NUCELLA LAPILLUS L.: IMPOSEX AFTER TRIBUTYLTIN (TBT) BAN AND POPULATION CONNECTIVITY STUDIES

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To my Family

"At about half way up I heard a wild yell from Dr. Mathewson, who was in the lead. A moment later the four of us were dancing around a huge black tortoise about 4 feet long. (…) I fell on his shell and embraced him."

Pilsbry, H. A. (1929) Notes from the Pinchot south sea expedition. The Nautilus 43: 37-38

"The Orion was a ship that had been ailing for a long time; in the course cruises thick layers of barnacles had collected on its keel to such a degree as to deprive it of half its speed; it had gone into the dry dock the year before this, in order to have the barnacles scraped off, then it had put to sea again (…)."

Hugo, V. (1868) The Miserables. Lacroix, A., Verboeckhoven & Ce. Brussel.

Rodolfo Barreiro Lozano and José Miguel Ruiz de la Rosa from the University of A Coruña

Certify:

That the following report entitled "*Nucella lapillus* L.: imposex after tributyltin (TBT) ban and population connectivity studies" written by Miss Belén Carro Espada has been prepared under their supervision in the Department of Biology at the Science Faculty of the University of A Coruña, within the framework of the Official PhD International Program DO*MAR Marine Science, Technology and Management regulated by RD 99/2011 and it meets the requirements to be defended and to aspire to the degree of PhD.

And for any legal statement, the present document is signed in A Coruña, January 20, 2021.

Dr. Rodolfo Barreiro Lozano Dr. José Miguel Ruiz de la Rosa

ABSTRACT

The gastropod *Nucella lapillus* is the most common predator in the European Atlantic intertidal rocky shores. Its populations have been exposed to the pollutant tributyltin (TBT) for years, which induced the development of male sexual characters onto females (imposex). At high concentrations, it produces sterility and can cause population to collapse. The use of TBT has been regulated until its total ban in 2003. *N. lapillus* is the sentinel organism for its biomonitoring. This thesis add new information to the data collected in Galician coast since 1996, and explores the effectiveness of the legislation. Furthermore, population recovery in the marine environment greatly rely on the arrival of new individuals. *N. lapillus* is a direct-developer and is assumed to have a limited dispersal. Here, AFLP markers were used to investigate the population genetic structure of *N. lapillus* to elucidate its actual dispersal capacity and thus its ability to recover. Sampling was designed to test if hydrodynamic conditions have an influence on the resulting genetic pattern. In addition, a genome scan for outlier loci was performed among the AFLP markers to explore if wave-exposure conditions were promoting local adaptation under isolation. Finally, the temporal genetic stability was investigated by sampling several generations.

RESUMEN

El gasterópodo *Nucella lapillus* es el depredor más común del intermareal rocoso del Atlántico Europeo. Sus poblaciones han estado expuestas durante años al tributilestaño (TBT), un contaminante que induce el desarrollo de caracteres sexuales masculinos en hembras de gasterópodos (imposex). A altas concentraciones conduce a la esterilidad, y finalmente a la extinción de las poblaciones. En 2003, y tras sucesivas regulaciones, se prohíbe su uso. *N. lapillus* es la especie bioindicadora oficial para su biomonitorización. Esta tesis añade nuevos datos a los recopilados en la costa gallega desde 1996 y comprueba la eficacia de la legislación implementada. En el medio marino, la recuperación de las poblaciones depende de la llegada de nuevos individuos. *N.lapillus* es una especie de desarrollo directo con una capacidad de dispersión limitada. Mediante el uso de marcadores AFLP, se investigó su estructura genética poblacional para deducir su capacidad real de dispersión y por tanto sus opciones de recuperación. El muestreo se diseñó para testar si las condiciones hidrodinámicas influyen en el patrón genético resultante. Además, se realizó un escaneo genómico en busca de loci atípicos entre los AFLPs para averiguar si la exposición al oleaje está promoviendo adaptación local bajo aislamiento. Por último, se investigó la estabilidad genética temporal mediante el muestreo de diferentes generaciones.

RESUMO

O gasterópodo *Nucella lapillus* é o depredador máis común do intermareal rochoso do Atlántico Europeo. As súas poboacións víronse durante anos expostas ó tributilestaño (TBT), un contaminante que induce o desenvolvemento de caracteres sexuais maculinos en femias de gasterópodos (imposex). A altas concentracións leva á esterilidade e finalmente á extinción das poboacións. No 2003, e tras sucesivas regulacións, prohíbese o seu uso. *N. lapillus* é a especie bioindicadora oficial para a súa biomonitorización. Esta tese engade novos datos ós recollidos na costa galega dende o 1996 e comproba a eficacia da lexislación implementada. No medio mariño, a recuperación das poboacións depende da chegada de novos individuos. *N. lapillus* é unha especie de desenvolvemento directo cunha capacidade de dispersión limitada. Mediante o uso de marcadores AFLP, investigouse a súa estrutura xenética de poboacións para deducir a súa verdadeira capacidade de dispersión e polo tanto as súas opcións de recuperación. A mostraxe deseñouse para testar se as condicións hidrodinámicas inflúen no patrón xenético resultante. Ademais, realizouse un escaneo xenómico na procura de loci atípicos entre os AFLPs para pescudar se a exposición á ondada promove adaptación local baixo illamento. Por último, investigouse a estabilidade xenética temporal mediante a mostraxe de diferentes xeracións.

CONTENTS

INTRODUCTION

THE MARINE SNAIL *NUCELLA LAPILLUS*

At low tide, we can find a dweller who is the most common predator in our intertidal rocky shores, from South Portugal to Northern Russia, spreading also along the Northern West Atlantic coast of North America (Crothers 1985, Fretter & Graham 1985). *Nucella lapillus* (Linnaeus 1758) is a marine snail belonging to the order Neogastropoda, which distinguishes it as a dioecious carnivore with a concentrated nervous system, siphon, and a spiral and horny operculum. Its habitat is full of barnacles and mussels, sedentary preys on which dogwhelks mostly feed, *Semibalanus balanoides* being its favorite (Crothers 1985). *N. lapillus* feeds on them mechanically and chemically drilling holes in their shells. The optimal size of a mussel for a 30 mm *N. lapillus* specimen is around 20 mm (Bayne & Scullard 1978). The average feeding rate is 0.7 of a mussel or 16 barnacles per week at 20°C (Largen 1967). This means that *N. lapillus* can dominate the distribution of its prey species (Crothers 1985, Ehlers *et al.* 2018) and shape the intertidal landscape at sites with pleasant conditions. In turn, they are predominantly devoured by crabs and seabirds. Fertilization is internal and females lay about 20-30 capsules strongly attached to the substrate in crevices, each one storing hundreds of eggs (Fretter 1953). This snail is a direct-developer, so from these capsules finally emerge ca. 30 tiny crawlaways (the majority acting as nurse eggs) after a period of time that goes from 2 to 7 months in South Britain and the White Sea, respectively (Fretter & Graham 1985).

Kigdom	Animalia
Phylum	Mollusca
Class	Gastropoda
Subclass	Prosobranchia
Order	Neogastropoda
Superfamily	Muricoidea
Family	Muricidae
Genus	*Nucella*
Specie	*Nucella lapillus*

Fig. 1. *Buccinum lapillus*. Linneo shell collection

After hatching, they grow making by spiraling clockwise from the apex (the vestigial protoconch) by adding material to the lip from the mantle edge. The mantle epithelium secretes conchiolin impregnated with calcium carbonate, and then adds calcium carbonate over the entire inner surface, which adopts the form of the snail body (i.e. of the visceral hump) whorl after whorl (Fretter & Graham 1994a). At maturity, they stop growing and thicken the outer lip, even developing teeth on it to close the entrance to predator attacks (a major concern in sheltered areas). They grow up to 20-35 mm long (Crothers 1985). Snails stop growing when they start to reproduce, usually when three years old. Dogwhelks can live 6 years or more, and females outlive males. As a result, females are the oldest, largest and most abundant in dogwhelk populations (Moore 1938, Feare 1970b, Fretter & Graham 1994a, Son & Hughes 2000). Mortality is around 90, 50 and 27% of the individuals in a population in the first, second and third years, respectively.

The taxon *N. lapillus* was proposed soon, and already in Linnaeus' masterpiece *Systema Naturae* it is named *Buccinum lapillus* (Linnaeus 1758) (Fig. 1). From the beginning, this snail attracted the attention of

scientists because of the variability of colors and banding patterns of its shell (Colton 1922, Moore 1936, 1938). *N. lapillus* displays pronounced polymorphisms that are correlated with environmental variables (Bantock & Cockaine 1975, Crothers 1985, Harris & Jones 1995, Rolán *et al.* 2004). Remarkable are the two morphotypes strongly linked to wave exposure (Fig. 2). Dogwhelks develop a shorter, squatter shell with a larger broad aperture on exposed shores, while they grow a longer, thicker shell and with narrower aperture on more sheltered ones (Crothers 1985). Larger apertures prevent dislodgement on exposed shores but lead to increased desiccation on sheltered ones no subjected to wave spray. Besides, larger apertures are especially disadvantageous on sheltered shores because of the risk of crab predation. Therefore, there is an advantage in having ecotypes adapted to different environmental conditions. When gene flow is prevented, as it might be for a poor disperser such as the dogwhelk, local genetic differences may accumulate. However, quantifying the relative importance of the genetic component and phenotypic plasticity in determining shell morphology in invertebrates is challenging. Phenotypic plasticity is often linked to species with a higher dispersal ability than might be anticipated for *N. lapillus*. On the other hand, some studies suggested a genetic basis for shell morphology (Rolán *et al.* 2004, Pascoal *et al.* 2012b), and even divergent selection could be acting at one or a few genomic loci (Guerra-Varela *et al.* 2009).

A

B

Fig. 2. *Nucella lapillus* shell polymorphism. Adults specimens from A, Malpica (Galicia, exposed coast); and B, Santa Cristina (Galicia, sheltered coast)

SETTING AN EYE ON TBT

A remarkable fact in the history of *Nucella lapillus* that led it to be in the spotlight is its relation with the pollutant tributyltin (TBT). TBT is a simple molecule (a tin atom covalently bound to three butyl substituents) whose toxicity was widely employed in paints for submerged structures (Bennett 1996). The function of these antifouling coatings is to delay the growth of organisms that can ruin these structures, resulting in economic losses. Biofouling on ships increases the roughness of the hull surface, leading to an increase in drag and fuel consumption and a decrease in top speed. The increase in fuel consumption of a ship after operating for 6 months in temperate waters was estimated to be 35-50% (see Schultz *et al.* 2011 for a rationale). Abbott *et al.* (2000) estimated fuel savings thanks to TBT of 7.3 million t per year (a saving equivalent of US $730 million), taking into account a major part of the world commercial fleet in 1989. The antifouling efficiency of TBT led to its use worldwide and, consequently, to its release in all marine waters.

Early, a direct link between TBT and many invertebrate malformations was established (Smith 1981a, b, Alzieu 1996, de Mora 1996). Diluted in seawaters worldwide, TBT has affected hundreds of non-target species (see Sousa *et al* 2014 for a review). There was further evidence that TBT was entering the food chain and might therefore adversely affect human health (Sousa *et al.* 2014). In fact, it was recognized as the most pollutant substance intentionally released into the marine environment by human hands (Goldberg 1986). However, it was not until the collapse of oyster culturing in France due to malformations and a lack of recruitment, the well-known episode of the Arcachon Bay in the early 1980s, that the use of TBT started to be partially regulated (see Alzieu 2000). Moreover, it was not until 2003 that TBT was totally prohibited in European coasts, that is, fifty years after the discovery of its biocidal properties in the early 50's. By 17 September 2008 it was banned for the Parties to the International Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention, adopted by the International Marine Organization -IMO- to protect the aquatic environment), twelve months after 25 states (representing over 25% of the world merchant shipping tonnage) had ratified it. Nowadays, the number of contracting states is 90, 96.13% of the gross tonnage of the world merchant fleet (IMO 2020).

Among the organisms severely affected by TBT, *N. lapillus* displays the best documented cause-effect, dose-dependent relationship (Gibbs *et al.* 1987), making it the preferred sentinel organism for TBT pollution monitoring (OSPAR 2003). The term imposex was coined by Smith

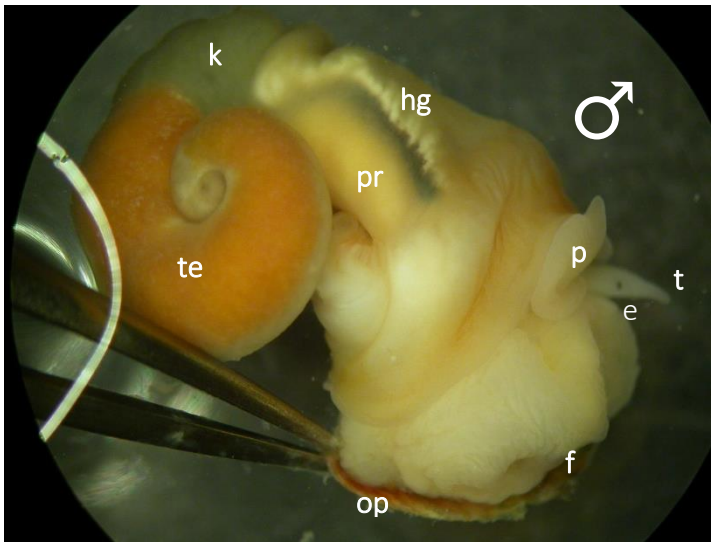
(1971) to denote the superimposition of male sexual characters onto females of marine dioecious gastropods (see Fig. 3 for a comparison between male and female specimens of *N. lapillus*). TBT was demonstrated to act as an endocrine disruptor, capable of mimicking or antagonizing the action of hormones and causing abnormal development of organisms. Underlying mechanisms were further studied with more up-to-date techniques by Pascoal *et al.* (2013), where they explored gene expression with next generation sequencing (NGS) in *N. lapillus* after TBT treatments. In addition to the three previously proposed metabolic pathways (i.e. steroid, neuroendocrine and retinoid, Sternberg *et al.* 2010) a fourth mechanism arose, the PPAR pathway (putative peroxisome proliferator-activated receptor). Their results also pointed out that TBT may be acting over both the immune and endocrine systems.

Imposex is a gradual phenomenon that can be quantified by a number of indexes. The *vas deferens* sequence index (VDSI) and the relative penis size index (RPSI) are the most prominent two. RPSI gives the relative size of the female penis to the male penis in a given population as a percentage. VDSI is the mean of the VDS of females in the population. The VDS employed for *N. lapillus* has six stages, from a healthy female with no trace of imposex (stage 0), through intermediate stages as the *vas deferens* extends and penis grows (stages 2 and 3), until the *vas deferens* is fully developed and the females penis resembles that of a male of the population (stage 4) (Fig. 4). With a high degree of exposure to TBT, the *vas deferens* grows up to clog the vulva

(stage 5) and prevent the release of egg capsules, which remain aborted inside the capsule gland (stage 6). At this point, females are sterile and the population will decrease and eventually become extinct. However, after the EU ban on TBT in 2003, we are in a scenario of progressive decrease of TBT. The BioCost research group of the Universidade da Coruña has been monitoring the presence of TBT in the Norhwest of the Iberian Peninsula since 1996 (Barreiro *et al.* 1998, Ruiz *et al.* 1998, Barreiro *et al.* 1999, Quintela *et al.* 2000, Barreiro *et al.* 2001, Barreiro *et al.* 2006, Quintela *et al.* 2006b, Ruiz *et al.* 2008, Ruiz *et al.* 2010). The data accumulated by the group provides a unique opportunity to assess the evolution of TBT from its earlier regulations to its total ban. In addition, after the European ban, TBT biomonitorization is mandatory as waters must meet European Quality Standards (EQS) (OSPAR 2004, EC 2008).

In a TBT-free environment, previously damaged dogwhelk populations could now recover by the arrival of new specimens from neighboring, less affected populations. The rate at which *N. lapillus* will be able to recolonize places where it had disappeared due to TBT pollution will be determined by its dispersal capacity. Until now, conflicting estimates of the dispersal capacity have been obtained using different molecular markers (Day *et al.* 1994, Colson & Hughes 2004). Considering the still open questions about the population dynamics of *N. lapillus*, a key member of the intertidal landscape, we would like to contribute new data to elucidate the true dispersal potential of dogwhelks.

A



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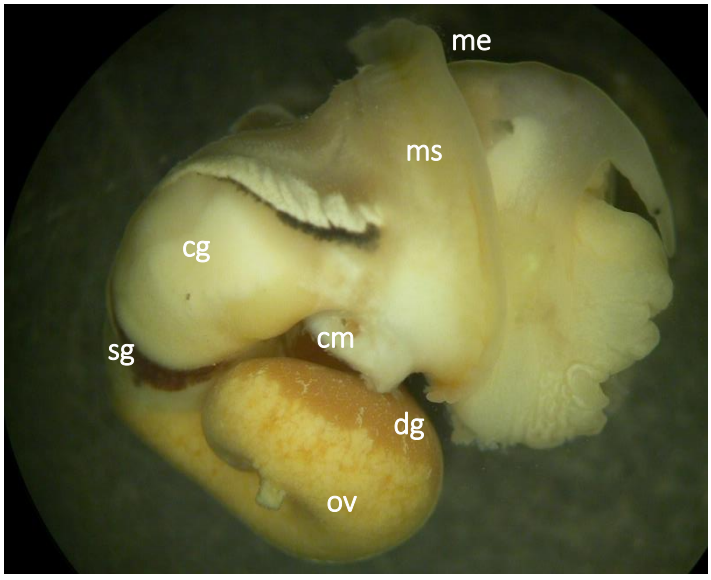
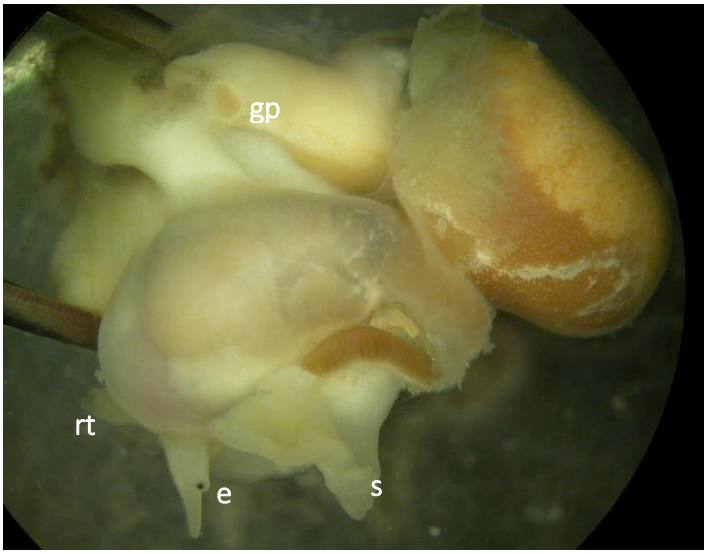


Fig. 3. *Nucella lapillus* removed from shell and seen from the right side. A, external features of mature male and B, mature female. Abbreviations: (cg) capsula gland, (cm) columellar muscle, (dg) digestive gland, (e) eye, (f) foot, (hg) hypobranchial gland, (k) kidney, (me) mantle edge, (ms) mantle skirt, (op) operculum, (ov) ovary, (p) penis, (pr) prostate, (sg) sperm-ingesting gland (t) tentacle, (te) testis.

A



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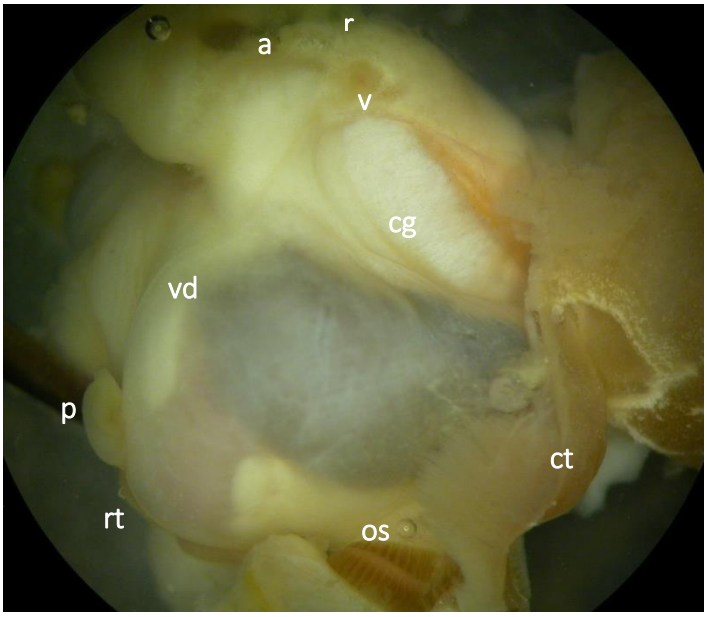

Fig. 4. *Nucella lapillus* removed from its shell with the mantle skirt cut longitudinally along the mid-dorsal line to display pallial structures. A, *vas deferens* stage (VDS) 0 that corresponds to a healthy female; and B, VDS 4 that corresponds to a female with proximal and distal sections of *vas deferens* fused and penis similar to that of a male. Abbreviations: (a) annus, (ct) ctenidium, (e) eye, (gp) genital papilla, (os) osphradium, (p) penis, (r) rectum, (rt) right tentacle, (s) siphon, (v) vulva, (vd) *vas deferens.*

STUDYING MARINE NATURAL POPULATIONS

The study of population dynamics is a major issue in the maintenance and management of natural communities and in the conservation of marine diversity (Hedgecock *et al.* 2007, G. P. Jones *et al.* 2007, Cowen & Sponaugle 2009).The increase and intensity of human threat to the oceans accelerates the loss and fragmentation of marine habitats, increasing the risk of extinction of marine species. The effective design of marine protected areas relies on understanding the dispersal mechanisms, key to determining the optimal size, configuration and location. Understanding the dynamics of fisheries resources facing several pressures (such as overfishing, eutrophication and habitat destruction), understanding and controlling the distribution of invasive species, and investigating the role of dispersal in the process of adapting species to changes in their range (such as those caused by climate change), is also critical (Palumbi 2003, Shanks *et al.* 2003, Bradbury *et al.* 2008). Moreover, assessing the degree of connectivity between different populations may have important implications for applied ecology (Grosberg & Cunningham 2001, Hellberg *et al.* 2002, Kinlan & Gaines 2003, Palumbi 2003). Tools to deal with this are divided into direct and indirect methods (Grosberg & Cunningham 2001, Palumbi *et al.* 2003). Direct methods imply observation and monitoring of individuals and propagules which in marine environment is, at the very least, arduous. The unmanageable size of the environment and the small size of the organisms make it difficult to access and monitor them (Hellberg *et al.* 2002). Therefore, interpretations based on the biology

of the organisms have often been used. The vast majority of marine species possess larvae that are released into the sea and are considered a means of dispersal over vast distances, which would keep populations connected. This gave rise to the traditional idea of open populations in the marine environment. Furthermore, even if individuals could be tracked, their impact in the host population remains elusive. Thus, alternative methods have been used to study population dynamics (Palumbi 2003). Among these, the analysis of the geographic patterns of genetic variation in populations can be used to estimate gene flow (Slatkin 1987, Bossart & Pashley Prowell 1998, Hellberg *et al.* 2002). Studying the genetic composition of individuals has the additional advantage that it only reports those that managed to reproduce and have an impact on the host population, and can provide a compendium of final information about the history and characteristics of natural populations (Hellberg *et al.* 2002). Using appropriate genetic markers and genetic population models, is possible to infer information about the biology of organisms, since the processes that affect populations (i.e. reproductive success, migration, population size, natural selection and historical events) leave an indelible signal in their genome (Sunnucks 2000, Grosberg & Cunningham 2001).

Despite the relatively long tradition of biological studies with *N. lapillus*, many uncertainties remain about key aspects of its biology. Its life-cycle attributes (direct development, apparently sedentary adults) suggest a minimal dispersal, and this is the conventional assumption in the older literature (Colton 1922, Moore 1936, 1938, Moore & Sproston 1940,

Staiger 1957, Berry & Crothers 1968, Feare 1970a, 1971, Hoxmark 1971, Largen 1971). However, studies with molecular markers have suggested a somewhat unexpectedly high dispersion capacity (Colson & Hughes 2003, Bell & Okamura 2005, Colson *et al.* 2006) even over huge distances of hundreds of kilometres (Day *et al.* 1994, Wares & Cunningham 2001, Colson & Hughes 2007). If we take *N. lapillus* as a model organism, elucidating its true dispersal capacity may contribute to understanding the population dynamics of marine invertebrates.

To investigate the population dynamic of *N. lapillus*, AFLP markers (Vos *et al.* 1995) were selected rather than other tools because of their ability to generate a high number of markers widely distributed along the entire genome of an organism. They are versatile and flexible and can be applied without any prior knowledge of the target species (Bensch & Akesson 2005, Paun & Schönswetter 2012). Furthermore, AFLPs are highly reproducible and robust, with a modest investment in development time, as well as cost-effective. The AFLP technique is based on PCR amplification of subsets of restriction fragments from a total digest of genomic DNA (Fig. 5). Nucleotide changes at restrictions sites (e.g. mutations, insertions) generate fragments of different length. The technique records the presence/absence of fragments of any given length that results in a unique fingerprint for each individual (Vos *et al.* 1995).

AFLP markers also have some drawbacks. They are dominant markers and it is not possible to discriminate between homozygotes and heterozygotes when a fragment of a given length is present. For this

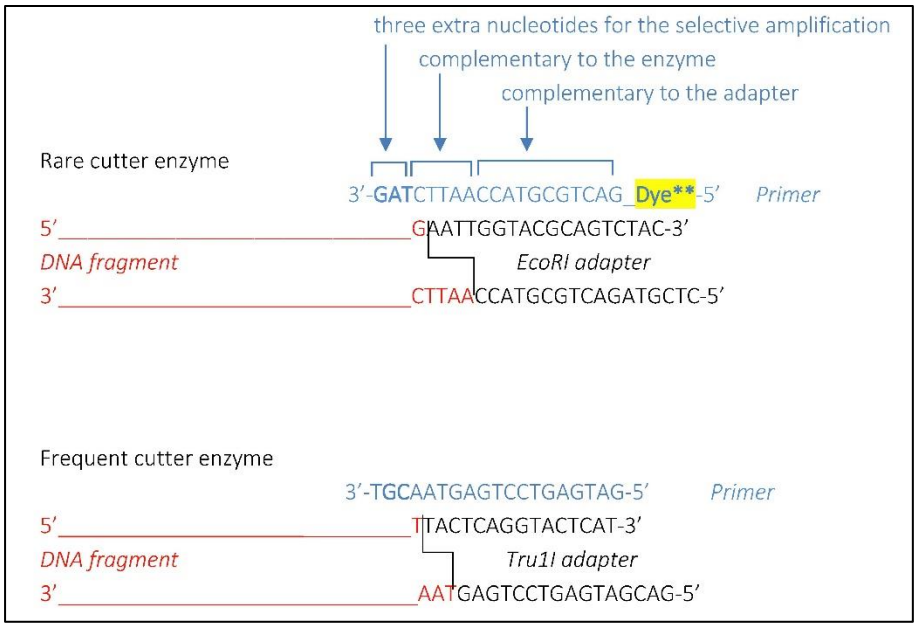

Fig. 5. AFLP principles. Restriction is made with two enzymes with different cutting rate. An adapter is ligated to the resulting sticky ends that supplies the sequence for anchoring the amplification primers. The design of these primers with selective bases inward to the fragment allows the amplification of a subset of fragments. This reduces the complexity of the resulting fingerprint.

reason, a minimum number of AFLPs should be analyzed with proper toolkits (Blears *et al.* 1998, Bonin *et al.* 2004, Bonin *et al.* 2007). In addition, to ensure the robustness and reproducibility of the technique, DNA has to be of certain quality to preserve the consistency of the restriction enzymes. Yet, by adhering to these guidelines, AFLPs may be one of the best options.

OBJECTIVES

- To study the evolution of imposex and biometric indices, and the relationship between them, in the marine gastropod *Nucella lapillus* over more than 10 years in populations along the Galician coast (Chapter I)
- To verify the achievement of the Ecological Quality Objective (EcoQO) stated by the European Union (EU) after the TBT ban (Chapter II)
- To verify the real dispersal capacity of *N. lapillus* by studying its population genetic structure with AFLP markers, as having restricted gene flow could compromise the survival of populations affected by TBT (Chapter III)
- To investigate if wave exposure is having an impact on the resulting pattern of genetic structure by sampling areas subjected to contrasting wave regimens (Chapter III)
- To evaluate the different contribution of phenotypic plasticity and divergent selection in shaping the pronounced morphtypes exhibited by *N. lapillus* in response to wave exposure (Chapter IV)
- To investigated if wave action could be promoting or constraining the exchange of individuals among populations in exposed shores by exploring the stability of the genetic structure over generations (Chapter V)
- To explore the underlying mechanism behind the different population genetic structure exhibited by *N. Lapillus* on exposed and sheltered coasts (Chapter V)

CHAPTER I

A DECADAL STUDY OF BIOMETRIC AND IMPOSEX INDICES IN *NUCELLA LAPILLUS* L.

ABSTRACT

We have monitored tributyltin (TBT) pollution in Galicia (NW Spain) for more than a decade by means of assessing gastropod imposex in populations of *Nucella lapillus* (N ≥ 34) at regular intervals. Several thousand specimens were processed to obtain their shell height (SH), penis length (PL) and *vas deferens* sequence (VDS); imposex indices (including the VDS index, VDSI) were subsequently calculated. The regional mean SH of both females and males has not changed significantly throughout the study. This also applies to the mean male PL. On the contrary, the regional mean female PL (MFPL) and all imposex indices significantly decreased. Results confirm previous conclusions based on the chemical analyses of tissues and partial imposex observations. In addition, the close correlations between MFPL and VDSI show some potential applications to TBT biomonitoring.

INTRODUCTION

Imposex (i.e. the superimposition of male sexual characteristics – penis included – onto female gastropods; Smith 1971) has been reported for hundreds of species worldwide (e.g. Shi *et al.* 2005). The firm link established between this anomaly and tributyltin (TBT) pollution from antifouling paints led OSPAR (the Convention for the Protection of the Marine Environment of the NE Atlantic) to include its recording within the mandatory monitoring programme (OSPAR 2004). The species recommended was *Nucella lapillus* L. (a rocky shore carnivore, see Bryan *et al.* 1986). Even if imposex is largely irreversible, its monitoring is an effective way of assessing changes in TBT effects at the population level. Many works have since shown imposex decrease as a result of legal restrictions, both within the OSPAR area (Guomundsdottir *et al.* 2011, Wilson *et al.* 2015, Laranjeiro *et al.* 2018) and beyond (Harding *et al.* 2013, Kim *et al.* 2017, Cacciatore *et al.* 2018).

In Galicia (NW Spain) we started monitoring TBT at a regional scale with *N. lapillus* in 1996. Results over more than one decade have proved substantial improvement as determined by both the TBT body burden (Ruiz *et al.* 2015) and some partial imposex observations (Ruiz *et al.* 2017). This continued effort has also compiled some considerable databases on biometric and imposex indices that deserve further exploration. Thus, the present work aims at detailing and summarizing this regional study to produce some insights on both basic marine biology features and their potential application to pollution monitoring.

MATERIALS AND METHODS

SAMPLING AND IMPOSEX ASSESSMENT

The baseline survey was conducted in July–September of 1996 and sampled 37 populations; their location is detailed in a print map and a Google one (see Ruiz *et al.* 1998, 2017, respectively), and their UTM coordinates are given in Table 1. All of them were revisited in the summer of 2003 and every 3 years thereafter, but unknown factors precluded the collection of a sufficient sample in one site per subsequent summer campaign (i.e. n = 37, 36, 35 and 34, respectively for 1996, 2003, 2006 and 2009). The collection and treatment of specimens is described in Ruiz *et al*. (1998, 2017), where full details (including maps, shell and penis measurements, and participation in QUASIMEME international ring tests) have been previously published. Briefly, sampling by hand during low tide aimed at collecting ≥30 toothed (i.e. adult) animals, which were within a few hours transported in a cooler to the laboratory. They were there kept in aquaria with seawater (35 psu, 15 ± 1 °C) for a minimum of 24 h before dissection under a stereomicroscope; evidently parasitized specimens were rejected. Finally, opercula were removed and each sex was pooled per site in vials that were frozen until the chemical analysis of female tissues (see results in Ruiz *et al*. 2015). Shell height (SH) was measured to the nearest 0.01 mm with digital callipers before extracting the soft parts for close examination; only specimens within an established SH range (from 17.5 to 32 mm, see Ruiz *et al.* 2017, for rationale) are dealt with here. Every penis length (PL) was gauged as above for SH and imposex

assessed as proposed by Gibbs *et al.* (1987) and recommended by OSPAR (2003); aphallic females were classified according to a very similar scheme by Barreiro *et al.* (1999). The parameters determined in individual samples were: (i) The incidence or percentage of females with any sign of imposex (I%). (ii) The mean female penis length (MFPL). (iii) The mean male penis length (MMPL). (iv) The relative penis size index (RPSI = 100 × [$MFPL^3/MMPL^3$]). (v) The *vas deferens* (VD) sequence index (VDSI): the arithmetic mean of all the *vas deferens* sequence (VDS) values (integers from 0 to 6) assigned to single females. The first indication of imposex in this species (and therefore the individual VDS score of 1) typically is the proximal section of the VD. The VDSI data reported here have been previously published by Ruiz *et al.* (2017).

STATISTICAL TREATMENT

After checking for normality and variance homogeneity, the mean shell height of both females and males (MFSH and MMSH, respectively) in the four surveys (n = 34–37) were subject to parametric ANOVA in order to explore differences within sex. Later, differences between sexes were checked with a pairwise contrast, either a parametric *t*-test when datasets were normally distributed or a Mann–Whitney (Wilcoxon) W-test (MWW) when sets were not so.

As for all the other variables considered (MFPL, MMPL, I%, RPSI and VDSI), their change over time was explored as in Ruiz *et al.* (2017) for VDSI with pairwise contrasts testing for differences between every couple of consecutive surveys. In addition, for these tests to be as robust as possible, the number of populations considered in each contrast

equalled to the lowest of each pair of successive surveys: n = 36 for 1996 vs. 2003, n = 35 for 2003 vs. 2006, and n = 34 for 2006 vs. 2009. The significance levels are depicted below as NS, *, ** and ***, respectively for $P > 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.001$. It has to be noted that non-parametric methods are generally considered to be simpler and more robust than parametric ones but, in turn, the latter are more powerful.

RESULTS

Biometric results for the four surveys (n = 37, 36, 35 and 34, respectively for 1996, 2003, 2006 and 2009) are shown in Table 1; they all refer to specimens of the selected size (i.e. SH from 17.5–32 mm). A total of 5063 individuals distributed over 142 samples were considered, and in only 8 out of them there were fewer than 6 representatives of either sex. The mean number of animals computed per site in each campaign ranged from 15 ± 5 to 22 ± 5 and from 12 ± 4 to 19 ± 6, respectively for females and males. The mean shell height for females (MFSH) and males (MMSH) ranged from 24.6 ± 2.5 to 25.5 ± 2.8 and from 23.9 ± 2.6 to 24.8 ± 2.9, respectively. This variation, however, did not result in significant MSH differences within sex along the four surveys (ANOVA, F = 0.64 and 0.68, respectively for MFSH and MMSH, $P > 0.5$ in both cases); the differences between sexes were neither statistically relevant (MWW test for n = 142, NS). As for the mean penis length, in 1996 the regional MFPL was 1.86 ± 0.66 (n = 37), and the situation showed little change by the onset of the 2003 EU total TBT ban (1.70 ± 0.61, *t*-test for n = 36, NS). However, this regulation quickly caused MFPL to decrease at virtually all sites, so that the regional average significantly diminished in

Table 1. Biometric data for samples of *Nucella lapillus* collected along four surveys, including code and site (with UTM coordinates) of the populations studied, sex (F for female and M for male) and number of individuals selected (n), mean shell height (MSH, mm) mean penis length (MPL, mm) and summary statistics.

