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Salinity adaptation of the invasive New Zealand mud snail (*Potamopyrgus antipodarum*) in the Columbia River estuary (Pacific Northwest, USA): physiological and molecular studies

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Abstract In this study, we examine salinity stress tolerances of two populations of the invasive species New Zealand mud snail *Potamopyrgus antipodarum*, one population from a high salinity environment in the Columbia River estuary and the other from a fresh water lake. In 1996, New Zealand mud snails were discovered in the tidal reaches of the Columbia River estuary that is routinely exposed to salinity at near full seawater concentrations. In contrast, in their native habitat and throughout its spread in the western US, New Zealand mud snails are found only in fresh water ecosystems. Our aim was to determine whether the Columbia River snails have become salt water adapted. Using a modification of the standard amphipod sediment toxicity test, salinity tolerance was tested using a range of concentrations up to undiluted seawater, and the snails were sampled for mortality at daily time points. Our results show that the Columbia River snails were more tolerant of acute salinity stress with the LC_{50} values averaging 38 and 22 Practical Salinity Units for the Columbia River and freshwater snails, respectively. DNA sequence analysis and morphological comparisons of individuals representing each population indicate that they were all *P. antipodarum*. These results suggest that this species is salt water adaptable and in addition, this investigation helps elucidate the potential of this aquatic invasive organism to adapt to adverse environmental conditions.

Keywords Gastropod · Invasive species · Columbia River · Salinity · Mitochondrial DNA

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Introduction

The fresh water New Zealand mud snail (*Potamopyrgus antipodarum*; Gray 1843) invaded American fresh water streams and lakes in the latter half of the 20th

century (Kerans et al. 2005). In the western United States, New Zealand mud snails (NZMS) have spread rapidly from tributaries of the Snake River, where they were first found (Bowler 1992), to waterways in all western states except New Mexico (Berson 2010). NZMS in the US are clonal, and three distinct genotypes have been identified, with one genotype representing the vast majority of the range in the western US (Dybdahl and Drown 2011). Despite the lack of genetic variation one would normally associate with environmental adaptation, this gastropod is highly adaptable and has successfully spread through lakes, fast rivers, and slow-flowing and brackish water ecosystems (Alonso and Castro-Diéz 2008). In 1999, NZMS were found during Environmental Monitoring and Assessment Program (EMAP) benthic sampling in oceanic reaches of both Rogue River and Columbia River estuaries (Stoddard 2006) and have been shown to be of the same clonal lineage as the population first documented in the Snake River (Dybdahl and Drown 2011). This would suggest that NZMS could have been spread overland via the movement of aquaculture products or possibly by anglers or biologists (Zaranko et al. 1997; Gangloff 1998; Dybdahl and Drown 2011). Since its discovery in the lower Columbia, NZMS have apparently spread within that estuary reaching extremely high densities (200,000 m^2) in Youngs Bay (Bersine et al. 2008). It has also spread into upriver reaches of the Alsea, Coos, Tillamook, Umpqua, and Yaquina estuaries in Oregon and was recently found in the Port Alberni Inlet on Vancouver Island, British Columbia (Davidson et al. 2008).

This species is euryhaline, able to establish populations in fresh and brackish water, and in fact has inhabited the brackish waters of Europe for over 150 years (Costil et al. 2001). The collection site for NZMS used in this study is approximately 2 km from the ocean on the Columbia river near Hammond Harbor marina, an estuarine area with PSU (Practical Salinity Units) reaching near full seawater salinity levels ($\sim$34 PSU). The presence of NZMS in oceanic reaches is an interesting phenomena, as published papers suggest that the maximum salinity tolerance of NZMS could be 26 (Winterbourn 1970) or 28 PSU (Costil et al. 2001). Winterbourn (1970) noted that NZMS could only survive a short exposure at these highest salinity levels by withdrawing into their shells and closing their opercula and that only when these stressed snails were placed into water of 3–5 PSU did they recover full motility. It is possible that the presence of NZMS in these estuaries represents the invasion of a second more salinity adapted genotype. First, the populations of NZMS found in Pacific Northwest (PNW) estuaries appear to be geographically isolated from fresh water populations in the western United States (http://nas.er.usgs.gov; Bersine et al. 2008). Secondly, research on freshwater versus brackish water NZMS in Denmark (Jacobsen et al. 1996) has identified two NZMS genotypes. In subsequent research, Jacobsen and Forbes (1997) conducted feeding studies on these clones with salinity as a cofactor. Their results demonstrated these genetically distinct genotypes exhibited differing responses in feeding rates, growth, and reproductive output with respect to exposure salinity. However, their experiment was designed to address genetic diversity questions rather than the determination of salinity tolerances. Lee and Peterson (2003) studied the effects of a salt water copepod (*Eurytemora affinis*) exposed to acute and acclimation low-salinity stress (showing that there were clear differences of developmental acclimation in response to low versus high salinity, in addition they showed there was a significant effect in clutch survival of *E. affinis* under low-salinity conditions, which could indicate a genetic component to low-salinity tolerance). Very few papers have addressed acute and acclimation salinity stress, and this study is the first to measure the salinity tolerances of a fresh water population versus a higher salinity population of any aquatic invasive species.

