

Physiological tolerance as a tool to support invasion risk assessment of tropical ascidians

Rosana M. Rocha^{1,*}, Giovanna C. Castellano², Carolina A. Freire³

¹Zoology Department, Universidade Federal do Paraná, CP 19020, 81531-980 Curitiba, PR, Brazil

²Graduate Program in Zoology, Universidade Federal do Paraná, CP 19020, 81520-980 Curitiba, PR, Brazil

³Physiology Department, Universidade Federal do Paraná, CP 19031, 81531-980 Curitiba, PR, Brazil

ABSTRACT: Physiological tolerance is a trait that may increase the invasion potential of transported species. A review of current literature, in which most species tested were from temperate or subtropical regions, shows that invasive ascidians can indeed tolerate a large range of salinities and temperatures. In this study, we used 4 tropical ascidians from Caribbean Panama as models to test survival of adults and their ability to maintain ionic gradients between blood and seawater in different salinities (10 to 45 ppt) and temperatures (15 and 29°C); we also measured early development success. We used these physiological data along with environmental information collected from ports to estimate the colonization pressure of these species in ports with shipping connections to Panama. Adults were more tolerant than gametes to both increased and decreased salinities; order of tolerance was *Ascidia sydneiensis* > *A. curvata* > *Phallusia nigra* > *A. panamensis*. All species were able to regulate ions (Cl⁻, Na⁺, Mg⁺⁺, K⁺) when tested at different salinities, indicating a mechanism for tolerance to varying environmental conditions. Preliminary colonization pressure analysis indicated that 31% of the ports we evaluated are at risk of colonization by the 3 most tolerant species, with a high of 78% risk by *A. sydneiensis*; only 22% of the ports studied were determined to be not at risk. We predict that *A. sydneiensis* will spread in the East Pacific and the expansion of the Panama Canal will increase opportunities for *A. curvata* (high probability) and *P. nigra* (lower probability) to be transported to the Pacific coast of America.

KEY WORDS: Colonization pressure · Invasion debt · Salinity tolerance · Temperature tolerance · Ion regulation · Exotic species · Phlebobranchia · Tunicata · Panama

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Ascidians are considered ideal models for studying biological invasions because under natural conditions they have very limited dispersal due to their non-feeding, very short-lived larvae (Zhan et al. 2015). Many species have been transported far from their presumed native regions by human-mediated vectors, including primarily aquaculture, fouling of hulls and sea chests on commercial and recreational vessels and (to a much lesser extent) possibly ballast water (Chu et al. 1997, Coutts & Dodgshun 2007, Simkanin et al. 2016). Introduced ascidians can have detrimental effects when they colonize new locations. Rapid growth

of successful invaders can cause changes in benthic communities by overgrowth of native species (Bullard et al. 2007) and competition for food (Colarusso et al. 2016). Additionally, some colonial ascidians inhibit recruitment of other benthic invertebrates (Dijkstra et al. 2007, Valentine et al. 2007). Ascidians also cause economic damage such as fouling of shellfish aquaculture (McKindsey et al. 2007, Sievers et al. 2013), especially by introduced ascidian species. In the order Phlebobranchia, *Ciona* is perhaps the most well-known invasive genus, and at least 3 species are important pests of bivalve aquaculture (Ramsay et al. 2008). Many other phlebobranchs have also recently invaded new regions (*Corella eumyota*: Lambert

2004, Bishop et al. 2015; *Ascidella aspersa*: Tatián et al. 2010; *Phallusia philippinensis*: Vandepas et al. 2015). Estimation of the introduction debt (i.e. the number of species likely to become introduced into a region) helps prioritize which species to monitor for risk assessment (Rouget et al. 2016) because successful eradication is more likely if small and spatially restricted populations are found (Wotton et al. 2004). Risk analyses of aquatic species introductions have focused on temperate regions and species (Ricciardi & Rasmussen 1998, Locke 2009, Jofré Madariaga et al. 2014) with a few studies in the tropics (Gardon et al. 2008). The most common approach is to evaluate the similarity between the donor and receptor environments for any potentially invasive species (Barry et al. 2008, David & Gollasch 2011, Keller et al. 2011). Tolerance limits of species are poorly studied, and donor environmental conditions are often used as a proxy of ideal environmental conditions for the species. The larvae of *Eudistoma olivaceum*, *E. hepaticum*, and *Ecteinascidia turbinata*, for example, metamorphose more quickly in salinities similar to that of their natural habitat than otherwise (Vázquez & Young 2000).

Measuring species' tolerances and performance over a gradient of conditions allows predictions of biological invasions (Osovitz & Hofmann 2007, Rius et al. 2014). A physiological approach can improve our understanding of the relationship between species and habitats (Osovitz & Hofmann 2007, Gröner et al. 2011), yet most physiological studies have examined terrestrial, rather than marine, systems (reviewed in Gaston 2003).

In marine environments, salinity and temperature are the most influential factors affecting the distribution of species (Dybern 1969, Barry et al. 2008). Greater physiological tolerance is believed to confer a greater potential to survive transport and colonize new sites (Epelbaum et al. 2009, Gröner et al. 2011). The widespread invasive species *Didemnum vexillum*, for example, can tolerate temperatures from below 0 to above 24°C and salinities of 10 to 34 ppt (Bullard et al. 2007, Valentine et al. 2007, Bullard & Whitlatch 2009). Studies of other invasive species, such as *Botryllus schlosseri*, *Botrylloides violaceus* (Dijkstra et al. 2008) and *Ciona savignyi* (Nomaguchi et al. 1997) have found large salinity and temperature tolerances relative to most tunicates, which tend to have low tolerance for fluctuating environmental conditions (Sims 1984, see Table S2 in Supplement 1 at www.int-res.com/articles/suppl/m577p105_supp1.pdf). However, most studies have focused on temperate or warm-temperate species (but see Dijkstra & Simkanin 2016 for a tropical example).

Panama is recognized as one of the Caribbean hotspots of diversity for ascidian fauna (Rocha et al. 2005), and the presence of the Panama Canal linking the Atlantic and Pacific oceans raises the opportunity for anthropogenic transport between them (Carman et al. 2011), including the exportation of species native to Panama to other regions. Marine species along the Caribbean coast of Panama experience a relatively constant environment (Paton 2015), so they might have a small range of tolerance which may limit their invasive ability.

In this study, we tested tolerances of 4 tropical phlebobranch ascidians to answer the following questions: (1) What are the physiological tolerance limits of these tropical ascidians? (2) Which species have the potential to invade other ports currently connected by shipping with Panamanian ports? (3) Are species that have a wide geographical range more tolerant than native and also geographically limited species?

MATERIALS AND METHODS

Species

Experiments were conducted using 4 tropical species (Fig. 1). *Ascidia sydneiensis* Stimpson, 1855 and *Phallusia nigra* Savigny, 1816 are widely distributed (Locke 2009), while *A. curvata* (Traustedt, 1882) and *A. panamensis* Bonnet & Rocha, 2011 are both native to the Caribbean Sea. *A. sydneiensis* occurs between latitudes 43°N and 40°S, and there are several introduction records for this species in various regions of the world, including the Caribbean (Bonnet & Rocha 2011a, Carman et al. 2011). *P. nigra* is widespread in the West Atlantic, and its distribution also includes the Mediterranean Sea, the Red Sea, and the Indian Ocean. Recent evidence indicates that Pacific records for this species were misidentifications and current distribution patterns favor the hypothesis of an Atlantic origin (Vandepas et al. 2015). *A. curvata* has the same latitudinal range in the West Atlantic as *P. nigra*, but the species is very rare on the Brazilian coast and has probably been introduced into southeastern Brazil (Dias et al. 2013). In contrast, *A. panamensis* occurs only in Panama on the Caribbean side (Bonnet & Rocha 2011a).

Physiological experiments

Salinity tolerance: adults

We tested survival under laboratory conditions of diluted seawater or increased salinity at ambient sea-

