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Natural variability in *Drosophila* larval and pupal NaCl tolerance

Craig A. L. Riedl^{*}, Sara Oster, Macarena Busto^{*}, Trudy F. C. Mackay[‡], and Marla B. Sokolowski^{*,#}

^{*}Biology Dept., University of Toronto at Mississauga, 3359 Mississauga Rd., Mississauga, Ont., L5C 1J6, Canada

[‡]Department of Biological Sciences, Box 7614, North Carolina State University, North Carolina, 27695, USA

[#]Department of Ecology and Evolutionary Biology, 25 Willcocks St., University of Toronto, Toronto, Ontario, M5S 3B2, Canada

Abstract

The regulation of NaCl is essential for the maintenance of cellular tonicity and functionality, and excessive salt exposure has many adverse effects. The fruit fly, *Drosophila melanogaster*, is a good osmoregulator and some strains can survive on media with very low or high NaCl content. Previous analyses of mutant alleles have implicated various stress signaling cascades in NaCl sensitivity or tolerance; however, the genes influencing natural variability of NaCl tolerance remain for the most part unknown. Here, we use two approaches to investigate natural variation in *D. melanogaster* NaCl tolerance. We describe four *D. melanogaster* lines that were selected for different degrees of NaCl tolerance, and present data on their survival, development, and pupation position when raised on varying NaCl concentrations. After finding evidence for natural variation in salt tolerance, we present the results of Quantitative Trait Loci (QTL) mapping of natural variation in larval and pupal NaCl tolerance, and identify different genomic regions associated with NaCl tolerance during larval and pupal development.

Abstract

Corresponding Author: Marla B. Sokolowski, Ph.D. F.R.S.C., Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks St., Toronto, Ontario, M5S 3B2, Canada. marla.sokolowski@utoronto.ca.

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Keywords

QTL; NaCl; natural variation; *Drosophila melanogaster*; pupation position

Introduction

The regulation of NaCl concentrations is critical for many cellular and tissue processes including proper nervous and muscle functioning. Reflecting this importance, many animals have evolved the ability to taste salt, and thus better regulate their intake (Skøtt 2003). While much research has been directed at identifying the mechanisms underlying salt tolerance in agriculturally or epidemiologically relevant species (Yamaguchi and Blumwald 2005; Sreenivasulu *et al.* 2007; Munns *et al.* 2006; Colmer *et al.* 2006; Cuartero *et al.* 2006; Coetzee and Sueur 1988; Bell *et al.* 1999; Phang *et al.* 2008; Li *et al.* 2011; Dodd and Perez-Alfocea, 2012), such organisms are often not ideal for laboratory study. The fruit fly, *Drosophila melanogaster*, however, has long been established as an excellent model organism for the study of Genetics and it has many genes and molecular systems that are structurally and functionally conserved in other invertebrates as well as vertebrates. *D. melanogaster* can survive on media with very low NaCl concentrations; in fact, flies can be cultured on defined media with no added NaCl, (*i.e.* it is present only as a contaminant of other ingredients: see Loeb 1915, Kalmus 1943). Furthermore, some strains express enhanced viability when reared under elevated NaCl conditions that are highly detrimental to other strains (Waddington 1959; Croghan and Lockwood 1960; Miyoshi 1961). Previous experiments have used selection to generate salt-tolerant strains, indicating that the underlying variation has a genetic component (Waddington 1959; Miyoshi 1961; Wallace 1982).

Elevated environmental NaCl concentrations may disrupt osmotic balance and thus impose stress on exposed organisms (Miyoshi 1961; Davies *et al.* 2014). Studies of the various cellular responses to environmental stresses, including salt and osmotic stress, have identified several stress-response signaling cascades such as p38 and JNK MAPK (Kyriakis *et al.* 1994; Kyriakis and Avruch 1996; Han S.-J. *et al.* 1998; Stronach and Perrimon 1999; Inoue *et al.* 2001; Craig *et al.* 2004; Gonzalez-Yanes *et al.* 2005; Bartels and Sunkar 2005; Sun *et al.* 2006; Coulthard *et al.* 2009; and see Davies *et al.* 2014 for review). The p38 kinase cascade, for example, represents a very ancient stress response system that is widely conserved across yeasts, plants, and animals (Brewster *et al.* 1993; Han S.-J. *et al.* 1998; Han Z. S. *et al.* 1998; Kültz 1998; Martín-Blanco 2000; Berman 2001; Kyriakis and Avruch 2001; Solomon *et al.* 2004; Teige *et al.* 2004; Lee *et al.* 2010; Xu *et al.* 2013). JNK MAPK signaling systems facilitate the transmission of information from the extracellular environment into the nucleus to induce transcriptional changes; it is therefore not surprising that many members of such cascades also play significant roles in development (Stronach and Perrimon 1999; Martín-Blanco 2000; Nebreda and Porras 2000; Shen *et al.* 2003; Wagner and Nebreda 2009). Similarly, Overend *et al.* (2012) showed that the gunylate clycase receptor Gyc76C and a peptide ligand, NPLP₁-VQQ modulate the innate immune pathway in response to salt stress. Such pleiotropic effects, however, may complicate

traditional mutagenesis-based genetic analysis of the stress response traits such as mutants suffering from developmental abnormalities.

