UNIVERSIDADE DA CORUÑA

Genetic aspects for the management of the stocks of the spiny spider crab *Maja brachydactyla*: heteroplasmy and multipaternity

Elba Rodríguez Pena

Doctoral Thesis 2020

International doctorate

Supervisors:

Andrés Martínez Lage

Luis Fernández Rodríguez

Doctoral Program in Marine Science, Technology and Management (DOMAR)

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Aspectos genéticos para la gestión de los stocks de la centolla *Maja brachydactyla*: heteroplasmia y multipaternidad

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Departamento de Biología

Los Dres. ANDRÉS MARTÍNEZ LAGE, profesor titular de universidad del área de genética, y LUIS FERNÁNDEZ RODRÍGUEZ, catedrático de universidad del área de biología animal, del Departamento de Biología de la Universidade da Coruña

INFORMAN:

Que la graduada Elba Rodríguez Pena del programa de doctorado en "Marine Science, Technology and Management (DOMAR)" ha realizado en el Departamento de Biología de la Facultad de Ciencias la tesis titulada "Genetic aspects for the management of the stocks of the spiny spider crab *Maja brachydactyla*: heteroplasmy and multipaternity" bajo nuestra supervisión. Considerándola finalizada permitimos su presentación bajo la modalidad internacional.

Y para que conste firmamos la presente en A Coruña a 7 de septiembre de 2020.

VºBª de los Directores

Fdo. Andrés Martínez Lage

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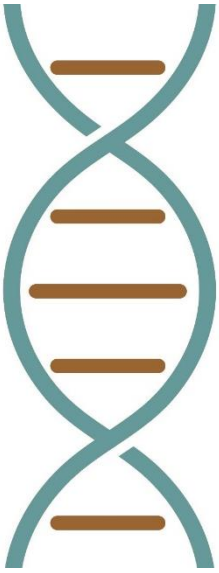
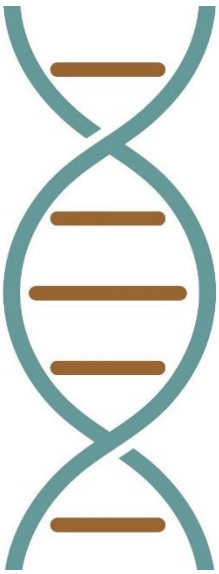
La doctoranda

Fdo. Elba Rodríguez Pena



Financial support and research stays



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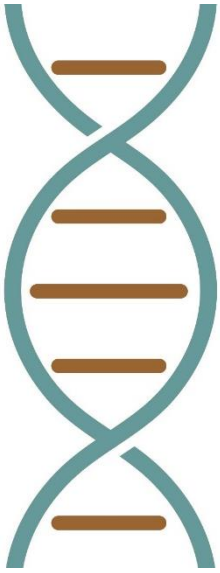
Part of the work included in this thesis was performed at the Marine Institute of Oranmore (Co. Galway, Ireland) supervised by Dr. Oliver Tully.



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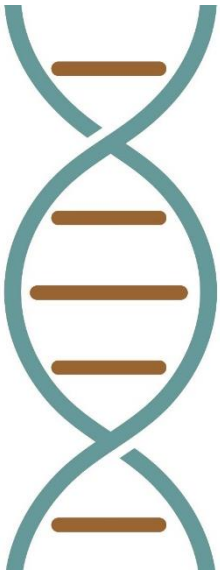

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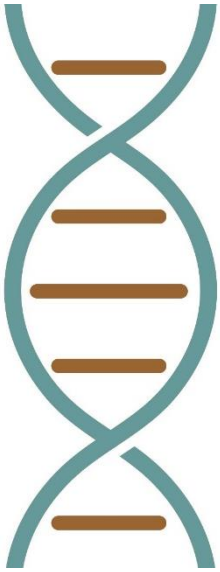
To all who have been there...

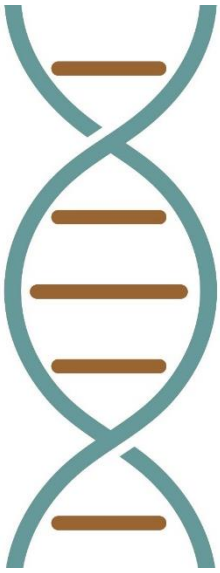
Thank you very much!

¡Muchas gracias!

¡Moitas grazas!



Short abstracts



Abstract

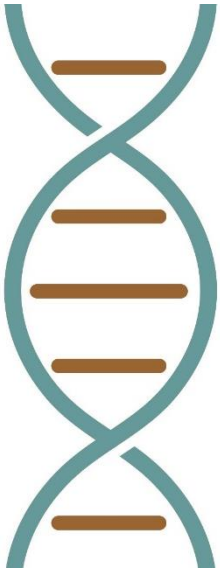
The spiny spider crab *Maja brachydactyla* is a decapod of high commercial interest. Despite the large number of studies conducted on this species, genetic research is still scarce. This doctoral thesis was focused on studying genetic aspects of the biology of *M. brachydactyla* relevant for a sustainable exploitation of stocks. As these studies require high-quality DNA, the first step consisted of performing methodological improvements of DNA extraction process, from choosing an adequate starting tissue to selecting the most effective protocol, both for adults and eggs. These improvements for DNA extraction facilitated the analysis of nuclear and mitochondrial markers. The analysis of microsatellites revealed multipaternity, although the prevalence differed between populations with different exploitation levels, probably due to the selective fishing focused on large males promoted by some fishing regulations. The analysis of mitochondrial genes disclosed heteroplasmy, likely caused by hybridisation with the congeneric species *Maja squinado*. Multipaternity and heteroplasmy, together with the high connectivity of the stocks, are probably responsible for the typical sperm limitation and genetic diversity loss of highly exploited populations not being detectable in this crab yet. However, if intense fishing continues, spider crab stocks will reach an unbridgeable bottleneck. For this reason, it is urgent to review the management measures that promotes male-selective fishing in *M. brachydactyla*.

Resumen

La centolla *Maja brachydactyla* es un decápodo de gran interés comercial. A pesar de los múltiples trabajos realizados sobre esta especie, los estudios genéticos son todavía escasos. Esta tesis doctoral se centró en el estudio de aspectos genéticos de la biología de *M. brachydactyla* relevantes para una explotación sostenible de los *stocks*. Dado que estos estudios requieren ADN de alta calidad, el primer paso consistió en realizar mejoras metodológicas en el proceso de extracción de ADN, desde la elección de un tejido inicial adecuado hasta la selección del protocolo más eficaz, tanto para adultos como para huevos. Estas mejoras para la extracción de ADN facilitaron el análisis de marcadores nucleares y mitocondriales. El análisis de microsatélites reveló multipaternidad, aunque la prevalencia varió entre poblaciones con diferentes grados de explotación, probablemente debido a la pesca selectiva de machos grandes promovida por algunas normas de regulación pesquera. El análisis de genes mitocondriales reveló heteroplasmia, posiblemente causada por la hibridación con la especie congénere *Maja squinado*. La multipaternidad y la heteroplasmia, junto con la alta conectividad de los *stocks*, son probablemente la causa de que la limitación de esperma y la pérdida de diversidad genética típicas de las poblaciones explotadas todavía no sean detectables en centolla. Sin embargo, si este intenso grado de explotación continúa, los *stocks* alcanzarán un cuello de botella insalvable. Por esta razón, es urgente revisar las medidas de gestión que promueven la pesca selectiva de machos en *M. brachydactyla*.

Resumo

A centola *Maja brachydactyla* é un decápodo de grande interese comercial. A pesar dos múltiples estudos realizados nesta especie, os estudos xenéticos son aínda escasos. Esta tese de doutoramento centrouse no estudo de aspectos xenéticos da bioloxía de *M. brachydactyla* relevantes para unha explotación sostible dos *stocks*. Dado que estes estudos requiren ADN de alta calidade, o primeiro paso consistiu en realizar melloras metodolóxicas do proceso de extracción de ADN, desde a elección dun tecido inicial adecuado ata a selección do protocolo máis eficaz, tanto para adultos como para ovos. Estas melloras para a extracción de ADN facilitaron a análise de marcadores nucleares e mitocondriais. A análise de microsatélites revelou multipaternidade, aínda que a prevalencia variou entre poboacións con distinto grao de explotación, probablemente debido á pesca selectiva de machos grandes promovida por algunhas normas de regulación pesqueira. A análise de xenes mitocondriais revelou heteroplasmia, posiblemente causada pola hibridación coa especie conxénere *Maja squinado*. A multipaternidade e a heteroplasmia, xunto coa elevada conectividade dos *stocks*, son probablemente a causa de que a limitación de esperma e a perda de diversidade xenética típica de poboacións explotadas aínda non sexan detectables en centola. Non obstante, se este elevado grao de explotación continúa, os *stocks* chegarán a un pescozo de botella insslvable. Por este motivo, é urxente revisar as medidas de xestión que promoven a pesca selectiva de machos en *M. brachydactyla*.



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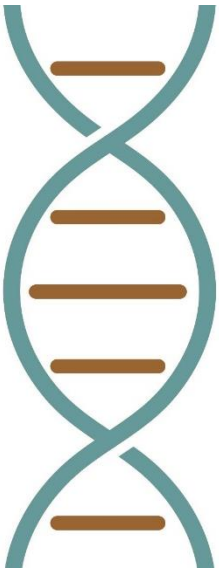

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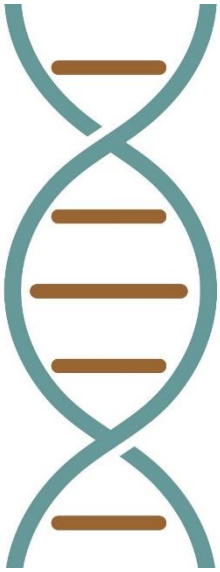
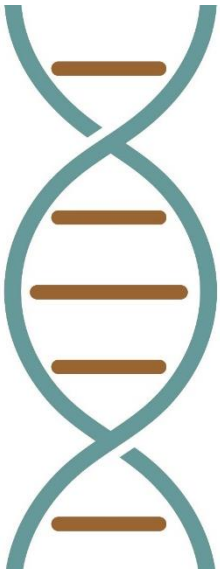
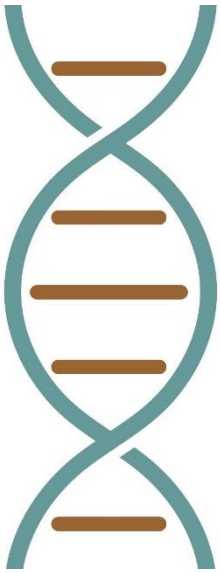


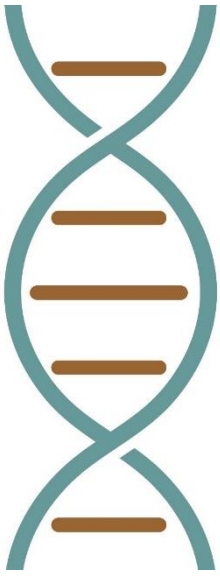
Abbreviations and symbols



ANOVA	Analysis of variance
ARMS-qPCR	Amplification Refractory Mutation System-Quantitative PCR
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CL	Carapace length
Co.	County
COI	Cytochrome c oxidase subunit I
DNA (ADN)	Deoxyribonucleic acid (Ácido desoxiribonucleico)
DUI	Doubly Uniparental Inheritance
EP	Exclusion probability
F1	First filial generation
FIS	Inbreeding coefficient
gDNA	Genomic deoxyribonucleic acid
H_e	Expected heterozygosity
H_o	Observed heterozygosity
HWE	Hardy-Weinberg equilibrium
LB-ampicillin	Lysogeny Broth- ampicillin
mtDNA (ADNmt)	Mitochondrial deoxyribonucleic acid (ácido desoxiribonucleico mitocondrial)
N	North
NE	Northeast
N_e	Effective population size
NGS	Next-Generation Sequencing
NUMT	Nuclear Mitochondrial DNA segment
NW (NO)	Northwest (Noroeste)
PCR	Polymerase Chain Reaction
PrDM	Probability of detecting multiple mating
qPCR	Quantitative Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal ribonucleic acid
SNP	Single-Nucleotide Polymorphism
SW	Southwest
W	West



List of tables and figures



1

General introduction

Distribution and biology of the spiny spider crab *Maja brachydactyla*

Maja brachydactyla Balss, 1922, is a decapod crustacean of the family Majidae (De Grave *et al.*, 2009; Ng and Richer de Forges, 2015), whose members are commonly known as spider crabs. This species inhabits the Northeast Atlantic from the British Isles to Senegal, including the archipelagos of the Azores, Madeira, the Canary Islands and Cape Verde (Hines *et al.*, 1995; Neumann, 1998; Udekem d'Acoz, 1999; Sotelo *et al.*, 2008b) (Fig. 1.1).

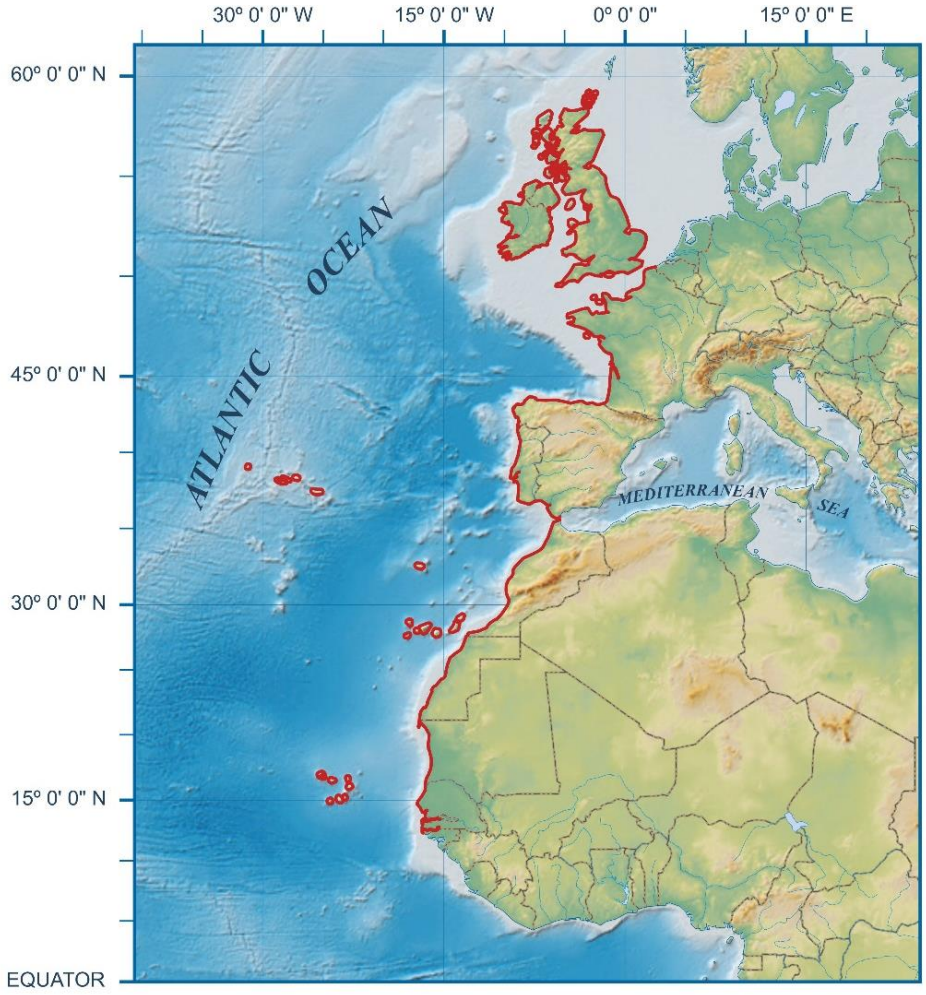


Fig.1.1. Geographic distribution of the spiny spider crab *Maja brachydactyla* (Neumann, 1998; Udekem d'Acoz, 1999; Sotelo *et al.*, 2008b).