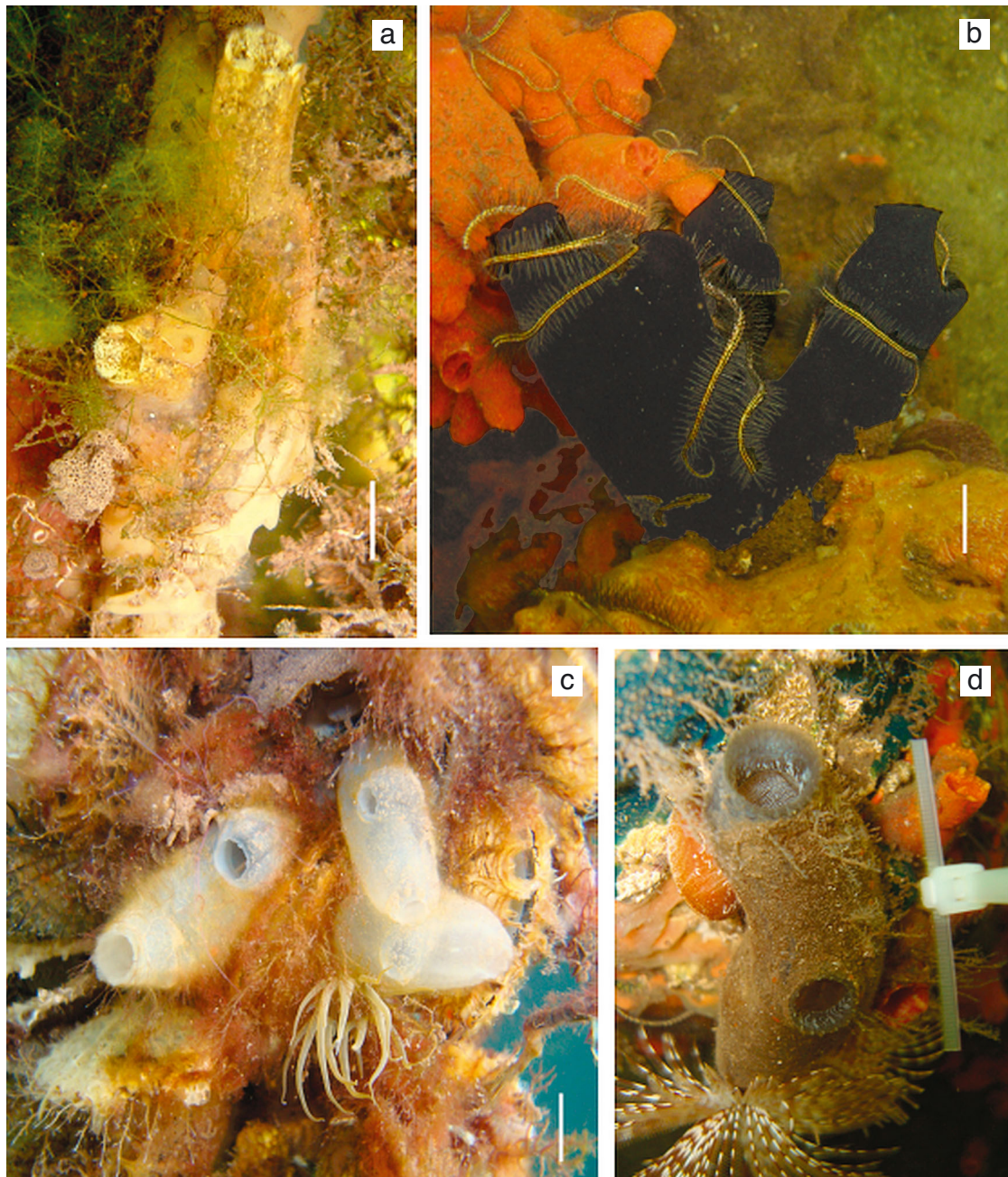


Fig. 1. Ascidian species included in this study: (a) *Ascidia sydneiensis*, (b) *Phallusia nigra*, (c) *A. curvata* and (d) *A. panamensis*. Scale bars: (a–c) = 1 cm; (d) = 5 cm

water temperature (around 29°C). Salinities were chosen within the gradient expected in the receiving ports, that are frequently located in estuaries or in closed embayments where low salinities are common after heavy rain. Thus, we focused the tests on salinity dilution challenges. Salinities as high as 45 ppt are uncommon but do occur in the Gulf of Suez (Keller et al. 2011) and the Persian Gulf (Russell 2013). Also, survival in increased salinity is seldom a problem

from the point of view of marine invasions, and in the source ports associated with Panamanian ports as destinations (see 'Materials and methods: Colonization pressure assessment in ports linked to Panama' below) only 2 out of 208 had mean salinity above 37 ppt.

Ascidians were collected in the field by snorkeling in Bocas del Toro region, on the Atlantic coast of Panama. Individuals were collected purposely from different locations in different years, so results would

not be due to a specific population. Animals were maintained along with their substrates whenever possible and placed in floating plastic colanders for up to 3 d in outdoor tanks (1000 l) with running, unfiltered seawater under environmental salinity and temperature conditions (~34 ppt, 27 to 29°C) before trials. This ensured that the animals used in the experiments were not damaged during collection. Previous experience showed that healthy solitary ascidians can survive for many days under these conditions; any damaged animals soon died and were removed.

Two to 4 individuals, one of each species, were placed in 4 different salinity treatments (10, 15, 20 and 45 ppt) in 10 l aquaria. Replicate measurements for each species within a treatment were taken from different tanks to avoid pseudoreplication. Due to availability of samples, experiments were carried out several times, with a few individuals each time, to attempt to balance the experimental design to include all species at all concentrations, with some replication (see Table 2 for final sample sizes).

To test osmotic limits of each species, we exposed them to salinities between 10 and 45 ppt for 4 and 8 h. Eight hours approximates a tidal cycle (during which low salinities are most likely to occur in an estuary), and is also the duration of typical transit through the Panama Canal (www.pan Canal.com). Survival after recovery was tested in a different treatment, in which ascidians were exposed to 20 ppt for 6 h and then returned to full-strength seawater (34 ppt) for 2 h, as a simulation of high tide return in a tidal cycle. Pilot experiments showed that most individuals of the 4 studied species would not survive many hours in low salinity (10 ppt), therefore we usually exposed them for 2 h at that level. *A. curvata* survived well, and was the only species exposed to 10 ppt for 8 h. At least 5 individuals of each species were tested in each of the low salinities (10, 15, 20) summing over time intervals, except *A. panamensis* in 10 ppt ($n = 2$). Three individuals of each of the 4 species were tested at 45 ppt. After immersion for the assigned time periods, each animal was rapidly dissected by a cut along the ventral margin of the tunic, slightly displaced to the right side of the animal. The tunic was opened to check for the heartbeat, then the body wall was opened. Approximately 1 cm² of the pharynx tissue was mounted on a glass slide in a drop of the seawater with a coverslip and placed under an optical microscope to observe cilia beating. Individuals were assigned scores of normal, weak or dead. A normal score indicated the cilia were beating rapidly. A weak score indicated the cilia were beat-

ing slowly or only a small portion of the cilia around stigmata was beating. Individuals whose cilia were not beating (or the only cilia beating were the small ones on primary papillae) were considered dead. One individual of each species remained in 34 ppt (control) to ensure that under typical seawater conditions the heart contracted and reversed the direction of blood flow, and the cilia continued beating for some minutes on the glass slide after the dissecting procedure, to show that this procedure worked correctly. All animals treated this way were found to be in the 'normal' category. All experimental animals damaged during dissection were discarded.

Ionic gradients: adults

To test if species could osmoregulate using ions (Na⁺, Cl⁻, Mg⁺⁺ and K⁺) found in their blood, we used a limited range of salinity and exposure time that would ensure survival of all or most individuals based on preliminary studies. Animals were exposed to combined conditions of salinity (15, 25, 34 and 38 ppt) and temperature (15 and 29°C) for 2 h in 3 l containers (1 animal container⁻¹).

All individuals tested were similar in size (~ 3 to 4 cm long), and most had mature gonads (at least the male gonad). At the end of each treatment, blood (100 to 200 µl) was taken from each animal (4 to 6 of each species for each condition) by piercing through the tunic into the heart with a hypodermic syringe. Blood samples were stored at -20°C. Na⁺ and K⁺ assays were performed by flame photometry (CELM-160), in samples diluted in deionized water. Cl⁻ and Mg⁺⁺ measurements were made using commercial colorimetric kits (Labtest). Absorbance was read with a spectrophotometer (Ultrospec 2100 PRO; Amersham Pharmacia Biotech). *A. panamensis* was not tested at 15°C because too few animals were available.

We calculated the difference between the ionic concentration in the blood and that in the surrounding water at each of the salinities tested. If the ion concentration in the blood was equal or proportional to that in the seawater at all salinities, then there was no ion regulation. In other words, in a regression of ion concentration differences by salinity, a slope of zero indicates no regulation. A negative slope, on the other hand, indicates regulation. Regressions were carried out in R (version 3.3.2, 2016) and figures in CoPlot (version 6.3). Results are shown as the observed values of ion concentration both in blood and seawater for clarity.

Salinity tolerance: fertilization and embryogenesis

The 4 species used in this study release gametes into the water, that then have to cope with local environmental conditions to become established. Gametes tend to be less tolerant of environmental conditions than adults (Pineda et al. 2012), and we tested them in the same range of salinities used for ionic gradients (15, 25, 34 [control] and 38 ppt).

Gametes were obtained by standard procedures of puncturing the gonadal ducts of animals that had been removed from the tunic. Oocytes were collected first to ensure that they were not self-fertilized before the sperm duct was punctured. Gametes were collected from 4 individuals of each species, except *A. panamensis*, for which only 2 individuals for each treatment were available. Oocytes from each replicate were stored in separate 50 ml beakers. Sperm from all individuals of one species were mixed together to increase the chance of fertilization. Male and female gametes were exposed separately to the salinity treatment for 30 min. The water in the oocyte beakers was then replaced with seawater (34 ppt). For sperm, after the 30 min exposure, 1 ml of the sperm solution was added to the water with oocytes at time zero of the fertilization trial. Thus, fertilization was allowed to occur in normal seawater (34 ppt), which simulated an incoming tide. The influence of salinity on gamete viability was measured by counting the percentage of eggs in the most advanced stage after 1 and 2 h, considering at least 30 eggs in each replicate.

Literature review

To compare our results with current understanding of ascidian tolerance to temperature and salinity variation, we comprehensively reviewed studies that experimentally tested the effects of temperature or salinity variation on survival, growth, metabolism or reproduction. We also compiled studies that reported the presence of healthy animals under extreme conditions of either salinity or temperature in the field, especially Phlebobranchia since all 4 species in this study belong to this order.

