TEMPERATURE-DEPENDENT RATES OF LARVAL DEVELOPMENT IN *LIMNOPERNA FORTUNEI* (BIVALVIA: MYTILIDAE)

DANIEL CATALDO[1,2,3], DEMETRIO BOLTOVSKOY[1,2,3], JOSE L. HERMOSA[4] AND CARLA CANZI[4]

[1]*Departamento de Ecología, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina*
[2]*Consejo Nacional de Investigaciones Científicas y Técnicas;*
[3]*Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia'*
[4]*Itaipu Binacional, Brasil-Paraguay*

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ABSTRACT

Laboratory-reared larvae of *Limnoperna fortunei* (Dunker) were monitored until the morula stage at 26°C, and from there on until settlement at 20, 25 and 28°C. The first polar lobe is produced *c.* 40 min after spawning, and the first division occurs 14 min later. Slightly over an hour after spawning, the second polar lobe appears and the second division yields a 4-cell stage. The third division occurs 90 min after spawning, and the fourth 115 min after spawning. The morula stage is reached 3.5 h after spawning. The trochophore stage (size: 95–110 μm) is reached 20, 6.3 and 6 h after spawning at 20, 25 and 28°C, respectively. Straight-hinged veligers (115–175 μm) start appearing at 45, 26, and 24 h after spawning; umboned veligers (156–220 μm) at 287, 165 and 118 h after spawning; and pediveligers (>200 μm) at 480, 313 and 265 h after spawning. Implications of these findings for the dispersion of the species and for its fouling impact are discussed.

INTRODUCTION

Limnoperna fortunei (Dunker, 1857) (Mytilidae) is a freshwater bivalve that constitutes a major fouling pest in Hong Kong, Korea, Japan and Taiwan (Morton, 1979; Ricciardi, 1998; Magara *et al*., 2001). Around 1990, it was introduced into Argentina and has since colonized much of the Río de la Plata watershed as far north as southern Brazil (Darrigran, 2002). In South America, *L. fortunei* has rapidly become a major nuisance for industrial and power plants located along waterways. Dense (over 150,000 individuals/m^2) populations attach bysally to filters, condensers, heat exchangers, sieves, valves, the inner surfaces of pipes, etc., retarding water flow and enhancing corrosion (Boltovskoy & Cataldo, 1999; Cataldo, Boltovskoy & Pose, 2003). As opposed to all other South American freshwater molluscs, which have either parasitic larvae (glochidia) or no free larval stage at all (e.g. Corbiculidae), *Limnoperna* has free-swimming larvae, which greatly facilitate rapid downstream dispersal. In this respect, as well as in many other ecological and biological traits, *Limnoperna* shares salient features with the North American invasive pest, the zebra mussel, *Dreissena polymorpha* (Dreissenidae): both are dioecious and have similar sizes, grow rapidly, attach to hard substrata by means of a strong byssus, and possess planktonic larvae (Morton, 1979; Ricciardi, 1998).

This work describes the stages that characterize the larval development of *Limnoperna* from fertilization to settlement and provides information on the rates of this process at 20, 25 and 28°C. Because geographic range expansion and fouling of industrial plants depend strongly on the location of the seeding parent populations and the time needed for the larva to complete its metamorphosis and settle on a substrate, information on development rates at different temperatures will contribute towards a better understanding of the mechanisms that govern the dispersal of the mollusc, as well as furnishing additional tools for the development of fouling control measures.

Correspondence: D. Cataldo; e-mail: daniel@bg.fcen.uba.ar

MATERIAL AND METHODS

Adult mussel broodstock was collected manually in the lower delta of the Paraná River, brought immediately to the laboratory in large vessels of water and held at room temperature (*c.* 26°C) in aerated 20-l fish tanks with dechlorinated tap water. From this stock, groups of approximately 20 animals were selected for the reproduction experiments, rinsed and placed in 2-l beakers, where they were acclimated to 19 ± 1°C for 48 h. Animals were then transferred to new 2-l beakers with water at 26°C and the chemical selected for inducing spawning (see below). Subsequently and until spawning, experimental chambers were monitored at 5 min intervals. Release of gametes is clearly indicated when water in the chamber turns suddenly milky. Upon appearance of this turbidity, a drop of water from the bottom of the chamber was examined under the microscope. In all cases this sample contained abundant sperm cells and ova. Adult specimens were then immediately removed from the beaker and the water with the gametes and fertilized ova thoroughly rinsed with clean water through a 26-μm sieve, and transferred to clean containers under different conditions. Three sets of containers (2-l aerated beakers) with developing larvae were incubated at 20, 25 and 28°C, respectively. Water temperature was maintained at ±1°C of the nominal setting with the aid of a digital thermostat (Every Control EC 3L20 N220). Lighting conditions were 12 h light: 12 h dark. These animals were not fed until the straight-hinged stage was reached, after which cultured *Chlorella* sp. were administered daily at a rate of *c.* 10,000–15,000 cells/ml. The fourth set of developing larvae was kept at room temperature (26°C) and used to monitor the early developmental stages (first 8 h).