Using data collected from CMOP (Center for Coastal Margin Observation and Prediction) stations, we have a detailed picture of the fluctuating salinity levels in this part of the estuary. These data show that this population of NZMS is routinely exposed to salinity levels over 20 PSU and are often exposed to daily maximums over 30 PSU. Even if, as Costil et al. (2001) observed, the maximum salinity NZMS could tolerate was 28 PSU, then these data indicate the Columbia River population is tolerating and indeed thriving in salinity levels that have previously not been observed.

Regardless of how accurate previous estimates of salinity tolerance are, assessment of salinity adaptation is needed to estimate the potential for this nuisance species to become established in the oceanic portions of Pacific Northwest Estuaries where they may pose ecological threats. In an invaded ecosystem, NZMS can impact native species through their high fecundity, rapid population growth, and fast spread. In addition,

they have a high consumption rate and together these factors produce a non-indigenous species that can displace native benthic species through competition (Alonso and Castro-Diéz 2008). The impact of NZMS in the Columbia River estuary has not been well defined; however, it is known that PNW coast estuaries are important nurseries and migration corridors for salmon and other fish species (Emmett et al. 2000). An ecological threat could emerge through the declining populations of native benthic organisms in which salmon and other fish forage. It has been shown that juvenile Chinook salmon (*Oncorhynchus tshawytscha*) do feed at low levels on NZMS in the Columbia River estuary (Bersine et al. 2008); however, another study documented that native fish in the Columbia River estuary received very little energetic value from the consumption of NZMS and there was an increased consumption of native amphipods and isopods in the presence of NZMS (Brenneis et al. 2011).

There is a broad belief that invasive species have the benefit of extensive physiological tolerances that enable them to colonize new habitats (Wolff 2000). To assess salinity tolerances and also to determine the potential limits of the environmental and geographic range of NZMS in the PNW, tests of acute and acclimation salinity stress were performed. Our primary question was whether snails were adapting to these higher salinity reaches or simply tolerating short exposures to higher salinity by closing up until the reduced salinities returned during low tides. To address this issue, in 2007 and again in 2008, we conducted salinity tolerance experiments on NZMS collected from a fresh water lake (Devils Lake, Lincoln City, OR) and from Hammond Marina near the mouth of the Columbia River Estuary. In addition, there is the potential that the higher salinity environment of the Columbia River population possesses a heritable molecular variation that allows for their tolerance of exceptional salinity levels. To evaluate that potential and to assess genetic similarity, we performed molecular analyses between the two populations.

Methods

Snail collection/morphology

In August 2007, snails were collected from a coastal freshwater lake (Devil's Lake, Lincoln City, OR; 44°97.131′N, 124°03.794′W), and in September 2007, they were collected at low tide from an estuarine site (Hammond Harbor, OR; 46°12.145′, 123°57.08′W) located approximately 2 km from the mouth of the Columbia River (Fig. 1). In August 2008, snails were collected from the same sites on consecutive days. At the Hammond Harbor site, average water temperature for September 2007 was 13.73c and for August 2008 was 15.2c. At both sites, snails were collected from the underside of rocks gathered from shallow water (<1 m) near the shorelines. Collected snails were placed on wet paper towels in zippered bags, which were then placed on ice in a cooler for transport to the lab. Upon arrival, all snails from each population were placed into small holding aquaria (38 l) containing filtered (25 μm), aerated water collected from the respective collection sites. Snails were maintained in this manner for ≤5 days before initial experimentation. Snail morphology was determined on 35 snails from each collection site, which were randomly selected from holding aquaria. On each snail, the number of whorls was counted and the length was measured using calipers.

Salinity levels in Hammond Harbor were based on data collection buoys deployed by the Center for Coastal Margin Observation and Prediction (CMOP) in Columbia River estuary. This includes the SATURN Observation Network, a network of buoys called Endurance Stations, which contribute to NANOOS, a regional association of the US Integrated Ocean Observing System. The closest data collection buoys to our NZMS collection site are Fort Stevens Wharf (US Coast Guard day mark 26) and Tansy Point station; these are endurance biochemical stations located 0.86 km (downstream) and 3.38 km (upstream) from Hammond Harbor. These stations provide continuous monitoring of baseline parameters, including salinity (data is collected by these stations once a minute, every minute).

2007 Experiment

Snails from both populations were tested for their salinity tolerance using 0, 5, 10, 15, 20, 25, 30, and ~33 PSU using a modification of the standard amphipod sediment toxicity test (Swartz et al. 1984). Snails placed in a salinity treatment were left in that particular salinity level for the duration of the experiment. Treatments were prepared from sand-filtered seawater that is