In general, decapod crustaceans are characterised by life cycles with planktonic larval phases and benthic post-larval phases. For this reason, their distribution and population dynamics are closely related both to processes of larval dispersal, mediated by physical

factors, and to processes of habitat selection, linked to movements and migrations in post-larval stages (Possingham and Roughgarden, 1990; Wahle and Steneck, 1991; Wahle, 1992; Botsford *et al.*, 1994, 1998; Pardieck *et al.*, 1999; Palma *et al.*, 1999).

The life cycle of the spider crab *M. brachydactyla* comprises three phases: the larval phase, the juvenile or growth phase, and the adult or reproductive phase. The planktonic larval phase lasts two to three weeks, depending on water temperature, and consists of two zoeal stages and one megalopal stage (Iglesias *et al.*, 2002; Guerao *et al.*, 2008). The beginning of the juvenile phase is marked by the post-larval recruitment. Juvenile growth through successive moults, with the frequency of moult being lower as the juvenile reaches larger sizes (Gónzalez-Gurriarán *et al.*, 1995; Guerao and Rotllant, 2009). During this phase, spider crabs inhabit shallow bottoms (> 15m) where they make limited movements (Gónzalez-Gurriarán and Freire, 1994; Hines *et al.,* 1995), reaching the adult phase after 2-3 years (Le Foll, 1993; Meyer, 1993), depending on when recruitment has occurred.

The end of this phase is marked by a terminal moult, after which individuals stop growing and the adult phase begins (Hartnoll, 1963; Sampedro *et al.*, 1999; Corgos and Freire, 2006). Adult females are characterised by a domed abdomen with well-developed pleopods suitable for the incubation of the brood, as opposed to the flat abdomen of the juvenile specimens. In contrast, in adult males there is a change in the size of the chelipeds with respect to the carapace, since these specialised appendages are used to hold the females during copulation (Brosnan, 1981; Rodhouse, 1984). In addition to possessing larger chelipeds, in the adult phase male spider crabs are distinguishable from females, first, by their narrow, elongated pleon and, second, by their pleopods modified in the form of copulatory appendages. Terminal moulting takes place in summer in the Ría de Arousa (Galicia, NW Spain) (González-Gurriarán *et al.*, 1995; González-Gurriarán *et al.* 2002; Sampedro *et al.*, 2003) and in early autumn in the Norman-Breton Gulf (France) (Le Foll, 1993; Meyer, 1993).

In the NE Atlantic adult spider crabs migrate to deep waters (30-100m) in autumn (Latrouite and Le Foll, 1989; Le Foll, 1993; González-Gurriarán and Freire, 1994; Hines *et al.*, 1995; Freire and González-Gurriarán, 1998; González-Gurriarán *et al.*, 2002), although differences between sexes have been observed. In the Golfo Ártabro (Galicia, NW Spain), males do not have a definite migration peak, moving to deep waters between September and October, while females have a definite peak in October. It has also been suggested that small males migrate earlier to avoid agonistic interactions with larger males and thus maximise their mating opportunities (Corgos *et al.*, 2006). Copulation occurs in these deep zones between hard-shelled individuals, without courtship or pre- or post-copulatory

guarding (González-Gurriarán *et al.*, 1998). Immediately after mating (in January and February), females carry out a return migration to shallow waters for the incubation of the first brood of the annual cycle (Kergariou, 1971; González-Gurriarán *et al.*, 1993; Le Foll, 1993). Although no data are available for males on this aspect, observations by fishermen indicate that they do not participate in the return migration to shallow waters, remaining in the wintering habitats (Corgos, 2004). This fact could be related to the presence of seminal receptacles in the females that allow them to store sperm from various copulations and use it for fertilisation of successive broods without the need for new mating (González-Gurriarán *et al.*, 1998).

The fertilisation of the brood in the spider crab is internal and occurs simultaneously to the extrusion of the eggs into the female abdominal cavity, remaining later attached to the pleopods until hatching (Diesel, 1991). During the incubation of the brood, the eggs go through three macroscopically distinguishable stages of development (González-Gurriarán *et al.*, 1993): stage A, with orange coloration and great amount of yolk; stage B, with brown coloration and primitive eye; stage C, with dark grey coloration, presence of chromatophores, well-developed eyes and with hardly any yolk (Fig. 1.2).

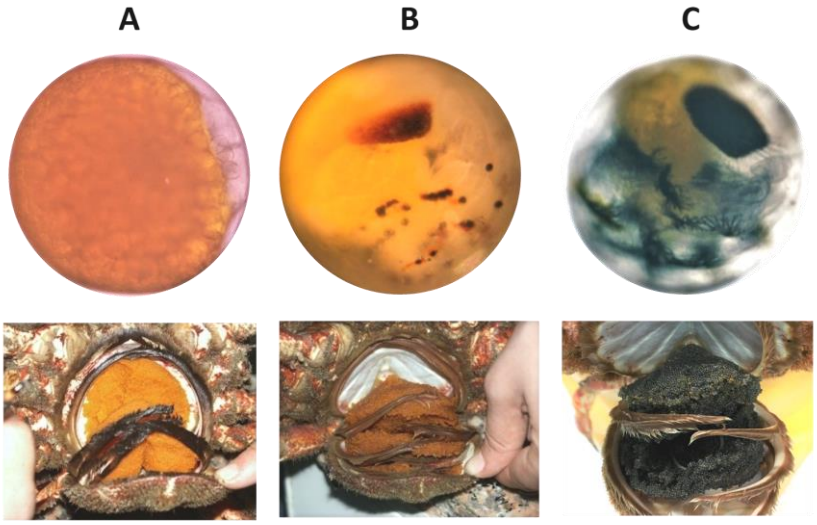


Fig. 1.2. Eggs (top) and broods (bottom) in the developmental stages A, B and C described by González-Gurriarán *et al.* (1993).

The incubation period of the brood ranges from 30 to 74 days depending on water temperature (Kergariou, 1975; Brosnan, 1981; Kergariou, 1984; Rodhouse, 1984; González-Gurriarán *et al.*, 1998; Iglesias *et al.*, 2002). For this reason, the number of broods that a female is capable of laying in each annual cycle varies with latitude: up to three broods in

Galicia (González-Gurriarán *et al.*, 1993, 1998), up to two on the French coast (Kergariou, 1975, 1984; Martin, 1983) and only one in Ireland (Brosnan, 1981; Rodhouse, 1984; Fahy, 2001). In Galicia, the presence of ovigerous females has been reported from December to November of the following year. In this region, the percentage of ovigerous females between March and September are approximately 75% (González-Gurriarán *et al.*, 1993, 1998).

Socioeconomic importance and management of *Maja brachydactyla* stocks

Maja brachydactyla is a species of high commercial interest in many countries, such as United Kingdom, Ireland, France, Spain, Portugal and Morocco (Kergariou, 1984; Le Foll, 1993; González-Gurriarán *et al.*, 1998). Along its distribution area, fishing of this species is carried out using multiple gears, although tangle and gill nets are most common in the south and traps in the north.

In Spain, an important socioeconomic activity is developed on this species, one of the most appreciated seafood in the country, Galicia being the region with the highest production. In the last 10 years, 3,314 t of spider crab were fished off the Galician coast, representing an income of almost 35 € million in the fish market (Xunta de Galicia, 2010-2019. URL: www.pescadegalicia.com/estadisticas). To try to control the disturbances that fishing cause on the size and quality of the stocks, in Galicia the spider crab is managed under a 3-S strategy (Season, Sex and Size Control). Specifically, there is a fishing closure that covers the months of June to November, fishing for ovigerous females is prohibited and there is a minimum landing size of 120 mm of carapace length for both sexes (DOG Resolution Nº133, 2019). However, it has been observed that regulations limiting fishing for one of the sexes can lead to imbalances in the sex ratio with consequent alterations in mating systems and population dynamics (Sato and Goshima, 2006; Fenberg and Roy, 2008; Alborés *et al.*, 2019). For this reason, it would be essential to evaluate the impact of this type of management on *M. brachydactyla* populations.

State-of-the-art of genetic studies in *Maja brachydactyla*

In recent decades, numerous studies have been conducted on the biology and population dynamics of *Maja brachydactyla* at different points along its distribution (Brosnan, 1981; Kergariou, 1984; Le Foll, 1993; González-Gurriarán *et al.*, 1998; Bernárdez *et al.*, 2005;

Corgos *et al.*, 2007; Corgos *et al.*, 2011; Thatje and Robinson, 2011; Verísimo *et al.*, 2011). The eagerness to achieve the breeding in captivity of this species has resulted in many of these studies focusing on its larval and juvenile phases (Iglesias *et al.*, 2002; Sampedro *et al.*, 2003; Andrés *et al.*, 2008; Guerao and Rotllant, 2009; Andrés *et al.*, 2010; Rotllant *et al.,* 2010; Alaminos, 2011; Andrés *et al.*, 2011; Rotllant *et al.*, 2013; Simeó *et al.*, 2013; Castejón *et al.*, 2019). Pazos *et al.* (2018) succeeded to close the complete cycle of the spider crab in captivity. However, the high mortality rate in the larval stages and during the settlement of the juveniles prevents the culture of *M. brachydactyla* from being profitable for the time being, so that all sales come from fishing in the wild. Adult spider crabs are sometimes kept in tanks for later sale, in some occasions even during months, but these specimens also come from natural populations and not from aquaculture.

Despite the large number of studies conducted on the biology of *M. brachydactyla*, genetic research is still scarce in this species. Genetic studies provides valuable information for a sustainable management of stocks, such as details on the structure and genetic diversity of the populations and on the mating system.

Sotelo *et al.* (2008a, 2008b, 2009) provided information about the phylogeography, taxonomy and population genetics of spider crab of great relevance for the management of its fisheries. Molecular analyses of mtDNA supported the distinction suggested by Neumann (1998) based on morphological characters between the Mediterranean spider crab *Maja squinado* (Herbst, 1788) and the NE Atlantic spider crab *M. brachydactyla*. The divergence rate for a fragment of the COI gene was 6.7% between these two taxa, while the divergence within *M. brachydactyla* was two orders of magnitude lower, confirming that these are two different species (Sotelo *et al.*, 2008b).

In another study, Sotelo *et al.* (2007, 2008a) used mitochondrial and nuclear (microsatellite) markers to infer population structure and genetic diversity in several spider crab populations along their distribution range. The results showed high genetic diversity and weak population structure, finding only very slight differences between the northernmost and southernmost populations. For this reason, it was considered that spider crab stocks are interconnected with each other forming a large metapopulation.

In addition, Sotelo *et al.* (2009) established the phylogenetic relationships between *M. brachydactyla* and its congeneric species from NE Atlantic by analysing mtDNA sequences. Mitochondrial markers showed a common origin for European species of the genus *Maja* (*M. goltziana* d'Oliveira, 1889; *M. brachydactyla* Balss, 1922, *M. squinado* (Herbst, 1788) and *M. crispata* Risso, 1827), *M. goltziana* being the basal species. This fact added to the

morphological differences with the other three species led Ng and Richer de Forges (2015) to relocate this species into a new genus under the name *Neomaja goltziana*. This basal species showed a sister lineage bifurcated into an Atlantic clade (*M. brachydactyla*) and a Mediterranean clade (*M. squinado* and *M. crispata*). The separation between *M. brachydactyla* and the Mediterranean clade would have taken place before the Messinian Salinity Crisis. It should be noted that two haplotypes belonging to *M. brachydactyla* have been found on the South African coast. This fact questions the validity of the species *M. cornuta* (Linnaeus, 1758, previously *M. capensis* Ortmann, 1894) that inhabits the coasts of southern Africa. They also suggested that the presence of these haplotypes in this area may be due to a hybridisation between *M. brachydactyla* and *M. cornuta*.

A few years later, Guerao *et al.* (2011) described a simple method to differentiate the four European species of the genus *Maja* (including the current *Neomaja goltziana*). They established a morphometric index that allows to discriminate the adult individuals of these species. Moreover, they developed a protocol based on a PCR-RFLP that also allows for the identification of these species, resulting especially useful in larval and juvenile stages, where morphometric identification is not very effective.

Using this morphometric and molecular method, together with morphological characters (Neumann, 1998), Abelló *et al.* (2014) reported the presence of individuals of *M. brachydactyla* in two localities from the Alboran Sea (western Mediterranean Sea). The RFLP analysis from one individual showed the specific bands of this species, together with a band of 330 bp that could correspond to one of the restriction fragments present in *M. squinado*. The authors attributed this extra band to incomplete digestion, although they did not completely exclude the existence of heteroplasmy or NUMTs as a possible explanation.

Despite efforts in recent years to determine the population structure of *M. brachydactyla* and its relationship with congeneric species, some aspects still remain surrounded by uncertainty. The possible hybridisations between sympatric species and the viability of their progeny remain to be clarified. Furthermore, reproductive aspects of interest to fisheries management, such as the existence of multiple paternity, have not yet been addressed in this species. To solve these questions it is necessary to perform analyses of molecular markers, which requires high-quality DNA as a starting point.

Objectives

The main aim of this thesis is to study in depth genetic aspects of the biology of *Maja brachydactyla* that are relevant for the management of the stocks and sustainable fishing of this species. Special emphasis has been placed on clarifying outstanding questions about the reproduction of spider crab, such as the possibility of multiple paternity or potential hybridisations of this species with congeneric ones.

To reach this global aim, the following specific objectives have been proposed:

- To optimise the process of DNA extraction from spider crab eggs in order to obtain high-quality DNA for molecular studies. To select the most effective extraction method and to determine the most suitable developmental stage for sampling and the best method for preservation.

- To find a non-invasive tissue for the extraction of quality DNA from adult spider crabs, which can be applied to other crustacean species, especially those that are endangered or cannot be slaughtered for other reasons.

- To analyse the paternity of broods of spider crabs from wild using microsatellite markers. Some of the reproductive characteristics of *M. brachydactyla*, such as the presence of seminal receptacles and the absence of post-copulatory guarding, could favour multiple paternity.

- To compare the prevalence of multiple paternity between two spider crab populations with different exploitation levels and management measures.

- To determine the effect of the maternal size on the paternity of the broods of *M. brachydactyla*.

- To analyse the mitochondrial variability and detect the possible existence of heteroplasmy in *M. brachydactyla*.