While the study of *D. melanogaster* mutants has been extremely successful in identifying roles of genes in many processes, the influence of these genes on the maintenance of natural phenotypic variation is often unclear. Natural variation of a trait within a species may result from subtle additive genetic effects. The genes involved in such natural variation are often more difficult to identify than genes affected by an induced mutation that has a very pronounced effect (Greenspan 1997; 2004). Elucidating those genes that harbor natural variants with direct contributions to phenotypic variations could provide guidance to researchers seeking to intervene in a signaling cascade while minimizing detrimental effects on other systems.

In this paper we use two approaches to investigate natural variation in *D. melanogaster* NaCl tolerance. In the first approach, we present our studies of a set of 4 strains that were selected over many years for varying degrees of salt tolerance. These strains were selected to survive on media comprised of 5%, 6%, 7.5%, or 9.5% NaCl by weight (for comparison, seawater averages approximately 3.5%). This demonstrates how natural genetic variation can be manipulated to elicit extreme phenotypic malleability; indeed, three of the strains can survive well on media up to 11 % NaCl by weight! We assay the strains for three traits: survival to adulthood, pupation latency, and pupation height on different media with a range of NaCl concentrations (from 0.05%, standard lab media, to 11% by weight). We expected the salt stress to result in delayed development and reduced viability and because *D. melanogaster* pupation site selection is sensitive to many environmental effects (Rizki and Davis 1953; Sokal *et al.* 1960; Markow 1979; Schnebel and Grossfield 1986; Guo *et al.* 1991; Rodriguez *et al.* 1992; Paranjpe *et al.* 2004; Riedl *et al.* 2007), we also predicted a difference in this behavior in response to salt stress. Specifically, we predicted that larvae from the salt selected strains, being better osmoregulators, would also be more tolerant of, or resistant to, the desiccating effects of the high-salt conditions and would therefore pupate farther from the surface of the food.

Given the above evidence for natural variation in salt tolerance, we employ a second approach, Quantitative Trait Loci (QTL) mapping, to explore the genetic architecture of variability in larval and pupal NaCl tolerance among a panel of recombinant inbred (RI) lines (Nuzhdin *et al.* 1997). These same RI lines have been used to identify QTL for a variety of phenotypes, including sensory bristle number (Gurganus *et al.*, 1998), sex combs tooth number (Nuzhdin and Reiwitich, 2000), courtship signal (Gleason *et al.* 2002), olfactory behavior (Fanara *et al.*, 2002), flight, respiration, energy metabolism (Montooth *et al.*, 2003), and adult longevity in various environmental contexts (Leips and Mackay, 2000; Nuzhdin *et al.*, 1997; Viera *et al.*, 2000).

Here, we focused our QTL analysis specifically on larval and pupal salt tolerance. *D. melanogaster* is a holometabolous insect with four distinct life phases: embryo, larva, pupa, and imago (adult). The first three developmental stages have defined durations of up to a few days at 25°C, and therefore are very convenient for survival studies as opposed to the longer-lived adults. Furthermore, unlike embryonic development which occurs in relative

isolation within the chorion, *Drosophila* larvae are highly exposed to their environment as they forage and feed on the food media. As a pupa, the animal is again relatively insulated from external conditions, but its survival is strongly dependent on its previous experiences as a foraging larva, including the quantity and quality of the food ingested and its pupation position. Thus, larval and pupal development was deemed most amenable to the QTL analysis which involves the simultaneous analysis of many RI strains on various salt media.

Materials and Methods

Selected NaCl tolerant strains

Four strains of NaCl tolerant flies were generated and kindly provided to us by J.S.F. Barker (University of New England, NSW, Australia). A brief description of the selection regime is as follows. On 16 Feb., 1966, two population cages, each containing approximately 1000 adults, were started on media containing 2% NaCl by weight. There are no known records of the originating strain. On 9 Mar., 1966, adults from one of these populations were transferred to media containing 5% NaCl by weight and were maintained on 5% NaCl media thereafter (strain 'Bss5'). Adults from the other population were transferred to media containing 0.5% increases, at approximately monthly intervals, in NaCl concentration until reaching 5% NaCl on 22 June, 1966. On that date, the population was divided with 500 adults used to start a third selected line. Selection continued on these two populations with increasing NaCl in 0.5% increments until the 6% selected strain ('Bss6') was stabilized and maintained at 6% NaCl from 9 Dec., 1966. The population was divided and NaCl levels were gradually increased in one group until reaching 7.5% on 18 Dec., 1967. This 7.5% NaCl selected strain ('Bss7.5') was again divided on 22 Sept., 1978 when a sample were transferred to media comprised of 8.5% NaCl. NaCl levels in this branch were increased in 0.5% increments until being stabilized at 9.5% on 3 Jan., 1979 (strain 'Bss9.5').

