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Research Article

Synergistic Effects of Salinity and Temperature on the Survival of Two Nonnative Bivalve Molluscs, *Perna viridis* (Linnaeus 1758) and *Mytella charruana* (d'Orbigny 1846)

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This study examined the combined salinity and temperature tolerance of two marine bivalve molluscs, *Perna viridis* and *Mytella charruana,* which have recently invaded the southeastern United States. It is essential to understand the role that these abiotic variables play in invasions and establishment of nonnative species. We simultaneously explored survival at three salinity ranges (5–9, 20–22.5, and 35–40 ppt) in both cold and warm water for juveniles and adults of both species. We determined that *Perna viridis* can survive at a wide range of temperatures (9–35°C) when the salinity is 35–37 ppt; however, as salinity decreased, the thermal survival range for *P. viridis* became narrower. With *M. charruana*, our data suggest that juvenile and adult individuals can survive at a wide range of salinities (5–40 ppt) at 20°C, but the salinity tolerance range narrowed as the temperature decreased or increased. Additionally, we observed that temperature rapidly impacted survival of *P. viridis* and *M. charruana* (within hours), while salinity impacts were more gradual (days to weeks). These data can be used to help predict successful introductions and future expansions of *P. viridis* and *M. charruana* in introduced habitats.

1. Introduction

Global transportation has provided a means for marine invertebrate species to be transferred to new locations [1–3]. Species can be transported as larvae in ballast water and as adults attached to ship hulls, through the aquarium trade, recreational boating, and aquaculture (e.g., [1, 4–8]). Globally, molluscs are infamous invaders in both fresh and saltwater, as they are easy to transport, difficult to identify, and difficult to eradicate once established [1, 9–12]. As a result, the number of invasive molluscs continues to increase [13]. For example, more than 88 species of molluscs have become established in the United States via ballast water and hull fouling [13]. Although modes of global dispersal are becoming better known for molluscs, less remains understood about the factors that affect establishment of these organisms in new locations.

Perna viridis and *Mytella charruana* are two species of nonnative, marine molluscs that have recently invaded Florida (USA) and are potential causes of concern along the southeastern United States. Both *P. viridis* and *M. charruana* have been documented on intertidal oyster reefs in Florida [14, 15] and have been found to negatively impact the eastern oyster *Crassostrea virginica* [12]. We are interested in understanding how the abiotic conditions of temperature and salinity independently, and in synergy, influence the survival (and subsequent establishment) of these two nonnative molluscs and speculate how these factors may further restrict distributions in estuaries along the eastern Atlantic Ocean from Florida to Nova Scotia (Canada) where the eastern oyster is located [16].

There is a wealth of information on the synergistic effects of temperature and salinity on native molluscs [17–19], while other researchers have examined the combined effects of these abiotic factors to better understand invasion potential of molluscs around the globe (e.g., [20, 21]). For example, Sarà et al. [21] investigated the invasive *Brachidontes pharaonis,* now found in the Mediterranean Sea, by simultaneously

testing a range of salinities and temperatures to determine its physiological tolerance for feeding. These researchers determined that the clearance rate of *B. pharaonis* declined under conditions of low salinity and low temperature. Likewise, His et al. [20] examined the combined effects of temperature and salinity on the invasive Japanese oyster *C. gigas* in France and determined that the oyster exhibited the best growth at higher temperatures.

Previous studies have independently investigated the role of salinity and temperature in survival of *P. viridis* and the role of salinity in survival in *M. charruana* [22–24]. *Perna viridis* has been documented in salinities ranging from 19 to 44 ppt in its native habitat [25]. Segnini de Bravo et al. [22] determined that *P. viridis* could tolerate an even wider range of salinities under laboratory conditions (25°C) and the lower and upper lethal salinities were 0 and 64 ppt, respectively. *Perna viridis* survived at temperatures ranging from 10 to 42°C in the laboratory [25–29]. This broad temperature range should enable *P. viridis* to survive in both tropical and temperate zones.

Mytella charruana survived in salinities ranging from 2 to 40 ppt at 20°C [24]. Not much is known about the thermal tolerance for *M. charruana*, so the first goal of our study was to address how temperature influenced survival in *M. charruana*, independent of salinity. By addressing this knowledge gap, we were then able to determine appropriate temperature ranges for our salinity-temperature interaction experiments.

The overall objective of this study was to investigate the simultaneous effects of salinity and temperature on nonnative *Perna viridis* and *Mytella charruana* to better understand their respective invasion potentials. Specifically, we addressed the following questions: (1) What is the temperature tolerance range for survival of *Mytella charruana*? (2) How do salinity and temperature interact to influence the survival of adult and juvenile *Perna viridis*? (3) How does the interaction of salinity and temperature influence the survival of adult and juvenile *Mytella charruana*? These results will aid in predicting suitable environments where adult and juvenile individuals of both molluscs may become established and predict future range expansions.

2. Materials and Methods

2.1. Study Species. Perna viridis, the Asian green mussel, is native to the Indo-Pacific region along the Southeast Asian coastline, from the Arabian Gulf to southern China and southern Japan [30] and was first documented in the United States in 1999 in the cooling system of an electrical power plant in Tampa, Florida [31]. Currently, *P. viridis* is well established in the United States and extends from the Ten Thousand Islands to the northern Gulf of Mexico on the west coast of Florida, and along the eastern United States coastline from Stuart, FL (27° 09.831′ N; 80° 11.604′ W) to northern Georgia (32° 01.206′ N; 80° 59.569′ W) [15, 32, 33]. In Tampa Bay, *P. viridis* had the highest density of all observed locations within the United States, with 2,505 individuals m^{-2} [34].

Mytella charruana, commonly known as the charru mussel or sururu, is native to the Galapagos Islands, the Pacific coast from Mexico to Ecuador [35–38] and the Atlantic coast from Argentina to Venezuela [39]. Like *P. viridis*, the discovery of *M. charruana* in the United States occurred at a power plant. *Mytella charruana* was reported in Jacksonville, FL, in 1987; it was considered to be locally extirpated by spring 1988 [39]. In 2004, however, *M. charruana* was found in Mosquito Lagoon, 212 km south of Jacksonville [14]. *Mytella charruana* has now been recorded along the southeastern United States coast from Titusville, FL (28° 37.221′ N; 80° 48.371′ W), to Charleston, SC (32° 46.452′ N; 79° 57.539′ W) [15]. Densities of this nonnative species in the United States have been measured up to ~12 individuals m^{-2} [15]. This density is much lower than that in their native habitats where *M. charruana* densities as high as 11,036 mussels m^{-2} have been reported [40].

2.2. Thermal Tolerance of Mytella charruana. Mytella charruana (20–54 mm) were collected in cold and warm months between December 2007 and October 2009 from two floating dock sites (Arlington Lions Club and Lonnie Wurn) in Jacksonville, FL (Table 1(a); Figure 1). The location of samples collected for our experiments varied because we could not collect the 300–400 mussels needed per experiment from a single site. Stenyakina et al. [41] found that *M. charruana* were sexually mature at 12.5 mm. For thermal tolerance trials, we used adult mussels that were ≥20 mm.