Following previous work (Matsutani & Nomura, 1982; Braley, 1985; Ram & Nichols, 1993; Vanderploeg, Liebig & Gluck, 1996), we first attempted to use serotonin for inducing spawning, but proportions of spawning individuals never exceeded 20%. We therefore experimented with several other stress-inducing substances. Of these, n-alkyl dimethylbenzyl ammonium

chloride, a quaternary ammonium commercially known as ClamTrol (Betz Chemicals), used for the control of zebra mussels and also tested for *L. fortunei* (Cataldo *et al.*, 2003), proved very effective at concentrations of 0.75 ppm (active ingredient), yielding ripe gametes in over 90% of the experimental beakers 30–180 min after exposure of the adults to the chemical. In the remaining 10% of mussels, treated gametes appeared up to 8 h after exposure, but these were not used for further observations.

RESULTS

Mature sperm cells measure about 4 μm (excluding the tail) and are highly mobile (Fig. 1B). Ova are 85–100 μm in diameter (Fig. 1A). Upon release these cells are still diploid, chromatic reduction taking place in the medium by production of the polar bodies (Fig. 1C).

Segmentation is similar to that observed in many invertebrates and in most molluscs: a spiral complete cleavage yields a characteristic trochophore larva. Segmentation starts about 40 min after spawning, producing the first polar lobe and, very shortly thereafter, the first cellular division (Fig. 1D–F). In a few minutes, the first polar lobe is resorbed by one of the daughter cells (Fig. 1F); at 26°C this first division, yielding two uneven blastomeres, occurs 54 min after spawning. Approximately 11 min later (slightly over 1 h after spawning) the second polar lobe appears (Fig. 1G) and the second division starts, yielding a 4-cell stage (Fig. 1H). The third division takes place along the equatorial plane, separating 4 micromeres from 4 macromeres; at 26°C this 8-cells stage occurs 90 min after spawning (Fig. 1I). Twenty-five minutes later (115 minutes after spawning) the fourth division yields the 16-cells stage (Fig. 2A). Approximately 3.5 h after spawning (at 26°C) the morula stage is reached (Fig. 2B). These organisms are ciliated and have limited, poorly coordinated movement, only occasionally leaving the bottom and venturing into the water column. Six hours and 20 min after spawning, the first trochophores appear (Fig. 2C); these larvae have a well developed apical tuft of cilia which allows them active and well coordinated swimming. Most of these larvae wander freely in the water column and rarely rest on the bottom.

Valves and the ciliated velum characteristic of the next stage, the veliger larva (Fig. 2D, E), start developing very early so that there is no clear-cut separation between the trochophore