In summary, whereas all strains originated from the same original population, the 6%, 7.5%, and 9.5% lines all derive from one founder cage of 1000 adults, and the 5% selected line originate from another. The Barker 5% salt selected line (Bss5) has been continuously reared on media containing 5% (or 0.86 M) NaCl since 9 Mar., 1966, the Barker 6% salt selected line (Bss6) has been reared on 6% (or 1.03 M) NaCl media since 9 Dec., 1966, the Barker 7.5% salt selected line (Bss7.5) has been reared since 18 Dec., 1967 on 7.5% (or 1.28 M) NaCl media, and finally, the 9.5% selected line (Bss9.5) has been reared since 3 Jan., 1979 on media containing 9.5% (or 1.62 M) NaCl.

The salt selected lines were reared on salt-supplemented semolina-treacle-yeast medium (Claringbold and Barker, 1961) until 1995 when they were acquired by the Sokolowski lab and henceforth reared on NaCl-supplemented yeast-agar-sucrose media (described below).

Prior to the experiments described herein, subject flies from all salt tolerant lines were maintained for two generations on a standard yeast-agar medium, which is 0.05% NaCl by weight (or 0.0086 M).

Wild-type strains

Because the original control strain for the salt selected lines cannot be established with certainty, we used two unrelated wild-type strains, Canton-S and our standard Natural Rover strain (de Belle and Sokolowski, 1987), as controls.

Recombinant inbred (RI) lines

The recombinant inbred (RI) lines were generated by, and are described in, Nuzhdin *et al.*, 1997. Briefly, two unrelated parental lines, Oregon R and 2b (Pasyukova and Nuzhdin, 1993), each with multiple *roo* transposable element insertions (45 and 47 respectively) spread across the three major chromosomes, were crossed. The Oregon R-derived chromosome 4 was marked with the recessive mutation *spa^{pol}*. F₁ progeny were backcrossed to 2b and the resulting progeny were randomly intermated for 4 generations. At generation 5, random pairs were isolated and mated and the RI lines were produced by 25 generations of full-sib matings within their progeny followed by a further 10 generations of small mass matings of 20 pairs each (Nuzhdin *et al.* 1997). The presence or absence of each of the 92 *roo* elements or the *spa* mutation provides information regarding the ancestral origin of the corresponding chromosomal regions in the RI lines and thus, *spa* and the *roo* elements were used as molecular markers for QTL mapping. QTL mapping was performed on the pupal and larval survival traits of RI strains reared on media containing approximately 0.05% (standard lab media) 1.75% and 3.8% NaCl.

Fly rearing conditions

Unless otherwise stated, flies were reared on standard yeast-agar-sucrose media (see recipe below) at 25°C, 12hr light : 12hr dark, with lights on at 08:00, at ambient pressure and humidity.

Standard yeast-agar-sucrose and NaCl-supplemented media

Food media containing different NaCl concentrations were made by adding varying amounts of salt to the base food recipe before autoclaving and mixing with a sterile yeast/water solution. To make 1L of the standard yeast-agar-sucrose media (approx. 0.05% or 0.0086M NaCl), the base food recipe contains: 800mL water, 100g sucrose, 16g agar, 8g KNa tartrate (C₄H₄KNaO₆), 1g KH₂PO₄, 0.5g each of NaCl, MgCl₂, CaCl₂, and Fe₂(SO₄)₃; and after autoclaving, this base mixture is combined with an autoclaved solution of 50g baker's yeast dissolved in 200mL water. Five different high NaCl media, 5% (0.856M), 6% (1.027M), 7.5% (1.283M), 9.5% (1.626M), 11% (1.882M), were used in the pupation distance experiments were made with modifications of the base recipe. Also, intermediate (1.75% or 0.3M) and high (3.8% or 0.65M) NaCl media were made for the QTL mapping experiments. All percentages refer to the amount of NaCl by weight of the media.

Pupation latency, survival, and pupation height assays of salt-selected strains

The 4 Bss strains and two wild-type controls (Natural Rover and Canton-S) were assessed on a range of elevated NaCl media. Media of six salt concentrations were used: standard media (0.05% b/w, 0.0086M), 5% (0.86M), 6% (1.03M), 7.5% (1.28M), 9.5% (1.62M), or 11% (1.89M). For each strain, 10 first instar larvae (0 to 4 hours post-hatch) were placed

into glass vials containing 6mL of carefully poured (such that there were no bubbles in the media and no media on the vial walls) yeast-agar (1.3% agar) culture media. Care was taken not to puncture the media as the larvae were placed on the surface. Each vial was plugged with a cotton ball, leaving approximately 65mm between the bottom of the cotton plug and the food surface. Five vials were prepared per strain and vials were randomized in racks across strains. All test vials were kept in the same incubator with lights attached directly above each shelf. Vials containing only media were placed around the outer edge of the racks ensuring that all test vials were surrounded by vials containing media; this reduced potential vial position effects due to uneven lighting. The vials were left undisturbed in an incubator at 25°C, 12 hour light:dark cycle with lights on at 08:00, until pupation. Mean survival (proportion of plated larvae that eclosed as adults, per replicate vial) and mean pupation latency (day post-plating that pupae were first observed, per replicate vial) were calculated per strain per salt treatment. Strain-by-treatment means were then calculated from the vial means and used in the comparative analyses. Pupation position was measured as described in Bauer and Sokolowski (1985). Once all the larvae had pupated, the distances between the anterior pupal spiracles and the food surface were measured, and mean distances within each vial were calculated. Strain pupation height means were calculated using 3 to 5 vials of 2 to 10 pupae each.