Articles were initially compiled from personal databases of references by R. M. Rocha and G. Lambert. We also searched Google Scholar using the following keywords: Ascidian salinity, tunicate salinity, ascidian temperature, tunicate temperature, ascidian physiology, tunicate physiology and ascidian tunicate tolerance, and considered results of the first 20 pages. After selecting an article based on its relevance (infor-

mation on either temperature or salinity tolerance limits observed in experiments or in the field) we also checked its list of references for additional articles.

Colonization pressure assessment in ports linked to Panama

Ports with connections to Atlantic Panamanian ports were determined following Keller et al. (2011). We used the number of voyages between ports in 2007 and 2008 to classify the connection between ports as high (≥ 50 voyages), medium (10 to 50), low (5 to 10) or very low (< 5).

The introduction of ascidians to new regions depends on transport (usually of adults) by a suitable vector. Nonetheless, establishment in the receiver region depends on the survival of all life stages, including gametes and larvae. Thus, we compared the most restrictive combinations of temperature and salinity suitable for both adults and early stages of each of our 4 study species with environmental conditions at receiving ports. A positive match indicated potential invasion risk. Salinity tolerance was established from our physiological experiments, and minimum tolerable temperature was estimated using the range limit of the species' current distribution: *A. curvata* (17.1°C), *A. panamensis* (25°C), *A. sydneyensis* (3.9°C), and *P. nigra* (16.9°C) (Table 1; www.seatemperature.org, accessed 14 Oct 2015). Environmental conditions of ports in the database (Keller et al. 2011) included average salinity (as for most ports, minimum and maximum salinity data were not provided), and the minimum temperature. The maximum temperature was not used because the maximum temperature of the ports is less than what the animals are able to withstand; only 3 ports—Jebel Ali in the United Arab Emirates, Jawaharlal Nehru in India and Bangkok in Thailand—have maximum temperatures slightly over 30°C, which is also the maximum experienced by ascidians in the Bocas del Toro region (Collin et al. 2009, see Table S3 in Supplement 2 at www.int-res.com/articles/suppl/m577p105_supp2.xls). Ports were analyzed in groups by their biogeographic realm (sensu Spalding et al. 2007).

Ports were classified in 4 risk categories of colonization pressure (= number of species that can invade a given region; Lockwood et al. 2009), which is one of the components of the invasion debt (Rouget et al. 2016): high (port environmental conditions match 3 of our 4 species tolerances); medium (port conditions permit survival of *A. sydneyensis* and *A. curvata*, while not favoring survival of the other 2 species); low (only

Table 1. Global distribution, origin and sites of probable introduction of *Ascidia curvata*, *A. panamensis*, *A. sydneiensis* and *Phallusia nigra*

Species Distribution	Origin	Sites of probable introduction	References
<i>Ascidia curvata</i> Bermuda, Caribbean Sea, Brazil	Tropical West Atlantic	South Brazil	Van Name (1945), Rocha & Kremer (2005), Bonnet & Rocha (2011a), Carman et al. (2011)
<i>A. panamensis</i> Bocas del Toro (Atlantic Panama)	Bocas del Toro	Not reported	Bonnet & Rocha (2011a)
<i>A. sydneiensis</i> Caribbean Sea, South Brazil, Cape Verde, Sierra Leon, South Africa (Atlantic), Japan, Philippines, Australia, Hawaii, Guam, Palau, Polynesia, (Pacific), Indian Ocean	Possibly West Pacific	Atlantic, Australia	Van Name (1945), Kott & Goodbody (1982), Monniot (1983), Monniot & Monniot (1987), Abbott et al. (1997), Lambert (2002), Rocha & Kremer (2005), Bonnet & Rocha (2011a,b), Carman et al. (2011)
<i>Phallusia nigra</i> Bermuda, Florida, Caribbean Sea, Brazil, Guinea, Angola, West Mediterranean, Red Sea, Gulf of Aden, Persian Gulf, India, Singapore	Possibly West Atlantic	India, Persian Gulf, Singapore, possibly the Red Sea	Van Name (1945), Pérès (1958), Lambert (2003), Bonnet & Rocha (2011b), Vandepas et al. (2015)

A. sydneiensis was likely to survive, while *A. curvata* was unlikely to survive or the probability was very low); and zero (environmental conditions beyond the physiological tolerances of all 4 species).

RESULTS

Physiological experiments

Salinity tolerance: adults

Most animals were either weak or dead after 2 h in very low salinity (10 ppt) and after 4 h in 15 ppt, except for *Ascidia curvata* (2 individuals survived 8 h in 10 ppt; Table 2). Some individuals of all species survived 4 h at 20 ppt, but surviving *A. panamensis* and *Phallusia nigra* had very weak cilia beats indicating strong stress. Only a few individuals of *A. curvata* were still normal after 8 h at 20 ppt. *P. nigra* was the most sensitive species; it was weak or dead within 4 h of immersion in 20 ppt or lower salinity, and did not recover even after 2 h at 34 ppt. In high salinity (45 ppt), again *P. nigra* was the most sensitive, with one dead after 4 h of exposure. The other species survived, but only *A. curvata* and *A. sydneiensis* maintained normal cilia beats.

Ionic gradients: adults

While the lower salinity treatments resulted in lower ionic concentrations in the blood than at standard salinity, the reduction was often much less than that expected under osmoconformality, both at 15 and 29°C (*A. sydneiensis*: Fig. 2, other species: Figs. S1–S3, Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m577p105_supp1.pdf). Negative regression slopes demonstrate that at low salinity, animals maintain a higher ion concentration in the blood, and at high salinity, a lower blood ion concentration compared to seawater. Thus, osmotic regulation is carried out in these ascidians.

In addition, at ambient water temperature (29°C), all species maintained gradients between the blood and seawater for sodium, magnesium and potassium with the exception of *A. panamensis*, which was unable to maintain the gradient for potassium. Only *A. sydneiensis* was able to maintain gradients for chloride at that temperature. Under temperature stress (15°C), most gradients were steeper and tighter (greater r^2 values) than at 29°C, except for potassium (Fig. 2, S1–S3, Table S1 in Supplement 1). *A. curvata* also maintained a potassium gradient and steeper gradients of all ions compared to the other species.

Table 2. Survival of ascidian adults exposed to different salinities. Each cell contains the number of animals in each category of response: normal: heart and pharyngeal cilia clearly beating; weak: heart and cilia very slowly beating or few cilia moving; dead: static (not moving) heart and cilia

Salinity (ppt)	Duration (h)	<i>Ascidia sydneiensis</i>			<i>Ascidia curvata</i>			<i>Phallusia nigra</i>			<i>Ascidia panamensis</i>		
		Normal	Weak	Dead	Normal	Weak	Dead	Normal	Weak	Dead	Normal	Weak	Dead
10	2	1	0	4	4	2	0	0	2	3	0	1	1
10	8	–	–	–	2	1	0	–	–	–	–	–	–
15	4	0	1	2	0	4	0	0	1	3	1	2	0
15	8	0	0	2	1	2	1	0	0	2	0	2	3
20	4	2	1	1	3	2	0	0	3	2	0	3	0
20	8	0	0	2	1	3	0	0	0	4	0	4	1
20	6 + 2 ^a	2	0	0	0	2	0	0	2	2	1	1	0
34 ^b	8	1	0	0	1	0	0	1	0	0	1	0	0
45	4	2	1	0	1	2	0	0	2	1	0	3	0

^aIn this treatment, animals stayed for 6 h in 20 ppt and 2 h for recovery in environment salinity (34 ppt), with the exception of *A. sydneiensis* which was tested for 4 h in 20 ppt and 2 h in 34 ppt; ^bcontrol salinity

Salinity tolerance: fertilization and embryogenesis

Fertilization and development over a period of 2 h were compared between control (34 ppt) and each experimental salinity (15, 25 and 38 ppt). Salinity of 15 ppt was the most detrimental to initial development for all species, but especially for *A. curvata* and *A. panamensis* (Table 3). In *A. curvata*, fertilization and cleavage were also hindered in 25 ppt salinity but were normal in 38 ppt. In *A. panamensis* and *P. nigra*, fertilization and cleavage were depressed in all experimental salinities, but were normal in *A. sydneiensis* at 25 and 38 ppt (Fig. S4 in Supplement 1 at www.int-res.com/articles/suppl/m577p105_supp1.pdf). Thus, the most euryhaline (i.e. tolerant of salinity change) gametes were of *A. sydneiensis*, and the most stenohaline (i.e. intolerant of salinity change) were of *A. panamensis* and *P. nigra*.