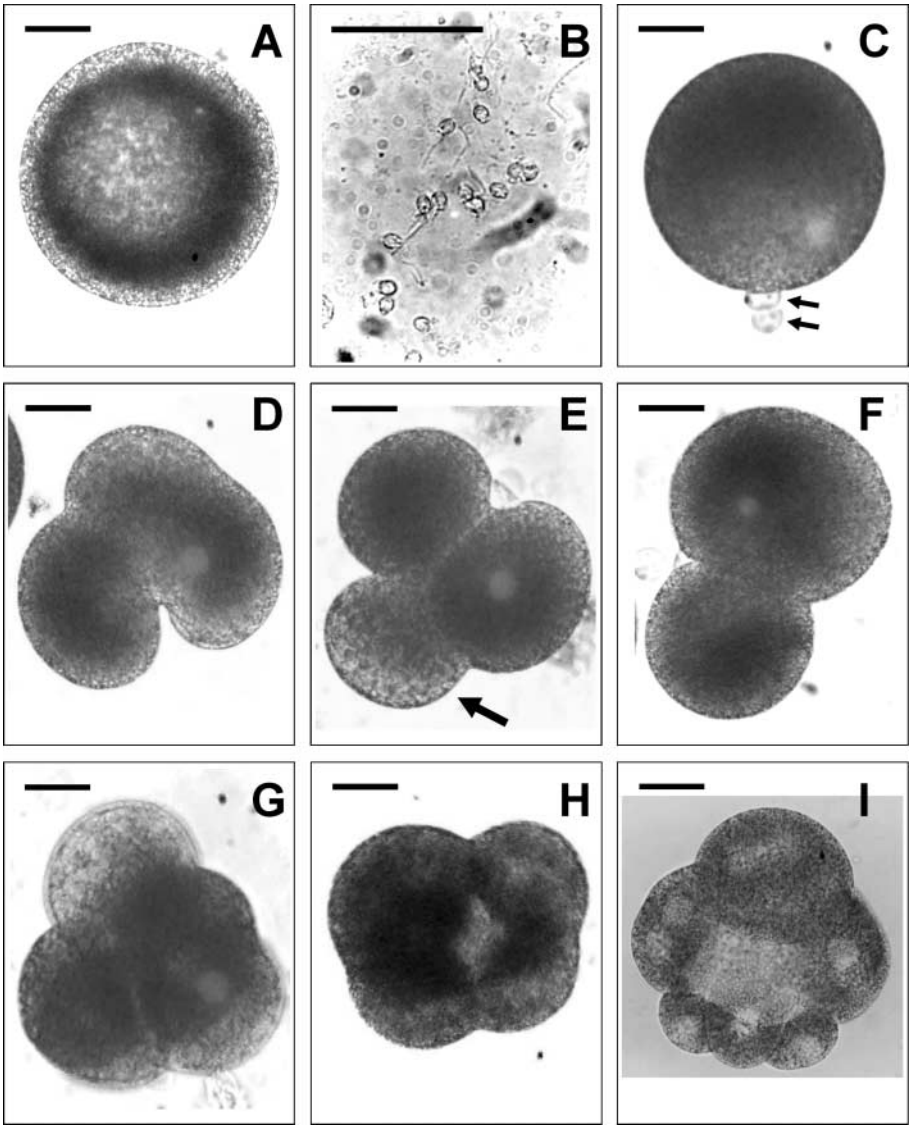

Figure 1. A. Mature oocyte. **B.** Sperm cells. **C.** Fertilized oocyte with two polar bodies (arrows). **D.** Development of first polar lobe and beginning of first division. **E.** 2-cell stage with remnants of first polar lobe (arrow). **F.** 2-cell stage. **G.** 4-cell stage and second polar lobe. **H.** 4-cell stage. **I.** 8-cell stage. Scale bars = 25 μm.

