GÖTEBORGS UNIVERSITET

Reproductive isolation at contact zones

Samuel Perini

Department of Marine Sciences

The Faculty of Science

University of Gothenburg

2021

Reproductive isolation at contact zones

Samuel Perini

Department of Marine Sciences
The Faculty of Science
University of Gothenburg
Tjärnö Marine Laboratory, 45296 Strömstad, Sweden

samuel.perini@gu.se

ISBN: 978-91-8009-210-4 (PRINT)
ISBN: 978-91-8009-211-1 (PDF)
Electronic version available at
Printed by Stema Specialtryck AB, Borås, Sweden 2021

ABSTRACT

Speciation is among the most important evolutionary processes that contribute to biodiversity. It involves the formation of new species that have become reproductively isolated through a process that is not linear and varies in time and space. For divergent populations that still exchange genes, questions remain on how selective forces and demographic history can influence the maintenance and/or accumulation of multiple barriers to gene flow. In this thesis, I tackle these questions using contact zones between genetically-differentiated populations.

The salinity gradient between the marine North Sea and the brackish Baltic Sea impacts heavily on the adaptation of marine species living in this area. Our review found 23 out of 24 marine species with genetic data have formed divergent populations over this gradient. Population differentiation is strongly driven by divergent selection and/or temporal or spatial segregation, and it seems to be facilitated by ancestral variation. Another excellent example of divergent populations that show barriers to gene flow across contact zones is the intertidal snail *Littorina saxatilis*. This species is abundantly distributed on the European and North American Atlantic coasts and it is known for the Crab and Wave ecotypes, the first adapted to crab predation and the second adapted to wave exposure. In *L. saxatilis*, patterns of divergence have been analyzed using single nucleotide polymorphisms (SNPs), the most common source of genetic variation. Polymorphic short insertions and deletions (INDELs) are the second most abundant variant type but they have been overlooked in speciation studies. My analyses suggest that INDELs are more affected by purifying selection than SNPs. However, they also show patterns of divergent selection and thus, have potential as genetic markers for studies of adaptation and population divergence.

In *L. saxatilis*, shell size is an adaptive trait that is also important for mating. I found that the probability of mating is size-dependent (assortative mating) and there is sexual selection for small male size. Given the different local optima of shell size, this mating pattern contributes to reproductive isolation between ecotypes and simulations showed that gene flow was more reduced by sexual selection than by assortative mating.

Barriers to gene flow may also occur during mating and determining when mating is successful requires knowing when sperm transfer starts. I found evidence suggesting that sperm transfer begins within a few minutes in *L. saxatilis* and seems largely independent of mating duration.

Reproductive isolation at contact zones may be strongly affected by divergent ecological selection and the species history, and without a large hybrid disadvantage, sexual selection may be a more important barrier than assortment. Whether these barrier effects will lead to complete reproductive isolation remains an open question.

Keywords: hybrid zone | gene flow | genetic variants | mating | local adaptation | marine species | speciation

POPULÄRVETENSKAPLIG SAMMANFATTNING

Artbildning är viktig för den bidrar med ny biologisk mångfald. Nya reproduktivt isolerade arter uppstår genom en process som varken är spikrak eller konstant i dimensioner av tid och rum. När utbyte av gener fortfarande sker mellan populationer uppstår frågor om hur naturlig selektion och artens demografiska historia upprätthåller genetiska barriärer. I denna avhandling studerar jag dessa frågor genom att undersöka vad som händer i kontaktzoner mellan genetiskt åtskilda bestånd.

Den gradient i salthalt som råder mellan Nordsjön och Östersjöns brackvatten har lett till anpassningar hos de marina arter som lever i området. Vi sammanställde genetisk data för 24 arter och 23 av dessa uppvisade skillnader mellan bestånden över gradienten. Dessa skillnader upprätthålls dels genom beståndens olika överlevnad i de olika salthalterna och dels genom att reproduktionen är separerad i tid och/eller rum. Den genetisk variation som behövts för att åstadkomma dessa skillnader har i många fall varit variation som fanns i arten redan innan Östersjön bildades.

Hos strandsnäckan *Littorina saxatilis* finns genetiska barriärer som begränsar genflödet över kontaktzoner mellan olika divergerande bestånd. Arten är vanlig längs Europas och Nordamerikas kuster av Atlanten, och speciellt tydliga skillnader finns mellan krabb och vågsnäckor. Krabbsnäckor lever i steniga miljöer med strandkrabbor medan vågsnäckorna lever på vågexponerade klipphällar. Tidigare kartläggningar visar att mutationer av enskilda kvävebaser (s.k. SNPs) gett upphov till genetiska skillnader mellan krabb- och vågsnäckor. En annan typ av mutation som kan uppstå är att små DNA fragment tillkommer eller faller bort (s.k. INDELS). Mina studier visar att INDELs rensas bort av selektion mer effektivt än SNPs men de uppvisar alltjämt skillnader mellan krabb- och vågsnäckor och kan användas för studier av anpassning och divergens.

En snäckas storlek är viktigt för anpassning till de olika miljöerna men också viktigt vid parning. Jag visar i min avhandling att parning sker oftare med snäckor av ungefär samma storlek, och att små hanar får fler parningar. Stora snäckor gynnas bland krabbor och små i vågexponering, och storleksberoende partnervalet utgör därför en barriär för genflöde. I simuleringar visar vi att både storleksval och att små hanar gynnas bidrar till en barriär men att det sistnämnda har större betydelse.

Barriärer till genetiskt utbyte påverkas också av hur framgångsrik varje parning är men för att förstå detta krävs att man vet när under parningen som en överföring av spermier sker. Mina resultat visar att spermier överförs även vid parningar så korta som några få minuter och att parningens längd inte tycks påverka spermieöverföringen.

Isolering mellan divergerande bestånd i kontakt med varandra tycks ofta vara ett resultat av naturlig selektion och påverkas av artens historia. Om hybrider som bildas överlever bra så kan parningsframgång snarare än partnerval utgöra viktiga barriärer. Om det till slut blir en komplett artbildning eller inte är trots att vi förstår vilka mekanismer som är inblandade, ofta svårt att fastställa.

LIST OF PAPERS

Paper I : Johannesson, K., A. Le Moan, S. Perini and C. Andre, 2020 A Darwinian laboratory of multiple contact zones. Trends Ecol Evol 35: 1021–1036.

Paper II: Perini, S., R. K. Butlin, K. Johannesson and A. M. Westram, Manuscript Evolutionary patterns of divergence using short INDELs and SNPs.

Paper III: Perini, S., M. Rafajlović, A. M. Westram, K. Johannesson and R. K. Butlin, 2020 Assortative mating, sexual selection, and their consequences for gene flow in *Littorina*. Evolution 74: 1482–1497.

Paper IV: Perini, S., R. K. Butlin, A. M. Westram and K. Johannesson, Manuscript Timing of the start of sperm transfer in *Littorina saxatilis*.

Supervisors: Professor Roger K. Butlin, Department of Animal and Plant Sciences, University of Sheffield; Professor Kerstin Johannesson, Department of Marine Sciences, University of Gothenburg; Dr Anja M. Westram, IST Austria

Examiner: Professor Per Jonsson, Department of Marine Sciences, University of Gothenburg

TABLE OF CONTENTS

INTRODUCTION 1
EVOLUTION OF REPRODUCTIVE ISOLATION UNDER GENE FLOW 1
BARRIERS REDUCING GENE FLOW 2
REPRODUCTIVE TRAITS ACTING AS BARRIERS 3
EVOLUTIONARY PATTERNS OF DIVERGENCE 6
STUDY SYSTEMS 7

SUMMARY OF RESULTS 9
PAPER I 9
PAPER II 14
PAPER III 16
PAPER IV 20

DISCUSSION 21

ACKNOWLEDGMENTS 24

REFERENCES 24

INTRODUCTION

Evolution of reproductive isolation under gene flow

Biodiversity is tightly connected with ecosystem functions (e.g., primary production and nutrient cycling) and it can be understood by studying how species form, migrate and go extinct (BUTLIN *et al.* 2009; CARDINALE *et al.* 2012). Investigating the first of these processes is the aim of speciation research and since the very first milestone for this field, *The Origin of Species* (DARWIN 1859), speciation research has explored the formation of biological diversity in three directions: examining the components of reproductive isolation, resolving the genetic basis of speciation and comparing speciation processes with patterns of biodiversity (COYNE AND ORR 2004; BUTLIN *et al.* 2011). In this thesis, I focus on the first two directions and examine reproductive barriers that reduce gene flow between divergent populations, evolutionary processes such as natural and sexual selection that produce these barriers and review the contribution to speciation of genomic features such as chromosomal rearrangements. In my effort to provide new insights to the field of speciation research, the evolution of reproductive isolation is key.

Speciation is the split of one species into two fully reproductively isolated species and it is a continuous process that can be measured quantitatively using a scale for the strength of reproductive isolation where 0 represents no reproductive isolation and 1 represents complete reproductive isolation (COYNE AND ORR 2004; SEEHAUSEN *et al.* 2014). At the low-end of this continuum there are taxa such as *Rhagoletis* spp. host races (FEDER 1998) and *Ophrys* spp. orchids (SCOPECE *et al.* 2007), at the middle two examples are *Heliconius* spp. butterflies (JIGGINS 2008) and *Mus musculus* subspp. house mice (BÍMOVÁ *et al.* 2011), and at the high-end there are nearly complete reproductively isolated species such as *Mimulus* spp. monkeyflowers (RAMSEY *et al.* 2003) and the annual plants in the genus *Linanthus* (SCHEMSKE AND GOODWILLIE 1996).

The evolution of reproductive isolation is best studied when different populations have evolved some, but not complete reproductive isolation. On one hand, we cannot tell whether these partially-isolated populations will ever evolve into separate species. On the other hand, we can identify which components contribute the most to the differentiation between these populations. In species with complete, or nearly complete reproductive isolation, it is more difficult to know which are the components that had large effects during differentiation because most of them could have accumulated either at later stages, still increasing reproductive isolation but with small contributions, or after reproductive isolation was complete (NOSIL AND SCHLUTER 2011). For instance, PRESGRAVES (2003) found about 200 genes that were involved in reproductive isolation of *Drosophila melanogaster* and *D. simulans* but probably only some of them had a large impact on promoting species differentiation. The rest had probably small effects and accumulated when reproductive isolation was almost complete.

Hybrid zones are excellent examples for studying the evolution of reproductive isolation as they consist of two divergent populations that meet and exchange genes at the overlap of their distributions (HEWITT 1988; ABBOTT *et al.* 2013). The way that genes are exchanged between these populations is through interpopulation crosses that produce hybrid individuals and successive back-crossings between hybrids and the pure individuals from either parental population. Populations at either side of the hybrid zone may have diverged *in situ* (primary contact) as a result of adaptive changes via divergent selection in the face of gene flow or may have diverged prior to contact during a period of isolation in which evolutionary changes may have been accumulated independently between populations via selection and/or drift (secondary contact) (BARTON AND HEWITT 1985). If divergence increases, the two populations will become reproductively isolated. Speciation can then be understood by studying how this gene flow between divergent populations is reduced (DOBZHANSKY 1937; MAYR 1942).

Barriers reducing gene flow

In both primary and secondary contact, traits that have diverged between populations (e.g., body size and mating duration) may also have diverged due to sexually antagonistic selection. This type of selection is predicted to promote reproductive isolation between different populations through rapid evolutionary divergence of reproductive traits (see below, section **Reproductive traits acting as barriers**). If divergent traits are then involved either indirectly or directly in reproductive isolation, they will act as isolating barriers reducing gene flow between these populations.

There are three classes of reproductive barriers (COYNE AND ORR 2004). Premating isolating barriers prevent gene flow before mating or pollination leading to assortative mating. For example, females may be more attracted to conspecific than heterospecific males or pollination may only occur in the habitat of origin or at a different season depending on which pollinators have been used. Postmating, prezygotic isolating barriers prevent gene flow when mating or pollination has already started but fertilization has not yet occurred. These are traits that are required for normal fertilization such as the evolution of seminal proteins that guarantee proper gamete transfer in one population but result in poor gamete transfer in the other population. Finally, postzygotic isolating barriers act after fertilization such as hybrid inviability and infertility. For example, hybrids may not develop due to genetic incompatibilities between parental genetic backgrounds or may develop normally but with intermediate phenotypes that are maladaptive to either of the parental habitats.

Over time, different types of barriers may accumulate and become associated to increase divergence between two locally-adapted populations. In the absence of gene flow, this association between divergent traits with barrier effects is automatic but in the presence of gene flow, recombination is likely to disrupt trait associations (FELSENSTEIN 1981; SMADJA AND BUTLIN 2011). Importantly, the number of traits contributing to reproductive isolation and the strength of their associations are excellent indicators for the build-up of divergence despite gene flow and thus, the likelihood of speciation (SMADJA AND BUTLIN 2011). Theory suggests that a low number of isolating traits and strong genetic correlations between them due to, for example, pleiotropy or tight physical linkage, can increase the overall barrier to gene flow because there are few opportunities for recombination to split apart advantageous combinations of alleles (FELSENSTEIN 1981; SERVEDIO AND BURGER 2018). A similar conclusion was also drawn after a review of empirical studies that simulated speciation in the face of gene flow. The evolution of reproductive isolation was strongly facilitated when a trait under divergent selection directly induced nonrandom mating via pleiotropy whereas it was rarely observed when hybrid fitness was artificially manipulated in order to reduce mating events between divergent populations via indirect selection or reinforcement (RICE AND HOSTERT 1993).

An excellent example of trait associations with strong contribution to reproductive isolation has been described in *Heliconius* butterflies (MERRILL *et al.* 2012; MERRILL *et al.* 2019). Wing mimetic color is a trait that is used for advertising distastefulness to predators and closely related taxa show different mimicry patterns which are maintained by strong selection due to high predation on non-mimetic colored butterflies (JIGGINS 2008). Hybridization between these different species of butterflies occurs but it is presumed to be rare because hybrids have been infrequently found in the wild, they are less attractive to either parental species and they also have low survival because they display intermediate warning patterns that may not be recognized as distasteful (MERRILL *et al.* 2012). Wing color pattern is therefore under divergent selection but it is also involved in nonrandom mating as it serves as a male mating signal to attract conspecific females (MERRILL *et al.* 2012). Furthermore, the male mating signal and the female preference are genetically correlated via very close physical linkage which strengthens the association between ecological and sexual barriers to gene flow and thus, promotes speciation (MERRILL *et al.* 2019).