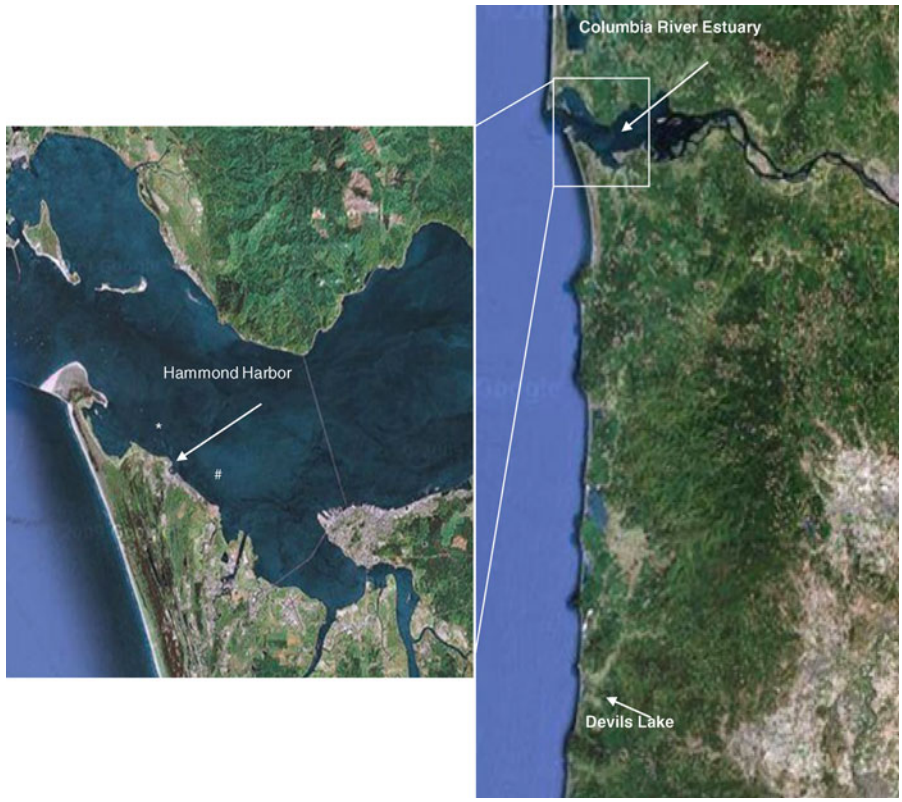

Fig. 1 Locations of the Hammond Harbor and Devil's Lake Populations, and the Fort Stevens Wharf (*) and Tansy Point (#) data collection buoys

supplied continuously within our laboratory and originate from near the bottom (within the salt wedge) of the Yaquina Estuary (Newport, OR, USA) and has a maximum salinity of ~33 PSU. In the first experiment (August, 2007) using Devil's Lake snails, treatment waters were prepared by diluting sand-filtered seawater with filtered (25 μm) water from Devil's Lake. For the test with snails collected from Hammond Harbor (September, 2007), treatment waters were prepared by diluting sand-filtered seawater with filtered (25 μm) dechlorinated tap water (0 PSU or >0.05 ppm dissolved salts). Target salinities were adjusted and verified using a salinity probe (YSI). After preparation, treatment waters were stored in large (~40 l) carboys in the same room in which experiments were conducted.

Each experimental unit consisted of ten snails contained in a graduated 1-l beaker containing ~750 ml of treatment water. Snails (100–200) were first removed from their holding aquaria using a small aquarium fish net and placed into a glass preparation dish (~250 ml) filled with control water (water from collection site). From this dish, ten snails were removed using forceps and placed into a Petri dish containing a minimum volume of control water and examined under a dissecting microscope to verify species and vitality. After verification, snails were washed into randomly selected 1-l beakers using a small amount of treatment water with the beaker filled to the 750 ml level with the treatment water. In each of these experimental units, a 1-ml disposal pipette provided aeration at the bottom of the beaker (2–5 bubble s^{-1}). A small (~1 cm^2) piece of Nori was place in the beaker for food and the beaker covered with a watch glass.

For the Devil's Lake 2007 test, seven experimental units were prepared at each test salinity. Following 1, 2, 3, and 9 days of exposure, one experimental unit from each treatment was sampled for mortality determination. Three experimental units were similarly sampled following 6 days of treatment. Based on the salinity sensitivity of the Devil's Lake population test, a short-term survival test was initiated on unused snails. These snails were tested with 20, 25, and 30 PSU seawater using the same pre-experimental methods. Seven experimental units at each of the above dilutions were prepared and sampled for mortality after 1, 2, 4, 6, 8, 14, and 24 h of exposure. Mortality was determined by examining snails that were not

attached to the beaker under a dissecting microscope for motility.

In 2007, Hammond Marina snails were tested using the same procedures used in the Devil's Lake test with the exceptions that mortalities were determined following 1 and 6 days of treatment with three experimental units examined at each treatment level on each of those experimental days. As high mortalities (50–80 %) occurred in the 0 PSU treatment, a subsequent test was performed using locally collected well water (three experimental units of Hammond Marina snails that were not used in the salinity tolerance test) to determine whether there was a problem with the dechlorinated tap water or if snails were sensitive to low PSU water. No mortality was observed in the 0 PSU treatment when well water was used as a diluent indicating that snails could tolerate low PSU water that the full strength dechlorinated tap water was toxic to snails. Further investigation revealed the laboratory water dechlorinator was not functioning properly, resulting in elevated chlorine concentrations, likely causing the snail mortalities.

In both test series, mortality was evaluated by removing all snails from the sampled experimental unit and placing them into control water in a Petri dish. These snails were then examined using a dissecting microscope with the number of active or dead snails noted. If a mortality determination could not be definitively made on an individual snail, the non-motile snail was placed back into the same sampled beaker containing the ~750 ml of the appropriate control water and rechecked for motility a minimum of 24 h later.

