Phenotypic Plasticity and Adaptation Potential to Salinity in Early Life Stages of the Tunicate, *Ciona intestinalis* spB

Doctorial Thesis

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Abstract

Species respond to environmental heterogeneity through a variety of mechanisms such as plasticity, genetic adaptation and phenotypic buffering. Determining how gene flow, scale of environmental heterogeneity and trait heritability influence these responses is important for understanding how these different mechanisms arise, which is a central task in the field of evolutionary biology.

For many marine organisms salinity is an important driver of environmental heterogeneity and physiological stress. As with many stressors, salinity stress is often more severe for early life-history stages such as embryos and larvae. The main aim of this thesis was to investigate underlying mechanisms that allow species to cope with environmental heterogeneities in their natural environment. More specifically, I focused on strategies to manage salinity differences in early life-history stages of the tunicate *Ciona intestinalis*. This marine invertebrate has a large geographical distribution and is considered highly invasive in some parts of the world. Plasticity in important fitness related traits is generally considered to promote invasiveness even though there is evidence that local adaptation also could play an important role in range expansions of invasive species.

Through investigations of population differences in larval performance, I wanted to understand what mechanisms allowed existing populations to inhabit different salinity regimes, focusing explicitly on the role of transgenerational phenotypic plasticity. Adult acclimation had a predominant effect on tolerance ranges of developing embryos and larvae, but there were also small signs of population differences that could be related to local adaptation and/or persistent environmental effects.

To better understand the potential for adaptation of larval salinity tolerance I used quantitative genetic methods to assess the extent to which larval performance in different salinities is a heritable trait. Heritable variation proved to be extremely low, suggesting limited potential for local adaption in investigated populations. The potential for local adaptation can be strongly influenced by gene flow between populations that inhabit different environments. *C. intestinalis* has pelagic larvae, which could disperse over relatively large areas, thereby preventing local genetic differentiation. Through a population genetic study we found that gene flow at times was restricted at much smaller scales than suggested by the dispersal potential of larvae. Population structures implied that physical barriers, such as density differences between water masses, restricted larval dispersal.

The study of sexual selection is an important field in evolutionary biology. Traditionally, it was assumed that sexual selection could not operate in sessile marine invertebrates with external fertilization. Today, however, there are many examples of mechanisms governing gamete interactions that allow eggs to "select" sperm. Our understanding of the underlying selective pressures, and indeed how these mechanisms affect fertilization success between individuals within a species, is however limited. I examined causes of variation in fertilization success in populations of *C. intestinalis*. I found significant variability in compatibility between parental genotypes, which indicated that this may be a way for individuals to avoid the negative effects of inbreeding.

Populärvetenskaplig sammanfattning *Swedish summary*

Organismer kan utveckla olika strategier för att hantera variationer i deras levnadsmiljö. De kan vara "specialister", dvs. populationer kan ha anpassats genetiskt till olika lokala miljöförhållanden. Alternativt kan de ha utvecklat egenskaper som gör dem till mer av "generalister", vilket innebär att varje individ kan anpassa sig till variation i miljön genom fysiologiska, morfologiska och beteendemässiga mekanismer. Hur dessa olika strategier uppstår i samspelet mellan organism och miljö är en central frågeställning inom evolutionsbiologin.

För marina organismer är salthalt en viktig miljöfaktor och möjligheten att kunna hantera salthaltsvariation är av stor betydelse för många arters utbredning. Den marina evertebraten *Ciona intestinalis* spB är en sjöpungsart som ofta utgör en dominerande del av den bottenlevande faunan i de områden arten förekommer. Den har en stor geografisk spridning och kategoriseras som en "invasiv" art på många platser. Invasiva arter har förmågan att sprida sig till nya områden där de får en stor utbredning och därmed kan utgör ett hot mot den inhemska faunan. Studier tyder på att dessa arter ofta har karaktärer som är av mer "generalist-typ". Detta kan vara en fördel då det medför att individer direkt kan anpassa sig till rådande miljöförhållanden istället för att det sker genom naturlig selektion över generationer. Denna förmåga kan dock vara fysiologiskt begränsad och kostsam och det finns bevis för att lokal genetisk anpassning kan vara viktigt för etablering i nya områden. Många marina evertebrater har yttre befruktning och frilevande larvstadier och dessa tidiga livsstadier är ofta mer känsliga för miljöstress än vuxna individer.

Syftet med mitt doktorandarbete var att undersöka hur tidiga livsstadier hos *C. intestinalis* spB kan klara olika salthalter. Genom acklimatiseringsexperiment kunde jag påvisa att det gick att förändra larvers tolerans genom att ändra föräldramiljön och att småskaliga skillnader i salthaltstolerans mellan populationer till största delen var relaterade till acklimatisering och inte lokal genetisk anpassning. För att förstå deras förutsättningar för genetisk anpassning, undersökte jag också "ärftlighet" relaterad till variation i larvers förmåga att hantera olika salthaltsmiljöer. Det visade sig att den ärftliga delen av variationen var ytterst liten, vilket tyder på en begränsad förmåga till genetisk anpassning för de undersökta populationerna. Genflöde mellan populationer som existerar i olika miljöer påverkar också deras möjlighet till lokal genetisk anpassning. Då *C. intestinalis* spB har frilevande larver, har de potential för spridning över relativt stora områden, vilket skulle kunna motverka att lokal genetisk anpassning uppstår. Genom en populationsgenetisk studie kunde vi dock visa att genflödet mellan populationer ibland var begränsat på en skala som man inte förväntar sig hos organismer med frilevande larver. Populationsstrukturerna tydde på att fysiska barriärer, som t.ex. språngskikt mellan vattenmassor, radikalt begränsar larvspridningen.