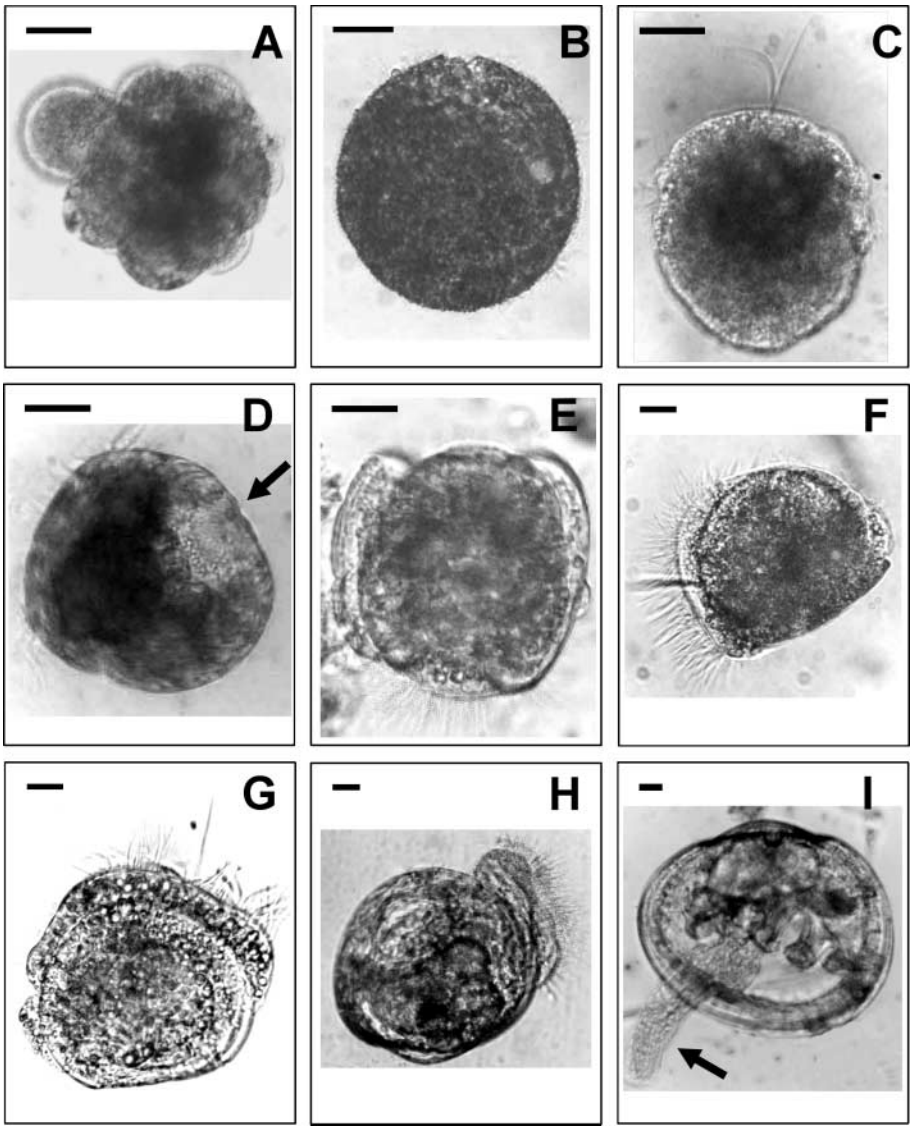

Figure 2. A. 16-cell stage. **B.** Morula. **C.** Trochophore. **D.** Veliger with rudimentary valves (arrow). **E.** Veliger. **F, G.** Straight-hinged veliger. **H.** Umboned veliger. **I.** Juvenile (arrow points at foot). Scale bars = 25 μm.

and the veliger, but a smooth, gradual transition. Valves first appear as small rosette-shaped structures on the dorsal side of the trochophore (Fig. 2D) and grow until they encompass the entire body (Figs 2E and 3). At first, the dorsal margin of the shell is straight (straight-hinged larva, Figs 2F, G; 3A, B), but gradually the umbo appears as a progressively more conspicuous formation (Figs 2H and 3C). Straight-hinged larvae are 115–175 μm in length. As the larva develops, swimming activity becomes progressively slower and the animals tend to spend more time on the bottom of the vessel. Shortly before settling, the larva reabsorbs its velum and develops a muscular, adhesive foot (Fig. 2I). At 25°C, plantigrade (metamorphosed) individuals 250–405 μm in size (based on five specimens) were observed on day 15 after fertilization, whereas at 28°C this stage was reached on day 13 at a size of 210–220 μm (five observations). At 20°C, metamorphosis was not observed.

Table 1 summarizes variations in the development times and sizes observed at the three temperatures assayed. As expected, fastest development rates were observed at the highest temperature (28°C), decreasing at 25°C and further at 20°C. Differences in developmental times for all the stages surveyed were almost two times greater between 20 and 25°C than between 25 and 28°C (Table 1). Size, on the other hand, varied little with temperature (Table 1).

DISCUSSION

Our data confirm that in *L. fortunei*, as in most other organisms, larval development rates increase at higher temperatures (Sprung, 1987; Kimura & Sekiguchi, 1996). Our results show that at 20°C (which is slightly above the lower thermal limit for reproduction for the species; Cataldo & Boltovskoy, 2000), the bivalve needs several hours to days more time to reach the same developmental stage than at 28°C (which are typically the highest summer water temperatures in the lower delta of the Paraná River and the Río de la Plata estuary). At 25°C, development rates are intermediate, yet closer to those at 28°C than at 20°C (Table 1). Extrapolation of these results to field populations, however, should be done with care because of differences between natural and laboratory conditions, in particular regarding food type and density (natural plankton *vs Chlorella* sp.).