Individuals were acclimated to the laboratory environment at 20°C for 13 days in a recirculating aquaria (151 L) with water from the collection site. This temperature was chosen as an intermediate for acclimation of all individuals as we previously determined that this species placed in water at this temperature always survived in high numbers, regardless of whether it represented a temperature increase or decrease from field conditions. In Florida, between 1981 and 2010, the temperature ranged from 3 to 26°C during January (coldest month) and from 19 to 35°C in July (warmest month) [42]. Thirteen days was used as the acclimation period as it represented sufficient time for any mortality associated with collection to occur. Prior to running these trials, we documented that individuals that survived at least 13 days would survive for months in 20°C waters (Yuan, pers. obs.). Additional details of each trial are listed in Table 1(a).

After acclimation, groups of 25 adult individuals ranging in length from 20 to 54 mm were placed in separate, aerated aquaria (21 L) that contained 14 L of water in a ratio of 25% collection site water: 75% artificial seawater (Instant Ocean® salts and deionized water) that maintained the salinity from the collection site. It was not practical to collect sufficient natural seawater for all trials. Survival in preliminary trials was 100% with this ratio of seawater to natural seawater (Yuan, pers. obs.). We divided the range of experimental temperatures into two seasons, cold for winter and warm for summer conditions. To avoid shocking the *M. charruana,* cold and warm experiments were correlated with field collection temperatures.

Cold experiment A was conducted in a walk-in freezer at 6°C at the University of Central Florida at 6, 9, 11, and 13°C. Warm experiment B examined survival at 20, 31, and 36°C, while experiment C investigated survival over a narrower

Table 1: (a) Summary of *Mytella charruana* thermal experiments. (b) Summary of *Perna viridis* and (c) *Mytella charruana* temperature-salinity interaction experiments. Acclimation period was the number of days in holding tanks prior to start of experiments. Adjustment period was the number of days to transition from holding tank temperature (20°C) to treatment temperatures. Experiment period was the number of days trial ran including adjustment period. Capital letters correspond to the experimental treatments presented in Figures 2, 3, and 5.

(a) *Mytella charruana*

Experiments	Collection sites	Latitude & longitude	Collection temperatures (°C)	Mussel size (mm)	Collection salinity (ppt)	Acclimation period (days)	Adjustment period (days)	Experiment period (days)	Mussels per tank/replicate tanks	Temperatures tested (°C)	Salinities tested (ppt)
Adult A: cold	Arlington Lions Club Jacksonville, FL	30.378272′ N 81.620828′ W	13	20–46	10	13	15	33	25/4	6, 9, 11, and 13	NA
Adult B: warm	Lonnie Wurn Jacksonville, FL	30.375798′ N 81.585165′ W	28	22–54	8	13	15	33	25/3	20, 31, and 36	NA
Adult C: warm	Lonnie Wurn Jacksonville, FL	30.375798′ N 81.525165′ W	27	25–46	10	13	15	33	25/4	20, 23, 28, and 31	NA

(b) *Perna viridis*

Experiments	Collection sites	Latitude & longitude	Collection temperatures (°C)	Mussel size (mm)	Collection salinity (ppt)	Acclimation period (days)	Adjustment period (days)	Experiment period (days)	Mussels per tank/replicate tanks	Temperatures tested (°C)	Salinities tested (ppt)
Juvenile A: cold	Huguenot Park Jacksonville, FL	30.405237′ N 81.40321′ W	10	12–38	36	5	15	28	10/3	6, 9, and 20	5, 20, 36
Juvenile B: warm	Huguenot Park Jacksonville, FL	30.405237′ N 81.40321′ W	10	12–38	36	5	15	28	10/3	20, 31, and 35	5, 20, 36
Adult C: cold	Municipal Marina St. Augustine, FL	29.891973′ N 81.310051′ W	18	58–114	35	5	15	28	10/3	6, 9, and 20	5, 20, 35
Adult D: warm	Municipal Marina St. Augustine, FL	29.891973′ N 81.310051′ W	28	57–113	37	5	15	28	10/3	20, 31, and 35	5, 20, 37

(c) *Mytella charruana*

Experiments	Collection sites	Latitude & longitude	Collection temperatures (°C)	Mussel size (mm)	Collection salinity (ppt)	Acclimation period (days)	Adjustment period (days)	Experiment period (days)	Mussels per tank/replicate tanks	Temperatures tested (°C)	Salinities tested (ppt)
Juvenile E: cold	Fire Station 38 Jacksonville, FL	30.390077′ N 81.641576′ W	17	5–12	5	7	15	28	10/3	6, 9, and 20	5, 22.5, 40
Juvenile F: warm	Fire Station 38 Jacksonville, FL	30.390077′ N 81.641576′ W	29	4–12	9	7	15	28	10/3	20, 31, and 33	5, 22.5, 40
Adult G: cold	Fire Station 38 Jacksonville, FL	30.390077′ N 81.641576′ W	16	20–38	5	7	15	28	10/3	6, 9, and 20	5, 22.5, 40
Adult H: warm	Arlington Lions Club Jacksonville, FL	30.378272′ N 81.620828′ W	28	20–31	5	7	15	28	10/3	20, 31, and 33	5, 22.5, 40