2008 Experiment

Snails from both populations were concurrently tested using similar treatments and procedures used in the 2007 test. The differences in the 2008 tests were that four experimental units from each treatment were evaluated for mortality following 6 and 9 days of exposure and that the local well water was used for dilution rather than dechlorinated tap water or Devil's Lake water. In addition, two site control treatments (four experimental units of each) were prepared using undiluted filtered water from Devil's Lake (for Devil's Lake snails) and Hammond Marina water (for Hammond Marina snails) and examined for mortality following 3, 6, 9, 12, 15, 18, 21, and 24 days of exposure. In these site control experimental units, water was exchanged each time the snails were examined by pouring the contents of beakers though a small plastic sieve (~1 mm mesh) that retained snails and any uneaten Nori. This material was then washed back into the same beaker using the appropriate site control water. After addition of a small amount of water, the beaker was swirled to dislodge any attached snails. The number of mortalities in the beaker was then assessed as live snails would quickly attach to the bottom of the beaker as soon as the swirling stopped. Snails which did not attach were removed, examined for mortality, and quickly returned to the same site control beaker if found to be alive. During the day 14 mortality check, Nori was again added to each experimental unit.

Concurrent with the above, an acclimation experiment was conducted to determine whether the survival of snails from the fresh water population could be improved if they were pre-adapted to higher salinities. At the initiation of this experiment, twenty Devil's Lake experimental units (200 snails) were prepared using well water amended with sea water to attain 10 PSU. The salinity in each experimental unit was then increased by 5 PSU every 3 days until full strength sea water (~34 PSU) was attained (day 18) with the surviving snails exposed to this salinity for 3 days at which time the number of mortalities was again determined. Surviving snails were then maintained in this high salinity water for an additional 6 days without water renewal. Mortalities in these high salinity beakers were determined on day 21 and 24, at which time the experiment was terminated. These experimental units were fed Nori at test initiation and after each water exchange.

Statistics

Differences in snail morphology between Devil's Lake and Hammond Marine snails were tested using a two-sample t test ($p < 0.05$). Salinity LC_{50} (lethal concentration for 50 % of exposed animals) were determined at each time interval using the SAS Probit Procedure (SAS Institute Inc., Cary, NC, USA).

DNA extraction

Upon collection, the snails were stored in 70 % EtOH. Prior to DNA extraction, the shells were removed, and

the body was rinsed in sterile water and blotted dry. Total genomic DNA was extracted from entire individuals using a modified cetyl trimethyl ammonium bromide (CTAB) plant genomic DNA extraction method (Ausubel et al. 2002): snail bodies were placed in 2-ml tubes containing a lysing matrix (lysing matrix "A", MP Biomedicals) with 600 µl CTAB extraction buffer and 60 µl 20 mg/ml Proteinase-K, bead-beaten for 20 s at speed setting 4 in a Qbiogene FP120 bead beater (Qbiogene Inc. Carlsbad, CA, USA). Samples were vortexed for 20 s, placed in a 55 °C water bath for 1 h with periodic mixing. Sample tubes were centrifuged at 12,000 rpm for 5 min, and supernatant transferred to a new 1.6 ml microcentrifuge tube. One volume of Chloroform/Isoamyl alcohol (24:1) was added, samples centrifuged at 12,000 rpm for 10 min, the aqueous phase transferred to a new tube, and 0.1 volume 10 % CTAB/NaCl solution added and mixed. One volume of Chloroform/Isoamyl alcohol (24:1) was added, mixed, and centrifuged at 12,000 rpm for 5 min. The aqueous phase was transferred to a new tube, one volume CTAB precipitation solution added and mixed, and 0.6 volume isopropanol added, mixed, and let stand overnight at room temperature. After another centrifugation at 12,000 rpm for 20 min, the supernatant was decanted and the pellet resuspended in 1 ml high salt TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 1 M NaCl). The tubes were gently shaken until the pellets dissolved, 550 µl isopropanol added, mixed, and DNA pelleted by centrifugation (12,000 rpm, 10 min). The supernatant was decanted, and the pellet gently washed with 1 ml 70 % ethanol. The DNA was air dried and resuspended in 100 µl of TE buffer.

DNA amplification

To reveal any potential sequence variation between the two populations, three genes were analyzed. Two separate regions of the 28s rDNA gene were PCR amplified: (1) primers P.aITS2F (5′-ACGTTCTAC CCATCGCTG) and P.a28sR (5′-CAAGACG GGTCGGATGGA) amplified a short region of the ITS2 sequence (37 bp) and 578 bp of the 28s rDNA, yielding an 615-bp product (referred as 28s rDNA-1); (2) Primers 220 (5′-GACCCGTCTTGAAACA CGGA) and 221 (5′-TCGGAAGGAACCAGCTAC TA; Litvaitis et al. 1994) amplified a 264-bp fragment of D3 expansion region of the 28s rDNA subunit that is downstream of the 28s rDNA-1 product (referred as 28s rDNA-2). A partial gene sequence of the mitochondrial 12s rDNA gene was analyzed using the primer set 12sF (5′-CTGGGATTAGATACCCC-ACTA)/12sR (5′- TGAGGAGGGTGACGGGCGGT; Crandall lab primer database, BYU-http://crandalllab.byu.edu/PrimerDatabase.aspx), which amplifies a 318-bp fragment (bp 4190–4508) of the mitochondrial gene. Also a partial gene fragment of the mitochondrial 16s rDNA gene was analyzed. The primer set 16Sar-H (5′ CCGGTCTGAATCCAGATCACG 3′) and 16Sar-L (5′ CGCCTGTTTATCAAAAACAR 3′; Palumbi et al. 1991) was used for PCR amplification, which amplifies a 526-bp fragment (bp 5378–5901) of the mitochondrial gene. All PCRs in this study were carried out in 20 µl volumes with one drop of mineral oil, and each 20 µl volume contained: approximately 10 ng genomic DNA template, 2 µl of Bioline 10× NH_4 reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 20pMol of each primer, and 0.75 units of Bioline Biolase DNA polymerase. Thermal cycling was carried out using Applied Biosystems Verity model #9902.