Reproductive traits acting as barriers

Single traits such as the wing mimetic color in *Heliconius* butterflies are called “multiple-effect” traits because they contribute to more than one barrier effect (SMADJA AND BUTLIN 2011) but they are also known as “magic traits” because they are simultaneously under divergent selection and mediate assortative mating (SERVEDIO *et al.* 2011). In *Heliconius* butterflies, assortative mating is based on a preference/trait rule: the female preference is separate from the male mating signal but the tight physical linkage between the two traits allows them to coevolve and both to be affected by divergent selection (KOPP *et al.* 2018). However, without such a strong genetic correlation between preference and signal, divergence may be more easily opposed by gene flow and recombination (SERVEDIO 2009; SMADJA AND BUTLIN 2011). Divergence will be less opposed if the preference and the signal are not separate and assortative mating is based on phenotypic similarity of a trait under divergent selection ("matching rule with magic trait") (SERVEDIO AND KOPP 2012; KOPP *et al.* 2018). This is the case of *Gasterosteus* sticklebacks where individuals mate with individuals of similar size and size differences are adaptive for foraging in different habitats (MCKINNON AND RUNDLE 2002).

In both *Heliconius* butterflies and *Gasterosteus* sticklebacks, the barrier to gene flow between populations is generated by divergent selection due to ecological differences between environments (i.e., ecological speciation) (NOSIL 2012). However, reproductive isolation may also evolve without divergence (one-allele mechanism as in habitat choice or flowering time) (FELSENSTEIN 1981; KOPP *et al.* 2018) or with divergence but not as a result of ecologically based divergent selection between environments. In the latter case, sexual selection may promote divergence while being uniform across populations as it may trigger a rapid antagonistic coevolution between female and male reproductive traits in each population.

Sexually antagonistic coevolution is the result of sexual conflict over mating which arises because females and males are not expected to share the same fitness optima: what may be beneficial and selected for in one sex may be costly and selected against in the other (PARKER 1979; PARKER 2006). Copulation duration and number of matings are well-known examples of sexual conflict because long and numerous matings are generally observed to increase male fitness but to decrease female fitness (reviewed in CHAPMAN *et al.* 2003).

The origin of sexual conflict is found in anisogamy. In general, males produce many small and inexpensive gametes with very little investment in offspring provisioning while females invest much more resource per gamete and in this way contribute more to offspring early in development (TRIVERS 1972; JANICKE *et al.* 2016). At the union of the different gametes, females and males gain the same benefit but for females the costs are much higher. Limited by the number of gametes that the females produce, males will compete for such resources and there will be selection for traits or behaviors that increase male reproductive success (BATEMAN 1948). If these selected traits or behaviors reduce female fertility or survival, sexual conflict will then arise. An excellent example of sexual conflict can be found in *Drosophila melanogaster* where males produce accessory gland proteins that, in elevated quantities, make the females die younger, lay eggs at a higher rate and become more reluctant to accept further matings (CHAPMAN *et al.* 1995; WOLFNER 1997). Females are expected to respond to this fitness reduction by evolving counteradaptations that allow them to move closer to their fitness optimum which will often be disadvantageous for males (HOLLAND AND RICE 1998; LESSELLS 2006). Male traits or behaviors that may coerce females to mate and female responses to male coercion have been extensively studied in a wide range of taxa (SMUTS AND SMUTS 1993; CLUTTON-BROCK AND PARKER 1995; LESSELLS 2006). This antagonistic coevolution between female and male traits has been compared to the arms race between harmful traits of pathogens and defensive traits of hosts (VAN VALEN 1973) and it has been predicted to have a significant impact on population divergence and speciation. However, whether sexual conflict is "an engine of speciation" is still unclear because both theoretical and empirical results have been contradictory (reviewed in GAVRILETS AND HAYASHI (2005); PARKER (2006); GAVRILETS (2014); LINDSAY *et al.* (2019)). For isolated populations, sexually antagonistic coevolution has been predicted to increase genetic and phenotypic divergence thereby promoting reproductive isolation (HOWARD *et al.* 1998; PARKER AND PARTRIDGE 1998; RICE 1998; GAVRILETS 2000). Under such a scenario, sexual conflict is expected to generate both pre-zygotic (e.g., assortative mating or sperm precedence) and post-zygotic (e.g., inviable or infertile hybrids) barriers to gene flow (PARKER AND PARTRIDGE 1998; COYNE AND ORR 2004). Sexual conflict has also been modelled in populations with ongoing gene flow and without prior isolation. Using a model for sexual conflict over mating rate, GAVRILETS AND WAXMAN (2002) predicted that reproductive isolation was established when female diversification was accompanied by male diversification and it was not established when coevolution between females and males ended because females reached their own fitness optimum while males were left in a condition of reduced mating success. Alternative outcomes to the one where sexually antagonistic coevolution led to reproductively isolated clusters have also been found in other models (reviewed in PERRY AND ROWE 2015).

On one hand, the theoretical prediction that speciation can be driven by sexual conflict has been supported by comparative analyses in insects (ARNQVIST *et al.* 2000a; KATZOURAKIS *et al.* 2001), passerine birds (BARRACLOUGH *et al.* 1995), amphibians (DE LISLE AND ROWE 2015) and by a few studies of experimental evolution where laboratory populations of insects were reared for many generations under elevated sexual conflict (MARTIN AND HOSKEN 2003; HOSKEN *et al.* 2009). There is also evidence that rapid evolution of female and male reproductive traits such as genital morphology in insects, may represent a strong barrier to gene flow between isolated populations (ARNQVIST AND NILSSON 2000b; ARNQVIST AND ROWE 2005; WOJCIESZEK AND SIMMONS 2013). On the other hand, the prediction that speciation can be driven by sexual conflict was not supported by other comparative studies in mammals, butterflies and spiders (GAGE *et al.* 2002), birds (MORROW *et al.* 2003), fish (RITCHIE *et al.* 2005) and by several experimental evolution studies mostly with insects (BACIGALUPE *et al.* 2007; GAY *et al.* 2009; GAGNON AND TURGEON 2011; PLESNAR-BIELAK *et al.* 2013; CARVALHO *et al.* 2020).

Evolutionary patterns of divergence

With the emergence of high-throughput and next-generation sequencing technologies, single nucleotide polymorphisms (SNPs) have been extensively used as markers for both neutral (e.g., gene flow) and selective processes (e.g., divergent selection) which, as we have seen above, may represent important evolutionary forces for the evolution of reproductive isolation (BRUMFIELD *et al.* 2003; MORIN *et al.* 2004).

Neutral processes (e.g., gene flow and drift, influenced by changes in population size) can be studied by measuring allele frequencies at neutral variants which are expected to segregate randomly in a population, and not influenced by the hitchhiking effect due to linkage disequilibrium with the target loci of selection. For example, under the assumption that a drastic reduction in population size (bottleneck) will cause the removal of rare alleles, we should observe that low-frequency neutral variants are significantly less common than expected under a model of constant population size. Processes such as purifying selection and divergent selection are instead expected to have locus-specific effects. For example, during the evolution of adaptive traits to different environments, purifying selection will prevent some variants from increasing in frequency due to their deleterious fitness effects while divergent selection will cause large allele-frequency differences (e.g., high F_{ST}) between populations. Neutral loci linked to the loci that are targets of selection will also segregate at low frequencies or show elevated F_{ST} and may reveal regions of the genome under selection. Hence, genetic variants can reflect the impacts of direct or indirect selection, which may differ according to the type of variants used. However, this genomic approach for identifying the effects of gene flow, divergent selection and reproductive isolation is not always straightforward (RAVINET *et al.* 2017).

Earlier, microsatellites were popular PCR-based markers for population genetic studies (JARNE AND LAGODA 1996; GOLDSTEIN AND SCHLÖTTERER 1999) but these were outcompeted by SNPs because of their lower mutation rate, higher abundance, broader genome coverage, easier bioinformatic automation and higher opportunity for comparative studies across taxa (SCHLÖTTERER 2004). In particular, the use of SNPs for comparative methods represents an excellent approach to search for global patterns in the evolution of isolating barriers.

However, SNPs are not the only category of common, small variants: Short insertions and deletions (INDELs $\leq$ 50 bp) could represent additional or alternative candidate genetic markers for evolutionary processes, as they are the most common type of genetic variants among the non-SNP variants (MONTGOMERY *et al.* 2013). In the nonmodel teleost fish Australasian snapper, non-SNP variants have been shown to exceed SNPs in regard to the number of bases affected at the genome-wide scale and among them, short INDELs covered a number of bases comparable to SNPs (CATANACH *et al.* 2019). Moreover, while calling longer variants such as duplications and inversions may require a different sequencing approach, calling short INDELs can be done using the same input sequences used for calling SNPs.

Recent advances in genome-wide and long-read sequencing have allowed to generate, also for nonmodel organisms, large amounts of data that can be used for understanding the general role of longer, structural variants such as chromosomal inversions and duplications in the evolution of reproductive isolation (LEE *et al.* 2016; WELLENREUTHER *et al.* 2019). The evidence across a wide range of taxa seems to support the theory that chromosomal rearrangements contain genes involved in local adaptation as well as genes that contribute to reproductive isolation (reviewed by WELLENREUTHER AND BERNATCHEZ 2018) . However, it remains unclear what mechanisms are involved that have allowed rearrangements to have such a role in speciation (reviewed by BUTLIN *et al.* 2011; JACKSON 2011; FARIA *et al.* 2019a) .

Thus, using different types of genetic variants that are directly or indirectly affected by evolutionary processes in a context where populations are partially isolated is expected to improve our understanding of the components and the genomic patterns of reproductive isolation.

Study systems

The North Sea–Baltic Sea transition of marine species

From the marine North Sea (North-East Atlantic) to the brackish Baltic Sea, salinity decreases gradually and in the Danish Straits the change is the steepest (Figure 1). The Baltic Sea formed about 8,500 years ago, following the last glaciation (~10,000–15,000 years ago), and was colonized about 8,000 years ago by a subset of marine species living in the North Sea (OJAVEER *et al.* 2010). Because marine species are not expected to be adapted to low salinity, the colonization of the Baltic Sea was likely driven by plastic responses with respect to salinity which were later followed by local adaptation (WEST-EBERHARD 2005). For many marine Baltic Sea species, there is empirical evidence for strong phenotypic plasticity (KAUTSKY *et al.* 1990; RENBORG *et al.* 2013; WOOD *et al.* 2014; JOHANSSON *et al.* 2017) and steep allele frequency change across the salinity gradient forming parallel hybrid zones in species of fish, invertebrates and macroalgae (SICK 1961; CHRISTIANSEN AND FRYDENBERG 1974; JOHANNESSON AND ANDRE 2006).

The formation of such contact zones is crucial for our understanding of how genetically-differentiated populations may coexist despite the homogenizing effect due to gene flow. By combining information from multiple single-taxon contact zones, it is possible to identify what types of barriers reduce gene flow between these populations and whether this reduction is due to single traits with large barrier effects or multiple traits with small barrier effects. In addition, it is possible to investigate the relationship between reproductive isolation and life-history traits or past demographic events that vary across different species. For example, are species with long-range dispersal less genetically differentiated than species with less mobile or sessile life cycles? What is the contribution to reproductive isolation of historical changes in population size and/or periods of spatial isolation prior to secondary contact? Finally, where information about the structure of the genome is available, it is also possible to ask which genomic features are more likely to reduce gene flow between divergent populations of multiple species that are in contact over the same environmental gradient.

The Wave–Crab microenvironment transition in a marine snail

An example of a single-species and narrow hybrid zone (from a few meters to tens of meters) can be found on rocky-shores between wave-exposed and crab-rich habitats. Here, *Littorina saxatilis*, an intertidal marine snail, forms phenotypically distinct ecotypes. Indeed, Wave and Crab ecotypes are encountered widely in wave-exposed and crab-rich habitats, respectively, over the species' North Atlantic distribution (PANOVA *et al.* 2006; JOHANNESSON *et al.* 2010a). Wave individuals live on cliffs, they have evolved a relatively large foot, thin shell, small size at maturation and a bold behavior, whereas Crab snails live among boulders, have developed a larger, thicker shell with a narrower foot and show a wary behavior. Trait differences between ecotypes are the result of divergent natural selection induced by wave exposure in the Wave habitat and crab predation in the Crab habitat (JOHANNESSON 1986; BOULDING *et al.* 2017; LE PENNEC *et al.* 2017).

Many genomic regions potentially involved in the divergence process in *L. saxatilis* have been identified using SNPs; these include several putative inversions (WESTRAM *et al.* 2018; MORALES *et al.* 2019; FARIA *et al.* 2019b). Nevertheless, it is not excluded that other regions of the genome may have participated in the ecotype differentiation and that classes of variants other than SNPs and inversions may contribute to divergence. For instance, in a few cases, sequence divergence and genetic isolation have been studied using INDELs (VETSIGIAN AND GOLDENFELD 2005; YANG *et al.* 2007), a type of variants that has been overlooked in *L. saxatilis*.

Divergent natural selection is a powerful barrier against gene flow between Wave and Crab ecotype snails and such a barrier is likely to have arisen in the face of continuous gene flow (BUTLIN *et al.* 2014). However, there is also evidence for other isolating components such as habitat choice, low dispersal (due to direct development) and size-assortative mating (JANSON 1983; ROLÁN-ALVAREZ *et al.* 1997; JOHANNESSON *et al.* 2016). Particularly, assortative mating has been investigated in empirical studies both in the field and the laboratory, and in modelling studies, and these results have shown that mating behavior in *L. saxatilis* is dominated by size-assortative mating, in the presence of sexual size dimorphism (JOHANNESSON *et al.* 1995; ERLANDSSON AND ROLÁN-ALVAREZ 1998; HULL 1998; ROLÁN-ALVAREZ *et al.* 1999; CRUZ *et al.* 2004; HOLLANDER *et al.* 2005; CONDE-PADÍN *et al.* 2008; SADEDIN *et al.* 2009; FERNÁNDEZ-MEIRAMA *et al.* 2017). These results were obtained using sexual isolation indices (e.g., Yule's V (GILBERT AND STARMER

1985) and I_{PSI} (ROLÁN-ALVAREZ AND CABALLERO 2000)) which divide a continuous variable, such as shell size, into discrete categories, such as large/small Crab snails and small/large Wave snails. In hybrid zones like the one in *L. saxatilis* where individuals with intermediate phenotypes are viable and fertile, excluding these hybrids from mating experiments risks biasing the inferred contribution of assortative mating to the overall barrier to gene flow between divergent populations (COYNE AND ORR 2004; IRWIN 2020). Hence, an approach that can quantify mating probability and the resulting strength of assortative mating for a continuous trait distribution is recommended in hybrid zone studies.