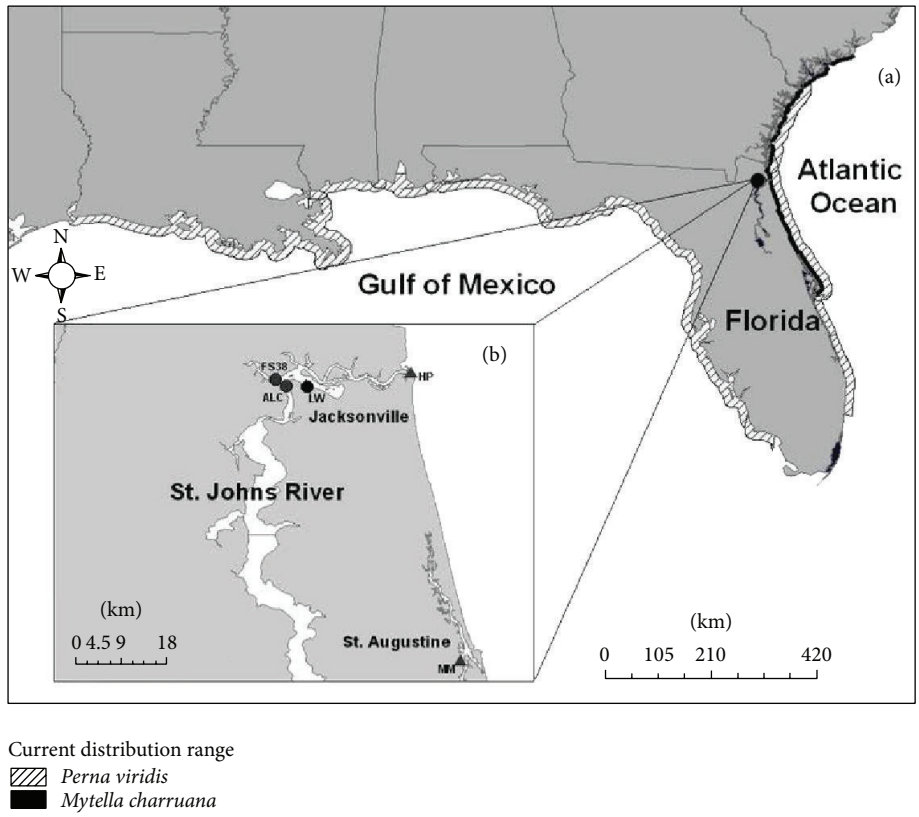

Figure 1: (a) Map of current distribution of *Perna viridis* and *Mytella charruana* and (b) collection sites for both species along Atlantic coast of Florida. ▲ indicates collection sites for *P. viridis* at Huguenot Park (HP) and Municipal Marina (MM). ● indicates collection sites for *M. charruana* at Fire Station 38 (FS38), Arlington Lions Club (ALC), and Lonnie Wurn (LW).

range of temperatures (20, 23, 28, and 31°C). Experimental temperatures were controlled by aquarium heaters (Hydor® 7.5 W, Top Fin® 25 W, or Finnex® 50 W heaters). All aquaria media started at their collection site salinity (see Table 1) and 20°C (control). Temperature adjustments occurred every 5 days for 15 days in equal increments depending on the difference between 20°C and the preplanned experimental temperatures (Table 1(a)). Once the treatment temperatures were reached on day 15, trials continued for 18 additional days. Salinities and temperatures were monitored and adjusted as needed twice each day for the duration of each trial.

There were four replicate aquaria for each temperature treatment for experiments A and C ($n = 4$) and three replicates for experiments B ($n = 3$), based on availability (Table 1(a)). Individuals were fed daily with a 10% algal solution made from an algal paste containing *Chaetoceros-B*, *Phaeodactylum tricornutum*, and *Nannochloropsis oculata* from Innovation Aquaculture (SPAT formula brand). Each tank of 25 individuals was fed 2.5 mL (0.1 mL per mussel); the volume of food per tank was reduced with mussel mortality. Mortality was checked daily during each trial. Individuals were considered dead if they remained gaping and did not respond to physical stimulus. Dead mussels were removed from tanks.

2.3. Perna viridis: Interaction of Salinity and Temperature. *Perna viridis* were collected in winter and summer months between February 2009 and September 2010 along the northeast coast of Florida from floating docks in St. Augustine, FL, and a jetty in Jacksonville, FL (Table 1(b), Figure 1). These locations were separated by 64 km. *Perna viridis* were placed in recirculating 210 L tanks in laboratory for acclimation using methods described above. Shell length for reproductive maturity in this species was 40 mm [43]; we, therefore, categorized *P. viridis* less than 40 mm as juveniles and greater than 40 mm as adults. Although adult *P. viridis* have been reported to reach over 220 mm in length along the southeastern Atlantic coastline, no mussels greater than 120 mm were used in our trials. Sizes for each trial are provided in Table 1(b). All were acclimated to aquarium conditions for 5 days prior to the start of each experiment.

After acclimation, the procedures were similar to the *M. charruana* thermal tolerance trials. We used 20°C as the starting temperature in both the cold and warm experiments to facilitate comparisons. The starting salinities for the experiments with *P. viridis* ranged from 35 to 37 ppt depending on collection site salinity (Table 1(b)). Each trial had nine salinity-temperature treatments, which combined three salinity levels (5–9, 20, and 35–37 ppt) and three temperature levels for the cold (6, 9, and 20°C) and warm (20, 31, and 35°C)