			1996 (n = 37)			2003 (n = 36)			2006 (n = 35)			2009 (n = 34)		
Code	Site (coordinates)	Sex	n	MSH	MPL	n	MSH	MPL	n	MSH	MPL	n	MSH	MPL
1	Ribadeo[a]	F	23	22.7	1.27	21	20.8	1.12	13	19.6	0.85	17	21.0	0.00
	(29TPJ587240)	M	7	22.0	2.80	21	20.4	1.96	12	19.7	1.63	18	19.8	1.62
2	Foz	F	25	21.7	1.41	33	20.3	0.97	14	20.1	0.58	29	20.7	0.00
	(29TPJ410267)	M	5	20	3	17	19.6	2.15	17	19.4	2.14	11	19.6	2.02
3	Viveiro	F	21	26.5	1.91	21	26.3	1.42	28	25.5	0.38	26	24.3	0.6
	(29TPJ119372)	M	9	26.5	4.02	20	26.2	2.46	16	24	2.72	14	22.7	2.78
4	Barqueiro	F	14	29.7	2.8	22	26.9	0.7	26	27.4	0.71	17	26.6	0.47
	(29TPJ065433)	M	12	29.7	4.18	19	26.4	2.59	18	27.1	2.6	21	26.9	2.28
5	Cariño	F	18	23	2.3	7	19.4	1.56	29	21.1	0.23	–	–	–
	(29TNJ914443)	M	11	22	4	7	21.5	2.93	16	20.8	2.84	–	–	–
6	Cedeira	F	22	25.1	1.93	18	28.9	2.03	21	28.3	1.25	18	27	0.27
	(29TNJ750344)	M	8	25.1	3.27	21	27.8	3.17	21	27.3	3.19	21	26.7	2.78
7	Prior	F	19	23.1	1.61	24	21.7	1.36	22	21.4	0.24	33	23.3	0.18
	(29TNJ564216)	M	11	22.1	3.82	19	21.3	2.8	20	20.3	2.84	10	20.5	2.54
8	Mugardos[a]	F	5	28.5	1.81	13	27.5	2.2	20	28.2	1.69	13	28.4	0.94
	(29TNJ591128)	M	11	26.5	3.08	15	26.6	3.19	21	27.8	3.21	23	28.5	2.72
9	Centroña[a]	F	10	28.8	1.38	19	29.4	1.43	16	25.8	0.53	16	29.7	0.38
	(29TNJ652070)	M	18	29.9	3.65	18	29.1	2.9	24	26.5	3.19	17	29.5	3.34
10	Perbes	F	13	26.8	1.01	25	26.6	1.1	26	23.7	0.17	19	27.7	0
	(29TNJ636029)	M	17	26	2.89	14	26.4	3.06	17	22.4	2.59	21	26.6	3.13
11	Sada	F	11	27.5	1.29	19	26.1	0.92	17	26	1.06	21	27.2	0.67
	(29TNJ610012)	M	8	27.9	1.98	26	24.9	2.6	24	25.5	2.3	18	27.3	1.68
12	Veigue	F	20	26.4	1.47	31	25.6	1.55	23	25.5	0.57	23	26.8	0.14
	(29TNJ573040)	M	9	26.1	2.57	18	25	2.59	20	24.3	2.3	19	26.5	2.71
13	Mera[a]	F	14	22.9	1.88	25	23.3	1.86	21	23.2	0.46	20	23.3	0.11
	(29TNJ522037)	M	15	22.2	2.62	19	22.4	2.66	21	23.3	2.84	21	23	2.57
14	Bastiagueiro[a]	F	10	23.1	2	19	23.2	1.94	20	26.3	0.38	22	25	0.23
	(29TNH520994)	M	20	23	2.39	22	22.4	2.79	21	24.3	3	19	24.5	2.59

15	Sta. Cristina	F	13	26	2.09	21	24.7	2.2	19	25	1.23	18	26.5	0.49
	(29TNH509992)	M	17	25.7	2.84	25	24	2.87	29	25.5	3.1	28	26.1	2.78
16	Dique[a]	F	5	18.6	1.33	4	19.3	1.06	12	20	0.12	16	20.7	0
	(29TNJ500022)	M	3	18.7	2.32	2	18.9	2	3	19.2	2.65	11	20.3	2.32
17	Langosteira[a]	F	9	18.7	1.14	17	19.3	0.85	23	22.4	0.09	20	19.4	0
	(29TNJ417012)	M	2	18	2.51	10	19.2	3.12	20	21.4	2.96	13	18.6	2.04
18	Malpica[a]	F	7	23.3	1.09	21	20.2	0.75	25	20.2	0.07	16	21	0
	(29TNH159970)	M	13	20.9	2.84	27	19.4	1.96	12	19	2.25	22	20.2	2.41
19	Laxe	F	17	24.7	2.59	24	23.7	2.18	20	24.9	0.6	19	25.9	0.11
	(29TNH008852)	M	13	24.4	3.79	15	23.4	3.08	22	24.3	3.33	21	24.5	2.89
20	Muxía	F	24	26.6	2.67	21	25	2.38	25	26.1	0.12	17	25.4	0
	(29TMH832724)	M	6	26	4.03	17	24	3.17	21	25.6	3.45	13	25.4	2.94
21	Corcubión[a]	F	18	25.6	2.22	19	26.2	1.92	30	26.2	0.63	21	24.3	0
	(29TMH821547)	M	12	23.7	3.96	22	25.6	3.32	18	24.3	3.28	19	23.6	3
22	Louro	F	18	25.9	1.8	9	26.7	2.5	25	25.9	0.47	24	24.6	0.06
	(29TMH908342)	M	11	24.7	3.38	31	25.6	3.7	19	24.9	3.14	14	24.2	2.66
23	Muros	F	16	28.9	2.81	16	28.7	2.15	20	27.9	1.12	15	26.9	0
	(29TMH958373)	M	9	27.5	4.27	31	28.6	3.69	23	28.1	3.01	25	26.8	2.72
24	Creo	F	12	27.6	2.9	11	29.9	1.33	–	–	–	–	–	–
	(29TNH019374)	M	17	27.1	3.52	23	29.9	3.54	–	–	–	–	–	–
25	Ribeira[a]	F	17	26.7	2.66	23	29.2	1.87	28	26.7	0.72	22	26.3	0
	(29TNH012113)	M	13	25.1	3.64	18	28.9	3.52	16	26.5	3.06	18	26.2	2.98
26	Vilagarcía[a]	F	12	29.4	1.63	8	30.2	3.08	18	27.2	1.84	10	28.9	0.26
	(29TNH181160)	M	11	28.4	3.21	10	30.8	4.23	23	26.2	3.63	17	29	3.35
27	Cambados	F	13	26.7	1.82	19	29	2.06	21	25.8	1.36	21	25.6	0.25
	(29TNH142072)	M	16	25	3.2	18	28	3.48	21	25.1	3.26	17	24.5	3.12
28	A Toxa[a]	F	6	29.4	2.41	7	29.7	2.78	21	26.8	1.2	19	27.9	0.04
	(29TNH128041)	M	11	28.1	3.44	21	28.7	3.41	15	25.5	3.25	21	26.6	2.91
29	Sanxenxo	F	11	25.6	1.64	17	26.4	2	28	24.6	0.47	29	23.7	0
	(29TNG153943)	M	19	24.5	3.08	22	26.1	2.61	15	24.4	2.12	11	23.8	2.36
30	Poio	F	9	29.3	1	–	–	–	–	–	–	–	–	–
	(29TNG259976)	M	19	28.3	3.35	–	–	–	–	–	–	–	–	–

(*Continued*)

Table 1. (*Continued*)

Code	Site (coordinates)	Sex	1996 (n = 37)			2003 (n = 36)			2006 (n = 35)			2009 (n = 34)		
			n	MSH	MPL	n	MSH	MPL	n	MSH	MPL	n	MSH	MPL
31	Marín	F	19	25.3	1.5	18	24.3	1.53	20	24	0.75	25	25.4	0.51
	(29TNG233933)	M	11	25.4	2.57	26	24.3	2.88	23	24	2.85	15	25.9	2.73
32	Rande[a]	F	11	28.6	3.12	21	28.8	2.44	22	24.8	1.78	23	26.3	1.06
	(29TNG280813)	M	13	26.6	3.99	17	27.2	3.64	20	24.4	2.98	17	25.8	3.02
33	Bouzas[a]	F	15	24.2	0.94	17	23.7	1.56	17	23	1	25	25.2	0.18
	(29TNG280813)	M	14	23.5	2.23	13	21.7	2.39	23	21.8	2.45	15	23.5	2.65
34	Samil[a]	F	16	24.5	0.66	20	25.3	1.5	27	24.2	0.26	26	24.9	0
	(29TNG184742)	M	13	24.2	2.83	11	24.4	3.01	19	21.7	2.74	14	24.3	2.64
35	Canido	F	17	25	1.44	22	25.3	0.82	26	23.6	0.84	28	25.2	0.49
	(29TNG169748)	M	11	24.9	3.01	19	24.7	2.75	18	23.2	2.22	12	24.4	2.17
36	Baiona	F	17	21.6	2.14	16	20.1	1.51	22	22.9	0.17	25	22.3	0.05
	(29TNG123636)	M	12	20.8	3.04	17	19.2	2.11	19	21.6	2.8	15	21.7	2.65
37	A Garda[a]	F	14	26.7	3.21	11	27.7	2.46	20	27.9	1.58	24	25.4	0.09
	(29TNG103390)	M	15	26.9	4.27	31	27.5	3.55	23	27.2	3.31	16	25.1	2.81
	Sum	F	541			659			765			717		
		M	442			682			670			587		
	Mean	F	15	25.5	1.86	18	25.3	1.7	22	24.6	0.73	21	25.1	0.22
		M	12	24.8	3.23	19	24.7	2.94	19	23.9	2.84	17	24.5	2.65
	SD	F	5	2.8	0.66	6	3.4	0.61	5	2.5	0.51	5	2.5	0.28
		M	4	2.9	0.62	6	3.4	0.55	5	2.6	0.45	4	2.8	0.41
	Max.	F	25	29.7	3.21	33	30.2	3.08	30	28.3	1.84	33	29.7	1.06
		M	20	29.9	4.27	31	30.8	4.23	29	28.1	3.63	28	29.5	3.35
	Min.	F	5	18.6	0.66	4	19.3	0.7	12	19.6	0.07	10	19.4	0
		M	2	18	1.98	2	18.9	1.96	3	19	1.63	10	18.6	1.62

Female n and MSH were first published by Ruiz *et al.* (2017).

[a]: Butyltin tissue concentrations were repeatedly determined in these 17 sites, see Ruiz *et al.* (2015).

2006 (0.73 ± 0.51, *t*-test for n = 35, ***) and further from here to 2009 (0.22 ± 0.28, MWW test for n = 34, ***), when 12 samples were for the first time composed of only penis-free females. On the other hand, MMPL appeared to decrease from 1996 (3.23 ± 0.62, n = 37) to 2003 (2.94 ± 0.55), but differences were not significant (MWW test for n = 36, NS); thereafter it remained quite similar in 2006 (2.84 ± 0.45, *t*-test for n = 35, NS) and 2009 (2.65 ± 0.41, *t*-test for n = 34, NS). This may be better appreciated in Figure 1 plotting the mean penis length in all samples of both sexes vs. their corresponding mean shell height; MMPL values in Table 1 have been multiplied by 2.2 for a better view. There was a significant linear relationship for males (n = 142, R^2 = 0.30, ***) and for each one of the three female groups as well: 1996–2003 (n = 73, R^2 = 0.20, ***), 2006 (n = 35, R^2 = 0.31, ***) and 2009 (n = 34, R^2 = 0.19, **).

Table 2 collects the values of all three imposex indices; those for RPSI in 1996 were first published by Ruiz *et al.* (1998), but some may be different here because now we have computed all females within the established SH range (see above). The evolution of I% and RPSI along these 13 years closely mirrors the VDSI decrease first published in Ruiz *et al.* (2017), and can be better appreciated in Figure 2. Values for I% were highest in 1996 (95 ± 12, n = 37) and 2003 (95 ± 9), with no significant differences between them (MWW test for n = 36, $P > 0.05$). However, they declined later in 2006 (63 ± 22, MWW test for n = 35, ***) and further from here to 2009 (52 ± 22, *t*-test for n = 34, *). Similarly, RPSI was topmost in 1996 and 2003 (22.10 ± 14.93 and

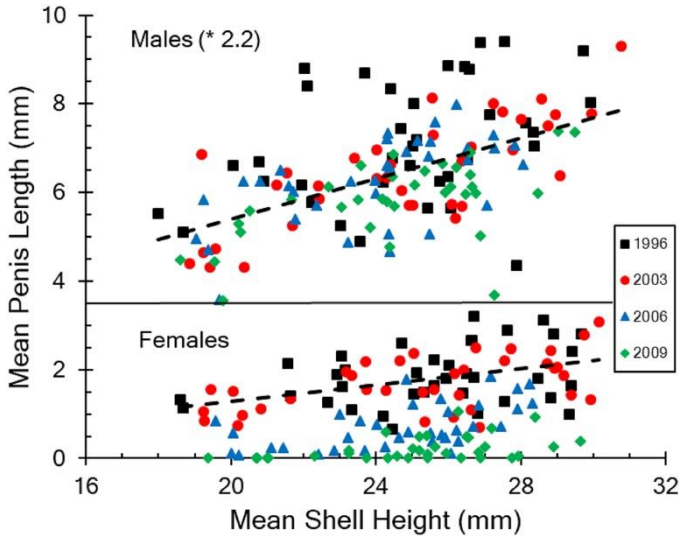

Fig. 1. Biometric and imposex indices. Plot of the mean penis length in males (MMPL, top) and females (MFPL, bottom) against their corresponding mean shell height in samples of *Nucella lapillus* collected along four surveys. Trend lines are shown only for males and the homogeneous female group resulting from pooling together data from the first two campaigns, see text for further explanation.

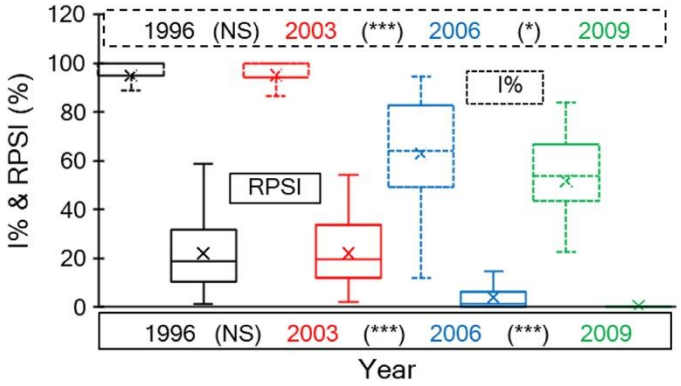

Fig. 2. Biometric and imposex indices. Box plot of the imposex incidence (I%, broken lines) and the relative penis size index (RPSI, solid lines) in samples of *Nucella lapillus* collected along four surveys. Boxes range from Q1 to Q3, with the median across, the mean represented with an x and the tails indicating the minimum and maximum values that are not outliers (not shown). The significance of statistical contrasts between pairs of consecutive surveys is indicated in parentheses, see text for further explanation.

22.12 ± 14.08, respectively, *t*-test for n = 36, NS) and decreased in 2006 (3.95 ± 5.33, MWW test for n = 35, ***) and again in 2009 (0.57 ± 1.43, MWW test for n = 34, ***). RPSI values in the first of these three groups (1996–2003, n = 73) did not correlate with the corresponding MFSH, but later there was a significant (if weak) relationship for both 2006 (n = 35, R^2 = 0.13, *) and 2009 (n = 34, R^2 = 0.13, *) (figures not shown). The same circumstance was observed when plotting VDSI vs. MFSH (Fig. 3): there was no relationship in 1996–2003 (n = 73) but subsequently the linear relationship was significant in 2006 (n = 35, R^2 = 0.13, *) and moderately strong in 2009 (n = 34, R^2 = 0.35, ***). Finally, the concomitance between imposex measurements in all samples is summarized in Figure 4. The close fit between I% and their corresponding VDSI is adequately described in two of the groups established (i.e. 1996–2003, and 2006) by a simple linear model, with similar slope (25.72 ± 1.39) and strength (R^2 > 0.99, ***) in either case. Linearity would also be appropriate for the third group (i.e. 2009), but data from this last study reach the strongest fit with a square root X function ($y = 47.74\ x^{0.5}$, R^2 > 0.98, ***). As for the MFPL, a squared X model fitted the correlation with VDSI in each of the three groups, with similar coefficients (0.11 ± 0.01 and 0.91 ± 0.02, respectively for the quadratic and the determination one). In addition, since individual curves overlapped consecutively and all together spanned the full VDSI range, data from all surveys were pooled to get the single curve displayed in Figure 4 (R^2 = 0.93, ***). A similar correlation was found between RPSI and VDSI (figure not shown).

Table 2. Imposex indices (I%: Percentage of imposex-affected females; RPSI: Relative Penis Size Index; VDSI: Vas Deferens Sequence Index) for samples of *Nucella lapillus* collected along 4 surveys, including summary statistics. VDSI values were first published by Ruiz *et al.* (2017).

			1996 (n = 37)			2003 (n = 36)			2006 (n = 35)			2009 (n = 34)		
Code	Site		I%	RPSI[a]	VDSI	I%	RPSI	VDSI	I%	RPSI	VDSI	I%	RPSI	VDSI
1	Ribadeo	[b]	100	9.24	3.22	100	18.33	4.00	82	14.02	3.08	29	0.00	0.65
2	Foz		100	10.28	4.18	100	9.06	4.00	88	2.00	2.79	34	0.00	0.45
3	Viveiro		86	10.66	3.57	100	19.32	4.02	32	0.27	1.18	69	0.99	2.31
4	Barqueiro		100	30.01	4.04	64	1.96	2.41	42	2.07	1.65	47	0.90	1.53
5	Cariño		100	19.08	4.17	100	15.13	4.00	48	0.05	1.76	-	-	-
6	Cedeira		100	20.63	4.07	100	26.52	4.03	71	5.97	2.71	61	0.09	1.78
7	Prior		95	7.49	3.03	100	11.34	3.88	59	0.06	2.14	55	0.03	1.42
8	Mugardos	[b]	100	20.21	4.30	100	32.54	4.12	90	14.57	3.55	69	4.08	2.62
9	Centroña	[b]	90	5.36	3.40	84	11.97	3.42	50	0.47	1.88	75	0.14	2.41
10	Perbes		77	4.25	2.77	88	4.64	3.38	31	0.03	1.19	42	0.00	0.89
11	Sada		100	27.47	4.05	79	4.50	3.05	94	9.65	3.65	67	6.32	2.07
12	Veigue		90	18.82	3.68	94	21.26	3.77	57	1.50	2.13	48	0.02	1.43
13	Mera	[b]	100	36.85	4.18	100	34.07	4.02	76	0.43	2.86	60	0.01	1.40
14	Bastiagueiro	[b]	100	58.78	4.50	95	33.63	3.79	65	0.20	2.35	59	0.07	1.45
15	Sta. Cristina	[b]	100	39.98	4.27	100	44.99	4.07	84	6.25	3.37	67	0.55	2.14
16	Dique	[b]	100	18.76	4.30	100	15.11	4.00	50	0.01	1.42	0	0.00	0.00
17	Langosteira	[b]	100	9.31	3.33	76	1.98	2.94	43	0.00	1.61	25	0.00	0.40
18	Malpica	[b]	100	5.64	3.57	100	5.57	3.74	12	0.00	0.44	6	0.00	0.06
19	Laxe		100	31.91	4.09	100	35.27	4.10	60	0.59	2.05	58	0.01	1.42
20	Muxía		100	29.05	4.04	100	42.21	4.05	24	0.00	0.88	6	0.00	0.06
21	Corcubión	[b]	100	17.63	4.28	100	19.39	4.03	83	0.72	2.97	76	0.00	1.43
22	Louro		100	14.96	3.89	100	30.87	4.06	64	0.33	2.20	54	0.00	1.23

23	Muros		100	28.38	4.25	94	19.78	3.59	60	5.17	2.20	53	0.00	1.33
24	Creo		100	55.41	4.58	100	5.31	3.64	-	-	-	-	-	-
25	Ribeira	[b]	100	38.95	4.29	96	15.04	3.87	75	1.32	2.75	23	0.00	0.36
26	Vilagarcía	[b]	100	13.14	4.46	100	38.83	4.19	94	12.93	3.72	80	0.05	1.50
27	Cambados		100	18.49	4.00	100	20.68	3.84	71	7.21	2.90	52	0.05	1.26
28	A Toxa	[b]	100	34.35	4.33	100	54.15	4.21	86	5.00	3.29	53	0.00	0.95
29	Sanxenxo		82	15.08	3.09	76	45.31	3.09	50	1.12	1.86	48	0.00	0.93
30	Poio		89	2.65	3.44	-	-	-	-	-	-	-	-	-
31	Marín		100	19.99	4.05	94	14.80	3.78	75	1.85	2.65	84	0.65	2.32
32	Rande	[b]	100	47.79	4.68	100	30.15	3.93	86	21.21	3.36	83	4.30	3.02
33	Bouzas	[b]	87	7.50	3.23	100	27.62	4.09	82	6.80	3.00	68	0.03	1.96
34	Samil	[b]	38	1.25	1.16	100	12.34	3.93	41	0.09	1.26	50	0.00	1.00
35	Canido		82	11.08	3.15	86	2.67	3.50	58	5.46	2.31	54	1.13	1.86
36	Baiona		100	34.71	4.00	100	36.50	4.09	27	0.02	0.86	36	0.00	0.68
37	A Garda	[b]	100	42.62	4.39	100	33.44	4.09	90	10.92	3.50	67	0.00	1.54
Mean			95	22.10	3.84	95	22.12	3.80	63	3.95	2.33	52	0.57	1.35
SD			12	14.93	0.67	9	14.08	0.40	22	5.33	0.88	22	1.43	0.76
Max.			100	58.78	4.68	100	54.15	4.21	94	21.21	3.72	84	6.32	3.02
Min.			38	1.25	1.16	64	1.96	2.41	12	0.00	0.44	0	0.00	0.00

[a] : RPSI values for this 1996 survey were first published by Ruiz *et al.* (1998). They are recalculated here for homogeneity with other surveys, see text for full details.

[b] : Butyltin tissue concentrations were repeatedly determined in these 17 sites, see Ruiz *et al.* (2015).

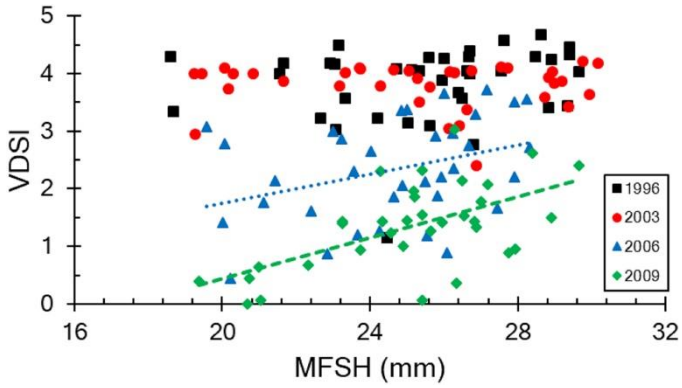

Fig. 3. Biometric and imposex indices. Plot of the *vas deferens* sequence index (VDSI) against their corresponding mean female shell height (MFSH) in samples of *Nucella lapillus* collected along four surveys. The lines represent the trend for 2006 (dotted blue) and 2009 (dashed green).

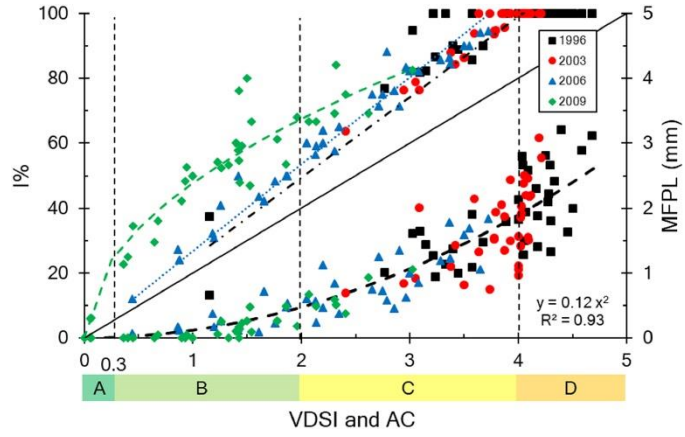

Fig. 4. Biometric and imposex indices. Plot of the imposex incidence (I%, left) and the mean female penis length (MFPL, right) against the *vas deferens* sequence index (VDSI) in samples of *Nucella lapillus* collected along four surveys. The bottom colour-coded scale refers to the OSPAR scheme (2004), with capital letters denoting the corresponding Assessment Class (AC). The I% lines represent the trend for 1996–2003 (dot-and-dash black), 2006 (dotted blue) and 2009 (dashed green).

DISCUSSION

This work details our effort to monitor TBT pollution in Galicia by gauging its biological effects in the common NE Atlantic intertidal gastropod *Nucella lapillus*. On the one hand, it shows that regional water quality improved following the 2003 EU total TBT ban, confirming previous conclusions based on the chemical analyses of tissues (Ruiz *et al.* 2015) and partial imposex observations (Ruiz *et al.* 2017). It thus conforms to other studies that, using the large geographic range of *N. lapillus*, have verified imposex decrease throughout Atlantic Europe: from the northernmost reaches such as Iceland (Guomundsdottir *et al.* 2011) and Norway (Schøyen *et al.* 2019), to mid-latitude areas such as the UK (Nicolaus & Barry 2015) and Ireland (Wilson *et al.* 2015), and further down to the southernmost limits in Portugal (Laranjeiro *et al.* 2018). Although international comparisons are not straightforward even within the OSPAR area due to national particularities (e.g. beginning and timing of surveys, application of TBT partial bans, number of sites in the sampling networks, etc.), all these studies agree in that the Ecological Quality Objective (EcoQO) set by OSPAR (2004) is generally met. In this respect, the recent report that all 2017 samples in the Norwegian network (n = 8) are free of imposex (Schøyen *et al.* 2019) constitute some most welcome news that will be hopefully mirrored elsewhere in the coming years. On the other hand, this work compiles biometric data extending over more than 10 years to offer novel insights on both basic marine biology features and their potential application to pollution monitoring, as follows.

BIOMETRICS

This snail is conspicuous in Atlantic Europe and other coasts and, therefore, have for long been the subject of many biological studies. More precisely, the finding that it suffers from imposex and it is valid to assess TBT pollution (e.g. Bryan *et al.* 1986) promoted it use in biomonitoring exercises over the last decades. However, while many such works included the customary measurement of the animals' shell height (SH) in addition to genital records, few have studied the relationship between both datasets (and hardly ever at a regional scale and over more than a sampling season). In doing so, the current results show negligible SH variation within sex, in concurrence with Galante-Oliveira *et al.* (2011). They reported no significant difference in MFSH when 12 populations of *Nucella lapillus* spread from north to south Portugal were repeatedly sampled (from 2003 to 2006 and 2008) and analysed together. In these homogeneous conditions, the assessment of imposex change can confidently be performed with no need to normalize biometric parameters by specimen size. In turn, the selection of our samples to comply with an established SH range (see Materials and methods above) precludes further inference on the medium term trend of this species size. On the other hand, our results on SH variation between sexes do not appear to agree with previous findings (see Son & Hughes 2000 and references therein) that *N. lapillus* females tend to be larger than males. However, our approach in the selection and treatment of samples was unlike theirs, probably inappropriate to discern this specific question.

With respect to the mean penis length, the observations that in females (MFPL) it decreased from the first two surveys to the last two was to be expected following the known beneficial effects of the 2003 EU total TBT ban. This is a rewarding result largely justified by our samplings being concentrated on the same calendar months to avoid the MMPL seasonal variation due to temperature-dependent sexual maturation stage (Gibbs *et al.* 1987), even though some other kinds of variation may be involved. For instance, the finding by Bailey and Davies (1991) that penis growth was stimulated in males (as well as in females) in response to field TBT exposure might justify the minor MMPL drop in *N. lapillus*. Moreover, gastropods' parasites are known to have a castrating impact and a reducing effect on male penis size, possibly even at their early stages (Morley 2006, Rato *et al.* 2009). Thus, while we discarded evidently infested specimens (see Materials and methods above), the possibility that immature infections went overlooked cannot be rejected. In addition, we were unaware that *N. lapillus* males close to egg capsules clusters had larger penises than those further away (Galante-Oliveira *et al.* 2010), and it is thus possible that our collections inadvertently contributed to male penis variability. Finally, extended handling time (Minchin & Davies 1998) and narcotization (Huet *et al.* 1995) lead to larger penis length in *N. lapillus*, but we never sedated these samples nor needlessly prolonged any assessment.

The correlations found between the mean penis length (MPL) and the mean shell height (MSH) in both sexes of *N. lapillus* (Fig. 1) have been reported for other gastropods (e.g. Vasconcelos *et al.* 2011, Castro &

Fillmann 2012). The strength, significance and slope of the linear trends are typically higher in males, reflecting that the relationship is a natural, ontogenetic one. However, imposex is known to be a dose-dependent anomaly (e.g. Bryan *et al.* 1987) whereby females mirror the genital development of males in their populations (Gibbs *et al.* 1987), and therefore the growth of their TBT-imposed penises is subject to some secondary SH dependence too. Figure 1 further shows that, while the relationship in males is consistent over time despite some MMPL variability (due to factors including those discussed above), MFPL gradually decreased after 1996–2003; as a consequence, its correlation with MFSH fades away so that in 2009 it is only sustained by fewer than two-thirds of the sites initially considered. There is little doubt that this association will eventually disappear as populations with no penis-bearing females proliferate in response to decreasing TBT pollution.

The expected MFPL decrease is progressive and uniform (Fig. 1). It has to be noted that this is a stenotopic species (i.e. with strict environmental requirements) of restricted mobility (e.g. Crothers 1985) and, because it accumulates TBT mostly from water (Bryan *et al.* 1989), it is regarded as a quickly reacting matrix (Ruiz *et al.* 2017). Individuals at a given site are hence exposed to relatively homogeneous conditions, populations narrowly reflecting the specific level of water TBT they suffer. Therefore, in a scenario of sustained and widespread pollution decrease *N. lapillus* respond as a consistent biomonitor, progressively in time and uniformly in space.

IMPOSEX

The temporal evolution of all measurements of imposex in *Nucella lapillus* were in phase along the study period (see Table 1 and Fig. 1 for MFPL, Table 2 and Fig. 2 for I% and RPSI, and Table 2, Fig. 3 and Ruiz *et al.* 2017, for VDSI). This sequential reduction of effects (from the early- to the mid- and then to the late-2000s) partially conforms to the pace of change in their causative agent as chemically determined in the same specimens: the TBT body burden differentiated between the first two surveys (1996–2003) and the last two (2006–2009) (see Ruiz *et al.* 2015). This concurrence confirms the quick success of the 2003 EU total TBT ban. It is likely due to the biological characteristics of this species, which enable both direct reaction to water TBT (see above) and rapid population renewal (individuals mature when 2–3 years old, see Gibbs 1999). As for the disagreement between both datasets on the changes occurred (or not) between 2006 and 2009, it is not thought to be due to false biological positives. Differences were clear-cut and significant in all imposex measurements, and MFPL and derived RPSI are free of specimens size caveats because there was no SH heterogeneity either within or between sex. We rather think the dissimilarity is caused by TBT desorption from sediments, a natural phenomenon expected to occur in environments where water quality begins to improve. We have previously discussed how *N. lapillus* is prone to be affected by such a passive diffusion of hydrophobic TBT so that persistent tissue concentrations in 2009 can be explained (Ruiz *et al.* 2015).

On the other hand, TBT pollution before the 2003 EU total ban was such that most populations showed a VDSI close to 4; as usual, RPSI was more variable (Table 2), but the interesting point is that both were irrespective of MFSH (see Fig. 3 for VDSI). Later, as soon as TBT declined, indices also decreased, but seemingly more pronouncedly in populations of smaller SH. This was repeated in the last survey, when the correlation between VDSI and MFSH was highly significant. In this respect, Galante-Oliveira *et al.* (2011) proved that individual VDS (and consequently the VDSI) in *N. lapillus* is dependent on SH. They thus confirmed a longstanding belief of plain reasoning: larger females within a given population are normally older, have been exposed to TBT pollution for a longer period, and therefore suffer more from its adverse effects (e.g. develop higher VDS). This relationship is critical when we are to assess imposex change in a scenario of decreasing TBT, and prompted our SH selection in samples (see Materials and methods). Now Figure 3 implies that VDSI is related to SH not only at the local but also at the regional scale, and that such association is masked when the sole responsible agent is high and widespread enough to homogenize the imposex response throughout the area. A similar relationship is less obvious for MFPL and derived RPSI because TBT is not the only determinant of penis length (see Fig. 1 and text above).

It is well known that the interaction between the phenotypic plasticity in *N. lapillus* and the physical action of waves results in exposed enclaves of shorter SH inhabiting the open coast, and sheltered populations of longer shells located inside estuaries (e.g. Crothers 1985, Gibbs 1993).

With the advent of antifouling TBT paints, this gradient overlapped with another one established by the dilution of the pollutant released in the more protected upper reaches (where activities releasing TBT such as marinas, shipyards, dry docks, etc. tend to concentrate) towards the mouth of the estuary and beyond (e.g. see Fig. 5 in Ruiz *et al.* 1998, showing such a TBT downstream gradient in the estuary of Vigo). For instance, the 5 left-most 2009 samples in Figure 3 (code 1, 2, 16, 17 and 18) can be considered as exposed, and the 5 right-most ones (code 8, 9, 10, 26 and 28) as sheltered (see Ruiz *et al.* 1998, for a print map or Ruiz *et al.* 2017, for a Google one). The average MFSH, imposex (MFPL, RPSI and VDSI) and TBT tissue concentration in 1996 in the former group (all data from Table 1 and Table 2, n = 5 except TBT, n = 4, see Ruiz *et al.* 2015, and references therein) were 21.0 ± 2.2 mm, 1.25 ± 0.13 mm, 10.65 ± 4.87, 3.72 ± 0.49 and 122 ± 134 ng Sn g^{-1} DW (or ppb Sn DW), respectively; in the latter they were 28.6 ± 1.1 mm, 1.65 ± 0.52 mm, 15.46 ± 12.37, 3.85 ± 0.74 and 402 ± 222 ppb Sn DW, respectively. In 2009 there were negligible MFSH differences in either the exposed or the sheltered group (20.6 ± 0.7 mm and 28.5 ± 0.8 mm, respectively), but imposex had decreased considerably in the latter (average MFPL, RPSI and VDSI were at 0.32 ± 0.38 mm, 0.85 ± 1.80 and 1.67 ± 0.80, respectively) and to a larger extent in the former one (there was no penis-bearing female in these 5 samples, and mean VDSI was 0.31 ± 0.27). There is no doubt these drops were caused by some huge TBT declines (down to 8 ± 5 and 55 ± 40 ppb Sn DW, respectively for the exposed and the sheltered sites). Variability between these two extreme groups may be considerable due to site singularity, but data

from all 34 samples in 2009 confirm a significant and consistent trend. Therefore, it is concluded that the association found between VDSI and MFSH would not come from a direct dependence but from the inverse relationship between other two latent, causative, variables: TBT pollution and wave exposure, respectively.