Literature review

Our literature review found that of 41 species evaluated, 23 had a history of introduction, 9 of which are invasive (*Ciona intestinalis*, *C. ro-*

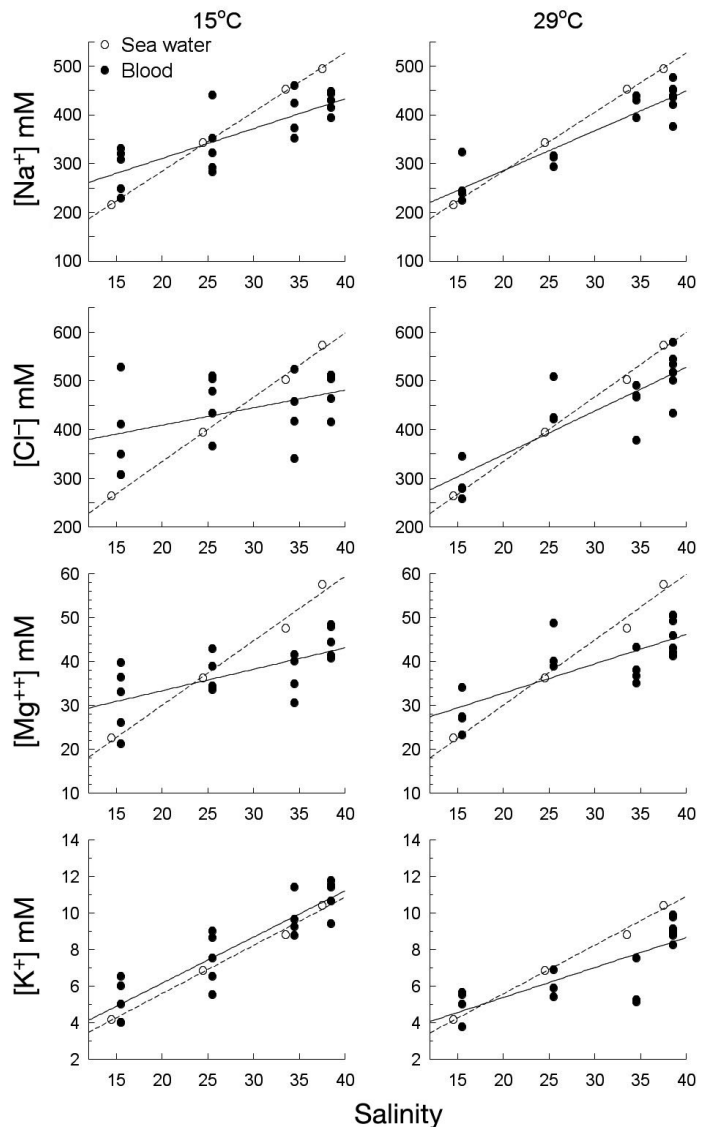


Fig. 2. Regression of ion concentration in *Ascidia sydneiensis* blood (solid line) and seawater (hatched line) against salinity challenges (15, 25 and 38; control: 34 ppt) in 15 and 29°C, after 2 h of immersion. Each dot represents a different individual. Test results for significance in Table S1. For these data for the other 3 species, please refer to Figs. S1–S3, Table S1 in Supplement 1 at www.int-res.com/articles/suppl/m577p105_supp1.pdf

Table 3. Fertilization and initial development of embryos, in which gametes were subject to salinity challenge for 30 min. Reports for the most advanced stage in any of the replicates in each time frame, considering at least 30 oocytes in each replicate

Species (no.)	Time after fertilization (h)	Salinities			
		15 ppt	25 ppt	34 ppt (control)	38 ppt
<i>Ascidia sydneiensis</i> (4)	1	12 % 2-cell stage	95 % 2-cell or 4-cell stage	100 % 2-cell or 4-cell stage	98 % 2-cell or 4-cell stage
	2	15 % many-cells stage	98 % many-cells stage	100 % many-cells stage	98 % many-cells stage
<i>Ascidia curvata</i> (4)	1	No fertilization	65 % 2-cell stage	80 % 4-cell stage	80 % 4-cell stage
	2	No fertilization	50 % many-cells stage	80 % many-cells stage	90 % many-cells stage
<i>Phallusia nigra</i> (4)	1	Fertilization	Fertilization	10 % 2-cell or 4-cell stage	10 % 2-cell stage
	2	Fertilization	30 % many-cells stage	40 % many-cells stage	17 % many-cells stage
<i>Ascidia panamensis</i> (2)	1	No fertilization	No fertilization	10 % many-cells stage	No fertilization in the only cross tested
	2	No fertilization	9 % many-cells stage	100 % many-cells stage	40 % many-cells stage

busta, *Styela clava*, *S. plicata*, *Botryllus schlosseri*, *Botrylloides violaceus*, *Microcosmus squamiger*, *Didemnum perlucidum*, *D. vexillum*; Tables 4, S2 in Supplement 1). Five species are able to tolerate dilution as low as 15 ppt (*C. intestinalis*, *B. violaceus*, *B. schlosseri*, *M. manhattensis* and *D. vexillum*), at least for a short period of time. Species with the greatest range in temperature tolerance (25°C or higher) comprised 5 with histories of introductions, 3 of which are known to be invasive (*B. violaceus*, *B. schlosseri* and *D. vexillum*). Among those 23 introduced species only 8 have tropical populations. Surprisingly, 11 of the 41 species have a salinity tolerance range greater than 20 ppt, and 10 have a temperature tolerance range of $\geq 20^\circ\text{C}$.

Colonization pressure assessment

Data compilation (Keller et al. 2011) indicated 7 ports on the Atlantic coast of Panama that are connected by ship routes to 208 ports in 59 different countries in 10 biogeographic realms (sensu Spalding et al. 2007). Most of these ports (132) were connected by < 5 voyages in 2007 and 2008, while 19 ports were connected by 5 to 10 voyages, 37 ports by 10 to 50 voyages and 19 ports by > 50 voyages (see Fig. 4A, Table S3 in Supplement 2).

Our experiments determined the combination of temperatures and salinities that allowed survival and reproduction of 4 species; *A. sydneiensis* was most tolerant to variable conditions while *A. panamensis* was the least tolerant (Fig. 3). Based on these results, we determined the number of species likely to sur-

vive and reproduce in each combination of salinity and temperature, and matched port environmental conditions to determine their risk of being invaded by those species (Fig. 4B, Table S3 in Supplement 2). Ports with salinities between 30 and 35 ppt and temperatures between 15 and 30°C may be invaded by 3 or 4 of the species we tested, and were considered to have high colonization risk. Ports with salinities 25 to 30 or 35 to 40 ppt, and temperatures of 15 to 30°C were considered at medium colonization risk. The combination of salinities between 15 to 40 ppt and temperatures between 5 and 15°C or salinities below 15 or above 40 ppt were considered of low risk. Ports with no risk had salinities below 15 ppt or temperatures below 5°C (Fig. S5 in Supplement 1).

Risk analysis indicated that around half of the ports evaluated are at risk of being invaded by at least 2 of the species—*A. sydneiensis* and *A. curvata* (ports with high and medium risk)—and another 21% are at risk of being invaded by at least *A. sydneiensis* (low risk; Table 5). Less than one-third of the ports are not at risk of being invaded by any of these species. Most of the ports analyzed are in the Tropical Atlantic (87), 13% of which are at high risk of establishment of any of the 4 ascidians, but 82% are at risk of being invaded by *A. sydneiensis* or *A. curvata*. In other tropical realms (Tropical Eastern Pacific: 18 ports; Central IndoPacific: 12 ports) 78 and 67% of the ports were at high risk of establishment of any of the 4 species, while $\sim 83\%$ of the ports may be invaded by *A. sydneiensis* or *A. curvata*. Temperate provinces (88 ports) included 10 that were warm enough to be invaded by 3 of the species studied, and 52 may be invaded by *A. sydneiensis*.

Table 4. Summary of the literature survey of salinity (S) and temperature (T) tolerances of ascidians. Results include information from both physiological tolerance experiments and natural environments of known geographical distributions. Zones: A: Arctic; Te: temperate; ST: subtropical; Tr: tropical. 'x' indicates species with reports of introduction. See text for method details and Table S2 in Supplement 1 at www.int-res.com/articles/suppl/m577p105_supp1.pdf for references