In *L. saxatilis*, copulation durations are highly variable (from a few minutes to hours) but without information on the timing of sperm transfer, this variation cannot be interpreted. For instance, JOHANNESSON *et al.* (2010b) suggested that in *L. saxatilis* mating frequency has been likely influenced by population density and costs of mating (e.g., predation risk) and these factors may also impact copulation duration and sperm transfer. Additionally, JOHANNESSON *et al.* (2016) found that both sperm competition and/or cryptic female choice may be involved during fertilization in *L. saxatilis* and it is expected that these two components may also influence copulation duration and sperm transfer. Finally, this variation in copulation duration and potentially also in sperm transfer, might impact on the contributions of assortative mating and sexual selection to the barrier to gene flow between the Crab and Wave ecotypes. In earlier studies on mating behavior, short copulations have been excluded from analysis because they were interpreted as failed mating attempts during which sperm transfer was unlikely (HOLLANDER *et al.* 2005; PERINI *et al.* 2020). This interpretation was based on the observation that sperm in *L. saxatilis* is slowly channeled into the female through a groove in the penis by ciliary movements and also the fact that males mount heterospecific individuals, conspecific males and juveniles for similar short durations (SAUR 1990; HOLLANDER *et al.* 2005). However, there is currently no evidence in *L. saxatilis* on when the males start transferring the sperm during copulation.

SUMMARY OF RESULTS

Paper I

Divergent populations with partially overlapping distributions form contact zones which offer great opportunities for studying barriers that reduce gene flow, evolutionary processes that produce these barriers and genomic features that are involved in reproductive isolation. Using single-species contact zones, it is possible to provide important details about the mechanisms implicated during divergence while using multispecies contact zones, where more species show divergent populations that are in contact over the same environmental gradient (e.g., salinity), it is possible to address the generality of patterns of reproductive isolation. It is also possible to test for a relationship between divergence and traits that are invariable within a species such as dispersal, generation time and mating system. A multispecies contact zone is found at the entrance of the Baltic Sea where the change in salinity is the steepest. Data on genetic variation across this North Sea–Baltic Sea transition was available for 23 marine species (15 fish, five invertebrates, two macroalgae, and one microalga) with different life histories and it consisted mostly of genome-wide SNP data (Table 1). This dataset was used for addressing questions about general patterns in the evolution of isolating barriers.

To test for the position of the contact between divergent populations along the salinity gradient, clines were fitted to the genetic data. For most of the species with enough data (ten out of 14 species), the center of the cline coincided with the steepest part of the salinity gradient, two were slightly shifted towards lower salinities and two were found at the high-salinity margin of the North Sea–Baltic Sea transition (Figure 1).

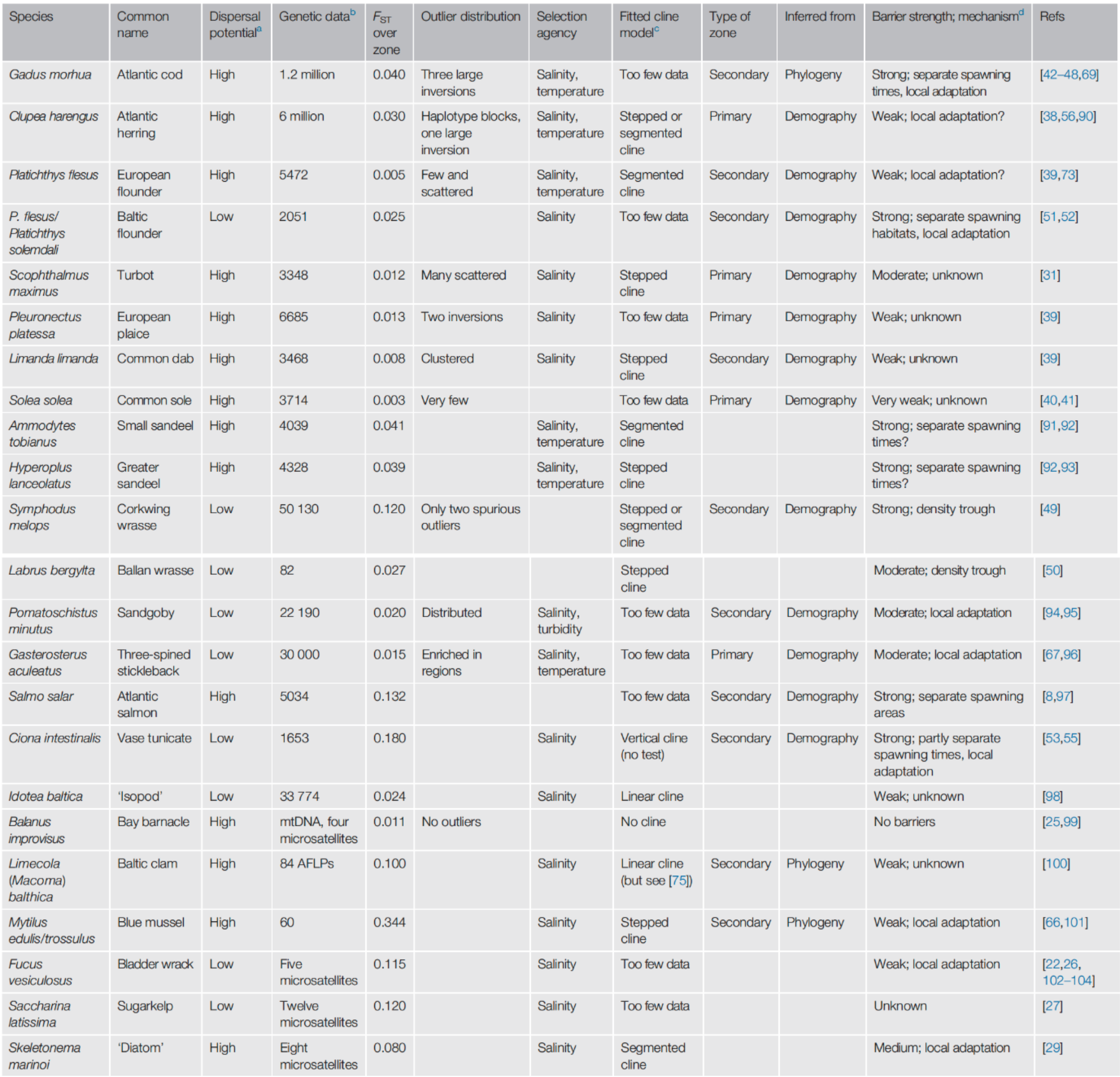
The origins of contact zones (primary versus secondary) can be reconstructed using demographic models to infer what the most likely scenario is given the observed patterns of divergence. In a comparative framework, this means that species with different demographic histories can be used to understand how, for example, primary and secondary contact may have influenced the evolution of reproductive isolation between divergent populations. However, results from demographic inference should be treated with caution as these models can be sensitive to the set of parameters used (MOMIGLIANO *et al.* 2020).

Over the North Sea–Baltic Sea transition, contacts were primary or secondary and they seemed to be independent of dispersal capacity (Table 1). For example, in several studies based on demographic models of five flatfish species (Pleuronectiformes), divergence was inferred to be both primary and secondary even though their dispersal capacity and migratory patterns are similar (DIOPERE *et al.* 2018; LE MOAN *et al.* 2019a; LE MOAN *et al.* 2019b; MOMIGLIANO *et al.* 2020).

Most genetic clines were centered at the steepest shift of the salinity gradient suggesting that once the contact zone is formed, divergent natural selection acts as a strong ecological (prezygotic) barrier to gene flow between different populations. There was evidence for divergence being driven by local adaptation and spatial or temporal segregation (Table 1). Additionally, in field and lab studies where individuals' performance was measured in the non-native environment, migrants were shown to perform worse than native individuals (RIGINOS AND CUNNINGHAM 2005; DEFAVERI AND MERILÄ 2014; MØLLER NIELSEN *et al.* 2016; JOHANSSON *et al.* 2017; RUGIU *et al.* 2018; BARTH *et al.* 2019). However, studies showing the impact of divergent selection on either development or fitness of hybrids (postzygotic barrier) were mostly lacking. In the

Atlantic cod (*Gadus morhua*), where first-generation hybrids are rarely found (SICK 1961; WEIST *et al.* 2019), postzygotic barrier effects are likely to reduce gene flow between divergent populations living in the North Sea–Baltic Sea transition.

Table 1. Summary for the reviewed species from the North Sea–Baltic Sea transition. This table and the references (Refs) are from Table 1 in JOHANNESSON *et al.* (2020a).



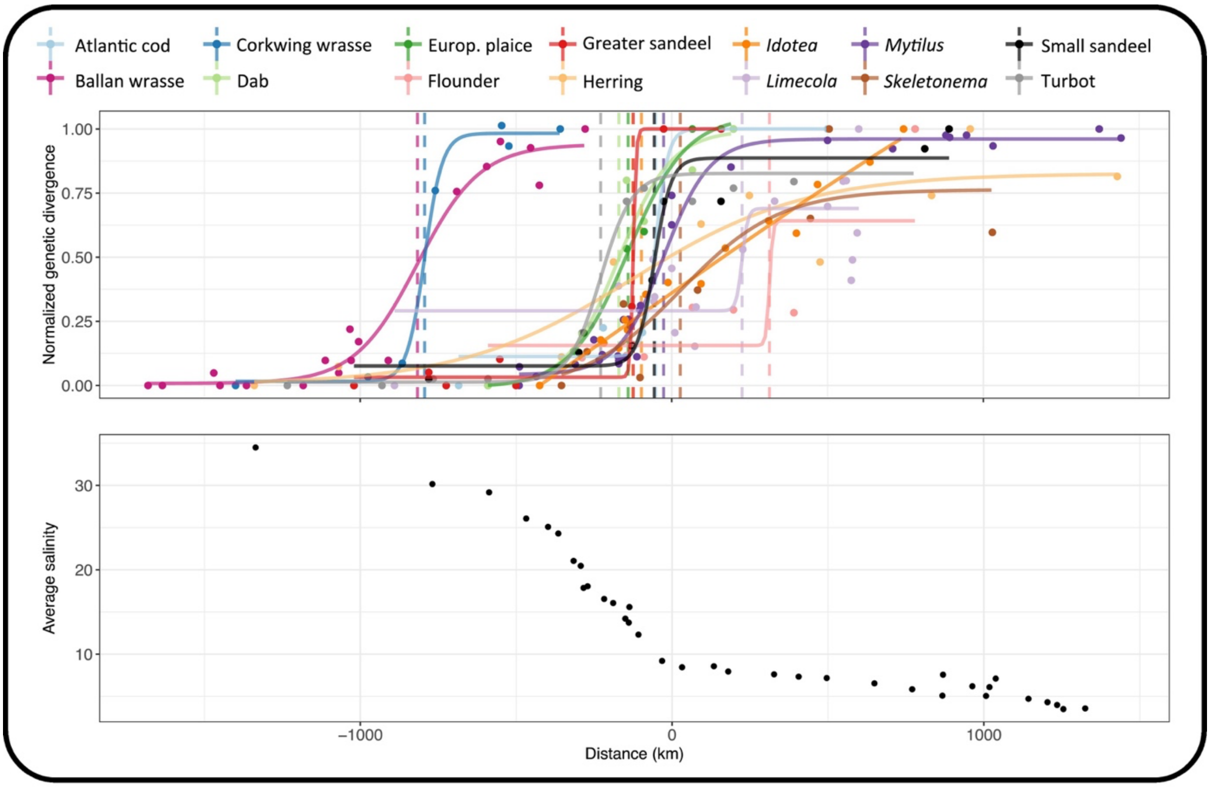
Species	Common name	Dispersal potential[a]	Genetic data[b]	F_{ST} over zone	Outlier distribution	Selection agency	Fitted cline model[c]	Type of zone	Inferred from	Barrier strength; mechanism[d]	Refs
Gadus morhua	Atlantic cod	High	1.2 million	0.040	Three large inversions	Salinity, temperature	Too few data	Secondary	Phylogeny	Strong; separate spawning times, local adaptation	[42–48,69]
Clupea harengus	Atlantic herring	High	6 million	0.030	Haplotype blocks, one large inversion	Salinity, temperature	Stepped or segmented cline	Primary	Demography	Weak; local adaptation?	[38,56,90]
Platichthys flesus	European flounder	High	5472	0.005	Few and scattered	Salinity, temperature	Segmented cline	Secondary	Demography	Weak; local adaptation?	[39,73]
P. flesus/ Platichthys solemdali	Baltic flounder	Low	2051	0.025		Salinity	Too few data	Secondary	Demography	Strong; separate spawning habitats, local adaptation	[51,52]
Scophthalmus maximus	Turbot	High	3348	0.012	Many scattered	Salinity	Stepped cline	Primary	Demography	Moderate; unknown	[31]
Pleuronectus platessa	European plaice	High	6685	0.013	Two inversions	Salinity	Too few data	Primary	Demography	Weak; unknown	[39]
Limanda limanda	Common dab	High	3468	0.008	Clustered	Salinity	Stepped cline	Secondary	Demography	Weak; unknown	[39]
Solea solea	Common sole	High	3714	0.003	Very few		Too few data	Primary	Demography	Very weak; unknown	[40,41]
Ammodytes tobianus	Small sandeel	High	4039	0.041		Salinity, temperature	Segmented cline			Strong; separate spawning times?	[91,92]
Hyperoplus lanceolatus	Greater sandeel	High	4328	0.039		Salinity, temperature	Stepped cline			Strong; separate spawning times?	[92,93]
Symphodus melops	Corkwing wrasse	Low	50 130	0.120	Only two spurious outliers		Stepped or segmented cline	Secondary	Demography	Strong; density trough	[49]
Labrus bergylta	Ballan wrasse	Low	82	0.027			Stepped cline			Moderate; density trough	[50]
Pomatoschistus minutus	Sandgoby	Low	22 190	0.020	Distributed	Salinity, turbidity	Too few data	Secondary	Demography	Moderate; local adaptation	[94,95]
Gasterosterus aculeatus	Three-spined stickleback	Low	30 000	0.015	Enriched in regions	Salinity, temperature	Too few data	Primary	Demography	Moderate; local adaptation	[67,96]
Salmo salar	Atlantic salmon	High	5034	0.132			Too few data	Secondary	Demography	Strong; separate spawning areas	[8,97]
Ciona intestinalis	Vase tunicate	Low	1653	0.180		Salinity	Vertical cline (no test)	Secondary	Demography	Strong; partly separate spawning times, local adaptation	[53,55]
Idotea baltica	'Isopod'	Low	33 774	0.024		Salinity	Linear cline			Weak; unknown	[98]
Balanus improvisus	Bay barnacle	High	mtDNA, four microsatellites	0.011	No outliers		No cline			No barriers	[25,99]
Limecola (Macoma) balthica	Baltic clam	High	84 AFLPs	0.100		Salinity	Linear cline (but see [75])	Secondary	Phylogeny	Weak; unknown	[100]
Mytilus edulis/trossulus	Blue mussel	High	60	0.344		Salinity	Stepped cline	Secondary	Phylogeny	Weak; local adaptation	[66,101]
Fucus vesiculosus	Bladder wrack	Low	Five microsatellites	0.115		Salinity	Too few data			Weak; local adaptation	[22,26, 102–104]
Saccharina latissima	Sugarkelp	Low	Twelve microsatellites	0.120		Salinity	Too few data			Unknown	[27]
Skeletonema marinoi	'Diatom'	High	Eight microsatellites	0.080		Salinity	Segmented cline			Medium; local adaptation	[29]

[a] 'High' stands for high potential for gene flow due to a life cycle where the pelagic larva stage lasts for several weeks and/or adults are highly mobile. 'Low' means that gene flow is expected to be low given that the pelagic larval (or zygote/spore) stage is short and adults are sessile or less mobile.

