.4% of nominal) during lethal exposures. The actual

Cu concentrations in the juvenile aquaria averaged 113.2 µg/L (90.6% of nominal) during sublethal exposures and 333.4 µg/L (88.9% of nominal) during lethal exposures.

2.4. Whole-body Cu and Na⁺

Following the 96-h lethal exposures, up to 20 surviving minnows from each group in each of the six experiments were sacrificed and weighed. Minnows were digested in 1 ml of concentrated nitric acid (Grippo and Dunson, 1991) and diluted with nanopure water. Larvae were analyzed for Cu using graphite furnace atomic absorption spectrometer. Whole-body Cu was measured in larvae, but not in juveniles.

Following the initial dilution, a sample was taken from each solution, diluted with a potassium chloride solution (2200 ppm) and then analyzed for Na⁺ with a flame atomic absorption spectrometer. Whole-body Na⁺ concentrations were obtained on all fish except for the 8-d-old larvae, which were too small for reliably measuring whole-body Na⁺.

2.5. Statistical analyses

Survival-time analysis (Dixon and Newman, 1991) was performed using the statistical program JMP 5.0 to evaluate larval and juvenile survival. The times at which individual deaths were recorded (time-to-death) were fit to models describing the relationship between survival and time of exposure to Cu. Minnows alive at the end of the exposures were recorded as right-censored. During each experiment, all of the minnows in the unexposed group were alive and therefore recorded as right-censored. Due to this lack of data for the unexposed groups, they could not be included in the survival time analysis. The various models for distributions of the time-to-death data (Weibull, log-normal or exponential) were all compared for each experiment, and the one with the greatest log-likelihood value was taken to be the model of best fit. Models assuming a Weibull distribution were selected for the 8-, 12- and 16-d-old larval experiments, while the model incorporating the log-normal distribution was selected for the 20-d-old larval experiment. In the first juvenile experiment, a model could not be selected because none of the three models fit the data. In the second juvenile experiment, the model assuming an exponential distribution was selected. The models included one covariate—exposure group. The effects of the covariate on survival time were tested by likelihood ratio tests, in which log-likelihoods of the model with and without the covariate were computed and the results were compared against a chi-square distribution.

Differences in whole-body Cu and Na⁺ between exposure groups were tested using one-way analysis of variance (ANOVA, Statview 5.0) followed by Newman–Keuls multiple comparison tests. Statistical significance throughout the experiments was assumed at $P \leq 0.05$.

3. Results

3.1. Acclimatory response in larvae

For each larval experiment, survival plots (Fig. 2) were generated and survival-time analysis was conducted to compare the survival of the naïve and episodic groups to that of the continuous group. In each experiment, the survival of the naïve group was significantly lower than that of the continuous group ($P < 0.001$ in each case; Table 1). In the 8-, 16- and 20-d-old experiments, the survival of the episodic group was significantly less than the survival of the continuous group ($P < 0.0001$ in each case; Table 1). However, in the 12-d-old experiment, there was no significant difference between the survival of the continuous and episodic groups ($P = 0.212$; Table 1).

Survivorship among the naïvely exposed larvae was not the same for all of the different age groups. Eight-day-old naïve larvae had significantly reduced survival relative to 12-, 16- and 20-d-old naïve larvae ($P < 0.001$ in each case; Table 2).

3.2. Acclimatory response in juveniles

For the juvenile experiments, survival plots (Fig. 3 and Fig. 4) were generated and survival-time analysis was conducted to compare the survival of the naïve and episodic groups to that of the continuous group. In experiment 1, the survival of the three experimental groups was virtually 100% until 48 h. From 48 to 96 h, significant mortality occurred; however, attempts to fit a model distribution to these curves were not successful (see Section 2). In experiment 2, the survival of fish in the naïve and episodic groups was significantly lower than that of fish in the continuous group ($P < 0.02$; Table 3).

3.3. Larval whole-body Cu

Whole-body Cu was measured in the larval, but not in the juvenile experiments. In each of the four larval experiments, the whole-body Cu of unexposed larvae was significantly lower than that of continuously exposed, episodically exposed and naïvely exposed larvae (Fig. 5). For 8-, 16- and 20-d-old larvae, no other significant differences occurred among the treatment groups. In the 12-d-old larval experiment, the whole-body Cu concentrations of episodically and naïvely exposed minnows were not significantly different from each other, but both groups had significantly lower concentrations than continuously exposed minnows.