Studier av sexuell selektion är ett betydande fält inom evolutionsbiologi. Traditionellt ansåg man dock inte att det fanns möjligheter till sexuell selektion hos fastsittande marina evertebrater med yttre befruktning. Det finns i dagsläget betydande kunskap om att mekanismer, som styr interaktionen mellan spermier och ägg, kan vara ett sätt för honor att "selektera" hanar. Kunskap om hur dessa mekanismer påverkar befruktningsframgången mellan individer inom olika arter och bakomliggande evolutionära "motiv" är dock begränsad. Genom att undersöka skillnader i befruktningsframgång inom populationer av *C. intestinalis* spB kunde jag se effekter som tydde på betydande kompatibilitetsskillnader mellan par och det fanns tecken på att detta kan vara ett sätt för honor att undvika negativa effekter av inavel.

List of papers

This thesis is based on the following papers:

Paper I: Renborg E, Johannesson K, Havenhand J (2013) Variable salinity tolerance in ascidian larvae is primarily a plastic response to the parental environment. Evolutionary Ecology:1-12

Paper II: Renborg E, Johannesson K, Laugen AT, Havenhand JN (Manuscript) Heritability of salinity tolerance in early life-history-stages of a widely distributed and highly invasive marine invertebrate species.

Paper III: Johannesson K, Ring AK, Johannesson KB, Renborg E, Jonsson PR, Havenhand JN (Manuscript) Strong physical barriers to gene flow promote subdivision of a coastal population of a tunicate species (*Ciona intestinalis* spB) with pelagic eggs and larvae.

Paper IV: Renborg E, Ring AK, Johannesson AK, Havenhand J (Manuscript) Coevolution of gamete and genetic compatibility suggests sexual selection to avoid inbreeding in the solitary tunicate *Ciona intestinalis*.

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Introduction

Coping with environmental heterogeneity: phenotypic plasticity vs. local adaptation

Species respond to environmental heterogeneity through genetic and phenotypic mechanisms. Populations can adapt through genetic change over generations, caused by natural selection i.e. local adaptation (Kawecki 2008), and individuals can alter their phenotypes in response to changes in the environment i.e. phenotypic plasticity (West-Eberhard 1986, 2005; Pigliucci 2001; DeWitt and Scheiner 2003). A plastic response does not involve any genetic change, but underlying mechanisms that facilitate plasticity may have evolved as genetic adaptations to selection pressures that favour plasticity over a fixed response (Via and Lande 1985; Harvell 1998; Pigliucci 2001; David et al. 2003).

According to grain-size theory, (Levins 1968), the spatial scale of environmental heterogeneity is an important determinant of the evolution of phenotypic plasticity. Plasticity will be favoured when the environment is perceived as fine-grained i.e. when the individual organism will encounter different environmental conditions. Plasticity could thus be a beneficial strategy under temporal environmental fluctuations (Pigliucci 2001; DeWitt and Scheiner 2003). The scale of environmental heterogeneity could furthermore influence the type of plastic response that would be adaptive. If the environmental conditions an individual will experience during it's lifetime are likely to be set at an early age, phenotypic plasticity would tend to be fixed and induced during early development (Meyers and Bull 2002; Piersma and van Gils 2010). In contrast, reversible phenotypic plasticity, (e.g. transformations in behaviour, physiology or morphology), is a more favourable strategy when the environment fluctuates over timescales shorter than a lifetime (Meyers and Bull 2002; Piersma and Drent 2003; Piersma and van Gils 2010). When the parental environment is positively correlated with the environment of the offspring, transgenerational plasticity will be adaptive (Galloway 2005; Whitman and Agrawal 2009). The reliability of environmental cues is fundamental for the evolution of all type of plasticity (theoretically modelled by Moran 1992). It is also commonly thought that underlying mechanisms enabling a plastic response may entail fitness costs, even though empirical evidence for this is scarce (Pigliucci 2001). Costs of plasticity would implicate that a fixed response could be favoured even when some degree of plasticity would be the optimum.

Substantial gene flow between populations inhabiting different environments would favour plasticity and counteract local adaptation (Pigliucci 2001; Berrigan and Scheiner 2003; Kawecki 2008; Sotka 2012). Pelagic eggs and larvae are the primary dispersal mechanism of marine invertebrate species that have a sessile or sedentary benthic adult stage. The presence and duration of pelagic larval stages has therefore been seen as an important determinant for population genetic structure and gene-flow (Grosberg & Cunningham 2001), and hence also for the relative importance of plasticity and local adaptation (Sotka 2012). The classical assumption has been that species that lack pelagic larvae ("direct developers") would be genetically substructured, whereas those with planktonic larvae may disperse over long-distances (Scheltema 1986; but see Johannesson 1988), resulting in low levels of genetic structuring at small geographic scales (Burton 1983; Hedgecock 1986). Similarly, plasticity has been shown to be more prominent in marine species with pelagic larvae than in species with short-lived larvae or direct development (Hollander 2008), although there are exceptions to this (Hollander et al. 2006). More recent studies have, however, shown no strong relationships between duration of pelagic dispersal and population genetic structure (Weersing & Toonen, 2009): species with high migratory potential often show genetic differentiation at sometimes surprisingly small geographic scales (Johannesson and André 2006; Sanford and Kelly 2011), and species with direct development often show a lack of genetic substructuring among distant populations (reviewed by Winston 2012). Furthermore, the relative

frequency of published examples of local adaptation in marine invertebrates does not support the assumption that adaptive genetic differentiation would be more common in direct developers compared to species with planktonic dispersal (Sotka 2012). Clearly, genetic structures of marine populations are influenced by more factors than just the potential for larval dispersal. The evolution of plasticity and local adaptation are also dependent on other aspects of the organism such as the amount of heritable genetic variation and genetic architecture of the traits under selection (Etterson and Shaw 2001).

