



Effects of temperature and salinity on juveniles of two ascidians, one native and one invasive, and their hybrids



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ABSTRACT

Human-mediated transports of species, i.e. biological introductions, promote secondary contacts between previously spatially isolated species. When the species are not reproductively isolated, hybridization is an important possible outcome of these secondary contacts, influencing the fate of both the native and non-native species. Such a situation is exemplified by the introduction of the warm-temperate tunicate *Ciona robusta*, native to Asia, into the European native range of its cold-temperate congener *Ciona intestinalis*. Despite an old divergence (ca. 4 Mya), the two species lack reproductive isolation with fertile F1-hybrids produced in laboratory. However, a recent population genomic study showed that hybrids are extremely rare in the wild and back-crossed individuals are absent, suggesting the existence of reproductive barriers between the two species in nature. Post-zygotic mechanisms like selection against hybrids by the environment were thus hypothesized. Because the two species have only been recently reclassified, data on their respective ecology and their hybrids are lacking. To investigate these environmental effects, we carried out 21 experimental crosses and examined survival and individual growth rate of ca. 1900 F1-juveniles produced with intra- and inter-specific crosses. Four treatments combining two temperatures (12 °C and 17 °C) and two salinity levels (25 and 35), representative of conditions found in nature, were applied to one-week old juveniles. After 28 days, a high survival rate was observed in the three F1 categories, with no difference between hybrids and parental species. As expected based on biogeography studies and field monitoring of the two species, the results showed that *C. robusta* performed particularly well in warm temperature with standard salinity condition. F1-hybrids did not show outbreeding depression, with growth rates similar to or higher than F1-*C. intestinalis*. Altogether the two species performed well under the tested environmental conditions, and post-zygotic ecological effects alone are unlikely to be effective against hybridization between the native and non-native species.

1. Introduction

Human-mediated transport of species outside their natural range of distribution, i.e. biological introductions, are responsible for secondary contacts between species previously isolated in allopatry. Consequently, in the absence of reproductive isolation between the native and introduced species, hybridization and introgression (i.e. introgressive hybridization, Harrison, 2012) can occur. Depending on the strength of pre- and post-zygotic barriers, the outcomes of hybridization processes are diverse (Abbott et al., 2013; Harrison and Larson, 2014). For instance, the differentiation between the two parental taxa can be maintained (e.g. through the establishment of a tension zone) or increased through the evolution of pre-mating barriers and reinforcement mechanisms. Novel genetic combination can also arise from introgression, a process which has attracted much interest in the context of human-mediated dispersal (Brumfield, 2010). Introgression between

introduced and native taxa can be fast as well documented with the spread of genes of the introduced Barred Tiger Salamander into the genome of its native and endangered relative the California Tiger Salamander, despite their old divergence (3–5 Mya), rising important conservation issues regarding the native salamander (Fitzpatrick et al., 2010). In other cases, introgression seems (so far) limited, questioning the role of hybridization in the invasive success in animals. This is exemplified by the introduction of *Mytilus galloprovincialis*, native to the Mediterranean Sea, in the native range of *M. trossulus*: in Central California, only few hybrids or recently back-crossed individuals had been observed with only a slightly higher level of introgression occurring from the native to the introduced species (Saarman and Pogson, 2015). With a few notable exception like the *Mytilus* species complex (Fraisette et al., 2016 and references therein), these hybridization patterns and processes have been poorly studied in marine animals as compared to terrestrial plants and animals (e.g. for review, Schierenbeck and

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Ellstrand, 2008; Schwenk et al., 2008). And yet, marine animals are particularly prone to such processes being easily transported by human activities and often free spawning, two factors favoring secondary contact and subsequent hybridization between introduced species and their native relatives.

In this context, the tunicates *Ciona robusta* (Hoshino & Tokioka, 1967) and *C. intestinalis* (Linnaeus, 1767), sensu Millar, are particularly interesting to examine. Based on genetic evidences and morphological features, these two species, formerly known as *C. intestinalis* type A and *C. intestinalis* type B, respectively, have been recently reclassified (Brunetti et al., 2015; Gissi et al., 2017). They are both short-lived, solitary, hermaphrodite and free spawning. They inhabit the same habitats and are notably conspicuous in the biofouling community established in artificial habitats and man-made structures like marinas and ports (Aldred and Clare, 2014). Interestingly, the two species had come recently (2000s) into contact in the English Channel, after the introduction of *C. robusta*, thought to be native to Asia, into the natural European distribution range of *C. intestinalis* (Bouchemousse et al., 2016a; Nydam and Harrison, 2010). In the English Channel, the two species are found in syntopy, mature at the same period and display similar life-cycle, with at least two generations per year (Bouchemousse et al., 2016b). In addition, despite an ancient divergence (ca. 4 Mya) the two species are only partially reproductively isolated. Experimental crosses between *C. intestinalis* and *C. robusta* sampled in the English Channel are successful under laboratory conditions although asymmetrically: oocytes of *C. intestinalis* can be efficiently fertilized by sperm of *C. robusta* but the fertilization rate is very low in the opposite direction (Bouchemousse et al., 2016b). F1-hybrids are viable, fertile and producing viable larvae from backcrosses to parental genotypes in both directions (Sato et al., 2014). Despite this high potential for hybridization, population genetic and genomic studies showed only very rare F1-hybrids (e.g. one out of 3048 individuals studied in 11 marina in the Atlantic Ocean and the English Channel) and no recent backcrossed individuals in nature (Bouchemousse et al., 2016b; Bouchemousse et al., 2016c). In the contact zone, the *Ciona* parental species apparently display different environmental preferendum: adult's *C. robusta* are more abundant in summer-autumn than in spring, thus under warmer conditions (Bouchemousse et al., 2016b). This result is congruent with the present-day distribution range of the two species. *C. robusta* is considered as a warm-temperate species whereas *C. intestinalis* is considered as a cold-temperate (Procaccini et al., 2011). Based on these results and the apparent absence of pre-zygotic isolation mechanisms (Sato et al., 2014), post-zygotic isolation mechanisms can be hypothesized, in particular outbreeding depression and selection by environmental factors against the F1-hybrids as compared to parental species, in the wild.