Species	Zone	I	S min. ^a	S max.	S range	T min.	T max.	T range
Phlebobranchia								
<i>Ascidia callosa</i>	A, Te		30					
<i>Ascidia ceratodes</i>	Te, ST		30	35	5	11	24.5	13.5
<i>Ascidia conchilega</i>	Te		30					
<i>Ascidia mentula</i>	Te		20					
<i>Ascidia virginea</i>	Te		20					
<i>Ascidia zara</i>	Te, ST	x	30	35	5	11	23	12
<i>Asciidiella aspersa</i>	Te	x	18			15	25	20
<i>Asciidiella scabra</i>	Te		18					
<i>Phallusia mamillata</i>	ST					7	25	18
<i>Ciona intestinalis</i>	Te	x	10	37	27	-0.6	20.5	20
<i>Ciona robusta</i>	Te, ST	x	5	45	40	10	25	15
<i>Ciona robusta</i> (larva)	Te, ST		32			15	25	10
<i>Ciona savignyi</i> (larva)	Te	x				12	25	13
<i>Corella parallelogramma</i>	Te		18					
<i>Ecteinascidia turbinata</i> (larva)	Tr	x	22					
Stolidobranchia								
<i>Styela clava</i>	Te	x	17.5	38.5	21			
<i>Styela montereyensis</i>	Te		17.5	38.5	21			
<i>Styela plicata</i>	Tr, ST	x	17.5	38.5	21			
<i>Styela plicata</i> (larva)	Tr, ST		30			18	30	12
<i>Styela rustica</i> (larva)	A, Te	x	16					
<i>Dendrodoa grossularia</i>	A, Te		7-8					
<i>Polyandrocarpa zorritensis</i>	Tr, ST, Te	x	26					
<i>Botrylloides leachi</i>	Te		16	38	22	3	28	25
<i>Botrylloides nigrum</i>	Tr, ST	x	24	35	11			
<i>Botryllus planus</i>	Tr, ST		20	35	15			
<i>Botrylloides violaceus</i>	Te	x	15	38	23	5	34	29
<i>Botryllus schlosseri</i>	Te, ST	x	14	44	30	3	29	26
<i>Halocynthia roretzi</i>	Te				11	5	25	20
<i>Halocynthia aurantium</i>	A, Te		25	34	9	8	20	12
<i>Microcosmus exasperatus</i>	Te, ST, Tr	x	33	45	12	12	32	20
<i>Microcosmus squamiger</i>	Te, ST	x				20	25	5
<i>Pyura dalbyi</i>	Te		23.5	33.4	10			
<i>Pyura herdmani</i>	ST, Tr	x				20	20	0
<i>Pyura stolonifera</i>	Te, ST	x				20	20	0
<i>Molgula manhattensis</i>	Te	x	9	40	31			
<i>Molgula socialis</i>	Te, ST	x	15	45	30	5	30	25
Aplousobranchia								
<i>Clavelina lepadiformis</i>	Te	x	14					
<i>Clavelina huntsmani</i>	ST		28	37.6	9.6	4	22.5	18.5
<i>Eudistoma hepaticum</i> (larva)	Tr		16					
<i>Eudistoma olivaceum</i>	Tr, ST		26					
<i>Distaplia occidentalis</i>	A, Te						24.5	6.5
<i>Didemnum perlucidum</i>	ST, Tr	x	27	39.5	12.5	13	30	17
<i>Didemnum vexillum</i>	Te, ST	x	10	34	24	-2	24	26
<i>Didemnum vexillum</i> (larva)	Te, ST					14	20	6
<i>Diplosoma listerianum</i>	Te, ST, Tr	x	34	34	0	9	29	20

^aFigures include maximum and minimum temperatures and salinity conditions in which the species survived, even if briefly, under conditions of the study. In many cases the authors did not test the species beyond their limits; thus, the numbers given are conservative and probably not true limits

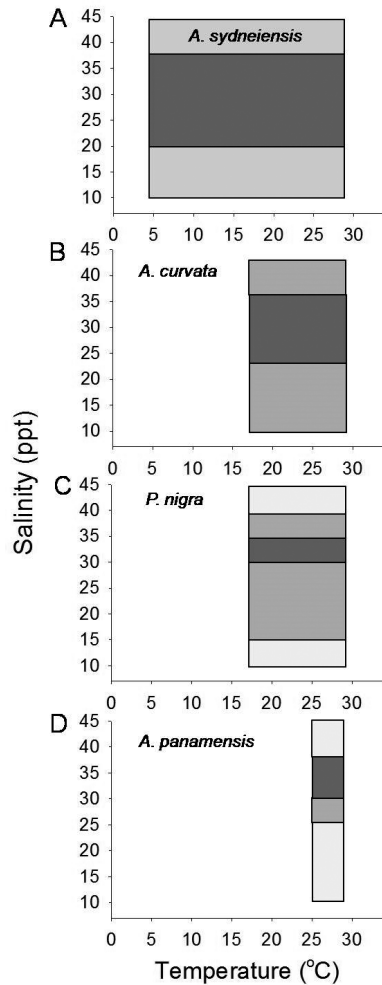


Fig. 3. Physiological limits for survival with respect to salinity and temperature for *Ascidia sydneienseis*, *A. curvata*, *Phallusia nigra* and *A. panamensis* based on adult survival and fertilization experiments (salinity), and on latitudinal limits of occurrence of species (temperature) (see 'Material and methods: Colonization pressure assessment in ports linked to Panama' for details). Dark colored areas represent optimal tolerance (adults + gametes); intermediate colored areas represent some tolerance (either adults or gametes); light colored areas represent low tolerance

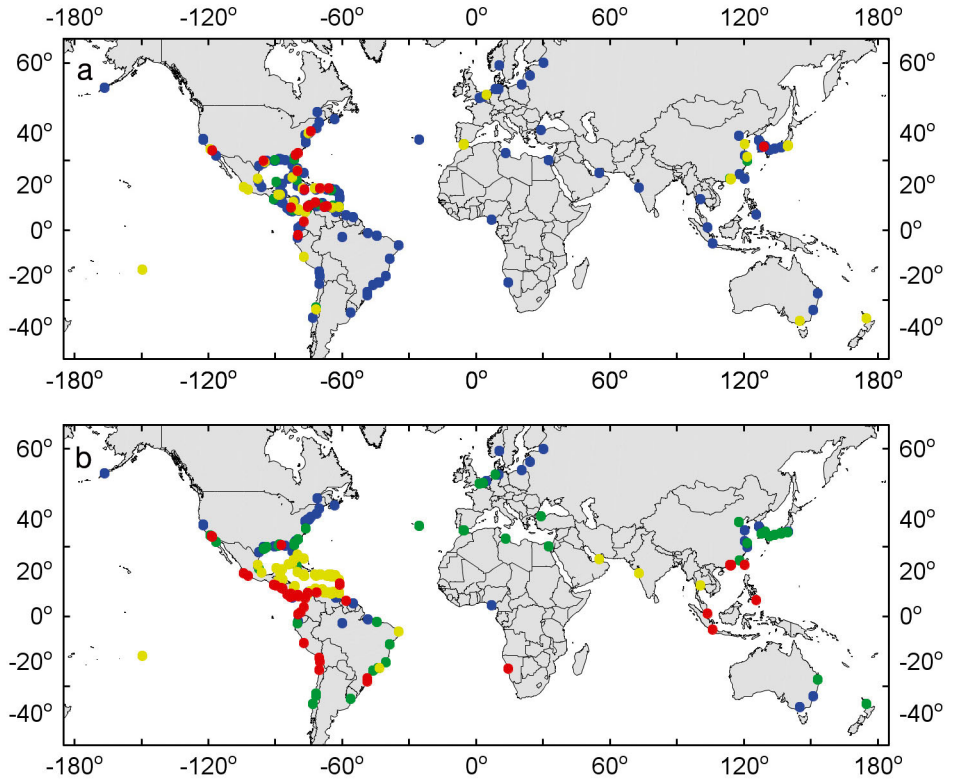
DISCUSSION

Despite the limited physiological tolerances of many ascidians (especially for dilute seawater) and the location of many shipping terminals within bays and estuaries with extremely variable salinity, ascidians are increasingly being reported as introduced around the globe (Lambert 2007, Shenkar & Swalla 2011, Simkanin et al. 2016). Here, we demonstrate invasion risk by tropical ascidians in ports connected to Panamanian ports by shipping, and show that this risk is a consequence of their wide tolerance to variations in salinity and temperature.

Our literature review found information on salinity and/or temperature tolerance for 8 tropical species. Here, we add information for another 4 species — 2 of them very tolerant, one native (*Ascidia curvata*) and one introduced (*A. sydneienseis*). *A. curvata* and *A. sydneienseis* adults were the most tolerant to both hypo- and hyper-salinity. The sequence of tolerance of gametes to low salinity was *A. sydneienseis* > *Phallusia nigra* > *A. curvata* > *A. panamensis*, while in high salinity *A. curvata* gametes were more tolerant than *P. nigra*. *A. curvata* and *A. sydneienseis* adults were also most able to maintain their ion gradients at ambient temperature (29°C). At lower temperatures, on the other hand, ion gradients were strong for all species. *A. sydneienseis* and *A. curvata* were thus the most euryhaline species compared to *A. panamensis* and *P. nigra*, while *A. sydneienseis* was the most eurythermic and *A. panamensis* the most stenothermic (Fig. 3). While our experiments measured only a few individuals and do not permit estimation of the proportion of each species that can survive extreme conditions, the fact that some individuals of each species can survive significant variation in such a small sample demonstrates that survival is often likely among individuals arriving in a new region.

The physiological capabilities described here for phlebobranchs are new results for the field of ascidian ion regulation related to osmotic control. A study of ion regulation found sodium and potassium gradients in adult *Styela clava*, *S. montereyensis*, and *S. plicata* (stolidobranch ascidians) after a challenge of reduced salinity (Sims 1984, see Table S2 in Supplement 1 at www.int-res.com/articles/suppl/m577/p105_supp1.pdf). Sodium and chloride gradients are not expected in osmoconformers (but see Vidolin et al. 2007), as these ions are the major contributors to extracellular fluid osmolality. These gradients are thought to be due to sudden changes in apparent permeability (e.g. siphon closure). We found sodium gradients in the phlebobranchs tested. While we did not find chloride gradients in environmental temperature, chloride channel genes have been found in the *Ciona* genome (Okamura et al. 2005), suggesting potential chloride regulation. The genus *Ciona* is believed to be the closest to the ancestral ascidian (Tsagkogeorga et al. 2009), which suggests that similar genes and potential for ion regulation may be found in other species. Indeed, even echinoderms show consistent ion gradients (Freire et al. 2011) suggesting some ion regulation is present in all deuterostomes. It is important to recognize that gradients in osmoconformers are not permanent but rather tend to dissipate with exposure time (Evans 2009), thus future research

Fig. 4. (a) Connectivity between Panamanian and other ports based on number of voyages in 2007 and 2008 (Keller et al. 2011). Red circles: >50 voyages (high connectivity); yellow: 10 to 50 (medium connectivity); green: 5 to 10 (low connectivity); blue: <5 (very low connectivity) (see Supplement 2 at www.int-res.com/articles/suppl/m577p105_supp2.xls). (b) Colonization pressure (no. of species that could establish in the port) for ports connected with Atlantic Panamanian ports (Supplement 2) determined by a combination of salinity and temperature with ascidian tolerance tested here (see Fig. 3, S5 in Supplement 1 at www.int-res.com/articles/suppl/m577p105_supp1.pdf). Red circles: possible establishment of *Ascidia sydneiensis*, *A. curvata* and *Phallusia nigra* or of all 4 species; yellow: possible establishment of *A. sydneiensis* and *A. curvata* with very low probability of introduction of *P. nigra* or *A. panamensis*; green: possible establishment of *A. sydneiensis* with very low probability of colonization of *A. curvata*; blue: no risk for establishment of any of the studied species



should examine how long ascidians can maintain ionic gradients under a variety of conditions.