Salinity, environmental heterogeneity and physiological stress
For many marine organisms salinity is an important driver of environmental heterogeneity and physiological stress (Tomanek 2011). Salinity can both directly and indirectly influence many different aspects of the organism such as osmotic balance, immune system response, metabolic rate, growth and reproduction (Kinne 1964, Butt et al 2006). As with most environmental stressors, salinity stress is often more severe for early life-history stages such as embryos and larvae than for adults (e.g. Kinne 1964, Dybern 1967, Anger 1998, Charmantier 1998). Salinity is often seen to be an important determinant of species boundaries in marine organisms (Ojaveer et al 2010) and there are several examples of local adaptation (Sanford & Kelly 2011) and even speciation (Pereyra et al. 2009) in response to salinity. Coastal waters are often characterized by temporal and spatial variation in salinity, driven by e.g. tides and terrestrial run-off. In the Skagerrak-Baltic Sea system, a strong salinity gradient spans the range from almost freshwater (inner Baltic Sea) to full ocean salinity (North Sea). In addition, Swedish coastal waters are stratified with a less saline surface layer above a deeper, high salinity, water mass. Seasonal weather patterns and wind-driven currents cause the surface water salinity to vary over time, whereas salinity of the deep water is more stable. These horizontal and vertical gradients provide an excellent test-bed for exploring mechanisms to cope with salinity variation.

Ciona intestinalis *spB – a broadly distributed and highly invasive tunicate species*
Ciona intestinalis has a cosmopolitan distribution and has therefore been considered to be able to cope with a wide range of environmental conditions. However, recent studies have shown that it is actually a species complex of at least four morphologically cryptic species (Caputi et al. 2007, Zhan 2010). Two of these species, spA and spB, (the latter being the species found in Swedish waters) have large and disjoint distributions and are considered highly invasive in many parts of the world (Zhan et al. 2010; Therriault and Herborg 2008). *C. intestinalis* is an important filter feeder in many benthic ecosystems where it often exists in very large numbers. Their life-cycle consists of a sessile hermaphroditic adult stage with broadcast-spawning, external fertilization and a short non-feeding pelagic larval stage of ≤ 5 days. This relatively short pelagic phase could occasionally be even more restricted as eggs sometimes are released and retained in mucus strings and thus metamorphose without a free-swimming stage (Svane & Havenhand 1993). In contrast, the worldwide distribution of *C. intestinalis* spB indicates long-distance dispersal, presumably mediated by anthropogenic vectors (Zhan et al. 2010, Therriault and Herborg 2008). Various levels of connectivity are also reflected by population genetic data, in which *C. intestinalis* spB sometimes shows rather low genetic structure at larger geographical scales, while at times having constrained gene-flow on smaller scales (Zhan et al. 2010, Zhan et al. 2012). *C. intestinalis* has been commonly used as a model chordate species in developmental biology, and the full genome of *C. intestinalis* spA has been sequenced (Dehal *et al.,* 2002). Comparatively little is known about their ecology and classical population genetic knowledge is still fragmentary (Procaccini et al 2011). Most ecological studies on *C. intestinalis* have actually been done in Scandinavian waters on fjord populations of *C. intestinalis* spB (e.g. Dybern 1965; 1967; Havenhand and Svane

1991, Svane and Havenhand 1993; Petersen and Svane 1993). In Swedish waters, *C. intestinalis* spB exists in a range of different salinities, from full salinity down to 11 PSU, which limits their distribution both vertically and horizontally, and has been suggested to be the minimum salinity for successful development (Dybern 1967). The width of the salinity tolerance norm appears to be related to the different life-history stages – adults having a wider range than larvae, and larvae being more phenotypically buffered than embryos (Dybern 1967). The tolerance norm of early life-history stages also seems to be correlated with the salinity regime of the parental population (Dybern 1967).

Sexual selection and the evolution of gamete compatibility in broadcast-spawners
The majority of marine invertebrate species reproduce by broadcast-spawning, i.e. they release eggs and sperm directly into the water column were fertilization takes place (Thorson 1950). While Darwin was the first to recognise the potential for sexual selection (Darwin, 1871), he found it unlikely that it would act in broadcast spawning marine invertebrates, which often were sedentary and lacked sexual dimorphism. With the realisation that sexual selection could take place after mating, but before fertilization (Parker 1970), and the recognition of cryptic female choice of fathers (Eberhard, 1996, Thornhill, 1983), views on sexual selection changed radically and today there is a growing body of knowledge about mechanisms for "mate-choice" in broadcast spawning species (reviewed by Evans and Sherman 2013). Mate-choice and mate-competition can operate at several stages of fertilization including sperm chemoattraction (Bishop et al., 2001, Evans et al., 2012), sperm-egg binding (Galindo et al., 2003, Palumbi, 1999), sperm penetration (Kresge et al., 2001), fusion of sperm and egg membranes (Vacquier and Swanson 2011) and fusion of pro-nuclei (Carré and Sardet 1984). How these various mechanisms mediate self-sterility is relatively well known (e.g. Sawada *et al.* 2014), and there is evidence that these mechanisms also can prevent heterospecific fertilizations (Lambert 2000). As female broadcast-spawners receive no non-genetic benefits from the male (such as offspring protection etc.), the sexual selection by females is constrained to maximising genetic gains from the male. Accordingly, there are hypotheses that females would choose either males with "good genes" or males with a "compatible genes" (Jennions and Petrie 2000; Zeh and Zeh, 1996; Tregenza and Wedell, 2000; Neff and Pitcher 2005; Evans and Sherman 2013). Marshall and Evans (2007) have shown support for the "good gene" hypothesis in the ascidian *Pyura stolonifera*. Support for the "compatible gene" hypothesis comes from numerous studies that show significant male-female interactions in *in vitro* fertilization experiments (e.g. Evans and Marshall 2005, Marshall and Evans 2005, Jiang & Smith 2005). Few studies have however linked these differences in fertilization success to differences in offspring fitness.