[b] Number of SNPs unless it is specified.

[c] The change in genetic divergence over the salinity gradient was analyzed for each taxon separately using three different models: a linear model compatible with isolation by distance, a stepped cline model compatible with a scenario with one contact (HARRISON 1993) and a regression model with segmented relationships (R package 'segmented' (MUGGEO 2008)) compatible with a scenario with more than one contact. The different models were compared using the Akaike Information Criterion.

[d] Suggested mechanisms that are not experimentally confirmed are followed by '?'. Note that other mechanisms, not yet investigated, might add to barriers.



Trends in Ecology & Evolution

Figure 1. Clines and sampling location of 14 species (this figure corresponds to Figure 2 in JOHANNESSON *et al.* (2020a)). Top panel: normalized pairwise allele frequency difference (F_{ST}) across distance from the entrance of the Baltic Sea. Clines (solid lines) and cline centers (dashed lines) were omitted for species without any contact (*Balanus improvisus*), species with vertical contact (*Ciona intestinalis*), species with contact inside the Baltic Sea (*Platichthys flesus/solemdali*), five species with poorly resolved geographic data (*Fucus vesiculosus, Saccharina latissima, Solea solea, Pomatoschistus minutus, Gasterosterus aculeatus*), and species without F_{ST} data (*Salmo salar*). Bottom panel: Sea-surface salinity (yearly average) against the same geographic distance.

Of the reviewed marine taxa, two wrasse species showed a sampling distribution that did not overlap with the region of the steepest salinity shift. For both species, the change in genetic composition was abrupt and the center of the cline coincided with a sandy habitat (~60 km long) which is an unsuitable environment for these wrasse species. Besides this barrier effect due to low population density, for one species (*Symphodus melops*), MATTINGSDAL *et al.* (2020) suggested that the barrier effect due to divergent selection was small compared to that possibly generated by founder events during colonization of the North Sea after the last glacial maximum (~21,000 years ago). Their conclusion was supported by the evidence of genome-wide patterns of divergence but with only a small number of loci under divergent selection. For the other wrasse species (*Labrus bergylta*), a demographic analysis is missing (SELJESTAD *et al.* 2020).

Genome-wide data and genome assemblies have become available for an increased number of non-model organisms and for the reviewed species in the North Sea–Baltic Sea transition, fish had the largest suite of genomic tools available. Patterns of divergence varied from extensive differentiation across the entire genome (e.g., Baltic populations of cod, turbot, herring, and demersal flounder) to a few genomic regions or loci showing high differentiation between populations (e.g., dab, plaice, pelagic flounder) (BERG *et al.* 2015; MOMIGLIANO *et al.* 2017; LE MOAN *et al.* 2019a; LE MOAN *et al.* 2019b; PETTERSSON *et al.* 2019). Such a diversity of genomic landscapes confirms that reproductive isolation forms a continuum and using multispecies contact zones like the one in the Baltic Sea, is a powerful comparative approach for understanding how the evolution of barriers to gene flow depends on characteristics such as life-history traits and genomic architecture that are variable across different species.

Variation in a key life-history trait, dispersal potential, is unlikely to explain the observed variation in genetic divergence. The presence of chromosomal rearrangements such as inversions is instead more likely to promote divergence through the accumulation of genes with barrier effects that cannot be disrupted by gene flow and recombination (BUTLIN 2005; KIRKPATRICK AND BARTON 2006; BUTLIN AND SMADJA 2018; FARIA *et al.* 2019a). However, inversions were only studied in a handful of species and so for this conclusion to be general, data from more species are required.

Inversions are also expected to contribute to reproductive isolation by promoting local adaptation via standing genetic variation. The support for this role comes from two reviews in which it was shown that inversions are often polymorphic and old (WELLENREUTHER AND BERNATCHEZ 2018; FARIA *et al.* 2019a). In the North Sea–Baltic Sea transition, three fish species were shown to be characterized by inversions that are older that the Baltic Sea and thus, likely to have facilitated rapid adaptive divergence via selection on preexisting genetic variation (BARTH *et al.* 2019; PETTERSSON *et al.* 2019; LE MOAN *et al.* 2020).

The importance of standing genetic variation as a source of adaptive loci for local adaptation and divergence was also found in the vase tunicate *Ciona intestinalis* which, differently from the other reviewed species, shows a vertical distribution over the transition. The brackish population lives in the surface water and it is genetically divergent from the high-salinity population which lives below 20 m (DYBERN 1967; JOHANNESSON *et al.* 2018). Using demographic models, HUDSON *et al.* (2020) suggested that the colonization of the brackish habitat may have been facilitated by ancestral divergence followed by gene flow between ancestral populations. Like for the vase tunicate, the two divergent populations of the Baltic flounder were also inferred to have colonized the new environment separately due to selection on preexisting genetic variation (MOMIGLIANO *et al.* 2017).

This review of species living along the salinity gradient of the North Sea–Baltic Sea transition summarized what types of barriers to gene flow are present in this multispecies contact zone. Most of the species that we reviewed showed population differentiation by divergent selection and/or temporal or spatial segregation. Ancestral variation seems to promote divergence but dispersal potential could not explain variation in the genetic difference across the salinity gradient.

Paper II

From the comparisons among species made in Paper I, the barrier effect due to divergent selection was clear. However, divergent selection does not operate alone on the available genetic variance and it may also affect types of genetic variants other than SNPs and chromosomal rearrangements. Polymorphic short insertions and deletions (INDELs ≤ 50 bp) are abundant, although less common than single nucleotide polymorphisms (SNPs) (MONTGOMERY *et al.* 2013). Evidence from model organisms shows INDELs to be more strongly influenced by purifying selection than SNPs, especially in coding regions (CORCORAN *et al.* 2017; BARTON AND ZENG 2019). Partly for this reason, INDELS are rarely used as markers for neutral demographic processes or to detect balancing or divergent selection. In Paper II, I compared INDELs and SNPs in the Wave and Crab ecotypes of the intertidal snail *Littorina saxatilis*. Snails were sampled from the rocky shore of three different islands on the Swedish west coast and each shore contained two rocky headlands separated by one boulder field, for a total of three populations (one Crab and two Wave) and two Crab-Wave hybrid zones per island. Using capture sequencing data from the six hybrid zones, I classified short INDELs and SNPs into coding and non-coding and found that the number of non-coding SNPs was five times higher than the number of non-coding INDELs. Non-coding SNPs were nearly 30 times more numerous than coding SNPs while non-coding INDELs were approximately 50 times more numerous than coding INDELs. The observed reduction in coding regions was significantly stronger for INDELs than for SNPs and it was consistent across populations. The different proportion of short INDELs and SNPs in non-coding and coding categories was the first evidence that purifying selection was stronger against short INDELs than against SNPs, as expected.

The second piece of evidence for the different impact of purifying selection on short INDELs and SNPs was obtained from comparing site frequency spectra (SFS) and Tajima's (1989) D and Fay and Wu's (2000) H summary statistics between variant types. Both SFS and summary statistics patterns suggested that short INDELs were more affected by purifying selection than SNPs (Figure 2). This effect was clearer in non-coding than coding variants, most likely due to the difference in sample size. As in the case of regulatory regions in *Drosophila* (KOHN *et al.* 2004) and wild house mice

(KOUSATHANAS *et al.* 2011), I found evidence that non-coding DNA may evolve under selective constrains also in *L. saxatilis*.

Patterns of summary statistics also reflected whether the impacts of positive and balancing selection differed between these variant types. The difference in the relative values of Tajima's D and Fay and Wu's H suggested that positive selection may act more strongly on SNPs than on short INDELs (Figure 2).

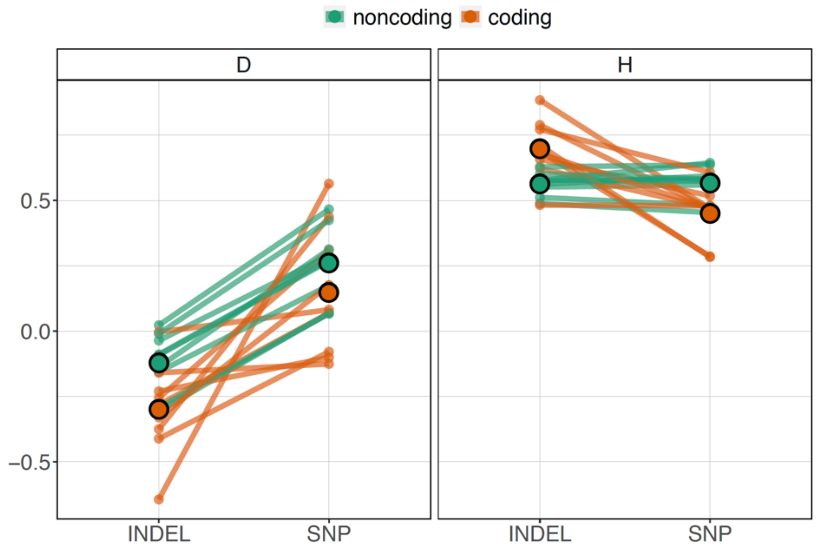


Figure 2. Patterns of Tajima's D (left panel) and Fay and Wu's H (right panel) between INDELs and SNPs of the non-coding (green) and coding (orange) category. All nine populations are shown (small dots connected by a line) in addition with the mean across populations (big dots).

Tajima's D, and Fay and Wu's H values are not only driven by demography and selective processes but they can also be influenced by molecular mechanisms such as allelic gene conversion (DURET AND GALTIER 2009) and/or polarization errors due to misidentification between insertions and deletions (HERNANDEZ *et al.* 2007). These effects were weak and were hard to distinguish from stochastic variation given the relatively small sample of variants.

I then assessed whether divergent selection may also affect differently these two types of variants. In Paper II, I have done this in two steps. First, I examined the distribution of short INDELs and SNPs across the genome as it can tell us whether these variants are similarly influenced by indirect effects of selection. Second, I analyzed patterns of divergence between Crab and Wave ecotypes by fitting clines to INDEL and SNP allele frequencies and compared cline estimates between the two types of variants. Positions of short INDELs and SNPs across the genome were similar and cline estimates were mostly shared between the variant types (Figure 3), suggesting that short INDELs and SNPs are likely to be influenced by loci under direct selection in an equivalent way. Clustering of short INDELs and SNPs is common in model species (TIAN *et al.* 2008) and one potential mechanism for its formation has been suggested to be hitchhiking and background selection (Huang *et al.* 2014). Still, I found putatively selected regions of the genome that were uniquely identified by outlier INDELs but these were a minority, as expected, given that there were overall fewer short INDELs than SNPs.

In conclusion, short INDELs can potentially be used as genetic markers alongside SNPs to study evolutionary processes because even though they cluster with SNPs across the genome, they can still provide information on genomic regions with important biological functions that are under purifying selection and also identify new regions that may be important for local adaptation or other barriers to gene flow as they are likely affected by divergent selection. However, depending on the focus of the study and the genomic resources available for the biological system(s), SNPs may still outperform short INDELs.

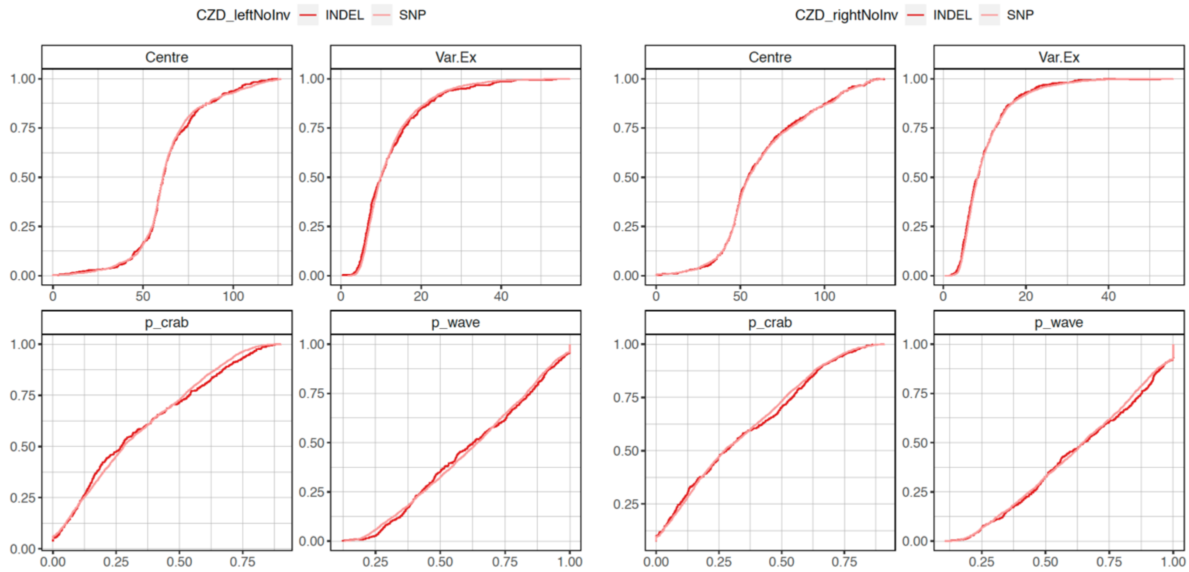


Figure 3. Empirical distribution functions of the cline estimates for short INDELs (red) and SNPs (pink) (CZD left and right hybrid zone as an example: for the other four hybrid zones, see Figure S6 in SI of Paper II). The cline estimates are the position of the cline center (Centre), the variance in the data explained by the cline model (Var.Ex) and the allele frequency at the cline end in the Crab habitat (p_crab) and in the Wave habitat (p_wave).