Salinity tolerance is frequently greater at lower temperatures, depending on the intensity and dura-

tion of the temperature stress, as found in amphipods and mollusks (Berezina & Panov 2004). Also, caridean shrimps that regulate magnesium in the haemolymph and that maintain low concentrations are better

adapted to withstand colder waters (Frederich et al. 2001), so it is interesting that tropical ascidian species can also regulate magnesium. In our experiments, the ionic gradients were greater at 15 than 29°C (control), suggesting that species were better able to maintain their osmotic balance at lower temperatures. Thermal stress at 15°C may have elicited siphon closure, thus diminishing flow between the internal and external medium, and this could explain why ascidians maintained the ionic gradient. Unfortunately, our experimental design did not allow testing this possibility because animals were inside an incubator chamber and could not be directly observed. This behavior has been recorded in other ascidians, such as 3 *Styela* species, under hypotonic conditions (Markus & Lambert 1983, Sims 1984) and *Ciona savi-*

Table 5. Colonization pressure analysis: percentage of ports connected to the Atlantic Panamanian ports and grouped by biogeographic realms (sensu Spalding et al. 2007) in risk of the establishment of *Ascidia curvata*, *A. panamensis*, *A. sydneiensis* and *Phallusia nigra*. List of ports is provided in Table S3 in Supplement 2 at www.int-res.com/articles/suppl/m577p105_supp2.xls. High: possible establishment of 3 or 4 species; Medium: possible establishment of *A. sydneiensis* and *A. curvata*, very low probability of introduction of the other 2 species; Low: possible establishment of *A. sydneiensis*, very low probability of introduction of *A. curvata*; Zero: no risk of establishment of any of the 4 species

Biogeographic realms	No. of ports analyzed	Percentage of ports in each colonization pressure classification			
		High	Medium	Low	Zero
Tropical Atlantic	87	13	69	9	9
Tropical Eastern Pacific	18	78	6	6	11
Central Indo-Pacific	12	67	17	17	0
Western Indo-Pacific	2	0	100	0	0
Temperate Northern Atlantic	46	2	0	46	52
Temperate South America	13	46	8	46	0
Temperate Southern Africa	1	100	0	0	0
Temperate Northern Pacific	25	8	0	56	36
Temperate Australasia	3	0	0	33	67
Arctic	1	0	0	0	100
Total	208	31.3	19.9	21.2	27.5

gnyi in the presence of an irritant (Moody et al. 1999). A similar mechanism is used by bivalves (HRS-Brenko 2006) and barnacles (Chan et al. 2001) that isolate themselves inside an external calcareous skeleton.

Avoiding stressful conditions is a good strategy for temporary events, such as severe storms in tropical environments (Kauffman & Thompson 2005) or salinity fluctuation during the tidal cycle. Nonetheless, apparent ion regulation possibly achieved by siphon closure may contribute to invasion capability in areas of fluctuating salinities over a period of hours, such as in estuaries, where most ports are located. Recovery after episodic events of decreased salinity has been observed in the colonial ascidians *Botrylloides nigrum* and *Botryllus planus* (Dijkstra & Simkanin 2016), the first of which has been introduced in many regions (Sheets et al. 2016).

The only other introduced phlebobranch ascidians with information regarding physiological tolerance for stressful environment conditions are *Asciidiella aspersa*, *Ciona robusta* (previously reported as *C. intestinalis* A; Brunetti et al. 2015), *C. intestinalis* (*C. intestinalis* B; Brunetti et al. 2015), and *C. savignyi* (Tables 4, S2 in Supplement 1). While *A. aspersa* can live in salinity as low as 18 ppt in its natural geographic range (Dybern 1969), salinity challenge experiments have not yet been carried out with this species. *C. robusta* has a widespread distribution, yet individuals tested in Chile did not survive in 15 ppt, while 30% of the individuals survived 25 ppt (Jofré Madariaga et al. 2014). In contrast, *C. intestinalis*, although less widespread than *C. robusta* and confined to the Northern Hemisphere, is tolerant of salinities as low as 12 ppt (Dybern 1969), yet juveniles did not survive at 20 ppt longer than 8 wk in experimental conditions (Vercaemer et al. 2011). Ascidians are known to adapt to local conditions and their tolerance range may change (Toop & Wheatly 1993); thus, the study of widespread populations, under a variety of environmental conditions, might reveal even greater ranges of tolerance than observed in most studies usually directed to spatially restricted populations (Tables 4, S2 in Supplement 1).

In contrast to the extreme examples cited above, most ascidians are stenohaline, as reflected by their exclusively marine distribution (Lambert 2005), and most species do not survive extended periods (i.e. days, weeks) of low salinity (Table S2). In our experiments, the longest duration of low salinity (≤ 20) exposure was 8 h, which resulted in high (but not complete) mortality in all species. Our experiment was not designed to test exactly how long individuals

may survive, but our results suggest lower mortality when animals are re-introduced to control salinity before 8 h of low salinity exposure (Table 2).

Greater physiological tolerance to salinity stress was expected in adults compared to gametes, due to isolation of the animal within the tunic in contrast to direct exposure of gametes (Pineda et al. 2012). Thus, adults may be better able to invade new environments than gametes or larvae, but whether they establish viable populations remains to be determined by the tolerances of the first life stages. The phlebobranchs used in this study all release gametes directly into the sea, and except for *A. sydneyensis*, their gametes were very sensitive to low salinity, even briefly (30 min; Table 3).

The hypothesis that species with a wide geographical range are more tolerant to variations in temperature and salinity than those that are spatially restricted was partially rejected. This hypothesis was based on a relationship between physiological tolerance, geographical distribution and invasive potential (Osovitz & Hofmann 2007, Epelbaum et al. 2009, Rius et al. 2014). Indeed, *A. sydneyensis*, probably introduced into the Caribbean Sea and the most geographically widespread (Locke 2009), also had the most tolerant gametes and very tolerant adults to variation in salinity (although adults were not as tolerant to very low salinity as the native *A. curvata*). In contrast, *P. nigra*, also very widespread (Vandepas et al. 2015), was not very tolerant either as adults or gametes. Thus, even stenohaline species can spread and invade new regions given the right conditions during transport and an environmental match between donor and receptor regions.

As expected, our risk analysis indicated a greater likelihood of establishment at tropical than temperate ports, even though establishment remains likely at 10 temperate ports because of the combination of water temperature and salinity. When contrasted with port connectivity, the ports most connected to Panama (higher number of voyages per year) were on both Atlantic and Pacific sides of tropical America. Among the environmentally adequate temperate ports, Long Beach in the United States had a high connectivity and Callao in Peru had a medium connectivity; the others were very low. It is important to note that only 22% of the ports were environmentally unsuitable for any of the species studied while 78% were likely to be colonized at least by *A. sydneyensis*, the most euryhaline and temperature-tolerant species in this study. There are many adequate ports for *A. sydneyensis* with high connectivity where the species has not yet become established (e.g. Pacific coast

of United States and Colombia, Ecuador, Peru, Chile, Atlantic coast of United States and Spain). Thus our classification of a port as of low colonization risk does not mean that monitoring or prevention should be relaxed, because the species that may arrive may also be the most harmful. This potential invasibility of many ports becomes even more alarming because ports have abundant artificial hard substrate, on which colonization by non-native ascidians is favored over that of natives (Airoldi et al. 2015). In addition, successfully establishing non-native species are tolerant of pollution, which is generally present in ports (Piola & Johnston 2008).

Our estimate of colonization pressure is probably conservative, even with the sample size available. Individuals of each species used in the experiments were collected from different locations at different times and therefore should include most of the population variation in their tolerance traits. It is unlikely that by chance, we found the only tolerant individuals of each species. Survival of a single adult can be sufficient for colonization, because phlebobranch ascidians are hermaphroditic and self fertile, at least to some extent. There is evidence that one adult colony was enough to colonize extensive regions, for *Ecteinascidia turbinata* (López-Legentil & Turon 2007) and *Clavelina oblonga* (Rocha et al. 2012), given the lack of genetic variation in CO1 among individuals of many populations. A more complete risk analysis would require additional information about survival in transit (Coutts et al. 2010), propagule size and number (Lockwood et al. 2009) and biotic resistance in the receiving region (Rius et al. 2014). Thus, while we cannot precisely predict introduction probability, we can anticipate the ability of the species to colonize once they arrive.