A GRAPHICAL SUMMARY

Lastly, Figure 4 plots I% and MFPL vs. VDSI for all four *Nucella lapillus* surveys; the time evolution of imposex is thus condensed in a single graph. Figures include the colour-coded Assessment Classes (ACs) established from the VDSI scale in *N. lapillus* (OSPAR 2004). This type of chart displaying the relationship between different measures is common practice in imposex works, from some of the first reports (Bryan *et al.* 1986) to the latest ones (e.g. Cacciatore *et al.* 2018). While the former employed primitive indices to illustrate the phenomenon in *N. lapillus* as it was unveiled, the latter propose using imposex in other gastropods as an indicator of the impact of TBT within the European Union Water Framework Directive.

The relationship between I% and VDSI comes as no surprise since the continuous former variable is fully dependent on the ordinal latter one: as soon as VDSI is determined, the I% can be calculated straight away (see Materials and methods above). Although the character of these plots is thus merely descriptive, they still offer some interesting insights, particularly when several surveys or species are compared. In the case of *N. lapillus* (Fig. 4), the simple linear model fitting the association in two of the I% (or VDSI) groups established (i.e. 1996–2003 and 2006)

renders the total I% at VDSI around 4 and beyond (AC D). These topmost values were never found in the third group (i.e. 2009) which, rather, includes the only three samples in AC A ever found (see Table 2). Thus, a square root X curve describes better how the shape of the relationship between imposex incidence and severity changed in this last regional sampling.

On the other hand, the existence of females with complete *vas deferens* but no penis at all (e.g. Barreiro *et al.* 1999) demonstrates that these two structures are somehow independent of each other. However, both are caused by TBT pollution, and therefore some correlation between them is to be expected (as was found elsewhere, e.g. Barroso *et al.* 2002). Here we prefer to deal with the absolute female penis length because it is the direct measure, free of interferences such as the unexplained MMPL variability presented above.

Finally, the relationship between both accessory organs (on the right in Fig. 4) sustains some useful applications since it allows the close estimate of a sample VDSI from the mere appraisal of its MFPL. Practitioners are aware that assessing penis length in sacrificed females is easier and quicker: penises (always located behind the right tentacle) have just to be measured, while the categorization of a vas deferens requires the meticulous examination of the whole floor of the pallial cavity. Besides, the VD cannot be observed in its entirety without shell removal and dissection (i.e. killing the animal), while PL can be gauged in living females (as was shown to be feasible, if delicate, by Gibbs 2005). Such a non-destructive procedure minimizes the impact of imposex

surveys, taking particular care of those populations previously depleted by TBT and/or other stressors; in retrospect, this technique could have been beneficial for some of our *N. lapillus* enclaves. Moreover, ACs can be directly guessed from penis length values. Figure 4 shows that most *N. lapillus* samples with MFPL from ~0.5 to 2 mm correspond to the yellow C, and samples above that range belong to the orange D; penises below 0.5 mm are more problematic to measure and interpret, but this seems of a lesser importance since in any green case (AC A or B) the EcoQO is met (OSPAR 2004). It is remarkable that a sample's AC can be consistently derived from its MFPL.

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CHAPTER II

EXTENDED IMPOSEX MONITORING IN N ATLANTIC SPAIN CONFIRMS PUNCTUAL ATTAINTMENT OF EUROPEAN ENVIRONMENTAL OBJECTIVES FOR TBT

ABSTRACT

Legislation in the European Union (EU) aimed at reaching by 2015 a Good Ecological Status in regard to tributyltin (TBT, the biocide used in traditional antifouling paints). With a view to check such an achievement in N Atlantic Spain, baseline monitoring of gastropod imposex (the recommended assessment tool) was extended up to that date. In Galicia (the Western part of the study area) the use of the rock snail *Nucella lapillus* since 1996 had shown this environmental objective to be met as soon as 2009, but new surveys reveal no further improvement thereafter.

INTRODUCTION

The organometallic compound tributyltin (TBT) has been worldwide used as a biocide in ship antifouling paints over the last half century, from its first marketing around 1960 (Clark *et al.* 1988) to its 2008 global ban by the International Maritime Organization (http://www.imo.org). TBT pollution had been shown to cause deleterious effects on marine life and, among them, imposex [i.e. the superimposition of male sexual characteristics (penis included) onto female gastropods (Smith 1971)] proved of paramount relevance to this long story. Imposex in field populations has been reported for hundreds of species, and in several cases it has been specifically linked to TBT. In Atlantic Europe these include *Nucella lapillus* (L.), a rocky shore carnivore (Bryan *et al.* 1986).

This unequivocal cause-effect relationship led OSPAR (the Convention for the Protection of the Marine Environment of the NE Atlantic) to include the recording of imposex within its mandatory monitoring program (OSPAR 2004). In addition, an Ecological Quality Objective (EcoQO) was formulated so that levels of imposex should agree with exposure to water annual average (AA) concentrations below the Environmental Quality Standard (EQS) set for TBT at 0.2 ng TBT/L (OSPAR 2011). As a member of the OSPAR Convention, the European Union (EU) has also assumed this EQS through the Water Framework Directive (WFD, EC 2008); this ambitious legislation set out an overall timetable according to which 2015 was the first deadline for achieving a Good Ecological Status in regard to priority hazardous substances such

as TBT (http://ec.europa.eu/environment/water/waterframework/info /timetable_ en.htm).

The imposex EcoQO, initially devised for the intertidal rock snail *Nucella lapillus*, is based on the development onto females of a male genital structure, the *vas deferens*: the values of the *vas deferens* sequence index (VDSI, first proposed by Gibbs *et al.* 1987) in samples define the Assessment Classes, and their lowest two indicate that the EcoQO has been met (see Table 1). The present work reports on the evolution of imposex in N Atlantic Spain, from a baseline established before the IMO's ban to the primary date targeted to meet EU environmental objectives for TBT (i.e. 2015).

MATERIAL AND METHODS

This study deals with the Spanish Northwest littoral, a neatly Atlantic stretch of coast. The preferred gastropod for imposex watch in Europe, *Nucella lapillus,* is well distributed in this area (http://www.marinespecies.org).

The baseline survey was conducted in the summer (July–September) of 1996 and, for the present work, sampled 9 populations. Their location is detailed in Ruiz *et al.* (1998) and in Figure 1. All of them were revisited in 2003 and every 3 years thereafter up to 2015. The collection, laboratory treatment and imposex assessment of animals is also described there, and summarised in Ruiz *et al.* (2017). The VDS in each female runs from 0 (unaffected animal) to 4 (individual with penis and full *vas deferens*) and further to 6 (top value with aborted egg capsules

inside the sterilized female), and so does the sample mean value or *vas deferens* sequence index (VDSI).

Fig. 1. Map of Galicia (NW Spain) showing the localization of the study populations. See also Table 2.

Since imposex monitoring should ideally compare adult females of the same age (or size as a surrogate), the shell height (SH) range we had previously defined was used and maintained (i.e. SH from 17.5 to 32 mm, see Ruiz *el al.* 2017). In addition, the SH homogeneity between surveys was checked by means of pairwise nonparametric Mann-Whitney (Wilcoxon) W-tests (MWW). These compared the medians of every couple of consecutive campaigns in Galicia, and found no statistically significant SH differences ($P > 0.05$) in 4 out of 5 contrasts. Specimens' size was then considered homogeneous within these 4 pairs

so that regional VDSI changes could be confidently assessed. This was done with contrasts considering a number of populations equal to the lowest involved, either a parametric *t*-test when VDSI data sets were normally distributed (2009 vs. 2012 –n = 9– and 2012 vs. 2015 –n = 8–) or a MWW test when sets were not so (2003 vs. 2006 and 2006 vs. 2009, n = 9 in both cases). The survey conducted in 1996 collected gastropods whose SH resulted heterogeneous when compared with that of 2003, and was therefore not subject to statistical imposex tests. The significance levels are depicted below as NS, * and **, respectively for $P > 0.05$, $P < 0.05$ and $P < 0.01$.

RESULTS AND DISCUSSION

Biometrical and VDSI results for the six *Nucella lapillus* surveys in Galicia are gathered in Table 2; they all refer to females of the selected size (i.e. SH from 17.5 to 32 mm). The evolution of imposex in this rock snail along these 20 years can be better appreciated in Figure 2, where VSDI is referred to both the numerical scale defined above and also to the OSPAR scheme in Table 1. In 1996, at the beginning of this study, the mean VDSI for the 9 samples considered was 4.08 ± 0.44, and the situation showed little change by 2003 (3.81 ± 0.55). However, the 2003 EU TBT ban quickly resulted in VDSI significant decreases, first detected in 2006 (2.71 ± 1.02, MWW test, **) and further from here to 2009 (1.63 ± 0.97, MWW test, *). These results agree with our previous report on concurrent surveys that considered 4 times as many populations (see Ruiz *et al.* 2017), suggesting that less intensive inspections may be equally representative of regional imposex evolution if sampling sites

Table 1. Imposex monitoring in N Atlantic Spain. OSPAR scheme for TBT-specific biological effects, where the values of the *vas deferens* sequence index (VDSI) in the rock snail *Nucella lapillus* are the criteria originally defining the Assessment Classes. The green colours in A and B mean that the Ecological Quality Objective is met (OSPAR, 2004).

Assessment Class	*Nucella lapillus* VDSI
A	< 0.3
B	0.3 – < 2.0
C	2.0 – < 4.0
D	4.0 – 5.0
E	> 5.0

are properly selected. Later on, the present work shows that VDSI has changed to no significant extent over the following 6 years (*t*-tests for both 2009 vs. 2012 and 2012 vs. 2015). According to the OSPAR scheme the overall improvement has also been considerable since most populations in Galicia, initially classified as D (VDSI from 4.0 to 5.0, orange colour) in 1996, they changed to C (VDSI from 2.0 to < 4.0, yellow) ten years later. Remarkably, the OSPAR EcoQO at this regional scale was achieved in 2009, when the majority of samples could be classed as B (VDSI from 0.3 to < 2.0, light green). Thereafter, however, there has been no further improvement and only a minor proportion of sites (up to 22% in 2012) have been ever classed as A (VDSI < 0.3, dark green).

General improvement does not certainly mean total recovery. The EcoQO is in 2015 still exceeded at sites under the influence of big ports

such as Rande (site 32 in Table 2). If this comes as no surprise in view of the intense shipping activity of this kind of areas and the expected TBT

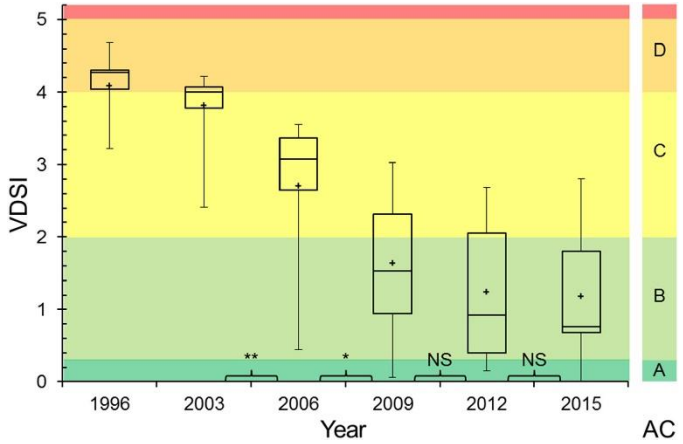

Fig. 2. Imposex monitoring in N Atlantic Spain. Box plot of the *vas deferens* sequence index (VDSI) in samples of the rock snail *Nucella lapillus* collected along 6 surveys in Galicia. Boxes range from Q1 to Q3, with the median across, the tails representing the minimum and maximum values, and the mean indicated with a cross. See text for significance of statistical contrasts between pairs of consecutive surveys. The right hand scale refers to the OSPAR colour-coded scheme, with capital letters denoting the corresponding Assessment Class (AC, see Table 1).

legacy in their sediments (e.g. Langston *et al.* 2015), the situation in Barqueiro (code 4 in Table 2) is rather more shocking. The VDSI of this dog-whelk population (located within a beautiful small estuary in the not so industrially developed Northern Galician coast) was consistently below the regional average in all surveys but 2015, when it abruptly tripled to set the top value at 2.80. We can propose no explanation other than TBT paints (perhaps the remnants of a pre-ban stock) being illegally used in nearby vessel facilities such as the port of Vicedo. In any case, this kind of distressing observation is not uncommon (in Europe

Table 2. Imposex monitoring in N Atlantic Spain. Biometrical and *vas deferens* sequence index (VDSI) data for samples of the rock snail *Nucella lapillus* collected in Galicia along 6 swurveys, including number of females selected (nF), shell height (SH, mm), and summary statistics. * denote years previously included in a more intensive study, see Ruiz *et al*. 2017.

		1996 *			2003 *			2006 *			2009 *			2012			2015		
Code	Site	nF	SH	VDSI	nF	SH	VDSI	nF	SH	VDSI	nF	SH	VDSI	nF	SH	VDSI	nF	SH	VDSI
1	Ribadeo	23	22.7	3.22	21	20.8	4.00	13	19.6	3.08	17	21.0	0.65	20	21.6	0.15	-	-	-
4	Barqueiro	14	29.7	4.04	22	26.9	2.41	26	27.4	1.65	17	26.6	1.53	20	24.6	0.93	20	23.0	2.80
8	Mugardos	5	28.5	4.30	13	27.5	4.12	20	28.2	3.55	13	28.4	2.62	19	29.3	2.68	14	28.5	0.71
15	Sta. Cristina	13	26.0	4.27	21	24.7	4.07	19	25.0	3.37	18	26.5	2.14	20	27.5	2.05	25	26.7	0.80
18	Malpica	7	23.3	3.57	21	20.2	3.74	25	20.2	0.44	16	21.0	0.06	18	20.8	0.17	18	20.3	0.00
21	Corcubión	18	25.6	4.28	19	26.2	4.03	30	26.2	2.97	21	24.3	1.43	20	25.7	0.80	16	24.4	0.63
28	A Toxa	6	29.4	4.33	7	29.7	4.21	21	26.8	3.29	19	27.9	0.95	20	23.6	0.40	13	25.3	0.69
31	Marín	19	25.3	4.05	18	24.3	3.78	20	24.0	2.65	25	25.4	2.32	19	25.8	1.37	25	25.8	1.68
32	Rande	11	28.6	4.68	21	28.8	3.93	22	24.8	3.36	23	26.3	3.02	18	28.9	2.61	20	29.0	2.15
	Mean	13	26.6	4.08	18	25.5	3.81	22	24.7	2.71	19	25.3	1.63	19	25.3	1.24	19	25.4	1.18
	SD	6	2.6	0.44	5	3.3	0.55	5	3.0	1.02	4	2.7	0.97	1	3.0	1.00	5	2.9	0.93
	Max.	23	29.7	4.68	22	29.7	4.21	30	28.2	3.55	25	28.4	3.02	20	29.3	2.68	25	29.0	2.80
	Min.	5	22.7	3.22	7	20.2	2.41	13	19.6	0.44	13	21.0	0.06	18	20.8	0.15	13	20.3	0.00

and elsewhere, e.g. Galante-Oliveira *et al.* 2011 and Batista *et al.* 2016, respectively) and calls for further action.

On the other hand, there is nowadays little doubt that TBT curtailed the population density and geographical distribution of gastropods suffering imposex (and probably other marine life, see Bryan & Gibbs 1991). This was first shown by some pioneering work on *Nucella lapillus* by Bryan *et al.* (1986) which prompted widespread legislation. The abatement of such a chemical control should, accordingly, facilitate the recovery of affected native species and the development of non-native ones. There are several detailed studies reporting on the former, mostly dealing with *N. lapillus* in Europe (see Morton 2009 and references therein). We do not have quantitative data ourselves, but have observed dog-whelk population growth at many sites through these monitoring surveys. As for the latter, Galicia is an important aquaculture area prone to receive alien species together with shellfish imports, and has recorded over the 2000s the introduction and establishment of gastropods known to suffer imposex elsewhere: *Hexaplex trunculus* (Quintas *et al.* 2005), *Rapana venosa* (Rolán & Bañón 2007) and *Bolinus brandaris* (Bañón *et al*. 2008). In addition, there are other imposex-affected species which, being traditionally established in N Atlantic Spain just in the Easternmost Basque reaches, were discovered in Galicia around 1990 but prospered in this region only by the mid 2000s: *Cyclope neritea* (Quintela *et al.* 2006a, Couceiro *et al.* 2012) and *Stramonita haemastoma* (Souto *et al.* 2008).

Finally, there are some interesting observations for Ribadeo. This is one of the less TBT-affected places in Galicia (see site 1 in Table 2), and used to host the Westernmost established population of *Nucella lapillus* in Spain: it was so abundant that in 1997 two people could collect within a low tide > 600 adults for a transplant experiment (see Quintela *et al.* 2000). However, parallel to the VDSI drop since the mid 2000s, the population density has been decreasing to the point that the visit by an experienced sampler in 2015 for the present study gathered only 5 specimens. They were insufficient for population imposex assessment and released on site in the hopes that the population could avoid definitive disappearance. Concurrently, individuals of *Stramonita haemastoma* (up to a dozen) were observed for the first time on this site. These findings agree with the opposed range shifts (towards contraction or expansion, respectively for septentrional and meridional species) reported in the Bay of Biscay for marine (Hemery *et al.* 2008) and, particularly, coastal organisms such as algae (Díez *et al.* 2012) and gastropods (Rubal *et al.* 2013). If widespread changes are largely ascribed to global warming, other interacting non-climatic forces deserve consideration at a more local scale (Hemery *et al.* 2008), (Martínez *et al.* 2012), and TBT pollution over the last half century is likely one of them.

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CHAPTER III

AFLPS REVEAL DIFFERENT POPULATION GENETIC STRUCTURE UNDER CONTRASTING ENVIRONMENTS IN THE MARINE SNAIL *NUCELLA LAPILLUS* L.

ABSTRACT

Dispersal has received growing attention in marine ecology, particularly since evidence obtained with up-to-date techniques challenged the traditional view. The dogwhelk *Nucella lapillus* L., a sedentary gastropod with direct development, is a good example: dispersal was traditionally assumed to be limited until studies with microsatellites disputed this idea. To shed some light on this controversy, the genetic structure of dogwhelk populations in northwest Spain was investigated with highly polymorphic AFLP markers giving special attention to the influence of hydrodynamic stress. In agreement with the expectations for a poor disperser, our results show a significant genetic structure at regional (< 200 km) and area scales (< 15 km). However, the spatial genetic structure varied with wave-exposure in the present case study: IBD was evident under sheltered conditions but absent from the exposed area where genetic differentiation was stronger. Our results provide evidences that differences in wave-exposure can exert a detectable influence on the genetic structure of coastal organisms, even in species without a planktonic larva.

INTRODUCTION

The quantification of dispersal ability and connectivity between populations is a major challenge in marine ecology with implications for the conservation and management of coastal environments (Hellberg 2006, Cowen & Sponaugle 2009). Dispersal determines where a species can be found and also its demographic structure (continuous or patchy, stable or unstable) (Slatkin 1987, Kinlan & Gaines 2003). Above all, dispersal supports the gene flow that acts either promoting evolution by transmitting successful alleles or constraining evolution by establishing homogeneity that limits the action of natural selection (Slatkin 1987). The type of development was long thought to be crucial for the final population structure of many species due to its direct relationship with dispersal ability (Grosberg & Cunningham 2001). In marine organisms, populations with a long meroplanktonic phase (weeks, months) are expected to have large gene flow even at extensive spatial scales (Palumbi 1994). This view has lately been challenged with the discovery that the actual dispersal distance of some species could be much smaller than previously thought due to the action of both physical (currents, habitat factors) and behavioral mechanisms (Chia *et al.* 1984, Johnson & Black 1984, Highsmith 1985, Knowlton 1993, G. P. Jones *et al.* 1999, Swearer *et al.* 1999, Taylor & Hellberg 2003, Selkoe *et al.* 2006, Shank & Halanych 2007, A. R. Wood & Gardner 2007, Galarza *et al.* 2009, Selkoe *et al.* 2010).

Estimating dispersal is challenging. Direct measures are only possible in few species (Shanks *et al.* 2003) and some of them can eventually be

misleading (Slatkin 1987). Thus, it is necessary to resort to alternative procedures (Palumbi *et al.* 2003). Among the latter, the genetic analysis of adult populations allows an indirect inference of dispersal without having to follow specific individuals. Besides, genetic data avoid uncertainties about the final destination of migrants and provide a time-integrated overview of dispersal patterns (Bohonak 1999). Nevertheless, the genetic approach is not free from limitations. An observed genetic structure can sometimes be explained as a consequence of different processes: gene flow, local selection, or even recruitment pattern (Bossart & Pashley Prowell 1998, Grosberg & Cunningham 2001, Marko & Hart 2011). Moreover, genetic estimates are often difficult to interpret in ecological terms, although recent developments in data analyses have opened the way to estimate dispersal at ecologically relevant time scales (i.e. events occurring in a year or a few years) under certain circumstances (Manel *et al.* 2005). In spite of these limitations, the lack of alternatives makes genetic estimates the only available option to get at least a rough idea of dispersal in many marine organisms.

The dogwhelk *Nucella lapillus* illustrates the uncertainties surrounding the assessment of dispersal in marine organisms. This snail is the most common neogastropod in Eastern Atlantic intertidal rocky shores ranging from south Portugal to northern Russia, and it even reaches the northern west Atlantic coast of North America (Crothers 1985). Since the mid-1980s, this species has been intensively studied due its high sensitivity to tributyltin (TBT), a biocide present in anti-fouling paints

that disrupts hormonal homeostasis in female dogwhelks causing imposex; i.e. a superimposition of male sexual organs. At its highest expression, imposex results in a blockage of the oviduct that renders the female functionally sterile (Gibbs & Bryan 1986, Barreiro *et al.* 1999). TBT resulted in the local extermination of *N. lapillus* in many areas throughout its European range (reviewed in Gibbs & Bryan 1996). Presumably, the demographic consequences of female sterility were exacerbated by the fact that juvenile dispersal, and thereby the arrival of recruits from distant populations, is limited in species with direct development (Crothers 1985, Fretter & Graham 1985). Moreover, although there are no definitive data on adult mobility and dispersal, traditional view is that adults probably spend their entire lives in the same area, and earlier studies found that both *N. lapillus* and its Pacific congener *N. emarginata* move only a few meters in time periods as long as one year (Palmer 1984, Crothers 1985). On the other hand, dogwhelks are rocky dwellers and their populations are often confined to enclaves separated by stretches of coast unsuitable for the species (e.g. soft sediment). Limited dispersal together with a fragmented habitat led to anticipate that, once TBT pollution declines, dogwhelks will be little able to recolonize sites from where they had formerly disappeared. Instead, studies using microsatellites have concluded that dogwhelks must disperse more than anticipated to explain the rapid recovery of genetic diversity in sites recolonized after the ban on TBT paints (Colson & Hughes 2004, 2007).

Here, we investigated the diversity and genetic structure of populations of *N. lapillus* in Galicia (NW Spain) to obtain indirect inferences about its dispersal ability. Sampling was designed to investigate the influence of wave exposure on genetic structure. We hypothesized that different hydrodynamic conditions could modify the dispersal capacity of the organisms with an impact on the resulting genetic structure. With this aim we sampled two different areas belonging to contrasting hydrodynamic environments.

AFLPs were chosen as molecular markers because of high reproducibility and potential to comprehensively scan the genotype (Vos *et al.* 1995, Blears *et al.* 1998, Bensch & Akesson 2005). Besides, AFLP markers can be more efficient than microsatellite loci in systems characterized by weak population structure (Campbell *et al.* 2003).

MATERIAL AND METHODS

SAMPLING AND DNA EXTRACTION

Ten samples consisting of 30 individuals each (15 males and 15 females) were collected in Galicia (NW Spain): five from sites scattered along a exposed stretch of coast (Laxe-F, LF; Laxe-C, LC; Santa Mariña, SM; Cabo Tosto, CT; Caldebarcos, C); and five from locations in a sheltered stretch, all of them within a single ria (i.e. a drowned river valley) (Chazo2, CH2; Chazo, CH; As Sinas, S; Isla de Arousa, A; Meloxo, M) (Fig. 1). Importantly, sites were selected so that the range of distances among locations (from 400 m to 15 km) was analogous within both areas. The aim of the study was to compare a sheltered and and exposed area in terms of their population structure (i.e. genetic differentiation between

sites, IBD). Therefore, sites within each area were not intended to be considered true independent replicates.

Only subadults, identified by their shell traits (Crothers 1985), were sampled in an effort to analyze

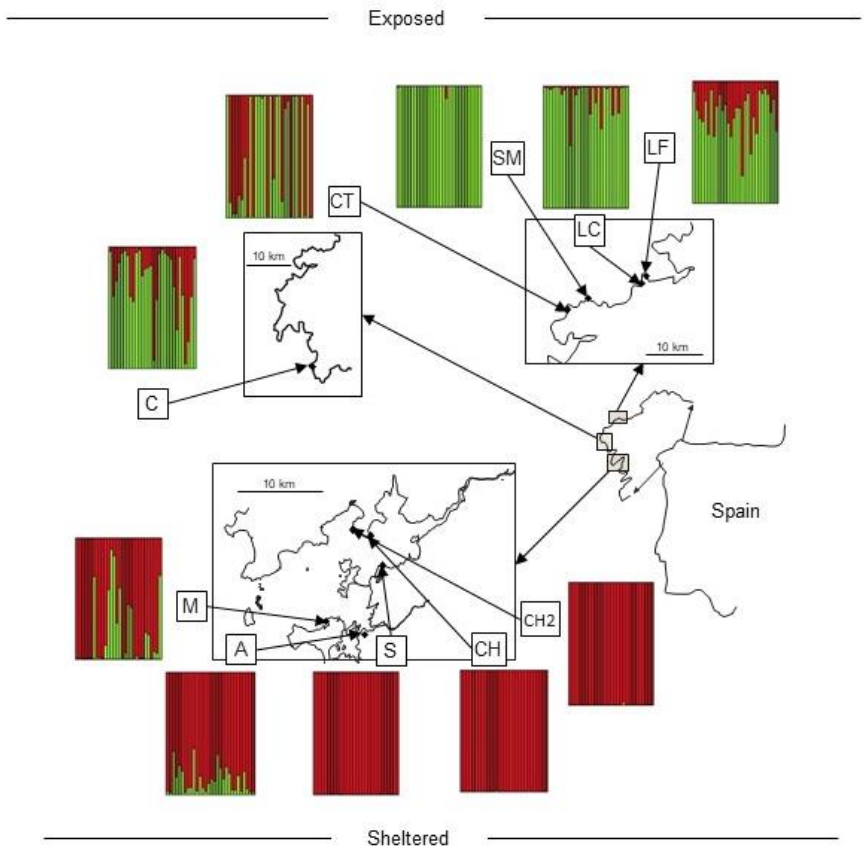

Fig. 1. Map combining *Nucella lapillus* sample locations and STRUCTURE output. Histograms are the STRUCTURE output; each individual is represented by a vertical line divided into segments of different color that represent the two clusters detected with a Bayesian approach.

individuals from a single cohort (Barreiro *et al.* 2006). Also, within each site, individuals were collected from the smallest possible area to

prevent mixing different breeding groups that could lead to a heterozygosity reduction due to Wahlund effect as seen in other studies with *N. lapillus* (Day 1990, Goudet *et al.* 1994). No specific permits were required for the described field studies. Also, no specific permissions were required for these locations/activities, none of the locations was privately-owned or protected in any way, and field studies did not involve endangered or protected species. Shells were opened with a vice and individuals were sexed under the stereomicroscope. Foot and mantle tissue were stored in 96º ethanol at 12ºC. To avoid cross contamination, each individual was dissected using disposable tools and/or flame sterilized material. Genomic DNA was extracted from some 3 mg (dry weight) mantle tissue using Qiagen DNeasy tissue kit following manufacturer instructions and stored in TE at -20°C.

Table 1. Primer sequences used for the AFLP selective amplification and number of loci generated

Primer combination	*EcoRI*-primer (5'-3')	*Tru1I*-primer (5'-3')	No. of loci
1	GACTGCGTACCAATTC+TAG	GATGAGTCCTGAGTAA+CGT	52
2	GACTGCGTACCAATTC+TAG	GATGAGTCCTGAGTAA+CAC	40
3	GACTGCGTACCAATTC+TAG	GATGAGTCCTGAGTAA+CTG	29
4	GACTGCGTACCAATTC+TCT	GATGAGTCCTGAGTAA+CGT	34
5	GACTGCGTACCAATTC+TCT	GATGAGTCCTGAGTAA+CAC	38
6	GACTGCGTACCAATTC+TCT	GATGAGTCCTGAGTAA+CTA	37

PRIMER SELECTION

We carried out a multistage study to select AFLP primer combinations that produced polymorphic, reproducible, and easily scorable patterns.

In a first stage, 24 combinations were tested on six individuals from two sites (sheltered and exposed coast, respectively). The 11 combinations that produced the most interpretable banding profiles were then re-tested on 24 individuals from six sites (four sites from the sheltered coast area and two sites from the exposed one; four individuals per site). The six combinations yielding the highest levels of polymorphism were thus retained and tested again on new, independent DNA extractions of the same 24 individuals to assess reproducibility (see primer sequences in Table 1).

AFLP REACTIONS

AFLP reactions were performed following the protocol of Vos *et al.* (1995) with minor modifications. Briefly, DNA extractions from each individual were diluted in TE buffer to a final concentration of 24-35 ngμl^{-1} (Blears *et al.* 1998); and 10 µl of diluted DNA were restricted with 2.5 units of *EcoRI* and *Tru1I* in a total volume of 20 µl containing 2X Tango buffer (Fermentas). After incubation, the product was added to 6 µl of a ligation solution containing 0.43 µM *EcoRI*-adapters (5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3'), 4.3 µM *Tru1I*-adapters (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3'), 0.52 units of T4 ligase (Fermentas) and 10X ligation buffer. Ligation products were diluted 10-fold in Milli-Q H_2O (Millipore Co.) and 10 µl were used for a preselective amplification with 0.3 µM *EcoRI*-primer with a T selective nucleotide (5'-GACTGCGTACCAATTC+T-3'), 0.3 µM *Tru1I*-primer with a C selective nucleotide (5'-GATGAGTCCTGAGTAA+C-3'), 2.5 mM $MgCl_2$, PCR buffer, 0.04 µgμl^{-1} BSA, 0.2 µM dNTPs and 0.04

units of *AmpliTaq* polymerase (Applied Biosystems). Ten µl from the 10-fold diluted product of the preamplification were finally used for selective amplifications with 0.6 µM of each *EcoRI* and *Tru1I* primers, both designed with three extra selective nucleotides added to the 3' end (*EcoRI*/*Tru1I*: TAG/CGT, TAG/CAC, TAG/CTG, TCT/CGT, TCT/CAC and TCT/CTA), 0.8 µM dNTPs, 2.5 mM $MgCl_2$, 0.04 $\mu g \mu l^{-1}$ BSA, PCR buffer, and 0.4 units of *AmpliTaq Gold* polymerase (Applied Biosystems). Preamplification was performed immediately after ligation whereas the products of the other reactions were kept overnight at -20ºC. PCR reactions were performed in a Hybaid thermocycler model PxE. The 5' end of the selective E-primers was labeled with FAM or NED fluorochromes.

Negative DNA extraction controls were regularly included to screen for cross contamination. Furthermore, DNA extractions of 10% of individuals were duplicated on different dates and run blindly along the other extracts to avoid bias scoring during reproducibility tests (Bonin *et al.* 2004). The estimated genotyping error (1.6%, error rate by primer combination: 1.1-1.9%) was consistent with former studies (Bonin *et al.* 2004); and none of the individual loci exceeded the maximum acceptable error rate (0.1) recommended by Bonin *et al.* (2007). Samples, negative controls, and replicates were randomly distributed in PCR plates. Reactants were always mixed in a laminar flow cabin; DNA and PCR product solutions were always added using filter tips to minimize the risk of cross contamination.

PCR fragments were separated on a 3130xl Genetic Analyzer (Applied Biosystems). Fingerprint patterns were processed with the software GeneMarker v1.70 (Softgenetics) using the options suggested for AFLP and following common recommendations for these markers (Bonin *et al.* 2005, Whitlock *et al.* 2008). Results were translated into a binary matrix of presence/absence of each band. Binary data from the six primer combinations were pooled to obtain a multilocus phenotype for each individual.

DATA ANALYSIS

Allele frequencies were estimated using the Bayesian method of Zhivotovsky (1999) implemented in AFLP-SURV v1.0 (Vekemans 2002) with the option of non-uniform prior distributions of allele frequencies. In contrast with alternative procedures (e.g. Lynch & Milligan 1994), this Bayesian approach does not imply a pruning of loci with low frequency of null-alleles and produces statistically unbiased estimates of diversity and genetic distances (Zhivotovsky 1999). Genetic diversity per sample was measured by assessing the number of polymorphic loci (5% criterion), Nei's gene diversity and the presence of private bands. Significant differences in gene diversity between samples were tested with the T' method for multiple unplanned comparisons between pairs of means based on equal sample sizes (Sokal & Rohlf 1995).

Allele frequencies were also used to estimate genetic differentiation between samples (F_{ST}). F_{ST} values were calculated for the whole data set as well as for each area (exposed and sheltered coast) following Lynch and Milligan (1994) and their significance tested with a permutation test

(10,000 pseudoreplicates). Nei's genetic distances between pairs of populations were used for UPGMA *(unweighted pair-group mean average)* cluster analysis and for Principal Coordinates Analysis (PCoA) with the help of MVSP v3.12d. (Kovach Computing Services) and GeneAlEx v6.1 (Peakall & Smouse 2006) respectively. To facilitate comparison with other AFLP studies, differentiation was also estimated by the analysis of molecular variance (AMOVA, Excoffier *et al.* 1992b) implemented in GeneAlEx v6.1. AMOVA calculates Φ_{PT}, an analogue of F_{ST}, using the squared Euclidean distance matrix between AFLP phenotypes and allows a hierarchical analysis of the genetic structures (e.g. differentiation between regions, between populations within regions). Φ_{PT} is a band-based approach that does not depend so critically on specific assumptions that could underestimate genetic variability (Excoffier *et al.* 1992b, Yan *et al.* 1999, Shank & Halanych 2007) and has been specifically recommended for AFLP data (Bonin *et al.* 2007).

The occurrence of isolation by distance (IBD) was explored with the Mantel test of correlation between the matrices of pairwise Φ_{PT} values and pairwise geographical distances (the shortest paths following the shore line between sites were measured with Google Earth). Both distances were used unprocessed and log-transformed in all possible combinations. Mantel test was performed with the online application *Isolation by Distance Web Service* (Jensen *et al.* 2005).