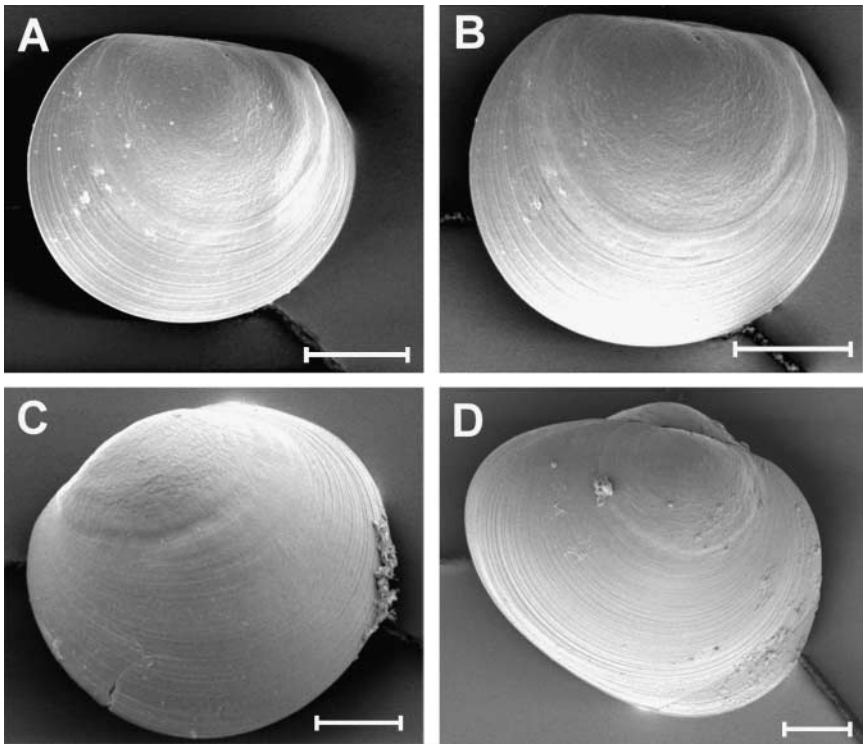

Figure 3. A, B. Straight-hinged veliger. **C.** Umboned veliger. **D.** Plantigrade. Scale bars = 50 μm.

To our knowledge, the only previous reports on the larval development of *L. fortunei* are by Choi & Kim (1985) and Choi & Shin (1985). These authors described three D-shaped (i.e. straight-hinged) stages: early, middle and late D-shaped larvae. The size ranges reported for these organisms (120–220 μm) are considerably higher than those recorded in our study (115–175 μm; see Table 1), and confirmed by earlier observations on plankton materials collected in the Río de la Plata estuary and in the lower and upper Paraná River (Cataldo & Boltovskoy, 2000; unpublished data). Although water temperatures at the sites of Choi & Kim's (1985) survey varied seasonally between *c.* 1 and 27–28°C, their larvae were obtained in August, when water temperatures were around 25°C (Choi & Shin, 1985). Thus, temperature is probably not responsible for these differences in size. Genetic drift between Korean and South American populations could conceivably account for these disagreements, but it is our feeling that methodological constraints are involved. For example, Choi & Kim's (1985) field-collected plankton samples (see below) may have contained larger particles misidentified for *L. fortunei* larvae, whereas our cultures were practically barren of materials other than the larvae.

Development times reported by Choi & Kim (1985) for the straight-hinged stage (23 h) overlap our data for 28°C (see Table 1), but their estimates for the unboned (10 days) and the pediveligers (18 days) are considerably longer than our results for the first appearance of these stages at 28°C (less than 5 and 11 days, respectively).

It should be noticed that precise estimation of development times were hindered in Choi & Kim's (1985) study by the fact that larvae were collected from the plankton (rather than obtained in the laboratory), and therefore development periods after spawning were unknown. Furthermore, no information was provided on the water temperature at which development was followed in the laboratory. Finally, according to Choi & Kim (1985), early D-shaped larvae are still devoid of a velum, which they only observed in the middle D-shaped stage. However, out observations indicate that by the time the dorsal side of the animal shows the straight line of the hinge between the two valves, the velum has already fully developed. This discrepancy might be due to the fact that plankton samples (on which Choi & Kim, 1985, based their observations) usually contain large proportions of stressed and dead larvae, especially immediately after collection with a plankton net. These animals show no or little mobility, and their velum is retracted and inconspicuous.