NaCl tolerance assays of RI lines

First instar larvae (2±2 hr post-hatch) were collected from each of the RI lines and placed in plastic culture vials containing 10mL of test media. Test media contained either standard (0.05% NaCl), intermediate (1.75% NaCl), or high (3.8% NaCl) salt media (described above under 'NaCl media'). Four vials of ten larvae were assayed on each test media for each RI line. Vials were then randomized across treatment and strain into bundles of twelve which were placed in an incubator at 25°C under a 12:12 light:dark regime at ambient pressure and humidity. Vials containing no larvae (media only) were placed around the outside of every bundle to reduce differences in light exposure among test vials. Upon pupation, the numbers of pupae per vial were counted and these values were used to calculate the larval survival of each strain on each salt treatment. After the pupae were counted, the vials were returned to the incubator and left until adults eclosed. Numbers of adults per vial were counted and compared to the numbers of pupae to calculate the pupal survival per strain per treatment. In some cases there were not enough larvae to test all treatments for all strains. 84 RI lines were assayed on the low (standard) NaCl media, 71 lines were tested on the intermediate NaCl media, and 87 lines were assayed on the high NaCl media. All experiments on the RI lines were completed in late 2001, 4-5 years after the lines were first published (Nuzhdin *et al.*, 1997).

QTL analysis

The genomic marker positions (*i.e.* the cytological insertion sites of the transposable *roo* elements) were mapped relative to each other using the recombination frequencies derived from the set of RI lines used in this study as compared to the two parental strains (Oregon R and 2b). QTL analysis was performed using QTL Cartographer (v. 1.17e) software (Basten *et al.* 1994; Basten *et al.* 2003a), as described in (Riedl *et al.* 2007). Specifically, a Kosambi mapping function was applied to the recombination map and composite interval mapping

(Zeng, 1994) was used. The likelihood ratio (LR) was calculated as $LR = -2 \ln(L_0/L_1)$ where L_0/L_1 is the ratio of the likelihood under the null hypothesis (no QTL in the interval) to the likelihood under the alternate hypothesis (that a QTL is present in the interval), and a conditioning window of 10cM was used. The 5% and 2.5% thresholds of significance was determined by either 10000 or 20000 random permutations of the data (Churchill and Doerge, 1994; Doerge and Churchill, 1996) (for further reference see the QTL Cartographer user's manual (Basten *et al.*, 2003)).

Statistics

Statistical analyses were performed using SAS software (SAS Institute, 1998). The General Linear Models (GLM) Procedure was used to analyze the pupation heights and survival of the salt-selected lines on various media, and the larval and pupal survival of the RI lines on the three test media. Significant differences were identified using the Student-Newman-Keuls (SNK) *post hoc* test (SAS Institute, 1998). $P=0.05$ was considered significant.

Results

Pupation latency, survival, and pupation position of salt selected lines

We assayed the development time (latency to pupate), survivorship (proportion of larvae surviving to adulthood), and the pupation position of the four salt selected lines as well as two wild-type strains (Canton-S and the lab standard natural rover strain) on a collection of media substrates with varying NaCl concentrations (0.05%, 5%, 6%, 7.5%, 9.5%, and 11% NaCl). High media NaCl concentrations impose an environmental stress that can affect fluid balance. Therefore, we hypothesized that selection for salt tolerance would concomitantly affect differences in behaviors responsive to such stresses. One such behavior is larval pupation position, which is influenced by various environmental cues such as humidity, light, and temperature (Rizki and Davis 1953; Sokal *et al.* 1960; Markow 1979; Schnebel and Grossfield 1986; Guo *et al.* 1991; Rodriguez *et al.* 1992; Paranjpe *et al.* 2004).

No larvae from either of the wild-type strains survived to adulthood on media having 5%, or greater, NaCl concentrations. Consequently, the responses of these strains cannot be directly compared to the salt-selected strains on elevated salt media. However, we can compare and correlate the responses of the salt-tolerant lines with the level of salt tolerance as an indication of phenotypic responses associated with the selection.