In an alternative approach, the genetic structure was further investigated with the Bayesian model-based clustering algorithms

implemented in STRUCTURE v2.3.3 (Pritchard *et al.* 2000, Falush *et al.* 2007, Hubisz *et al.* 2009) under a model assuming admixture and independent allele frequencies. Sampling locations were used as prior information to assist the clustering, as recommended when the signal of structure is weak. Ten runs with a burn-in period of 100,000 replications and a run length of 1,000,000 Markov chain Monte Carlo (MCMC) iterations were performed for a number of clusters (*K*) ranging 1-10. The *ad hoc* summary statistic Δ*K* of Evanno *et al.* (2005) was used to select the value of *K* with the uppermost hierarchical level of population structure in our data. Anticipating that a superstructure might hide other structures at smaller spatial scales, STRUCTURE was run for the whole data set as well as for the populations within each area separately (Evanno *et al.* 2005).

RESULTS

The six primer combinations produced a total of 230 AFLP loci, ranging from 29 to 52 per combination (Table 1), 99 (43%) of which were polymorphic (5% criterion) for the whole data set; every single individual produced a distinct multilocus genotype. The percentage of polymorphic loci per site was similar across sites except for S and CT, with the lowest and the highest values respectively (Table 2). Likewise, Nei's gene diversity ranged from 0.123 ± 0.010 in S to 0.224 ± 0.011 in CT; these two extreme values were significantly different from estimates obtained at the other locations ($P < 0.01$, test T'). Sites CH2 and LF showed one private band each, although with no evident link to any other parameter.

Both F_{ST} (0.172) and Φ_{PT} (0.254) statistics revealed the presence of highly significant genetic differences among sites *(P* < 0.0001 and *P* < 0.001 respectively) (Table 3A); as expected, Φ_{PT} values exceeded those of F_{ST}. Genetic differentiation was also significant when sheltered and exposed sites were considered separately but F_{ST}/Φ_{PT} estimates were clearly higher for the complete data set than for each area, suggesting that genetic distances might follow a hierarchical structure linked to geographical distance. Despite comparable geographic distances within the two areas, genetic differentiation between exposed coast sites was twice as high as within the ria. Hierarchical AMOVA (Table 3B) confirmed

Table 2. *Nucella lapillus* summary of AFLP markers and Nei's gene diversity for every sample

Site	N[a]	Polymorphic loci[b]	Nei's gene diversity (± S.E.)	No. private bands
Sheltered coast				
Chazo2 (CH2)	29	93 (40.4%)	0.167 ± 0.010	1
Chazo (CH)	30	89 (38.7%)	0.156 ± 0.010	0
As Sinas (S)	30	68 (29.6%)	0.123 ± 0.010	0
Arousa (A)	30	93 (40.4%)	0.174 ± 0.011	0
Meloxo (M)	30	96 (41.7%)	0.182 ± 0.010	0
Exposed coast				
Laxe_F (LF)	30	96 (41.7%)	0.194 ± 0.011	1
Laxe_C (LC)	30	85 (37.0%)	0.158 ± 0.010	0
St_Mariña (SM)	30	91 (39.6%)	0.165 ± 0.010	0
C_Tosto (CT)	30	113 (49.1%)	0.224 ± 0.011	0
Caldebarcos (C)	29	94 (40.9%)	0.186 ± 0.011	0

[a]: Average number of individuals takes into account missing data for some primer combinations.

[b]: 5% criterion applied to Bayesian estimates of allele frequencies (Zhivotovsky 1999).

the occurrence of a hierarchical structure as differences between areas explained a higher fraction of the genetic variation (26%, Φ_{RT} = 0.258, P < 0.001) than differences between populations within the same area (8%, Φ_{PR} = 0.110, P < 0.001). Pairwise Φ_{PT} differentiation was always highly significant *(*P < 0.01 after Bonferroni correction for multiple testing) and ranged from 0.021 for the comparison CH-CH2 (two sheltered sites separated 400 m) to 0.506 for SM-CH2 (exposed vs. sheltered site, over 100 km apart). Similarly, pairwise F_{ST} differentiation ranged from 0.014 for CH-CH2 to 0.405 for SM-S (Table 4).

Table 3. *Nucella lapillus* genetic structure

A) Wright's fixation index (F_{ST}) and Φ_{PT} values.

	F_{ST}	Φ_{PT}
All populations (10)	0.172	0.254
	P < 0.0001	P< 0.001
Sheltered coast populations (5)	0.049	0.082
	P < 0.0001	P < 0.001
Exposed coast populations (5)	0.095	0.134
	P < 0.0001	P < 0.001

B) Hierarchical AMOVA with populations grouped in two areas (exposed and sheltered).

Source	df	SS	Estimated variance	Φ Statistics	P
Between areas	1	871.2	5.38 (26%)	Φ_{RT} = 0.258	< 0.001
Between populations	8	517.8	1.70 (8%)	Φ_{PR} = 0.110	< 0.001
Within populations	29	3,987.2	13.75 (66%)	Φ_{PT} = 0.340	< 0.001

The hierarchical structure was clearly illustrated both by the UPGMA cluster analysis (Fig. 2) and by the Principal Coordinates Analysis (PCoA, Fig. 3) of Nei's genetic distances between pairs of populations. Samples consistently formed two distinct, non-overlapping groups of exposed and sheltered coast sites. Separation between samples within each

group was smaller than the distances found between samples from different areas. Furthermore, the position of sheltered coast sites within the PCoA ordination was highly consistent with their geographical arrangement. The same hierarchical structure could also be recognized when Euclidean genetic distances between individuals (not populations) were used to construct the PCoA plot (results not shown). Individuals

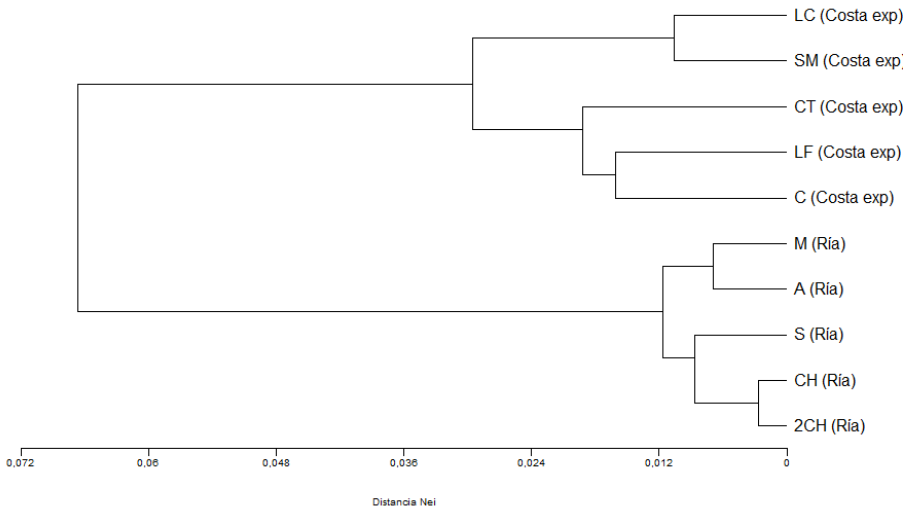

Fig. 2. *Nucella lapillus* UPGMA analysis based on Nei's genetic distances between sites.

from sheltered and exposed coast locations clearly separated into two compact groups along coordinate one. The only exceptions were (i) a small number of individuals collected in M (8) and A (2) that clustered with individuals from exposed coast sites, and (ii) 14 individuals from CT, four from C, and one from LF (exposed coast sites) that were plotted alongside with animals sampled within the ria.

The occurrence of hierarchical structure suggested that *N. lapillus* might follow a model of isolation by distance. Mantel test confirmed this hypothesis (Fig. 4) and the correlation between the logarithm of genetic distance and the logarithm of geographical distance was highly significant (reduced major axis regression: $\Phi_{PT} = 0.0330\ Dist^{0.4911}$, $r^2 = 0.639$, $P < 0.001$ for 1,000 randomizations). The correlation was still evident when the test was restricted to sheltered coast sites ($\Phi_{PT} = 0.0317\ Dist^{0.5030,}$ $r^2 = 0.773$, $P < 0.051$) but disappeared when only samples from exposed coast were considered in the analysis ($r^2 = 0.014$, $P < 0.43$) (figure not shown).

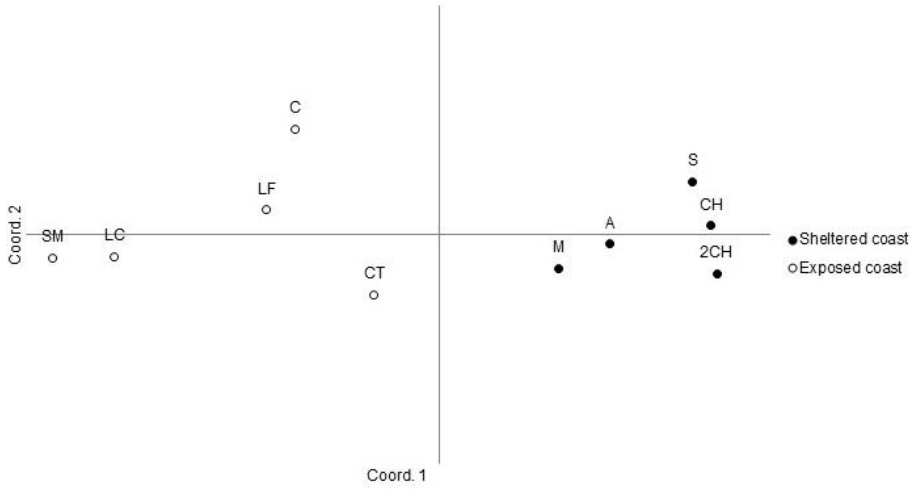

Fig. 3. *Nucella lapillus* Principal Coordinates Analysis. Nei's distances between populations are used for the PCoA analysis. The first coordinate explains 79.9% of the variation; the second coordinate explains 6.4%

Using the complete data set, STRUCTURE detected two clusters that matched the two studied areas (Fig. 1). The cluster that drew together the sheltered coast sites showed some individuals with mixed ancestry.

These individuals, which show a minor fraction of their membership belonging to the other cluster, were sampled within the two sites in the outermost part of the ria (M and A). In contrast, most of the individuals in the cluster corresponding to the exposed coast showed mixed ancestry except in SM (only one individual with mixed ancestry) and in CT (some individuals were entirely assigned to the sheltered cluster). Separate analysis for sheltered coast sites returned identical results, whereas the analysis of the exposed coast revealed a genetic structure matching the sampling locations.

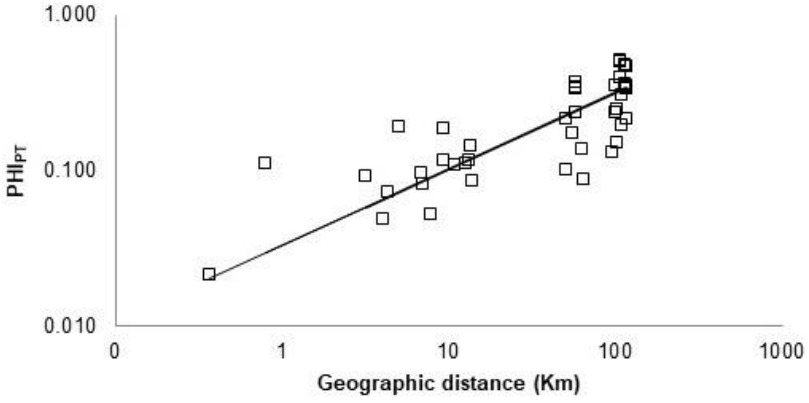

Fig. 4. *Nucella lapillus* correlation analysis. Linear relationship between geographic and genetic distance between pairs of samples in all the sites ($r^2 = 0.587$, $P < 0.001$). Line is the major axis regression between the two variables

Table 4. *Nucella lapillus*. Pairwise F_{ST} between populations (lower diagonal) and pairwise geographic distance in km (upper diagonal).

	Sheltered coast					Exposed coast				
	CH2	CH	S	A	M	C	CT	SM	LC	LF
CH2		0.370	4.280	6.800	13.240	57.830	101.390	106.510	115.370	115.430
CH	0.0139***		4.020	7.001	12.800	57.490	101.080	106.740	115.610	115.760
S	0.0576***	0.0434***		3.150	10.860	58.070	102.480	107.600	116.400	116.405
A	0.0368***	0.0390***	0.0540***		7.800	58.120	103.420	108.040	117.000	117.005
M	0.0569***	0.0749***	0.0806***	0.0308***		50.330	95.640	100.220	109.190	109.270
C	0.2223***	0.2132***	0.2228***	0.1500***	0.1332***		50.130	54.710	63.760	63.910
CT	0.1394***	0.1489***	0.1791***	0.0916***	0.0812***	0.0765***		5.000	13.700	13.780
SM	0.3698***	0.3810***	0.4046***	0.2983***	0.2579***	0.1219***	0.1426***		9.300	9.400
LC	0.3398***	0.3444***	0.3671***	0.2642***	0.2307***	0.1176***	0.1233***	0.0519***		0.800
LF	0.2253***	0.2190***	0.2405***	0.1494***	0.1392***	0.0638***	0.0611***	0.1149***	0.0747***	

*** $P < 0.001$ (significance after 10,000 permutations).

DISCUSSION

GENETIC EVIDENCES OF LOW DISPERSAL IN *NUCELLA LAPILLUS*

The dogwhelk *Nucella lapillus* possesses attributes typical of a low dispersal organism. The absence of a planktonic larva together with a sedentary adult stage constrained to rocky intertidal enclaves are traits linked to limited dispersion (Bohonak 1999, Shanks *et al.* 2003, Lester *et al.* 2007). In agreement with this, the conventional genetic analysis of our AFLP data (F_{ST}, IBD) found a significant genetic differentiation among sites, indicating that *N. lapillus* does not form the single panmictic population expected in organisms with long-distance dispersal. Instead, genetic differentiation was strong between areas (sites separated by 50-120 km) as well as between sites within a single area (distances < 15 km). Even sheltered sites separated as little as 400 m along an uninterrupted rocky intertidal suitable for *N. lapillus* showed significant genetic differences. Our F_{ST} estimates are comparable to those obtained for other gastropods with direct development (Hoskin 1997, Bell 2008, Lee & Boulding 2009, Hoffman *et al.* 2011). Moreover, differentiation increased with geographical distance following an IBD pattern with a steep slope that suggests that dispersal must be low in this species. IBD was likewise evident at area scale within the sheltered ria, but not in the exposed coast where it was replaced by a seemingly unordered pattern.

The slopes of the isolation by distance lines can be grossly translated into estimates of mean dispersal distances using the theoretical model proposed by Kinlan and Gaines (2003) based on an idea of Palumbi

(2003). These estimates suggest that dogwhelks disperse, on average, between circa 300 meters per year (sheltered sites only) to 700 (exposed and sheltered sites analyzed together). From a compendium of genetic studies of marine organisms, Kinlan and Gaines (2003) concluded that estimates of dispersal distances obtained for species lacking both planktonic larval phase and other secondary mechanisms of dispersion (as *N. lapillus*) typically were << 1 km whereas the average distances in organisms without planktonic phase but with secondary dispersal mechanisms (e.g. adults who are able to raft attached to floating structures, drift or have reproductive fragments) would reach some 10 Km. Therefore, the mean dispersal distances estimated for *N. lapillus* fall well within the typical range found in species with very low dispersal capacity. Our results are also in agreement with a pioneer allozyme work conducted on *N. lapillus* (Day & Bayne 1988) where interpopulation variability, both for allele frequencies and heterozygosity, fitted the expectations for a species with direct development. Later, Goudet *et al.* (1994) reanalyzed allozyme data finding overall F_{ST} estimates (0.3326) and a three level structure that provided further support to the low dispersal of the dogwhelks.

The low gene flow and restricted dispersal inferred with AFLP and allozyme data contrasts with the more recent proposal that dogwhelks must have larger dispersal ability and move longer distances than previously thought (Colson & Hughes 2004). The reasons for this discrepancy are uncertain, but they may partly lie in the different approach of the data analysis used in each study. Colson and Hughes

(2004) based their proposal in the comparison of microsatellite genetic diversity and structure between continuous and recolonized sites after a partial ban on TBT antifouling paints imposed in 1987. Their finding that genetic diversity was non-significantly lower in recolonized populations was interpreted as evidence that recolonization of vacant sites had been accomplished by a relatively high number of individuals originating from several source populations. Later work based on quantitative genetic variation in shell form produced similar results (Colson *et al.* 2006). However, genetic diversity is known to be notably resilient to reductions in effective population size and short bottlenecks must be very severe to have a substantial impact on heterozygosity. For example, conventional population genetics theory predicts that a bottleneck of size two still retains 75% of initial heterozygosity after one generation (Allendorf *et al.* 2007). In this regard, the small declines in average gene diversity observed by Colson and Hughes (see Table 3 in Colson & Hughes 2004) in sites recolonized by *N. lapillus*, albeit statistically non-significant, are compatible with recolonization by a very low number of colonist (between 3 and 35) provided that census sizes were rapidly recovered after a short founding effect. Similarly, Piñeira *et al.* (2008) did not find evidence of severe bottleneck effects (i.e. reductions in genetic variation) in populations of *Littorina saxatilis*, another marine gastropod with direct development that had recovered from a massive oil spill.

Our genetic markers reveal that, in addition to being genetically differentiated, the exposed and sheltered areas differed in the intensity and spatial pattern of their genetic structure. Exposed populations displayed stronger genetic differences among them but these differences did not conform to any spatial pattern. In comparison, populations inside the ria were genetically closer to each other but their differences fitted the expectations of an IBD pattern. Accordingly and after a hierarchical approach, STRUCTURE was still not able to resolve among sheltered IBD-patterned sites (Pritchard *et al.* 2010), but it did detect some structuring among the non-IBD patterned exposed ones. This variation in the genetic structure of exposed and sheltered areas is not unique to *N. lapillus*. The netted dogwhelk *Nassarius reticulatus* also showed a stronger genetic structure between exposed populations than between protected ones (Barreiro *et al.* 2006). Therefore, our results seem consistent with the hypothesis that the environmental conditions of exposed sites may promote higher levels of genetic variability between local populations in both gastropods.

Despite these similarities, there are still interesting differences between *N. lapillus* and *N. reticulatus*. Specifically, the populations from inside the ria are genetically indistinguishable in *N. reticulatus* while they retain significant levels of genetic differentiation in *N. lapillus*. Since both gastropods were studied in the same geographical context (NW Spain), it seems reasonable to presume that these differences in the intensity of the genetic structure in sheltered sites are possibly linked to

the contrasting dispersal ability of each species. Thus, while veligers of *N. reticulatus* spend 1-2 months in the plankton and its adults are very mobile (Lebour 1931, Tallmark 1980, Lambeck 1984), *N. lapillus* is a snail with direct development and a reputation for very sedentary adults (see Fretter & Graham 1994 and references therein). Hence, the stronger genetic structure and IBD pattern found inside the ria for *N. lapillus* seems consistent with its life history traits and provides further support to the conclusion that this dogwhelk must disperse little, at least in sheltered areas with low hydrodynamics.

Part of the genetic differences detected inside the ria is due to a gradual increase of individuals with a distinct genetic composition towards its mouth. Both PCoA and STRUCTURE reveal that the two enclaves located in the lower reaches of the ria (M, A) are genetically closer to open-coast populations than those from the upper reaches (S, CH, CH2). STRUCTURE showed that the admixture proportion is higher in the individuals sampled in site M (closer to the entrance of the ria) which genetic composition assigned to the cluster dominating in exposed populations. The origins of the gradual increase of a distinct genetic composition towards the mouth of the ria are uncertain. Two hypotheses can be invoked to explain the admixture proportion found in individuals in the mouth of the ria. One explanation is that a fraction of the genome could have been inherited from a migrant ancestor. Alternatively, the occurrence of dogwhelks from two genetic clusters within the same location has formerly been related to the existence of individuals belonging to two different shell morphotypes (exposed and

sheltered) (Guerra-Varela *et al.* 2009). Although sheltered and exposed morphs are typically found at localities with contrasting levels of exposure to wave action, they also occur sympatrically in some NW Spain sites (Rolán *et al.* 2004, Guerra-Varela *et al.* 2009). In the latter, Guerra-Varela *et al.* (2009) found significant microsatellite differentiation between the two morphs and each morphotype was mostly assigned to a different genetic cluster by STRUCTURE. Nevertheless, their results show that dogwhelks from localities with moderate protection comparable to our M and A sites are also divided into the two genetic groups even though they all share the sheltered morph.

The situation is very different in exposed populations. Their unordered arrangement of genetic differences resembles the structure found in other coastal organisms studied at small-to-moderate spatial scales (Barreiro *et al.* 2006, Selkoe *et al.* 2006, González-Wangüemert *et al.* 2007, Hogan *et al.* 2010, Selkoe *et al.* 2010) and adds to the growing body of evidence that suggests that geographic distance is often a poor predictor of gene flow in coastal systems (White *et al.* 2010). Traditionally, this "chaotic genetic patchiness" (Johnson & Black 1982) has been interpreted as evidence that larval pools and recruitment are heterogeneous, and several mechanisms (limited mixing of larvae from distinct sources, patchy environmental selection, sweepstakes reproductive success, or even a combination of local recruitment with low dispersion) have been proposed to explain this heterogeneity (see Selkoe *et al.* 2006 and references threrein). However, recent studies

suggest that the genetic patterns of coastal organisms possibly reflect more than just larval dispersal. Hence, habitat factors (e.g. size of suitable habitat) have been found to be more informative predictors of the genetics for different species than oceanographic features (temperature, currents, distance) suggesting that the genetic structure may be driven predominantly by variations in effective population sizes (*Ne*) instead of migration rates (*m*) (Selkoe *et al.* 2010). Further work is needed to corroborate whether these conclusions apply to other coastal systems. Yet, the proposal that the genetic structure might not be tightly linked to dispersal among populations would explain why organisms with substantial differences in their potential to disperse (e.g. *N. lapillus* in this study vs. *N. reticulatus* in Barreiro *et al.* (2006) manage to develop a similarly unordered pattern along wave-exposed coasts (for other examples of discrepancies between life traits and genetic differentiation see Galarza *et al.* 2009 and references therein).

Most efforts to explain chaotic genetic patchiness have focused on recruitment from a larval pool, sometimes from distant sources, since this pattern is mostly detected in organisms with planktonic larvae. However, dogwhelks are direct-developers and our results suggest that dispersal is restricted, at least under some conditions. If limited dispersal were also applicable to wave-exposed populations, they should be mostly maintained by self-recruitment. Under this hypothesis, we would predict a spatially stable pattern of genetic differences over time and/or across age groups (Hancock 2000). Alternatively, if stronger wave action enhances dispersal, exposed populations would be

maintained by both self-recruits and immigrants resulting in spatio-temporal changes in the genetic structure. Finally, spatial structure may depend on variations of *Ne* rather than *m* as proposed by Selkoe *et al.* (2010). We are not aware of any studies on changes of *Ne* over time in *N. lapillus*. However, some long-term monitoring surveys suggest that dogwhelks may experience appreciable changes in local abundance that, more importantly, may not be synchronous between sites (Burrows *et al.* 2002). Should these changes have an impact on N_e, we would anticipate variations in the spatial genetic structure over time. Future work comparing population structure across age groups to test this prediction seems warranted.

In summary, using a sizeable number of reproducible AFLP markers, we found significant interpopulation differentiation at both regional (< 200 km) and area (< 15 km) scales. Genetic differences followed an IBD pattern at regional scale as well as among sheltered populations within a ria, indicative of restrictions to gene flow. Thus, our results support the conventional view of *N. lapillus* as a poor disperser and seem consistent with earlier allozyme studies as well as with predictions derived from its life history features (Chia *et al.* 1984, Day & Bayne 1988, Day 1990, Day *et al.* 1994, Fretter & Graham 1994a, Goudet *et al.* 1994). Nevertheless, the variation in the genetic arrangement observed within the two areas (sheltered and exposed) analyzed in this study seems consistent with an impact of the hydrodynamic regime on the spatial genetic structure. The correlation between spatial and genetic distances detected among our sheltered sites vanished in wave-exposed

populations. The latter showed the unordered arrangement of genetic distances typical of other coastal organisms. The mechanisms behind this unordered patchiness are not clear and further work is required to unravel them.

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CHAPTER IV

WAVE EXPOSURE AS A DRIVER OF ISOLATION BY ENVIRONMENT IN THE MARINE GASTROPOD *NUCELLA LAPILLUS* L.

ABSTRACT

The way adaptive and neutral genetic variation is shaped by environmental factors is crucial for evolutionary biology. To investigate whether wave exposure can enhance local adaptation on littoral snails, AFLP markers were scanned across ten populations of *Nucella lapillus* from contrasting habitats (protected vs. exposed). As some 6% of the analysed loci deviated from neutral expectations, it was suggested that wave exposure could be a strong selective agent shaping genetic variation. Neutral markers described a pattern of "Isolation by distance (IBD) only" with no signature of Isolation by environment (IBE), whereas loci under divergent selection followed a pattern of "IBD and IBE", as Partial Mantel tests detected a significant IBD after accounting for environmental differences. The topology of genetic networks revealed a substantial gene flow at neutral markers (i.e. dense net with edges connecting similar and contrasting habitats), whereas few connections were established between contrasting environments at loci under divergent selection. Furthermore, loci correlated to phenotype (shell shape; i.e. a morphological biomarker of wave exposure) explained up

to ca 11% of the variance of this trait. Altogether, our results suggest that, even in a context of gene flow, local adaptation could outline a feature such as shell shape.

INTRODUCTION

Seascape genetics is the marine analogous to the terrestrial landscape genetics, and aims to evaluate the impact of environmental features on spatial genetic divergence in marine organisms (H. M. Galindo *et al.* 2006, 2008, Riginos & Liggins 2013). The transposition of terrestrial approaches to the marine environment faces challenges because of the oceanography dynamism, and the subtle genetic structure emerging from high larval dispersal coupled with large effective population sizes (Hellberg *et al.* 2002, Selkoe *et al.* 2008). The distribution of genetic variation among and within populations of a species is determined by the interaction between neutral evolutionary processes such as drift or gene flow, and natural selection. Divergent natural selection can cause phenotypic differentiation either by its direct action on the additive genetic component of quantitative variation (Coyne & Orr 1989, Schluter 2000, 2004, Dieckmann *et al.* 2004), or by phenotypic plasticity (Via 1993, Via *et al.* 1995, Kingsolver *et al.* 2002). Unravelling which traits are subjected to divergent selection - and under which conditions they experience diversification - are essential steps towards understanding local adaptation, i.e. “the fine-tuning of populations to their local environments via natural selection” (Lenormand 2002, Kawecki & Ebert 2004, Räsänen & Hendry 2008, Kavanagh *et al.* 2010, Sanford & Kelly 2011). Genetic drift together with gene flow may disturb the effects of divergent selection and hamper local adaptation (e.g. Slatkin 1987, García-Ramos & Kirkpatrick 1997, Lenormand 2002, Kawecki & Ebert 2004, Garant *et al.* 2007). However, in situations where

selection is strong, local adaptation can evolve albeit the presence of gene flow (e.g. Koskinen *et al.* 2002, Conover *et al.* 2006, Yeaman & Jarvis 2006, Peccoud *et al.* 2009), and eventually give rise to new species (Schluter 2009).

Local adaptation has long been studied in terrestrial and freshwater habitats, but an increasing body of literature has been devoted to the marine environment as well. In this context, intertidal areas provide a valuable scenario to explore the mechanisms that account for local adaptation and speciation due to their variable environmental conditions, both in spatial and temporal terms. Although differentiation is usually stronger among direct-developing species (Bohonak 1999), many marine species and, in particular, a significant fraction of the marine invertebrates with planktonic dispersal show evidences of local adaptation (see Sanford & Kelly 2011 for a review). Littoral snails have also long been a frequent study system to address local adaptation due to the remarkable shell phenotypic polymorphism they exhibit in shape, thickness, colour, spotting (e.g. Vermeij 1982, Crothers 1985, Boulding 1990, Etter 1996, B. Johannesson & Johannesson 1996). Probably, the most thoroughly studied snail is the intertidal *Littorina saxatilis*, which shows pairs of ecologically divergent ecotypes in multiple sites along the North Atlantic shores (e.g. Wilding *et al.* 2001, Panova *et al.* 2006, Rolán-Álvarez 2007, K. Johannesson *et al.* 2010, Martínez-Fernández *et al.* 2010, Chrismas *et al.* 2014, J. Galindo *et al.* 2014, Westram *et al.* 2014, Rolán-Álvarez *et al.* 2015, Tirado *et al.* 2016, Kess *et al.* 2018, J. Galindo *et al.* 2019). Furthermore, this species has been considered a very

suitable model to investigate ecological speciation in the face of gene flow (e.g. Rolán-Álvarez 2007, Butlin *et al.* 2008, Schluter 2009, K. Johannesson *et al.* 2010, Nosil & Feder 2012, Butlin *et al.* 2014). In Galicia, the region of our study, two ecotypes have been described in connection to the shell shape: ridged-banded and smooth-unbanded, which are found associated with different levels of the intertidal and dominated by different ecological pressures (Rolán-Álvarez 2007). These ecotypes are characterized by a great divergence in morphology (Carvajal-Rodríguez *et al.* 2005, Conde-Padín *et al.* 2007, Conde-Padín *et al.* 2008), gene expression (Martínez-Fernández *et al.* 2010), protein expression (Martínez-Fernández *et al.* 2008) and behaviour (Erlandsson *et al.* 1998). Interestingly, in spite that both forms show a little neutral genetic differentiation (K. Johannesson *et al.* 1993, Rolán *et al.* 2004, Quesada *et al.* 2007, J. Galindo *et al.* 2009), an AFLP genome scan between them detected some 3% loci presumably affected by selection (directly or indirectly) and highly divergent between them (J. Galindo *et al.* 2009). Therefore, all the evidences suggest that ecologically based divergent natural selection is mostly responsible for the evolution of partial reproductive isolation between the aforementioned ecotypes (see Rolán-Álvarez 2007 for a review). More recently, a third ecotype with a distinctive shell morphology and associated to very sheltered coastal sites has been described (Tirado *et al.* 2016, J. Galindo *et al.* 2019). Similarly, the Australian intertidal snail *Bembicium vittatum* shows heritable differences in shell shape among habitats indicating local adaptation (Johnson & Black 2008). In contrast, the phenotypic

different patterns found in the shells of the Antarctic limpet *Nacella concinna*, seem a purely plastic response (Hoffman *et al.* 2010).

The dogwhelk *Nucella lapillus* (Linnaeus 1758) is a predatory gastropod with a Northern amphi-Atlantic distribution that occurs abundantly on rocky shores. This dioecious species is a direct-developer with limited dispersal capability and a marked spatial variation in shell morphology (Kitching *et al.* 1966). Its prominent shell polymorphism in terms of shape, color, ornamentation and size has attracted the interest of researchers since the beginning of 20th century (Colton 1922, Moore 1936, Berry & Crothers 1968, 1974, Bantock & Cockaine 1975, Kitching 1977, Vermeij 1982, Kitching 1985, Day & Bayne 1988, Kirby *et al.* 1994, Etter 1996) making this organism a suitable model to study genetic variability (Castle & Emery 1981), even before the raise of molecular-based techniques. Shell shape has been associated with the degree of wave exposure as exposed ecotypes are typically squatter, with relatively larger apertures and thinner shells than the sheltered ecotypes (Crothers 1985). Kitching *et al*. (1966) showed experimentally that the large-mouthed form was better suited to attach to the rock surface than the more elongated, sheltered form, therefore presenting empiric evidence of positive selection for the short-squat type on exposed shores (Berry & Crothers 1968). Although shell-shape variation has been shown to have an important genetic component (Guerra-Varela *et al.* 2009), *N. lapillus* may also display phenotypic plasticity in response to environmental cues such as wave action (Crothers 1983, Etter 1996) or predators (Vermeij 1982, Crothers 1983, Palmer 1990)

"Isolation by Distance" (IBD) is the term used to refer to the increase of genetic differentiation with geographic distance as a result of restricted gene flow and drift (Wright 1943, Malécot & Blaringhem 1948, Slatkin 1993, Rousset 1997). Its underlying process is "Isolation by Dispersal Limitation" (IBDL), an essentially neutral process unrelated to any change in the environment leading to differential individual success (Orsini *et al.* 2013). Nonetheless, adaptive processes can also play a part when contrasting environmental conditions between source and receptor habitats promote barriers to gene flow through local adaptation. In these cases, the reduced gene flow under local adaptation may also increase the genetic divergence at neutral loci through genome-wide divergence via genetic drift in a process known as "Isolation by Adaptation" (IBA) (Nosil *et al.* 2007, Nosil 2009). IBA can result in "Isolation by Environment" (IBE) (I. J. Wang & Bradburd 2014), a pattern in which genetic differentiation increases with environmental differences irrespective of geographic distance (I. J. Wang & Bradburd 2014). In extreme cases, ecological speciation might arise when divergent selection reduces gene flow (Schluter 2000, Rundle & Nosil 2005), and eventually results in a complete reproductive isolation (Nosil 2012) through mechanisms such as selection against migrants (Hendry 2004, Thibert-Plante & Hendry 2009), matching habitat choice (Edelaar *et al.* 2008) or assortative mating (Weissing *et al.* 2011). Recent meta-analyses highlight a prevalence of IBE across taxa in different ecosystems (Shafer & Wolf 2013, Sexton *et al.* 2014). Moreover, the co-occurrence of IBD and IBE poses interesting challenges such as i) disentangling the relative strengths of IBD and IBE in the observed patterns of genetic

differentiation when geographic distance and environmental differences are usually correlated (Shafer & Wolf 2013, I. J. Wang *et al.* 2013, Nanninga *et al.* 2014); and ii) determining the processes that generate and maintain the observed patterns.

To assess the role of selection in generating diversity in *N. lapillus*, we performed an Amplified Fragment Length Polymorphism (AFLP) scan on ten populations sampled along a gradient of wave exposure in Galicia (NW Spain) aiming to detect and quantify the proportion of the genome under divergent selection. AFLPs are popular markers to conduct scans for species with no genome information because they are easy to genotype, the protocols are transferable between closely related taxa (Blignaut *et al.* 2013), the technique is robust and repeatable (Meudt & Clarke 2007), and the cost is affordable (Mattersdorfer *et al.* 2012, Westberg *et al.* 2013). In particular, AFLP scans have been successfully used with gastropods (e.g. Grahame *et al.* 2006, H. M. Wood *et al.* 2008, J. Galindo *et al.* 2009, Chen *et al.* 2010, Quintela *et al.* 2014). We also explored the variation in shell morphology related to wave exposure by studying the association of AFLP band frequencies with habitat categories as implemented in the spatial analysis method (SAM) (Joost *et al.* 2008). The way geographic distance and environmental features (wave exposure) structure adaptive and nonadaptive genetic variation was assessed through a network approach (Dyer & Nason 2004). Finally, the correlation between genetic and phenotypic traits was investigated with the NOIA model (Álvarez-Castro & Carlborg 2007, Le Rouzic & Álvarez-Castro 2008).