The Panama Canal is an important hub in the international maritime trade, allowing passage of ca. 13 000 vessels yr⁻¹, and its new expansion will double capacity (Muirhead et al. 2015). Because of the geographic location of Panama, tropical ports in the American continent are likely to be invasion destinations by these ascidian species. *A. sydneyensis* is the only species that has already established populations on the Pacific side of Panama (Carman et al. 2011) but not yet in other countries in the eastern Pacific, where we predict that it will spread. We also predict that *A. curvata* will also be able to cross the Panama Canal barrier, given the high tolerance of adults to low salinity and its already widespread distribution, which suggests that it is transportable by anthropogenic vectors. Even though salinity drops to 0 ppt during the passage through the Panama canal, that

will not affect ascidians attached to the walls of ballast tanks, and the current distribution of many species confirms that the barrier can be crossed (Carman et al. 2011, Sheets et al. 2016). Currently, ships take only 10 h (or less) to transit the canal (www.pancanal.com), and with its expansion this time will decrease, thereby increasing the probability of adult survival. This is also likely to increase the opportunities for *P. nigra* to be transported, despite it being less tolerant of low salinity. *A. panamensis* has the lowest chance of invading new regions because of the sensitivity of its gametes to salinity variation, small population size and limited present distribution. Previously, tropical species have been absent from most physiological studies of tolerance to stressful environmental conditions, but here we demonstrate that some phlebobranch ascidians tolerate fluctuations in salinity and temperature, at least for a few hours, and because of that tolerance, they are likely to spread to many other regions.

Acknowledgements. The authors thank all the staff at Bocas del Toro Research Station and especially its director Rachel Collin who made this study possible. We also thank Gretchen Lambert for valuable comments on the manuscript, English revision and indication of articles for the literature review, and James J. Roper for English revision and help with Figs. 2, 4, S2, S3, and S4. The National Council of Technological and Scientific Development – CNPq financed research grants for R.M.R. (200914/2008-1, 305201/2014-0) and C.A.F. (306630/2011-7), and a doctoral scholarship for G.C.C.

LITERATURE CITED

- Abbott DP, Newberry AT, Morris KM (1997) The reef and shore fauna of Hawaii. Section 68: Ascidians (Urochordata). Bishop Museum, Honolulu, HI
- ✦ Airoldi L, Turon X, Perkol-Finkel S, Rius M (2015) Corridors for aliens but not for natives: effects of marine urban sprawl at a regional scale. *Divers Distrib* 21:755–768
- ✦ Barry SC, Hayes KR, Hewitt CL, Behrens HL, Dragsund E, Bakke SM (2008) Ballast water risk assessment: principles, processes, and methods. *ICES J Mar Sci* 65:121–131
- ✦ Berezina NA, Panov VE (2004) Distribution, population structure and salinity tolerance of the invasive amphipod *Gmelinoides fasciatus* (Stebbing) in the Neva Estuary (Gulf of Finland, Baltic Sea). *Hydrobiologia* 514:199–206
- ✦ Bishop JDD, Wood CA, Yunnice ALE, Griffiths CA (2015) Unheralded arrivals: non-native sessile invertebrates in marinas on the English coast. *Aquat Invasions* 10:249–264
- Bonnet NYK, Rocha RM (2011a) The family Ascidiidae Herdman (Tunicata: Ascidiacea) in Bocas del Toro, Panama. Description of six new species. *Zootaxa* 2864:1–33
- Bonnet NYK, Rocha RM (2011b) The Ascidiidae (Ascidiacea, Tunicata) in coastal Brazil. *Zool Stud* 50:809–825
- ✦ Brunetti R, Gissi C, Pennati R, Caicci F, Gasparini F, Manni L (2015) Morphological evidence that the molecularly determined *Ciona intestinalis* type A and type B are dif-

- ferent species: *Ciona robusta* and *Ciona intestinalis*. *J Zool Syst Evol Res* 53:186–193
- ✦ Bullard SG, Whitlatch RB (2009) *In situ* growth of the colonial ascidian *Didemnum vexillum* under different environmental conditions. *Aquat Invasions* 4:275–278
- ✦ Bullard SG, Lambert G, Carman MR, Byrnes J and others (2007) The colonial ascidian *Didemnum* sp. A: current distribution, basic biology and potential threat to marine communities of the northeast and west coasts of North America. *J Exp Mar Biol Ecol* 342:99–108
- ✦ Carman MR, Bullard SG, Rocha RM, Lambert G and others (2011) Ascidiaceans at the Pacific and Atlantic entrances to the Panama Canal. *Aquat Invasions* 6:371–380
- ✦ Chan BKK, Morritt D, Williams GA (2001) The effect of salinity and recruitment on the distribution of *Tetraclita squamosa* and *Tetraclita japonica* (Cirripedia; Balanomorpha) in Hong Kong. *Mar Biol* 138:999–1009
- ✦ Chu KH, Tam PF, Fung CH, Chen QC (1997) A biological survey of ballast water in container ships entering Hong Kong. *Hydrobiologia* 352:201–206
- ✦ Colarusso P, Nelson E, Ayvazian S, Carman MR, Chintala M, Grabbert S, Grunden D (2016) Quantifying the ecological impact of invasive tunicates to shallow coastal water systems. *Manage Biol Invasions* 7:33–42
- Collin R, D’Croz L, Gondola P, Rosario JB (2009) Climate and hydrological factors affecting variation in chlorophyll concentration and water clarity in the Bahia Almirante, Panama. *Smithson Contrib Mar Sci* 38:323–334
- ✦ Coutts AD, Dodgshun TJ (2007) The nature and extent of organisms in vessel sea-chests: a protected mechanism for marine bioinvasions. *Mar Pollut Bull* 54:875–886
- ✦ Coutts ADM, Piola RF, Hewitt CL, Connell SD, Gardner JPA (2010) Effect of vessel voyage speed on survival of biofouling organisms: implications for translocation of non-indigenous marine species. *Biofouling* 26:1–13
- David M, Gollasch S (2011) Risk assessment as a decision supporting tool in ballast water management. In: International Conference on Traffic Science—ICTS 2011, Portorož, Slovenia. Conference Proceedings, 1–6
- ✦ Dias GM, Rocha RM, Lotufo TMC, Kremer LP (2013) Fifty years of ascidian biodiversity research in São Sebastião, Brazil. *J Mar Biol Assoc UK* 93:273–282
- ✦ Dijkstra JA, Simkanin C (2016) Intraspecific response of colonial ascidiaceans to variable salinity stress in an era of global change. *Mar Ecol Prog Ser* 551:215–225
- ✦ Dijkstra JA, Sherman H, Harris LG (2007) The role of colonial ascidiaceans in altering biodiversity in marine fouling communities. *J Exp Mar Biol Ecol* 342:169–171
- ✦ Dijkstra JA, Dutton A, Westerman E, Harris LG (2008) Heart rate reflects osmotic stress levels in two introduced colonial ascidiaceans *Botryllus schlosseri* and *Botrylloides violaceus*. *Mar Biol* 154:805–811
- Dybern BI (1969) The ascidiaceans of the Baltic Proper. Distribution and ecology. *Limnology* 7:27–36
- ✦ Epelbaum A, Herborg LM, Theriault TW, Pearce CM (2009) Temperature and salinity effects on growth, survival, reproduction, and potential distribution of two non-indigenous botryllid ascidiaceans in British Columbia. *J Exp Mar Biol Ecol* 369:43–52
- Evans DH (2009) Osmotic and ionic regulation: cells and animals. CRC Press, New York, NY
- ✦ Frederich M, Sartoris F, Poertner HO (2001) Distribution patterns of decapod crustaceans in polar areas: A result of magnesium regulation? *Polar Biol* 24:719–723
- ✦ Freire CA, Santos IA, Vidolin D (2011) Osmolality and ions of the perivisceral coelomic fluid of the intertidal sea urchin *Echinometra lucunter* (Echinodermata: Echinoidea) upon salinity and ionic challenges. *Zoologia* 28:479–487
- Gaston KJ (2003) The structure and dynamics of geographic ranges. Oxford University Press, Oxford
- ✦ Gardon CG, Walters LJ, Quintana-Ascencio PF, Mccauley LA, Stam WT, Olsen JL (2008) Predicting risks of invasion of macroalgae in the genus *Caulerpa* in Florida. *Biol Invasions* 10:1147–1157
- ✦ Gröner F, Lenz M, Wahl M, Jenkins SR (2011) Stress resistance in two colonial ascidiaceans from the Irish Sea: the recent invader *Didemnum vexillum* is more tolerant to low salinity than the cosmopolitan *Diplosoma listerianum*. *J Exp Mar Biol Ecol* 409:48–52
- Hrs-Brenko M (2006) The basket shell, *Corbula gibba* Olivi, 1792 (bivalve mollusk) as a species resistant to environmental disturbances: a review. *Acta Adriat* 47:49–64
- ✦ Jofré Madariaga DJ, Rivadeneira MM, Tala F, Thiel M (2014) Environmental tolerance of the two invasive species *Ciona intestinalis* and *Codium fragile*: their invasion potential along a temperate coast. *Biol Invasions* 16:2507–2527
- Kauffman KW, Thompson RC (2005) Water temperature variation and the meteorological and hydrographic environment of Bocas del Toro, Panama. *Caribb J Sci* 41:392–413
- ✦ Keller RP, Drake JM, Drew MB, Lodge DM (2011) Linking environmental conditions and ship movements to estimate invasive species transport across the global shipping network. *Divers Distrib* 17:93–102
- Kott P, Goodbody I (1982) The ascidiaceans of Hong Kong. In: Proc 1st Int Mar Biol Workshop: the marine flora and fauna of Hong Kong. Hong Kong University Press, Hong Kong, p 504–554
- ✦ Lambert G (2002) Nonindigenous ascidiaceans in tropical waters. *Pac Sci* 56:291–298
- Lambert G (2003) Marine biodiversity of Guam: the Ascidiaceae. *Micronesica* 35:588–597
- ✦ Lambert G (2004) The south temperate and Antarctic ascidian *Corella eumyota* reported in two harbours in north-western France. *J Mar Biol Assoc UK* 84:239–241
- ✦ Lambert G (2005) Ecology and natural history of the protochordates. *Can J Zool* 83:34–50
- ✦ Lambert G (2007) Invasive sea squirts: a growing global problem. *J Exp Mar Biol Ecol* 342:3–4
- ✦ Locke A (2009) A screening procedure for potential tunicate invaders of Atlantic Canada. *Aquat Invasions* 4:71–79
- ✦ Lockwood JL, Cassey P, Blackburn TM (2009) The more you introduce the more you get: the role of colonization pressure and propagule pressure in invasion ecology. *Divers Distrib* 15:904–910
- ✦ López-Legentil S, Turon X (2007) Lack of genetic variation in mtDNA sequences over the amphiatlantic distribution range of the ascidian *Ecteinascidia turbinata*. *Mol Phylogenet Evol* 45:405–408
- ✦ Markus JA, Lambert CC (1983) Urea and ammonia excretion by solitary ascidiaceans. *J Exp Mar Biol Ecol* 66:1–10
- ✦ McKindsey CW, Landry T, O’Beirn FX, Davies IM (2007) Bivalve aquaculture and exotic species: a review of ecological considerations and management issues. *J Shellfish Res* 26:281–294
- Monniot C (1983) Ascidiaceae littorales de Guadeloupe II: Phlébobranches. *Bull Mus Natl Hist Nat Sect A* 5(1):51–71