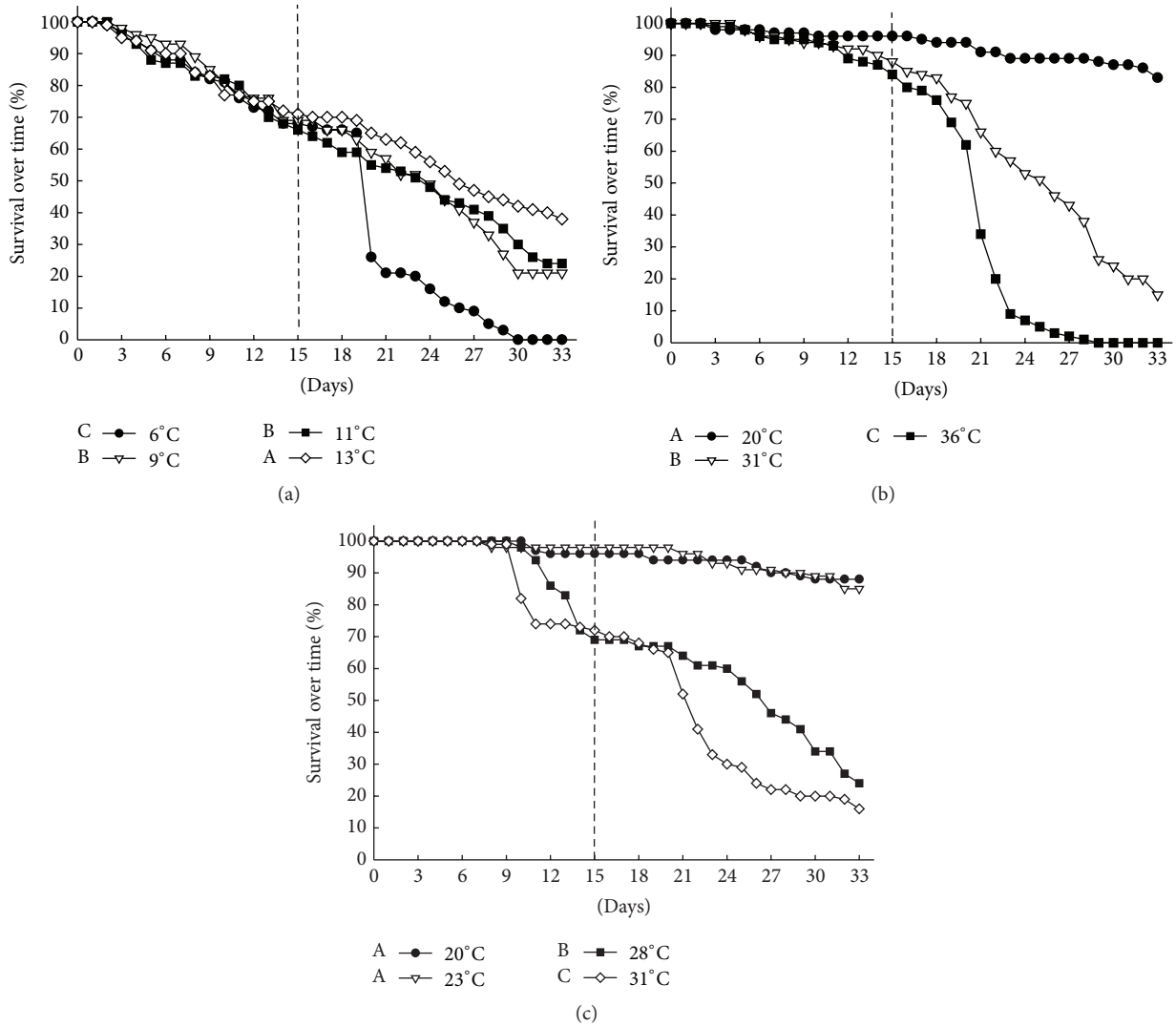

FIGURE 2: Survivorship curves of *Mytella charruana* for temperature experiments. Shell lengths: 20–54 mm. Capital letters correspond to the experimental treatments presented in Table 1(a). (a) Cold experiment (temperatures: 6, 9, 11, and 13°C); (b) warm experiment (temperatures: 20, 31, and 36°C); and (c) warm experiment (temperatures: 20, 23, 28, and 31°C). Upper case letters indicate significant differences in survivorship ($p \leq 0.05$) among temperature treatments. Dotted lines show the end of the 15-day temperature adjustment periods.

trials. Each treatment had three replicate aquaria ($n = 3$) and each aquarium held 10 individuals. Adjustments in salinity and temperature occurred every 5 days during the adjustment period and depended on the difference between the collection site and the preplanned experimental treatments. Individuals remained in their experimental tanks throughout each trial. The final salinity-temperature treatments were reached at the same time for all combinations at 15 days. Then, each experiment ran for an additional 13 days. Additional details are provided in Table 1(b). Throughout the experiments, *P. viridis* were fed with an algal solution as described above (0.4 mL per adult; 0.2 mL per juvenile mussel).

2.4. Mytella charruana: Interaction of Salinity and Temperature. *Mytella charruana* were collected in warm and cold months between February 2009 and September 2010 on floating docks in Jacksonville (Table 1(c), Figure 1). Individuals were placed in recirculating aquaria (76–151 L) with water from collection sites. All samples were held for 7 days before experiments began. *Mytella charruana* was fed with an algal solution of 0.1 mL per mussel throughout the experiment.

The experimental procedures were similar to the *P. viridis* interaction trials. For *M. charruana*, the starting salinities in the estuaries at the time of collection ranged from 5 to 9 (Table 1(c)). Each trial had nine salinity-temperature treatments of three salinity levels (5–9, 22.5, and 40 ppt) and three temperature levels for the cold (6, 9, and 20°C) and warm (20, 31, and 33°C) trials. The final salinity-temperature treatments were reached at the same time for all combinations at 15 days and then the experiments were run for 13 additional days. Each aquarium contained 10 individuals and there were three replicate aquaria for each treatment combination. Additional details are provided in Table 1(c).

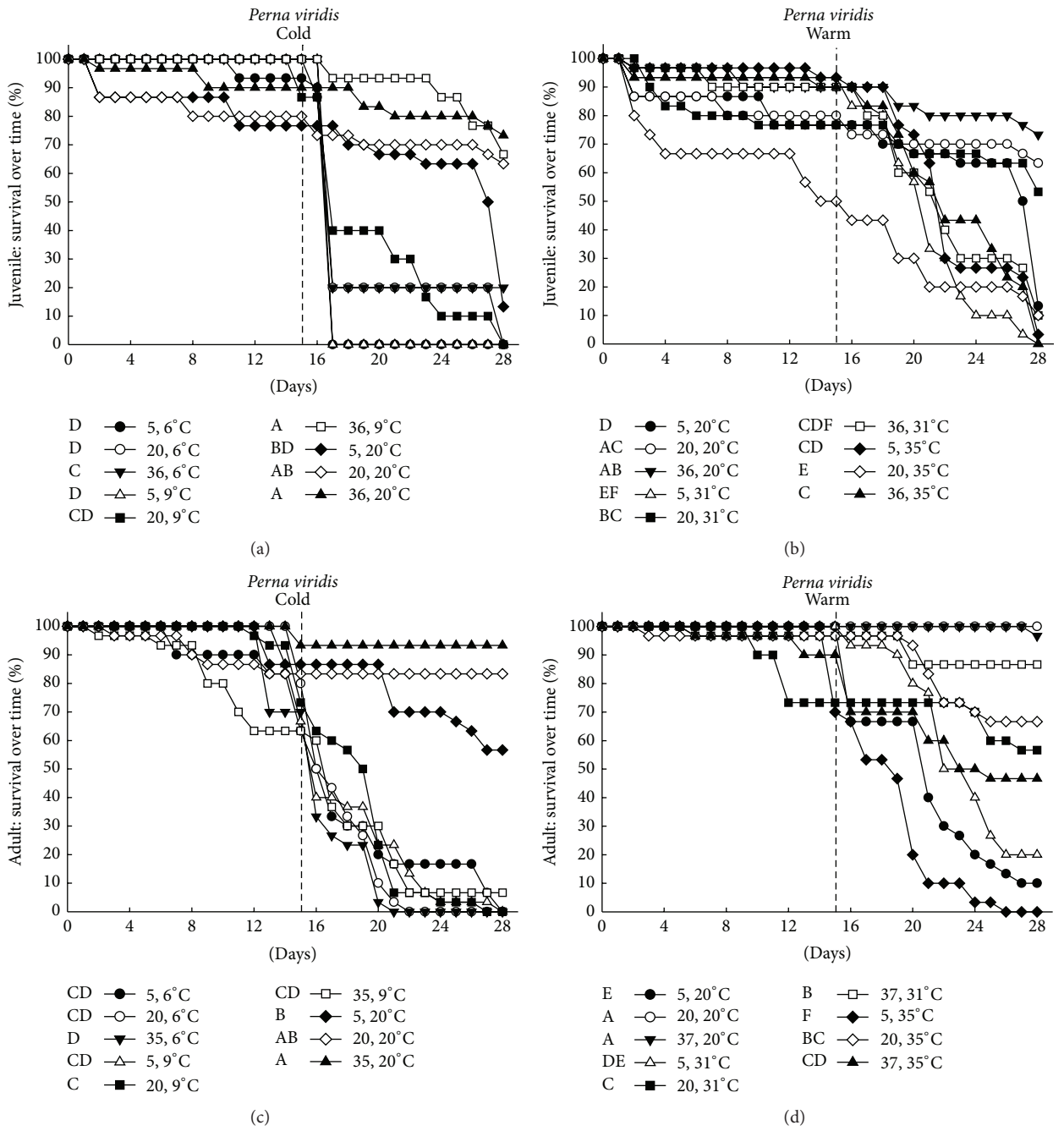

Figure 3: Survivorship curves over 28 days for *Perna viridis* in the temperature-salinity interaction experiments. (a) Juvenile, cold experiment (temperatures: 6, 9, and 20°C; salinities: 5, 20, and 36 ppt); (b) juvenile, warm experiment (temperatures: 20, 31, and 35°C; salinities: 5, 20, and 36 ppt); (c) adult, cold experiment (temperatures: 6, 9, and 20°C; salinities: 5, 20, and 35 ppt); and (d) adult, warm experiment (temperatures: 20, 31, and 35°C; salinities: 5, 20, and 37 ppt). Upper case letters indicate significant differences of $p \leq 0.05$ among treatments using survivorship analysis. Dotted lines show the end of the 15-day temperature adjustment periods.