MATERIAL AND METHODS

SAMPLING AND AFLP (AMPLIFIED FRAGMENT LENGTH POLYMORPHISM) GENOTYPING

Ten samples consisting of 30 individuals each (15 males and 15 females) were collected in Galicia (NW Spain, Fig. 1). Five of the sampling sites (CH2, CH, S, A, M) were found in sheltered coast locations within a ría (i.e. coast inlet formed by the partial submergence of an unglaciated river valley) whereas the five remaining ones (LF, LC, SM, CT, and C) were located in open and exposed sites. Importantly, sites were selected so that the range of geographic distances among locations (from 400 m to 15 km) was analogous within both areas. The degree of exposure of the sites allowed classifying them into five types defined by the vegetation of the site and wave exposure, with lower numbers depicting higher exposure. Thus, open coast sites were assigned to classes 1 to 3 whereas sheltered sites were classified in classes 4 and 5 (Table 1). Only subadults, identified by their shell traits (Crothers 1985), were sampled in an effort to gather individuals from a single cohort (Barreiro *et al.* 2006) to avoid the confounding effects of possible temporal genetic instability (Johnson & Black 1991). Furthermore, individuals were collected from the smallest possible area per site to avoid mixing different breeding groups, which might lead to a heterozygosity reduction due to Wahlund effect, as reported in former studies with the same species (Day 1990, Goudet *et al.* 1994).

Table 1. Information about the sampling sites: area in which they are located, code of the site, category of shore protection (Exposure) with higher numbers depicting more protected areas, coordinates, number of individuals sampled (N), and distribution of frequencies (%) per population for the 11 candidate loci for directional selection.

						Frequency of AFLP locus (%)										
Area	Site	Exposure	Latitude	Longitude	N	AGAC314	AGAC345	CTTA166	AGGT187	CTGT115	CTTA190	AGAC228	CTAC180	CTTA245	AGAC323	AGTG82
Sheltered	CH2	5	42.606581	-8.862765	30	0	0	0	7	3	3	7	14	93	100	100
	CH	5	42.606669	-8.858151	30	7	0	0	0	7	7	23	7	93	100	100
	S	4	42.578366	-8.825678	30	10	0	0	0	13	13	23	20	100	100	100
	A	4	42.548874	-8.833489	30	37	7	3	3	50	13	67	50	73	100	97
	M	4	42.491891	-8.889548	30	47	27	23	30	43	23	37	43	83	90	93
Open	C	2	42.84436	-9.12749	30	90	57	55	53	86	59	93	93	28	40	50
	CT	3	43.187825	-9.165893	30	70	50	50	47	63	70	60	57	50	57	57
	SM	3	43.195657	-9.110091	30	97	97	97	97	100	100	100	100	0	0	7
	LC	1	43.226764	-9.011101	30	97	97	97	97	100	100	100	100	30	30	23
	LF	1	43.232676	-9.012217	30	97	67	63	63	97	73	97	100	23	47	57

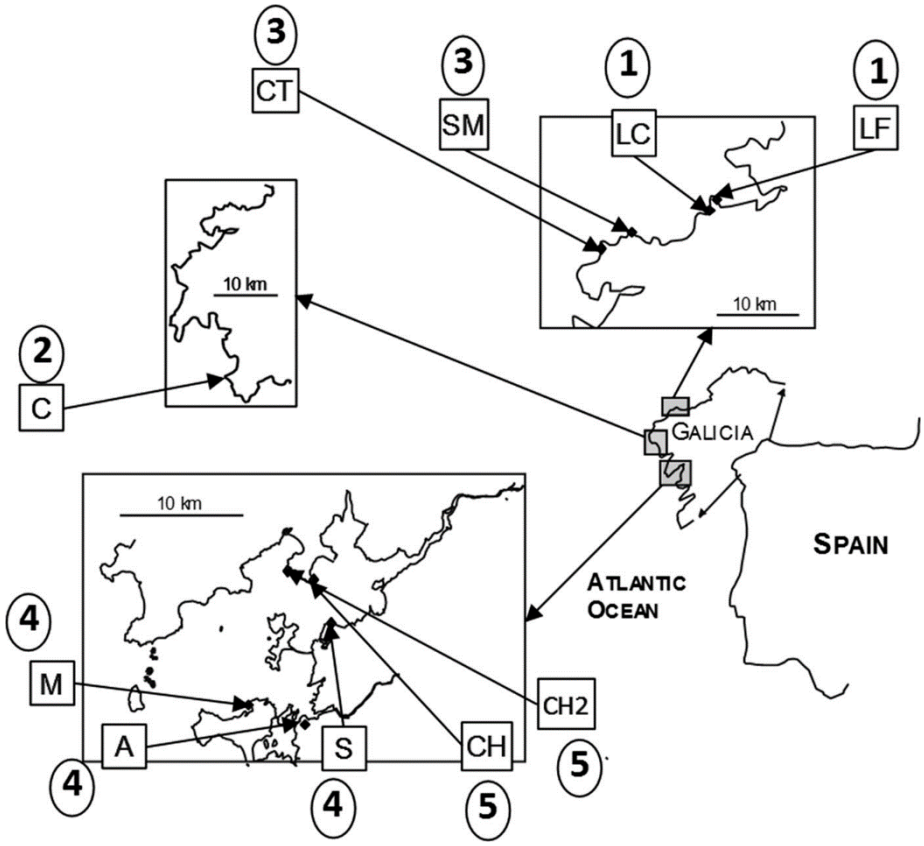

Fig. 1. Map of Galicia (NW Spain) showing the localization of the study area. Sample sites located in open coast (above) and in sheltered coast within a ría (below). Numbers depict the categories of wave exposure (the lower the number the higher the exposure). See the text for further information and Table 1 for full names of the sampling sites.

In the laboratory, shell length (L) and aperture height (Ap) were recorded for each individual. The relationship between both parameters (L/Ap) has been used as a proxy of shell shape as in Crothers (1973) (Table 1). Shells were opened with a vice and individuals were sexed. Foot and mantle tissue were stored in 96º ethanol at 12º C. To avoid cross contamination, each specimen was dissected using disposable scalpels and tweezers that were flame sterilized between individual

samples. Genomic DNA was extracted from some 3 mg (dry weight) mantle tissue using Qiagen DNeasy tissue kit following manufacturer instructions and stored in TE at -20 ° C.

We followed Tru1I /EcoRI protocol of Vos *et al.* (1995) and excluded all loci that did not produce consistent genotypes for positive controls across plates. Although AFLP profiles usually display high levels of reproducibility, it is widely recommended that reproducibility should be tested for each batch of reactions by using replicates; also, negative controls should be routinely run along samples to avoid systematic contamination (Bonin *et al.* 2004, Paun & Schönswetter 2012). Consequently, a very stringent approach was adopted to ensure the reproducibility/reliability of our data set by filtering-out any potentially unreliable loci, and which ended with 218 repeatable loci from six selective primer combinations, with 20 - 52 loci produced per primer pair.

OUTLIER DETECTION

Two different basic approaches allow screening for loci putatively under selection: (i) correlative methods that investigate associations between genetic variation and environmental features (Foll & Gaggiotti 2006, Joost *et al.* 2007, Eckert *et al.* 2010) and (ii) methods to detect outlier loci showing unexpectedly low or high differentiation (Beaumont & Nichols 1996, Foll & Gaggiotti 2008, Excoffier *et al.* 2009). To minimize the risk of detecting false positives, we used three different procedures to detect loci deviating from neutrality. First, we used the hierarchical Bayesian method described in Beaumont & Balding (2004) and

implemented in BayeScan software (Foll & Gaggiotti 2008), which is grounded on the basis that genetic differentiation among populations from contrasting environments is expected to be different for those loci under selection than for the rest of the genome. The program was run by setting sample size to 10000 and the thinning interval to 50 as suggested by Foll & Gaggiotti (2008), resulting in a total chain length of 600000 iterations. Loci with a posterior probability over 0.999 were retained as outliers, corresponding to a Bayes Factor > 3 and providing substantial support for the model. Secondly, we used the Fdist approach by Beaumont & Nichols (1996) and implemented in Mcheza (Antao & Lopes 2011) in which loci with an unusually high F_{ST} are considered to be putatively under divergent selection. We simulated the neutral distribution of F_{ST} with 1000000 iterations at a significance level of 0.001. This method also implements a multitest correction based on false discovery rates (FDR) to avoid high overestimation of the percentage of outliers (e.g. 1% of false positive with a threshold of 99%). Finally, we used the Spatial Analysis Method (SAM) described by Joost *et al*. (2007) based on the evaluation of the incidence of spatial or environmental coincidence. SAM identifies alleles associated with environmental variables without the need of defining populations. However, it must be combined with the above-mentioned methods to differentiate between divergent and stabilizing selection. SAM calculates logistic regressions between all possible marker-environmental pairs and determines if a model including an environmental variable is more informative than a model including only the constant. We tested for the effect of wave exposure and shell shape

under a restrictive approach, which considered a model significant only if both G and Wald Beta 1 tests rejected the corresponding null hypothesis at the 0.000103093 confidence level after Bonferroni correction, corresponding to P = 0.01. The aforementioned analyses were restricted to loci with a dominant allele frequency between 5% and 95% across the whole dataset. The aim of restricting the analyses to those loci was to decrease the probability that differentiation, at a given locus, would be mistakenly identified as a signature of selection merely because it stood out against low levels of background genetic variation resulting from the inclusion of low-polymorphic markers (Richter-Boix *et al.* 2011).

GENETIC ANALYSIS AND POPULATION CONNECTIVITY

Subsequent to outlier detection, the original data set was partitioned into two subsets: neutral markers and loci under divergent selection. Genetic differentiation among populations was estimated separately for both data sets by the Analysis of Molecular Variance (AMOVA, Excoffier *et al.* 1992a) implemented in the program GeneAlEx v6.4 (Peakall & Smouse 2006). Pairwise F_{ST} was calculated using AFLP-SURV, with significance based on 10000 permutations.

We performed network analyses to investigate genetic connectivity among sites. A population graph is a network that represents a landscape of discrete habitat patches as a set of nodes (sites) interconnected by edges (gene flow) between them (Minor & Urban 2007). The length of an edge is inversely proportional to the genetic covariance between both populations, and, conversely, pairs of

populations that are isolated from each other due to lack of gene flow are not linked in the graph. A relatively small graph distance between spatially distant sites indicates long-distance migration (extended edges) whereas geographical or ecological barriers that impede migration relative to other localities with similar distance generate relatively high graph distances (compressed edges; Dyer *et al.* 2010). Conditional genetic distance (cGD) derived from a population graph was used as a measure of genetic distance between pairs of populations (see Dyer & Nason 2004, Garroway *et al.* 2008), and can be defined as the minimal topological distance that connects them. Dyer *et al*. (2010) demonstrated that cGD outperforms traditional measures of genetic distance, such as F_{ST}, for describing spatial genetic structure due to its homoscedasticity. Networks were constructed using Population Graphs v2, and the software Genetic Studio (Dyer 2009) was used to derive cGD for all pairs of populations. We compared the genetic networks generated by neutral markers and by loci under divergent selection. A binomial test for the existence of two subgraphs within the dataset was conducted in each network to determine whether there was restricted gene flow between the populations from exposed and protected habitats. In partially reproductively isolated populations, the expected outcome would be to find two subgraphs for the loci under divergent selection but not for the neutral ones (Dyer & Nason 2004, Giordano *et al.* 2007).

ISOLATION BY DISTANCE (IBD) AND ISOLATION BY ENVIRONMENT (IBE)

Neutral and adaptive genetic variation distributed across space are net products of different evolutionary mechanisms (genetic drift, gene flow and selection), and it is important not to confound these components and the mechanisms shaping their spatial distribution. The relationships among genetic distance, local environmental conditions and geographical distances were examined using Mantel tests. To evaluate whether genetic drift may explain the spatial genetic patterns found in neutral markers, we performed a simple Mantel (1967) test between the matrices of genetic distance (estimated as pairwise F_{ST}) and geographical distance (measured with GoogleEarth as the shortest paths following the shore line between sites), expecting to find IBD due to restricted gene flow. An identical approach was used by substituting F_{ST} with the conditional genetic distance (cGD) to test for the existence of Isolation-By-Graph Distance (IBGD). This test allows us to detect the existence of genetic barriers to gene flow among populations. Assuming a homogeneous IBD process, graph distances and spatial distances should be proportional, but the relationship between expected edge length and spatial distance may change if migration is heterogeneous (Dyer & Nason 2004, Dyer 2009).

Environmental distance to test for IBE was calculated as the Euclidean pairwise differences of wave exposure. Likewise, a matrix of phenotypic distance was created using the differences in shell shape (estimated as shell length/aperture height, L/Ap). Mantel tests were conducted with

PASSaGE (Rosenberg & Anderson 2011) and significance was tested after 10000 permutations.

CORRELATION GENOTYPE-PHENOTYPE AND PHENOTYPE-ENVIRONMENT

The relationships between allele frequencies and phenotypes were assessed using the NOIA model implemented in the R-package "noia" (Le Rouzic *et al.* 2015), which uses linear regressions between individual phenotypes and genotypes (Álvarez-Castro & Carlborg 2007, Le Rouzic & Álvarez-Castro 2008). Allele frequencies per population for both the set of neutral markers and the set of loci under divergent selection were inferred with GenAlEx. Significant differences were declared when the allele effect was significant at level $P < 0.05$, after implementing a Bonferroni correction for multiple testing. Individual phenotypes for shell shape were obtained from the relation between length and aperture, L/Ap. Likewise, to investigate the correlation between phenotype and environment, we fitted a linear model with phenotype as a response to a five–levels environmental factor (exposure 1 to 5).

RESULTS

OUTLIER DETECTION

The six combinations of primers yielded a total of 218 AFLP loci ranging from 29 (combination AGTG) to 52 (AGGT). Each of the 300 individuals analyzed from the ten populations presented a unique multilocus genotype. Of the 218 repeatable AFLP loci, 97 loci with dominant allele frequencies ranging between ≥ 5 and ≤ 95% were retained for outlier detection analyses. The set of loci under divergent selection was

composed by the 11 markers that were simultaneously detected by BayeScan ($\log_{10}$(BF) > 3), Mcheza (P = 0.005) and SAM (P = 0.01) (Table 2). The distribution of allele frequencies per population of these loci showed that three of them were linked to sheltered sites (CTTA245, AGAC323, AGTG82) whereas the other eight showed higher frequencies in exposed sites (Table 1). We used a likewise restrictive approach to build the subset of neutral loci as it consisted of 27 markers that showed no sign of selection (neither divergent nor balancing) under any of the aforementioned outlier procedures.

Table 2. Candidate loci for directional selection simultaneously detected by: MCHEZA at a significance P value of 0.005, BayeScan at $\log_{10}$(BF) > 3, and SAM for coast exposure (P values for G and Wald Beta 1 with a significance threshold set to 95% after Bonferroni correction).

	MCHEZA		BAYESCAN		SAM	
Locus	F_{ST}	P(Simul F_{ST} < Sample F_{ST})	F_{ST}	$\log_{10}$(BF)	P value for G	P value for Wald Beta 1
AGAC323	0.531296	0.997675	0.4395	1000	0	0
CTAC180	0.532547	0.998575	0.40437	1000	0	0
CTGT115	0.500909	0.996000	0.39448	1000	0	0
CTTA166	0.461034	0.989350	0.39405	1000	0	0
AGAC345	0.450732	0.985925	0.39013	1000	0	0
AGAC314	0.445558	0.980900	0.38803	1000	0	0
CTTA190	0.510060	0.996025	0.38535	1000	0	0
AGTG82	0.474638	0.990075	0.37772	3.9999	0	2.2204E-16
AGGT187	0.442826	0.983650	0.37125	3.9999	0	0
AGAC228	0.465542	0.988375	0.37037	3.3008	0	1.1102E-16
CTTA245	0.384704	0.930302	0.34821	3.6988	0	0

GENETIC ANALYSES

The percentage of variation attributed to habitat (sheltered vs. exposed) by AMOVA (Table 3) took the highest value, 59%, at loci under divergent selection in contrast with the 16% found at neutral markers; whereas the differentiation among populations was similar in both cases (8 - 10%). Overall population differentiation was highly significant and higher for loci under selection (0.662, $P < 0.0001$). Pairwise F_{ST} estimates based on the 27 neutral AFLP markers ranged from 0.0084^{NS} to 0.1766*** (Table 4), whereas this range was larger for loci under selection (0.0000^{NS} to 0.8873***). Interestingly, F_{ST} estimates among populations from contrasting habitats were statistically significant in 96% of the pairwise comparisons for loci under selection (ranging from 0.2150 to 0.8873); whereas only 20% of the comparisons were significant for neutral loci (0.1185 - 0.1511).

Table 3. Hierarchical AMOVA differentiating between sheltered (within ría) and open coast (outside ría) for the 27 neutral markers and the 11 loci under directional selection. Probabilities (*P*) have been estimated based on 9999 permutations.

Markers	Source	df	SS	Estimated variance	Φ Statistics	*P*
Neutral	Between sheltered and open coast	1	101.75	0.587 (16%)	Φ_{RT} = 0.155	< 0.0001
	Among populations	8	109.15	0.360 (10%)	Φ_{PR} = 0.113	< 0.0001
	Within populations	290	821.13	2.831 (75%)	Φ_{PT} = 0.251	< 0.0001
Directional selection	Between sheltered and open coast	1	341.79	2.213 (59%)	Φ_{RT} = 0.586	< 0.0001
	Among populations	8	79.05	0.287 (8%)	Φ_{PR} = 0.184	< 0.0001
	Within populations	290	370	1.276 (34%)	Φ_{PT} = 0.662	< 0.0001

Table 4. Pairwise F_{ST} between populations calculated with AFLP-SURV for the 27 neutral loci (lower diagonal) and the 11 loci under directional selection (upper diagonal). Significance (values in bold-type face) is based on 10000 permutations.

		Sheltered coast					Open coast				
		CH2	CH	S	A	M	C	CT	SM	LC	LF
Sheltered coast	CH2		0.0000	0.0199	**0.1592**	**0.1487**	**0.5314**	**0.3754**	**0.8873**	**0.8261**	**0.6106**
	CH	**0.0242**		0.0071	**0.1261**	**0.1311**	**0.5110**	**0.3560**	**0.8759**	**0.8136**	**0.5923**
	S	0.0084	**0.0409**		**0.1521**	**0.1412**	**0.5127**	**0.3634**	**0.8728**	**0.8094**	**0.5904**
	A	**0.0621**	**0.0924**	**0.0399**		**0.0283**	**0.2813**	**0.1737**	**0.7323**	**0.6581**	**0.3685**
	M	**0.0349**	**0.0960**	**0.0343**	0.0229		**0.2150**	**0.0818**	**0.6635**	**0.5874**	**0.3072**
Open coast	C	**0.1582**	**0.1511**	**0.0833**	**0.1245**	**0.1392**		**0.0604**	**0.3407**	**0.2686**	0.0032
	CT	**0.1047**	**0.1366**	**0.1238**	**0.0800**	**0.0856**	**0.1758**		**0.4719**	**0.3958**	**0.1389**
	SM	**0.1709**	**0.1766**	**0.1182**	**0.0890**	**0.1157**	**0.1260**	**0.0996**		0.0000	**0.2610**
	LC	**0.1260**	**0.0973**	**0.0803**	**0.0603**	**0.1063**	**0.1394**	**0.0988**	**0.0866**		**0.1860**
	LF	0.0494	**0.0369**	**0.0740**	**0.0609**	0.0715	**0.1459**	**0.0731**	**0.1096**	**0.0749**	

The network that best fitted the set of neutral markers (Fig. 2a, Table 5) contained 21 edges (out of the 45 possible ones) connecting the ten populations, a density of edges very similar to the 17 found in the divergent network (Fig. 2b, Table 5). As the number of connections between nodes provides an indication of the amount of gene flow, the aforementioned numbers of edges suggest a similar and fair amount of gene flow in both cases. However, genetic exchange between contrasting habitats was lower at loci under divergent selection than at neutral ones, as suggested by four vs. eight connecting edges respectively. Likewise, markers significantly correlated with shell shape according SAM (Fig. 2c, Table 5) outlined a network with a lower number of edges (14) and a bridge between habitats of just two connections. When testing the possibility of distinct genetic groups according to sheltered and exposed coast, the null hypothesis stated that the probability of obtaining an edge connecting both subgraphs in the graph was $P = 0.5556$ (binomial test). Within graphs, a significant deficiency of edges between habitats was found at loci under divergent selection ($P = 0.0076$) and at loci correlated with shell shape ($P = 0.0019$) but not at neutrals ($P = 0.0826$) (Table 5). Connections between contrasting habitats (Fig. 2) lacked compressed edges in all data sets, whereas connections within similar habitats showed very few extended ones (just 8% at divergent loci). In general, the most common type of edge was extended, ranging from 50% (loci under divergent selection) to 75%

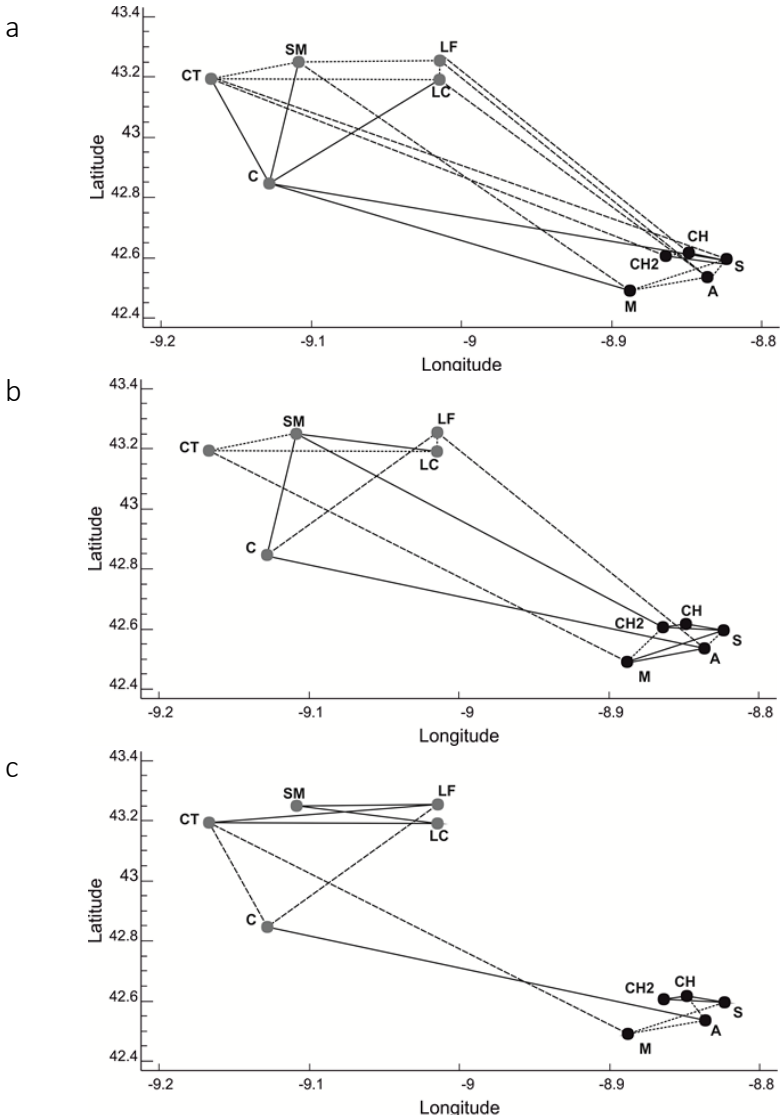

Fig. 2. Graphs of the genetic networks created with (a) the 27 neutral loci, (b) the 11 loci under divergent selection, and (c) the eight loci under divergent selection associated to shell shape according SAM. Black circles represent sites in sheltered shore whereas grey circles depict sites in exposed habitats. Site pairs in a network connected by lines are considered to exchange migrants exhibiting significant conditional genetic covariance. Solid lines indicate connections assuming an IBD process where genetic distances and spatial distances are proportional. Dotted lines (**…**) represent compressed edges with relatively higher conditional genetic distance (cGD) related to spatial distance (suggesting geographical or ecological barriers), whereas dashed lines (**---**) depict extended edges indicating long distance migration processes (Dyer 2015).

Table 5. Results of the correlation to assess isolation by graph distance (IBGD) and of the structure analyses testing for the presence of two subgraphs in the network determined by sheltered and open coast. Analyses were performed for the following data sets: 27 neutral markers, 11 loci under divergent selection and 8 loci under divergent selection associated to shell shape according SAM. Probabilities in bold depict the existence of two subgraphs in the network.

Network	IBGD, r (*P*)	Testing for two subgraphs					
		Bridge probability between habitats	Separating edge set between habitats	p(X≤ Kbth)	Full Graph |Nodes|	Full Graph |Edges|	Edge probability
Neutral loci	**0.2741 (0.002)**	0.5556	8	0.0826	10	21	0.2222
Loci under divergent selection	**0.5156 (0.004)**	0.5556	4	**0.0076**	10	17	0.2222
Loci under divergent selection associated to shell shape	**0.5251 (0.004)**	0.5556	2	**0.0019**	10	14	0.2222

(neutrals) thus indicating that populations were further apart than expected by the IBGD.

ISOLATION BY DISTANCE (IBD) AND ISOLATION BY ENVIRONMENT (IBE)

Simple Mantel tests revealed a pattern of IBD both at neutral markers and at markers under divergent selection. Interestingly, neutral markers did not correlate neither with environmental distance (wave exposure) nor with phenotypic distance (shell shape) whereas markers under divergent selection showed a significant correlation with both variables (Table 6). The strength of the aforementioned correlations was higher for genetic differentiation measured as cGD than as $F_{ST.}$

At neutral markers, IBD was not lost when holding the effects of environmental distance constant through Partial Mantel tests; likewise, the lack of correlation between genetic differentiation and exposure did not change when correcting by geographic distance (Table 6). However, the correlation between exposure and genetic differentiation (as F_{ST}) at loci under divergent selection became non-significant when the effects related to geographic distance were removed.

CORRELATIONS GENOTYPE-PHENOTYPE AND PHENOTYPE-ENVIRONMENT

NOIA analysis revealed that all loci under divergent selection were significantly correlated with phenotype, explaining a percentage of variance ranging between ca. 3 and 11% (Table 7). After Bonferroni correction, 64% of them remained significant. Interestingly, the divergent loci pointed out by SAM as correlated with shell shape explained the largest percentages of variance (> 5%), and all of them

Table 6. Two-tailed Mantel tests comparing matrices of geographic distance (Geo), environmental distance (Exp) and shell shape (Shape) versus genetic distance measured either as pairwise F_{ST} (estimated with AFLP-SURV) or pairwise Conditional Genetic Distance (cGD, estimated with GeneStudio) for the 27 neutral markers (N) and the 11 loci under divergent of selection (Div). Numbers in bold depict significant values after 9999 permutations.

		F_{ST}			Conditional Genetic Distance (cGD)		
		Matrices	Mantel´s r	*P*-value	Matrices	Mantel´s r	*P*-value
Simple Mantel tests	Geographic distance	NF_{ST}-Geo	**0.3185**	**0.0207**	NcGD-Geo	**0.3971**	**0.0056**
		DivF_{ST}-Geo	**0.7253**	**0.0021**	DivcGD-Geo	**0.7554**	**0.0011**
	Environmental distance (wave exposure)	NF_{ST}-Exp	0.1693	0.1894	NcGD-Exp	0.1932	0.1592
		DivF_{ST}-Exp	**0.5817**	**0.0049**	DivcGD-Exp	0.6827	**0.0006**
	Shell shape	NF_{ST}-Shape	0.1664	0.2066	NcGD-Shape	0.1513	0.1656
		DivF_{ST}-Shape	**0.2996**	**0.0453**	DivcGD-Shape	**0.3570**	**0.0178**
Partial Mantel tests	Correcting by environmental distance	NF_{ST}-Geo(Exp)	**0.2774**	**0.0342**	NcGD-Geo(Exp)	**0.3627**	**0.0069**
		DivF_{ST}-Geo(Exp)	**0.5649**	**0.0020**	DivcGD-Geo(Exp)	**0.5673**	**0.0096**
	Correcting by geographic distance	NF_{ST}-Exp(Geo)	-0.0473	0.4379	NcGD-Exp(Geo)	-0.0864	0.3662
		DivF_{ST}-Exp(Geo)	0.2222	0.0905	DivcGD-Exp(Geo)	**0.3958**	**0.0092**

continued to be significant after Bonferroni correction (Table 7). In comparison, no neutral markers were significantly correlated with phenotype after Bonferroni. Exposure regime and phenotype were significantly correlated (r^2 = 0.17, $P = 2.2 \cdot 10^{-11}$), suggesting that 17% of the variation in phenotype could be explained by exposure regime.

Table 7. Linear regression results from NOIA model, exploring the relationship between phenotype (i.e. shell shape measured as shell length/aperture) (Le Rouzic & Álvarez-Castro 2008) and genotype determined by loci under divergent selection. We provide the additive genetic effect, its standard deviation, *P*-value and percentage of the phenotypic variance explained. Significant values highlighted in boldface type, being **B** significant after Bonferroni correction. Shaded cells depict loci significantly correlated with shell shape according SAM. Locus names in bold italics are the markers from the subset of 11 loci under divergent selection that were also significant under SAM approach.

Locus	*R*	SD	*P*-value	Variance explained (%)
CTTA245	0.0113	0.0043	**0.0089**	2.60
AGTG82	0.0135	0.0045	**0.0033**	3.18
AGAC323	0.0142	0.0045	**0.0016**	3.63
AGGT187	-0.0148	0.0043	**0.0007**	4.14
AGAC228	-0.0149	0.0043	**0.0006**	4.18
CTTA166	-0.0160	0.0043	**0.0002**^{B}	4.76
AGAC345	-0.0168	0.0043	**0.0001**^{B}	5.25
AGGT303	-0.0169	0.0042	**0.0001**^{B}	5.47
AGGT240	-0.0188	0.0044	**0.0000**^{B}	6.16
AGAC314	-0.0180	0.0042	**0.0000**^{B}	6.17
CTTA190	-0.0190	0.0042	**0.0000**^{B}	6.84
CTAC180	-0.0212	0.0042	**0.0000**^{B}	8.24
CTGT115	-0.0235	0.0041	**0.0000**^{B}	10.20
CTAC251	-0.0236	0.0041	**0.0000**^{B}	10.44

DISCUSSION

OUTLIER DETECTION

Assessing the ways in which neutral and genetic variation are shaped by environmental features in natural populations requires an efficient way to identify loci merely influenced by random genetic drift, migration and mutation from those under selection (either divergent or balancing). The accurate detection of such outliers is not free from difficulties and, as the analyses performed with BayeScan and Mcheza (DFDIST kernel) yielded different results, we decided to build the set of loci under divergent selection with 11 markers that were simultaneously detected by all the approaches. A situation that is comparable to Nunes *et al.* (2011) when DFDIST and BayeScan detected a similar proportion of outliers (3-4%), but with only a few loci in common. Caballero *et al.* (2008) also raised several concerns about the sensitivity of DFDIST, and a simulation study by Pérez-Figueroa *et al*. (2010) showed that BayeScan performed more efficiently under a wide range of scenarios than DFDIST and DETSELD. However, when the ultimate aim of a genome scan is targeting candidate loci influenced by selection to conduct further research, combining different methods to minimize the risk of selecting potentially ineffective candidates seems a wise alternative.

Using this conservative threshold for detecting signatures of divergent selection, we found that divergent selection seemed to operate on the populations of *N. lapillus* in Galicia over a gradient of wave exposure. Our AFLP scan revealed that some 11% of the genome could be responsible for the adaptive differences found among populations from

protected and exposed habitats. This value is consistent with the 5-10% proportion of the genome likely to be under selection according to the growing body of AFLP genome scans performed on different taxa (Bonin *et al.* 2006, Nosil *et al.* 2007, Nosil *et al.* 2009, Freedman *et al.* 2010, Paris *et al.* 2010, Nunes *et al.* 2011, Tollenaere *et al.* 2011, Quintela *et al.* 2014). Also in the Galician coast, some 3% of the genome seemed to be responsible for the adaptive differences between ecotypes associated to shore levels and habitats in populations of *Littorina saxatilis* (J. Galindo *et al.* 2009). Also in *L. saxatilis*, about 5% of the loci showed greater differentiation than expected in a cline of shell shape across vertical gradients of rocky shores in England, providing evidence of the effects of selection, either directly or indirectly through linkage (Wilding *et al.* 2001).

GENETIC NETWORKS

Population graphs have not yet been extensively used albeit they proved useful for examining specific topological characteristics in terms of the relationships among individual populations, and can provide essential information about how evolutionary processes have affected interacting populations (Dyer & Nason 2004, Fortuna *et al.* 2009, Murphy *et al.* 2010, Sork *et al.* 2010, Richter-Boix *et al.* 2011, Quintela *et al.* 2014, I. J. Wang & Bradburd 2014). The use of conditional genetic distances obtained from population network topology can improve the estimation of IBD and IBE (Meirmans 2012) and, combined with information about phylogeographic history, may help disentangling the

effects of historical and contemporary barriers to gene flow (Dyer *et al.* 2010, Dyer 2015).

The fact that neutral and candidate selective markers depict different network patterns is in concordance with the idea of a genetic mosaic at the initial stages of ecological speciation with gene flow (Via 2009). A significant deficiency of edges (i.e. limited gene flow) linking two subgraphs corresponding to contrasting habitats was found at loci under divergent selection (P = 0.0076) and at loci correlated with shell shape (P = 0.0019), but not at neutral markers (Fig. 2, Table 6). This suggests that wave exposure is a strong environmental axis and that local adaptation has contributed to the spatial genetic structure between habitats by conditioning the genetic composition of populations on both sides of the range. The observation that connections were mostly restricted to populations within habitats is in agreement with former studies that reported the existence of a relationship between habitat features and functional genetic variation (e.g. Bonin *et al.* 2006, Kane & Rieseberg 2007, Steiner *et al.* 2007, Storz & Kelly 2008, Richter-Boix *et al.* 2011, Quintela *et al.* 2014).

ISOLATION BY DISTANCE (IBD) AND ISOLATION BY ENVIRONMENT (IBE)

In a recently published meta-analysis, Orsini *et al*. (2013) described the single and combined scenarios that would account for patterns such as Isolation by Dispersal Limitation (IBDL), Isolation by Adaptation (IBA) or Isolation by Colonization (IBC). In their theoretical framework, Isolation by Environment (IBE) would involve the lack of correlation between

genetic differentiation and geographic distance among populations coupled with a positive correlation between genetic differentiation and environmental dissimilarity between them. On the other hand, Isolation by Distance (IBD) would require the positive correlation between genetic differentiation and geographic distance irrespective of environmental variables. Our study aimed to disentangle the relative contribution of geographic and ecological distance (measured in terms of wave exposure) to the structuring of neutral and adaptive genetic differentiation in the marine snail *Nucella lapillus*. Thus, confronting our results with the above-mentioned framework (see Fig. 1 in Orsini *et al*. 2013) shows that our neutral data set followed a pattern of IBDL because genetic differentiation (assessed as either F_{ST} or cGD) was positively correlated with geographic distance but not with environmental distance (as coastal exposure). On the other side, the set of loci under divergent selection provided evidence for both IBE and IBD, given their positive correlation with geographic and environmental distance respectively. In Orsini's framework, this scenario is regarded as a combined pattern IBDL+IBA. An identical (or extremely similar) situation can be found across the literature in a number of animal species such as Atlantic herring *Clupea harengus* (Limborg *et al.* 2012), Atlantic salmon *Salmo salar* (Bourret *et al.* 2013), ocellated lizard *Lacerta lepida* (Nunes *et al.* 2011), or plants like *Arabis alpina* (Poncet *et al.* 2010). In these cases, environmental selection driving adaptation was the explanation invoked by the authors to account for the observed patterns. However, from Orsini's framework perspective, the interpretation is either a combined scenario of IBDL coupled with local

adaptation (LA) or, alternatively, a combined scenario of IBC (serial colonization) + LA. A taxonomically close example to our study system is *Littorina saxatilis*, a marine snail that shares habitat with *N. lapillus* and shows an almost identical pattern to ours (Wilding *et al.* 2001, Grahame *et al.* 2006). This pattern, which had been interpreted as divergent selection affecting a small proportion of the genome (IBA), was reinterpreted as a combined scenario IBDL + LA or IBC (serial colonization) + LA (Orsini *et al.* 2013). The IBDL/IBC component in this reinterpretation is in agreement with the observation that *L. saxatilis*, like *N. lapillus*, is a poor disperser that lacks a planktonic stage. This possibly explains that the genetic structure of populations separated by tens of km in Galicia matched their geographical distribution (Quesada *et al.* 2007). It seems likewise consistent with the finding that our set of loci under divergent selection correlated positively with both environmental and geographic distance, suggesting that a portion of the differences detected in our outlier loci might also reflect stochastics differences generated among geographically fragmented populations.