Latency to pupation increased with increasing salt concentrations (Figure 1a). The strain selected for highest salt tolerance (Bss9.5) exhibited the lowest increase in latency across media treatments. Generally, the observed increase in latency to pupation across salt treatments is inversely correlated with the degree of salt tolerance. This trend is most evident when the strains are reared on media containing 9.5% NaCl, or greater. On standard media ("0.05%"), there were no significant differences between the pupation latencies of the salt-selected or wild-type strains. However, Bss9.5 showed significantly lower latencies to pupate than other strains when reared on media containing 7.5% NaCl or greater (ANOVA/SNK, 0.05% NaCl: $F_{5,24} = 1.68$, $p = 0.18$; 5% NaCl: $F_{3,16} = 2.67$, $p = 0.08$; 6% NaCl: $F_{3,16} =$ not applicable (first pupae appeared in all sample vials on day 6); 7.5% NaCl: $F_{3,16} = 6.48$,

$p < 0.01$; 9.5% NaCl: $F_{3,16} = 47.11$, $p < 0.01$; 11% NaCl: $F_{3,16} = 15.81$, $p < 0.01$). Furthermore, Bss9.5 larvae expressed no significant delay in their pupation latency until NaCl levels reached 9.5% or greater, whereas all other strains experienced significant increases in pupation latency when reared on any salt media compared to standard media (ANOVA/SNK, Bss5: $F_{5,24} = 171.43$, $p < 0.01$; Bss6: $F_{5,24} = 152.32$, $p < 0.01$; Bss7.5: $F_{5,24} = 67.07$, $p < 0.01$; Bss9.5: $F_{5,24} = 31.65$, $p < 0.01$) (Figure 1a).

Survival to adulthood, measured as the proportion eclosed, was significantly reduced in Bss5 when reared on 9.5% and 11% NaCl media (ANOVA/SNK $F_{5,24} = 24.15$, $p < 0.01$) and Bss6 survival was significantly reduced on 11% NaCl media (ANOVA/SNK $F_{5,24} = 20.84$, $p < 0.01$). Bss7.5 and Bss9.5 experienced no significant reduction in survival on any salt media (ANOVA/SNK, Bss7.5: $F_{5,24} = 1.91$, $p = 0.13$; Bss9.5: $F_{5,24} = 0.63$, $p = 0.68$). Furthermore, when reared on the highest salt media, 11%, the strains selected for higher salt tolerance have progressively greater survival, with Bss9.5 expressing a significantly higher survival rate than the other strains (ANOVA/SNK, $F_{5,24} = 11.87$, $p < 0.01$) (Figure 1b).

Besides survival to adulthood and latency to pupate, selection for salt tolerance also influences pupation position (Figure 1c). When reared on standard media, the salt-selected lines all pupate significantly further from the media than either wild-type strain (ANOVA/SNK $F_{5,23} = 13.68$, $p < 0.01$). Pupation distance increases with the degree of selected salt tolerance; the more NaCl tolerant strains pupated further from the media on all NaCl treatments (ANOVA/SNK, 5% NaCl: $F_{3,16} = 29.53$, $p < 0.01$; 6% NaCl: $F_{3,16} = 33.86$, $p < 0.01$; 7.5% NaCl: $F_{3,16} = 6.15$, $p < 0.01$; 9.5% NaCl: $F_{3,16} = 8.56$, $p < 0.01$; 11% NaCl: $F_{3,16} = 4.25$, $p < 0.01$). Bss5 and Bss6 strains both express reduced pupation distances when raised on salt-supplemented media, but there were no significant changes in pupation distance among the more salt tolerant lines, Bss7.5 and Bss9.5 (ANOVA/SNK, Bss5: $F_{5,22} = 8.49$, $p < 0.01$; Bss6: $F_{5,24} = 4.65$, $p < 0.01$; Bss7.5: $F_{5,21} = 1.68$, $p = 0.18$; Bss9.5: $F_{5,24} = 0.27$, $p = 0.92$).

Natural variation in NaCl tolerance among the RI lines

As noted above, 5% NaCl media is lethal to the wild-type strains that had not undergone selection for salt tolerance, it was therefore necessary to establish an appropriate NaCl concentration to stress the RI larvae without killing them. Furthermore, to facilitate QTL mapping, we desired a level of NaCl stress at which maximal variation in larval and pupal survival would be observed across the RI lines. To accomplish this, we assayed larval and pupal survival on range of three media NaCl concentrations: 0.05% (standard media, 84 RI Lines tested), 1.75% (71 RI lines tested), and 3.8% (87 RI lines tested).

Larval survival, expressed as the proportion of larvae surviving to pupal formation, was significantly reduced only on media containing the highest NaCl concentration, 3.8% (ANOVA/SNK $F_{2,243} = 365.84$, $p < 0.01$) (Figure 2a). Pupal survival, the proportion of pupae that eclosed, was reduced significantly when the lines were reared on either the intermediate (1.75%) or high (3.8%) NaCl media (ANOVA/SNK $F_{2,242} = 139.01$, $p < 0.01$) (Figure 2b). These results indicated that the chosen NaCl concentrations are sufficient to stress the RI lines while permitting at least some animals to complete development. The parental strains (2b and Oregon-R) were found to have significantly different larval survival

on the 3.8% salt media (ANOVA/SNK $F_{1,6} = 6.39$, $p < 0.0448$) but did not differ in pupal survival (ANOVA/SNK $F_{1,6} = 2.45$, $p < 0.1682$) on this high salt media.