2.5. Data Analysis. Kaplan-Meier survival analysis [44] was used to compare survivorship curves over the entire length of each experiment using pairwise comparisons to determine if significant differences were present among treatments [45]. Additionally, survival at the end of each trial for *M. charruana* in the thermal tolerance experiments was analyzed with one-way ANOVA with tank as the experimental unit. Similarly, the interaction of salinity and temperature at the end of each trial for *P. viridis* and *M. charruana* was analyzed with two-way ANOVA. Both tests were run using JMP v.10 [44].

Table 2: One-way ANOVA on the final survival for *Mytella charruana* thermal tolerance experiments.

Experiments	Source	DF	MS	F	p
Cold A	6, 9, 11, & 13°C	3	61.56	3.98	0.0352*
	Error	12	15.48		
Warm B	20, 31, & 36°C	2	489.09	251.53	<0.0001*
	Error	9	1.94		
Warm C	20, 23, 28, & 31°C	3	371.56	35.18	<0.0001*
	Error	12	10.56		

*Significantly different at $p < 0.05$ level.

Homogeneity of variances was checked using Levene's test and normality was checked using Shapiro-Wilk test; transformations of data were performed as required. Tukey's *a posteriori* tests were used to determine significant differences among treatments.

3. Results

3.1. Mytella charruana: Temperature Tolerance Experiments. Cold experiment A examined survival at 6, 9, 11, and 13°C; treatments were significantly different based upon survivorship analysis ($p < 0.001$, Figure 2(a)). The 13°C treatment had a steady and gradual decline in survival but still maintained the highest survival throughout this experiment (Figure 2(a)). The 6°C treatment had high mortality 5 days after reaching 6°C and 100% mortality on day 30 (Figure 2(a)). End-point survival also revealed significant differences among temperature treatments ($F_{3,12} = 3.98$; $p = 0.035$, Table 2). Overall, *M. charruana* had low survival in cold temperatures with 38% as the highest mean final survival at 13°C.

Warm experiments B and C investigated survival over time at 20–36°C and documented significant differences among treatments in both experiments ($p < 0.001$, Figure 2(b); $p < 0.0001$, Figure 2(c)). The 20°C (and 23°C) treatment maintained the highest survival over 33 days, followed by the warmer temperature treatments (Figures 2(b) and 2(c)). Treatments at 28, 31, and 36°C experienced dramatic mortality after reaching their respective experimental temperatures (Figures 2(b) and 2(c)). End-point survival in warm experiments B and C showed that all treatments were significantly different from each other when compared with Tukey's *a posteriori* tests (Exp. B, $F_{2,12} = 251.53$, $p < 0.0001$; Exp. C, $F_{3,9} = 35.18$, $p < 0.0001$; Table 2). The 20°C (and 23°C) treatment had the highest final survivals at 83–88%, followed by the higher temperatures, ranging from 0 (at 36°C) to 24% (at 28°C) (Figures 2(b) and 2(c)).

3.2. Juvenile Perna viridis: Salinity-Temperature Interaction Experiments. Survivorship over time in the cold experiment was significantly different overall for the tested salinity-temperature combinations ($p < 0.001$, Figure 3(a)). The survivorship patterns showed low mortality during the adjustment period (15 days) with 77% or greater survival for all treatment combinations (Figure 3(a)). Two days after the adjustment period ended, all juveniles at 6°C experienced a dramatic decline in survival (Figure 3(a)). At 9°C, no individuals kept at salinities of 5 and 20 ppt survived past day 28, but individuals held at a salinity of 20 ppt showed a more gradual decline. At 20°C, survival was highest at salinities of 36 and 20 ppt, respectively, and at salinity of 5 ppt, mortality increased greatly from day 26.

In the warm experiment with juvenile *P. viridis*, survival analysis showed a significant difference among the salinity-temperature treatments ($p < 0.001$, Figure 3(b)). The survivorship patterns showed a gradual decline during the 15-day adjustment period for all treatments except for a salinity of 20 ppt at 35°C, where survival declined more rapidly, with only 50% alive on day 15 (Figure 3(b)). Slopes steadily declined to 100% mortality for low salinity and high temperature combinations (Figure 3(b)). From both cold and warm experiments for juvenile *P. viridis*, the survival patterns indicated that this species responded more quickly to the effect of temperature than to salinity.

When considering end-point survival for juvenile *P. viridis*, there was no interaction between salinity and cold temperature ($F_{4,18} = 2.132$, $p = 0.119$; Table 3). However, the data in the cold experiment showed a strong temperature effect ($F_{2,18} = 7.755$, $p = 0.004$) and an even stronger salinity effect ($F_{2,18} = 9.940$, $p < 0.001$). Individuals in the cold experiment had the highest survival at the upper salinity and temperature combinations and 100% mortality in the lower salinity and temperature combinations (Figure 4). Opposite to the cold experiment, a significant interaction ($F_{4,18} = 3.366$, $p = 0.032$; Table 3) between salinity and warm temperature was observed for final survival with juvenile *P. viridis* (Figure 4).

3.3. Adult Perna viridis: Salinity-Temperature Interaction Experiments. We found significant differences among treatments in the survivorship of adult *P. viridis* in the cold experiment ($p < 0.001$, Figure 3(c)). The combinations that included 6 and 9°C media gradually declined until day 15, followed by a more rapid decline to 6% survival or less by day 28 (Figure 3(c)). The survivorship pattern for 20°C treatments showed that low salinity had a negative effect on adult *P. viridis* and that the difference in survivorship became more apparent during the last 8 days of the cold experiment (Figure 3(c)).

For the warm experiment, there was also a significant difference among treatments in the survivorship of adult *P. viridis* ($p < 0.001$, Figure 3(d)). The warm temperature survivorship pattern showed minimal decline during the adjustment period, except at a salinity of 20 ppt at 31°C (Figure 3(d)). Once the adjustment period ended at day 15, more rapid declines in survival were observed at all tested treatments when salinity was 5 ppt (Figure 3(d)). Adult *P. viridis* in both warm and cold experiments showed that temperature affected survival more rapidly than changes in salinity.

The final survival of adult *P. viridis* in the cold experiment showed that there was a significant interaction ($F_{4,18} = 3.480$, $p = 0.028$) between salinity and cold temperature (Table 3). The highest mortality was observed at the combination

Table 3: Two-way ANOVA tables: interaction between salinity and temperature on nonnative mussels.