IBE was shown to be the strongest pattern observed among invertebrates, with some evidences for IBD (Sexton *et al.* 2014). To account for IBD in models of IBE, the commonest approach is the partial Mantel test (Mantel 1967, Shafer & Wolf 2013, Sexton *et al.* 2014) as it allows holding the effects of geographic distance matrix constant. Environmental data is often spatially autocorrelated (Legendre & Fortin 2010) and, therefore, when IBD is detected in a study, it is crucial to isolate spatial population structure when assessing the correlation

between molecular markers and environmental variables (Meirmans 2012). Our system study also faces this challenge as there is a significant correlation between geographic and environmental distance (r = 0.639, P = 0.0028). In a comprehensive meta-analysis, Sexton *et al*. (2014) described the evidences for IBD/IBE patterns. According to their expectations, our neutral data clearly followed a pattern of "IBD only" because there was no sign of IBE, whereas the loci under divergent selection described a pattern of "IBD and IBE" as Partial Mantel tests still detected a significant IBD after accounting for environmental differences (Table 5), and also IBE after accounting for geographic distance. The latter condition was only met when genetic differentiation was based upon cGD (r = 0.396, P = 0.0092) but not on F_{ST} (r = 0.222, P = 0.0905). In this regard, cGD is known to outperform traditional measures of genetic distance, such as F_{ST}, when describing the spatial genetic structure; mostly because the homoscedasticity of cGD improves the estimation of IBD and IBE (Dyer *et al.* 2010, Dyer 2015). Similar examples were found in the mustelidae *Martes americana* in which elevation shaped the environmental gradient (Wasserman *et al.* 2010), in the three-spined stickleback, *Gasterosteus aculeatus*, inhabiting freshwater and maritime environments (McCairns & Bernatchez 2008), or when assessing thermal adaptation in the freshwater snail *Radix balthica* in a volcanic lake in Iceland (Quintela *et al.* 2014).

CORRELATIONS GENOTYPE-PHENOTYPE

Across more than twenty articles, Crothers published an exhaustive set of observations on shell polymorphism in *N. lapillus* along European and American coasts showing that the ratio between shell length and shell aperture (L/Ap) was directly correlated to wave exposure (*X*) as assessed by Ballantine's (1961) scale. Moreover, the relationship could be described as L/Ap = 1.214 + 0.036 *X*, *P* = 0.001 (Crothers 1973), and this relationship was shown to be conserved in the southernmost limit of the species' European range (Crothers 1977) where our study area was placed. Indeed, by applying the same approach to our data set, the correlation also proved significant with L/Ap = 1.411 + 0.017*X* (*P* < 0.0001).

We thus used L/Ap as a proxy for the phenotype, and employed the NOIA model, an approach formerly used on both domesticated (Álvarez-Castro *et al.* 2008, Le Rouzic *et al.* 2008) and wild species (Besnier *et al.* 2010, Richter-Boix *et al.* 2013), to investigate the existence of any correlation with the individual genotype. Interestingly, while no neutral marker was significantly correlated to phenotype after Bonferroni corrections, the loci under divergent selection linked to shell shape by SAM remained significant and explained the highest percentages of variance (> 5%). The portion of explained variance (5-10%) was in the range reported for tameness with QTLs in rats (7-10%; Albert *et al.* 2009). Moreover, although our estimate is below the 9.8-36.4% found for pigmentation traits in beach mice (Hoekstra *et al.* 2006), it should be noted that the correlation genotype-phenotype is usually investigated

with candidate genes which are expected to account for a larger portion of variance than the merely anonymous AFLPs (Hoekstra *et al.* 2004, Storz *et al.* 2007, McCracken *et al.* 2009, Mullen *et al.* 2009, Chan *et al.* 2010). On the other hand, the non-neglectable plastic component of shell polymorphism (Crothers 1973, 1977, Lam & Calow 1988, Kirby *et al.* 1994, Parsons 1997, Johnson & Black 1998, Trussell & Etter 2001, Wilding *et al.* 2001, Cotton *et al.* 2004, Grahame *et al.* 2006, Pfenninger *et al.* 2006, Conde-Padín *et al.* 2007, Lakowitz *et al.* 2008, Hollander & Butlin 2010, Brönmark *et al.* 2011, Cuña *et al.* 2011, Pascoal *et al.* 2012a, Pascoal *et al.* 2012b) might also reduce the variance explained by genetic variation. Direct-developers such as *N. lapillus* might be expected to adapt to local heterogeneity through selection on genes affecting shell and phenotypic variation. Indeed, Colson *et al*. (2006) and Guerra-Varela *et al*. (2009) assessed the genetic component of shell-shape variation and showed that the two ecotypes (exposed and protected) found in Galician were distinctly adapted to differences in wave exposure. Recent studies with another direct-developer (*L. saxatilis*) also revealed high heritability estimates for shell traits in embryos, but still suggested that phenotypic plasticity possibly plays a part in the ecotypic differentiation of adult snails (Galindo *et al.* 2019; but see also Hollander & Butlin 2010).

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CHAPTER V

USING A TEMPORAL APPROACH TO DISENTANGLE THE POPULATION GENETIC PATTERNS DISPLAYED BY THE DIRECT-DEVELOPER *NUCELLA LAPILLUS* L.

ABSTRACT

The analysis of population genetic structure is a common alternative for elucidating connectivity among populations when direct measurements are beyond reach. However, interpreting genetic results is sometimes challenging because factors other than migration can also shape the resulting pattern. One example is *Nucella lapillus* (Linnaeus 1758), a direct developer marine gastropod whose population genetic structure has been found to vary depending on wave exposure. In exposed coast, populations show an unordered arrangement compatible with the chaotic genetic patchiness seen in other coastal organisms with larger dispersal abilities. To elucidate this seemingly paradoxical situation, we investigated the temporal stability of the genetic patterns displayed by *N. lapillus* under contrasting wave exposure conditions. A total of 480 whelks from 16 samples, covering three sympatric generations, were analysed with AFLP markers revealing that both the isolation by distance pattern found in the sheltered area and the unordered pattern found in the wave exposed sites persisted through generations (Φ_{PT} = -0.064). Since the latter is at odds with the expectations of chaotic genetic

patchiness, either the environmental segregation of ecotypes adapted to different wave exposure conditions or, more plausibly, genetic drift are discussed as explanations to the pattern displayed in wave exposed coast. As each alternative has very different implications for connectivity among populations, this study highlights the subtleties of its inference from spatial genetic structure.

INTRODUCTION

The study of the population genetic structure of a species is the most widely employed strategy to elucidate key information for the sustainable management of natural richness and resources such as how populations are arranged and connected, what kind of forces act over them, and towards what they are evolving (Bohonak 1999, Palumbi 2003). The genetic approach is currently preferred over other alternatives as it provides a compendium of many forces acting over the time (Bossart & Pashley Prowell 1998, Bohonak 1999). Its use becomes critical in the marine environment given the impossibility of applying direct measures to most species (i.e. following specific individuals) and the uncertainty of mating success, the only way for migrants to have an impact on next generations (Hellberg *et al.* 2002, Palumbi 2003). However, interpreting genetic results is sometimes arduous, given the difficulty of discriminating among the several causes that can shape the genetic structure, and the fact that they often act in combination (Bossart & Pashley Prowell 1998, Bohonak 1999, Grosberg & Cunningham 2001). But although managing scarce data involving behaviour, ecology or biogeography could lead to multiple interpretations of the genetic model obtained (Bohonak 1999), the strength and pattern of gene flow might still stand as the main shaping force (Bohonak 1999, Haye *et al.* 2014). Dispersal ability delimits the way marine populations are genetically connected. In marine sessile organisms, dispersal mainly relies on two basic life trait strategies: to have or not a planktonic larval stage, respectively attributed to

generalist or specialist species (K. Johannesson 2003). Being a direct-developer means that both adults and descendants have limited dispersal capacity and spend their entire lives at the same place. Therefore, the exchange of individuals among populations is often very low and populations become genetically differentiated by a combination of genetic drift and selection of the alleles best suited to local conditions, generation after generation. By contrast, adopting a planktonic larval strategy allows new habitats to be colonised as well as a wider range (depending on the length of the larval stage). Therefore, planktonic larvae offer the opportunity to escape from a changing hostile environment, although at the cost of never being fully adapted to a particular habitat as well as of wasting progeny. As planktonic larvae can potentially reach almost anywhere, they tend to homogenize the genetic structure as distant populations receive new alleles through migration. In practice, however, the dispersal of planktonic larvae is subordinated to barriers to dispersal, confinement by ocean currents and the suitability of the new substrate. Therefore, it is not uncommon for species with a planktonic larval stage to show genetic differentiation between populations that resembles the one found in direct developers (Taylor & Hellberg 2003, Zhan *et al.* 2009, Hoffman *et al.* 2012, Owen & Rawson 2013, K. Johannesson *et al.* 2018). However, unlike direct developers, their population genetic composition often shows some temporal instability (González-Wangüemert *et al.* 2007, Hogan *et al.* 2010, Kesäniemi *et al.* 2014). The latter is attributable to stochastic environmental events leading to a stochastic composition of recruitments (changes in source populations, mortality), meaning that

the successful contribution to the next generation varies among adults (Hedgecock 1994b, Hellberg *et al.* 2002, Lee & Boulding 2009, Hogan *et al.* 2010). By contrast, species with limited dispersion are expected to display temporal stability. Therefore, exploring the temporal changes in population genetic composition and arrangement may allow a discrimination between a scenario of high or limited dispersion (Hedgecock 1994a, Hellberg *et al.* 2002, Hellberg 2006). Most studies of changes in genetic structure over time have focused on species with planktonic larvae stages, in an effort to explain Johnson and Black (1982) so-called "chaotic genetic patchiness" (González-Wangüemert *et al.* 2007, Ng *et al.* 2009, Byrne *et al.* 2013, Owen & Rawson 2013; but see Lee & Boulding 2009). However, what about a direct-developer that exhibits a different genetic arrangement depending on the hydrodynamic regime? Is wave action having an effect on the temporal genetic stability of populations?

In a previous study (Carro *et al.* 2012), we surveyed the spatial genetic arrangement and composition of dogwhelks, *Nucella lapillus* (Linnaeus 1758), in the Northwest Iberian Peninsula (NWIP). Dogwhelks are intertidal rocky dwellers, and they are the commonest predator in European Atlantic shores, with a range that spans from southern Portugal to northern Russia, and even reaches the north coast of America (Crothers 1985). *N. lapillus* is a dioecious species with internal fertilisation and direct development: females release up to 50 egg capsules strongly attached to the substratum in crevices, from which dozens of crawl-away juveniles emerge (Crothers 1985, Fretter &

Graham 1994b). Using AFLP neutral markers, our results agreed with the expectations for a poor disperser, as we found a significant genetic differentiation even between populations only 400 m apart. However, the arrangement and strength of the genetic differences among populations varied with wave exposure. Whereas genetic variation along the sheltered coast followed the isolation by distance (IBD) pattern typical of poor dispersers, the unordered arrangement of the genetic structure found in wave-exposed populations was compatible with the chaotic genetic patchiness seen in other coastal organisms with larger dispersal abilities (Barreiro *et al.* 2006, Selkoe *et al.* 2006, Hogan *et al.* 2010, Selkoe *et al.* 2010). Moreover, later work suggested that wave exposure can be a strong selective agent in shaping the genetic variation and, even in a context of gene flow, local adaptation may outline traits such as shell shape (Carro *et al.* 2019). Many inferences of connectivity among populations from their genetic structure are based on samples taken at a particular point of time, drawing conclusions that assume a stable genetic composition (Byrne *et al.* 2013). However, a pre-requisite to discriminate among several hypothesis is to perform a repeated sampling over time and/or generations to investigate the temporal stability of genetic structure (Hellberg *et al.* 2002, Hellberg 2006). In this study, the stability over time of the genetic pattern of *N. lapillus* in NWIP was investigated to discriminate among three putative scenarios: (i) wave stress prevents dispersal so that populations are mainly maintained by self-recruitment; (ii) waves promote a stochastic exchange of individuals between populations resembling what occurs in organisms with more dispersal potential; and (iii) it is the variations in

the effective population size (*Ne*), rather than the migration rate (m), that cause changes in spatial genetic structure over time. We assume that the spatial genetic pattern would be stable over time under the first scenario but would shift under the second one. Alternatively, the third scenario would imply that *Ne* would vary with time and/or among wave-exposed sites.

MATERIAL AND METHODS

SAMPLING AND DNA EXTRACTION

Six intertidal rocky shores sites were repeatedly sampled at low tide in Galicia (NWPI) in 2007 (generation 07) and 2009 (generation 09). Three of them were located in wave-exposed coast (Laxe_C, LC; Cabo Tosto, CT; Santa Mariña, SM) and three were sheltered within a ria, i.e. a drowned river valley, (Chazo, CH; Arousa, A; Meloxo, M) (Fig. 1). The study covered different geographic scales: the range of distances among sites (from 5 to 14 km) was analogous within both areas, and the two areas were separated > 100 km. Samples consisted of 30 subadults (generation S), 15 males and 15 females per site. Besides, 30 juveniles (generation J) were also collected in four of these six sites (LC, SM, CH, and M) in 2009. At each site, individuals were collected from the smallest possible area to avoid mixing different breeding groups that may lead to a heterozygosity reduction due to Wahlund effect, as seen in other studies with *N. lapillus* (Day 1990, Goudet *et al.* 1994). Once in the laboratory, shells were broken with a vice after being measured to the nearest 0.01 mm with a digital calliper. Subadults and juveniles were easily discernible by their shell characteristics (Crothers 1985), and

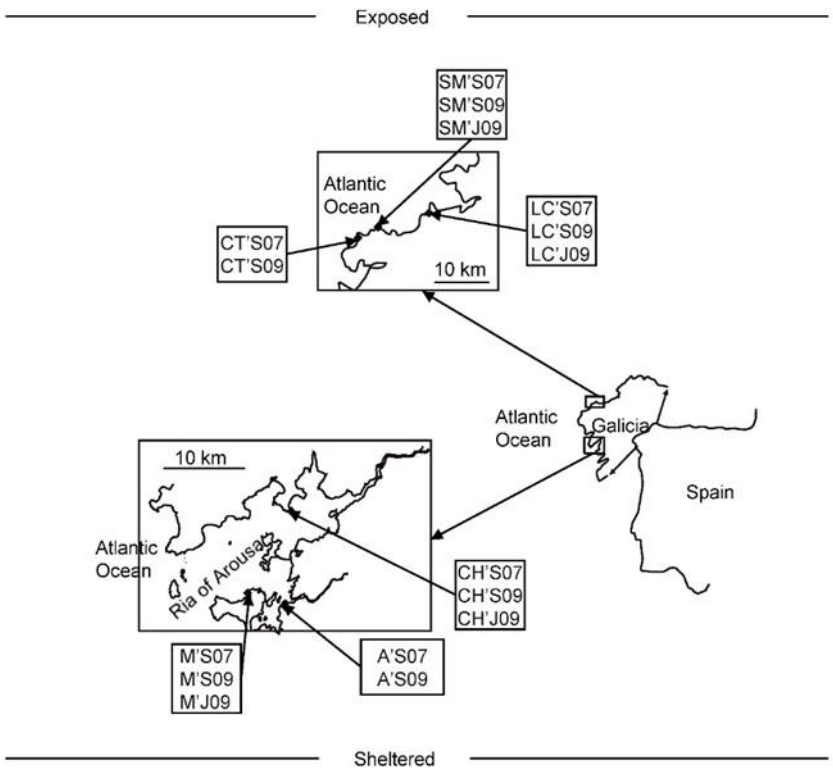


Fig. 1. *Nucella lapillus* sampling sites along an exposed and a sheltered stretches of coast of NW Iberian Peninsula. Sample codes indicate abbreviation of site name, whether it was made of subadults (S) or juveniles (J), and sampling year (2007, 2009).

allowed us to get two different cohorts in the same year (Barreiro *et al.* 2006) (Fig. 2). Naked animals were sexed under the stereomicroscope when possible (juveniles younger than one year were too immature for reliable sexing). Dissection of each individual was made with disposable tools and/or flame sterilized material to avoid cross contamination. Mantle and foot tissue from each individual were kept in ethanol 96° at 12°C until processed. Genomic DNA was extracted from 3 mg (dry

weight) mantle tissue using Qiagen DNA tissue kit following manufacture instructions and stored in TE at -20°C.

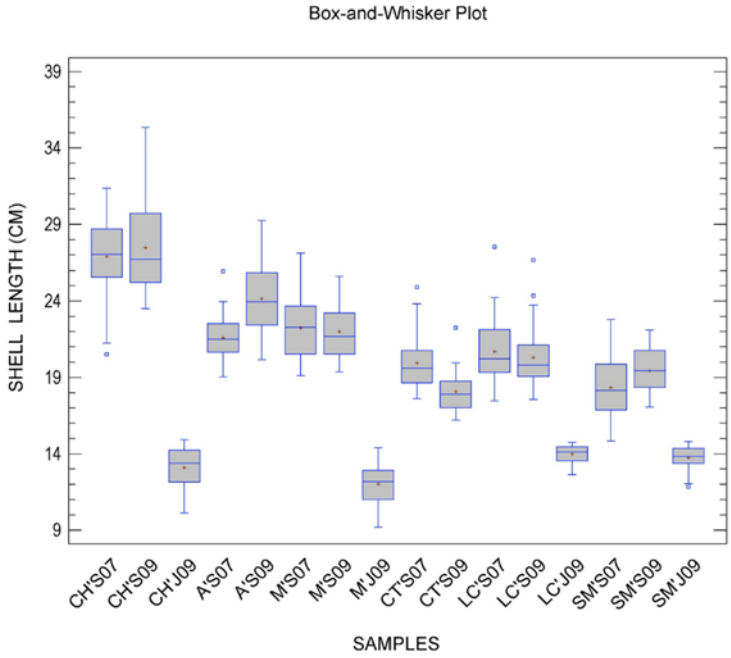


Fig. 2. *Nucella lapillus* size variation among samples. Box-and-whistle plots for shell length (L, in cm). Sample codes as in Fig. 1.

AFLP REACTIONS

AFLP reactions were performed following the protocol of Vos *et al.* (1995) with minor modifications. About 250 ng of DNA (Blears *et al.* 1998) were restricted with 2.5 units of each *EcoRI* and *Tru1I* enzymes in 2X Tango buffer (Fermentas) yielding a pool of three different kinds of fragments based on their ends. The digested DNA was adapter ligated in the presence of 0.1 µM *EcoRI*-adapters (5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3'), 1 µM *Tru1I*-adapters (5'-

GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3'), 0.52 units of T4 ligase (Fermentas) and 0.2X ligation buffer. Ligation product was diluted 10-fold in Milli-Q H_2O (Millipore Co.). A preselective amplification was conducted with 0.3 µM *EcoRI*-primer with a T selective nucleotide (5'-GACTGCGTACCAATTC+T-3'), 0.3 µM *Tru1I*-primer with a C selective nucleotide (5'-GATGAGTCCTGAGTAA+C-3'), 2.5 mM $MgCl_2$, 1X PCR buffer, 0.04 $\mu g \mu l^{-1}$ BSA, 0.2 µM dNTPs and 0.04 units of *AmpliTaq* polymerase (Applied Biosystems) to narrow the diversity of fragments. Finally, a subset of fragments was still selective amplified with 0.6 µM of each *EcoRI* and *Tru1I* primers, both with three extra selective nucleotides (*EcoRI*/*Tru1I*: TAG/CGT, TAG/CAC, TAG/CTG, TCT/CGT, TCT/CAC and TCT/CTA), 0.8 µM dNTPs, 2.5 mM $MgCl_2$, 0.04 $\mu g \mu l^{-1}$ BSA, 1X PCR buffer, and 0.4 units of *AmpliTaq Gold* polymerase (Applied Biosystems). PCR reactions were performed in a Hybaid thermocycler model PxE. The 5' end of the selective *EcoRI*-primers was labeled with FAM or HEX fluorochromes.

Negative DNA extraction controls were regularly included to screen for cross contamination. DNA extractions of 10% of individuals were repeated on different dates and run blindly along other extracts to avoid a biased scoring during reproducibility tests (Bonin *et al.* 2004). The estimated genotyping error (0.78%, error rate by primer combination: 0.5-1%) was consistent with former studies (Bonin *et al.* 2004); none of the individual loci exceeded the maximum acceptable error rate (0.1) recommended by Bonin *et al.* (2007). Samples, negative controls and replicates were randomly distributed in PCR plates. Reactants were

always mixed in a laminar flow cabin; DNA and PCR product solutions were always added using filter tips to minimize the risk of cross contamination.

PCR fragments were separated on a 3130xl Genetic Analyzer (Applied Biosystems) at the Molecular Biology Unit (UBM) from Universidade da Coruña (A Coruña, Spain). Fingerprint patterns were processed with the software GeneMarker v1.70 (Softgenetics) using the options suggested for AFLPs and following common recommendations for these markers (Bonin *et al.* 2005, Whitlock *et al.* 2008). Results were translated into a binary matrix of presence/absence of each band. Binary data from the six primer combinations were pooled to obtain a multilocus phenotype for each individual.

DATA ANALYSIS

Individuals were grouped into 16 samples according to their sampling site and generation: CH'S07, CH'S09, CH'J09, A'S07, A'S09, M'S07, M'S09, M'J09, CT'S07, CT'S09, SM'S07, SM'S09, SM'J09, LC'S07, LC'S09, LC'J09. Allele frequencies were estimated using the Bayesian method of Zhivotovsky (1999) implemented in AFLP-SURV v1.0 (Vekemans 2002) with the option of non-uniform prior distributions of allele frequencies. In contrast with alternative procedures (e.g. Lynch & Milligan 1994), this Bayesian approach does not involve the pruning of loci with low frequency of null-alleles and produces statistically unbiased estimates of diversity and genetic distances (Zhivotovsky 1999). Genetic diversity per sample was measured by assessing the number of polymorphic loci (5% criterion), Nei's gene diversity and the presence of private bands.

Significant differences in gene diversity between samples were tested with the T' method for multiple unplanned comparisons between pairs of means based on nearly equal sample sizes (Sokal & Rohlf 1995).

Allele frequencies were used to estimate genetic differentiation (F_{ST}) between samples. Overall F_{ST} values were calculated for the whole data set as well as for each zone (exposed and sheltered coast) following Lynch and Milligan (1994) and their significance tested with a permutation test (10,000 pseudoreplicates). In addition, samples were grouped in three generations (i.e.: 'S07, 'S09 and 'J09). Nei's genetic distances between pairs of samples were used for Principal Coordinates Analysis (PCoA) using GeneAlEx v6.1 (Peakall & Smouse 2006). To facilitate the comparison with other AFLP studies, differentiation was also estimated by the analysis of molecular variance (AMOVA, Excoffier *et al.* 1992b) implemented in GeneAlEx v6.1. AMOVA calculates Φ_{PT}, an analogue of F_{ST}, using the squared Euclidean distance matrix between AFLP phenotypes and allows a hierarchical analysis of the genetic structure (e.g. differentiation between regions, between sites within regions). Φ_{PT} is a band-based approach that does not depend so critically on specific assumptions that could underestimate genetic variability (Excoffier *et al.* 1992b, Yan *et al.* 1999, Shank & Halanych 2007) and has been specifically recommended for AFLP data (Bonin *et al.* 2007). Several AMOVAS were performed with samples grouped per generation and, for each generation, by geographical layout (sheltered vs. exposed).

In an alternative approach, the genetic structure was further investigated with the Bayesian model-based clustering algorithms

implemented in STRUCTURE v2.3.3 (Pritchard *et al.* 2000, Falush *et al.* 2007, Hubisz *et al.* 2009) under a model assuming admixture and correlated allele frequencies. Sampling information was used both to assist the clustering and to test for migrants. Ten runs with a burn-in period of 100,000 replications and a run length of 1,000,000 Markov chain Monte Carlo (MCMC) iterations were performed for a number of clusters (K) ranging 1-19, being 19 the maximum number of samples plus 3 (Evanno *et al.* 2005). The ad hoc summary statistic ΔK of Evanno *et al.* (2005), implemented in STRUCTURE HARVESTER (Earl & Vonholdt 2011), was used to select the value of K with the uppermost hierarchical level of population structure in our data. Anticipating that a superstructure might hide other structures at smaller spatial scales, STRUCTURE was also run separately for samples within each geographical zone (i.e. sheltered and exposed). In that case, K ranged from 1 to 11. Runs for each geographical zone were repeated after removing individuals that identified as likely immigrants from the other geographical area in the first analyses to check whether their presence interfered with the detection of population structure on a finer scale (Evanno *et al.* 2005). After the best clustering arrange was calculated, the CLUMPP (Jakobsson & Rosenberg 2007) program was run for aligning the multiple replicate analyses of the same data set. The FullSearch algorithm was used as the authors ensure that it finds the optimal alignment of clusters across multiple runs. Finally, distruct program (Rosenberg 2004) was used to visualize the results obtained from CLUMPP.

To test the hypothesis that variations on effective population size (*Ne*), rather than on migration rate per generation (m), could account for the changes in spatial genetic structure over time, *Ne* was estimated for each sample with Colony 2.0 (O. R. Jones & Wang 2010). The sibship assignment method was used to infer *Ne* as it has been shown to be more accurate than alternative strategies (e.g. heterozygote excess, linkage disequilibrium or temporal method) (J. Wang 2009). The program was run with the full likelihood analysis method and the options of very high precision, polygamous breeding systems for both sexes, and sibship complexity prior.

RESULTS

The 218 AFLP loci obtained ranged from 26 (selective nucleotides combination TAG/CTG) to 49 (TAG/CGT); 75 loci (34.4%) were polymorphic (5% criterion) for the whole data set. Every single individual produced a unique multilocus genotype. For each site, the percentage of polymorphic loci was similar in generations J09 and S09 while generation S07 showed between 20 and 40% more polymorphic loci. CT was the site with the highest values (Table 1). Nei's gene diversity showed a similar pattern: estimates for generations J09 and S09 revealed little to no differences within the same site while estimates for generation S07 were significantly higher ($P < 0.01$, test T'). Samples CH'S09 and M'J09 had one private band each, although they were detected only in a couple of individuals and with no evident link to any other parameter

Table 1. Summary of AFLP markers and Nei's gene diversity for every sample.

Site	Generation	n[a]	Polimorphic loci[b]	Nei's gene diversity (± S.E.)	Nº private bands
Sheltered coast					
Chazo (CH)	S07	30	80 (36.7%)	0.149 ± 0.010	0
	S09	30	68 (31.2%)	0.132 ± 0.010	1
	J09	30	66 (30.3%)	0.132 ± 0.011	0
Arousa (A)	S07	30	87 (39.9%)	0.170 ± 0.011	0
	S09	29	70 (32.1%)	0.143 ± 0.010	0
Meloxo (M)	S07	30	90 (41.3%)	0.180 ± 0.011	0
	S09	30	64 (29.4%)	0.137 ± 0.011	0
	J09	30	64 (29.4%)	0.131 ± 0.010	1
Exposed coast					
CaboTosto (CT)	S07	30	109 (50.0%)	0.228 ± 0.012	0
	S09	30	85 (39.0%)	0.181 ± 0.012	0
St Mariña (SM)	S07	30	81 (37.2%)	0.156 ± 0.011	0
	S09	29	66 (30.3%)	0.127 ± 0.010	0
	J09	30	69 (31.7%)	0.130 ± 0.010	0
Laxe C (LC)	S07	30	81 (37.2%)	0.159 ± 0.011	0
	S09	30	64 (29.4%)	0.130 ± 0.011	0
	J09	30	65 (29.8%)	0.139 ± 0.011	0

[a] average number of individuals takes into account the presence of missing data for some primer combinations.
[b] 5% criterion applied to Bayesian estimates of allele frequencies (Zhivotovsky 1999)

As expected, Φ_{PT} values exceeded those of F_{ST}. Nonetheless, both F_{ST} (0.242) and Φ_{PT} (0.336) statistics revealed highly significant genetic differences among all samples *(*$P < 0.0001$ and $P < 0.001$ respectively) (Table 2). Pairwise comparisons relevant to the present study were those comparing sites within a given generation or those comparing generations from the same site (Table 3). Among the latter (within blue boxes Table 3), comparisons between generations J09 and S09 were consistently non-significant across all sites, while most intra-site comparisons between generation S07 and the generations sampled in

Table 2. *Nucella lapillus* genetic structure. F_{ST} and Φ_{PT} estimates for the whole data set (all samples) and for samples within each area (sheltered and wave-exposed areas).

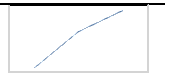
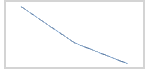
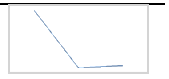
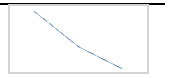
	All generations grouped		S07		S09		J09		Φ_{PT} trend over generations
	F_{ST}	Φ_{PT}	F_{ST}	Φ_{PT}	F_{ST}	Φ_{PT}	F_{ST}	Φ_{PT}	
All samples	0.242 ($P < 0.0001$)	0.336 ($P < 0.001$)	0.192 ($P < 0.0001$)	0.271 ($P < 0.001$)	0.291 ($P < 0.0001$)	0.393 ($P < 0.001$)	0.335 ($P < 0.0001$)	0.463 ($P < 0.001$)	
Between areas		0.409 ($P < 0.001$)		0.253 ($P < 0.001$)		0.474 ($P < 0.001$)		0.526 ($P < 0.001$)	
Between samples within areas		0.097 ($P < 0.001$)		0.123 ($P < 0.001$)		0.086 ($P < 0.001$)		0.064 ($P < 0.001$)	
Sheltered area samples	0.037 ($P < 0.0001$)	0.069 ($P < 0.001$)	0.049 ($P < 0.0001$)	0.087 ($P < 0.001$)	0.025 ($P < 0.0001$)	0.056 ($P < 0.001$)	0.030 ($P < 0.0001$)	0.057 ($P < 0.001$)	
Exposed area samples	0.084 ($P < 0.0001$)	0.119 ($P < 0.001$)	0.118 ($P < 0.0001$)	0.156 ($P < 0.001$)	0.074 ($P < 0.0001$)	0.105 ($P < 0.001$)	0.051 ($P < 0.0001$)	0.072 ($P < 0.001$)	

Table 3. Heatmap of pairwise genetic comparisons. F_{ST} values below the diagonal; Φ_{PT} values above the diagonal. Blue boxes indicate inter-generation comparisons within a single site. Non-significant values ($P < 0.05$) in bold italics.

		Sheltered								Exposed							
		CH'J09	CH'S09	CH'S07	A'S09	A'S07	M'J09	M'S09	M'S07	CT'S09	CT'S07	SM'J09	SM'S09	SM'S07	LC'J09	LC'S09	LC'S07
Sheltered	CH'J09		***0.000***	0.037	0.049	0.090	0.057	0.048	0.102	0.463	0.254	0.578	0.543	0.536	0.545	0.562	0.493
	CH'S09	***0.000***		0.040	0.052	0.092	0.058	0.054	0.104	0.469	0.253	0.584	0.550	0.542	0.550	0.569	0.498
	CH'S07	0.022	0.019		0.069	0.086	0.109	0.088	0.121	0.457	0.240	0.575	0.542	0.531	0.543	0.561	0.489
	A'S09	***0.011***	***0.017***	0.039		0.027	0.068	0.062	0.081	0.410	0.200	0.526	0.494	0.480	0.494	0.512	0.439
	A'S07	0.037	0.044	0.036	***0.014***		0.101	0.103	0.054	0.346	0.146	0.465	0.432	0.416	0.430	0.452	0.371
	M'J09	0.029	0.032	0.072	***0.033***	0.060		***0.004***	0.066	0.462	0.252	0.566	0.530	0.519	0.538	0.558	0.486
	M'S09	0.026	0.029	0.055	0.029	0.054	***0.007***		0.060	0.455	0.245	0.563	0.528	0.519	0.534	0.553	0.481
	M'S07	0.056	0.057	0.078	0.056	0.031	0.031	0.026		0.311	0.129	0.418	0.383	0.366	0.388	0.408	0.324
Exposed	CT'S09	0.315	0.323	0.318	0.299	0.236	0.329	0.314	0.209		0.106	0.119	0.115	0.128	0.087	0.114	0.115
	CT'S07	0.157	0.163	0.150	0.146	0.089	0.178	0.165	0.082	0.072		0.229	0.210	0.192	0.197	0.217	0.156
	SM'J09	0.444	0.453	0.446	0.433	0.357	0.450	0.440	0.315	0.069	0.175		***0.000***	0.041	0.072	0.093	0.153
	SM'S09	0.445	0.454	0.447	0.432	0.356	0.445	0.440	0.307	0.075	0.172	***0.001***		0.029	0.060	0.082	0.119
	SM'S07	0.412	0.420	0.408	0.396	0.320	0.409	0.405	0.277	0.080	0.155	0.019	0.019		0.086	0.108	0.103
	LC'J09	0.412	0.420	0.418	0.405	0.331	0.422	0.411	0.291	0.063	0.157	0.051	0.045	0.055		***0.000***	0.055
	LC'S09	0.431	0.440	0.438	0.424	0.353	0.442	0.430	0.308	0.083	0.174	0.060	0.061	0.068	***0.007***		0.064
	LC'S07	0.362	0.370	0.363	0.353	0.278	0.370	0.361	0.243	0.063	0.131	0.074	0.060	0.048	0.032	***0.024***	

2009 were statistically significant (8 out of 10). The genetic structure of the population was similar across generations (Table 2); i.e. F_{ST}/Φ_{PT} estimates were higher for the complete data set than for the samples from each area (sheltered or exposed) taken separately. Nonetheless, genetic differentiation between samples was still significant when sheltered and exposed sites were analyzed separately. Moreover, despite comparable geographical distances within the two zones, genetic differentiation between exposed sites was always higher than between sheltered ones (often, twice as high or even higher). However, although this pattern was maintained over the generations, its intensity changed gradually from the oldest to the most recent one. Thus, from S07 to J09, differentiation between areas became stronger while differentiation between samples within each area became weaker (Table 2, miniplots). The latter was particularly apparent for the exposed area where Φ_{PT} estimates went from 0.156 for S07, to 0.105 for S09, and 0.072 for J09. By comparison, most of the weakening in the sheltered area happened between S07 (Φ_{PT} = 0.087) and the two generations sampled in 2009 (0.056 for S09 and 0.057 for J09). Hierarchical AMOVA comparing generations (Table 4) revealed no significant differentiation between them (0%, Φ_{RT} = -0.064, P < 1.000), while differences between samples within the same generation explained a high fraction of the variation (36%, Φ_{PR} = 0.364, P < 0.001).