Paper III

Divergent populations with partially overlapping distributions that still exchange genes are excellent systems for studying how selection creates and maintains barriers to gene flow. Theory predicts that single traits with multiple barrier effects may promote divergence between populations and thus, reproductive isolation, because gene flow cannot disrupt their joint effects through recombination (Servedio *et al.* 2011; Smadja and Butlin 2011; Kopp *et al.* 2018). For example, a trait such as body size in *Gasterosteus* sticklebacks makes a strong contribution to reproductive isolation because it is under divergent selection and it is also involved in assortative mating (McKinnon and Rundle 2002). This association increases the overall barrier to gene flow because it cannot be opposed by gene flow and recombination (Smadja and Butlin 2011). There are other examples of multiple-effect or magic traits but their impact on gene flow has been determined in only few cases (SERVEDIO *et al.* 2011; SMADJA AND BUTLIN 2011). Additionally, assortative mating is often calculated from experiments where matings are only possible between individuals of the same or divergent populations without accounting for the presence of intermediate phenotypes (GILBERT AND STARMER 1985; ROLÁN-ALVAREZ AND CABALLERO 2000). In hybrid zone studies where divergent populations show incomplete reproductive isolation and form hybrids with intermediate phenotypes, failing to include hybrids may lead to overestimation of the strength of assortative mating as a barrier to gene flow (IRWIN 2020).

In Paper III, I investigated patterns of assortment and their barrier effects in *L. saxatilis* hybrid zones with respect to shell size. Shell size is a multiple-effect trait that is under divergent selection between Crab and Wave ecotypes and contributes to assortative mating. I built a model from a large dataset of mating experiments (~4,000 trials) to describe the probability of mating given an encounter between a male and a female with specified phenotypes (e.g., shell size and shape). I used this model to infer strengths of assortative mating and sexual selection in the field, at any point in the hybrid zone, and used simulations created by a colleague to determine the likely barrier effects, given these estimates.

"Transect" snails were sampled intensively along four transects on four small islands on the Swedish west coast and their positions on the transect was recorded. "Reference" snails were sampled at a different island in Crab and Wave habitats away from the contact zone and were used as partners in mating trials. In each mating trail, one transect snail was paired with one reference snail of the opposite sex. Male mounting position was used for determining whether a mating occurred or not (HOLLANDER *et al.* 2005).

For the analysis of the mating trials, I fitted the binary mating response using logistic regression to a skew normal function of the female to male size ratio. The mating function was fitted to the data from all four islands combined and it showed that the probability of mating followed a right-skewed distribution with optimal size ratio equal to pairs in which the male was ~25% smaller than the female (Figure 4). The probability rapidly approached zero as the male became larger than the female but it declined more slowly as the male became smaller than the female (Figure 4). Such an asymmetry indicated a mating advantage for smaller compared to larger males even when the mean size ratio is equal to the mating optimum. The mating pattern did not show significant differences between ecotypes and islands and for this reason, assortative mating, sexual selection and their barrier effects were estimated based on a mating pattern that was invariant in time and space.

After estimating the parameters of the mating function using data from the mating experiment, I predicted the consequences of such a mating pattern (i.e., assortative mating and sexual selection) in natural conditions based on the distributions of male and female size across the sampled transects. Mating encounters were not real but were generated using a custom script for predetermined positions along each transect.

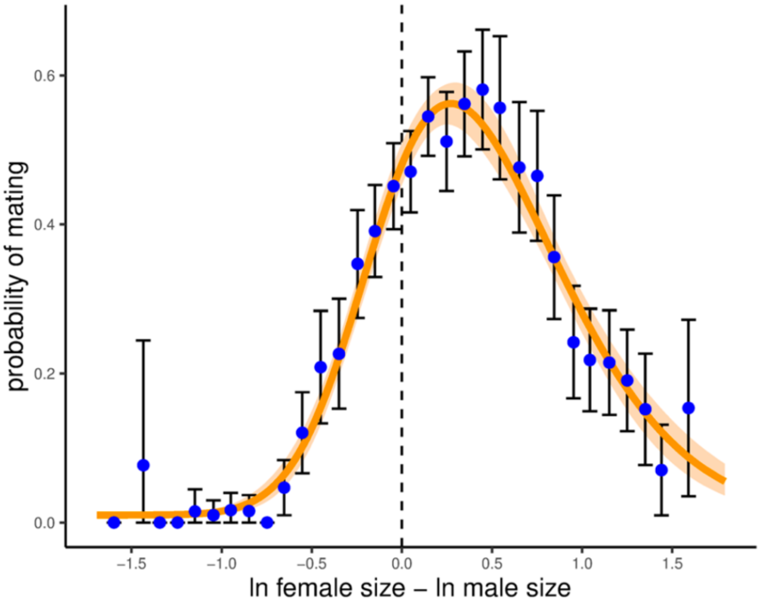


Figure 4. The asymmetric mating pattern across all islands. Fitted curve and 95% CIs in orange are superimposed on the observed proportions of matings (blue dots—proportions of trials resulting in mating for size ratio bins; black error bars—2.5th and 97.5th percentiles).

Size-assortative mating was positive for all transect positions in all four islands, confirming previous results on size-assortative mating in *L. saxatilis* and in other marine gastropods (NG *et al.* 2019). Its strength was predicted to vary along the transects, proportionally to the variance in size distribution (Figure 5 and Figure S4 in the Supplementary Information of Paper III). Sexual selection on male size was predicted to favor smaller values and lower variance in all the islands and like size-assortative mating, sexual selection varied along the transects depending on the variance in size distribution (Figures 5 and S4).

Computer simulations were then performed to understand the contribution of assortative mating and sexual selection to the barrier to gene flow between ecotypes. These contributions were analyzed separately and also together. Size evolution was simulated using cline models across a hybrid zone and the width of the cline was taken as a measure of the barrier to gene flow (BARTON AND GALE 1993).

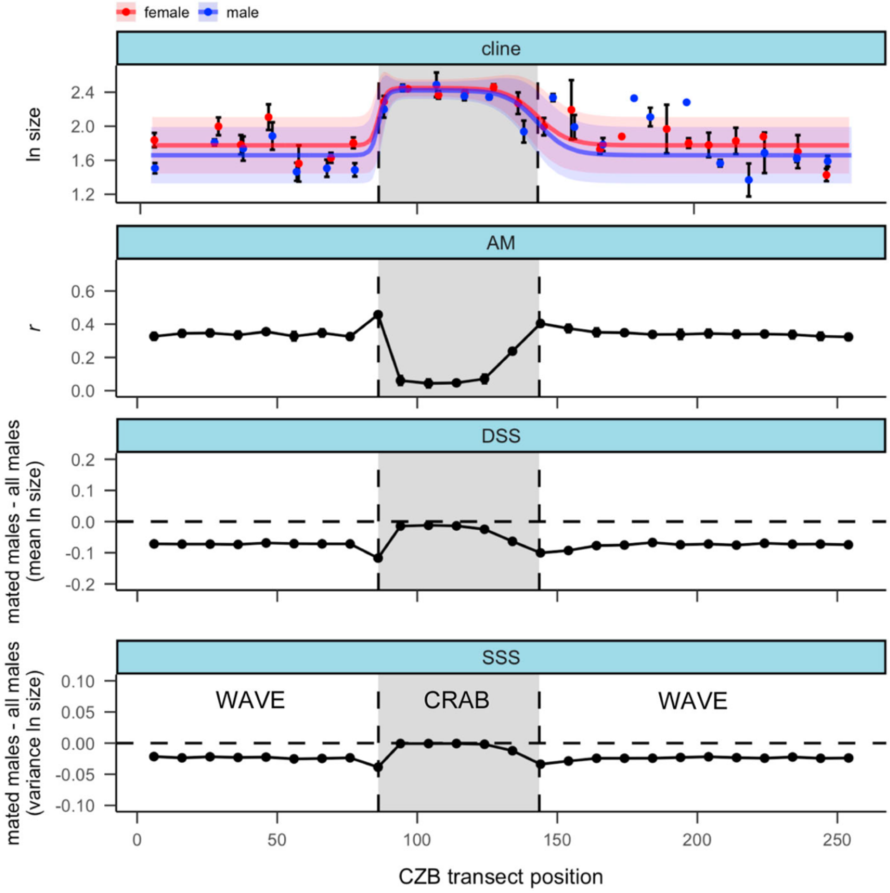


Figure 5. Predicted assortative mating and sexual selection (CZB transect as an example: for the other three transects, see Figure S4 in the SI of Paper III). Habitat boundaries are marked by black vertical dashed lines, the Crab habitat is the region inside (gray fill), and the Wave habitat is outside (white fill) the two dashed lines. Cline facet: ln(size) of transect snails in bins (dots with 95% CIs) and fitted clines (solid lines ± SD) for females (in red) and males (in blue). AM facet: strength of assortative mating measured as the Pearson correlation coefficient (*r*) between female and male ln(size) of mated pairs. DSS facet: directional component of sexual selection measured as the difference in mean ln(size) of mated males compared to mated plus nonmated males. The black horizontal dashed line indicates where this component is absent. SSS facet: stabilizing component of sexual selection calculated as the difference in variance between mated male ln(size) and mated plus nonmated male ln(size). The black horizontal dashed line indicates where this component is absent.

A cline model for assortative mating alone showed a clear barrier effect but the reduction in gene flow was stronger for a cline model that included sexual selection. The contribution of sexual selection was due to the displacement of the size ratio from the natural selection optimum and, more importantly, the asymmetry of the mating function. These two directional components of sexual selection on males are opposed by natural selection and, at equilibrium, the result is stronger net stabilizing selection. The barrier to gene flow between the two *L. saxatilis* ecotypes is then strengthened because this stronger overall selection is associated with a difference in male fitness optima between Crab and Wave habitat even though small males have a mating advantage in both habitats (i.e., sexual selection is uniform and not divergent).

The result that assortative mating alone is a weak barrier compared to divergent selection in a hybrid zone supported a recent simulation study (IRWIN 2020) and emphasized the need to include individuals of intermediate phenotypes whenever the barrier effect of assortative mating is estimated in partially isolated populations.

Paper IV

Sexual selection can be a powerful force driving trait evolution and divergence. It may affect traits that are important for assortative mating and adaptation (Paper III) as it may also affect traits that are involved in fertilization (ANDERSSON 1994). Sperm characteristics such as quantity and morphology are well-known to evolve under strong sexual selection as a result of competition for access to the eggs (reviewed by SIMMONS AND FITZPATRICK 2012). For instance, in the common dung fly, males that copulated for longer transferred a larger quantity of ejaculate which was suggested to increase their reproductive success (MARTIN AND HOSKEN 2002). However, long copulations do not necessarily mean that more sperm are transferred as males may guard the female to impede other males from fertilizing her eggs (reviewed in SIMMONS (2001)). Finally, sperm transfer may also be influenced by population density and predation risk which are factors that might differ between habitats of a species. As a consequence, the timing of sperm transfer may diverge between populations living in different habitats, eventually acting as a barrier to gene flow.

In the highly promiscuous intertidal snail *L. saxatilis*, copulations vary substantially in duration, from less than a minute to more than an hour, and it has been assumed that copulations of a few minutes do not result in any sperm being transferred. In paper IV, I examined timing of sperm transfer performing time-controlled copulation trials using *L. saxatilis* virgin females and males. I measured sperm transfer indirectly based on the relationship between proportion of developing embryos carried by the females and copulation duration interrupted at one, five and ten or more minutes.

I showed that very short copulations were sufficient for the sperm transport into the female to begin (Figure 6). Similar timing of sperm transfer was also found in the opisthobranch sea hare *Aplysia parvula* (YUSA 1994). However, experimental evidence in other gastropods is mostly unclear and limited to a few species (reviewed by WEGGELAAR *et al.* 2019).

Costs of mating due to high predation risk have been shown in other species to be related with short copulation durations and thus, with rapid start to sperm transfer (e.g., WING 1988; KARLSSON *et al.* 2010). In *L. saxatilis*, there is also evidence for high predation risk (JOHANNESSON *et al.* 2010b) which may explain why copulations interrupted at one minute were effective for initiating sperm transfer. However, what is not explained is the combination of rapid start to sperm transfer but long average copulation duration (~20 minutes). Hence, in addition to predation risk, other factors such as sperm competition and/or cryptic female choice may influence copulation duration in *L. saxatilis*.

I also showed that females involved in interrupted copulations at short, medium and long duration did not carry different proportions of developing embryos (Figure 6). This lack of change in proportion of developing embryos with increasing copulation duration might suggest that sperm transfer is completed quickly and most of the copulation duration is guarding time. In *L. saxatilis*, this seems unlikely because guarding for 20 minutes (average copulation time) is probably not an effective strategy when females are year round reproductively active. Because I did not measure sperm directly, I cannot exclude the possibility that more sperm were transferred in longer matings. Sperm transfer may continue and influence male reproductive success and/or female fitness over longer time-scales and in the presence of sperm competition.