Experiment		Source	*Perna viridis*					*Mytella charruana*				
			DF	MS	*F*	*p*	Significance	DF	MS	*F*	*p*	Significance
Juvenile	Cold	Salinity	2	43.370	9.940	<0.001	*	2	1.009	7.977	0.003	*
		Temperature	2	55.593	7.755	0.004	*	2	0.127	63.372	<0.001	*
		Salinity × temperature	4	11.926	2.132	0.119	ns	4	0.041	2.605	0.071	ns
		Error	18	5.593				18	0.016			
Juvenile	Warm	Salinity	2	47.815	8.208	0.003	*	2	1.8	2.978	0.076	ns
		Temperature	2	30.704	12.782	<0.001	*	2	0.103	52.269	<0.001	*
		Salinity × temperature	4	12.593	3.366	0.032	*	4	0.031	0.914	0.477	ns
		Error	18	3.741				18	0.034			
Adult	Cold	Salinity	2	1.764	5.160	0.017	*	2	0.229	2.528	0.108	ns
		Temperature	2	0.048	190.56	<0.001	*	2	0.037	148.361	<0.001	*
		Salinity × temperature	4	0.032	3.480	0.028	*	4	0.037	1.653	0.205	ns
		Error	18	0.009				18	0.013			
Adult	Warm	Salinity	2	0.218	56.194	<0.001	*	2	0.471	109.87	<0.001	*
		Temperature	2	1.29	9.500	0.002	*	2	0.936	55.348	<0.001	*
		Salinity × temperature	4	0.088	3.839	0.020	*	4	0.215	25.217	<0.001	*
		Error	18	0.023				18	0.009			

*Significantly different at $p < 0.05$ level.

of lowest salinities (5, 20 ppt) and lowest temperatures (6, 9°C) (Figure 4). As for the warm experiment, a significant interaction ($F_{4,18} = 3.839$, $p = 0.020$) was also observed in the final survival between salinity and warm temperature (Table 3). At 20°C, final survival was highest for all tested salinities. Unlike the survival of juvenile *P. viridis*, adults had moderate to high survival at warm temperatures and high salinities (Figure 4).

3.4. Juvenile Mytella charruana: Salinity-Temperature Interaction Experiments. Survival analysis showed significant differences among treatments in cold temperature and salinity on the survivorship for juveniles ($p < 0.001$, Figure 5(a)). In this experiment, the survivorship pattern showed a gradual decline in survival for all treatment combinations through to the end of the adjustment period (15 days), with 67% or higher survival (Figure 5(a)). All 6°C treatments experienced rapid declines to 23% survival or lower during the postadjustment period (Figure 5(a)). An intermediate gradual decline was observed at 9°C with the highest survival in the lowest salinity treatment (Figure 5(a)). At 20°C, the survival pattern remained greater than 70% for all tested salinities (Figure 5(a)).

In the warm experiment, survival analysis showed significant differences among treatments on survivorship for juvenile *M. charruana* ($p < 0.001$, Figure 5(b)). Similar gradual survivorship patterns were observed for all treatments before the adjustment period ended (Figure 5(b)). Treatments at 31 and 33°C experienced a rapid decline in survival after adjustment (Figure 5(b)). At 20°C, survival was 90% or higher on day 28. The mortality of juvenile *M. charruana* increased as the temperature increased, with the highest mortality in the uppermost tested salinity.

With final survival in the cold experiment, there were no significant interactive effects between salinity and temperature on juvenile *M. charruana* ($F_{4,18} = 2.605$, $p = 0.071$; Table 3). There were, however, a significant main effect of cold temperature and a significant salinity effect on juveniles ($F_{2,18} = 63.372$, $p < 0.001$; $F_{2,18} = 7.977$, $p = 0.003$, resp.; Table 3). In the warm experiment, there were also no significant interactive effects between salinity and temperature on the final survival for juvenile *M. charruana* ($F_{4,18} = 0.914$, $p = 0.477$), but a significant main effect of temperature ($F_{2,18} = 52.269$, $p < 0.001$). The final survival of juvenile *M. charruana* showed that they can endure temperatures that ranged from 6 to 33°C at a salinity of 5 ppt (Figure 4). The highest survival was at 20°C and salinity of 22.5 ppt, but mortality became greater as temperature and salinity decreased or increased (Figure 4). The highest mortality for juvenile *M. charruana* was observed at the combination of the highest salinity with lowest and highest tested temperatures.

3.5. Adult Mytella charruana: Salinity-Temperature Interaction Experiments. Survival analysis showed significant differences within treatments of cold temperature and salinity ($p < 0.001$, Figure 5(c)) on the survivorship of adult *M. charruana.* Before the adjustment period ended, mortality was only observed at 6°C at salinities of 5 and 40 ppt. After adjustment, 6°C treatments all showed declines to 7% survival or less by day 28, with survival at the highest salinity declining most rapidly (Figure 5(c)). High survival (>93%) was observed at the end of the experiment at 20°C at all tested salinities (Figure 5(c)).

In the warm experiment with adult *M. charruana*, survival analysis showed significant differences within treatments on survivorship ($p < 0.0001$, Figure 5(d)). There