The persistence of the genetic structure over the generations was illustrated by the Principal Coordinates Analysis (PCoA) (Fig. 3). Regardless of the generation, the samples formed two distinct non-

overlapping groups of exposed and sheltered sites. Within each group, the samples from the various generations collected at each site tended

Table 4. Hierarchical AMOVA with samples grouped by generations

Samples	Source of variation	Φ Statistics (P-value)
All samples	Between generations	-0.064 ($P > 0.05$)
	Between samples within generation	0.364 ($P < 0.001$)
	Within samples	0.323 ($P < 0.001$)
Sheltered area samples	Between generations	-0.003 ($P > 0.05$)
	Between samples within generations	0.071 ($P < 0.001$)
	Within samples	0.068 ($P < 0.001$)
Exposed area samples	Between generations	-0.009 ($P > 0.05$)
	Between samples within generations	0.125 ($P < 0.001$)
	Within samples	0.117 ($P < 0.001$)

to fall near each other, indicating that there was more genetic variability between sites than between generations within each site. Samples were similarly scattered in the two groups (exposed, protected). In both cases, the samples from one of the sites were more separated from the rest of the samples in the group. However, the distance of CT to other sites in the exposed group was greater than the separation of M to other sites in the protected group, which would explain why F_{ST}/Φ_{PT} estimates were higher in the exposed than in the sheltered area.

Using the complete data set, STRUCTURE only detected two clusters that matched the two wave exposure areas, albeit some individuals showed mixed ancestry. A'S07, M'S07, LC'S07, CT'S09, and CT'S07 were

the samples with largest proportion of mixed individuals. In particular, the exposed CT'S07 and the sheltered M'S07 included individuals that

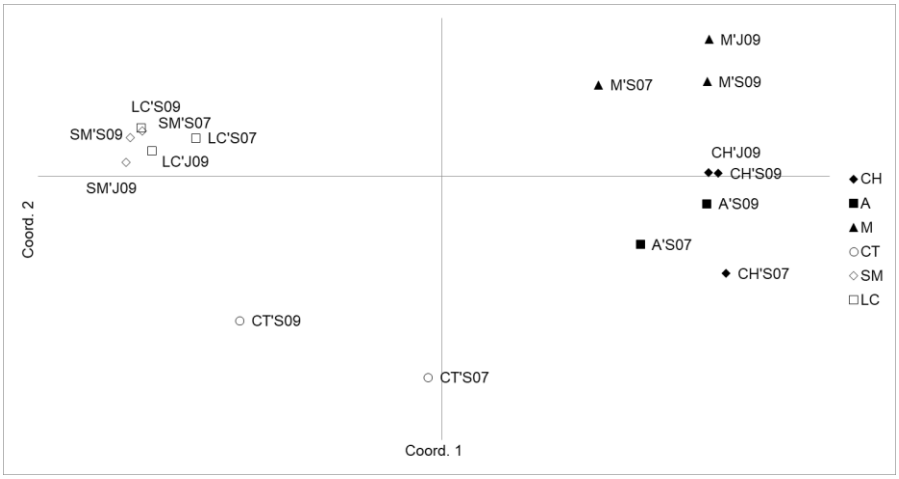


Fig. 3. *Nucella lapillus* Principal Coordinates Analysis. Nei's distances between populations are used for the PCoA analysis. The first coordinate explains 89.25% of the variation; the second coordinate explains 3.79%. Sample codes as in Fig. 1. Black symbols stand for sites in sheltered coast, blank symbols stand for sites in exposed coast.

clearly belonged to the other cluster. Separate analysis for sheltered and exposed coast sites after filtering immigrants returned similar results; most individuals showed a pure ancestry but some of them showed a minor proportion assigned to a different ancestry. These individuals occurred essentially in the same samples with mixed ancestry in the joint analysis (A'S09, A'S07, M'S07, LC'S07, CT'S09, and CT'S07) (Fig. 4).

Point *N*e estimates were low with narrow 95% confidence intervals for all the samples (Fig. 5). The lowest values were 6 for CT'S07 and 11 for M'S07, while the highest ones were 38 for SM'J09 and 36 for both M'J09 and M'S09. In most sites, estimates were very similar across

generations. The only exceptions were CT and M where *N*e estimates for generation S07 were clearly lower than those obtained for the generations sampled in 2009.

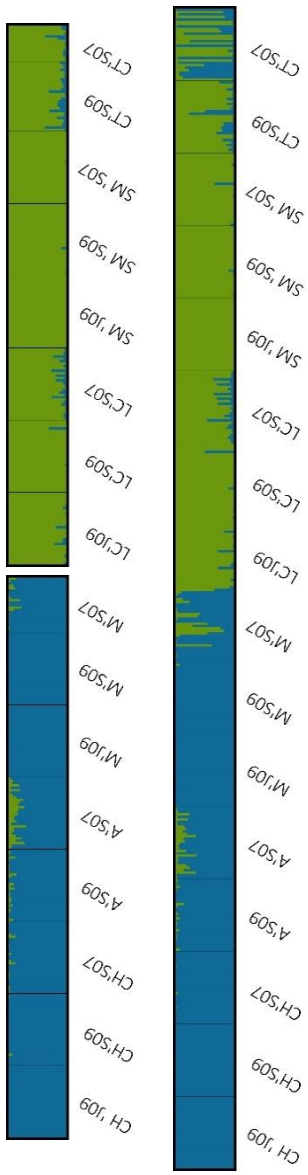


Fig. 4. *Nucella lapillus* STRUCTURE output. Each individual is represented by a vertical line divided into segments of different colour. Each segment represents the posterior probability of belonging to the clusters detected with Bayesian approach.

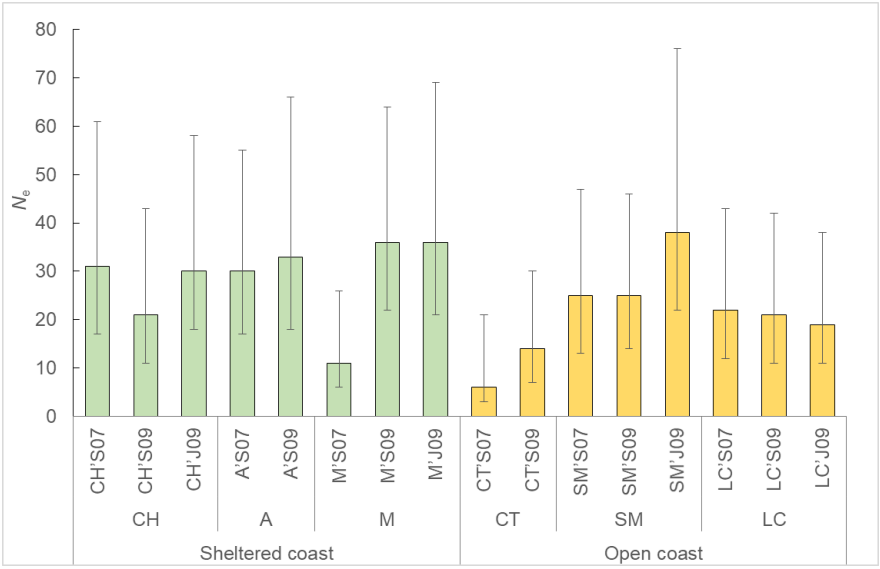


Fig. 5. *Nucella lapillus* *N*e and 95% confidence intervals for each sample inferred with sibship assignment method.

DISCUSSION

Population genetics has become a widely used insightful addition to the toolkit available for inferring dispersal in marine organisms (Palumbi *et al.* 2003). However, to what extent dispersal is actually responsible for the genetic structure displayed by the populations of some organisms may still be uncertain in some cases. For instance, research on organisms with planktonic larvae has often found more structure than might be anticipated when the dispersal stage has the potential to travel long distances depending on the length of the planktonic period (A. R. Wood & Gardner 2007, Hoffman *et al.* 2012, Owen & Rawson 2013). This seemingly contradictory situation has been attributed to a number of mechanisms that range from early proposals based on pre- and post-settlement selection (Crisp 1978, Johnson & Black 1984) to more recent

hypothesis based on geographic affiliation mediated by larvae retention, ocean currents promoting a stochastic mixing of larval contributions from different sources, biogeographical barriers, or divergence times and demographic changes (Palumbi 2003, Ayre *et al.* 2009, Hart & Marko 2010, Broquet *et al.* 2013, Eldon *et al.* 2016). Likewise counterintuitive has been the finding that direct developers sometimes exhibit high levels of gene flow even though they are presumably constrained to stay at their birthplace for their entire lives due to the lack of an appropriate dispersal stage (K. Johannesson 1988, Cumming *et al.* 2014, González-Wevar *et al.* 2018, Donald *et al.* 2020).

In our case, we studied a direct developer with a genetic population structure that varies depending on wave exposure. While dogwhelk populations in sheltered coast show the IBD pattern expected in a poor disperser, exposed coast ones display a disordered genetic differentiation reminiscent of what is seen in organisms with higher dispersal capacity (Carro *et al.* 2012, 2019). To explain this unordered differentiation, we proposed several scenarios, one of which is that dogwhelk populations in wave-swept coasts might exchange more individuals than could be anticipated from their lack a planktonic stage. Certain traits of the dogwhelk and observations in other benthic organisms suggest that this scenario might not necessarily be implausible. Dogwhelks hatch as tiny snails or 'crawlaways' (Crothers 1985) which, thanks to their very small size (around 1 mm long), might easily be transported to some distance by waves. Indeed, hatchlings of the congener *Nucella emarginata* have been reported to actively

disperse in the water column with the help of mucus threads that provide buoyancy (Martel & Chia 1991), and other marine invertebrates without a planktonic larval stage also disperse by drifting in the water column when very small (see Gosselin & Chia 1995 and references therein). Even adult individuals might be able to successful reach remote destinations by floating and/or rafting (Highsmith 1985, Cumming *et al.* 2014, González-Wevar *et al.* 2018, Donald *et al.* 2020). As chaotic genetic patchiness is characterized by temporal changes in the genetic structure of the populations (Johnson & Black 1982), we predicted that the spatial genetic pattern of *N. lapillus* should shift over generations if the cause for its genetic patchiness in exposed coast was that waves actually promote a stochastic exchange of individuals among populations. Contrary to this prediction, the arrangement of genetic differences among our populations of *N. lapillus* was notably stable across generations both regionally (exposed vs. sheltered areas) as well as within each area. Moreover, our analyses persistently revealed significant differences among sites within each generation but failed to detect differences among the various generations sampled at each site. Therefore, rather than a shifting, ephemeral structure, the stable genetic patchiness found in *N. lapillus* seems incompatible with the temporal variation in the source of recruits seen in other marine organisms as a likely explanation for their genetic patchiness (Johnson & Black 1982, Eldon *et al.* 2016). Instead, subadults and juveniles sampled several years apart at the same site are closely related to each other, as might be expected if exposed coast populations were maintained primarily by self-recruitment.

Finding that the unordered genetic structure of *N. lapillus* on exposed coast lacks the ephemeral character typical of a chaotic genetic patchiness (sensu Johnson & Black 1982) still leaves us with the need to find an alternative explanation as to why it differs from the IBD pattern detected in sheltered coast. Diversifying selection on a local spatial scale has been proposed as a plausible explanation for other cases of patternless genetic structure (Johnson & Black 1982, Eldon *et al.* 2016). In fact, direct developing marine gastropods such as *N. lapillus* and *L. saxatilis* provide some of the best known examples of phenotypic segregation in response to environmental factors such as shore level in the intertidal and wave exposure (Crothers 1985, Day 1990, K. Johannesson 2003). In *N. lapillus*, the degree of exposure to wave action has been shown to influence phenotypic traits such as shell shape and allozyme variation (Day 1990, Guerra-Varela *et al.* 2009). Even chromosome number has been reported to change with wave intensity in some regions (Day 1990, Pascoe & Dixon 1994), although the geographical variation in karyotypic forms throughout the species' range indicates that this is a complex phenomenon whose explanation remains elusive (Pascoe 2006). Shell shape, a trait typically used to differentiate *N. lapillus* ecotypes adapted to different degrees of wave exposure (Crothers 1985), has a genetic component and might be affected by divergent selection (Guerra-Varela *et al.* 2009). Additionally, shell ecotypes can leave an imprint on the genetic differentiation estimates obtained with neutral markers (Guerra-Varela *et al.* 2009, Carro *et al.* 2019). Moreover, microsatellite and AFLP data show that dogwhelk populations in semi-exposed sites sometimes include a

variable proportion of individuals assignable to genetic groups possibly related to the separation between exposed and sheltered shell ecotypes (Guerra-Varela *et al.* 2009, Carro *et al.* 2012). Since effective exposure to waves on exposed coast can vary on a meter scale depending on the microtopography, it seems feasible that segregation processes in response to this fine-grained mosaic of wave stresses might account for the apparently disordered pattern detected in these areas. If so, genetic information would be of limited use to infer connectivity on exposed coast because segregation processes might influence the differentiation between sites whether they exchange individuals or not. Natural selection against immigrants would be a barrier to gene flow via habitat-associated fitness (Nosil *et al.* 2005). Indeed, recent studies found that some AFLP loci correlate with shell shape despite gene flow, and reflect environmental differences rather than spatial distances (Carro *et al.* 2019). However, after pruning AFLP loci under divergent selection, a pattern of a pure IBD for the whole range studied emerged for remaining neutral loci, and results from network analysis suggested barriers to gene flow between our sites from exposed coast (Carro *et al.* 2019). Similarly, the homogeneous lack of wave stress that characterizes sheltered coasts would leave little room for environmental segregation. Moreover, it would make it more likely that migrant exchange and genetic differentiation between sites would depend mostly on the spatial distance between them, leading to a pattern typical of low dispersal organisms such as IBD.

Neutral processes could also explain the unordered pattern found in exposed coast. Theory predicts that genetic drift is stronger when local breeding groups are small. Life-history traits such as low-mobility adults and internal fertilization suggest that this might be the case in *N. lapillus* (Crothers 1985). Our *Ne* estimates are in line with these expectations as they are always in the range of a few tens of individuals, being similar to estimates reported for other intertidal invertebrates with low dispersal potential (Haye *et al.* 2014). Even taking into account the confidence intervals of our point estimates, they are below the number of animals usually found in any site. On the other hand, there was no clear evidence that *Ne* changed significantly between generations within a site. Hence, we found little support for our third scenario. However, effective population size alone cannot explain the different spatial patterns detected in each area, as low *Ne* values were not exclusive to the exposed coast. Differentiation by genetic drift also requires that dispersion among sites is not strong enough to homogenize their genetic variability (Eldon *et al.* 2016). Again, this might be the case for *N. lapillus* if, as anticipated for a direct-developer, most individuals recruit in the population where they were produced. Moreover, direct-developers are generally considered to have low fecundity but high survival. This, along with low immigration rates, might allow for a genetic structure stable over time such as the one reported here for *N. lapillus* (Lee & Boulding 2009).

In summary, by comparing the genetic structure over several generations we have shown that it is stable over time both on exposed

and sheltered coasts. Therefore, the unordered spatial pattern detected on exposed coast lacks the shifting nature inherent to chaotic genetic patchiness. Both environmental segregation of ecotypes adapted to different wave exposure conditions and genetic drift might explain the pattern seen in exposed coast. However, these two alternatives have different implications for connectivity since genetic drift would require little migration among populations while environmental segregation could even occur with an exchange of individuals among them. Meanwhile, this study highlights the challenges of inferring population connectivity from the spatial genetic structure.

Submitted as Carro, B., Quintela, M., Ruiz, J. M. & Barreiro, R. (xxxx) Using a temporal approach to disentangle the population genetic patterns displayed by the direct-developer *Nucella lapillus*. Malacologia

GENERAL CONCLUSIONS

As the marine snail *Nucella lapillus* is a rocky intertidal dweller, it has been subjected to TBT pollution for many years until the total ban of this strong biocide on European coasts in 2003. From 2008, it has also been prohibited worldwide. Exposure to this contaminant causes imposex in females of this dioecious gastropod, which is the recommended biomarker for TBT biomonitoring.

The study of imposex along the Galician coast for 13 years allowed us to verify the quick decrease of all imposex indices since the European Union (EU) total ban onwards, highlighting the success of the actions implemented. The simultaneous collection of biometric measures allowed us to investigate the relationship between imposex and biometric indices. Among them, the correlation found between VDSI and MFPL opens the possiblility of using a non-destructive procedure to survey the imposex, as measuring the penis length does not require the removal of the shell.

Further, by adding data from subsequent surveys (i.e. 2012 and 2015), we corroborated that the improvement in water quality seen from 2003 to 2006 and thereafter until 2009, has not changed significantly in the following 6 years. This stagnation could be attributed to subsidiary TBT from the sediments of the large ports and, exceptionally, to an illegal use of TBT. In addition, we proved that the Ecological Quality Objective (EcoQO) set by the OSPAR was achieved in 2009 on this regional scale, with the majority of samples meeting class B, which means having

achieved the Good Ecological Status aimed by the European Union (EU) before 2015 .

Now that we are in a scenario of declining TBT, populations from where *N. lapillus* had previously disapeared could receive new specimens and recover. However, this relies on the dogwhelk's dispersal capacity. Genetic analysis using our AFLP data suggest that *N. Lapillus* has a limited dispersal capacity as expected from a direct developer, and showed a significant genetic structure on both exposed and sheltered coasts. But, while it followed an isolation by distance (IBD) pattern on sheltered coast, the structure showed an unordered pattern with stronger genetic differentiation among populations on more exposed one.

The two genetic patterns displayed by *N. Lapillus* depending on wave exposure suggest that environmental features have an impact on genetic variation. Dogwhelks exhibit two ecotypes linked to wave exposure and, given the limited dispersion found here, it could be speculated that genetic differences may accumulate. Throughout a scan of our AFLP loci, we identified and separated those subjected to divergent selection (6%) from neutral ones and performed separated analysis for each group. We aimed to disentangle the relative contribution of geographic and ecological distance to the structure of neutral and adaptive genetic differentiation. The neutral data set followed an IBD pattern while loci under divergent selection correlated with geographic and environment distance. The loci under divergent selection also correlated with phenotype and seemed linked to shell

shape. As expected for a direct developer, *N. lapillus* might adapt to local heterogeneity through selection on genes affecting shell and phenotypic variation.

Although *N. lapillus* has proved to be a poor disperser, what is triggering the unordered pattern found on exposed coast, as opposed to the IBD displayed on sheltered one, remains unresolved. A chaotic genetic patchiness is typical of a higher dispersal capacity. But if this was the case, one would also expect a temporal instability in this pattern. Instead, the stability of the population genetic pattern across the three generations studied by us seems compatible with either a scenario where local adaptation by divergent selection (as a portion of the genome has shown to be subject to) would preclude newcomers from managing to reproduce, or with a lack of exchange of individuals between populations because wave action would prevent it. The latter could lead to the differentiation of populations by genetic drift which whould be promoted by genetic isolation.

RESUMEN EXTENDIDO

EL CARACOL MARINO *NUCELLA LAPILLUS*

Uno de los depredadores más comunes del intermareal rocoso europeo es un caracol que encontramos en nuestras costas con la marea baja. Se extiende desde el sur de Portugal hasta el norte de Rusia, y a lo largo de la costa atlántica norteamericana (Crothers 1985, Fretter & Graham 1985). *Nucella lapillus* (Linnaeus 1758) es un caracol marino perteneciente al orden Neogastropoda, que lo distingue como un carnívoro dioico con un sistema nervioso concentrado, sifón y un opérculo córneo en espiral. Su hábitat está lleno de bellotas de mar y mejillones, presas sedentarias que conforman su dieta, siendo *Semibalanus balanoides* su favorita (Crothers 1985). *N. lapillus* se alimenta de ellos haciendo agujeros mecánica y químicamente en sus conchas. El tamaño óptimo de un mejillón para una *N. lapillus* de 30 mm es de aproximadamente 20 mm (Bayne & Scullard 1978). Su tasa de alimentación media es de 0,7 mejillones o 16 bellotas de mar a la semana a 20°C (Largen 1967). Esto quiere decir que, en sitios de clima favorable, *N. lapillus* puede dominar la distribución de sus presas (Crothers 1985, Ehlers *et al.* 2018) y ser la responsable del paisaje intermareal. A su vez, es devorada por cangrejos y aves marinas, principalmente. Su fecundación es interna y las hembras ponen 20-30 cápsulas fuertemente adheridas al sustrato en las grietas de las rocas, cada una de las cuales almacena cientos de huevos (Fretter 1953). Este caracol es de desarrollo directo, de las cápsulas emergen arrastrándose unos 30 minúsculos caracolillos (la mayoría restante actúa como huevos

nodriza) después de un período de tiempo que va de 2 a 7 meses en el sur de Gran Bretaña y el Mar Blanco, respectivamente (Fretter & Graham 1985).

A partir de ese momento, crecen haciendo una espiral en el sentido de las agujas del reloj desde el ápice (protoconcha vestigial), añadiendo material al labio desde el borde del manto. El epitelio del manto segrega conquiolina impregnada con carbonato cálcico, y luego agrega carbonato cálcico en toda su superficie interna, lo que hace que la concha adopte la forma del cuerpo del caracol (es decir, de la joroba visceral) a medida que crece vuelta a vuelta (Fretter & Graham 1994a). Cuando alcanzan la madurez, dejan de crecer y engrosan el labio externo, llegando incluso a desarrollar dientes para cerrar la entrada al ataque de los depredadores (en zonas protegidas suponen una verdadera amenaza). Crecen hasta alcanzar 20-35 mm (Crothers 1985). Dejan de crecer a los tres años desde que fueron depositados como huevo, y entonces se reproducen. Pueden vivir 6 o más años, y las hembras sobreviven a los machos. Ellas son más viejas, más grandes y más numerosas (Moore 1938, Feare 1970b, Fretter & Graham 1994a, Son & Hughes 2000). La mortalidad es de aproximadamente el 90, 50 y 27% de los individuos dentro de una población en el primer, segundo y tercer año, respectivamente.

El taxón *N. lapillus* fue propuesto pronto, y ya en la obra maestra de Linneo, *Systema Naturae*, es bautizado como *Buccinum lapillus* (Linnaeus 1758). Desde el principio, este caracol atrajo la atención de los científicos por los colores y el bandeado tan variable de su concha

(Colton 1922, Moore 1936, 1938). *N. lapillus* exhibe un polimorfismo muy pronunciado relacionado con las variables medioambientales (Bantock & Cockaine 1975, Crothers 1985, Harris & Jones 1995, Rolán *et al.* 2004). Pero son sobre todo notables los dos morfotipos ligados a la exposición al oleaje. *N. lapillus* desarrolla una concha más pequeña, achaparrada y con una abertura más ancha en costa expuesta, mientras que crece como un caracol más alargado, con la concha más gruesa y una abertura más estrecha en costa protegida (Crothers 1985). La abertura más ancha evita que los caracoles sean arrancados del sustrato por el oleaje en costa expuesta, pero provocan una mayor desecación del animal en costa protegida, a donde no llega el spray de las olas. Además, es también en costa protegida donde tener una abertura más grande es especialmente contraproducente por el riesgo elevado de depredación por cangrejos. Así que supone una gran ventaja exhibir ecotipos adaptados a diferentes condiciones medioambientales. Cuando el flujo genético se ve obstaculizado, como puede suceder a un animal de desarrollo directo como *N. lapillus*, las diferencias genéticas locales acaban acumulándose. Es difícil cuantificar la importancia relativa del componente genético frente a la plasticidad fenotípica en la determinación morfológica de la concha de los invertebrados. Pero la plasticidad fenotípica como responsable del morfotipo está relacionada con especies con una capacidad de dispersión más elevada que aquella que se espera de *N. lapillus*. Por otro lado, algunos estudios sugieren la existencia de una base genética para la morfología de la concha (Rolán *et al.* 2004, Pascoal *et al.* 2012b), e incluso que un proceso de selección

divergente podría estar actuando sobre uno o unos pocos loci genómicos (Guerra-Varela *et al.* 2009).

El tributilestaño (TBT)

Un hecho importante en la historia de *Nucella lapillus* que la llevó a estar en el punto de mira es su relación con el contaminante tributilestaño (TBT). El TBT es una molécula simple (un átomo de estaño unido covalentemente a tres sustituyentes butilo) cuya elevada toxicidad fue bien aprovechada en pinturas para estructuras sumergidas (Bennett 1996). La función de estas cubiertas antiincrustantes es retrasar el crecimiento de organismos que puedan arruinar estas estructuras, lo que se traduce en elevadas pérdidas económicas. La bioincrustación en los barcos, por ejemplo, aumenta la rugosidad de la superficie del casco, lo que causa un incremento en la resistencia y el consumo de combustible, y una disminución de la velocidad máxima. El aumento de consumo de combustible de un buque después de operar durante 6 meses en aguas templadas se estimó en un 35-50% (véase Schultz *et al.* 2011 para una justificación). Abbott *et al.* (2000) calcularon un ahorro de combustible gracias al TBT de 7,3 millones de toneladas al año (lo equivalente a 730 millones de dólares) en 1989, teniendo en cuenta una parte importante de la flota comercial mundial. La eficiencia del TBT como biocida llevó a su uso generalizado en todo el mundo y, por consiguiente, a su difusión en todas las aguas del globo. Muy pronto se estableció un vínculo directo entre el TBT y un gran número de malformaciones que empezaron a desarrollar muchos invertebrados marinos (Smith 1981a, b, Alzieu 1996, de Mora 1996), que suponían

cientos de especies no objetivo (véase Sousa *et al.* 2014 para una revisión). Todo apuntaba, además, a que el TBT estaba entrando en la cadena trófica y, por lo tanto, podría afectar negativamente a la salud humana (Sousa *et al.* 2014). De hecho, el TBT es reconocido como la sustancia más tóxica liberada premeditadamente al medio marino por la mano del hombre (Goldberg 1986). Sin embargo, no fue hasta que el cultivo de las ostras colapsó en Francia, debido a las malformaciones y a la falta de reclutamiento (el conocido episodio de la Bahía de Arcachon a principios de los 80), que se empieza a restringir parcialmente su uso en 1982 (ver Alzieu 2000), y no fue hasta 2003 que se prohibió completamente en las costas europeas, cincuenta años después del descubrimiento de sus propiedades biocidas a principios de los años 50. El 17 de septiembre de 2008 se prohíbe para todos los estados miembros a través del Convenio Internacional sobre el control de los sistemas antiincrustantes perjudiciales en los buques (Convenio AFS), adoptado por la Organización Marítima Internacional (IMO) para proteger el medio ambiente acuático. Eso sucede doce meses después de que 25 estados (más del 25% del tonelaje de la marina mercante mundial) lo hubiesen ratificado. A día de hoy, el número de estados firmantes es de 90, que suponen el 96,13% del tonelaje bruto de la flota mercante mundial (IMO 2020).

Entre los organismos afectados por el TBT, *N. lapillus* muestra la relación causa-efecto, dosis-dependiente mejor documentada (Gibbs *et al.* 1987), lo que lo convierte en el organismo bioindicador de referencia para biomonitorizar la contaminación por TBT (OSPAR 2003). El término

imposex fue acuñado por Smith (1971) para denominar la superimposición de caracteres sexuales masculinos sobre hembras de gasterópodos marinos dioicos. Se demostró que el TBT actuaba como un disruptor endocrino, capaz de imitar o antagonizar la acción de las hormonas y causar un desarrollo anormal de los organismos a través de 3 rutas metabólicas (i.e. esteroide, neuroendocrina y retinoide, Sternberg *et al.* 2010). Más tarde, mediante el uso de técnicas más actualizadas, Pascoal *et al.* (2013) exploraron la expresión genética con secuenciación masiva (NGS) en *N. lapillus* tras someterla a diferentes tratamientos con TBT, apareciendo un cuarto mecanismo, la ruta PPAR (receptor activado por el proliferador del peroxisoma). Sus datos también apuntan a que el TBT estaría actuando sobre el sistema inmune además de sobre el endocrino.

El imposex es un fenómeno gradual que se desarrolla a medida que la concentración de TBT en el medio aumenta, y que puede medirse mediante una serie de índices. El índice de la secuencia del vaso deferente (VDSI) y el índice del tamaño relativo del pene (RPSI) son dos de los principales. El RPSI representa el tamaño relativo (en %) del pene de las hembras con respecto al de los machos de una población dada. El VDSI es la media del VDS de las hembras de una población. El VDS empleado para *N. lapillus* tiene seis estadios, desde una hembra sana sin rastro de imposex (estadio 0), pasando por estadios intermedios a medida que el conducto deferente se extiende y el pene crece (estadios 2 y 3), hasta que el conducto deferente se desarrolla completamente y el pene de la hembra alcanza el aspecto del pene de un macho de la

misma población (estadio 4). A una concentración aún mayor de TBT, el conducto deferente crece hasta obstruir la vulva (estadio 5) impidiendo la liberación de cápsulas de huevos, que pueden aparecer abortadas en la glándula de la cápsula (estadio 6). En este punto, las hembras son estériles y la población disminuirá, pudiendo incluso extinguirse. Sin embargo, tras la prohibición mundial del TBT a partir de 2008, nos encontramos en un escenario de disminución progresiva de su presencia en el medioambiente. El grupo de investigación de BioCost de la Universidade da Coruña ha estado biomonitorizando su presencia en el Noroeste de la Península Ibérica desde 1996 (Barreiro *et al.* 1998, Ruiz *et al.* 1998, Barreiro *et al.* 1999, Quintela *et al.* 2000, Barreiro *et al.* 2001, Barreiro *et al.* 2006, Quintela *et al.* 2006b, Ruiz *et al.* 2008, Ruiz *et al.* 2010). Los datos acumulados por el grupo ofrecen una oportunidad única para estudiar su evolución y la eficacia de las regulaciones a las que ha estado sometido hasta su prohibición total. Además, la biomonitorización del TBT es obligatoria en Europa en donde las aguas deben alcanzar unos niveles de calidad ambiental mínimos (OSPAR 2004, EC 2008).

Una vez libres de TBT, las poblaciones que en otro tiempo se vieron afectadas podrían ahora recuperarse con la llegada de nuevos especímenes desde poblaciones vecinas. La capacidad de dispersión de *N. lapillus* determina la velocidad a la que podría recolonizar los lugares de donde desapareció debido a la contaminación por TBT. Sin embargo, hasta ahora, en lo que respecta a su capacidad de dispersión, se han obtenido resultados contradictorios usando diferentes marcadores

moleculares (Day *et al.* 1994, Colson & Hughes 2004). Teniendo en cuenta las cuestiones aún abiertas sobre su dinámica de poblaciones, se hace imprescindible elucidar el verdadero potencial de dispersión de esta especie emblemática del paisaje intermareal.

El estudio de las poblaciones marinas

El conocimiento de la dinámica de poblaciones de las especies marinas es clave para mantener y gestionar las comunidades naturales y para conservar la diversidad del medio marino (Hedgecock *et al.* 2007, G. P. Jones *et al.* 2007, Cowen & Sponaugle 2009). El aumento y la intensidad de la amenaza humana sobre los océanos aceleran la pérdida y la fragmentación de los hábitats marinos, lo que eleva el riesgo de extinción de las especies. El diseño eficaz de áreas marinas protegidas depende en gran medida de la comprensión de los mecanismos de dispersión de los organismos, clave para delimitar el tamaño, la configuración y la ubicación óptimos de las mismas. Además, es fundamental comprender la dinámica de los recursos pesqueros (que se enfrentan a fuertes presiones como la sobrepesca, la eutrofización y la destrucción del hábitat); comprender y controlar la distribución de las especies invasoras, e investigar el papel de la dispersión en la adaptación de las especies a cambios en su área de distribución (como es el cambio climático) (Palumbi 2003, Shanks *et al.* 2003, Bradbury *et al.* 2008). Los métodos disponibles para su estudio se dividen en directos e indirectos (Grosberg & Cunningham 2001, Palumbi *et al.* 2003). Los métodos directos implican la observación y el seguimiento de individuos y propágulos que en medio marino se vuelve una tarea,

cuando menos, ardua. El tamaño inabarcable del medio, y el pequeño tamaño de los organismos dificultan el acceso a ellos y su vigilancia (Hellberg *et al.* 2002). Por ello, se ha recurrido a menudo a interpretaciones basadas en la biología de los organismos. La gran mayoría de las especies marinas poseen larvas que se liberan al mar y son consideradas un medio de dispersión a grandes distancias, y que mantendría las poblaciones conectadas. Esto ha dado lugar a la idea tradicional de poblaciones abiertas en medio marino. Además, incluso aún pudiendo rastrear a los individuos, su impacto en la población anfitriona sigue siendo difícil de determinar. La solución pasa por emplear métodos alternativos (Palumbi 2003) como es el análisis del patrón geográfico de la variación genética de las poblaciones, con el que se puede estimar el flujo genético (Slatkin 1987, Bossart & Pashley Prowell 1998, Hellberg *et al.* 2002). El estudio de la composición genética de los individuos tiene la ventaja de que sólo tiene en cuenta los individuos que han logrado reproducirse y tener un impacto en la población anfitriona, a la vez que proporciona un compendio de información final sobre la historia y las características de las poblaciones naturales (Hellberg *et al.* 2002). El uso de marcadores y modelos genéticos de población apropiados puede proporcionar a su vez información sobre la biología de los organismos, ya que los procesos que afectan a las poblaciones (i.e. el éxito reproductivo, la migración, el tamaño de la población, la selección natural y los acontecimientos históricos) dejan una señal indeleble en su genoma (Sunnucks 2000, Grosberg & Cunningham 2001).

A pesar de la tradición relativamente larga de estudios biológicos con *N. lapillus*, siguen existiendo muchas incertidumbres sobre aspectos clave de su biología. Las características de su ciclo de vida (desarrollo directo, adultos aparentemente sedentarios) sugieren una capacidad de dispersión mínima, a menudo asumida en la literatura más clásica (Colton 1922, Moore 1936, 1938, Moore & Sproston 1940, Staiger 1957, Berry & Crothers 1968, Feare 1970a, 1971, Hoxmark 1971, Largen 1971). Sin embargo, algunos trabajos con marcadores moleculares sugieren una capacidad de dispersión algo inesperada (Colson & Hughes 2003, Bell & Okamura 2005, Colson *et al.* 2006), incluso a distancias enormes de cientos de kilómetros (Day *et al.* 1994, Wares & Cunningham 2001, Colson & Hughes 2007). Considerando a *N. lapillus* como un organismo modelo, elucidar su verdadera capacidad de dispersión puede contribuir a comprender la dinámica de las poblaciones de invertebrados marinos; además, evaluar el grado de conectividad entre las diferentes poblaciones tiene importantes implicaciones para la ecología aplicada (Grosberg & Cunningham 2001, Hellberg *et al.* 2002, Kinlan & Gaines 2003, Palumbi 2003).

Para el presente estudio de la dinámica de poblaciones de *N. lapillus*, se seleccionaron marcadores genéticos AFLP (Vos *et al.* 1995) por la capacidad que tienen de generar un gran número de marcadores ampliamente distribuidos a lo largo de todo el genoma del organismo. Son versátiles y flexibles y pueden aplicarse sin ningún conocimiento previo de la especie (Bensch & Akesson 2005, Paun & Schönswetter 2012). Son reproducibles y robustos y no requieren un período largo de

desarrollo. Tienen, además, una buena relación coste-beneficio. Los AFLPs se fundamentan en la amplificación por PCR de subconjuntos de fragmentos de restricción a partir de la digestión total del ADN genómico. Un cambio de nucleótidos en el sitio de restricción (e.g. mutaciones, inserciones) genera fragmentos de diferente longitud entre individuos. La técnica registra la presencia/ausencia de fragmentos de distintas longitudes que dan lugar a una huella genética única para cada individuo (Vos *et al.* 1995).