Sequence analysis

Both of the 28s rDNA, and the 12s and 16s mitochondrial rDNA PCR products were directly sequenced in both directions. Purification and sequencing were performed at the High-Throughput Genomics Unit, department of Genome Sciences at the University of Washington. For the 28s rDNA-1 product, 13 individual snails from the Devils Lake and 15 individuals from the Hammond Marina populations were sequenced, and for the 28s rDNA-2 product, 10 individuals from both populations were sequenced. For the 12s mtDNA product, 10 individuals from each population were sequenced. For the 16s rRNA product, 13 individual snails from the Devil's Lake and 14 individuals from Hammond Harbor populations were sequenced. DNA from the same set of individual snails was used for sequencing these three genes, and not all genes are represented by the same number of individuals due to inconsistencies in PCR or sequencing results. The forward and reverse sequences were aligned into contigs and corrected manually using Sequencher™ sequence assembly program. The sequences were compared against the GenBank database using BLAST (Altschul et al. 1997). Sequences of other Gastropod

species were obtained from GenBank and aligned with the *P. antipodarum* sequences using Sequencher™.

The phylogenetic tree based on the 16s rRNA gene fragments was constructed using MacVector™. The tree was constructed using NZMS sequences from the Devil's Lake and Hammond Harbor populations, along with sequences from the most closely related species (Subfamily Hydrobiidae) found in GenBank. The Neighbor-joining method was used for the model with bootsrap (1,000 replications), and Best Tree calculation methods were used to create the phylogram.

Results

The data collection buoys near Hammond Harbor were used to determine the fluctuating salinity levels in this part of the Columbia River estuary. A figure of the monthly mean PSU with standard deviations and monthly high and low PSU values at these two buoys are shown (Fig. 2). Salinity levels at the buoys ranged from 0.3 to 31.0 PSU monthly based on tidal influence with a yearly mean at ~12 PSU.

The morphology of the two snail populations appeared to be identical as the mean ± standard