3.4. Larval whole-body Na^+

The average whole-body Na^+ of unexposed larvae from the 12-, 16- and 20-d-old experiments was 51.4 ± 1.3 $\mu\text{mol/g}$ (S.E.), 47.9 ± 0.9 $\mu\text{mol/g}$ and 52.1 ± 1.2 $\mu\text{mol/g}$, respectively (Fig. 6). In all larval experiments, the whole-body Na^+ of continuously exposed larvae was never significantly less than that of controls. In contrast, the whole-body Na^+ of naïve fish was significantly lower than that of the unexposed and continuously exposed fish in each experiment. Whole-body Na^+ of 12-d-old episodically exposed fish was not significantly different from that of 12-d-old unexposed or continuously exposed fish. However, 16- and 20-d-old episodically exposed larvae had significantly reduced levels of whole-body Na^+ relative to unexposed and continuously exposed larvae.

3.5. Juvenile whole-body Na^+

The average whole-body Na^+ of unexposed fish in experiments 1 and 2 was 53.7 ± 1.3 (S.E.) $\mu\text{mol/g}$ and 47.2 ± 1.6 $\mu\text{mol/g}$, respectively (Fig. 7). In both experiments, whole-body Na^+ concentrations of continuously exposed, episodically exposed and naïvely exposed minnows were significantly lower than that of the unexposed minnows, but were not significantly different from one another.

4. Discussion

The first objective was to determine if the length of exposure necessary for acclimation to develop in larval fathead minnows is different than that for juveniles. The second objective was to determine whether the acclimatory response, as determined by organism survival, is consistent with acclimation as determined by whole-body Na^+ . The results of this study show that larvae and juveniles differ in their ability to acclimate. Larvae develop acclimation after only a 4-d sublethal exposure, while in contrast, juveniles require a 16-d exposure. Furthermore, acclimated larvae were able to maintain normal whole-body Na^+ concentrations when subjected to a survivability test. Interestingly, this was not the case for juveniles.

4.1. Development and persistence of acclimation in larvae and juveniles

In each larval experiment, survival of continuously exposed fish was significantly higher than that of naïvely exposed fish indicating that fish in the continuous group had acclimated (Fig. 2; Table 1). Furthermore, these results indicate that an 8-d continuous exposure to Cu induces acclimation in larval fathead minnows. At 12-d-old, the survival of larvae in the episodic group did not differ from the survival of larvae in the continuous group. This demonstrates that larvae given a 4-d sublethal exposure to Cu acclimate to it. This rapid development of metal acclimation has been documented for at least one other larval fish species; Chang et al. (1998) found that tilapia larvae (*Oreochromis mossambicus*) were acclimated to Cd after a 3-d subacute exposure.

In contrast to the results from larvae, juvenile fathead minnows showed no evidence of acclimation when exposed for 12 d, but were acclimated after 16 d of exposure (Fig. 3 and Fig. 4; Table 3). This is consistent with the findings of previous studies. Fathead minnows were found to require a 14-d exposure for acclimation when juveniles were exposed to Zn (Hobson and Birge, 1989). Adult fathead minnows also appear to require a long exposure to acclimate. For example, Benson and Birge (1985) found acclimation that adults required a 14-d exposure to Cd for acclimation to occur.

While the onset of acclimation appears to occur more rapidly in larval fathead minnows relative to juveniles and adults, the rate of subsequent attenuation of acclimation appears to be similar. Twelve-day-old episodically exposed larvae were still acclimated following an 8-d depuration period. In contrast, 16- and 20-d-old larvae given 12- and 16-d depuration periods, respectively, had not acclimated relative to continuously exposed fish. These results suggest that acclimation in larvae persists for 8 d after return to clean water, but attenuates within 12 d. These findings are consistent with several studies conducted on other fish species. Dixon and Sprague (1981) found that acclimation of rainbow trout parr to Cu attenuates within 7 d following transfer to clean water. Similarly, adult fathead minnows acclimated to Cd and given a depuration period of 7d were not capable of maintaining their acclimatory response (Benson and Birge, 1985). Likewise, juvenile rainbow trout (*Salmo gairdneri*) acclimated to Zn lose acclimation after a 7-d depuration period (Bradley et al., 1985; Anadu et al., 1989).