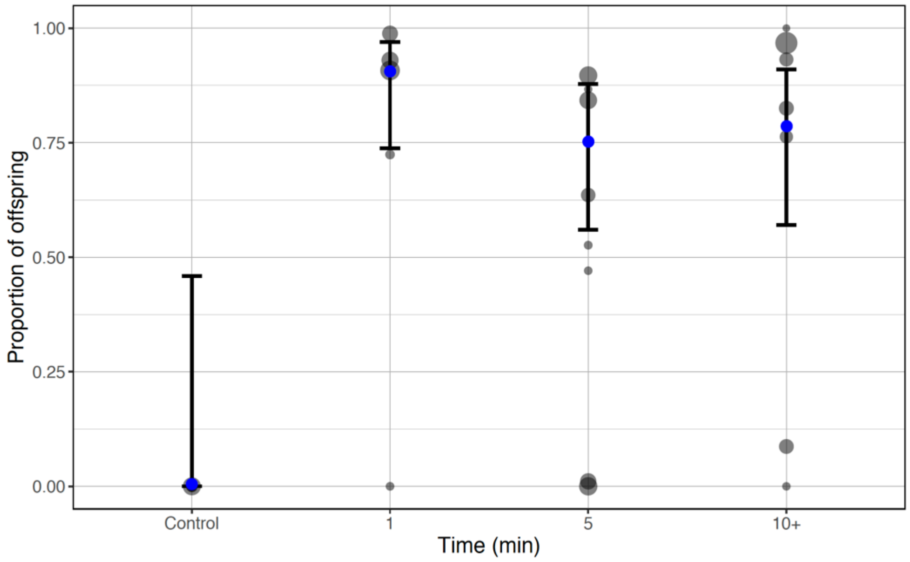


Figure 6. Proportion of developing offspring in the control and treatments. For each female (black points), the proportion (y axis) was calculated as the number of developing embryos divided by the total number of embryos (size of the black points ∝ total number of embryos, range 3-432). For the control group and each time treatment (x axis), the fitted value (blue points) and 95% confidence intervals (black bars) were calculated using a beta-binomial model and back-transformed on the scale for proportions (0 to 1).

DISCUSSION

Contact zones are geographic areas where differentiated populations of the same taxon or of closely related taxa meet and exchange genes. They are expected to coincide with habitat transitions, especially when two populations have diverged *in situ* due to local adaptation, but they can also be found in the absence of environmental gradients when two populations, prior to contact, have undergone independent evolution (BARTON AND HEWITT 1985; SWENSON AND HOWARD 2005). In this thesis, I focus on taxa showing differentiated populations with partially overlapping distributions over two environmental transitions: the North Sea–Baltic Sea salinity gradient where multiple marine species were found to form contact zones, and the wave exposure–crab predation transition where the Crab and Wave ecotypes of one species, *L. saxatilis*, were examined. Contact zones are excellent study systems for investigating how different populations coexist despite ongoing gene flow. Multispecies contact zones can be used for assessing what barrier effects, evolutionary processes, genomic architectures and life-history traits that are identified in single-species contact zone, are the most common and thus, important for reproductive isolation. Ultimately, understanding the processes and components involved in local adaptation and population differentiation over contact zones will provide us with a clearer picture of how biodiversity is formed, both within and between species.

In both examples of transitions, there is strong evidence for differences between environments at either side of the contact being a source of divergent selection. This is also true for other multispecies contact zones (DIBATTISTA *et al.* 2015; STANLEY *et al.* 2018; EL AYARI *et al.* 2019; but see PATARNELLO *et al*. 2007). Divergent traits such as egg buoyancy (NISSLING AND WESTIN 1997), spawning season (CHRISTENSEN *et al.* 2008) and shell size (JOHANNESSON 1986) were shown to be favored by differences in selection. In studies where isolating traits are not known, the evidence for divergent ecological selection causing reproductive isolation came instead from inferences about reductions in gene flow based on immigrant fitness (JOHANSSON *et al.* 2017; RUGIU *et al.* 2018; BARTH *et al.* 2019). In all these cases, divergent selection generates prezygotic barriers to gene flow by reducing interpopulation matings in space and time (i.e., assortative mating). However, concluding that the most important barrier effects over the studied contact zones are prezygotic would be erroneous. Estimates of the strength of the barriers are mostly missing (but see Paper III) and data on postzygotic isolating barriers (low hybrid fitness) are also lacking. In Atlantic cod, for example, postzygotic barrier effects are plausible but the specific traits have not been investigated (WEIST *et al.* 2019). *L. saxatilis* instead, might represent an exception for the presence of postzygotic barriers. Embryo abortion, a potential postzygotic barrier due to genetic incompatibilities, was suggested to have a negligible effect on gene flow between Crab and Wave ecotypes (JOHANNESSON *et al.* 2020b). Strong incompatibilities are usually found between populations that prior to contact have accumulated genetic differences in isolation whereas ecotype formation in *L. saxatilis* is likely to have occurred under continuous gene flow (BUTLIN *et al.* 2014), constraining the evolution of genetic incompatibilities. However, it is still expected for relatively weak postzygotic barrier effects to evolve between differentiated populations due to the multiple interactions that genes under divergent selection have with other genes (KULMUNI AND WESTRAM 2017). In addition, from past reviews of multiple components of reproductive isolation, both prezygotic and postzygotic barriers appeared to be generated by divergent selection and thus, be common during speciation (FUNK *et al.* 2002; NOSIL *et al.* 2005; LOWRY *et al.* 2008; MATSUBAYASHI *et al.* 2010). I expect these different types of barriers also to be present in the systems examined in this thesis.

Barrier effects generated by divergent ecological selection are likely to be strong in both environmental transitions. The genomic patterns of divergence are clear and while SNPs were the most common type of variants that was used for analyzing these patterns, I showed in *L. saxatilis* that short INDELs can also be used for identifying genomic regions under selective constraints or important for adaptation and population divergence. Using both types of variants can help to understand how different processes such as purifying and divergent selection may interact and affect genomic variation in the presence of gene flow.

The genomic landscapes are diverse and for the species across the salinity gradient, this variation appear to be independent of dispersal potential. This seems to be in contrast with the expectation that dispersal potential should be negatively correlated with genetic differentiation (BOHONAK 1999) but dispersal is not the only factor affecting genetic variation. For example, differences in the strength of divergent selection, the demographic history or the genomic architecture are likely to intervene in complex ways during the evolution of reproductive isolation (RAVINET *et al.* 2017). Importantly, inversions and demographic processes, also examined in *L. saxatilis* (BUTLIN *et al.* 2014; FARIA *et al.* 2019b), seem to contribute more to the difference in patterns of divergence. The contribution to reproductive isolation of inversions is broadly known and the theory is supported by empirical studies (WELLENREUTHER AND BERNATCHEZ 2018; FARIA *et al.* 2019a). However, in the case of the North Sea–Baltic Sea transition, the limited number of species in which inversions have been investigated makes it difficult to understand how common it is for inversions to promote reproductive isolation. With respect to demographic processes instead, more data are available which suggest that divergence can be facilitated by segregation of genetic polymorphisms in ancestral populations. Standing genetic variation can be maintained by gene flow between ancestral populations and/or balancing selection on regions of low recombination (e.g., inversions). Such preexisting genetic variation can then be selected for in favorable conditions, promoting rapid adaptation that may act as a barrier to gene flow between populations in different environments (MARQUES *et al.* 2019).

Finally, of the evolutionary processes that generate barrier effects at contact zones, sexual selection is an important candidate. In simulations of the *L. saxatilis* mating pattern, gene flow between ecotypes was more reduced by sexual selection than by assortative mating alone. A weak barrier effect of assortment is likely due to the opportunity for hybrids to mate inside the hybrid zone and back-cross with either Crab or Wave populations. This result is similar to what was found in recent simulation studies that assortative mating is a less effective barrier than natural selection in a hybrid zone (IRWIN 2020; METZLER *et al.* 2020). Hybrid formation and barrier strength of assortment and sexual selection are largely unexplored in the North Sea–Baltic Sea transition, possibly because for most marine species, a detailed description of the mating pattern is unattainable given the challenges of maintaining such species under laboratory conditions.

In this thesis, I investigated which types of barriers reduce gene flow between divergent populations, which selective and demographic processes produce these barriers and which genomic features are more likely to drive the evolution of reproductive isolation at contact zones. While there is sufficient knowledge of the impact of divergent ecological selection on population differentiation, direct evidence of how it causes reproductive isolation is not well understood. Finding the traits that act as barriers and their genetic basis would be ideal and it can be achieved with more experimental work and the available genomic tools. How divergent ecological selection interacts with other evolutionary processes such as sexual selection and demography is also poorly understood. However, the development of a framework and guidelines for the study of reproductive isolation provides us with a solid base to test empirically how different species form and coexist.

ACKNOWLEDGMENTS

I am really grateful to my supervisors, Roger Butlin, Kerstin Johannesson and Anja Westram for being very helpful, insightful and patient throughout my doctoral study. Grazie mille. I feel truly lucky for the opportunity of working with them. Being part of the *Littorina* team has been a wonderful experience and I thank all of its members for having shared ideas and knowledge during those long days and nights in and outside the lab. Everyone that is listed in this website https://littorina.group.shef.ac.uk/people/ has had a positive influence during my doctoral study. I also thank the people that have been involved with the Swedish Bioinformatics Advisory Program organized by SciLifeLab and with the Graduate Research School in Genomic Ecology GENECO for feedback and assistance on genomic techniques. I am also grateful to the researchers and staff at the Tjärnö Marine Laboratory who have made me experience a friendly working environment where coffee, tea and food were never missing. Finally, I owe a big thank to my family and friends for their unconditional support.

This doctoral study was funded by the European Research Council (ERC-2015-AdG-693030- BARRIERS "The evolution of barriers to gene exchange").

REFERENCES

Abbott, R., D. Albach, S. Ansell, J. W. Arntzen, S. J. Baird *et al.*, 2013 Hybridization and speciation. Journal of Evolutionary Biology 26: 229–246.

Andersson, M., 1994 *Sexual selection*. Princeton University Press, Princeton, New Jersey.

Arnqvist, G., M. Edvardsson, U. Friberg and T. Nilsson, 2000a Sexual conflict promotes speciation in insects. Proceedings of the National Academy of Sciences of the United States of America 97**:** 10460–10464.

Arnqvist, G., and T. Nilsson, 2000b The evolution of polyandry: multiple mating and female fitness in insects. Animal Behaviour 60**:** 145–164.

Arnqvist, G., and L. Rowe, 2005 *Sexual conflict*. Princeton University Press.

Bacigalupe, L., H. Crudgington, F. Hunter, A. Moore and R. Snook, 2007 Sexual conflict does not drive reproductive isolation in experimental populations of *Drosophila pseudoobscura*. Journal of Evolutionary Biology 20**:** 1763–1771.

Barraclough, T. G., P. H. Harvey and S. Nee, 1995 Sexual selection and taxonomic diversity in passerine birds. Proceedings of the Royal Society of London. Series B: Biological Sciences 259**:** 211–215.

Barth, J. M., D. Villegas-Ríos, C. Freitas, E. Moland, B. Star *et al.*, 2019 Disentangling structural genomic and behavioural barriers in a sea of connectivity. Molecular Ecology 28**:** 1394–1411.

Barton, H. J., and K. Zeng, 2019 The impact of natural selection on short insertion and deletion variation in the great tit genome. Genome Biology and Evolution 11**:** 1514–1524.

Barton, N. H., and K. S. Gale, 1993 Genetic analysis of hybrid zones, pp. 13–45 in *Hybrid zones and the evolutionary process*, edited by R. G. Harrison. Oxford University Press, Oxford.

Barton, N. H., and G. M. Hewitt, 1985 Analysis of hybrid zones. Annual Review of Ecology and Systematics 16**:** 113–148.

Bateman, A. J., 1948 Intra-sexual selection in *Drosophila*. Heredity 2**:** 349–368.

Berg, P. R., S. Jentoft, B. Star, K. H. Ring, H. Knutsen *et al.*, 2015 Adaptation to low salinity promotes genomic divergence in Atlantic cod (*Gadus morhua* L.). Genome Biology and Evolution 7**:** 1644–1663.

Bímová, B. V., M. Macholán, S. J. Baird, P. Munclinger, P. Dufková *et al.*, 2011 Reinforcement selection acting on the European house mouse hybrid zone. Molecular Ecology 20**:** 2403–2424.

Bohonak, A. J., 1999 Dispersal, gene flow, and population structure. The Quarterly Review of Biology 74**:** 21–45.

Boulding, E. G., M. J. Rivas, N. González-Lavín, E. Rolán-Alvarez and J. Galindo, 2017 Size selection by a gape-limited predator of a marine snail: Insights into magic traits for speciation. Ecology and Evolution 7**:** 674–688.

Brumfield, R. T., P. Beerli, D. A. Nickerson and S. V. Edwards, 2003 The utility of single nucleotide polymorphisms in inferences of population history. Trends in Ecology & Evolution 18**:** 249–256.

Butlin, R., J. Bridle and D. Schluter, 2009 *Speciation and patterns of diversity*. Cambridge University Press, Cambridge, England.

Butlin, R., A. Debelle, C. Kerth, R. R. Snook, L. W. Beukeboom *et al.*, 2011 What do we need to know about speciation? Trends in Ecology & Evolution 27**:** 27–39.

Butlin, R. K., 2005 Recombination and speciation. Molecular Biology 14**:** 2621–2635.

Butlin, R. K., M. Saura, G. Charrier, B. Jackson, C. André *et al.*, 2014 Parallel evolution of local adaptation and reproductive isolation in the face of gene flow. Evolution 68**:** 935–949.

Butlin, R. K., and C. M. Smadja, 2018 Coupling, reinforcement, and speciation. The American Naturalist 191**:** 155–172.

Cardinale, B. J., J. E. Duffy, A. Gonzalez, D. U. Hooper, C. Perrings *et al.*, 2012 Biodiversity loss and its impact on humanity. Nature 486**:** 59–67.

Carvalho, A. P. S., R. A. St Laurent, E. F. A. Toussaint, C. Storer, K. M. Dexter *et al.*, 2020 Is sexual conflict a driver of speciation? A case study with a tribe of brush-footed butterflies. Systematic Biology 0**:** 1–8.

Catanach, A., R. Crowhurst, C. Deng, C. David, L. Bernatchez *et al.*, 2019 The genomic pool of standing structural variation outnumbers single nucleotide polymorphism by threefold in the marine teleost *Chrysophrys auratus*. Molecular Biology 28**:** 1210–1223.

Chapman, T., G. Arnqvist, J. Bangham and L. Rowe, 2003 Sexual conflict. Trends in Ecology & Evolution 18**:** 41–47.

Chapman, T., L. F. Liddle, J. M. Kalb, M. F. Wolfner and L. Partridge, 1995 Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. Nature 373**:** 241–244.

Christensen, A., H. Jensen, H. Mosegaard, M. St. John and C. Schrum, 2008 Sandeel (*Ammodytes marinus*) larval transport patterns in the North Sea from an individual-based hydrodynamic egg and larval model. Canadian Journal of Fisheries and Aquatic Sciences 65**:** 1498–1511.

Christiansen, F. B., and O. Frydenberg, 1974 Geographical patterns of four polymorphisms in *Zoarces viviparus* as evidence of selection. Genetics 77**:** 765–770.