Just as self-sterility and incompatibility between species prevent fertilization by non-compatible genomes, gamete compatibility systems acting *within* species could prevent negative effects of different levels of relatedness, such as inbreeding depression. Sexual incompatibility mechanisms related to biparental inbreeding avoidance are well studied in terrestrial plants (e.g. Wasser and Price; Souto 2002), but there are few if any examples in sessile broadcast spawning marine invertebrates (reviewed by Evans and Sherman 2013; but see Bishop et al. 1996 and Pemberton et al. 2004 for "spermcasting" species). Given that sessile broadcast-spawners typically have dispersive larvae, it's perhaps unusual to expect that they would be selected for inbreeding avoidance. However such species can have high levels of local recruitment (e.g. *Ciona intestinalis*, Svane and Havenhand 1993; Petersen and Svane 1993), and consequently there is a risk that they may encounter sperm from close relatives, and hence inbreeding.

Aims of thesis

Widely distributed species are likely to experience a broad range of environmental heterogeneities on different temporal and spatial scales. These species are interesting model organisms to study how, and under what circumstances, different plastic, specialist and generalist strategies to handle variations in the environment may evolve. The fact that the dispersal of *C. intestinalis* seems to vary, with evidence of high local recruitment but also potential for dispersal through pelagic larval stages and anthropogenic means, makes them particularly interesting models to study the balance between plasticity and genetic differentiation in relation to gene-flow. Although marine invertebrates with external fertilization appear to be excellent candidate species for examining the evolution of gamete compatibility systems, few studies have done this, and no studied have addressed gamete compatibility in relation to parental relatedness (Evans and Sherman 2013).

The main aim of this thesis was to investigate the underlying mechanisms that allow a species to cope with environmental heterogeneities in its natural environment. More specifically, I focused on strategies to manage salinity differences in early life-history stages of the tunicate *Ciona intestinalis*. Through investigations of *between*-population variation in larval performance, I attempted to identify the mechanisms that enable existing populations to inhabit different salinity regimes, specifically focusing on the role of transgenerational phenotypic plasticity. Investigating sources of *within*-population variation, and the extent to which larval performance in different salinities is a heritable trait, allowed for assessments of the potential for local genetic adaptation. I also wanted to explore population genetic structures, as adaptive genetic differentiation between populations inhabiting different environments would be facilitated if the gene-flow between these populations were restricted.

Sexual incompatibility mechanisms related to inbreeding avoidance are well studied in terrestrial plants, but have rarely been studied in marine invertebrates. There are however reasons to believe that selection pressures for analogous mechanisms to avoid inbreeding are present. I therefore wanted to explore the hypothesis that gamete compatibility systems have evolved to reduce negative fitness consequences of parental relatedness.

Methods

Animal sampling and adult acclimation

All animals used in the experiments were collected by SCUBA divers and transported to the laboratory in bags containing seawater from the sampling depth conserved in cool boxes. Adult acclimation (Paper I) was conducted using fully aerated recirculating seawater systems equipped with mechanical and biological filters. Animals were fed twice a day with a mixture of microalgae, and once a day with newly hatched *Artemia* nauplii. Feeding was adjusted to provide excess food without negatively affecting water quality.

Fertilization and larval experiments

All gametes used in the experiments (Papers I, II, IV) were collected through strip-spawning. As mature sperm and eggs are stored in the gonoducts of adult *C. intestinalis,* gametes can be collected through strip-spawning without risking using immature gametes (for detailed descriptions see Havenhand 1991). *C. intestinalis* is a simultaneous hermaphrodite, however individuals were used only once in each experiment – either as a functional male or female. Fertilization was performed at adult salinity and the newly fertilized eggs were subsequently

moved to Petri dishes containing the different treatment salinity. Petri dishes were incubated in a humidity chamber on a shaking table to minimize the risk of water evaporation and stratification. To maintain good water quality, partial exchanges of the treatment water were performed during development. Fertilization and later development were scored from high-resolution digital macro photographs of Petri dishes using Adobe Photoshop[©].

Breeding design and quantitative genetic analysis
Quantitative genetics is the study of inheritance of inter-individual differences in quantitative traits (Lynch and Walsh 1998). To be able to break down phenotypic variance of a trait into different genetic and environmental components requires knowledge of relatedness between individuals. We used a North-Carolina II breeding design (Papers II and VI, Lynch and Walsh 1998). This is a factorial, and hence statistically powerful, design that we applied to replicate blocks of $3\male \times 3\female$ crosses. The number of cells in each block was constrained by the average number of eggs that were possible to collect from each female. The proportion of phenotypic variance due to additive genetic effects and non-genetic maternal effects was estimated using an animal model, a form of mixed-effect model that explicitly takes into account the resemblance among all relatives. This procedure models an individual's phenotype as a function of a number of fixed and random factors (Wilson et al 2010, Postma et al. 2011). We used Bayesian Markov chain Monte Carlo (MCMC) techniques to determine variance components. For detailed descriptions of the fitted model see Paper II.