Early detection and monitoring of colonization by *Limnoperna* requires effective identification of the larvae in the water column. Lakes and rivers of southern South America host between 60 (Argentina, Uruguay) and slightly over 100 (Brazil) species of bivalves, yet most of these have either parasitic larvae (glochidia or are viviparous (Castellanos & Landoni, 1995; Rocha, 2000). In addition, with few exceptions (e.g. *Corbicula fluminea* in the lower delta of the Paraná River; Boltovskoy, Correa, Cataldo, Stripeikis & Tudino, 1997; Cataldo & Boltovskoy, 1998), these molluscs are seldom abundant, for which reason the planktonic larvae of *L. fortunei* are usually easy to identify. *Corbicula fluminea* releases (from its incubating gills) juveniles that settle immediately on the bottom. Occasionally, these animals can be picked up by currents and dwell in the water column for short periods of time, yet they are larger than the larvae of *L. fortunei* (190–250 μm for *C. fluminea*, *vs* 100–160 μm for *L. fortunei*) and have a well developed foot (Kraemer & Galloway, 1986; Nichols & Black, 1993).

Infestation of industrial plants and water treatment facilities occurs because of the free-swimming larvae of *Limnoperna*, whose size allows them to evade grids and filters in the intake pipelines. Susequently, these larvae settle on filters, the inner surfaces of pipes, heat exchangers, valves, etc. causing severe fouling problems. Because the residence time of raw (river or lake) cooling water within the plant is usually low (< 1 h), only very late larvae, i.e. those that are provided with a muscular foot and are ready to settle, are retained in the plant, whereas all others pass through. The development times reported here indicate that the dense populations usually present on the outer and inner hard surfaces of the plants, are innocuous from the point of view of

Table 1. Development times (ranges for the numbers of observations indicated) and size ranges of *Limnoperna fortunei* at the three temperatures assayed.

	20°C			25°C			28°C			
	Time after spawning (h)	Size (μm)	*n*	Time after spawning (h)	Size (μm)	n	Time after spawning (h)	Size (μm)	*n*	Characteristics
Trochophore	20–48	95–110	12	6.3–30	95–110	10	6–26	95–110	12	Ciliated and motile
Straight-hinged veliger	45–336	115–160	12	26–168	125–165	10	24–120	125–175	12	Feeds and swims with ciliated velum, Straight-hinged shell.
Umboned veliger	287–504	160–200	12	165–336	156–216	10	118–288	175–220	12	Umbo developed, ciliated velum present.
Pediveliger	480	>200	5	313	>200	6	265	>200	5	Foot developed

further infestation, unless cooling-water flow within and around the plant is highly irregular and abundant 'dead-water sites' with significantly higher water residence times are present. On the other hand, considering water temperature, flow speed and mode, these development times may aid in pinpointing the areas that host the seed populations affecting the plant (Stoeckel *et al*., 1996). For example, since the Paraná has a mean flow speed of about 0.3 m/s, the core of the seeding populations for the biofouling affected plants located in Buenos Aires, along the Río de la Plata estuary, is some 250 km (in summer, when water temperatures are above 25°C) to 500 km (in the autumn and spring, when water temperatures are below 20°C) upstream in the Paraná and/or Uruguay Rivers (the two major tributaries of the Río de la Plata). Thus, knowledge of temperature-dependent development times can contribute to the assessment of the fouling mechanisms and towards the development of sound control measures.

Comparison of *Limnoperna*'s larval development rates with those reported for the North American freshwater invader, *Dreissena polymorpha*, indicates that both are roughly similar: the zebra mussel reaches the D-shaped stage in *c*. 30–70 h (Sprung, 1987; Stoeckel *et al*., 1996; Nichols, 1993), whereas our data for *Limnoperna* indicate 24–50 h. Vanderploeg *et al*. (1996) reported for *D. polymorpha* an age of 15–22 days at settling; in *Limnoperna* the pediveliger stage appears on days 11–20.