2

Optimisation of DNA extraction in the spiny spider crab *Maja brachydactyla*

Introduction

The spiny spider crab *Maja brachydactyla* Balss, 1922, is a decapod crustacean which occurs in the northeastern Atlantic, from southern North Sea to Senegal, including the Azores, Madeira, the Canary Islands and Cape Verde (Hines *et al.*, 1995; Neumann, 1998; Udekem d´Acoz, 1999; Sotelo *et al.*, 2008b). The life cycle of *M. brachydactyla* consists in a larval phase, with two zoeal and one megalopal stages, (Iglesias *et al.*, 2002; Guerao *et al.*, 2008), a juvenile phase (Le Foll, 1993) and an adult or reproductive phase (Sampedro *et al.*, 1999; Corgos *et al.*, 2006). The fertilisation is internal and occurs during the extrusion of the brood to the female abdominal cavity, where the incubation occurs. During this process, the eggs go through three macroscopically distinguishable stages (González-Gurriarán *et al.*, 1993, 1998): stage A (orange eggs with much yolk), stage B (brown eggs, visible eyes) and stage C (eggs with dark coloration, many chromatophores, well-developed eyes and hardly yolk).

Although many aspects of the spiny spider crab biology have already been studied, many others are unknown yet. Methodological studies focused on improving DNA extraction and sample storage (Moorad *et al.*, 1997; Bitencourt *et al.*, 2007; Steele *et al.*, 2009; Palero *et al.*, 2010; Moreira *et al.*, 2013) are an important previous step for multiple molecular studies, such as paternity (Urbani *et al.*, 1998; Mckeown and Shaw, 2008; Yue *et al.*, 2010; Bailie *et al.*, 2011; Pardo *et al.*, 2016; Hill *et al.*, 2017), population and phylogenetic studies (Schubart *et al.*, 2000; Fratini and Vannini, 2002; Puebla *et al.*, 2008; Sotelo *et al.*, 2008a; Guerao *et al.*, 2011).

In the present work, the DNA extraction process from eggs and adult tissues of *M. brachydactyla* was optimised. For this purpose, the most efficient sample conservation method as well as the best developmental stage for DNA extraction from eggs were determined. In addition, the best adult tissue to obtain a large amount of high-quality DNA without sacrificing or causing serious damages to the specimens was searched.

Materials and methods

Sample collection and DNA extraction

First of all, several DNA extraction methodologies were tested in eggs of ten females from the Golfo Ártabro and the Ría de Arousa (Galicia, NW Spain), both fresh and preserved in ethanol. These methods were applied to individual eggs and included incubation in 10% bleach, heat shock, mechanical methods (cutting, puncture and homogenisation), Chelex 100 resin (Bio-Rad), hypotonic shock and the NucleoSpin Tissue kit (Macherey-Nagel).

Eggs from another six *M. brachydactyla* females were used to determine the most suitable developmental stage and sampled conservation method for the posterior DNA extraction. These specimens were captured in the Ría de Arousa (Galicia, NW Spain) and their eggs were conserved for 15 years in three different ways: freezing, lyophilisation and storage in absolute ethanol. Two ovigerous females for each developmental stage were selected and, from each brood, two replicas of frozen eggs, two of lyophilised eggs and two of eggs preserved in ethanol were analysed.

Samples of nine females captured in the Golfo Ártabro (Galicia, NW Spain) were also collected. From each of these females, pleopod setae, pereiopod setae and one endite of the second pair of maxillipeds were sampled. These tissues were conserved in absolute ethanol and were used to determine the most suitable starting sample for the DNA extraction from adults of *M. brachydactyla*.

These samples were processed using the commercial kit. The DNA was extracted according to the manufacturer's protocol for animal tissues, both for eggs and adults, with an incubation in proteinase K overnight. Nevertheless, the beginning of the extraction process varied depending on the tissue.

From each brood, a pool of 20 eggs were selected and the lisis buffer and the proteinase K were added before sample homogenisation.

One endite was extracted from the second pair of maxillipeds and was weighed. Next, it was cut into small pieces and homogenised together with the lisis buffer and the proteinase K.

Pleopod setae were cut from the external branch of these structures and 10 mg were placed into a microtube in small pieces with the lisis buffer and the proteinase K.

Pereiopods were brushed before sampling in order to minimising the contamination. Next, their surface was scraped with a scalpel and 20 mg of setae were placed into a microtube together with the lisis buffer and the proteinase K.

DNA quantification and statistical analysis

The DNA concentration was quantified using the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). After that, two ANOVA were conducted using the DNA concentration data: the first one to determine the best egg developmental stage and the best sample conservation method for the DNA extraction; the second one to identify the adult tissue that provides more DNA. All the statistical analysis was performed using the software R 3.0.2 (R Core Team, 2017).

DNA quality testing

DNA quality was checked by electrophoresis in 1% agarose gel. Furthermore, was amplified using the primers 16L29-F (5´-YGCCTGTTTATCAAAAACA-3´) and 16HLeu-R (5´-CATATTATCTGCCAAAATAG-3´) (Schubart, 2009). The PCR was performed with the NZYTaq 2x Green Master Mix kit, separate $MgCl_2$ (Real Laboratory) in a final volume of 25 µL. Each reaction contained approximately 25 ng of DNA, 12.5 µL of Green Master Mix, 4mM of $MgCl_2$ and a concentration of 0.5 µM for each primer. The PCR consisted in an initial denaturalisation of 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 53.1 °C and 45 s at 72 °C, and a final extension of 5 min at 72 °C. The amplification success was tested by electrophoresis in 1% agarose gels.

The PCR products of the nine females captured in the Golfo Ártabro were sequenced after being purified with ExoSAP-IT (USB). Reactions were conducted in a capillary DNA sequencer (3130xl Genetic Analysis System, Applied Biosystems). The obtained sequences were checked using the tool BLAST of NCBI (National Centre for Biotechnology Information).

Results

The rate of visualisation by electrophoresis of DNA obtained using mechanical methods, thermal shock and hypotonic shock was low or null. Within the mechanical methods, homogenisation was the most effective. The treatment of eggs with 10% bleach was not successful in any case. This is probably due to bleach is too aggressive and damaged the DNA. Although in other studies Chelex 100 resin has been effective in extracting DNA from eggs of marine species (Estoup *et al.* 1996; McKeown and Shaw, 2008), no DNA was visible in the agarose gels for the sample processed with this method. The commercial kit unquestionably showed the highest success rate (100%), providing large amounts of high-quality DNA.

Once established the commercial kit as the best DNA extraction method, it was applied to the eggs stored by 15 years and to the adult tissues. Table 2.1 shows the summary of the descriptive statistics analysis of the DNA concentration for these samples.

Table 2.1. Descriptive analysis of the DNA concentration for the samples processed with the NucleoSpin Tissue kit.

	Min.	Q1	Median	Mean	Q3	Max.	SD
Endite	73.85	229.80	365.00	590.80	602.80	2,359.82	701.04
Pleopod setae	165.40	252.90	477.50	446.80	607.40	726.50	205.24
Pereiopod setae	23.75	48.15	69.15	86.76	156.40	168.40	57.64
Overall (adult tissues)	23.75	107.77	250.70	374.81	502.55	2,359.82	460.30
Stage A	1.65	2.77	20.84	76.19	45.65	366.60	135.02
Stage B	64.36	133.10	170.70	211.10	324.00	371.90	113.74
Stage C	27.38	50.23	70.37	136.30	256.60	372.80	122.38
Lyophilised	1.83	92.41	170.70	184.10	278.80	366.60	130.48
Preserved in ethanol	1.65	35.75	101.80	153.50	307.00	372.80	150.96
Frozen	2.82	39.66	54.15	85.95	65.66	323.40	103.90
Overall (eggs)	1.65	39.14	75.01	141.21	267.98	372.77	132.80

Min.: minimum, Q1: fist quartile, Q3: third quartile, Max.: maximum, SD: standard deviation. Units for adult tissues: ng DNA/g tissue. Units for eggs: ng DNA/µL (20 eggs in 100 µL of elution buffer).

The mean value of DNA concentration ($\overline{x}$) from eggs was 141.21 ng DNA/µL and the standard deviation (SD), 132.80 ng DNA/µL. Nevertheless, the DNA obtained from eggs varied between the three developmental stages. The mean values of DNA concentration for the developmental stages A, B and C were 76.19, 211.10 and 136.30 ng DNA/µL, respectively, and the dispersion values were high and similar for the three developmental stages (SD = 135.02, 113.74 and 122.38, respectively).

The mean DNA concentration provided by the lyophilised eggs was 184.10 ng DNA/µL, by the eggs stored in ethanol 153.50 ng DNA/µL and by the frozen eggs 85.95 ng DNA/µL. The values of standard deviation were high for the three conservation methods (SD = 130.48, 150.96 and 103.90 ng DNA/µL, respectively), as for the developmental stages.

Regarding the adult tissues, the mean amount of DNA was 374.81 µg DNA/g tissue and the standard deviation 460.30 DNA/g tissue. The mean concentration of DNA extracted from pereiopod setae ($\overline{x}_{pereiopod} = 86.57$ µg DNA/g tissue) was low in comparison with that of the other tissues ($\overline{x}_{endite} = 590.80$, $\overline{x}_{pleopod} = 446.80$ µg DNA/g tissue). Although both endites and pleopod setae provided high amounts of DNA, the values for endite sample was very variable (73.85-2359.82 µg DNA/g tissue), while the DNA extracted from pleopod setae varied in a narrower range (165.40-726.50 µg DNA/g tissue). However, the wide range for the endites is considerably reduced by removing the highest value (73.85-811.46 µg DNA/g tissue).

Regarding these data and the box plots for DNA concentration (Fig. 2.1), it seems that eggs in stage B are the best choice for DNA extraction. However, differences between the three conservation methods and the three adult tissues are more difficult to establish. For this reason, a comparison of the means of each group were performed in order to identify the statistically significant differences.

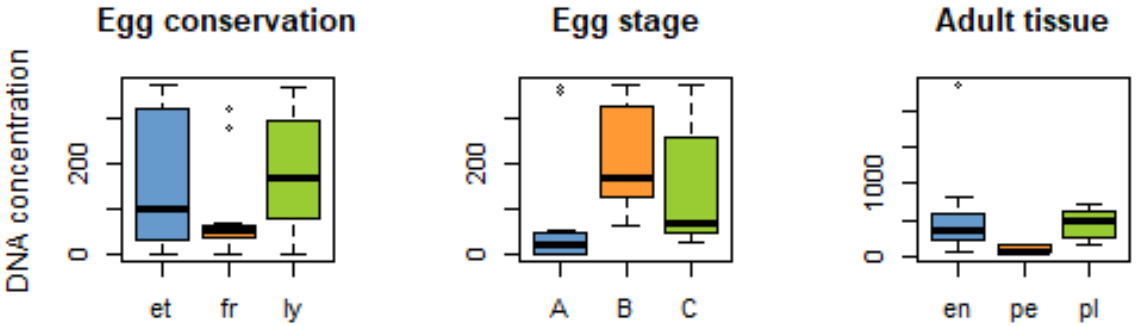


Fig. 2.1. Box plots of DNA concentration for developmental stage, conservation method and type of adult tissue. A: stage A, B: stage B, C: stage C; et: eggs stored in ethanol, fr: frozen eggs, ly: lyophilised eggs; en: endite; pe: pereiopod setae, pl: pleopod setae. Units for adult tissues: ng DNA/g tissue. Units for eggs: ng DNA/µL (20 eggs in 100 µL of elution buffer).

Significant differences between the amount of DNA extracted from eggs in different developmental stages (p-value = 3.9×10^{-9}) and conserved by different methods (p-value = 0.031) were detected. In addition to this, differences between the amount of DNA obtained from different adult tissues were found (p-value = 4.53×10^{-5}).

Once detected significant differences for DNA concentration, Tukey tests were conducted to pairwise comparisons. Next, the 95% confidence intervals were represented (Fig. 2.2). The Tukey plot for developmental stage (Fig. 2.2a) showed that the differences between the three stages are statistically significant. Moreover, the developmental stage that provides the highest quantity of DNA is the stage B.

The plot for conservation method (Fig. 2.2b) showed that there are only statistically significant differences between the amounts of DNA obtained from lyophilised eggs and from frozen eggs, being lower in the second ones. Finally, the plot for adult tissue (Fig. 2.2c) showed that pereiopod setae provide an amount of DNA significantly lower than the two other tissues.

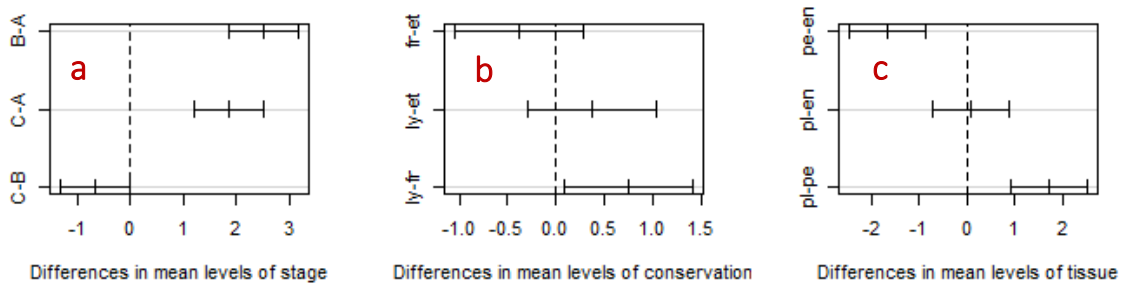


Fig. 2.2. Confidence intervals at 95% for the differences in mean levels of DNA concentration for developmental stage (a), conservation method (b) and type of adult tissue (c). The intervals which do not cross the discontinued line of 0 indicate statistically significant differences. When the interval covers positive values, the first sample type of the pair provides in mean more DNA than the second one. A: stage A, B: stage B, C: stage C; et: storage in ethanol, en: endite, fr: frozen, ly: lyophilised, pe: pereiopod setae, pl: pleopod setae.

Regarding the quality of the DNA, the amplification of 16S gene was successful and sequences of about 600 bp of 16S gene of *M. brachydactyla* were obtained, both from eggs and adult tissues.

Discussion

Optimisation of DNA extraction from eggs

Extraction methods using silica column kits are the most effective of all the tested methods for obtaining DNA from spider crab eggs. In addition, these kits have the advantage of providing high-purity DNA that can be quantified by spectrophotometry and used for amplification of target genes.

The DNA concentration per egg increases as the number of cells of the embryo rises (Verísimo, 2015; Espeland *et al.*, 2017), so eggs in stage C would be expected to contain more DNA. However, B was the developmental stage which provided greater amounts. This may be because of the presence of substances in the stage C that interfere in the extraction process, or hard structures that limit the access to the DNA or that obstruct the membrane of the columns of the extraction kit.

In terms of preservation, *a priori*, freezing would seem the most suitable method for the posterior DNA extraction. Nevertheless, a higher quantity of DNA from lyophilised eggs and eggs stored in ethanol was obtained. A possible explanation is that part of the DNA has been lost during egg handling. The frozen eggs are even more delicate than the fresh ones, while

lyophilisation and conservation in ethanol induce tissue hardening, which facilitates their handling. For these reasons, and since the Tukey test does not detected significant differences between lyophilisation and storage in ethanol, the latter one is the most recommendable conservation method, as it is simpler and does not require specialised equipment.