4.2. Cu sensitivity at different larval ages

Eight-d-old larvae episodically exposed to Cu had significantly lower survival than continuously exposed fish suggesting that these fish were not acclimated (Fig. 2; Table 1). One possible explanation for this is that episodic exposures do not induce an acclimatory response sufficient enough to overcome a heightened Cu sensitivity in the 8-d-old larvae. A comparison of the survival of naïvely exposed larvae in each age group revealed that the survival of naïve 8-d-old larvae was significantly lower than the survival of naïve larvae at 12-, 16- or 20-d-old suggesting that Cu sensitivity is age-dependent (Table 2). This finding is consistent with studies examining the age-specific sensitivity of larvae exposed to various metals. At 7-d-old, mummichogs (*Fundulus heteroclitus*) are more sensitive to Cd than at 1- or 14-d-old (Middaugh and Dean, 1977). Similarly, Wright et al. (1985) reported that 7-d-old striped bass (*Morone saxatilis*) had increased Cd sensitivity relative to 6-week-old bass. Hwang et al. (1995) found that 3-d-old tilapia larvae were more sensitive to Cd than 0-, 1-, 2- or 7-d-old larvae. Age-specific differences in metal sensitivity are most likely related to developmental differences between fish of different ages. McNulty et al. (1994) suggested that larvae are most sensitive to metal exposures as they transition from cutaneous to branchial respiration. During this transition period, larvae may experience an increase in Cu uptake because both respiratory pathways are being utilized concurrently. Transition from cutaneous to branchial respiration and the associated increase Cu uptake may be the explanation for the increased Cu sensitivity measured in the 8-d-old larvae in this experiment.

4.3. Larval whole-body Cu

In the larval experiments, the whole-body Cu of naïvely, episodically and continuously exposed fish did not differ, except for in the 12-d-old experiment where the continuously exposed fish had significantly higher whole-body Cu concentrations than the naïvely exposed fish (Fig. 5). It is interesting that fish with the same body burden of Cu differ so dramatically in terms of survival. The absolute amount of Cu accumulated in the larvae was not correlated with tolerance. A study by Hobson and Birge (1989) supports this finding. In their study, it was determined that whole-body Zn concentrations were not correlated with acclimation-induced tolerance in fathead minnows. However, other studies have yielded conflicting results. Cu-tolerant fathead minnows accumulate significantly higher whole-body Cu concentrations than Cu susceptible fish (Kolok et al., 2002), while Cd-tolerant killifish (*Heterandria formosa*) have significantly lower levels of whole-body Cd relative to susceptible fish (Xie and Klerks, 2004). A possible explanation for these discrepancies is that the toxic mechanisms of Cu, Cd and Zn differ. Cd and Zn impair Ca^{2+} ionoregulation, while Cu impairs Na^{+} ionoregulation (Reid and McDonald, 1988; Hogstrand et al., 1994). While this may explain some of the inconsistencies in the literature, in general the relationship between whole-body metal concentration and metal tolerance in subacutely exposed fish remains unclear.

4.4. Whole-body Na^{+}

Acclimation to Cu arises via recovery of ionoregulatory function at the gills where physiological alterations occur to restore Na^{+} influx (Laurén and McDonald, 1987; McGeer et al., 2000). Several studies have shown that acclimated fish maintain whole-body Na^{+} concentrations relative to unexposed fish. Laurén and McDonald (1987) found that upon initial exposure to subacute Cu concentrations, a reduction in whole-body Na^{+} occurred. However, 7 d after initiation of the exposure, whole-body Na^{+} returned to pre-exposure concentrations. Similarly, McGeer et al. (2000) found that following an initial decline in whole-body Na^{+} , rainbow trout sublethally exposed to Cu, Cd or Zn showed complete restoration of whole-body Na^{+} concentrations.

The results obtained on larval whole-body Na^{+} in our study are in line with previous studies. When subjected to a potentially lethal survival test, continuously exposed larvae maintained whole-body Na^{+} relative to unexposed larvae (Fig. 6). In contrast, naïvely exposed larvae experienced a significant reduction in whole-body Na^{+} . Episodically exposed larvae maintained whole-body Na^{+} levels similar to those of unexposed larvae at 12-d-old, but not at 16- or 20-d-old. These results support the findings of the 96-h survival tests, which indicate that the continuously exposed larvae and 12-d-old episodically exposed larvae are acclimated while the remaining groups are not. For larvae, the ability to maintain whole-body Na^{+} during a survivability test, directly correlates with the fishes ability to acclimate.