We next analyzed the degree of phenotypic variation across the RI lines reared on the three different media. The greatest variation, in both larval and pupal survival, was observed across the RI lines raised on the high (3.8%) NaCl media (Figure 3). At 3.8%, pupal and larval survivorship ranged from complete survival in some RI lines to near complete lethality in others. Linear regression of pupal NaCl tolerance against larval NaCl tolerance indicates that larval tolerance is not predictive of pupal tolerance of any of the NaCl treatments tested ($r^2_{0.05\%} = 0.097$, $r^2_{1.75\%} = 0.0913$, $r^2_{3.8\%} = 0.066$). Similarly, regression of the larval and pupal NaCl tolerance of the RI lines across the various salt media revealed only weak correlations (0.05% vs 1.75% NaCl medium: $r^2_{\text{larval}} = 0.236$, $r^2_{\text{pupal}} = 0.247$; 0.05% vs 3.8% NaCl medium: $r^2_{\text{larval}} = 0.138$, $r^2_{\text{pupal}} = 0.191$; 1.75% vs 3.8% NaCl medium: $r^2_{\text{larval}} = 0.109$, $r^2_{\text{pupal}} = 0.201$).

Salt tolerance may result in changes in desiccation tolerance, as salt and water can be balanced through common osmoregulatory mechanisms. Since pupation position is influenced by hydration, we hypothesized that pupation position may also be correlated with NaCl tolerance. We used data from a previous study of the pupation positions of the RI lines used in this study to test the correlation between pupation position and NaCl tolerance (Riedl *et al.* 2007). Regression analysis identified little correlation between pupation position and neither larval nor pupal survival on the 3.8% NaCl media ($r^2_{\text{larval}} = 0.134$ and $r^2_{\text{pupal}} = 0.011$).

QTL mapping of larval and pupal NaCl tolerance

To gain insight into the genetic roots of the variability in NaCl tolerance among the RI lines, we performed QTL analyses of their larval and pupal survival when reared on media with defined NaCl concentrations. When the RI lines were raised on standard (0.05%) or intermediate (1.75%) NaCl media, no significant QTL were observed for either larval or pupal survival (0.05%: larval 5% significance threshold = 12.86, larval peak likelihood ratio = 9.44, pupal 5% significance threshold = 33.15, pupal peak likelihood ratio = 15.11; 1.75%: larval 5% significance threshold = 12.32, larval peak likelihood ratio = 5.94, pupal 5% significance threshold = 18.23, pupal peak likelihood ratio = 9.68; significance thresholds were calculated from 10000 random permutations of the respective datasets) (Figure 4a-d).

When the lines were reared on media containing the highest (3.8%) NaCl concentration, significant QTL were observed for both larval and pupal survival (Figure 4e-f). For larval survival data, the likelihood ratio (LR) representing the 5% and 2.5% levels of significance (20000 permutations) were calculated as 15.52 and 17.32, respectively.

Based on these thresholds, the likelihood ratios identify two statistically significant larval NaCl tolerance QTL. The first is at 69D-70C (peak likelihood ratio: 15.71), and the proportion of the phenotypic variance explained by the QTL, r^2 , is 0.155, or 15.5% (Basten *et al.* 2003b). The second QTL was at 71E (peak likelihood ratio: 17.75), and accounted for 23.7% of the RI phenotypic variance. 2LOD support intervals were calculated (Lander and Botstein, 1989; van Ooijen, 1992) and defined overlapping regions. Therefore, the peaks are

more conservatively interpreted as describing a single larval salt tolerance QTL covering polytene region 69D-76A. The direction of the phenotypic response in the RI lines based on their genotypes at the peaks of the QTL for larval salt tolerance (at both local maxima within the 2LOD interval) was consistent with the larval salt-tolerance phenotypes of the parental lines.

Two significant QTL affecting pupal NaCl tolerance were also identified. After 20000 random permutations, the likelihood ratios representing the 5% and 2.5% thresholds of significance were calculated to be 14.59 and 16.22, respectively. Few QTL studies apply a correction for multiple testing, perhaps because there is no agreed upon approach to do so, here we present a more conservative 2.5% threshold as a reasonable correction for the multiple QTL testing. Statistically significant likelihood ratios identified QTL at genomic locations 93A and 96A (respective peak likelihood ratios of 14.67 and 17.19) with non-overlapping 2LOD support intervals of 92A-93B and 94D-96A, respectively. Because the 2LOD support intervals do not overlap, the results indicate that there are two separate pupal salt tolerance QTL. The QTL at 93A explains approximately 10% of the RI phenotypic variance, while the QTL at 96A explained approximately 12%.

Both parental genotypes at each of the QTL-linked *roo* marker positions (3 markers were located within the single larval survival QTL and 5 markers were within the two pupal survival QTL) were well represented among the RI lines.