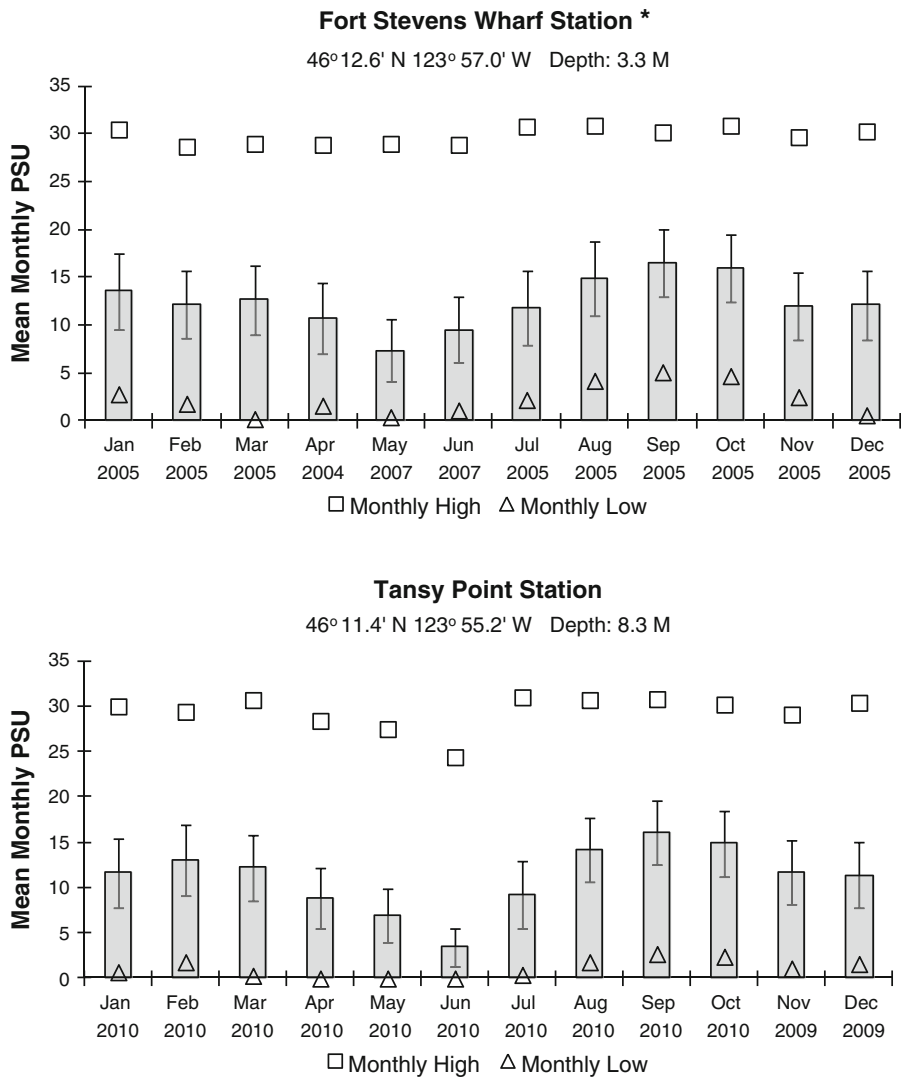

Fig. 2 Monthly mean salinity values (with standard deviations) from data collection buoys nearest Hammond Harbor, including buoy location and sampling depth. *Asterisk* for the Fort Stevens Wharf Station, some months of 2005 had incomplete data, so the closest year with complete data for that month was used

deviation of the Devil's Lake snails (4.25 ± 0.29 mm) was not statistically different (*t* test) from that of the Hammond Marina snails (4.19 ± 0.30 mm), nor were the number of whorls statistically different (Devils Lake: 5.6 ± 0.5, Hammond Marina: 5.9 ± 0.3). Morphology measurements were made the first year in an effort to determine whether the populations were different. As they were not, we did not make similar measurements the following year.

Sequence analysis showed that within the 28s, 12s, and 16s rRNA gene fragments, the Columbia River and Devil's Lake populations were identical. In addition, phylogenetic analyses performed with closely related gastropods revealed that the two *Potamopyrgus* populations were monophyletic with no sequence differences, and separation from other species was strongly supported with bootstrap values of >80 (Fig. 3). The 16s rDNA gene of genus *Potamopyrgus* is well represented in GenBank, and five different *Potamopyrgus* species were included in our phylogenetic analyses. In contrast, there are no *Potamopyrgus* 12s rDNA sequences in public DNA databases, and besides the sequences submitted from this study, there is one 28s rDNA *Potamopyrgus* submission (GenBank accession: EF417135.1). Therefore, phylogenetic analyses were performed with mtDNA and rDNA sequences from closely related gastropods (Litorinimorpha, Tateinea). Analysis of the 28s rDNA-1 and 12s mtDNA sequences revealed that the two *Potamopyrgus* populations were monophyletic with no sequence differences, and separation from the other species was strongly supported with bootstrap values of 98–100 (results not included here).

The Hammond Marina NZMS population density qualitatively appeared to increase substantially at our collection site from 2007 to 2008. In 2007, it took several hours for six people to collect the required number of snails for the initial salinity tolerance tests. In contrast, two people collected a greater number of snails from the same site in a few minutes the following year.

In both the 2007 and 2008 studies, Hammond Marina snails survived greater salinities than snails collected from Devil's Lake (Fig. 4). In the 2007, Devil's Lake test, almost all of these mortalities occurred during the first day of exposure to the higher salinities as evidenced by the similarity of LC_{50} values between the day 1 and day 9 determinations. Based on the consistency in LC_{50} values, we estimate that Devil's Lake snails were adversely affected at salinities greater than 20 PSU.

In contrast, no mortalities were observed in any treatments the first day of testing on the Hammond Marina snails in 2008 (Fig. 4). Estimated LC_{50} values determined after 6 and 9 days of exposure were at or above the highest salinity tested and were much more variable than observed in the Devil's Lake population (Fig. 4). Similar results were observed in 2007 with no mortalities observed in the 5–33 PSU treatments on the first day of exposure. However, high mortalities (50–80 %) occurred in the 0 PSU treatment (2007),

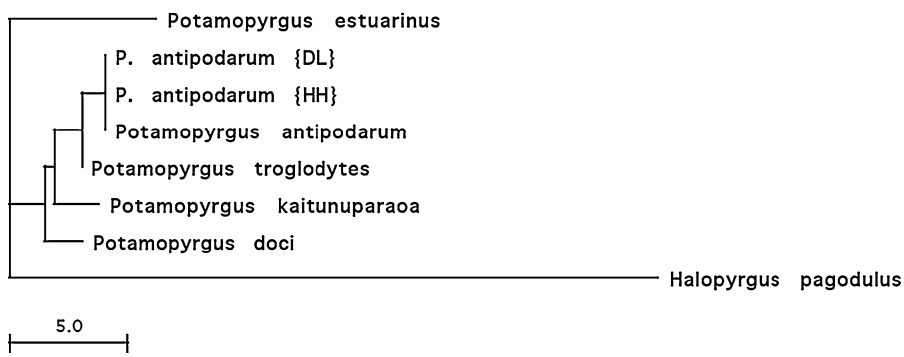

Fig. 3 Phylogram based on partial (477 bp) mitochondrial 16s rDNA sequences representing the relationship between snail species from the Genus *Potamoprygus* and rooted in an outgroup from Family Hydrobiidae. Neighbor-joining method used for model, with distances representing the absolute number of differences. Gaps are distributed proportionally and model is derived from 2,000 replications. Best Tree (shown) and Bootstrap trees were congruent with each other and current taxonomic descriptions. Associated GenBank accession numbers: *P. antipodarum* {HH} JN639014, *P. antipodarum* GQ996423, *P. troglotytes* AY634103, *P. kaitunuparaoa*, AY634083, *P. doci* AY634105, *H. pagodulus* AY634092