Molecular markers, estimates of relatedness/heterozygosity and population genetic analysis
To explore population structures (Paper III) and assess parental relatedness and estimated offspring heterozygosity (Paper IV) we used microsatellite markers. Microsatellites generally have high mutation rates and are commonly used in population genetic analysis, and to address questions regarding kinship (Selkoe 2006). Only five to six of the many microsatellites that we tried gave consistent results, which could be related to the fact that they originally were developed from the genome of the sister species, *C. intestinalis* spA. Although small sample sizes can reduce the power of estimations of relatedness (Csillery *et al.*, 2006), the methods were selected to minimise these effects (Wang, 2011, Wang, 2002). Relatedness '*W*' (Wang, 2002) between parents of each cross was determined from allele frequences at each of the five loci. Estimated heterozygosity of offspring, H_{EST}, (Primmer *et al.* 2003) was also determined from parental allele frequencies. To demonstrate the presence of population structure and infer individual ancestry we used a structure analysis. In this procedure, each sampled individual is assigned into a user-defined number of populations (K) (presented in Fig. 3 below, Pritchard et al. 2000). For more detail of statistical methods used and detailed description of the models see Papers II and IV.

Biophysical model
To better understand the population structures identified from the genetic data (Paper III) and explore possible barriers to gene-flow, we used biophysical modeling to estimate connectivities among study sites attributable to larval dispersal of *C. intestinalis*. Briefly, we combined an oceanographic circulation model that produces water velocity fields with an off-line trajectory model (parameterized with biological data) that calculates individual dispersal paths based on the velocity fields. Dispersal probabilities between sites were then estimated as the proportion of released particles from one site that reached another site and *vice versa*. We also calculated multi-generation connectivity based on stepping-stone dispersal. Due to the fact that the study area is

highly open, a significant amount of larvae were lost to outside the model domain and connectivities then approached zero with increasing number of generations. Consequently we only interpreted relative magnitudes of multi-generation connectivity between study sites. For more detailed descriptions of the models and parameterizations see Paper III.

Main Results & Discussions

Transgenerational plasticity – a way to cope with environmental heterogeneity in early life-history stages (Paper I):

Previous studies have found large differences in salinity tolerance of early life-history stages among *C. intestinalis* populations from different salinity regimes (Dybern 1967). This paper investigated whether such differences in salinity tolerance could be explained by transgenerational phenotypic plasticity. In a common garden experiment we used populations from different depths, exploring vertical differences in salinity. Adult acclimation salinity had a predominant, and statistically significant, effect on larval performance: the larval salinity tolerance range almost fully matched the parental acclimation salinity, independent of parental origin (deep, high saline, or shallow, low saline environments, Fig. 1). However we also detected minor population differences, (e.g. deep populations showed better larval performance at the higher end of the salinities tested, Fig 1). These could indicate local adaptation, or persistent environmental effects (incomplete acclimation of the parents). Rapid responses to changes in salinity are likely to be generally beneficial for *C. intestinalis* as most coastal areas are characterized by small-scale temporal and spatial salinity variation. If the parental environment is a relatively good predictor of the environment of the offspring, transgenerational acclimation effects will be a suitable way for early life-history stages to cope with environmental heterogeneities (Galloway 2005; Whitman and Agrawal 2009). Plasticity in important fitness related traits could also promote invasiveness (Richards et al. 2006; Muth and Pigliucci 2007). Serafini et al. (2011) showed that *C. intestinalis* responded faster to thermal stress than the related *Ciona savignyi*, indicating greater plasticity in stress-response of *C. intestinalis*, which also has the broadest distribution. Thus, a high degree of plasticity in larval salinity tolerance might have importance for the widespread distribution and invasive character of *C. intestinalis* spB.
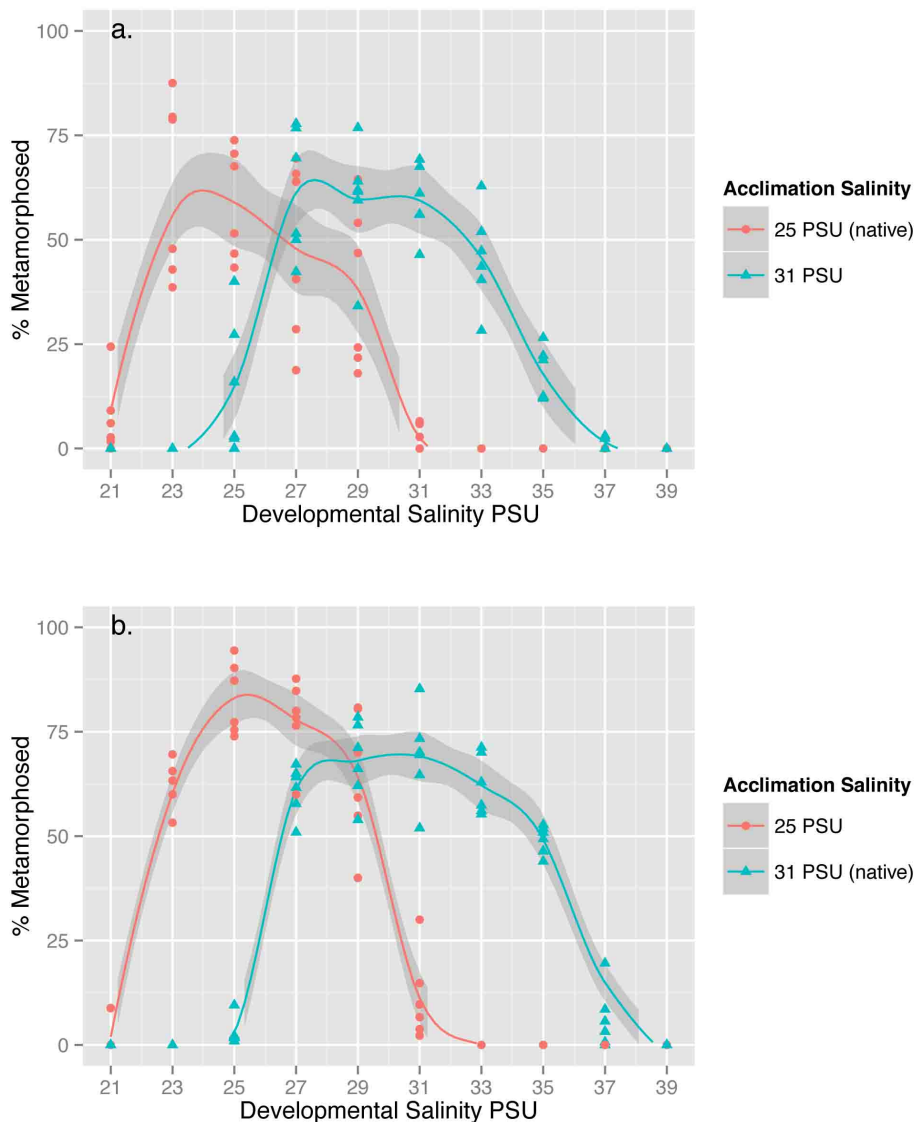
Fig. 1 (Paper I): Salinity tolerance of metamorphosis in *C. intestinalis* larvae from shallow (a) and deep (b) populations acclimated to native and non-native salinities (25 and 31 PSU). Data points are for individual replicates (pooled across sites, P = 0.53). Curves are LOESS smooths (span = 0.5). Grey shading indicates 95 % confidence intervals for each curve.