Clutton-Brock, T. H., and G. A. Parker, 1995 Sexual coercion in animal societies. Animal Behaviour 49**:** 1345–1365.

Conde-Padín, P., R. Cruz, J. Hollander and E. Rolan-Alvarez, 2008 Revealing the mechanisms of sexual isolation in a case of sympatric and parallel ecological divergence. Biological Journal of the Linnean Society 94**:** 513–526.

Corcoran, P., T. I. Gossmann, H. J. Barton, C. Great Tit HapMap, J. Slate *et al.*, 2017 Determinants of the efficacy of natural selection on coding and noncoding variability in two passerine species. Genome Biology and Evolution 9**:** 2987–3007.

Coyne, J. A., and H. A. Orr, 2004 *Speciation*. Sinauer Associates, Sunderland, Massachusetts.

Cruz, R., M. Carballo, P. Conde-Padín and E. Rolán-Alvarez, 2004 Testing alternative models for sexual isolation in natural populations of Littorina saxatilis: indirect support for by-product ecological speciation? Journal of Evolutionary Biology 17: 288–293.

Darwin, C. R., 1859 *The origin of species*. Murray, London, United Kingdom.

De Lisle, S. P., and L. Rowe, 2015 Independent evolution of the sexes promotes amphibian diversification. Proceedings of the Royal Society of London. Series B: Biological Sciences 282: 20142213.

DeFaveri, J., and J. Merilä, 2014 Local adaptation to salinity in the three-spined stickleback? Journal of Evolutionary Biology 27: 290–302.

Diopere, E., S. G. Vandamme, P. I. Hablützel, A. Cariani, J. Van Houdt *et al.*, 2018 Seascape genetics of a flatfish reveals local selection under high levels of gene flow. ICES Journal of Marine Science 75: 675–689.

Dobzhansky, T., 1937 *Genetics and the origin of species*. Columbia University Press, New York.

Duret, L., and N. Galtier, 2009 Biased gene conversion and the evolution of mammalian genomic landscapes. Annual Review of Genomics and Human Genetics 10: 285–311.

Dybern, B. I., 1967 The distribution and salinity tolerance of *Ciona intestinalis* (L.) F. *typica* with special reference to the waters around southern Scandinavia. Ophelia 4: 207–226.

Erlandsson, J., and E. Rolán-Alvarez, 1998 Sexual selection and assortative mating by size and their roles in the maintenance of a polymorphism in Swedish *Littorina saxatilis* populations, pp. 59–69 in *Aspects of Littorinid Biology*. Springer.

Faria, R., P. Chaube, H. E. Morales, T. Larsson, A. R. Lemmon *et al.*, 2019b Multiple chromosomal rearrangements in a hybrid zone between *Littorina saxatilis* ecotypes. Molecular Ecology 28: 1375–1393.

Faria, R., K. Johannesson, R. K. Butlin and A. M. Westram, 2019a Evolving inversions. Trends in Ecology & Evolution 34: 239–248.

Fay, J. C., and C.-I. Wu, 2000 Hitchhiking under positive Darwinian selection. Genetics 155: 1405–1413.

Feder, J. L., 1998 The apple maggot fly, *Rhagoletis pomonella*: Flies in the face of conventional wisdom about speciation?, pp. 130–144 in *Endless Forms: Species and Speciation*, edited by D. J. Howard and S. H. Berlocher. Oxford University Press, New York.

Felsenstein, J., 1981 Skepticism towards Santa Rosalia, or why are there so few kinds of animals? Evolution: 124–138.

Fernández-Meirama, M., A. Carvajal-Rodríguez and E. Rolán-Alvarez, 2017 Testing the role of mating preference in a case of incomplete ecological speciation with gene flow. Biological Journal of the Linnean Society 122: 549–557.

Funk, D. J., K. E. Filchak and J. L. Feder, 2002 Herbivorous insects: model systems for the comparative study of speciation ecology, pp. 251–267 in *Genetics of Mate Choice: From Sexual Selection to Sexual Isolation*. Springer.

Gage, M. J., G. A. Parker, S. Nylin and C. Wiklund, 2002 Sexual selection and speciation in mammals, butterflies and spiders. Proceedings of the Royal Society of London. Series B: Biological Sciences 269: 2309–2316.

Gagnon, M. C., and J. Turgeon, 2011 Sexual conflict in *Gerris gillettei* (Insecta: Hemiptera): intraspecific intersexual correlated morphology and experimental assessment of behaviour and fitness. Journal of Evolutionary Biology 24: 1505–1516.

Gavrilets, S., 2000 Rapid evolution of reproductive barriers driven by sexual conflict. Nature 403: 886–889.

Gavrilets, S., 2014 Is sexual conflict an "engine of speciation"? Cold Spring Harbor Perspectives in Biology 6: a017723.

Gavrilets, S., and T. I. Hayashi, 2005 Speciation and sexual conflict. Evolutionary Ecology 19: 167–198.

Gavrilets, S., and D. Waxman, 2002 Sympatric speciation by sexual conflict. Proceedings of the National Academy of Sciences of the United States of America 99: 10533–10538.

Gay, L., P. E. Eady, R. Vasudev, D. J. Hosken and T. Tregenza, 2009 Does reproductive isolation evolve faster in larger populations via sexually antagonistic coevolution? Biology Letters 5: 693–696.

Gilbert, D., and W. Starmer, 1985 Statistics of sexual isolation. Evolution 39: 1380–1383.

Goldstein, D. B., and C. Schlötterer, 1999 *Microsatellites: Evolution and applications*. Oxford University Press, New York.

Harrison, R. G., 1993 *Hybrid zones and the evolutionary process*. Oxford University Press, New York.

Hernandez, R. D., S. H. Williamson and C. D. Bustamante, 2007 Context dependence, ancestral misidentification, and spurious signatures of natural selection. Molecular Biology and Evolution 24: 1792–1800.

Hewitt, G. M., 1988 Hybrid zones-natural laboratories for evolutionary studies. Trends in Ecology & Evolution 3: 158–167.

Holland, B., and W. R. Rice, 1998 Perspective: Chase-away sexual selection: Antagonistic seduction versus resistence. Evolution 52: 1–7.

Hollander, J., M. Lindegarth and K. Johannesson, 2005 Local adaptation but not geographical separation promotes assortative mating in a snail. Animal Behaviour 70: 1209–1219.

Hosken, D., O. Martin, S. Wigby, T. Chapman and D. Hodgson, 2009 Sexual conflict and reproductive isolation in flies. Biology Letters 5: 697–699.

Howard, D. J., M. Reece, P. G. Gregory, J. Chu and M. L. Cain, 1998 The evolution of barriers to fertilization between closely related organisms, pp. 279–288 in *Endless forms: Species and speciation*, edited by D. J. Howard and S. H. Berlocher. Oxford University Press, New York.

Huang, W., A. Massouras, Y. Inoue, J. Peiffer, M. Ramia *et al.*, 2014 Natural variation in genome architecture among 205 *Drosophila melanogaster* Genetic Reference Panel lines. Genome Research 24: 1193–1208.

Hudson, J., K. Johannesson, C. D. McQuaid and M. Rius, 2020 Secondary contacts and genetic admixture shape colonization by an amphiatlantic epibenthic invertebrate. Evolutionary Applications 13: 600–612.

Hull, S., 1998 Assortative mating between two distinct micro-allopatric populations of *Littorina saxatilis* (Olivi) on the northeast coast of England, pp. 79–88 in *Aspects of Littorinid Biology*. Springer.

Irwin, D. E., 2020 Assortative mating in hybrid zones is remarkably ineffective in promoting speciation. The American Naturalist 195: E150–E167.

Jackson, B. C., 2011 Recombination-suppression: How many mechanisms for chromosomal speciation? Genetica 139: 393–402.

Janicke, T., I. K. Häderer, M. J. Lajeunesse and N. Anthes, 2016 Darwinian sex roles confirmed across the animal kingdom. Science Advances 2: e1500983.

Janson, K., 1983 Selection and migration in two distinct phenotypes of *Littorina saxatilis* in Sweden. Oecologia 59: 58–61.

Jarne, P., and P. J. Lagoda, 1996 Microsatellites, from molecules to populations and back. Trends in Ecology & Evolution 11: 424–429.

Jiggins, C. D., 2008 Ecological speciation in mimetic butterflies. BioScience 58: 541–548.

Johannesson, B., 1986 Shell morphology of *Littorina saxatilis* Olivi: the relative importance of physical factors and predation. Journal of Experimental Marine Biology and Ecology 102: 183–195.

Johannesson, K., and C. Andre, 2006 Life on the margin: Genetic isolation and diversity loss in a peripheral marine ecosystem, the Baltic Sea. Molecular Ecology 15: 2013–2029.

Johannesson, K., A. Le Moan, S. Perini and C. Andre, 2020a A Darwinian laboratory of multiple contact zones. Trends in Ecology & Evolution 35: 1021–1036.

Johannesson, K., M. Panova, P. Kemppainen, C. André, E. Rolán-Alvarez *et al.*, 2010a Repeated evolution of reproductive isolation in a marine snail: unveiling mechanisms of speciation. Philosophical Transactions of the Royal Society B: Biological Sciences 365: 1735–1747.

Johannesson, K., A.-K. Ring, K. B. Johannesson, E. Renborg, P. R. Jonsson *et al.*, 2018 Oceanographic barriers to gene flow promote genetic subdivision of the tunicate *Ciona intestinalis* in a North Sea archipelago. Marine Biology 165: 126.

Johannesson, K., E. Rolán-Alvarez and A. Ekendahl, 1995 Incipient reproductive isolation between two sympatric morphs of the intertidal snail *Littorina saxatilis*. Evolution 49: 1180–1190.

Johannesson, K., S. H. Saltin, G. Charrier, A.-K. Ring, C. Kvarnemo *et al.*, 2016 Non-random paternity of offspring in a highly promiscuous marine snail suggests postcopulatory sexual selection. Behavioral Ecology and Sociobiology 70: 1357–1366.

Johannesson, K., S. H. Saltin, I. Duranovic, J. N. Havenhand and P. R. Jonsson, 2010b Indiscriminate males: mating behaviour of a marine snail compromised by a sexual conflict? PLoS One 5: e12005.

Johannesson, K., Z. Zagrodzka, R. Faria, A. Marie Westram and R. K. Butlin, 2020b Is embryo abortion a post-zygotic barrier to gene flow between *Littorina* ecotypes? Journal of Evolutionary Biology 33: 342–351.

Johansson, D., R. T. Pereyra, M. Rafajlovic and K. Johannesson, 2017 Reciprocal transplants support a plasticity-first scenario during colonisation of a large hyposaline basin by a marine macro alga. BMC Ecology 17: 14.

Katzourakis, A., A. Purvis, S. Azmeh, G. Rotheray and F. Gilbert, 2001 Macroevolution of hoverflies (Diptera: Syrphidae): the effect of using higher-level taxa in studies of biodiversity, and correlates of species richness. Journal of Evolutionary Biology 14: 219-227.

Kautsky, N., K. Johannesson and M. Tedengren, 1990 Genotypic and phenotypic differences between Baltic and North Sea populations of *Mytilus edulis* evaluated through reciprocal transplantations. I. Growth and morphology. Marine Ecology Progress Series: 203–210.

Kirkpatrick, M., and N. Barton, 2006 Chromosome inversions, local adaptation and speciation. Genetics 173: 419–434.

Kohn, M. H., S. Fang and C. I. Wu, 2004 Inference of positive and negative selection on the 5' regulatory regions of *Drosophila* genes. Molecular Biology and Evolution 21: 374–383.

Kopp, M., M. R. Servedio, T. C. Mendelson, R. J. Safran, R. L. Rodríguez *et al.*, 2018 Mechanisms of assortative mating in speciation with gene flow: Connecting theory and empirical research. The American Naturalist 191: 1–20.

Kousathanas, A., F. Oliver, D. L. Halligan and P. D. Keightley, 2011 Positive and negative selection on noncoding DNA close to protein-coding genes in wild house mice. Molecular Biology and Evolution 28: 1183–1191.

Kulmuni, J., and A. M. Westram, 2017 Intrinsic incompatibilities evolving as a by-product of divergent ecological selection: Considering them in empirical studies on divergence with gene flow. Molecular Ecology 26: 3093–3103.

Le Moan, A., D. Bekkevold and J. Hemmer-Hansen, 2020 Evolution at two time-frames: Ancient and common origin of two structural variants involved in local adaptation of the European plaice (*Pleuronectes platessa*). BioRxiv: 662577.

Le Moan, A., O. Gaggiotti, R. Henriques, P. Martinez, D. Bekkevold *et al.*, 2019a Beyond parallel evolution: When several species colonize the same environmental gradient. BioRxiv: 662569.

Le Moan, A., B. Jiménez-Mena, D. Bekkevold and J. Hemmer-Hansen, 2019b Fine scale population structure linked to neutral divergence in the common sole (*Solea solea*), a marine fish with high dispersal capacity. BioRxiv: 662619.

Le Pennec, G., R. K. Butlin, P. R. Jonsson, A. I. Larsson, J. Lindborg *et al.*, 2017 Adaptation to dislodgement risk on wave-swept rocky shores in the snail *Littorina saxatilis*. PloS one 12: e0186901.

Lee, H., J. Gurtowski, S. Yoo, M. Nattestad, S. Marcus *et al.*, 2016 Third-generation sequencing and the future of genomics. BioRxiv: 048603.

Lessells, C. M., 2006 The evolutionary outcome of sexual conflict. Philosophical Transactions of the Royal Society B 361: 301–317.

Lindsay, W. R., S. Andersson, B. Bererhi, J. Hoglund, A. Johnsen *et al.*, 2019 Endless forms of sexual selection. PeerJ 7: e7988.

Lowry, D. B., J. L. Modliszewski, K. M. Wright, C. A. Wu and J. H. Willis, 2008 The strength and genetic basis of reproductive isolating barriers in flowering plants. Philosophical Transactions of the Royal Society B: Biological Sciences 363: 3009–3021.

Marques, D. A., J. I. Meier and O. Seehausen, 2019 A combinatorial view on speciation and adaptive radiation. Trends in Ecology & Evolution 34: 531–544.

Martin, O., and D. Hosken, 2002 Strategic ejaculation in the common dung fly *Sepsis cynipsea*. Animal Behaviour 63: 541–546.

Martin, O. Y., and D. J. Hosken, 2003 The evolution of reproductive isolation through sexual conflict. Nature 423: 979–982.