Los marcadores AFLP no están exentos de inconvenientes. Son marcadores dominantes y no es posible discriminar entre homocigoto y heterocigoto cuando está presente un fragmento de una determinada longitud. Para ello, se deben analizar un número mínimo de AFLPs con herramientas adecuadas (Blears *et al.* 1998, Bonin *et al.* 2004, Bonin *et al.* 2007). Además, para garantizar la robustez y la reproducibilidad de la técnica, el ADN tiene que ser de cierta calidad para asegurar la consistencia de las enzimas de restricción.

Por todo lo anterior, los OBJETIVOS que nos planteamos alcanzar en esta tesis se pueden concretar en:

- Estudiar la evolución del imposex, los índices biométricos, y la relación entre ambos, en el gasterópodo marino *N. lapillus* a lo largo de más de 10 años en las poblaciones de la costa gallega (Capítulo I)
- Averiguar hasta qué punto se ha alcanzado el objetivo de calidad ecológica (EcoQO) de la EU tras la prohibición del uso del TBT (Capítulo II)

- Verificar la capacidad real de dispersión de *N. lapillus* mediante el estudio de su estructura genética poblacional ya que un flujo genético restringido podría comprometer la supervivencia de las poblaciones afectadas por el TBT (Capítulo III)
- Investigar si la exposición al oleaje tiene un impacto en el patrón de la estructura genética mediante el muestreo de zonas sometidas a un régimen hidrodinámico opuesto (Capítulo III)
- Evaluar la diferente contribución de la plasticidad fenotípica y la selección divergente en el desarrollo de los morfotipos que exhibe *N. lapillus* vinculados a la exposición al oleaje (Capítulo IV)
- Investigar si el oleaje podría estar promoviendo o restringiendo el intercambio de individuos entre las poblaciones de costa más expuesta mediante la exploración de la estabilidad de la estructura genética a través de las generaciones (Capítulo V).
- Explorar el mecanismo tras la diferente estructura genética de población exhibida por *N. lapillus* en costa expuesta y en costa protegida (Capítulo V)

Estudio a lo largo de más de diez años de los índices biométricos y el imposex en *Nucella lapillus* L.

En Galicia (NO España) hemos biomonitorizado la contaminación por tributilestaño (TBT) durante más de una década a intervalos regulares mediante el estudio del imposex en poblaciones de *Nucella lapillus* ($n \geq 34$). Se procesaron miles de ejemplares de los que se registraron la longitud de la concha (SH), la longitud del pene (PL) y la secuencia del vaso deferente (VDS); posteriormente se calcularon los índices de

imposex (incluido el índice VDS, VDSI). La media regional de SH tanto de las hembras como de los machos no cambió significativamente a lo largo del estudio. Lo que también se aplica a la media del PL de los machos. Sin embargo, la media regional del PL de las hembras (MFPL) y todos los índices de imposex disminuyeron significativamente. Los resultados confirman unas conclusiones previas basadas en los análisis químicos de los tejidos y en observaciones parciales de imposex. Además, la estrecha correlación entre el MFPL y el VDSI proporciona una potencial aplicación para la biomonitorización del TBT.

Una ampliación del estudio del imposex en el Atlántico español confirma la consecución puntual de los objetivos medioambientales europeos para el TBT

La legislación de la Unión Europea (UE) tenía como objetivo alcanzar un buen estatus ecológico para 2015 en lo que respecta al tributilestaño (TBT, el biocida utilizado en las pinturas antiincrustantes tradicionales). Con miras a comprobar ese logro en el Atlántico español, se amplió el seguimiento hasta esa fecha del imposex en *Nucella lapillus* (el instrumento de evaluación recomendado). En Galicia, el uso de este caracol desde 1996 mostró que este objetivo ambiental se alcanzó ya en 2009, pero nuevos estudios no revelan ninguna otra mejora desde entonces.

LOS AFLPS REVELAN UNA ESTRUCTURA GENÉTICA DE POBLACIÓN DIFERENTE EN EL CARACOL MARINO *NUCELLA LAPILLUS* L. BAJO CONDICIONES AMBIENTALES OPUESTAS

La dispersión ha recibido una creciente atención en Ecología marina, en particular desde que con técnicas más actuales se obtuvieron evidencias que desafiaban la visión tradicional. El caracol marino *Nucella lapillus* L., un gasterópodo sedentario de desarrollo directo, es un buen ejemplo: tradicionalmente se pensaba que su capacidad de dispersión era limitada hasta que estudios con marcadores microsatélites cuestionaron esta idea. Para arrojar algo de luz sobre esta discrepancia, se investigó la estructura genética de poblaciones de los caracoles en el NO de España con marcadores AFLP altamente polimórficos, prestando especial atención a la influencia del estrés hidrodinámico. De acuerdo con lo que se espera de un organismo con baja capacidad de dispersión, nuestros resultados muestran una estructura genética significativa a escala regional (< 200 km) y local (< 15 km). Sin embargo, la estructura genética espacial varió con la exposición al oleaje en el presente estudio: el aislamiento por distancia (IBD) fue evidente en condiciones protegidas, pero no así en zona expuesta, donde la diferenciación genética fue más elevada. Nuestros resultados aportan evidencias de que la diferente exposición al oleaje puede ejercer una influencia detectable en la estructura genética de los organismos costeros, incluso en especies que carecen de larva planctónica.

La exposición al oleaje como causa de aislamiento por el medio ambiente en el gasterópodo marino *Nucella lapillus* L.

La forma en que la variación genética adaptativa y neutral es moldeada por factores ambientales es crucial para la Biología evolutiva. Para investigar si la exposición al oleaje puede incrementar la adaptación local en los caracoles del litoral, se escanearon marcadores AFLP en diez poblaciones de *Nucella lapillus* de hábitats opuestos (protegidos frente a expuestos). Cerca del 6% de los loci analizados se desviaron de las expectativas de neutralidad, lo que sugiere que la exposición al oleaje podría ser un fuerte agente selectivo que moldea la variación genética. Los marcadores neutrales describieron únicamente un patrón de aislamiento por distancia (IBD), sin rastro de aislamiento por el medio (IBE), mientras que los loci sometidos a selección divergente siguieron un patrón de IBD e IBE, ya que los test de Mantel parciales detectaron un IBD significativo tras tener en cuenta las diferencias ambientales. La topología de las redes genéticas reveló un flujo genético sustancial en marcadores neutrales (i.e. una densa red con ejes que conectaban hábitats similares y opuestos), mientras que se establecieron pocas conexiones entre ambientes opuestos en los loci sometidos a selección divergente. Además, los loci que correlacionaron con el fenotipo (forma de concha; i.e. un biomarcador morfológico de exposición al oleaje) explicaron hasta cerca del 11% de la varianza de este rasgo. En conjunto, nuestros resultados sugieren que, incluso en un contexto de flujo genético, la adaptación local podría perfilar un rasgo como la forma de la concha.

USANDO UN ENFOQUE TEMPORAL PARA DESENTRAÑAR LOS PATRONES DE LA GENÉTICA DE POBLACIONES EXHIBIDOS POR EL CARACOL DE DESARROLLO DIRECTO *NUCELLA LAPILLUS* L.

El análisis de la estructura genética de poblaciones es una alternativa común para dilucidar la conectividad entre poblaciones cuando las medidas directas están fuera del alcance. Sin embargo, la interpretación de los resultados genéticos es a veces complicada ya que otros factores distintos a la migración podrían estar también moldeando el patrón resultante. Un ejemplo es *Nucella lapillus* (Linnaeus 1758), un gasterópodo marino de desarrollo directo cuya estructura genética de poblaciones se ha comprobado que varía en función de la exposición al oleaje. En costa expuesta, las poblaciones muestran un patrón desordenado compatible con el patrón a manchas caótico que se observa en otros organismos costeros con mayor capacidad de dispersión. Para desentrañar esta situación aparentemente paradójica, investigamos la estabilidad temporal de los patrones genéticos mostrados por *N. lapillus* en condiciones opuestas de exposición al oleaje. Se analizaron un total de 480 caracoles de 16 muestras, que abarcaron tres generaciones simpátricas, con marcadores AFLP que revelaron que tanto el patrón de aislamiento por distancia encontrado en zona protegida como el patrón desordenado encontrado en sitios expuestos al oleaje persistía a través de las generaciones (Φ_{PT} = -0.064). Dado que esto último no concuerda con lo esperado de un patrón a manchas caótico, se plantean como explicaciones al patrón resultante en costa expuesta tanto la segregación ambiental de ecotipos

adaptados a diferentes condiciones de exposición como, más plausiblemente, la deriva genética. Como cada alternativa tiene implicaciones muy diferentes para la conectividad entre las poblaciones, este estudio pone de relieve las sutilezas de su inferencia a partir de la estructura genética espacial.

RESUMO ESTENDIDO

O CARACOL DE MAR *NUCELLA LAPILLUS*

Un dos depredadores máis comúns do intermareal rochoso europeo é un caracol que coa marea baixa atopamos nas nosas costas. Esténdese desde o sur de Portugal ata o norte de Rusia, e ao longo da costa atlántica norteamericana (Crothers 1985, Fretter & Graham 1985). *Nucella lapillus* (Linnaeus 1758) é un caracol mariño pertencente á orde Neogastropoda, que o distingue como un carnívoro dioico cun sistema nervioso concentrado, sifón e un opérculo córneo en espiral. O seu hábitat está cheo de arneiros e mexillóns, presas sedentarias que conforman a súa dieta, sendo *Semibalanus balanoides* o seu favorito (Crothers 1985). *N. lapillus* aliméntase deles facendo buratiños mecánica e químicamente nas súas cunchas. O tamaño óptimo dun mexillón para unha *N. lapillus* de 30 mm é de máis ou menos 20 mm (Bayne & Scullard 1978). A súa taxa de alimentación media é de 0,7 mexillóns ou 16 arneiros á semana a 20° C (Largen 1967). Isto quere dicir que, en sitios de clima favorable, *N. lapillus* pode dominar a distribución das súas presas (Crothers 1985, Ehlers et al. 2018) e ser a responsable da paisaxe intermareal. Á súa vez, é devorada por cangrexos e aves mariñas principalmente. A súa fecundación é interna e as femias poñen 20-30 cápsulas fortemente adheridas ó substrato nas gretas das rochas, cada unha das cales almacena centos de ovos (Fretter 1953). Este caracol é de desenvolvemento directo, das cápsulas emerxen arrastrándose uns 30 minúsculos caracois (a maioría restante actúa como ovos ama de cría) despois dun período de tempo que vai de 2 a 7

meses no sur de Gran Bretaña e o Mar Blanco, respectivamente (Fretter & Graham 1985).

A partir dese momento, crecen facendo unha espiral no sentido das agullas do reloxo desde o ápice (vestixio da protocuncha), engadindo material ó beizo desde a marxe do manto. O epitelio do manto segrega conquiolina impregnada con carbonato cálcico, e logo agrega carbonato cálcico en toda a súa superficie interna, o que fai que a cuncha adopte a forma do corpo do caracol (é dicir, da xiba visceral) a medida que crece volta a volta (Fretter & Graham 1994a). Cando alcanzan a madurez deixan de crecer e engrosan o beizo externo, chegando mesmo a desenvolver dentes para pecha-la entrada ó ataque dos depredadores (en zonas protexidas supoñen unha verdadeira ameaza). Crecen ata alcanzar 20-35 mm (Crothers 1985). Deixan de crecer ós tres anos desde que foron depositados como ovo, e entón reprodúcense. Poden vivir 6 ou máis anos, e as femias sobreviven ós machos. Elas son máis vellas, máis grandes e máis numerosas (Moore 1938, Feare 1970 b, Fretter & Graham 1994a, Son & Hughes 2000). A mortalidade é de aproximadamente o 90, 50 e 27% dos individuos dentro dunha poboación no primeiro, segundo e terceiro ano respectivamente.

O taxón *N. lapillus* propúxose pronto, e xa na obra mestra de Linneo, *Systema Naturae*, é bautizado como *Buccinum lapillus* (Linnaeus 1758). Desde o principio, este caracol atraeu a atención dos científicos polas cores e o bandeado tan variable da súa cuncha (Colton 1922, Moore 1936, 1938). *N. lapillus* exhibe un polimorfismo moi pronunciado relacionado coas variables ambientais (Bantock & Cockaine 1975,

Crothers 1985, Harris & Jones 1995, Rolán *et al.* 2004). Pero son sobre todo notables os dous morfotipos ligados á exposición ás ondas. *N. lapillus* desenvolve unha cuncha máis pequeña, achaparrada e cunha abertura máis ancha en costa exposta, mentres que crece como un caracol máis alongado, coa cuncha máis grosa e unha abertura máis estreita en costa protexida (Crothers 1985). A abertura máis ancha evita que os caracois sexan arrincados do substrato polas ondas en costa exposta, pero provocan unha maior desecación do animal en costa protexida, onde non chega o spray das ondas. Ademais, é tamén en costa protexida onde ter unha abertura máis grande é especialmente contraproducente polo risco elevado de depredación por cangrexos. Así que supón unha gran vantaxe exhibir ecotipos adaptados a diferentes condicións ambientais. Cando o fluxo xenético se ve obstaculizado, como pode suceder a un animal de desenvolvemento directo como *N. lapillus*, as diferenzas xenéticas locais acaban acumulándose. É difícil cuantifica-la importancia relativa do compoñente xenético fronte á plasticidade fenotípica na determinación morfolóxica da cuncha dos invertebrados. Pero a plasticidade fenotípica como responsable do morfotipo está relacionada con especies cunha capacidade de dispersión máis elevada que aquela que se espera de *N. lapillus*. Doutra banda, algúns estudos suxiren a existencia dunha base xenética para a morfoloxía da cuncha (Rolán *et al.* 2004, Pascoal *et al.* 2012 b), e mesmo que un proceso de selección diverxente podería estar a actuar sobre un ou uns poucos loci xenómicos (Guerra-Varela *et al.* 2009).

O TRIBUTILESTAÑO (TBT)

Un feito importante na historia de *Nucella lapillus* que a levou a estar no punto de mira é a súa relación co contaminante tributilestaño (TBT). O TBT é unha molécula simple (un átomo de estaño unido covalentemente a tres substituíntes butilo) de elevada toxicidade que foi ben aproveitada en pinturas para estruturas mergulladas (Bennett 1996). A función destas cubertas antiincrustantes é atrasa-lo crecemento de organismos que poidan arruinar estas estruturas, o que se traduce en elevadas perdas económicas. A bioincrustación nos barcos, por exemplo, aumenta a rugosidade da superficie do casco, o que causa un incremento na resistencia e o consumo de combustible, e unha diminución da velocidade máxima. O aumento de consumo de combustible dun buque despois de operar durante 6 meses en augas tépedas estimouse nun 35-50% (véxase Schultz *et al.* 2011 para unha xustificación). Abbott *et al.* (2000) calcularon un aforro de combustible grazas ó TBT de 7,3 millóns de toneladas ó ano (o equivalente a 730 millóns de dólares) en 1989, tendo en conta unha parte importante da frota comercial mundial. A eficiencia do TBT como biocida levou ó seu uso xeneralizado en todo o mundo e, por conseguinte, á súa difusión en tódalas augas do globo. Moi pronto se estableceu un vínculo directo entre o TBT e un gran número de malformacións que empezaron a desenvolver moitos invertebrados mariños (Smith 1981a, b, Alzieu 1996, de Amora 1996), que supoñían centos de especies non obxectivo (véxase Sousa *et al.* 2014 para unha revisión). Todo apuntaba, ademais, a que o TBT estaba a entrar na cadea trófica e, por tanto, podería afectar

negativamente á saúde humana (Sousa *et al.* 2014). De feito, o TBT é recoñecido como a substancia máis tóxica liberada premeditadamente ó medio mariño pola man do home (Goldberg 1986). Con todo, non foi ata que o cultivo das ostras colapsou en Francia, debido ás malformacións e á falta de recrutamento (o coñecido episodio da Baía de Arcachon a principios dos 80), que se empeza a restrinxi-lo seu uso (Alzieu 2000). E non foi ata 2003 que se prohibiu completamente nas costas europeas, cincuenta anos despois do descubrimento das súas propiedades biocidas a principios dos anos 50. O 17 de setembro de 2008 prohíbese para tódolos estados membros a través do Convenio Internacional sobre o control dos sistemas antiincrustantes prexudiciais nos buques (Convenio AFS), adoptado pola Organización Marítima Internacional (IMO) para protexe-lo medio ambiente acuático. Iso sucede doce meses despois de que 25 estados (máis do 25% da tonelaxe da mariña mercante mundial) o ratificasen. A día de hoxe, o número de estados asinantes é de 90, que supoñen o 96,13% da tonelaxe bruta da frota mercante mundial (IMO 2020).

Entre os organismos afectados polo TBT, *N. lapillus* amosa a relación causa-efecto, dose-dependente mellor documentada (Gibbs *et al.* 1987), o que o converte no organismo bioindicador de referencia para biomonitoriza-la contaminación por TBT (OSPAR 2003). O imposex foi acuñado por Smith (1971) para denomina-la superimposición de caracteres sexuais masculinos sobre femias de gasterópodos mariños dioicos. Demostrouse que o TBT actuaba como un disruptor endocrino, capaz de imitar ou antagonizar a acción das hormonas e causar un desenvolvemento anormal dos organismos. Os mecanismos detrás

deste trastorno fisiolóxico foron estudados máis a fondo con técnicas de última xeración por Pascoal *et al.* (2013), onde exploraron a expresión xenética con secuenciación masiva (NGS) en *N. lapillus* tras sometela a diferentes tratamentos con TBT. Ademais das tres vías metabólicas que se propuxeran con anterioridade (i.e. esteroide, neuroendocrina e retinoide, Sternberg *et al.* 2010), aparece un cuarto mecanismo, a ruta PPAR (receptor activado polo proliferador do peroxisoma). Os seus datos tamén apuntan a que o TBT estaría a actuar sobre o sistema inmune ademais de sobre o endocrino.

O imposex é un fenómeno gradual que aumenta o seu desenvolvemento na medida que o fai a concentración de TBT no medio, e que pode medirse mediante unha serie de índices. O índice da secuencia do vaso deferente (VDSI) e o índice do tamaño relativo do pene (RPSI) son dous dos principais. O RPSI representa o tamaño relativo (en %) do pene das femias con respecto ó dos machos dunha poboación dada. O VDSI é a media do VDS das femias dunha poboación. O VDS empregado para *N. lapillus* ten seis estadios, desde unha femia san sen rastro de imposex (estadio 0), pasando por estadios intermedios a medida que o conduto deferente esténdese e o pene crece (estadios 2 e 3), ata que o conduto deferente desenvólvese completamente e o pene da femia alcanza o aspecto do pene dun macho da mesma poboación (estadio 4). A unha concentración aínda maior de TBT, o conduto deferente crece ata obstruí-la vulva (estadio 5) impedindo a liberación de cápsulas de ovos, que poden aparecer abortadas na glándula da cápsula (estadio 6). Neste punto, as femias son estériles e a poboación diminuirá e mesmo pode extinguirse. Con todo, trala

prohibición mundial do TBT a partir de 2008, atopámonos nun escenario de diminución progresiva da súa presenza no medioambiente. O grupo de investigación BioCost da Universidade da Coruña estivo biomonitorizando a súa presenza no Noroeste da Península Ibérica desde 1996 (Barreiro *et al.* 1998, Ruiz *et al.* 1998, Barreiro *et al.* 1999, Quintela *et al.* 2000, Barreiro *et al.* 2001, Barreiro *et al.* 2006, Quintela *et al.* 2006 b, Ruiz *et al.* 2008, Ruiz *et al.* 2010). Os datos acumulados polo grupo ofrecen unha oportunidade única para estuda-la súa evolución e a eficacia das regulacións ás que estivo sometido ata a súa prohibición total. Ademais, a biomonitorización do TBT é obrigatoria en Europa onde as augas deben alcanzar uns niveis de calidade ambiental mínimos (OSPAR 2004, EC 2008).

Unha vez libres de TBT, as poboacións que noutro tempo se viron afectadas poderían agora recuperarse coa chegada de novos especímenes desde poboacións veciñas. A capacidade de dispersión de *N. lapillus* determina a velocidade á que podería recoloniza-los lugares de onde desapareceu debido á contaminación por TBT. Con todo, ata o de agora, no que respecta á súa capacidade de dispersión, obtivéronse resultados contraditorios usando diferentes marcadores moleculares (Day *et al.* 1994, Colson & Hughes 2004). Tendo en conta as cuestións aínda abertas sobre a súa dinámica de poboacións, faise imprescindible elucida-lo verdadeiro potencial de dispersión desta especie emblemática da paisaxe intermareal.

O ESTUDO DAS POBAOCIÓNS MARIÑAS

O coñecemento da dinámica de poboacións das especies mariñas é clave para manter e xestiona-las comunidades naturais e para conserva-la diversidade do medio mariño (Hedgecock *et al.* 2007, G. P. Jones *et al.* 2007, Cowen & Sponaugle 2009). O aumento e a intensidade da ameaza humana sobre os océanos aceleran a perda e a fragmentación dos hábitats mariños, o que eleva o risco de extinción das especies. O deseño eficaz de áreas mariñas protexidas depende en gran medida da comprensión dos mecanismos de dispersión dos organismos, clave para delimita-lo tamaño, a configuración e a localización óptimos das mesmas. Ademais, é fundamental comprende-la dinámica dos recursos pesqueiros (que se enfrontan a fortes presións como a sobrepesca, a eutrofización e a destrución do hábitat); comprender e controlar a distribución das especies invasoras, e investiga-lo papel da dispersión na adaptación das especies a cambios na súa área de distribución (como é o cambio climático) (Palumbi 2003, Shanks *et al.* 2003, Bradbury *et al.* 2008). Os métodos dispoñibles para o seu estudo divídense en directos e indirectos (Grosberg & Cunningham 2001, Palumbi *et al.* 2003). Os métodos directos implican a observación e o seguimento de individuos e propágulos que no medio mariño vólvese unha tarefa, cando menos, ardua. O tamaño inabarcable do medio, e o pequeno tamaño dos organismos dificultan o acceso a eles e á súa vixilancia (Hellberg *et al.* 2002). Por iso, recorreuse a miúdo a interpretacións baseadas na bioloxía dos organismos. A gran maioría das especies mariñas posúen larvas que se liberan ó mar e son consideradas un medio de dispersión

a grandes distancias, e que mantería as poboacións conectadas. Isto deu lugar á idea tradicional de poboacións abertas no medio mariño. Ademais, mesmo aínda podendo rastrexar ós individuos, o seu impacto na poboación anfitrioa segue sendo difícil de determinar. A solución pasa por empregar métodos alternativos (Palumbi 2003) como é a análise do patrón xeográfico da variación xenética das poboacións, co que se pode estima-lo fluxo xenético (Slatkin 1987, Bossart & Pashley Prowell 1998, Hellberg *et al.* 2002). O estudo da composición xenética dos individuos ten a vantaxe de que só ten en conta os individuos que lograron reproducirse e ter un impacto na poboación anfitrioa, á vez que proporciona un compendio de información final sobre a historia e as características das poboacións naturais (Hellberg *et al.* 2002). O uso de marcadores e modelos xenéticos de poboación apropiados pode proporcionar á súa vez información sobre a bioloxía dos organismos, xa que os procesos que afectan ás poboacións (i.e. o éxito reprodutivo, a migración, o tamaño da poboación, a selección natural e os acontecementos históricos) deixan un sinal indeleble no seu xenoma (Sunnucks 2000, Grosberg & Cunningham 2001).

A pesar da tradición relativamente longa de estudos biolóxicos con *N. lapillus*, seguen existindo moitas incertezas sobre aspectos clave da súa bioloxía. As características do seu ciclo de vida (desenvolvemento directo, adultos aparentemente sedentarios) suxiren unha capacidade de dispersión mínima, a miúdo asumida na literatura máis clásica (Colton 1922, Moore 1936, 1938, Moore & Sproston 1940, Staiger 1957, Berry & Crothers 1968, Feare 1970a, 1971, Hoxmark 1971, Largen

1971). Con todo, algúns traballos con marcadores moleculares suxiren unha capacidade de dispersión algo inesperada (Colson & Hughes 2003, Bell & Okamura 2005, Colson *et al.* 2006), mesmo a distancias enormes de centos de quilómetros (Day *et al.* 1994, Wares & Cunningham 2001, Colson & Hughes 2007). Considerando a *N. lapillus* como un organismo modelo, elucida-la súa verdadeira capacidade de dispersión pode contribuír a comprende-la dinámica das poboacións de invertebrados mariños; ademáis, avalia-lo grao de conectividade entre as diferentes poboacións ten importantes implicacións para a ecoloxía aplicada (Grosberg & Cunningham 2001, Hellberg *et al.* 2002, Kinlan & Gaines 2003, Palumbi 2003).

Para o presente estudo da dinámica de poboacións de *N. lapillus*, seleccionáronse marcadores xenéticos AFLP (Vos *et al.* 1995) pola capacidade que teñen de xerar un gran número de marcadores amplamente distribuídos ó longo de todo o xenoma do organismo. Son versátiles e flexibles e poden aplicarse sen ningún coñecemento previo da especie (Bensch & Akesson 2005, Paun & Schönswetter 2012). Son reproducibles e robustos e non requiren un período longo de desenvolvemento. Teñen, ademais, unha boa relación custo-beneficio. Os AFLPs fundaméntanse na amplificación por PCR de subconxuntos de fragmentos de restrición a partir da dixestión total do ADN xenómico. Un cambio de nucleótidos no sitio de restrición (e.g. mutacións, insercións) xera fragmentos de diferente lonxitude entre individuos. A técnica rexistra presenza/ausencia de fragmentos de distintas

lonxitudes que dan lugar a unha pegada xenética única para cada individuo (Vos *et al.* 1995).

Os marcadores AFLP non están exentos de inconvenientes. Son marcadores dominantes e non é posible discriminar entre homocigoto e heterocigoto cando está presente un fragmento dunha determinada lonxitude. Para iso, débense analizar un número mínimo de AFLPs con ferramentas adecuadas (Blears *et al.* 1998, Bonin *et al.* 2004, Bonin *et al.* 2007). Ademais, para garanti-la robustez e a reproducibilidade da técnica, o ADN ten que ser de certa calidade para asegura-la consistencia das encimas de restrición.

Por todo o anterior, os OBXECTIVOS que nos propuxemos acadar nesta tese pódense concretar en:

– Estuda-la evolución do imposex, os índices biométricos, e a relación entre ambos, no gasterópodo mariño *N. lapillus* ó longo de máis de 10 anos nas poboacións da costa galega (Capítulo I)

– Pescudar ata que punto se alcanzou o obxectivo de calidade ecolóxica (EcoQO) da EU trala prohibición do uso do TBT (Capítulo II)

– Verifica-la capacidade real de dispersión de *N. lapillus* mediante o estudo da súa estrutura xenética poboacional xa que un fluxo xenético restrinxido podería compromete-la supervivencia das poboacións afectadas polo TBT (Capítulo III)

– Investigar se a exposición á ondada ten un impacto no patrón da estrutura xenética mediante a mostraxe de zonas sometidas a un réxime hidrodinámico oposto (Capítulo III)

– Avalia-la diferente contribución da plasticidade fenotípica e a selección diverxente no desenvolvemento dos morfotipos que exhibe *N. lapillus* vinculados á exposición á ondada (Capítulo IV)

– Investigar se a ondada podería estar a promover ou restrinxir o intercambio de individuos entre as poboacións de costa máis exposta mediante a exploración da estabilidade da estrutura xenética a través das xeracións (Capítulo V).

– Explora-lo mecanismo trala diferente estrutura xenética de poboación exhibida por *N. lapillus* en costa exposta e en costa protexida (Capítulo V)

ESTUDO Ó LONGO DE MÁIS DE DEZ ANOS DOS ÍNDICES BIOMÉTRICOS E IMPOSEX EN *NUCELLA LAPILLUS* L.

En Galicia (NO España) biomonitorizamo-la contaminación por tributilestaño (TBT) durante máis dunha década a intervalos regulares mediante o estudo do imposex en poboacións de *Nucella lapillus* (n ≥ 34). Procesáronse miles de exemplares dos que se rexistraron a lonxitude da cuncha (SH), a lonxitude do pene (PL) e a secuencia do vaso deferente (VDS); posteriormente calculáronse os índices de imposex (incluído o índice VDS, VDSI). A media rexional de SH tanto das femias como dos machos non cambiou significativamente ó longo do estudo. O que tamén se aplica á media do PL dos machos. Con todo, a media

rexional do PL das femias (MFPL) e tódolos índices de imposex diminuíron significativamente. Os resultados confirman unhas conclusións previas baseadas nas análises químicas dos tecidos e en observacións parciais de imposex. Ademais, a estreita correlación entre o MFPL e o VDSI proporciona unha potencial aplicación para a biomonitorización do TBT.

UNHA AMPLIACIÓN DO ESTUDO DO IMPOSEX NO ATLÁNTICO ESPAÑOL CONFIRMA A CONSECUCIÓN PUNTUAL DOS OBXECTIVOS AMBIENTAIS EUROPEOS PARA O TBT

A lexislación da Unión Europea (UE) tiña como obxectivo alcanzar un bo status ecolóxico para 2015 no que respecta ó tributilestaño (TBT, o biocida utilizado nas pinturas antiincrustantes tradicionais). Con miras a comprobar ese logro no Atlántico español, ampliouse o seguimento ata esa data do imposex en *Nucella lapillus* (o instrumento de avaliación recomendado). En Galicia, o uso deste caracol desde 1996 amosou que este obxectivo ambiental alcanzouse xa en 2009, pero novos estudos non revelan ningunha outra mellora desde entón.

OS AFLPS REVELAN UNHA ESTRUTURA XENÉTICA DE POBOACIÓN DIFERENTE NO CARACOL MARIÑO *NUCELLA LAPILLUS* L. BAIXO CONDICIÓNS AMBIENTAIS OPOSTAS

A dispersión recibiu unha crecente atención en Ecoloxía mariña, en particular desde que con técnicas máis actuais obtivéronse evidencias que desafiaban a visión tradicional. O caracol mariño *Nucella lapillus* L., un gasterópodo sedentario de desenvolvemento directo, é un bo

exemplo: tradicionalmente pensábase que a súa capacidade de dispersión era limitada ata que estudos con marcadores microsatélites cuestionaron esta idea. Para botar algo de luz sobre esta discrepancia, investigouse a estrutura xenética de poboacións dos caracois no NO de España con marcadores AFLP altamente polimórficos, prestando especial atención á influencia do estrés hidrodinámico. De acordo co que se espera dun organismo con baixa capacidade de dispersión, os nosos resultados amosan unha estrutura xenética significativa a escala rexional (< 200 km) e local (< 15 km). Con todo, a estrutura xenética espacial variou coa exposición á ondada no presente estudo: o illamento por distancia (IBD) foi evidente en condicións protexidas, pero non así en zona exposta, onde a diferenciación xenética foi máis elevada. Os nosos resultados achegan evidencias de que a diferente exposición á ondada pode exercer unha influencia detectable na estrutura xenética dos organismos costeiros, mesmo en especies que carecen de larva planctónica.

A EXPOSICIÓN Á ONDADA COMO CAUSA DO ILLAMENTO POLO MEDIO AMBIENTE NO GASTERÓPODO MARIÑO *NUCELLA LAPILLUS* L.

A forma en que a variación xenética adaptativa e neutral é moldeada por factores ambientais é crucial para a Bioloxía evolutiva. Para investigar se a exposición á ondada pode incrementar a adaptación local nos caracois do litoral, escaneáronse marcadores AFLP en dez poboacións de *Nucella lapillus* de hábitats opostos (protexidos fronte a expostos). Preto do 6% dos loci analizados desviáronse das expectativas de neutralidade, o que suxire que a exposición á ondada podería ser un

forte axente selectivo que moldea a variación xenética. Os marcadores neutrais describiron unicamente un patrón de illamento por distancia (IBD), sen rastro de illamento polo medio (IBE), mentres que os loci sometidos a selección diverxente seguiron un patrón de IBD e mais IBE, xa que os test de Mantel parciais detectaron un IBD significativo tras ter en conta as diferenzas ambientais. A topoloxía das redes xenéticas revelou un fluxo xenético substancial en marcadores neutrais (i.e. unha densa rede con eixos que conectaban hábitats similares e opostos), mentres que se estableceron poucas conexións entre ambientes opostos nos loci sometidos a selección diverxente. Ademais, os loci que correlacionaron co fenotipo (forma da cuncha; i.e. un biomarcador morfolóxico de exposición á ondada) explicaron ata preto do 11% da varianza deste rasgo. En conxunto, os nosos resultados suxiren que, mesmo nun contexto de fluxo xenético, a adaptación local podería perfilar un rasgo como a forma da cuncha.

USANDO UN ENFOQUE TEMPORAL PARA DESENTRAÑA-LOS PATRÓNS DA XENÉTICA DE POBOACIÓNS EXHIBIDOS POLO CARACOL DE DESENVOLVEMENTO DIRECTO *NUCELLA LAPILLUS* L.

A análise da estrutura xenética de poboacións é unha alternativa común para dilucida-la conectividade entre poboacións cando as medidas directas están fóra do alcance. Con todo, a interpretación dos resultados xenéticos é ás veces complicada xa que outros factores distintos á migración poderían estar tamén moldeando o patrón resultante. Un exemplo témolo en *Nucella lapillus* (Linnaeus 1758), un gasterópodo mariño de desenvolvemento directo do cal comprobouse

que a súa estrutura xenética de poboacións varía en función da exposición á ondada. En costa exposta, as poboacións mostran un patrón desordenado compatible co patrón a manchas caótico que se observa noutros organismos costeiros con maior capacidade de dispersión. Para desentrañar esta situación aparentemente contraditoria, investigámo-la estabilidade temporal dos patróns xenéticos mostrados por *N. lapillus* en condicións opostas de exposición á ondada. Analizáronse un total de 480 caracois de 16 mostras, que abarcaron tres xeracións simpátricas, con marcadores AFLP que revelaron que tanto o patrón de illamento por distancia atopado en zona protexida como o patrón desordenado atopado en sitios expostos á ondada persistía a través das xeracións (Φ_{PT} = -0.064). Dado que isto último non se axusta ó esperado dun patrón a manchas caótico, expóñense como explicacións ó patrón resultante en costa exposta tanto a segregación ambiental de ecotipos adaptados a diferentes condicións de exposición como, máis plausiblemente, a deriva xenética. Como cada alternativa ten implicacións moi diferentes para a conectividade entre poboacións, este estudo salienta as sutilezas da súa inferencia a partir da estrutura xenética espacial.

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NUCELLA LAPILLUS L.: IMPOSEX AFTER TRIBUTILTIN BAN (TBT) AND POPULATION CONNECTIVITY STUDIES.

DOCTORAL THESIS, 2021

Belén Carro Espada

Nucella lapillus L. is one of the most common neogastropods on the European Atlantic intertidal coast. Populations have been affected by TBT pollution for years. From 2008, the use of this strong pollutant is banned in international waters. The recovery of marine species populations relies on their ability to disperse. *N. lapillus* is a direct-developer which implies limited dispersal potential. This study reports the results of TBT biomonitoring and population genetic studies.

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