Heritability of larval salinity tolerance (Paper II)

Our previous experimental work (Paper I) showed that transgenerational plasticity to a large extent explained differences in larval salinity tolerance *between* populations, but that differences at the extremes of the tolerance ranges might indicate a degree of local adaptation. To determine the potential for local genetic adaptation, it is essential to investigate the extent to which *within*-population variance in a trait is related to additive genetic variance and thus is available for natural selection. We used a quantitative genetics approach to assess the importance of additive genetic effects for variation in metamorphic success within populations of *C. intestinalis* in a range of different salinities. Overall heritabilities for metamorphic success were very low for both populations tested (e.g. fjord population, Fig. 2). There were no indications of substantial differences in the expression of genetic variability between salinities, although low sample sizes caused the 95% credible intervals to be relatively wide when analysing salinities separately (Fig.

2). It is generally believed that most traits display significant genetic variation in natural populations (Mousseau and Roff 1987, Houle 1992, Merilä and Sheldon 1999), even though heritabilties tend to be lower in traits highly related to fitness (reviewed by Mousseau and Roff 1987, Visscher *et al.* 2008). There is, however, evidence that evolution of traits can be constrained by low levels of heritable genetic variation (Hoffmann *et al.* 2003, Kellermann *et al.* 2009, Kelly *et al.* 2011). The extremely low heritability of metamorphic success across salinities, suggests little capacity for local adaptation of larval salinity tolerance in the investigated populations.
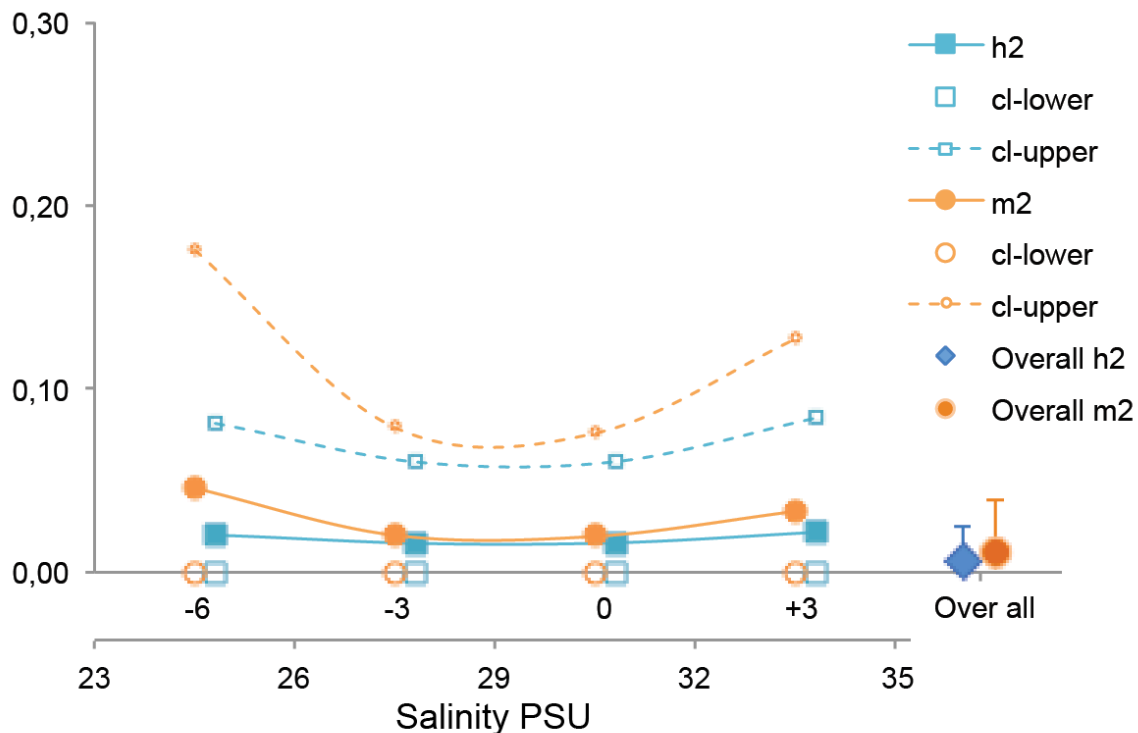
Fig. 2 (Paper II): Estimated heritabilities (h^2, solid blue lines) and maternal effects (m^2, solid orange lines) with 95% credible intervals (dotted lines) for metamorphosis success of larvae in a fjord population of *C. intestinalis* in Sweden. Overall m^2 and h^2 are pooled across salinities.