With respect to juveniles, the only acclimated fish were the continuously exposed fish from experiment 2. These fish had significantly greater survival when challenged with an 8-d survivability test, but failed to maintain whole-body Na^{+} (Fig. 7). In juvenile fathead minnows exposed to Cu, whole-body Na^{+} does not correlate well with the ability to acclimate. This difference between larvae and juvenile whole-body Na^{+} following a survival test further underscores our contention that the acclimatory response of juvenile fathead minnows is different than that of larvae.

Acknowledgements

This study was partially supported by the University of Nebraska at Omaha (UNO) Department of Biology, the UNO Graduate College and by the Nebraska Water Center. Additional support was given by NIH National Institute of Environmental Health Science, Grant #1 R15 ES11788-01 and by NIH Grant #1 P20 RR16469 from the BRIN Program

of the National Center for Research Resources. Its contents are solely the responsibility of the authors and do not necessarily represent the views of any of the funding agencies.

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Figure 1.

Exposure regimes for minnows in the naïve (N), continuous (C) and episodic (E) treatment groups. Exposure concentrations are represented as follows: solid line (—), sublethal Cu exposure (125 µg/L); dashed line (---), no Cu exposure; dotted line (·····), lethal exposure (375 µg/L).

Figure 2. Cumulative survivorship for continuous (—), episodic (---) and naïve (····) groups from the 8-, 12-, 16- and 20-d-old larval experiments. Lines extending beyond 96 h represent larvae that survived the 4-d test.

Figure 3. Cumulative survivorship for continuous (—), episodic (- - -) and naïve (····) groups of juvenile experiment 1. Lines extending beyond 96 h represent larvae that survived the 4-d test.

Figure 4. Cumulative survivorship for continuous (—), episodic (- - -) and naïve (····) groups of juvenile experiment 2. Lines extending beyond 192 h represent larvae that survived the 8-d test.

Figure 5. Whole-body Cu for unexposed, continuous, episodic and naïve groups from the 8-, 12-, 16- and 20-d-old larval experiments. Numbers in brackets denote the number of larvae in each group. Error bars represent positive standard error.

Figure 6. Whole-body Na^+ for unexposed, continuous, episodic and naïve groups from the 8-, 12-, 16- and 20-d-old larval experiments. Numbers in brackets denote the number of larvae in each group. Error bars represent positive standard error.

Figure 7. Whole-body Na^+ for unexposed, continuous, episodic and naïve groups from juvenile experiments 1 and 2. Numbers in brackets denote the number of juveniles in each group. Error bars represent positive standard error.

Table 1

Summary of survival-time analysis for the larval experiments

Experiment	Depuration period (d)	Parameter	χ^2	P-value
8-d-old	4	Intercept	1255.780	<0.0001
		Group		
		Naïve	38.509	<0.0001
12-d-old	8	Episodic	41.242	<0.0001
		Intercept	366.103	<0.0001
		Group		
16-d-old	12	Naïve	17.540	<0.0001
		Episodic	1.556	0.212
		Intercept	1021.775	<0.0001
20-d-old	16	Group		
		Naïve	13.363	0.0003
		Episodic	17.318	<0.0001
		Intercept	1167.380	<0.0001
		Group		
		Naïve	33.007	<0.0001
		Episodic	35.617	<0.0001

Chi-square (χ^2) values are from likelihood ratio tests of the effect of treatment group, and associated probabilities are based on one degree of freedom each.

Table 2

Summary of survival-time analysis for the naïve larvae at 8-, 12-, 16-and 20-d-old

Parameter	χ^2	P-value
Intercept	704.818	<0.0001
Age (d)		
12	6.954	0.0084
16	36.345	<0.0001
20	18.726	<0.0001

Chi-square (χ^2) values are from likelihood ratio tests of the effect of treatment group, and associated probabilities are based on one degree of freedom each.

Table 3

Summary of survival-time analysis for the juvenile experiment 2

Parameter	χ^2	P-value
Intercept	673.020	<0.0001
Group		
Naïve	8.945	0.0028
Episodic	5.501	0.0190

Chi-square (χ^2) values are from likelihood ratio tests of the effect of treatment group, and associated probabilities are based on one degree of freedom each. Data from juvenile experiment 1 are not given because a model could not be fit to the data (see text).