Minimisation of the damage caused in adults by sampling

The most widely used tissue for DNA extraction in crustaceans is the muscle (Sainte-Marie *et al.*, 1999; Streiff *et al.*, 2004; McKeown and Shaw, 2008; Palero *et al.*, 2010; Baggio *et al.*, 2011), although other tissues have been used such as mouthparts (Urbani *et al.*, 1998), pleopods (Yue *et al.*, 2010; Wang *et al.*, 2011) or internal tissues, like gills and hepatopancreas (Bitencourt *et al.*, 2007). However, one of the main purposes of this study is to provide alternative tissues for sampling without causing death or serious damages to the specimens.

DNA extraction without death enables to continue researching with the same specimens or even their liberation after sampling. Moreover, DNA extraction from structures that do not involve mutilation (e.g. setae) reduces the probability of posterior death or infections caused by sampling, and prevents animal suffering. The stimulation of autotomy of appendages has been proposed as a sampling method that reduces animal suffering (Toonen, 2004). Nevertheless, the extraction or loss of useful structures decreases the fitness of the specimens.

Setae could be an interesting alternative tissue for DNA extraction. These structures provides enough DNA to amplify and sequence the 16S rDNA in *M. brachydactyla*. Although endites and pleopod setae provide a higher amount of DNA than pereiopod setae, these tissues have some drawbacks. The extraction of an endite from an alive specimen can be a complicated task and can leave sequelae. On the other hand, males only have two pairs of pleopods transformed into copulatory appendages, so DNA extraction from pleopod setae is only possible in females. In the case of females, the best tissue for DNA extraction without slaughter or mutilation is indisputably the pleopod setae. In order to minimise the sampling impact, setae of the pleopods must be collected from the external branches, since internal branches play an important role offering support to the brood.

The setae which cover the carapace and the pereiopods of many decapods have mainly a sensorial function (Derby, 1982; Laverack, 1988; Derby, 1989). They also play an important role in the camouflage (Hartnoll, 1993; Wicksten, 1993; Woods, 1995; Fürböck and Patzner, 2005), serving as anchorage point to algae, sponges, tubeworms and other organisms that

help the animal to go unnoticed (Fernández *et al.*, 1998). Nevertheless, camouflage lose importance in adult individuals and the sampling required for DNA extraction would involve such a small area that it would hardly affect the life of the animal.

To summarise, setae are a good choice to extract DNA avoiding death or fitness reduction of the crabs. The use of these structures enables the sampling and posterior return of the specimens to the marine environment. This is especially relevant for sampling in endangered species of crustaceans, since it allows to obtain genetic information without affecting natural populations. Furthermore, an adequate choice of the egg developmental stage, of the adult tissue and of the sample conservation method, allows to obtain high-quality DNA from adult and embryonic tissues. This DNA can be used in molecular studies, especially in those that requires DNA from progenitors and their offspring, such as paternity analyses. Molecular research based on DNA analysis provides valuable information for sustainable management and conservation of crustacean resources.

3

Differences in prevalence of multiple paternity between two populations of *Maja brachydactyla* with different exploitation levels and management measures

Introduction

For an adequate management of commercial species, a detailed understanding of their mating systems and biological traits, such as demographic distributions and the intensity of sexual selection, is essential (Emlen and Oring, 1977). In marine species, the selective mortality caused by fishing produces disturbances in these mating systems (Hankin, 1997; Gosselin *et al.*, 2005; Sato *et al.*, 2010; Robertson and Butler, 2013; Rains *et al.*, 2016; Pardo *et al.*, 2017). When management measures are aimed at protecting particular sizes or a specific sex, these disturbances are accentuated and a decreasing in the reproductive potential and an alteration of the structure of the population may occur. This is the case of several crustacean fisheries regulated by minimum landing sizes and prohibitions on fishing for ovigerous females. In these fisheries, the largest males, with a high commercial value, are the main target (Orensanz *et al.*, 1998), resulting in female-biased sex ratios and changes in the relative male/female size (Kennelly, 1992; Pillans *et al.*, 2005; MacDiarmid and Sainte-Marie, 2006; Fenberg and Roy, 2008; Pardo *et al.*, 2015; Alborés *et al.*, 2019). In female-biased populations, males have greater mating opportunities. In such situations, sperm reserves may not recover between copulations (Kendall and Wolcott, 1999; Sato *et al.*, 2005; Sainte-Marie, 2007; Pardo *et al.*, 2015) or males may invest small amounts of sperm across a number of mates (Rondeau and Sainte-Marie, 2001; Pardo *et al.*, 2015). In addition, the removal of the largest males will force many females to copulate with smaller males, that are unable to fill their seminal receptacles and whose sperm plugs may be too small to be effective in preventing further inseminations. This situation causes sperm limitation and promote promiscuity of females and the consequent multiple paternity of their clutches (Kendall *et al.*, 2002; Gosselin *et al.*, 2003; Hines *et al.*, 2003; Gosselin *et al.*, 2005; Hill *et al.*, 2017). Furthermore, it has been suggested that overexploited selectively populations use multiple paternity as a compensation mechanism to maintain genetic diversity (Morán and García-Vázquez, 1998; Jennions and Petrie, 2000) and effective population size (Sugg and Chesser, 1994; Martínez *et al.*, 2000; Pearse and Anderson, 2009), as well as to reduce inbreeding (Stockley *et al.*, 1993; Yasui, 1998). Moreover, it has been reported that in populations where there is selective extraction of males, the remaining ones invest less time in mate guarding and they are smaller than the female they protect (Wilber, 1989; Rondeau and Sainte-Marie, 2001; Pardo *et al.*, 2016), increasing the probability of polyandry and the risk of injury or death of females (Sainte-Marie and Hazel, 1992; Sainte-Marie *et al.*, 2008).

Maja brachydactyla (Balss, 1922) is a brachyuran crab of family Majidae (De Grave *et al.*, 2009; Ng and Richer de Forges, 2015) that occurs in the northeastern Atlantic, from the

south of the North Sea to Senegal, including Madeira, the Azores, the Canary Islands and Cape Verde (Monod, 1966; Kergariou, 1984; Neumann, 1998; Udekem d'Acoz, 1999; Sotelo *et al.*, 2008b, McLay and Becker, 2015). Females of eubrachyuran crabs have a pair of seminal receptacles that allow them to store sperm from different copulations and use it for fertilisation of successive broods without the need for new matings (Hartnoll, 1968; Diesel, 1991; González-Gurriarán *et al.*, 1998; McLay and López Greco, 2011). The number of broods per annual cycle and the breeding season of *M. brachydactyla* varies with latitude due to differences in water temperature. Females lay up to three broods in Galicia (NW Spain) (González-Gurriarán *et al.*, 1998), up to two in France (Kergariou, 1984) and one in Ireland (Rodhouse, 1984; Fahy, 2001).

M. brachydactyla is a species of great commercial value fished in several countries, such as Spain, United Kingdom, Ireland, France, Portugal and Morocco (Kergariou, 1984; Le Foll, 1993; González-Gurriarán *et al.*, 1998). The present study is focused on two populations of spiny spider crabs: one in the Golfo Ártabro (43°21'49" N 8°21'54" W, Galicia, Spain) and another one in Carna (53°19'13" N 9°50'36" W, Co. Galway, Ireland). These two regions differs in several characteristics, such as the fishing pressure (Table 3.1) and the management measures for *M. brachydactyla*. Galicia is the principal fishing region of this species in Spain, with around 330 t landed annually (Xunta de Galicia, 2010-2019. URL: www.pescadegalicia.com/estadisticas). In the Ría de Arousa (Galicia), it has been suggested that more than 90% of newly mature specimens are caught each year (Freire *et al.*, 2002). In Galicia, there is a fishing closure from June to November, coinciding with the period of terminal moult and reproductive migration (González-Gurriarán *et al.*, 1993, 1998; Bernárdez *et al.* 2005; Corgos *et al.*, 2006), and the capture of ovigerous females is not permitted during the whole annual cycle. In addition to this, there is a minimum legal fishing size of 120 mm of carapace length (CL) for both sexes (DOG Nº133, 2019). In contrast, in Ireland, the only regions with an established spider crab fishery to date are Brandon Bay, Tralee Bay and some regions of the south east coast (Tully *et al.*, 2006; Fahy and Carroll, 2009; Tully, 2017). In Carna, the spiny spider crab are only caught in small amounts as by-catch in fisheries targeting *Homarus gammarus* and *Cancer pagurus*. Some targeted fishing for large spider crabs occurs periodically but populations are not heavily exploited and the species has expanded its range in Ireland in recent decades. On the Irish coast, the only restriction on spider crab fishing is a minimum landing size of 130 mm of CL for males and 125 mm for females (Kelly *et al.*, 2003; Fahy and Carrol, 2009).

Table 3.1. Landings of *Maja brachydactyla* (in tonnes) for Galicia and County Galway from 2004 to 2016 (Source: Marine Institute Ireland; Xunta de Galicia, 2010-2019).

Year	Landings in Galicia	Landings in Co. Galway
2004	214.90	16.27
2005	352.52	11.60
2006	382.31	13.23
2007	244.33	9.86
2008	290.43	63.80
2009	342.60	70.64
2010	259.59	51.15
2011	276.41	13.00
2012	334.01	2.22
2013	213.88	0.62
2014	241.00	46.09
2015	291.96	2.03
2016	272.40	6.95

The aim of this work is to analyse the paternity of the broods of the spider crab *M. brachydactyla* in two populations with different fishing intensity and management strategies, and to evaluate the effect of maternal size on genetic polyandry.

Materials and methods

Study specimens

For paternity analysis, 23 ovigerous females with different carapace lengths were captured. Thirteen of these were caught by divers in the Golfo Ártabro and 10 were caught by local fishermen in Carna. The 13 Spanish ovigerous females were captured in January-February, when they were carrying the first brood of the annual cycle.

In addition to this, another 118 adult specimens were collected for microsatellite characterisation. Sixty of these spider crabs were bought from local fishermen in Galicia, 44 females from the Ría de Arousa (42°35'00" N 8°53'02" W) and 4 females and 12 males from the Golfo Ártabro. The remaining 58 spider crabs, 29 females and 29 males, were imported from Rosslare (52°15'26" N 6°20'28" W, Co. Wexford, Ireland) by Airmar SL (Spain).

Sample collection

The females from Arousa and Rosslare were dissected and gonads were sampled. All these specimens were anesthetised at -20 °C for 10 min before being slaughtered. From the remaining adult individuals, including the ovigerous females, autotomy of a pereiopod was stimulated by performing a puncture in one of its joints in order to collect a muscle sample. Ovigerous females were kept in captivity until their first brood reached the developmental stage B described by González-Gurriarán *et al.* (1993). The egg samples were collected at this developmental stage, the most suitable for DNA extraction according to Rodríguez-Pena *et al.* (2017). For brood sampling, the eight egg packages adhered to the pleopods were numbered from one to eight, and eggs were collected in a zigzag pattern from pleopods 1, 4, 5 and 8 (Fig. 3.1). This sampling method allowed us to assess if there was an orderly distribution of the eggs fertilised by different males in the abdominal cavity of the female. If a pattern existed, sampling eggs from different areas of the brood would be more appropriate for detection of multiple paternity than completely random sampling.

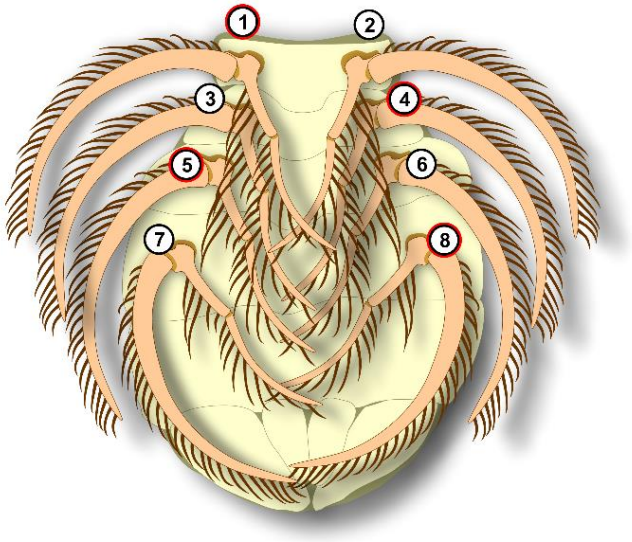


Fig. 3.1. Abdomen of a spider crab female with numbered pleopods for sample collection in zigzag. The egg samples for paternity analysis were collected from pleopods 1, 4, 5 and 8.

The egg samples of the females from the Golfo Ártabro were preserved in absolute ethanol until the time of DNA extraction. On the other hand, the samples collected in Carna (Ireland) were frozen and lyophilised to facilitate their transport.

DNA extraction

DNA was extracted from the muscle or gonad of the 141 adult individuals, including the 23 ovigerous females. In addition to this, egg pools and individual eggs were processed. For six of the females from the Golfo Ártabro, DNA from pools of five eggs were extracted for each sample collected in zigzag, having a total of four pools per female (20 eggs per female). Moreover, the DNA from 8-12 individual eggs were extracted for all the ovigerous females.

DNA extraction was conducted using the NZY Tissue gDNA Isolation kit (NZYTech, Portugal), following the protocol indicated by the manufacturer for DNA extraction from animal tissues. After adding the lysis buffer and proteinase K, the samples were homogenised and incubated at 55 °C overnight.

Microsatellite development and analysis

Sotelo *et al.* (2007) described specific microsatellites for *M. brachydactyla*. These microsatellite markers were tested but there were problems in amplifying them correctly. In order to develop new specific markers, a microsatellite-enriched genomic library from a gonad sample was constructed at AllGenetics & Biology SL (A Coruña, Spain). The library was prepared using Nextera XT DNA Library Preparation kit (Illumina), following the manufacturer's instructions, and was enriched with the microsatellite motifs AC, AG, ACG, and ATCT. Once enriched, the library was sequenced in the Illumina MiSeq PE300 platform (Macrogen Inc., Seoul, Korea). A total of 4,035,302 reads were obtained and processed using Geneious 11.1.2 (Kearse *et al.*, 2012) and in-house developed scripts. For primer design, sequences containing microsatellite loci were selected. Primer pairs were designed using Primer3 (Untergasser *et al.*, 2012) for PCR amplification of 500 microsatellite loci, with primers hybridising at the flanking regions of tandem repeats.