Temperature and salinity are reported as important environmental factors influencing growth and survival of ascidians particularly at early stages. This influence has been for instance reported on *Styela plicata* during embryonic development, larval metamorphosis and post-larval growth (Pineda et al., 2012; Thiyagarajan and Qian, 2003), *Botryllus schlosseri* and *Botrylloides violaceus* during growth of laboratory-raised juvenile colonies (Epelbaum et al., 2009), *Eudistoma olivaceum*, *Eudistoma hepaticum* and *Ecteinascidia turbinata* on larval settlement, metamorphosis, and juvenile mortality (Vázquez and Young, 2000). In *Ciona* spp. temperature has been shown to affect growth rate: for example, Yamaguchi (1975) showed that doubling the body size requires nine days in winter (13 °C) and four days in summer (25 °C). A recent study of salinity effects on *C. robusta* found that 75% of juveniles died after 10 days at salinity 25 whereas at salinity 45, 9% died (Jofré Madariaga et al., 2014). Note however that in several cases the species identity (i.e. *C. intestinalis* or *C. robusta*) can only be hypothesized based on the locality of the sampling as the two species are introduced in many place around the world and, for a long time, were not distinguished (see Supplementary Material of Gissi et al. (2017) for uncertainties and challenges due to this taxonomic revision). No previous

study has examined the ecology of the two species at once, and in the same region. Consequently, their respective ecology has still to be ascertained, in particular in their new range of sympatry. In addition, no previous study examined the influence of temperature and salinity on their hybrids, although a decreased in hybrids fitness may be an efficient barrier to introgression in the wild.

Using experimental crosses, the objectives of this study were two-fold: 1) study the effects of temperature and salinity on growth rate and survival of juveniles *C. robusta* and *C. intestinalis* in their range of sympatry; 2) and to examine the fitness of the hybrids at juvenile stage in order to test if temperature and salinity explain the almost complete absence of F1-hybrids in the wild. We chose to target juveniles known to be particularly prone to high mortality (Gosselin and Qian, 1997), making those stages a sensible step in the life cycle in marine invertebrates.

2. Materials and methods

2.1. Sampling and design of laboratory crosses

Adults were collected at Bloscon harbor (Roscoff, Finistère, France) and Trebeurden marina (Côte d'Armor, France) located 82 nautical miles from Brest, for *Ciona intestinalis* and *C. robusta* respectively. These two marinas were selected because of large abundance of one or the other of the two species, thus facilitating the sampling of mature and healthy individuals. Back to the laboratory, species identification was confirmed using diagnostic morphological traits (Brunetti et al., 2015; Sato et al., 2012). In addition, after production of offspring, diagnostic molecular markers were used to confirm species identification (following protocols given in Bouchemousse et al., 2016b).

Following their collection in the field, mature individuals were isolated for a few days before being used in crosses. Bi-parental crosses were made to control the maternal and paternal lineages. Previous experiments showed that selfing is rarely successful in the study species (Bouchemousse et al., 2016b). However, to avoid potential selfing through contamination of oocytes by sperm, oocytes were first collected with a needle, taking care to avoid the spermiduct. In addition, while counting the oocytes (see below), we checked for cell division (i.e. cleaved eggs due to selfing) under a dissecting microscope. Only one pool of oocytes (out of 29) showed signs of cell division on a limited number of oocytes; this pool of oocytes (and the individual) was discarded from the experiment. Note also that other crossing experiments (Malfant et al. in prep.) using the exact same protocol and for which parentage analyses were done with molecular markers (including on hybrids) never showed any signs of selfing. Three categories of offspring were produced: F1-*C. intestinalis*, F1-*C. robusta* and hybrids; for the later, oocytes of one parent *C. intestinalis* were fertilized by sperm of one parent *C. robusta*, a direction showing a fertilization rate similar to those observed with intra-specific crosses (Bouchemousse et al., 2016b). To decrease genetic and/or maternal effects, for each category, we pooled offspring produced with seven independent assays, each with a different parental pair. Note that for each of the seven assays, one *C. intestinalis* and one *C. robusta* used for the intra-specific crosses were also used for the inter-specific cross to minimize parental effects across categories. A total of 21 experimental bi-parental crosses, involving 28 parents, was thus done to produce the three categories (see Fig. 1.A).

2.2. Production of F1 individuals up to early juvenile stage under standard conditions

We used standard conditions (17 °C, salinity 35) to obtain 10 day-old offspring before starting the different temperature and salinity treatments in order to test for two extrinsic post-zygotic barriers, namely temperature and salinity. Fertilization was done in 88 mm petri dishes, at room temperature in natural filtered (0.45 µm) seawater at salinity 35, using gametes collected from spermiduct and oviduct, as

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