Physical barriers to gene flow and population structure (Paper III)

According to grain-size theory (Levins 1968) the dispersal distance in relation to the scale of environmental heterogeneity, should be an important predictor of the balance between local genetic adaptation and phenotypic plasticity. To explore patterns of connectivity between populations, we used genetic data from 6 highly polymorphic microsatellite markers. We investigated connectivity between populations from different depths in the same location (i.e. from shallow, low saline, and deep, high saline environments) and also populations from different locations along 110 km of coast (including both fjord and open-coast). The low level of genetic differentiation between deep open-coast populations (illustrated by a structure analysis, Fig. 3) suggests that connectivity may be high over relatively large distances. However, the high level of population differentiation between deep and surface populations from the same location, as well as between fjord and open-coast populations (Fig. 3, F_{ST} estimates – Paper III), points to the

existence of strong barriers to gene-flow. Long water residence times in this fjord (16-26 days for surface water, 40 days for deep water, Arneborg 2004) relative to the maximum pelagic period of eggs and larvae (7 days, Svane & Havenhand 1993) would most likely prevent dispersal between fjord and open-coast populations. The low connectivity between deep and shallow populations could be due to the density-driven discontinuity ("pycnocline") that separates the low-salinity surface water layer from high-saline deep water. Pycnoclines can be effective barriers to passively dispersing particles such as eggs and embryos, and there is evidence that tunicate larvae may also actively avoid passing through salinity gradients (Vazquez & Young 1996). Thus, the high level of phenotypic plasticity seen in larval salinity tolerance from shallow, low saline, and deep, high saline environments (Paper I) is probably not related to larvae experiencing a high degree of environmental heterogeneity while dispersing between depths. Instead, it is possible that the effects of transgenerational plasticity may rather reduce gene flow between depths, as larvae will be acclimated to the specific native conditions. Consequently, larvae that do cross the pycnocline may have reduced fitness ("phenotype-environment mismatch", Marshall *et al.* 2010). Such a mismatch may also drive the development of larval responses to salinity gradients mentioned above.
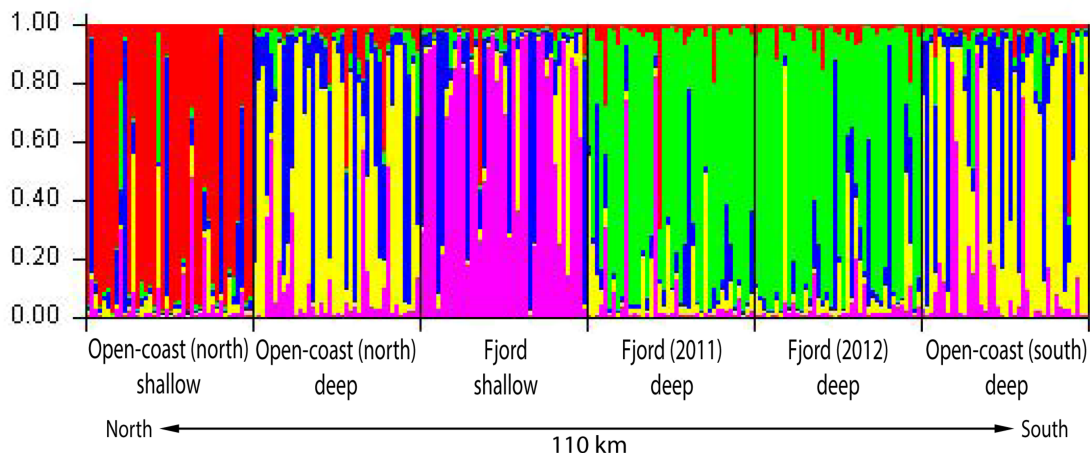
Fig. 3 (Paper III): Population structure as illustrated by a Structure analysis in which individuals are assigned to one of 5 clusters.

Gamete compatibility - a mechanism for biparental inbreeding avoidance? (Paper IV)
For broadcast-spawners, which release sperm and eggs freely into the water column, sperm-egg interaction is the only possible mechanism of sexual selection. To test whether gamete compatibility systems operate in populations of *Ciona intestinalis* spB we used a blocked factorial North Carolina II breeding design replicated over two demographically independent populations, one from a fjord, and one from an open-coast site. Most of the variation in fertilization success was due to the effect of male-female interactions, and none was related to male effects. This supports the hypothesis that gamete recognition mechanisms mediate selection for males with "compatible genes" rather than "good genes". Variation in larval fitness (measured as percent metamorphosis) was mostly due to male-female interactions in the fjord population, but much less so in the open-coast population. This suggests the existence of genetic compatibility effects on larval fitness. We found a negative relationship between parental relatedness and larval fitness in the open-coast population (Fig. 4), indicating that more closely related individuals had reduced

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offspring fitness. The same trend was seen in the fjord population, however this relationship was not significant. The fjord population did, however, show a significant negative effect of relatedness on fertilization, and that fertilization success was positively correlated to metamorphosis success (Fig. 4). These relationships are not statistically independent, but both support the hypothesis that gamete compatibility has evolved as a mechanism to select for genetically compatible mates. The effect of relatedness also indicates that gamete compatibility systems may act to prevent inbreeding, as mentioned this phenomenon has rarely been investigated in sessile aquatic invertebrates with external fertilization (reviewed by Evans and Sherman 2013). That these relationships were found in the fjord, but were absent in the open-coast population, may be related to a stronger selection pressure for inbreeding avoidance in the seemingly more closed fjord population. (For discussions of mean offspring heterozygosity see Paper IV.)