After preliminary studies, nine microsatellite loci (GeneBank accession numbers: MT211631 - MT211639) were selected for their amplification in 73 individuals from the Galician sampling location and 68 from the Irish location. PCRs were performed in a final reaction volume of 12.5 µL, containing 1 µL of DNA (10 ng/µL), 6.25 µL of the Type-it Microsatellite PCR Kit (Qiagen), 4 µL of PCR-grade water and 1.25 µL of the primer mix. The optimal PCR protocol consisted in an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 57 °C for 90 s, 72 °C for 30 s; 8 cycles of 95 °C for 30 s, 53 °C for 90 s, 72 °C for 30 s; and a final extension step at 68 °C for 30 min. All PCR batches included a negative control to check for potential cross-contamination. Oligonucleotide tails were attached to the 5' ends of the primers to allow for fluorescent labelling. The oligonucleotide tails used were the universal sequences M13 (5´-GGAAACAGCTATGACCAT-

3´) and CAG (5´-CAGTCGGGCGTCATC-3´). The two oligonucleotides were labelled with the HEX dye and the FAM dye, respectively. The software Geneious 11.1.2 was used for the analysis of fluorescent profiles and to identify the allele peaks. For each locus (except AG_Mbr_215) and study area, number of alleles (N_a), observed (H_o) and expected (H_e) heterozigosity, deviations from Hardy-Weinberg equilibrium (HWE) and inbreeding coefficient (FIS) were estimated using GenAlEx 6.5 (Peakall and Smouse, 2006). Null allele frequency was estimated using FreeNA (Chapuis and Estoup, 2007).

Considerations for the paternity analysis

Firstly, the PrDM software (Neff and Pitcher, 2002) was used to determine the probability of detecting multiple mating in a brood. This program is based on a Monte Carlo simulation, so the probability of detect multipaternity may vary slightly in each run. To minimise this effect, ten iterations were performed for each case and the average was computed. Simulations were performed varying the number of loci, the prevalence of multiple paternity, the percentage of contribution of the different sires and the number of eggs analysed. In addition to this, the software GERUD 2.0 (Jones, 2005) was used to calculate the exclusion probabilities for different loci combinations. The method described by Veliz *et al.* (2017) for species with high fecundity was used to evaluate the statistical power at population level. In this case, the following parameters were modified: the number of females analysed, the number of eggs analysed per female, the prevalence of multiple paternity and the contribution of male 1 (the one that fertilises most of the brood) and male 2 (other males that participate in the fertilisation of the brood). This method takes into account the fecundity of the study species. Verísimo *et al.* (2011) determined that the fecundity of female spider crabs ranged from 125,081-530,309 eggs, with an average of 300,192 ± 92,912 eggs. For simulations used here, the approximate value of 300,000 eggs was used.

Paternity analysis

Three loci (AG_Mbr_050, AG_Mbr_317, AG_Mbr_438) were selected for the paternity analysis. In a first phase, the egg pools of the six females were analysed to determine if multiple paternity existed in *M. brachydactyla*. In a second phase, the individual eggs of the 23 broods were genotyped. The 23 corresponding mothers were also genotyped from muscle samples. The PCR protocol was the same as that described for the amplification of the nine microsatellite loci in the subsection "Microsatellite development and analysis".

Genotypes were established using Geneious 11.1.2. The number of broods with multiple paternity, the number of sires of each brood and the most likely paternal genotypes were

determined using GERUD 2.0 and the allele frequencies of each study area. Taking into consideration the most probably parents, the egg genotypes were checked to determine how the alleles of the different sires were distributed throughout the brood.

Statistical analysis

Possible differences in the prevalence of multiple paternity between the two study populations and the effect of maternal size were studied using a multiple logistic regression analysis conducted with the statistical program R 3.6.3 (R Core Team, 2020).

Results

Microsatellite characterisation

Preliminary microsatellite analysis showed that the number of alleles varied between 2 and 15, being quite similar in both regions (Table 3.2). Moreover, the alleles deviating significantly from the Hardy-Weinberg equilibrium were the same in both areas, except for AG_Mbr_317 which was only significant in NW Spain. The low FIS values indicates that there is no inbreeding in either population.

Paternity analysis

For the nine loci, the exclusion probability was calculated by indicating that the genotype of one progenitor was known (Table 3.3). Three loci with a high individual exclusion probability were selected and their combined exclusion probability was compared with the combined exclusion probability of the nine loci. The exclusion probability for the three selected loci (AG_Mbr_317, AG_Mbr_050 and AG_Mbr_438) was > 0.9 for the NW Spain and the SW Ireland (0.9427 and 0.9166, respectively). Although by including the nine loci the exclusion probability increases slightly, this does not compensate for the large increase in costs that would result from tripling the number of loci analysed. For this reason, only three loci were amplified for the paternity analysis.

Table 3.2. Characterisation of the nine selected microsatellite loci for *Maja brachydactyla*.

Locus name	Accesion no.	Primer sequence 5′-3′	Dye	Repeat motif	Allele size (bp)	NW Spain							SW Ireland						
						N	N_a	H_o	H_e	PHWE	FIS	Null	N	N_a	H_o	H_e	PHWE	FIS	Null
AG_Mbr_050	MT211631	CTTCCCTTCAGATAGCCCGC GCTTGTTAACAACCCGCCTG	HEX	AC	218-230	64	6	0.516	0.591	0.852	0.128	0.248	66	5	0.485	0.516	0.013	0.060	0.116
AG_Mbr_177	MT211632	CGGAGCGTGACTACCTATCC GACTGAAGAGGCCGCTTAGG	6-FAM	AC	109-119	65	3	0.877	0.505	0.000*	-0.736	0.130	68	2	0.838	0.497	0.000*	-0.686	0.000
AG_Mbr_204	MT211633	TCCCTCCACTACCCTTCCTT CAAACACCACGACAACCACC	HEX	ATC	129-183	48	2	0.979	0.500	0.000*	-0.959	0.347	50	2	1,000	0.500	0.000*	-1,000	0.265
AG_Mbr_235	MT211634	ATCGAGCTGCGGGTTAAGAG GGTACCACGGGACCACTGTA	HEX	AC	107-121	53	4	0.415	0.553	0.000*	0.249	0.440	57	5	0.456	0.610	0.000*	0.252	0.322
AG_Mbr_317	MT211635	ATGCACACTTCTTCCTCCGC CCTGCCGATATCAGCTGACT	HEX	GT	97-125	67	15	0.716	0.857	0.000*	0.164	0.179	67	11	0.761	0.818	0.321	0.069	0.070
AG_Mbr_325	MT211636	ACACTGTTGGATTCCTGCCT CTCTGCCTACACAGGAGGGT	6-FAM	TG	94-102	43	4	0.209	0.584	0.000*	0.642	0.617	36	4	0.139	0.610	0.000*	0.772	0.679
AG_Mbr_415	MT211637	AACACTCCCATAACGCCACT CGGCCGAACAGTGAACAGTT	6-FAM	GT	102-116	52	4	0.981	0.519	0.000*	-0.891	0.292	54	4	0.889	0.515	0.000*	-0.725	0.230
AG_Mbr_438	MT211638	GCGTATGCTCCCAACTTTACC TCGCTTGCTCTTTGCCTACT	HEX	TTG	259-292	59	11	0.864	0.846	0.216	-0.021	0.236	62	12	0.871	0.827	0.015	-0.053	0.131
AG_Mbr_215	MT211639	GTGTAGCTCAGGCCCTGTAA AAAGTCCTCCTTGACGCTGG	HEX	CA	220-244	9	7	-	-	-	-	-	9	6	-	-	-	-	-

Accession no.: GeneBank accession number; N: sample size; N_a: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; PHWE: Hardy-Weinberg equilibrium p-values; FIS: inbreeding coefficient; Null: null allele frequency. *Significant departure from HWE after the sequential Bonferroni correction ($P < 0.00625$). H_o, H_e, PHWE, FIS and null allele frequency were not estimated for AG_Mbr_215 due to the small sample size (N = 9).

Table 3.3. Exclusion probability (EP) when the genotype of one progenitor is known. EPs were calculated for each individual locus, for a combination of three loci (AG_Mbr_317, AG_Mbr_050, AG_Mbr_438) and for the combination of the nine loci, for both study areas.

Locus	EP NW Spain	EP SW Ireland
AG_Mbr_415	0.2121	0.2105
AG_Mbr_204	0.1874	0.1875
AG_Mbr_317	0.7094	0.6519
AG_Mbr_050	0.3500	0.2931
AG_Mbr_177	0.1968	0.1868
AG_Mbr_235	0.2565	0.3327
AG_Mbr_325	0.3237	0.3245
AG_Mbr_438	0.6967	0.6612
AG_Mbr_215	0.4454	0.5067
AG_Mbr_317 AG_Mbr_050 AG_Mbr_438	0.9427	0.9166
All loci	0.9918	0.9903

In the initial phase of egg pool analysis, the existence of multiple paternity in *M. brachydactyla* was confirmed. More than three non-maternal alleles were detected in four of the six broods examined. For the individual eggs, the results obtained with GERUD 2.0 indicated that at least 10 of the 23 broods analysed had been fertilised by more than one male (Table 3.4). In the broods with multiple paternity, the minimum number of sires was always two, except in one brood where alleles of three males were detected. The contribution of each male to the fertilisation of the broods were calculated over the total number of eggs that could be unequivocally assigned to one father. In the case of broods fertilised by two males, the contribution of the main male ranged from 50-87.5% (Fig. 3.2). For the brood with three sires, GERUD 2.0 offered four solutions with the same likelihood, three with equal contribution of the males (33.33% each one) and one with a contribution of 40% for the males 1 and 2 and a contribution of 20% for the male 3. However, these percentages are an approximation since only a small proportion of the whole brood was analysed. Once the eggs from different pleopods were assigned to each father, it was found that, in at least six of the ten broods, the eggs fertilised by different males were partially or totally mixed in the female abdomen. In the remaining four broods, the existence of a patron of the paternal alleles could not been discarded.

Table 3.4. Paternity results for the analysis of individual eggs from 23 broods.

							Male 1		Male 2		Male 3		
Population	**Fem. code**	**Fem. CL (mm)**	**N**	**No. un. eggs**	**MP**	**Min. sires**	**No. eggs**	**Pl.**	**No. eggs**	**Pl.**	**No. eggs**	**Pl.**	**Egg pattern**
Golfo Ártabro	Sp1	170.89	10	10	Yes	2	6	1, 4, 5, 8	4	1, 4, 5	-	-	No
Golfo Ártabro	Sp2	129.86	12	12	No	1	12	-	-	-	-	-	-
Golfo Ártabro	Sp3	171.09	11	8	Yes	2	7	1, 4, 5, 8	1	8	-	-	Possible
Golfo Ártabro	Sp4	122.33	10	10	Yes	2	8	1, 4, 5, 8	2	8	-	-	Possible
Golfo Ártabro	Sp5	179.34	12	9	Yes	2	5	1, 4, 8	4	1, 4, 8	-	-	No
Golfo Ártabro	Sp6	124.37	12	12	No	1	12	-	-	-	-	-	-
Golfo Ártabro	Sp7	153.89	6	A: 5; B, C, D: 6	Yes	3	A, B, C, D: 2	A: 1, 8; B: 1; C: 1; D: 1	A, B, C, D: 2	A: 1, 5; B: 4, 5; C: 4; D: 4, 8	A: 1; B, C, D: 2	A: 4; B: 4, 8; C: 5, 8; D: 4, 5	A,B,D: No; C: Possible
Golfo Ártabro	Sp8	153.09	6	6	No	1	6	-	-	-	-	-	-
Golfo Ártabro	Sp9	146.11	8	8	No	1	8	-	-	-	-	-	-
Golfo Ártabro	Sp10	131.66	8	8	Yes	2	6	1, 4, 5, 8	2	1, 5	-	-	No
Golfo Ártabro	Sp11	134.24	8	8	Yes	2	7	1, 4, 5, 8	1	5	-	-	No
Golfo Ártabro	Sp12	152.95	6	6	Yes	2	4	1, 5, 8	2	4, 8	-	-	No
Golfo Ártabro	Sp13	179.76	8	8	Yes	2	4	1, 4, 5	4	4, 5, 8	-	-	No
Carna	Ir1	132.60	7	7	No	1	7	-	-	-	-	-	-
Carna	Ir2	147.80	8	8	No	1	8	-	-	-	-	-	-
Carna	Ir3	145.66	8	6	Yes	2	4	4, 5, 8	2	1, 4	-	-	Possible
Carna	Ir4	153.10	8	8	No	1	8	-	-	-	-	-	-
Carna	Ir5	149.36	8	8	No	1	8	-	-	-	-	-	-
Carna	Ir6	121.92	8	8	No	1	8	-	-	-	-	-	-
Carna	Ir7	145.04	8	8	No	1	8	-	-	-	-	-	-
Carna	Ir8	127.05	8	8	No	1	8	-	-	-	-	-	-
Carna	Ir9	133.37	8	8	No	1	8	-	-	-	-	-	-
Carna	Ir10	125.87	7	7	No	1	7	-	-	-	-	-	-

Fem. code: female code; Fem. CL: female carapace length in mm; N: number of analysed eggs per female; No. un. eggs: number of unambiguous eggs (eggs that could be unequivocally assigned to one father); MP: detection of multiple paternity; Min. sires: minimum number of sires; No. eggs: number of eggs assigned to a particular male; Pl.: pleopods in which eggs assigned to a particular male were detected; Egg pattern: organised distribution of the eggs fertilised by different males throughout the brood. This table shows the solutions with the highest likelihood provided by GERUD 2.0. For female Sp7 there are four solutions with the same likelihood (A, B, C and D).

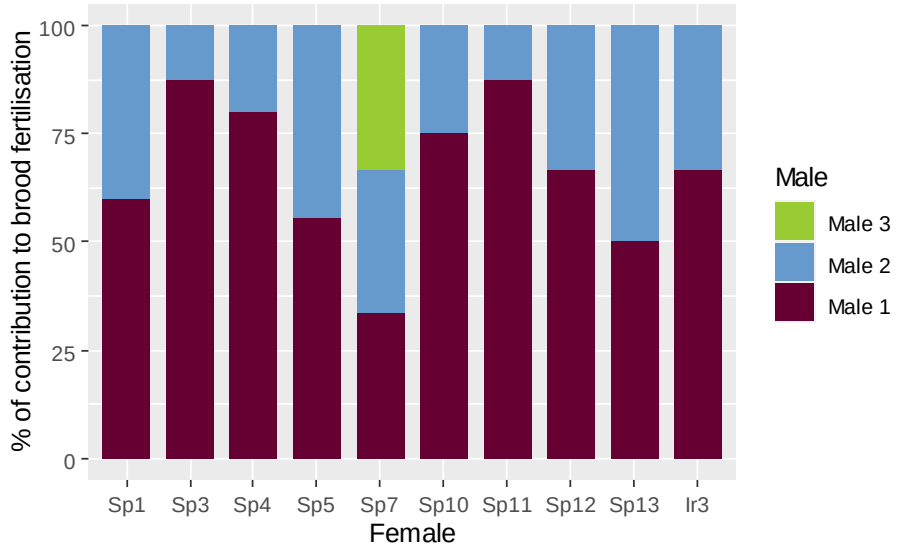


Fig. 3.2. Percentage of contribution of each sire in the broods with multiple paternity. For the female Sp7, GERUD 2.0 offers another solution with the same likelihood, with contributions to brood fertilisation of 40:40:20 (see Table 3.4).