- Monniot C, Monniot F (1987) Les ascidies de Polynésie française. *Mém Mus Natl Hist Nat* 136:1–155
- ✦ Moody R, Davis SW, Cubas F, Smith WC (1999) Isolation of developmental mutants of the ascidian *Ciona savignyi*. *Mol Gen Genet* 262:199–206
- ✦ Muirhead JR, Minton MS, Miller WA, Ruiz GM (2015) Projected effects of the Panama Canal expansion on shipping traffic and biological invasions. *Divers Distrib* 21: 75–87
- ✦ Nomaguchi TA, Nishijima C, Minowa S, Hashimoto M, Haraguchi C, Amemiya S, Fujisawa H (1997) Embryonic thermosensitivity of the ascidian *Ciona savignyi*. *Zool Sci* 14:511–516
- ✦ Okamura Y, Nishino A, Murata Y and others (2005) Comprehensive analysis of the ascidian genome reveals novel insights into the molecular evolution of ion channel genes. *Physiol Genomics* 22:269–282
- ✦ Osovitz CJ, Hofmann GE (2007) Marine macrophysiology: studying physiological variation across large spatial scales in marine systems. *Comp Biochem Physiol A Mol Integr Physiol* 147:821–827
- Paton S (2015) Meteorological and oceanographic summary for the Bocas del Toro Research Station. http://biogeodb.stri.si.edu/physical_monitoring/research/bocas (accessed 27 Nov 2016)
- Pérès J (1958) Ascidiées recoltées sur les côtes Méditerranéennes d'Israël. *Bull Res Counc Isr* 7B:143–150
- ✦ Pineda MC, McQuaid CD, Turon X, López-Legentil S, Ordóñez V, Rius M (2012) Tough adults, frail babies: an analysis of stress sensitivity across early life history stages of widely introduced marine invertebrates. *PLOS ONE* 7:e46672
- ✦ Piola RF, Johnston EL (2008) Pollution reduces native and increases invader dominance in marine hard-substrate communities. *Divers Distrib* 14:329–342
- ✦ Ramsay A, Davidson J, Landry T, Arsenault G (2008) Process of invasiveness among exotic tunicates in Prince Edward Island, Canada. *Biol Invasions* 10:1311–1316
- ✦ Ricciardi A, Rasmussen JB (1998) Predicting the identity and impact of future biological invaders: a priority for aquatic resource management. *Can J Fish Aquat Sci* 55: 1759–1765
- ✦ Rius M, Clusella-Trullas S, McQuaid CD, Navarro RA and others (2014) Range expansions across ecoregions: interactions of climate change, physiology and genetic diversity. *Glob Ecol Biogeogr* 23:76–88
- ✦ Rocha RM, Kremer LP (2005) Introduced ascidians in Paranaguá Bay, Paraná, southern Brazil. *Rev Bras Zool* 22:1170–1184
- Rocha RM, Faria SB, Moreno TR (2005) Ascidians from Bocas del Toro, Panama. I. Biodiversity. *Caribb J Sci* 41: 600–612
- ✦ Rocha RM, Kremer LP, Fehlaue-Ale KH (2012) Lack of COI variation for *Clavelina oblonga* (Tunicata, Ascidiacea) in Brazil: Evidence for its human-mediated transportation? *Aquat Invasions* 7:419–424
- ✦ Rouget M, Robertson MP, Wilson JRU, Hui C, Essl F, Renteria JL, Richardson DM (2016) Invasion debt – quantifying future biological invasions. *Divers Distrib* 22:445–456
- ✦ Russell MP (2013) Echinoderm responses to variation in salinity. *Adv Mar Biol* 66:171–212
- ✦ Sheets EA, Cohen CS, Ruiz GM, Rocha RM (2016) Investigating the widespread introduction of a tropical marine fouling species. *Ecol Evol* 6:2453–2471
- ✦ Shenkar N, Swalla BJ (2011) Global diversity of Ascidiacea. *PLOS ONE* 6:e20657
- ✦ Sievers M, Fitridge I, Dempster T, Keough MJ (2013) Biofouling leads to reduced shell growth and flesh weight in the cultured mussel *Mytilus galloprovincialis*. *Biofouling* 29:97–107
- ✦ Simkanin C, Fofonoff PW, Larson K, Lambert G, Dijkstra JA, Ruiz GM (2016) Spatial and temporal dynamics of ascidian invasions in the continental United States and Alaska. *Mar Biol* 163:163
- ✦ Sims LL (1984) Osmoregulation capabilities of three macro-sympatric stolidobranch ascidians, *Styela clava* Herdman, *S. plicata* (Lesueur) and *S. montereyensis* (Dall). *J Exp Mar Biol Ecol* 82:117–129
- ✦ Spalding MD, Fox HE, Allen GR, Davidson N and others (2007) Marine ecoregions of the world: a bioregionalization of coastal and shelf areas. *Bioscience* 57:573–583
- Tatián M, Schwindt E, Lagger C, Varela MM (2010) Colonization of Patagonian harbours (SW Atlantic) by an invasive sea squirt. *Spixiana* 33:111–117
- ✦ Toop T, Wheatly MG (1993) Some behavioural and physiological responses of the ascidian *Styela plicata* (Lesueur) during acclimation to low salinity. *Mar Behav Physiol* 24: 33–44
- ✦ Tsagkogeorga G, Turon X, Hopcroft RR, Tilak K and others (2009) An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. *BMC Evol Biol* 9:187–203
- ✦ Valentine PC, Carman MR, Blackwood DS, Heffron EJ (2007) Ecological observations on the colonial ascidian *Didemnum* sp. in a New England tide pool habitat. *J Exp Mar Biol Ecol* 342:109–121
- Van Name WG (1945) North and South American ascidians. *Bull Am Mus Nat Hist* 84:1–476
- ✦ Vandepas LE, Oliveira LM, Lee SSC, Hirose E, Rocha RM, Swalla BJ (2015) Biogeography of *Phallusia nigra*: Is it really black and white? *Biol Bull (Woods Hole)* 228:52–64
- ✦ Vázquez E, Young CM (2000) Effects of low salinity on metamorphosis in estuarine colonial ascidians. *Invertebr Biol* 119:433–444
- ✦ Vercaemer B, Sephton D, Nicolas JM, Howes S, Keays J (2011) *Ciona intestinalis* environmental control points: field and laboratory investigations. *Aquat Invasions* 6: 477–490
- ✦ Vidolin D, Santos-Gouveia IA, Freire CA (2007) Differences in ion regulation in the sea urchins *Lytechinus variegatus* and *Arbacia lixula* (Echinodermata: Echinoidea). *J Mar Biol Assoc UK* 87:769–775
- ✦ Wotton DM, O'Brien C, Stuart MD, Fergus DJ (2004) Eradication success down under: heat treatment of a sunken trawler to kill the invasive seaweed *Undaria pinnatifida*. *Mar Pollut Bull* 49:844–849
- ✦ Zhan A, Briski E, Bock DG, Ghabooli S, MacIsaac HJ (2015) Ascidians as models for studying invasion success. *Mar Biol* 162:2449–2470