A *Fjord*   **B** *Open-coast*
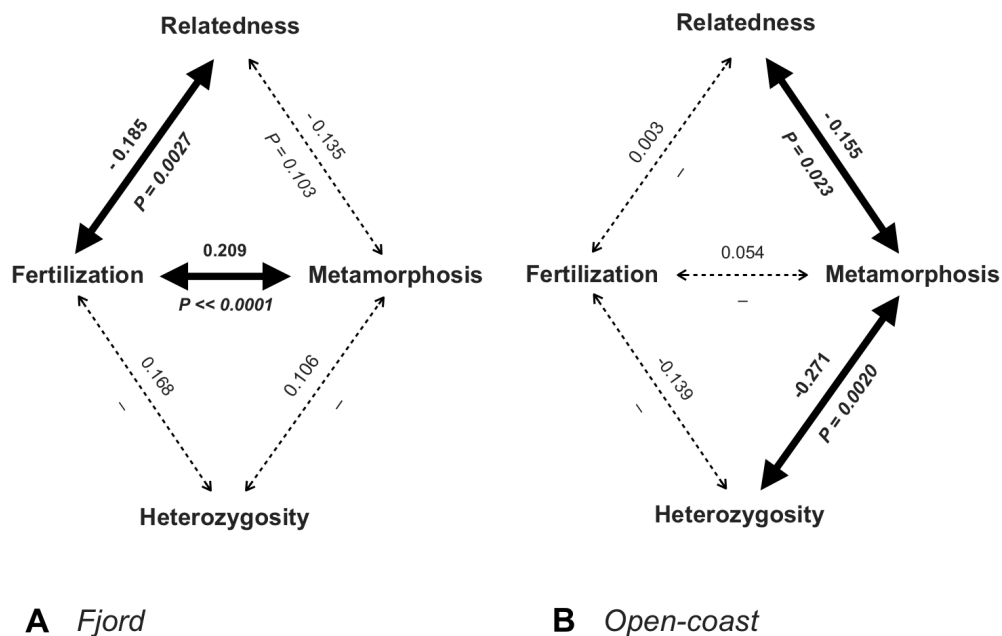
Fig. 4 (Paper IV)**:** Summary of statistical relationships among variables. "Relatedness" = '*W*', (Wang, 2002), "Heterozygosity" = H_{EST} (Primmer et al. 2003), "Fertilization" = fraction of eggs dividing 2h after fertilization, "Metamorphosis" = fraction of dividing eggs that successfully metamorphosed after 96h. Solid arrows indicate statistically significant effects. Numbers above arrows indicate estimated effect size of mean relationship.

Conclusions and future prospects

Transgenerational plasticity appears to be the main mechanism allowing early life-history stages in *C. intestinalis* spB to cope with spatial salinity differences, at least within the salinity range tested here (Paper I). A high degree of plasticity also mediates tolerance to temporal differences in salinity, something that characterizes most coastal areas. Previous studies suggest that populations from more extreme salinities at the species range-limit do not survive adult acclimation to full saline environments and more central populations cannot cope with salinities experienced in these marginal habitats (Dybern 1967). It would be interesting to investigate this issue further, and look for signs of local adaptation of salinity tolerance in more marginal habitats.

The population genetics data (Paper III) showed genetic subdivisions on small geographical scales, indicating restricted gene-flow between *C. intestinalis* spB fjord and open coast populations and populations from above and below the pycnocline. In contrast, there was a high level of connectivity between deep-water open coast populations that were separated by a relatively large distance (110 km). Long water retention times in the fjord would explain the lack of gene-flow between the fjord and open-coast. The exact mechanism of how the pycnocline could prevent gene flow is not clear, as larvae could – in principle – swim through the pycnocline. Two possibilities that would be interesting to investigate further are: 1) phenotypic-environment mismatch (Marshall 2010) related to the different salinity tolerance norms of early life-history stages; and 2) larval behaviour in relation to the pycnocline (Vasquez & Young 1996).

The scale of gene-flow relative to the spatial scale of environmental heterogeneities is considered an important determinant of the balance between phenotypic plasticity and local genetic differentiation. The genetic data (Paper III) suggest there would be possibilities for local adaptation of *C. intestinalis* spB to the different salinity regimes above and below the pycnocline. However, for genetic adaptation to take place there also needs to be heritable variance in traits under selection. As there was very little heritable genetic variance in larval salinity tolerance (Paper II), I do not expect this trait to show local genetic differentiation. It would however be interesting to investigate other traits to see if they show adaptive genetic differentiation on such small geographical scales as between depths.

I showed that in a fjord population of *C. intestinalis* spB, gamete compatibility appeared to have evolved as a mechanism by which individuals could avoid negative fitness effects arising from mating with close relatives, and thus "choose" genetically more compatible mates (Paper IV). There was not, however, an effect of parental relatedness on fertilization success in the open-coast population. This latter result may have been due to insufficient statistical power, but as the more closed fjord population also could have a higher selection pressure for inbreeding avoidance it would be interesting to investigate this further. Marine invertebrates with external fertilization appear to be ideally suited for studying the evolution of gamete compatibility systems, yet few studies have examined the evolutionary drive of such systems acting within populations (Evans and Sherman 2013).

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