Discussion

Development, survival, and pupation position of salt selected lines

D. melanogaster are very strong osmoregulators. This may be advantageous for their lifestyle as a flying insect exposed to continuous cycles of dehydration and rehydration (Albers and Bradley, 2004). Remarkably, some *Drosophila* larvae can maintain stable hemolymph osmolarities when raised on food containing as much as 7% NaCl (Croghan and Lockwood 1960); for reference, the salinity of seawater is around 3.5%. We observe that selection for survival in media with high NaCl concentrations affects not only larval developmental rate and survival, but also pupation site selection behavior. In general, the degree of salt tolerance is directly related to viability and inversely related to developmental delay when the strains are reared on very high NaCl media. The more salt tolerant lines also express higher eclosion rates and reduced developmental delay when reared on high NaCl media; the magnitudes of these differences generally correspond to the degree of selected salt tolerance.

Differences in pupation site selection behavior are related with the degree of NaCl tolerance among the selected lines. The more salt tolerant lines tend to pupate further from the moist food source. This may be because the salt-selected lines are stronger osmoregulators and thus less sensitive to dehydrating conditions imposed on them as larvae foraging in the high NaCl media. The selection of an environmentally appropriate pupation position has been previously demonstrated to have a significant impact on survival (Rodriguez *et al.* 1992). Humidity is an important factor, along with genetic and other environmental effects, in both the choice of pupation position and pupal survivability. In dry environments, larvae tend to

pupate near the (moist) media and those larvae pupating further afield may desiccate more quickly and have reduced viability. Conversely, in very moist conditions, most larvae tend to pupate further from the media and those pupating too close may drown or suffer microbial attack and thus incur reduced survival rates (Rodriguez *et al.* 1992).

Among the salt-selected strains, the inverse relation between pupation distance and media NaCl levels supports the hypothesis NaCl presents a desiccating stress and would therefore present a selective pressure toward desiccation-tolerance, which is also consistent with the significantly increased pupation distances compared to wild-type strains when assayed on standard, low-NaCl, media.

NaCl tolerance of RI lines

As a step towards finding candidate genes that contribute to natural variation in NaCl tolerance, we investigated larval and pupal NaCl tolerance in a set of RI lines derived from two genomically-marked parental strains (2b and Oregon-R). Our results indicate that larval NaCl tolerance is not always predictive of pupal tolerance, possibly implying different tolerance mechanisms. For example, some larvae may be able to more efficiently regulate NaCl via excretion with waste or via their anal plates (Edwards, 1983; te Velde *et al.* 1988), or they may alter their feeding or movement patterns to reduce NaCl exposure. Pupae, often removed from the media, immobilized, and enclosed in their pupal cases, do not have such options. They must tolerate any elevation of internal NaCl and thus, for example, strains with relatively high pupal NaCl tolerance may be more adept at NaCl sequestration or ion regulation.

Elevated levels of sodium salts are generally known to influence cellular osmotic regulation, and the high NaCl concentration used in this study are several fold higher than that estimated to be isosmotic with NaCl in Diptera larval hemolymph (Wigglesworth, 1938; Croghan and Lockwood 1960; Miyoshi, 1961). As confirmed by the severely reduced survival of some RI strains, these high salt levels present a significant stress to the animals. Previous studies have identified that activity in various general stress response systems affects tolerance of high NaCl conditions (Kyriakis *et al.* 1994; Kyriakis and Avruch 1996; Han S.-J. *et al.* 1998; Stronach and Perrimon 1999; Inoue *et al.* 2001; Craig *et al.* 2004).

QTL mapping of larval and pupal NaCl tolerance

Our QTL analysis of larval and pupal NaCl tolerance identified different genomic regions affecting tolerance at each developmental stage: one larval QTL is at polytene position 69D-76A and two pupal survival QTL are at 92A-93B and 94D-96A. The identification of different QTL for larval or pupal NaCl tolerance combined with the lack of correlation between larval and pupal tolerances implies that different mechanisms may underlie NaCl tolerance at different life stages. However, each of the QTL likely contain many genes that are associated with interacting signaling cascades and together they may describe a common salt stress tolerance system regulated at different points throughout development. This may also be the case for the salt selected lines described above.

The panel of RI lines used in this study has also been used to identify QTL associated with adult longevity. None of the QTL identified by Nuzhdin et al (1997) correspond to the QTL identified here. Intriguingly, analysis of longevity combined with exposure to environmental stresses (high temperature, low temperature, heat shock, and starvation conditions) identified 17 significant QTL (Viera et al. 2000), four of which map to within the larval salt tolerance QTL: QTL *Ls11*, at 65D-68B (relevant to the low temperature stress), *Ls12* at 68B-69D (relevant to high temperature stress and general female longevity), *Ls13* at 69D-70C (female-specific QTL relative to the 4 stress treatment), and *Ls14* at 69D-71E (relevant to starvation and heatshock stresses). Subsequent analysis of candidate loci among the starvation resistance QTL implicated members of Notch signaling cascades (Harbison et al. 2004). That the regulation of salt-tolerance is stage specific, might help alleviate the pleiotropic effects that many stress-signaling genes have on development.