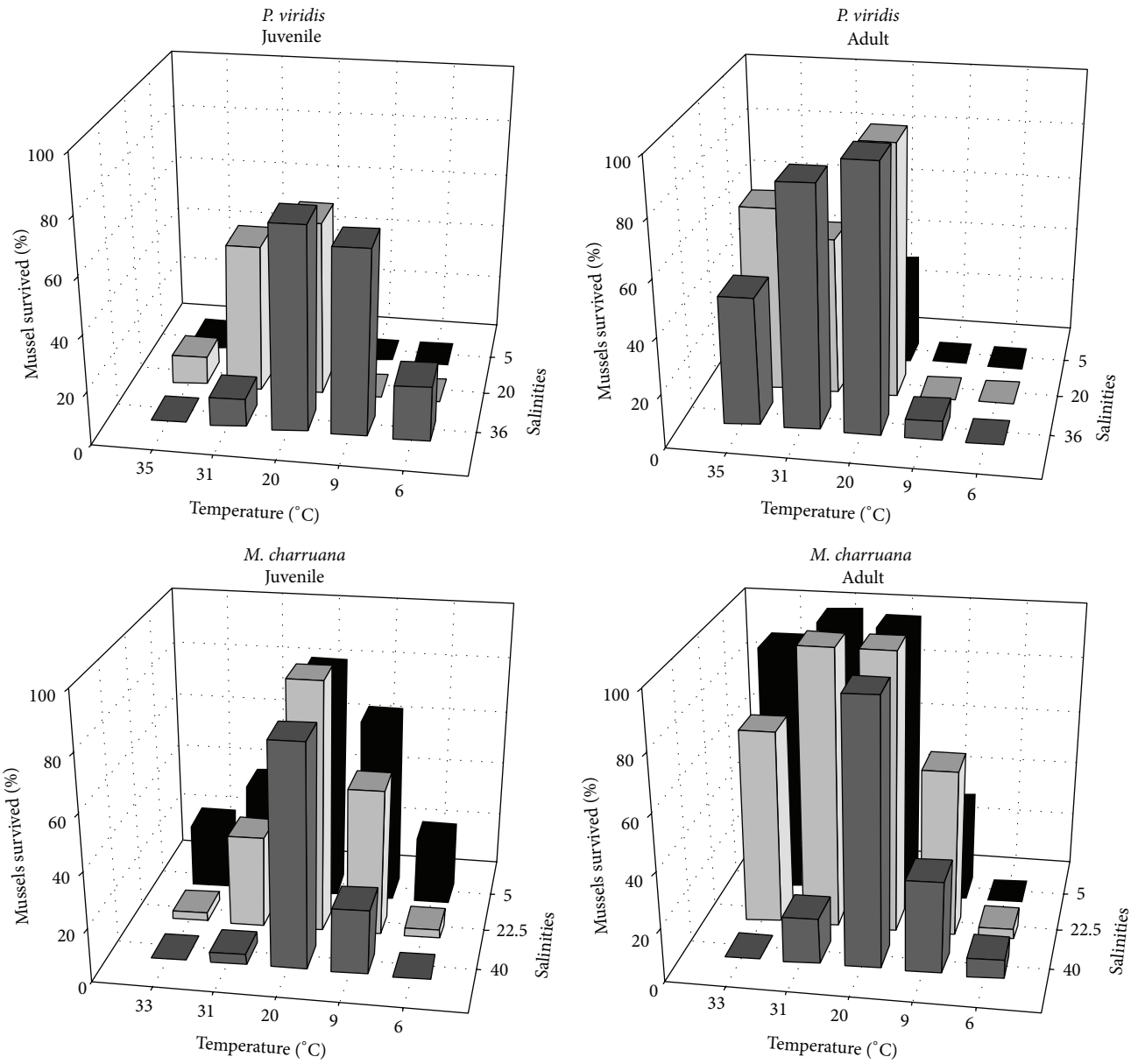

FIGURE 4: Final survival of *Perna viridis* in the salinity-temperature interaction experiments for juveniles and adults. Final survival of *Mytella charruana* in the salinity-temperature interaction experiments for juveniles and adults.

was no mortality before the end of the adjustment period (Figure 5(d)). Individuals kept in warmer temperatures and salinity of 40 showed rapid declines in survival beginning on day 17. Survival was 97% or higher in all treatments at 20°C (Figure 5(d)).

For the final cold survival experiment, there was no significant interaction ($F_{4,18} = 1.653$, $p = 0.205$) between salinity and temperature for adult *M. charruana*, though a significant temperature effect ($F_{2,18} = 148.361$, $p < 0.001$) was observed (Table 3). In the warm temperature experiment, a significant interaction effect between salinity and temperature ($F_{4,18} = 25.217$, $p < 0.001$) on the final survival of adult *M. charruana* was observed (Table 3). Survival approached 100% at 20°C; survival decreased both with reduced temperature and with increased salinity (Figure 4). The highest mortality was observed at combinations of high salinity and high temperature and of low salinity and low temperature.

4. Discussion

Environmental factors influence every species differently and their impact can change throughout the life history of an organism depending on body size and life stage [46, 47] and is particularly relevant for nonnative species following introduction (e.g., [48–50]). It is important to evaluate the synergistic effects of abiotic factors of all life stages to enable researchers to predict range expansions and survival of invasive species [51–53] and to determine whether species

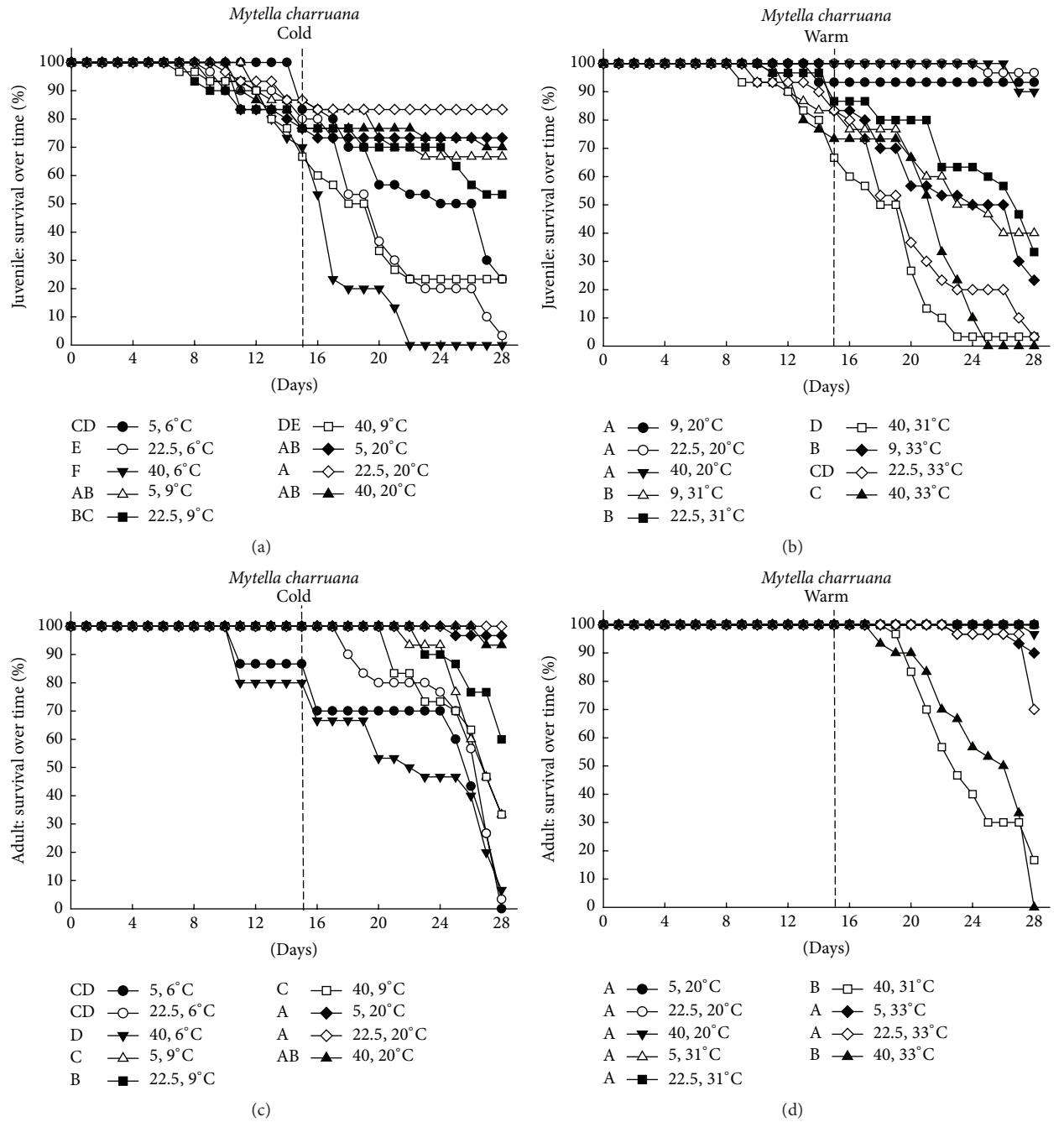

Figure 5: Survivorship curves over 28 days for *Mytella charruana* in the salinity-temperature interaction experiments. (a) Juvenile, cold experiment (temperatures: 6, 9, and 20°C; salinities: 5, 22.5, and 40 ppt), (b) juvenile, warm experiment (temperatures: 20, 31, and 33°C; salinities: 9, 22.5, and 40 ppt), (c) adult, cold experiment (temperatures: 6, 9, and 20°C; salinities: 5, 22.5, and 40 ppt), and (d) adult, warm experiment (temperatures: 20, 31, and 33°C; salinities: 5, 22.5, and 40 ppt). Upper case letters indicate significant differences of $p \leq 0.05$ among treatments using survival analysis. Dotted lines show the end of the 15-day temperature adjustment periods.