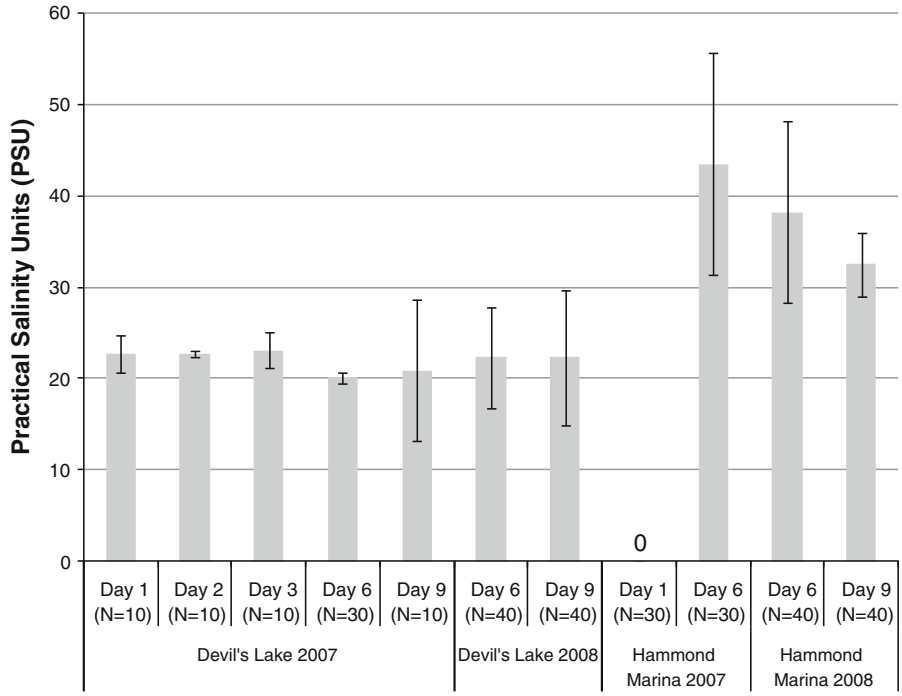

Fig. 4 Acute salinity tolerance LC50 values for Devil's Lake and Hammond Marina New Zealand mud snails. Salinity LC50 (standard deviation) values (PSU) were determined by Probit analysis using eight salinity treatments from 0 to full strength sea water. Day denotes the time period of exposure to each salinity treatment. *N* is the number of snails exposed for each day/treatment salinity. 0 = no mortalities

and those values were excluded from Probit analysis. The high mortalities observed in the 0 PSU control (2007) suggested that either the Hammond Marina snails were sensitive to low PSU water or the dechlorinated tap water was toxic to snails. Subsequent testing using 0.2 PSU water from a local well indicated that the snails were not sensitive to low PSU water (100 % survival). In addition, the lack of snail mortality on day one of exposure to a range of PSU treatments in 2007 suggests that the toxic component of dechlorinated tap water was easily diluted to sublethal levels.

Mortalities from acute salinity stress in the 2007 Devil's Lake tests usually occurred during the first day of the test. This was confirmed by the follow-up salinity tolerance test in which snails were exposed to 20, 25, and 30 PSU. In that test, the estimated LC_{50} for snails exposed to 25 and 30 PSU treatments was 22.8 and 13.8 h, respectively. All snails exposed to the 20 PSU treatment survived for the entire 24-h test.

Snails maintained in water from their collection sites for 24 days exhibited minimal mortalities. In both site control tests, 97.5 % of the Hammond Marina and Devil's Lake snails survived the full 24 days.

Although there were obvious and reproducible differences in the acute salinity tolerance of the Devil's Lake snails when compared to Hammond Marine NZMS, these salinity tolerance differences were most likely due to acute salinity stress as Devil's Lake snails when slowly acclimated, survived high salinities (Table 1). When slowly acclimated, these snails did not show a spike in mortalities until 34 PSU (essentially full strength seawater) was attained which resulted in 11, 18, and 22 % mortality after 18, 21, and 24 days of exposure, respectively. The 24-day

Table 1 Survival of Devil's Lake NZMS in 2008 salinity acclimation test

Day	Salinity (PSU)	# Alive	$\sum$ Survival (%)	Internal survival (%)
3	10	200	100.0	100.0
6	15	200	100.0	100.0
9	20	199	99.5	99.5
12	25	199	99.5	100.0
15	30	196	98.5	98.5
18	34	175	87.5	89.3
21	34	161	80.5	92.0
24	34	153	76.5	95.0

#Alive = number of snails that survived during each 3 days interval of the test. $\sum$Survival = % of t_0 snails alive at the end of each test interval. Interval Survival = % of snail that survived from the start to end of each test interval

survival rate of these Devil's Lake snails (76.5 %) was greater than the survival rate of Hammond Marina snails exposed for 9 days to full strength seawater in the 2007 (63 %) and 2008 (30 %) acute salinity tolerance tests. Acclimation tests were not performed on Hammond Marina snails.