The calculation of the probability of detecting multiple mating in a brood made with the PrDM software indicated that this probability was considerably higher when analysing three loci (AG_Mbr_438, AG_Mbr_317, AG_Mbr_050) than when analysing two (AG_Mbr_317, AG_Mbr_050), but the increment was moderate using five loci (AG_Mbr_325, AG_Mbr_438, AG_Mbr_317, AG_Mbr_050, AG_Mbr_215) instead of three. Table 3.5 shows only cases in which two males were considered, since in similar conditions, as the number of sires increase, the probability of detecting multiple mating also increases. For equal contributions from two males, three loci are sufficient to obtain a PrDM around 0.9 analysing 8 eggs and higher than 0.95 analysing 12 eggs. Nevertheless, for a skewed contribution, the probability of detecting multiple mating decreases considerably.

To estimate the statistical power at the population level, the method described by Veliz *et al.* (2017) was used, which takes into account the fecundity of the species and the number of females analysed. For the simulations it was assumed that 50% of the broods in the population were fertilised by more than one male and that the male contribution was biased (95:5), taking into account data reported in other crustacean species (Veliz *et al.*, 2017). For 10 females, the statistical power was 0.8416 analysing 8 eggs and 0.9266 analysing 12. By increasing the number of females to 20, the statistical power also increased, being 0.9749 for 8 eggs and 0.9946 for 12.

Table 3.5. Probability of detecting multiple mating (PrDM) in a brood. Simulations were performed for 8 and 12 eggs, using two, three and five loci, and for equal (50:50) and skewed (10:90) contribution of two males. PrDM were calculated for both study populations.

No. eggs	No. loci	Loci names	Male contribution	PrDM (NW Spain)	PrDM (SW Ireland)
8	2	AG_Mbr_317, 050	50:50	0.7936	0.7173
	2	AG_Mbr_317, 050	10:90	0.3928	0.3448
	3	AG_Mbr_438, 317, 050	50:50	0.9339	0.8998
	3	AG_Mbr_438, 317, 050	10:90	0.4953	0.4665
	5	AG_Mbr_325, 438, 317, 050, 215	50:50	0.9633	0.9518
	5	AG_Mbr_325, 438, 317, 050, 215	10:90	0.5253	0.5115
12	2	AG_Mbr_317, 050	50:50	0.8678	0.8008
	2	AG_Mbr_317, 050	10:90	0.5232	0.4659
	3	AG_Mbr_438, 317, 050	50:50	0.9730	0.9509
	3	AG_Mbr_438, 317, 050	10:90	0.6451	0.6129
	5	AG_Mbr_325, 438, 317, 050, 215	50:50	0.9884	0.9827
	5	AG_Mbr_325, 438, 317, 050, 215	10:90	0.6740	0.6612

Influence of maternal size and population on the prevalence of multiple paternity

Nine of the ten broods fertilised by several males came from the Golfo Ártabro (NW Spain) while only one came from Carna (W Ireland). To determine if this apparent difference between populations was significant and to check if multiple paternity depends on maternal size, a multiple logistic regression model was proposed. The results showed that the broods from the two study populations differed significantly from each other in terms of paternity of their broods (p-value = 0.031). On the other hand, the maternal size was not significantly correlated with the existence of multiple paternity (p-value = 0.146), thereby a Fisher's exact test was performed removing the maternal size from the statistical model. This test confirmed that the probability of multiple paternity is significantly higher in the Golfo Ártabro than in Carna (Table 3.6). The probability that a brood randomly taken in the Golfo Ártabro is fertilised by more than one male is 0.69, while in Carna it is 0.1. Therefore, it is seven times more likely to find a brood with multiple paternity in the Spanish population than in the Irish one.

Table 3.6. Number and percentages of broods with single and multiple paternity in both study populations.

	Multiple paternity		Single paternity		Fisher's exact test p-value
	N	%	N	%	
Carna (Ireland)	1	10	9	90	0.0065*
Golfo Ártabro (Spain)	9	69.2	4	30.8	
Total	10	43.5	13	56.5	

N: number of broods

Moreover, a simple logistic regression model was used to check if the size of the females was correlated with their population of origin. The results correlation between these variables was not significant (p-value = 0.127).

Discussion

In decapods, broods fertilised by more than one male are the norm (Dennenmoser and Thiel, 2015; Mclay and Dennenmoser, 2020). Table 3.7 summarises the results of several paternity studies conducted in recent years. In general, the prevalence of multiple paternity is higher in species without specialised sperm storage structures, such as carideans and anomurans (in 7 of the 9 species, the prevalence was higher than 73%). However, as for the only one analysed species from Achaelata, this showed a low proportion of broods fertilised by several males despite lacking sperm storage organs. Within astacids, the species with annulus ventralis also showed high levels of prevalence (87% on average), while in species with thelycum, the percentage of broods fertilised by more than one male was considerably lower (28% on average). Regarding the analysed brachyurans, which had ventral type seminal receptacles, multiple paternity was not reported in 22% of the species, and in those that was detected, its prevalence was 37% on average. Paternity studies in species with dorsal seminal receptacles are scarce. One example is the study by Hill *et al*. (2017), in which they analysed the paternity of one *Callinectes sapidus* clutch. They detected multipaternity, but genotyping of the ejaculates suggested that the true population prevalence is far from 100%. For not having more precise data, *C. sapidus* was excluded from Table 3.7.

Furthermore, male bias in brood fertilisation is very evident in taxa with internal fertilisation, while it is not so obvious in groups without organs specialised in sperm storage. The increase in the complexity of these organs is usually associated with an increase in fecundity, since sperm storage allows for a greater number of eggs to be fertilised. This implies that, in paternity studies on species with more developed sperm storage organs, the proportion of offspring analysed are usually lower.

Table 3.7. Paternity studies in decapod crustaceans with different strategies of sperm storage.

Infraorder	Species	Reference	Fecundity (no. eggs per brood)	Seminal receptacle	No. females	No. loci	Multiple paternity (prevalence)	Minimun no. of sires	Contribution of single male* (%)
Caridea	*Acanthephyra pelagica*	(Paegelow, 2014)	560-3,700	No	19	4	100	2-4	ND
	Alpheus angulosus	(Mathews, 2007)	> 200	No	53	5	31	1-2	ND
	Caridina ensifera	(Yue and Chang, 2010)	16	No	20	4	100	2-11	54
	Palaemonetes pugio	(Baragona *et al.*, 2000)	95-265	No	10	2	80	ND	ND
	Rhynchocinetes typus	(Bailie *et al.*, 2014)	1,700	No	15	3	73.3	1-4	65
Anomura	*Munida rugosa*	(Bailie *et al.*, 2011)	32,000	No	25	3	84	1-3	68
	Munida sarsi	(Bailie *et al.*, 2011)	2,000	No	5	2	100	2-4	50
	Paralithodes camtschaticus	(Vulstek *et al.*, 2013)	7,900-450,000	No	24	3	0	1	100
	Petrolisthes cinctipes	(Toonen, 2004)	1,300	No	10	2	80	1-3	ND
Achaelata	*Palinurus cygnus*	(Loo *et al.*, 2018)	300,000-700,000	No	24	7	8.33	1-3	ND
Astacidea	*Orconectes obscurus*	(Kahrl *et al.*, 2014)	250	Yes (annulus ventralis)	3	4	100	2-3	72
	Orconectes placidus	(Walker *et al.*, 2002)	250	Yes (annulus ventralis)	15	3	60	1-4	83
	Orconectes sanbornii	(Kahrl *et al.*, 2014)	250	Yes (annulus ventralis)	5	4	100	2-3	68
	Procambarus clarkii	(Yue *et al.*, 2010)	276	Yes (annulus ventralis)	30	4	96.7	1-4	74
	Homarus americanus	(Gosselin *et al.*, 2005)	4,800-38,300	Yes (open thelycum)	108	4	13	1-3	70
	Homarus gammarus	(Sørdalen, 2012)	2,000-40,000	Yes (open thelycum)	73	4	17.8	1-2	ND
	Nephrops norvegicus	(Streiff *et al.*, 2004)	2,600	Yes (open thelycum)	11	2	54.5	1-3	51
Brachyura	*Cancer pagurus*	(McKeown *et al.*, 2008)	26,600-2,836,000	Yes (ventral type)	18	3	0	1	100
	Chionecetes opilio	(Sainte-Marie *et al.*, 2008)	10,000	Yes (ventral type)	20	1	12.5	1-2	79
	Chionoecetes opilio	(Urbani *et al.*, 1998)	10,000	Yes (ventral type)	7	2	0	1	100
	Chionoecetes opilio	(Roy, 2003)	10,000	Yes (ventral type)	79	ND	3.8	ND	ND
	Dissodactylus primitivus	(Josart *et al.*, 2014)	200	Yes (ventral type)	18	4	61.1	1-6	86
	Maja brachydactyla	**This study**	**300,000**	**Yes (ventral type)**	**23**	**3**	**43.5**	**1-3**	**66[b]**
	Metacarcinus edwardsii	(Pardo *et al.*, 2016)	> 1,000,000	Yes (ventral type)	31	6	0	1	100
	Metacarcinus edwardsii	(Rojas-Hernández *et al.*, 2014)	> 1,000,000	Yes (ventral type)	5	8	0	1	100
	Metacarcinus magister	(Jensen and Bentzen, 2012)	1,000,000-2,000,000	Yes (ventral type)	10	3	40	1-3	98[a]
	Scopimera globosa	(Koga *et al.*, 1993)	4,300	Yes (ventral type)	ND	ND	ND	ND	94
	Uca mjoebergi	(Reaney *et al.*, 2012)	> 3,200	Yes (ventral type)	38	3	56	1-2	98
	Ucides cordatus	(Baggio *et al.*, 2011)	108,000	Yes (ventral type)	10	6	40	1-2	ND

*For species with multiple paternity, the broods with single paternity were removed from the calculation of contribution of single male, while for species with single paternity, the 100% of contribution of the male was indicated.

[a]A brood in which the contribution of single male was 64% was removed from the calculation.

[b]The brood fertilised by three males was removed from the calculation.

ND: no data

It should be highlighted that in the spider crab broods, the contribution of single male was relatively low (66% even excluding the brood with three sires) compared to that observed in other brachyurans (> 79%), which could be explained by the arrangement of the sperm masses and the potential sperm mixing in the seminal receptacles. González-Gurriarán *et al.* (1998) discovered that in *Maja brachydactyla*, when there are multiple sperm masses, they are arranged parallel to the major axis of the receptacles, and when these are very full, the masses are not organised. Moreover, own observations made during dissections of females mated in captivity with various males showed that, when copulations occur in short periods of time (from hours to weeks between copulations), it is not possible to distinguish defined sperm masses in the seminal receptacles of the female. Furthermore, in *M. brachydactyla* copulation occurs between hard-shelled individuals, there is no post-copulatory guarding, and the existence of sperm plugs has not been reported (González-Gurriarán *et al.*, 1998). In addition to this, it is a species with terminal moult (Kergariou, 1984; Le Foll, 1993; Sampedro *et al.*, 1999), so sperm stored in the seminal receptacles is not susceptible to be lost during moulting. Moreover, Corgos *et al.* (2006) suggested that small males migrate to mating areas earlier than large ones, probably to maximise their mating opportunities by avoiding agonistic interactions with larger males (Sainte-Marie *et al.*, 1997; 1999; Rondeau and Sainte-Marie, 2001; Correa *et al.*, 2003). These small males provide females with small amounts of sperm that are insufficient to fertilise all their eggs. This set of reproductive traits of *M. brachydactyla* favours polyandry and multiple paternity of broods.

In some of the female spider crabs where both pools and individual eggs were analysed, the results obtained differed. In one of these females, two sires were detected in the pools but only one in the individual eggs. This may be due to the larger sample size (20 *vs.* 12 eggs) or due to randomness if the contribution of both males to the fertilisation of the brood is skewed. In two another females, multiple paternity was not detected using the pool method but was found analysing individual eggs. This could be because of one male with a low contribution to brood fertilisation will show a low peak in the electropherogram which may go unnoticed during genotyping of the eggs of the pools.

The zigzag sampling method did not serve to increase the probability of detecting multiple paternity. Although in four of the ten multiple sired broods an organised patron of the parental alleles could not be excluded, in six of them there was an evident mixing. This lack of organisation of the paternal alleles had already been reported in some decapod species with less complex sperm storage organs, such as in *Orconectes placidus* (Walker *et al.*, 2002) or *Homarus americanus* (Gosselin *et al.*, 2005). This could be due to the mixing of different sperms into the seminal receptacles, the mixing of the eggs fertilised by different males in

the abdomen of the female prior to their adhesion to the pleopods or, more likely, to a combination of both.

Own observations in specimens in captivity confirmed that, during oviposition, the eggs are free in the female abdomen where they mix with each other before adhering to the pleopods. On the other hand, in species where the oviduct is connected dorsally to the sperm storage organs, as in *Callinectes sapidus* (Johnson, 1980), oocytes must pass through the receptacles to reach the vagina. In this case, it would be expected that the sperm of the first male which copulated have priority in fertilising the oocytes, although the entry of the oocytes into the receptacle could also favour multiple paternity (Mclay and Becker, 2015). In contrast, in brachyurans such as *Chionoecetes opilio* (Sainte-Marie *et al.*, 2000) and *Inachus phalangium* (Diesel, 1990), which have ventral type seminal receptacles, the oviduct is connected to the sperm storage organs at a point near the vagina, and the masses are stored dorsoventrally when the receptacles are not very full. These characteristics promote that the sperm from the last male to copulate, closest to the oviduct, is the first to be used for fertilisation of the brood. Although *M. brachydactyla* also has ventral type seminal receptacles, the spatial arrangement of the sperm masses described in this species by González-Gurriarán *et al.* (1998) suggests that there is a mixing of the sperm of several males, which would explain the absence of a pattern of the parental alleles along the brood. Koga *et al.* (1993) proposed that, if there is sperm mixing in the seminal receptacles, the male contribution should be equal (50:50), but this would only be true if two males supplied the female with equal quantities of sperm with similar spermatozoa concentration and viability. However, large males tend to provide greater amounts of ejaculate to females (Sato and Goshima, 2007; Butler *et al.*, 2011), not only sperm but also seminal liquid which promotes mixing (Pardo *et al.*, 2018). For this reason, when the sires of one female differ in size, it would be expected that the lager male dominate the fertilisation of the brood.

In the case of Galician spider crabs, first broods of the annual cycle were analysed. This ensured that females carried high amounts of sperm in their receptacles, maximising the probability of taking samples from a fully fertilised brood. During the breeding season, female spider crabs incubate their broods in shallow waters, while males remain in the mating areas at a depth of 30-100m (Le Foll, 1993; Hines *et al.*, 1995; Freire and González-Gurriarán, 1998; González-Gurriarán *et al.*, 2002). Although direct observations by fishermen in the Golfo Ártabro indicate that sometimes mating occurs in shallow areas after the return migration, the mating of the spider crab on the Galician coast occurs mainly in deep waters in winter (Corgos, 2004). Furthermore, although González-Gurriarán *et al*.

(2008) documented copulations by egg-bearing females, our observations in captivity mating experiments did not record any copulation by ovigerous females.