Similarly, Leips and Mackay (2000) used this set of RI lines to identify QTL associated with adult longevity after rearing at low or high larval densities. Of the six QTL identified, two (67D-68C and 71E) overlap with the QTL affecting larval salt tolerance identified here. This is intriguing because the strains in our assay were reared at very low densities (10 larvae per vial), though the rearing densities of previous generations were not controlled, as they were by Leips and Mackay. It is unclear what the relation there is, if any, between larval density, adult lifespan, and larval salt tolerance. Montooth et al. (2003) used the same panel of RI lines to investigate metabolic phenotypes and found a number of QTL's that overlap with our larval (69D-70E) and our pupal salt tolerance QTL (92A-93B and 94D-96A). For example, glycogen synthase activity at 94D-96A overlapped with our pupal QTL, a large QTL for flight velocity at 67D-87B overlapped with our larval QTL and metabolic rate at 69D-76F overlapped with our larval QTL. Identification of the genetic loci underlying these QTL will help determine if and how metabolism might play a role in larval and pupal salt tolerance.

Although the 4 salt-selected lines differ in their pupation position on various media, the NaCl tolerance QTL do not coincide with previously identified pupation position QTL (Riedl et al. 2007). Different QTL may have been identified because pupation position QTL were identified using larvae reared on standard media; whereas, here we assay pupation position on elevated NaCl media. Although, the corresponding effects of pupation height and salt tolerance in the four salt-selected lines suggest that the phenotypes are linked, it may be that though pupation position is sensitive to many stresses, regulatory variation affecting pupation height has evolved at different loci than those controlling salt tolerance. It should be remembered, however, that three of the four salt-selected lines were established via branching from a single selection process and therefore are not truly independent. Future experiments will resolve the relationship between genes effecting the natural genetic variation in the salt tolerance and pupation position phenotypes.

Overall, our results indicate that adaptation to high salt conditions can also effect changes in behaviors, such as pupation site selection, which may significantly impact survival in other environmental contexts. The elucidation of factors influencing natural variation in NaCl tolerance may help to clarify some of the impacts associated with tolerance of other environmental stresses. Finally, the identification of conserved functionality in genes

influencing natural differences in tolerance or resistance to environmental stresses will be relevant for other species.

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Highlights

1. *Drosophila melanogaster* lines were selected for very high salt tolerance.
2. Salt-tolerant lines grow and survive better than controls when reared on salty media.
3. Choice of pupation position is also correlated with the degree of salt-tolerance.
4. QTL for larval and pupal salt tolerance were found using recombinant inbred lines.
5. Pupal and larval salt tolerance is uncorrelated and each trait has distinct QTL.

Figure 1.

Variation in pupation latency, survival, and pupation position of four salt selected lines and two wild-type controls reared on six different media with increasing NaCl concentrations. No individuals from either wild-type strain survived on media with 5% or higher salt. (A) Pupation latency among the salt lines increases with media NaCl concentrations. This increased developmental time is generally less in the lines selected for tolerance of higher salt concentrations. We counted pupation once a day. If all vials had larvae pupate on the same day the error bars were zero. (B) Survival to adulthood was affected by media salt concentrations. Eclosion rates among flies reared on the highest salt concentration are less depressed in strains selected for the highest salt tolerance. (C) Strains selected for higher levels of salt tolerance also tend to pupate farther from their growth

media. When reared on standard media (0.05% NaCl), salt selected lines pupate significantly farther from the media compared to the wild-type strains. On salt-supplemented media, pupation distances generally increase with salt tolerance across strains.

Figure 2.

Mean larval and pupal survival of pooled RI lines reared on three different salt concentrations. (A) Larval survival, the proportion of larvae surviving to pupation, was significantly reduced only on media containing the highest NaCl concentration, 3.8%. (B) Pupal survival, the proportion of pupae that eclosed, was significantly reduced when the lines were reared on either the intermediate (1.75%) or high (3.8%) NaCl media.

Figure 3.

Phenotypic variation among the RI lines reared on three different salt concentrations. Mean proportion of larvae that pupated (▲) and the mean proportion of pupae that eclosed (□) for each RI line reared on media of (A) 0.05% NaCl, (B) 1.75% NaCl, or (C) 3.8% NaCl. RI lines are ranked independently in each chart according to their larval survival. In all salt treatments, the larval and pupal survival rates are not correlated.

Figure 4.

Results of composite interval mapping for the identification of QTL affecting larval and pupal survival on three different salt media. In each chart, the relative positions of the *roo* transposable elements used as genomic markers are indicated by the triangles positioned along the horizontal axis, which in turn indicates genomic position as calculated by recombination mapping the markers among the RI strains used in each experiment. Each chart is divided into four sections representing linkage groups: chromosomes 1 and 3 each form single linkage groups and chromosome 2 contains two groups. Plotted against this recombination map is the likelihood ratio (LR) as calculated by composite interval mapping. The horizontal solid and dashed lines across the plots indicate 5% and 2.5% levels of significance, respectively. No significant QTL were detected for larval (A) or pupal (B) survival on the standard media (0.05% NaCl). Similarly, on the intermediate (1.75% NaCl) salt media, no significant QTL were detected for larval (C) or pupal (D) survival. However, significant QTL were detected on chromosome 3 which affect either larval (E) or pupal (F) survival on media containing the high NaCl concentration (3.8%).