Matsubayashi, K. W., I. Ohshima and P. Nosil, 2010 Ecological speciation in phytophagous insects. Entomologia Experimentalis et Applicata 134: 1–27.

Mattingsdal, M., P. E. Jorde, H. Knutsen, S. Jentoft, N. C. Stenseth *et al.*, 2020 Demographic history has shaped the strongly differentiated corkwing wrasse populations in Northern Europe. Molecular Ecology 29: 160–171.

Mayr, E., 1942 *Systematics and the origin of species*. Columbia University Press, New York.

McKinnon, J. S., and H. D. Rundle, 2002 Speciation in nature: The threespine stickleback model systems. Trends in Ecology & Evolution 17: 480–488.

Merrill, R. M., P. Rastas, S. H. Martin, M. C. Melo, S. Barker *et al.*, 2019 Genetic dissection of assortative mating behavior. PLoS Biology 17: e2005902.

Merrill, R. M., R. W. Wallbank, V. Bull, P. C. Salazar, J. Mallet *et al.*, 2012 Disruptive ecological selection on a mating cue. Proceedings of the Royal Society B: Biological Sciences 279: 4907–4913.

Metzler, D., U. Knief, J. V. Peñalba and J. B. W. Wolf, 2020 Assortative mate choice and epistatic mating-trait architecture induces complex hybrid-zone movement. bioRxiv: 2020.2003.2010.985333.

Møller Nielsen, M., C. Paulino, J. Neiva, D. Krause-Jensen, A. Bruhn *et al.*, 2016 Genetic diversity of *Saccharina latissima* (Phaeophyceae) along a salinity gradient in the North Sea–Baltic Sea transition zone. Journal of Phycology 52: 523–531.

Momigliano, P., A.-B. Florin and J. Merilä, 2020 Biases in demographic modelling affect our understanding of the process of speciation. bioRxiv: 2020.2006.2003.128298.

Momigliano, P., H. Jokinen, A. Fraimout, A.-B. Florin, A. Norkko *et al.*, 2017 Extraordinarily rapid speciation in a marine fish. Proceedings of the National Academy of Sciences of the United States of America 114: 6074–6079.

Montgomery, S. B., D. L. Goode, E. Kvikstad, C. A. Albers, Z. D. Zhang *et al.*, 2013 The origin, evolution, and functional impact of short insertion-deletion variants identified in 179 human genomes. Genome Res 23: 749–761.

Morales, H. E., R. Faria, K. Johannesson, T. Larsson, M. Panova *et al.*, 2019 Genomic architecture of parallel ecological divergence: Beyond a single environmental contrast. Science Advances 5: eaav9963.

Morin, P. A., G. Luikart, R. K. Wayne and S. N. P. w. g. the, 2004 SNPs in ecology, evolution and conservation. Trends in Ecology & Evolution 19: 208–216.

Morrow, E. H., T. E. Pitcher and G. Arnqvist, 2003 No evidence that sexual selection is an 'engine of speciation' in birds. Ecology Letters 6: 228–234.

Muggeo, V. M., 2008 Segmented: An R package to fit regression models with broken-line relationships. R News 8: 20–25.

Ng, T. P., E. Rolán-Alvarez, S. S. Dahlén, M. S. Davies, D. Estévez *et al.*, 2019 The causal relationship between sexual selection and sexual size dimorphism in marine gastropods. Animal Behaviour 148: 53–62.

Nissling, A., and L. Westin, 1997 Salinity requirements for successful spawning of Baltic and Belt Sea cod and the potential for cod stock interactions in the Baltic Sea. Marine Ecology Progress Series 152: 261–271.

Nosil, P., 2012 *Ecological speciation*. Oxford University Press, New York.

Nosil, P., and D. Schluter, 2011 The genes underlying the process of speciation. Trends in Ecology & Evolution 26: 160–167.

Nosil, P., T. H. Vines and D. J. Funk, 2005 Reproductive isolation caused by natural selection against immigrants from divergent habitats. Evolution 59: 705–719.

Ojaveer, H., A. Jaanus, B. R. MacKenzie, G. Martin, S. Olenin *et al.*, 2010 Status of biodiversity in the Baltic Sea. PLoS one 5: e12467.

Panova, M., J. Hollander and K. Johannesson, 2006 Site-specific genetic divergence in parallel hybrid zones suggests nonallopatric evolution of reproductive barriers. Molecular Ecology 15: 4021–4031.

Parker, G. A., 1979 Sexual selection and sexual conflict, pp. 123–166 in *Sexual selection and reproductive competition in insects*, edited by M. S. Blum and N. A. Blum. Academic Press, New York.

Parker, G. A., 2006 Sexual conflict over mating and fertilization: An overview. Philosophical Transactions of the Royal Society B: Biological Sciences 361: 235–259.

Parker, G. A., and L. Partridge, 1998 Sexual conflict and speciation. Philosophical Transactions of the Royal Society B: Biological Sciences 353: 261–274.

Perini, S., M. Rafajlović, A. M. Westram, K. Johannesson and R. K. Butlin, 2020 Assortative mating, sexual selection, and their consequences for gene flow in *Littorina*. Evolution 74: 1482–1497.

Perry, J. C., and L. Rowe, 2015 The evolution of sexually antagonistic phenotypes. Cold Spring Harbor Perspectives in Biology 7: a017558.

Pettersson, M. E., C. M. Rochus, F. Han, J. Chen, J. Hill *et al.*, 2019 A chromosome-level assembly of the Atlantic herring genome—detection of a supergene and other signals of selection. Genome Research 29**:** 1919–1928.

Plesnar-Bielak, A., A. M. Skrzynecka, Z. M. Prokop, M. Kolasa, M. Dzialo *et al.*, 2013 No evidence for reproductive isolation through sexual conflict in the bulb mite *Rhizoglyphus robini*. PLoS One 8**:** e74971.

Presgraves, D. C., 2003 A fine-scale genetic analysis of hybrid incompatibilities in *Drosophila*. Genetics 163**:** 955–972.

Ramsey, J., H. Bradshaw Jr and D. W. Schemske, 2003 Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57**:** 1520–1534.

Ravinet, M., R. Faria, R. K. Butlin, J. Galindo, N. Bierne *et al.*, 2017 Interpreting the genomic landscape of speciation: A road map for finding barriers to gene flow. Journal of Evolutionary Biology 30**:** 1450–1477.

Renborg, E., K. Johannesson and J. Havenhand, 2013 Variable salinity tolerance in ascidian larvae is primarily a plastic response to the parental environment. Evolutionary Ecology 28**:** 561–572.

Rice, W. R., 1998 Intergenomic conflict, interlocus antagonistic coevolution, and the evolution of reproductive isolation, pp. 261–270 in *Endless forms: Species and speciation*, edited by D. J. Howard and S. H. Berlocher. Oxford University Press, New York.

Rice, W. R., and E. E. Hostert, 1993 Laboratory experiments on speciation: What have we learned in 40 years. Evolution 47**:** 1637–1653.

Riginos, C., and C. W. Cunningham, 2005 Local adaptation and species segregation in two mussel (*Mytilus edulis* × *Mytilus trossulus*) hybrid zones. Molecular Ecology 14**:** 381–400.

Ritchie, M. G., S. A. Webb, J. A. Graves, A. E. Magurran and C. Macias Garcia, 2005 Patterns of speciation in endemic Mexican Goodeid fish: Sexual conflict or early radiation? Journal of Evolutionary Biology 18**:** 922–929.

Rolán-Alvarez, E., and A. Caballero, 2000 Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution 54**:** 30–36.

Rolán-Alvarez, E., J. Erlandsson, K. Johannesson and R. Cruz, 1999 Mechanisms of incomplete prezygotic reproductive isolation in an intertidal snail: Testing behavioural models in wild populations. Journal of Evolutionary Biology 12**:** 879–890.

Rolán-Alvarez, E., K. Johannesson and J. Erlandsson, 1997 The maintenance of a cline in the marine snail *Littorina saxatilis*: The role of home site advantage and hybrid fitness. Evolution 51**:** 1838–1847.

Rugiu, L., I. Manninen, E. Rothäusler and V. Jormalainen, 2018 Tolerance and potential for adaptation of a Baltic Sea rockweed under predicted climate change conditions. Marine Environmental Research 134**:** 76–84.

Sadedin, S., J. Hollander, M. Panova, K. Johannesson and S. Gavrilets, 2009 Case studies and mathematical models of ecological speciation. 3: Ecotype formation in a Swedish snail. Molecular Ecology 18: 4006–4023.

Saur, M., 1990 Mate discrimination in *Littorina littorea* (L.) and *L. saxatilis* (Olivi) (Mollusca: Prosobranchia). Hydrobiologia 193: 261–270.

Schemske, D. W., and C. Goodwillie, 1996 Morphological and reproductive characteristics of *Linanthus jepsonii* (Polemoniaceae), a newly described, geographically restricted species from northern California. Madroño: 453–463.

Schlötterer, C., 2004 The evolution of molecular markers—just a matter of fashion? Nature Reviews Genetics 5: 63–69.

Scopece, G., A. Musacchio, A. Widmer and S. Cozzolino, 2007 Patterns of reproductive isolation in Mediterranean deceptive orchids. Evolution: International Journal of Organic Evolution 61: 2623–2642.

Seehausen, O., R. K. Butlin, I. Keller, C. E. Wagner, J. W. Boughman *et al.*, 2014 Genomics and the origin of species. Nature Reviews Genetics 15: 176–192.

Seljestad, G. W., M. Quintela, E. Faust, K. T. Halvorsen, F. Besnier *et al.*, 2020 "A cleaner break": Genetic divergence between geographic groups and sympatric phenotypes revealed in ballan wrasse (*Labrus bergylta*). Ecology and Evolution 00: 1–16.

Servedio, M., 2009 The role of linkage disequilibrium in the evolution of premating isolation. Heredity 102: 51–56.

Servedio, M. R., and R. Burger, 2018 The effects on parapatric divergence of linkage between preference and trait loci versus pleiotropy. Genes 9: 217.

Servedio, M. R., and M. Kopp, 2012 Sexual selection and magic traits in speciation with gene flow. Current Zoology 58: 510–516.

Servedio, M. R., G. S. Van Doorn, M. Kopp, A. M. Frame and P. Nosil, 2011 Magic traits in speciation: 'Magic' but not rare? Trends in Ecology & Evolution 26: 389–397.

Sick, K., 1961 Haemoglobin polymorphism in fishes. Nature 192: 894–896.

Simmons, L. W., 2001 *Sperm competition and its evolutionary consequences in the insects*. Princeton University Press, Princeton, New Jersey.

Simmons, L. W., and J. L. Fitzpatrick, 2012 Sperm wars and the evolution of male fertility. Reproduction 144: 519–534.

Smadja, C. M., and R. K. Butlin, 2011 A framework for comparing processes of speciation in the presence of gene flow. Molecular Ecology 20: 5123–5140.

Smuts, B. B., and R. W. Smuts, 1993 Male aggression and sexual coercion of females in nonhuman primates and other mammals: Evidence and theoretical implications. Advances in the Study of Behavior 22: 1–63.

Stanley, R. R., C. DiBacco, B. Lowen, R. G. Beiko, N. W. Jeffery *et al.*, 2018 A climate-associated multispecies cryptic cline in the northwest Atlantic. Science Advances 4: eaaq0929.

Swenson, N. G., and D. J. Howard, 2005 Clustering of contact zones, hybrid zones, and phylogeographic breaks in North America. The American Naturalist 166: 581–591.

Tajima, F., 1989 Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585–595.

Tian, D., Q. Wang, P. Zhang, H. Araki, S. Yang *et al.*, 2008 Single-nucleotide mutation rate increases close to insertions/deletions in eukaryotes. Nature 455: 105–108.

Trivers, R. L., 1972 Parental investment and sexual selection, pp. 136–179 in *Sexual selection and the descent of man*, edited by B. Campbell. Aldine-Atherton, Chicago.

Van Valen, L. M., 1973 A new evolutionary law. Evolutionary Theory 1: 1–30.

Vetsigian, K., and N. Goldenfeld, 2005 Global divergence of microbial genome sequences mediated by propagating fronts. Proceedings of the National Academy of Sciences of the United States of America 102: 7332–7337.

Weggelaar, T. A., D. Commandeur and J. M. Koene, 2019 Increased copulation duration does not necessarily reflect a proportional increase in the number of transferred spermatozoa. Animal Biology 69: 95–115.

Weist, P., F. M. Schade, M. Damerau, J. M. Barth, J. Dierking *et al.*, 2019 Assessing SNP-markers to study population mixing and ecological adaptation in Baltic cod. PLoS One 14: e0218127.

Wellenreuther, M., and L. Bernatchez, 2018 Eco-evolutionary genomics of chromosomal inversions. Trends in Ecology & Evolution 33: 427–440.

Wellenreuther, M., C. Merot, E. Berdan and L. Bernatchez, 2019 Going beyond SNPs: The role of structural genomic variants in adaptive evolution and species diversification. Molecular Ecology 28: 1203–1209.

West-Eberhard, M. J., 2005 Developmental plasticity and the origin of species differences. Proceedings of the National Academy of Sciences of the United States of America 102: 6543–6549.

Westram, A. M., M. Rafajlovic, P. Chaube, R. Faria, T. Larsson *et al.*, 2018 Clines on the seashore: The genomic architecture underlying rapid divergence in the face of gene flow. Evolution Letters 2: 297–309.

Wojcieszek, J. M., and L. W. Simmons, 2013 Divergence in genital morphology may contribute to mechanical reproductive isolation in a millipede. Ecology and Evolution 3: 334–343.

Wolfner, M. F., 1997 Tokens of love: Functions and regulation of *Drosophila* male accessory gland products. Insect Biochemistry and Molecular Biology 27: 179–192.

Wood, H. L., G. Nylund and S. P. Eriksson, 2014 Physiological plasticity is key to the presence of the isopod *Idotea baltica* (Pallas) in the Baltic Sea. Journal of Sea Research 85: 255–262.

Yang, S., K. Jiang, H. Araki, J. Ding, Y. H. Yang *et al.*, 2007 A molecular isolation mechanism associated with high intra-specific diversity in rice. Gene 394: 87–95.

Yusa, Y., 1994 Factors regulating sperm transfer in an hermaphroditic sea hare, *Alypsia parvula* Mörch, 1863 (Gastropoda: Opishobranchia). Journal of Experimental Marine Biology and Ecology 181**:** 213–221.