In general, both within a brood and at the population level, the simulations performed indicated that the probability of detecting multiple paternity was very high for the number of loci, eggs and females analysed in this study. However, the number of analysed females may be insufficient to establish a relationship between the existence of multipaternity and the maternal size. It would be expected that larger females would be able to store the sperm from more copulations in their receptacles. In addition, as they present a higher fertility (Verísimo *et al.* 2011), larger females would need to use a greater quantity of the stored sperm, being more likely to find eggs fertilised by different males in their broods. For this reason, we attribute the absence of a relationship between multiple paternity and maternal size to an insufficient sample size. Therefore, the analysis of a larger number of females, covering the entire size range of both populations, is necessary to clarify this aspect.

The importance of analysing an adequate number of broods has been highlighted in studies conducted in the same species but whose results differs (see Table 3.7). Urbani *et al.* (1998) analysed seven broods of *C. opilio* and found single paternity in all of them. In contrast, Roy (2003) and Sainte-Marie *et al.* (2008) analysed the offspring of 79 and 20 females of this species, detecting the existence of multiple paternity in 3.8 and 12.5% of them, respectively. In the case of *Metacarcinus edwardsii*, the two paternity studies performed (Rojas-Hernández *et al.*, 2014; Pardo *et al.*, 2016) agreed that all the broods analysed (5 and 31) were fertilised by a single male. However, in this case, Pardo *et al.* (2016) determined that, with the loci and number of eggs analysed in each of the study locations, the PrDM was > 0.99. Therefore, they concluded that in the five *M. edwardsii* populations analysed there was really no multiple paternity, its prevalence was negligible or the contribution of the additional sires was too low to be detected, independently of their fishing intensity.

Although a prevalence of multiple paternity similar to that in other brachyurans has been recorded in M. brachydactyla (43.5%), it is remarkable the differences found between the population of the Golfo Ártabro and that of Carna. This fact indicates that the paternity of the broods is also influenced by the socio-sexual context, and not only by the reproductive biology of the species. Below, we discuss a series of hypotheses that aim to explain the differences found in the paternity of these two populations:

a) Differences in population density. Carna is located at the upper limit of distribution of the spiny spider crab. This fact and the absence of a permanent fishery of this resource in the area (Tully, 2017) suggests that the spider crab population there

may be less dense than in the Golfo Ártabro. The low density of specimens would limit the availability of sexual partners (Jensen *et al.*, 2006) and, therefore, the level of polygamy. However, several studies have provided data that suggest the presence of a dense population of spider crabs on the west coast of Ireland, questioning the validity of this hypothesis (Bates, 1981; Rodhouse, 1984; Kelly *et al.*, 2003).

b) Differences in female fecundity. As already mentioned, in Galicia females can lay up to three broods in a breeding cycle while in Ireland they only lay one. Furthermore, fecundity per brood also decreases as latitude increases (Kergariou, 1971, 1984; Verísimo *et al.*, 2011). Since the annual fecundity of Galician females is higher than that of Irish ones, their demand for sperm should also be higher. For this reason, it would be expected that Spanish females will require a greater number of copulations to fill their seminal receptacles and to complete the fertilisation of their three broods, while for Irish females one copulation per year may be enough. Although females could carry sperm from previous years in their seminal receptacles, it has demonstrated that sperm stored for more than ten months shows high levels of genetic damage and has low viability (Rodríguez-Pena *et al.*, unpublished data), so multipaternity is improbable with only one annual copulation. To the best of our knowledge, there is no previous works suggesting that females with high fecundity actively search for more copulations. Nevertheless, the maturation of the gonads that follows the first hatching of the annual cycle, a process influenced by water temperature, could lead to females mating again.

c) Different levels of sex- and size-selective exploitation. Differences in the level of polyandry between areas with different fishing intensity have been reported previously in other decapods (Gosselin *et al.*, 2005). Galicia is the region of Spain where exploitation of *M. brachydactyla* is most intense, while in Carna there is no stable fishery focused on spider crab.

   Moreover, in Galicia, *M. brachydactyla* is managed under a 3-S strategy (Season, Sex and Size Control), while in Ireland the only measure in effect is size control. Management measures that prohibit fishing for ovigerous females result in exploitation focused on males, especially those of larger size with high commercial value (Fahy and Carroll, 2009). In addition, the individuals of extreme sizes are the scarcest in the population, so they are the most susceptible to disappear due to overexploitation. Several studies have provided data on the alteration of the sex-ratio and population size structure caused by size- and sex-selective fishing of spider crab (Brosnan, 1981; Rodhouse, 1984; Fox, 1985; Fahy, 2001; Kelly *et al.*, 2003; Corgos *et al.*, 2006; Fahy and Carroll, 2009). Within populations lightly

exploited, males far outnumber and outsized females. However, in areas with intense and selective fishing, the proportion of males and females tends to be similar, as does the average size of both sexes. This alteration of the population structure forces females to copulate with smaller males, leading to sperm limitation (Hines *et al.*, 2003; Sato *et al.*, 2007, 2010; Pardo *et al.*, 2015). It is then that multiple mating emerges as a solution to ensure the fertilisation of the broods.

On the other hand, when females mate with multiple small males, even assuming that they obtain sufficient amount of sperm, the trend over generations will be downwards in the average size of the population (Fenberg and Roy, 2008), provided that the size at terminal moult has a high genetic heritability. This will lead to a reduction of the reproductive potential at population level (Sato *et al.* 2007; Butler *et al.*, 2011; Verísimo *et al.*, 2011). Moreover, our observations under laboratory conditions confirm that small males have difficulties in copulating with females that far outsized them. Therefore, the removal of large males from the population could cause the number of partners compatible with large females to fall.

Taking these considerations into account, the presence of several sires in most of the broods of the Golfo Ártabro could be an indicator that the Galician population is being self-regulating in response to a selective overexploitation.

In the present study, multiple paternity was reported for the first time in *M. brachydactyla*, with a prevalence of 43.5% and a moderate bias between sires, unlike in other brachyurans. However, the frequency of multiple paternity was almost seven times higher in the intensely exploited Golfo Ártabro than in Carna, where exploitation levels are low. This difference is probably due to the intense and biased fishing towards large males in the Galician coast, which leads to polyandry to prevent sperm limitation caused by copulations with small males. To test this hypothesis it would be informative to analyse the paternity in broods from the Magharees region (Ireland). This population is characterised by intense exploitation focused on large males (Rodhouse, 1984; Fahy and Carroll, 2009) and by an annual cycle of one brood. A low prevalence of multiple paternity, similar to that found in Carna, would confirm that the annual fecundity of females is a factor that can influence the level of polygamy in the population. However, a high frequency of multipaternity would indicate that polyandry emerged to prevent sperm limitation caused by sex- and size-fishing.

Although at the moment polyandry seems to be effective, if intense exploitation continues in Galicia, multiple mating could be an insufficient mechanism to overcome sperm limitation. Faced with a situation of sperm limitation, the present management measures in

Galicia for spider crab could be counterproductive if they are not combined with the protection of sperm stocks and of the largest specimens. A possible measure to solve this would be the establishment of a maximum landing size, as it has been proposed or it is in effect for other decapods of commercial interest (NOAA, 2016; Gnanalingan and Butler, 2018). This regulation could be also applied to the Magharees fishery, where the removal of large males has also become apparent in recent years (Rodhouse, 1984; Fox, 1985; Fahy, 2001; Fahy and Carroll, 2009). In Galicia, another possibility would be the revocation of the prohibition on fishing for ovigerous females also suggested by Pardo *et al.* (2015) for *Metacarcinus edwardsii*, provided that it were combined with other measures such as the reduction of the maximum fishing quota. In some crab species under non-selective fishing pressure, it has been suggested a low risk of sperm limitation in females (Gardner and William, 2002; Xuan *et al.*, 2014).

4

High incidence of heteroplasmy in the mtDNA of a natural population of the spider crab *Maja brachydactyla*

Introduction

Mitochondria are mostly inherited by maternal via, that is, only mitochondria from eggs are retained in the embryos. Uniparental transmission of cytoplasmic genomes has had multiple and independent origins and it must involve a strong evolutionary advantage over biparental transmission (Burt and Trivers, 2006; Zouros, 2013). However, this general assumption of uniparentally transmitted, homoplasmic and non-recombining mitochondrial genomes is becoming more and more controversial (Rokas *et al.*, 2003; Tsaousis *et al.*, 2004; Barr *et al.*, 2005). Until now, the presence of different sequences of mtDNA within a cell or individual was a rare phenomenon in animals, but new detection methods based on NGS (Next-Generation Sequencing) or qPCR (quantitative Polymerase Chain Reaction) are allowing for their detection (Machado *et al.*, 2015; Burgstaller *et al.*, 2018; Mitrofanov *et al.*, 2018; Santibanez-Koref *et al.*, 2019). Nowadays, heteroplasmy has been reported in many organisms such as insects (Sherengul *et al.*, 2006; Nunes *et al.*, 2013), crustaceans (Doublet *et al.*, 2008; Williams *et al.*, 2017; Koolkarnkhai *et al.*, 2019), molluscs (Fisher and Skibinski, 1990), fishes (Gold and Richardson, 1990; Magoulas and Zouros, 1993) frogs (Radojičic *et al.*, 2015) birds (Crochet and Desmarais, 2000; Kvist *et al.*, 2003), mice (Gyllensten *et al.*, 1991) and humans (Schwartz and Vissing, 2002; Payne *et al.*, 2013), being the paternal leakage the primary cause of it. The existence of heteroplasmy in the studied organisms can provide erroneous results in researches that use mitochondrial markers. This factor must be taken into consideration before interpreting the obtained results. For example, the assumption of maternal mitochondrial inheritance in species with multiple mtDNAs per individual can result in an estimation of the effective population size larger than it really is as well as create false negatives in lineage determination.

There may be five main reasons for the presence of heteroplasmy (Breton and Stewart, 2015):

a) Occurrence of a *de novo* mutation in somatic line or germ line cells. In the first case, heteroplasmy is not transmitted to the offspring, while in the second case, it is. Studies in the crustacean *Daphnia pulex* estimate the frequency of *de novo* mutations at 1.63×10^{-7} / site / generation (Xu *et al.*, 2012).
b) Recombination events. However, despite the high number of mitochondrial molecules that exist in an individual, the possibility of being able to detect this type of heteroplasmy is limited, since its frequency is usually very low. In humans, recombination intermediates were detected in the mtDNA, varying their abundance between tissues (high in heart, intermediate in skeletal muscle or placenta, but low or absent in cultured cells) (Kajander *et al.*, 2001).

c) Paternal leakage as it has been reported in fruit fly (Nunes *et al.*, 2013) and hen (Alexander *et al.*, 2015). There are several mechanisms to prevent the transmission of paternal mtDNA to offspring in animals: (I) degradation of paternal mtDNA before or after fertilisation, (II) blocking paternal mitochondria from entering the oocyte, (III) elimination of paternal mitochondria by autophagy and/or ubiquitin-proteasome systems and (IV) uneven distribution of paternal mitochondria with remaining paternal mtDNA during embryogenesis (Sato and Sato, 2017). In the nematode *Caenorhabditis elegans*, paternal mitochondria and their mtDNA degenerate almost immediately after fertilisation and are selectively degraded by 'allophagy' (allogeneic [non-self] organelle autophagy) (Sato and Sato, 2012). In the fruit fly *Drosophila melanogaster*, paternal mtDNA is largely eliminated by an endonuclease G-mediated mechanism. Paternal mitochondria are subsequently removed by endocytic and autophagic pathways after fertilisation (Politi *et al.*, 2014). The reason of the existence of mechanisms to prevent the transmission of paternal mtDNA is not well understood yet. The most plausible explanation is that the retaining of two different, but individually fully functional, mtDNAs within a cell can cause mitochondrial dysfunction due to a potentially lethal genome conflict (Hurst and Hamilton, 1992; Sharpley *et al.*, 2012). It has also been argued as an adaptation to anisogamy, which prevents sperm mtDNA, damaged from intense respiration activity, entering the egg (Allen, 1996). Nevertheless, in some cases, a breakdown of mechanisms to recognise and remove paternal mtDNA may occur resulting in paternal leakage.

d) Biparental inheritance. The concepts of parental leakage and biparental inheritance may not be clearly delimited, depending on the definition of the second one. While some authors consider incidental paternal leakage constitutes a form of biparental mtDNA transmission, Breton and Stewart (2015) define "true" biparental transmission as the systematic transfer of mitochondrial genomes from both parents (or two different mating types) to zygotes as part of normal reproductive processes within a species, followed by the persistence of both parental mitochondrial types throughout development. So far, following this narrow definition, it has not been reported any organism that present this type of biparental mtDNA inheritance.

e) Doubly uniparental inheritance (DUI), typically observed in some bivalvian molluscs (Zouros *et al.*, 1992; Breton *et al.*, 2007; Theologidis *et al.*, 2007; White *et al.*, 2008; Zouros, 2013). Many bivalves have a sperm-transmitted mitochondrial genome (M), along with the standard egg-transmitted one (F). During embryonic

development of the mussel *Mytilus*, sperm mitochondria disperse randomly among blastomeres in females, but form an aggregate in the same blastomere in males. Consequently, in adults, somatic tissues of both sexes are dominated by the F mitochondrial genome, sperm contains only the M genome, and eggs contain the F genome (and perhaps traces of M).

In addition, events of duplications of some mitochondrial genes (White *et al.*, 2008) or the presence of Nuclear Mitochondrial DNA segments (NUMTs) (Bensasson *et al.*, 2001; White *et al.*, 2008) may also be interpreted erroneously as heteroplasmy. NUMTs are described as a transposition of mitochondrial DNA into the nuclear genome that can retain close homology to the original mitochondrial genes (Lopez *et al.*, 1994).

The spiny spider crab *Maja brachydactyla* Balss, 1922, is a decapod crustacean of the Majidae family (De Grave *et al.*, 2009; Ng and Richer de Forges, 2015) very common on the European Atlantic coast. This species was initially classified within the species *Maja squinado* (Herbst 1788), but in recent years morphological (Neumann, 1996; Neumann, 1998) and molecular (Sotelo *et al.*, 2008b) differences between the Atlantic and Mediterranean populations have confirmed that it is a different species. After this separation, its distribution is considered to be limited to the northeastern Atlantic, from the North Sea to Senegal, including Madeira, the Azores, the Canary Islands and Cape Verde (Hines *et al.*, 1995; Neumann, 1998; Udekem d´Acoz, 1999; Sotelo *et al.*, 2008b).

This species presents a complex life cycle, with planktonic larval and benthic post-larvae phases, which determines that its distribution and population dynamics are closely related to larval dispersion processes, mediated by physical factors, as well as habitat selection, linked to movements and migrations in post-larval stages (Pardieck *et al.*, 1999; Pascual *et al.*, 2001). The life cycle of *M. brachydactyla* consists of three main phases: larval phase, juvenile or growth phase, and adult or reproductive phase. The planktonic larval phase lasts from two to three weeks and consists of two zoeal and one megalopal stages (Iglesias *et al.*, 2002; Guerao *et al.*, 2008). The juvenile phase lasts from two to three years (Le Foll, 1993), inhabiting shallow bottoms (< 15 m) where they perform limited, non-directional, small-scale movements of less than 10 m per day (Hines *et al.*, 1995; González-Gurriarán *et al.*, 2002; Bernárdez *et al.*, 2003). Their growth occurs through successive moults and, after terminal moult, which takes place in spring and summer, individuals reach sexual maturity and begin the adult phase.