Comparison of our results with reported development times for other marine mytilids is complicated by the modulating effects of temperature, which are not always adequately accounted for. For some species, the rates described are generally within the ranges found for *L. fortunei* (e.g. *Perna viridis*, Tan, 1975; Siddall, 1980; *Mytilus platensis*, Penchaszadeh, 1980; *Modiolus modiolus*, De Schweinitz & Lutz, 1976), whereas for others they are significantly slower (e.g. *Mytilus edulis* reaches the veliger stage in up to 35 days, and may take over 6 months to complete its metamorphosis; Bayne, 1976). Other marine bivalves with free-swimming larvae seem to have slower development rates. According to data summarized by Thorson (1961), over 80% of the 37 marine bivalves surveyed have development times of 3 weeks (504 h) or more, whereas at the lowest temperature assayed *L. fortunei* reaches the pediveliger stage in only 480 h (Table 1). Only 5% of these marine species have development times shorter (168 h) than those of *L. fortunei* at 28°C (265 h). This contrast may represent an adaptational trait in the life cycle of the only mytilid that has managed to colonize fresh waters. Indeed, whereas marine benthic organisms can gain from extended larval periods, including the very long-living teleplanic larvae (Scheltema, 1986), for freshwater riverine animals longer larval stages ensure wider geographic dispersion, but also the danger of loss to the ocean becomes significantly higher. Modulation of the vertical distribution as an aid in reducing seaward flushing, as has been described for estuarine larvae (e.g. Cronin, 1982), is most probably unfeasible in riverine turbulent flow conditions. Thus, the relatively short development times of *L. fortunei* are probably a balance between the species' marine inheritance and the advantages for dispersion of a free larval stage, and the expatriation hazards associated with slow metamorphosis rates.

These considerations also pose some interesting questions regarding the speed of colonization of South American inland waters by *Limnoperna*. As noted above, the bivalve entered the subcontinent through the Río de la Plata and has been expanding its range northward upstream along the Paraná and, to a lesser extent, the Uruguay rivers. Historical data show that it took *Limnoperna* 6 years to cover the first *c*. 180 km upstream in the Río de la Plata estuary-Paraná River, from Bagliardi, where it presumably was first established around 1990 (Darrigran, 2002), to Lima, where it was found in the installations of the nuclear power plant Atucha I in 1996. A few years later and further upstream, the speed of colonization increased to over 1000 km in less than 3 years (from Asunción in Paraguay, where it arrived around 1997, to the Pantanal in Brazil, where it was first found in 2000; Darrigran, 2002). While these differences are probably associated with several factors (e.g. mean water temperature, boat traffic, food and substrate availability), we contend that geographic location of the pioneer populations plays a major role. Indeed, almost all the larvae produced by the first invaders in Bagliardi, located upstream and only 100–200 km from brackish waters with occasional very strong oceanic influence, must have been wasted through flushing into the Atlantic Ocean. On the other hand, none of the eggs fertilized in Asunción are advected to a hostile environment by the time they metamorphose and are ready to settle. Thus, it seems reasonable to anticipate that *Limnoperna*'s geographic range expansion is accelerating as the overall area covered by the animal enlarges.

The above discussion indicates that *Limnoperna*'s geographic expansion is achieved by means of isolated long-distance transport events or 'jump dispersal' (the so-called 'stratified diffusion' model, as opposed to the 'reaction-diffusion' model, characterized by gradual dispersion from a localized epicentre; Hengeveld, 1989; Shigesada, Kawasaki & Takeda, 1995; MacIsaac, Grigorovich & Ricciardi, 2001). Upstream dispersal is basically dependent on the availability of long-distance dispersal agents, which in the case of *Limnoperna* are represented by ship traffic. This conclusion is supported by the fact that 12 years after entry in the Río de la Plata estuary, along the Paraná–Paraguay waterway, which hosts intense boat traffic, *Limnoperna* has moved upstream at an average rate of 250 km per year, whereas along the Uruguay River, where boat traffic is restricted to the lowermost 200 km section, upstream colonization is almost 10 times slower. This suggests that upstream displacement through attachment to vessels is by far the most important dispersion mechanism.

These considerations may also explain why *Limnoperna* did not invade South America (or, for that matter, other continents) earlier. International trade (and the number and frequency of

cargo ships, whose ballast water is presumed to have brought *Limnoperna* to South America) between Buenos Aires and Asia had started growing long before 1990. It is therefore conceivable that there have been many previous immigrations attempts, but since all Argentine and Uruguayan ports that support sea-going ship traffic are too close to estuarine brackish waters for drifting larvae to complete their metamorphosis before being flushed into the sea, none of these immigrants managed to establish a permanent population.

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