are evolving in their invaded range based on altered abiotic conditions (e.g., [54]). In light of the recently characterized negative impacts of *Perna viridis* and *Mytella charruana* on the ecologically and economically important eastern oyster *Crassostrea virginica* [12], we compare our results to other studies and the implications of these results to the long-term persistence and spread of these invasive species.

Studies that considered body size of the invasive mussel *Perna viridis* found that larger individuals could withstand abiotic stressors better than small-sized mussels [27, 48]. This pattern has also been shown for *Perna viridis* when exposed to cold air temperatures that coincided with low tides; higher survival was documented for larger individuals [29]. In contrast, Yuan et al. [24] found that small *M.*

charruana survived a broader range of salinities than larger individuals, further indicating the importance of considering body size in understanding environmental gradients. Our current study found that adult *P. viridis* survived differently across salinities and temperatures than juveniles (Figure 4). The most dramatic differences were that adult *P. viridis* did not survive or barely survived at the colder temperatures regardless of salinity, whereas juveniles were able to survive at colder temperatures in the high salinity trials. Furthermore, survival was lowest for juvenile *P. viridis* at the highest tested temperature and lowest tested salinity (Figure 4). This is the opposite of Urian et al. [29], who found large-sized *P. viridis* had a greater survival than small-sized individuals when exposed to cold air temperatures. The difference between our study and Urian et al. [29] could reflect submersion (our study) versus aerial exposure.

Survival across salinities and temperatures appears to be species-specific for marine molluscs (e.g., [16, 20, 21]). Kinne [47] found several sessile and low-mobility invertebrates had greater survival success at low salinity and low temperatures than all other relative temperature and salinity combinations. Likewise, Andrews et al. [55] considered the oyster *Crassostrea virginica* to survive for long time periods in a state of "narcosis" in low salinity and low temperature conditions. Other studies have shown the reverse, where survival was higher at low salinity/high temperature and high salinity/low temperature combinations (e.g., [56, 57]). For example, a study on the nonnative mollusc *Mytilopsis leucophaeata* in Europe found that embryonic mortality was high with the combination of high salinity and low temperature as well as with low salinity and high temperature [57]. Salinity and temperature impacted the survival of *P. viridis* and *M. charruana* in different ways. *Perna viridis* was influenced by both salinity and temperature. *Perna viridis* survived at a wide range of temperatures from when the salinity was high; yet, as salinity decreased, the range of temperatures at which the species survived became narrower. In contrast, we found that the survival of *M. charruana* was primarily driven by temperature. Interestingly, both species had some survival at all tested salinities (*P. viridis*: 5–36 ppt; *M. charruana*: 5–40 ppt) and at all tested temperatures (6–33/35°C; Figure 4). Little is known about the salinity and temperature ranges of *Mytella charruana* in their native habitats, but *P. viridis* had been described in waters where salinities ranged from 27 to 33 ppt and where temperatures ranged from 26 to 32°C in the Philippines [25].

Temperature changes killed more rapidly than salinity for both species. By analyzing survivorship curves, rather than just final survival, for a suite of temperatures and salinities found within the southeastern United States, we determined that temperature affected survival more quickly than salinity for both species investigated here. The temperature effect was expressed as a sharp drop in a survivorship curve shortly after a salinity-temperature adjustment, whereas salinity effects tended to result in slower declines in survivorship. Many published studies that examine the importance of abiotic variables on marine invertebrates have focused on the final response by the organism without considering the rate of mortality. For example, survival, physiological response, reproduction, and growth are often recorded, but these results do not incorporate the timing of the response to each abiotic effect [19, 21, 57–60]. Schneider [61], however, investigated the effects of temperature and exposure to air on the survivorship curves for two intertidal species (*Mytilus galloprovincialis*, *M. trossulus*) and found species-specific differences, with rapid survival declines evident at the highest tested temperatures.

Many nonnative species are more tolerant of environmental stressors than their native taxonomically related equivalents. The success of nonnative species was demonstrated in Lenz et al. [62], where the authors compared five native and nonnative marine invertebrate pairs on a global scale covering five biogeographic regions and found that the nonnatives (bivalves: *P. viridis*, *Isognomon bicolor*, and *Crassostrea gigas*; ascidian: *Didemnum vexillum*; and crustacean: *Gammarus tigrinus*) had higher survival and wider tolerance ranges than the paired native species (bivalves: *Brachidontes exustus*, *Perna*, and *Saccostrea glomerata*; ascidian: *Diplosoma listerianum*; and crustacean: *Gammarus tigrinus*). Similar patterns have been found in other taxa (e.g., mammals and birds [63]; plants [64]). *Perna viridis* and *M. charruana* were found along the southeastern USA Atlantic coast in shallow, subtidal regions on docks alongside the native bivalve molluscs *Brachidontes exustus* and *Geukensia demissa*, as well as with the native oyster *Crassostrea virginica*. Our study showed that *P. viridis* and *M. charruana* did not have wider survival tolerance ranges for salinity and temperature than those of these native species [65], while Brodsky et al. [66] showed that native *G. demissa* continued to produce byssal threads at lower temperatures than *M. charruana*. These results suggest that *P. viridis* and *M. charruana* are not adapted to local environmental extremes. In the invaded region, a field study on *P. viridis* and *M. charruana* reported that no live individuals were found during the winter of 2009/2010 at locations where both were previously established [15]. That winter was one of the coldest on record and had an unusually long duration of freezing temperatures in the southeastern United States [67]. Both species were subsequently reported in the southeastern United States after this cold event [15], but whether these species reestablished via newly introduced propagules or survived the cold weather event is unknown.

In summary, our study provides a greater understanding of how abiotic factors interactively affect the survival of nonnative *P. viridis* and *M. charruana*. Both species were tolerant of many salinity-temperature combinations found in the southeastern United States and thus should be able to both persist in their present invaded range and expand into new estuaries and bays along the Atlantic coastline of Florida and west within the Gulf of Mexico. Freezes, however, could thwart expansions into colder waters. Given that both species negatively impact the eastern oyster *C. virginica* [12] and possibly many other native species, it is important to continue to study their physiological ecology and any changes in distributions over time.

Competing Interests

The authors declare there is no conflict of interests regarding the publication of this paper.

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