Discussion

There were differences in acute salinity tolerances between the Devils Lake NZMS and those collected in Hammond Marina; however, when Devils Lake snails were slowly acclimated to higher salinities, these differences disappeared. This result is consistent with the idea that the NZMS that invaded PNW estuaries may be of the same genotype(s) inhabiting western US fresh water lakes and streams and that these snails may have sufficient genetic or epigenetic plasticity allowing them to tolerate a large range of environmental conditions (Dybdahl and Kane 2005).

The invasive NZMS in North America and even in their native habitat have been shown to have high phenotypic plasticity (Dybdahl and Kane 2005), and in this study, though not statistically significant, there were differences in morphological metrics between populations. Nonetheless, a comparative DNA sequence analysis was performed to ensure accuracy of the morphological assessment and to identify potential genetic variation between the two populations. The results of the sequence analysis were congruent with the morphological measurements indicating that individuals from the freshwater population in Devil's Lake and the euryhaline population in the Columbia River estuary represented one species (*P. antipodarum*). Although there were no DNA sequence differences between the populations, more extensive genetic analyses are required to determine whether the populations represent the same genotype. Future studies involving genetic diversity of NZMS could reference Dybdahl and Drown (2011), who have developed a protocol to measure genotypic diversity that combines genotypic data from allozymes, mitochondrial DNA, and microsatellite DNA into a multilocus genotype. The mitochondrial gene Cytochrome *b* and ribosomal RNA Internal Transcribed Spacers 1 and 2 (ITS1, ITS2) are commonly used in genetic analysis to establish phylogenetic relationships. However, in *P. antipodarum*, these genes have been shown to have a high level of variation between populations, among populations and even within individuals (Hoy and Rodriguez, unpublished data; Nieman et al. 2011), which could present difficulties when making phylogenetic inferences.

Besides the fact that they are highly tolerant of a wide range of salinities, it has also been shown that NZMS possess the following traits that can contribute to their spread as an invasive species: (1) high fecundity (Zaranko et al. 1997; Richards 2002); (2) reproduce almost exclusively via parthenogenesis (Lively 1992), which is an attribute shared by many successful invasive species (Lynch 1984; Jacobsen and Forbes 1997; Haag and Ebert 2004); (3) low susceptibility to native predators due to its hard shell and solid operculum (Vinson and Baker 2008); (4) ability to colonize a wide range of habitats (estuaries, lakes, rivers, streams) (Alonso and Castro-Diéz 2008). Moreover, unlike their name suggests, they dwell not just on mud but an assortment of substrata, including aquatic macrophytes, clay and fine sand (Marshall and Winterbourn 1979; Weatherhead and James 2001) and tolerate a range of abiotic conditions (Møller et al. 1994; Alonso and Camargo 2003). Although not tolerant of temperatures below 0 °C at any salinity (Hylleberg and Siegismund 1987), NZMS are adept at withstanding periods of cold or desiccation by burying themselves in the sediment (Duft et al. 2003).

At present, NZMS have only been found in PNW estuaries described as river dominated (Lee II and Brown 2009), where they are usually present in the more brackish water reaches (http://nas.er.usgs.gov). Even though salinities near the Hammond Marina collection site often exceeded 30 PSU, mean yearly salinity measures at these nearby sites was ~12 PSU. Earlier research indicated that the NZMS cannot reproduce in waters greater than 12–18 PSU (Duncan and Klekowski 1967); however, the numbers of snails we observed in Hammond Marina and the apparent orders of magnitude increase in this population density observed at the collection site between 2007 and 2008 indicate that the snails are able to reproduce and thrive within a more saline regime. This result needs to be rigorously verified using life cycle tests to help evaluate the potential for the spread of NZMS into the more ocean-dominated reaches of PNW estuaries.

The spread of NZMS to other estuarine systems along the west coast of North America is a definite possibility. There are several mechanisms for transport, and the ability to survive for at least a few days in

full strength sea water suggests that hitch-hiking on the hulls of commercial and recreational vessels and in the ballast water of commercial vessels would be a likely vector. This theory is supported by data in our study showing that after 6 days of exposure to full strength seawater (34 PSU) NZMS had only 23 % mortality (Table 1, days 18–24). Other transport vectors include sport fishing gear such as waders or boots (ANS 2007), transportation of aquaculture products, and even within mud attached to the bills or legs of birds or inside the gut of birds or fishes (Alonso and Castro-Diéz 2008).

The brackish water Columbia River population of *P. antipodarum* is more adapted to acute salinity stress as evidenced by the substantially lower LC_{50} values than that of the freshwater population from Devil's Lake in our toxicity tests. There are very few reports of freshwater aquatic organisms adapting to and colonizing habitats with significantly higher salinities than their original habitat. *P. antipodarum* and *Tarebia granifera* (Thiaridae: Mollusca; a freshwater snail that has invaded estuarine systems in South Africa and by accounts has a similar history to *P. antipodarum*) are species capable of this transition (Jacobsen and Forbes 1997; Costil et al. 2001; Alonso and Castro-Diéz 2008; Miranda et al. 2010). Given the high degree of adaptability, the NZMS could provide an exceptional model for non-native invasion with regard to the geographic range and the scope of different aquatic habitats it encompasses.

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