Maja brachydactyla is a species of high commercial interest in several countries, especially in Spain. Galicia (NW Spain), with around 300 vessels devoted to spider crab fishing,

represents the main exploitation region of the country, both in terms of production (73%) and incomes (more than 80%). In 2018, the captures of *M. brachydactyla* reached the 525,934 kg in Galician coast, which represented around 4,640,000 € in sales (Xunta de Galicia, 2010-2019. URL: www.pescadegalicia.com/estadisticas).

During the course of a study with mitochondrial molecular markers in *M. brachydactyla*, double peaks were detected in some of the electropherograms obtained. The studies by Abelló *et al.* (2014) already suggested the possible existence of heteroplasmy in this species. The present work confirms the existence of heteroplasmy in the spiny spider crab and delves into this aspect to determine the magnitude and possible origin of this phenomenon.

Materials and methods

Specimens of *M. brachydactyla* were captured in two locations of the Galician coast (NW Spain): the Golfo Ártabro (43° 25' N 8° 21' W) and the Ría de Arousa (42° 33' N 8° 54' W). Most of the spider crabs were captured by the scuba divers of the Aquarium Finisterrae of A Coruña (Spain). The remaining specimens were directly bought at the fish market. All the specimens were anesthetised at -20 °C for 10 min before being slaughtered. Regarding the Golfo Ártabro, pereiopod muscle from 27 males and pleopod setae from 23 females were collected. Fertilised eggs from 11 of these females were also sampled (2-17 eggs depending on the female). Furthermore, different tissues and organs (pleopod setae, integument, eye, pereiopod muscle, gonad, heart, nervous tissue, hepatopancreas, stomach, gill, intestine, pereiopod setae and mouth-parts) from four of these females from the Golfo Ártabro were collected. In addition to this, gonadal tissue were sampled from 33 females from the Ría de Arousa. All samples were preserved in absolute ethanol until the time of DNA extraction. The sampling of individuals of both sexes and broods of known females was performed in order to determine the origin and the inheritance mechanism of the heteroplasmy in *M. brachydactyla*. The analysis of different tissues of the same individual provides information about the distribution of the mtDNA sequences in early development stages and the extension of the heteroplasmy in the different tissues in adults.

DNA extraction was conducted using the NZY Tissue gDNA Isolation kit (NZYTech, Portugal). All the laboratory material used in this process (scissors, tweezers, pestles) was washed in absolute ethanol between consecutive samples. Two different mtDNA fragments were amplified by PCR: the 16S rDNA and the cytochrome c oxidase subunit I (COI). While COI was analysed in all the samples, 16S was only sequenced for the 11 ovigerous females from the Golfo Ártabro and their eggs. The PCR conditions for the amplification of 16S rDNA

were described in Rodríguez-Pena *et al.* (2017). COI fragment was amplified using the primers COIMaja_F (5´-GAATGGCCGGAACATCTTTA-3´) and COIMaja_R (5´-CCACCAGCTGGATCAAAGAA-3´) and the NZYTaq 2x Green Master Mix separate $MgCl_2$ kit (NZYTech) according to the manufacturer´s protocol in a final concentration of 1.5 mM of $MgCl_2$ and 0.5 μM of each primer. The PCR consisted in a denaturalisation step of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 48.5 °C and 60 s at 72 °C, and a final extension of 5 min at 72 °C. All PCRs yielded single-band patterns, so that the resultant amplicons were directly sequenced in both directions.

Some of the PCR products of 16*S* rDNA were cloned using StrataClone PCR Cloning Kit according to the manufacturer´s protocol in order to determine the number of nucleotide combinations present in heteroplasmic samples. Cells were spread on an LB-ampicillin plate and let it grow overnight at 37 °C. Transformant colonies were selected, and insert size was checked by PCR. Plasmids were purified using the NZYMiniprep kit (NZYTech, Portugal), and they were sequenced using the M13 forward and reverse primers. The PCR consisted in a denaturation step of 5 min at 94 °C, 35 cycles of 60 s at 94 °C, 60 s at 55 °C, 60 s at 72 °C, and a final extension of 5 min at 72 °C.

The sequencing profiles were examined using BioEdit 7.0.9.0 (Hall, 1999). In order to detect the presence of double peaks in the PCR products, reads in both directions were checked. The DNA extraction, amplification and sequencing were repeated to verify these double peaks were not due to errors in the PCRs. The frequency of each nucleotide was estimated as the average of the peaks of the two reads. From the unambiguous sequences, a consensus sequence was established. Next, all the amplicons were aligned using the tool Muscle (Edgar, 2004) of MEGA 6.06 (Tamura *et al.*, 2013). The variable sites, the haplotypes and their frequencies were calculated using the DnaSP 6.10.04 (Rozas *et al.*, 2017) and the mitochondrial genetic code of *Drosophila*.

Results

The sequence alignments of 16S mtDNA allowed to detect the existence of three variable sites: 365, 442 and 580. Two of them are parsimony informative (365 and 580), while 442 is a singleton. Table 4.1 shows the three different haplotypes detected (GenBank accession numbers: MN006155 - MN006157).

Table 4.1. Detected haplotypes for the 16S gene and adult individuals in which they were observed.

Haplotype	N	Individual			
Hap-16S-1	8	MbraAG-M2-pleopod	MbraAG-M3-pleopod	MbraAG-P7-pleopod	MbraAG-P8-pleopod
		MbraAG-3IN-pleopod	MbraAG-4DN-pleopod	MbraAG-5IN-pleopod	MbraAG-G6-pleopod
Hap-16S-2	1	MbraAG-2DN-pleopod			
Hap-16S-3	2	MbraAG-M26-pleopod	MbraAG-P6-pleopod		
Total	11				

N: number of haplotypes. Sequences with double peaks were not included in this analysis.

We identified double peaks in the electropherograms of 16S mtDNA of the following adult females: MbraAG-M26 (at positions 365 and 580), MbraAG-P6 and MbraAG-5IN (at position 365), and MbraAG-P7 (at position 580) (Table 4.2, Fig. 4.1). After repeating DNA extractions, PCRs and sequencing, we detected again these ambiguous sites at the same positions of the same samples.

Regarding the fertilised eggs of these 11 ovigerous females, we detected heteroplasmy in all the eggs of MbraAG-P6, in five out of seven of MbraAG-P7 and in one out of two of MbraAG-5IN (Table 4.2) at least at one of the two positions (365 and/or 580). In addition to this, a double peak was also detected at position 365 of the egg MbraAG-2DN-1, in whose mother no type of polymorphism were previously detected.

Table 4.2. Nucleotidic proportions (%) at positions 365 and 580 of the 16S rDNA fragment for all the samples analysed.

		Position			
		365		580	
Sample	Origin	T	C	T	A
MbraAG-M2-pleopod	female	0	100	100	0
MbraAG-M2-1	egg	0	100	100	0
MbraAG-M2-2	egg	0	100	100	0
MbraAG-M3-pleopod	female	0	100	100	0
MbraAG-M3-1	egg	0	100	100	0
MbraAG-M3-2	egg	0	100	100	0
MbraAG-M26-pleopod	**female**	**78.4**	**21.6**	**19.4**	**80.6**
MbraAG-M26-1	egg	0	100	100	0
MbraAG-M26-2	egg	0	100	100	0
MbraAG-M26-3	egg	0	100	100	0
MbraAG-M26-4	egg	0	100	100	0
MbraAG-M26-5	egg	0	100	100	0
MbraAG-M26-6	egg	0	100	100	0
MbraAG-M26-7	egg	0	100	100	0
MbraAG-M26-8	egg	0	100	100	0
MbraAG-M26-pleopod-C1	clon female	100	0	0	100
MbraAG-M26-pleopod-C2	clon female	0	100	100	0
MbraAG-M26-pleopod-C3	clon female	100	0	100	0
MbraAG-M26-pleopod-C4	clon female	100	0	0	100
MbraAG-M26-pleopod-C5	clon female	100	0	0	100
MbraAG-M26-pleopod-C6	clon female	100	0	0	100
MbraAG-M26-pleopod-C7	clon female	100	0	0	100
MbraAG-M26-pleopod-C8	clon female	100	0	0	100
MbraAG-M26-pleopod-C9	clon female	100	0	0	100
MbraAG-P6-pleopod	**female**	**93.5**	**6.5**	**0**	**100**
MbraAG-P6-mouth-part	female	100	0	0	100
MbraAG-P6-muscle	female	100	0	0	100
MbraAG-P6-setae	**female**	**12.2**	**87.8**	**80.6**	**19.4**
MbraAG-P6-1	egg	87.3	12.7	15.5	84.5
MbraAG-P6-2	egg	65.9	34.1	26.1	73.9
MbraAG-P6-3	egg	85.0	15.0	11.1	88.9
MbraAG-P6-4	egg	68.0	32.0	26.3	73.7
MbraAG-P6-5	egg	68.7	31.3	29.1	70.9
MbraAG-P6-6	egg	74.1	25.9	19.9	80.1
MbraAG-P6-7	egg	91.4	8.6	0	100
MbraAG-P6-8	egg	90.2	9.8	0	100
MbraAG-P6-9	egg	57.6	42.4	83.6	16.4
MbraAG-P6-10	egg	92.9	7.1	0.0	100.0
MbraAG-P6-11	egg	90.6	9.4	0.0	100.0
MbraAG-P6-12	egg	87.2	12.8	9.8	90.2
MbraAG-P6-13	egg	75.8	24.2	21.8	78.2
MbraAG-P6-14	egg	72.9	27.1	23.9	76.1
MbraAG-P6-15	egg	83.7	16.3	12.5	87.5
MbraAG-P6-16	egg	75.1	24.9	22.2	77.8

Proportions were calculated by dividing the height of each peak in the electropherogram by the summation of the height of both peaks. The eggs and the clones correspond to the female that precedes them in the list. All samples tagged as "pleopod" correspond to pleopod setae from adult females. The samples MbraAG-P6-pleopod, MbraAG-P6-mouth-part, MbraAG-P6-muscle and MbraAG-P6-setae, correspond respectively to pleopod setae, one endite, pereiopod muscle and pereiopod setae of the same female. Adult heteroplasmic individuals are indicated in bold.

Table 4.2 (Cont.). Nucleotidic proportions (%) at positions 365 and 580 of the 16S rDNA fragment for all the samples analysed.

		Position			
		365		580	
Sample	Origin	T	C	T	A
MbraAG-P7-pleopod	**female**	**0**	**100**	**43.5**	**56.5**
MbraAG-P7-1	egg	0	100	28.0	72.0
MbraAG-P7-2	egg	0	100	0	100
MbraAG-P7-3	egg	0	100	57.9	42.1
MbraAG-P7-4	egg	0	100	64.5	35.5
MbraAG-P7-5	egg	0	100	35.7	64.3
MbraAG-P7-6	egg	0	100	19.8	80.2
MbraAG-P7-7	egg	0	100	0	100
MbraAG-P8-pleopod	female	0	100	100	0
MbraAG-P8-1	egg	0	100	100	0
MbraAG-2DN-pleopod	female	0	100	100	0
MbraAG-2DN-1	egg	16.7	83.3	100	0
MbraAG-2DN-2	egg	0	100	100	0
MbraAG-3IN-pleopod	female	0	100	100	0
MbraAG-3IN-1	egg	0	100	100	0
MbraAG-3IN-2	egg	0	100	100	0
MbraAG-4DN-pleopod	female	0	100	100	0
MbraAG-4DN-1	egg	0	100	100	0
MbraAG-4DN-2	egg	0	100	100	0
MbraAG-5IN-pleopod	**female**	**19.2**	**80.8**	**100**	**0**
MbraAG-5IN-1	egg	10.3	89.7	100	0
MbraAG-5IN-2	egg	0	100	100	0
MbraAG-G6-pleopod	female	0	100	100	0
MbraAG-G6-1	egg	0	100	100	0
MbraAG-G6-2	egg	0	100	100	0

Proportions were calculated by dividing the height of each peak in the electropherogram by the summation of the height of both peaks. The eggs and the clones correspond to the female that precedes them in the list. All samples tagged as "pleopod" correspond to pleopod setae from adult females. The samples MbraAG-P6-pleopod, MbraAG-P6-mouth-part, MbraAG-P6-muscle and MbraAG-P6-setae, correspond respectively to pleopod setae, one endite, pereiopod muscle and pereiopod setae of the same female. Adult heteroplasmic individuals are indicated in bold.

Due to the existence of double peaks at positions 365 and 580, two to four combinations of different mitochondrial molecules may be present. To clarify this aspect, the PCR products of the pleopod setae of the female MbraAG-M26, in which the double peaks were well-defined, were cloned and sequenced. In this case, it was detected that at least three different mitochondrial combinations are present in the pleopod setae of this female (Table 4.2).

```
                          365          442          580
MbraAG-M26-pleopod   : ATTTYTTTTG     CTAAGAGAGA     GATTWACCAG
MbraAG-M26-1         : ....C.....     ..........     ....T.....
MbraAG-M26-2         : ....C.....     ..........     ....T.....
MbraAG-M26-3         : ....C.....     ..........     ....T.....
MbraAG-M26-4         : ....C.....     ..........     ....T.....
MbraAG-M26-5         : ....C.....     ..........     ....T.....
MbraAG-M26-6         : ....C.....     ..........     ....T.....
MbraAG-M26-7         : ....C.....     ..........     ....T.....
MbraAG-M26-8         : ....C.....     ..........     ....T.....
MbraAG-M26-pleopod-C1: ....T.....     ..........     ....A.....
MbraAG-M26-pleopod-C2: ....C.....     ..........     ....T.....
MbraAG-M26-pleopod-C3: ....T.....     ..........     ....T.....
MbraAG-M26-pleopod-C4: ....T.....     ..........     ....A.....
MbraAG-M26-pleopod-C5: ....T.....     ..........     ....A.....
MbraAG-M26-pleopod-C6: ....T.....     ..........     ....A.....
MbraAG-M26-pleopod-C7: ....T.....     ..........     ....A.....
MbraAG-M26-pleopod-C8: ....T.....     ..........     ....A.....
MbraAG-M26-pleopod-C9: ....T.....     ..........     ....A.....
MbraAG-M2-pleopod    : ....C.....     ..........     ....T.....
MbraAG-M2-1          : ....C.....     ..........     ....T.....
MbraAG-M2-2          : ....C.....     ..........     ....T.....
MbraAG-M3-pleopod    : ....C.....     ..........     ....T.....
MbraAG-M3-1          : ....C.....     ..........     ....T.....
MbraAG-M3-2          : ....C.....     ..........     ....T.....
MbraAG-P6-pleopod    : ..........     ..........     ....A.....
MbraAG-P6-mouth-part : ....T.....     ..........     ....A.....
MbraAG-P6-muscle     : ....T.....     ..........     ....A.....
MbraAG-P6-setae      : ..........     ..........     ..........
MbraAG-P6-1          : ..........     ..........     ..........
MbraAG-P6-2          : ..........     ..........     ..........
MbraAG-P6-3          : ..........     ..........     ..........
MbraAG-P6-4          : ..........     ..........     ..........
MbraAG-P6-5          : ..........     ..........     ..........
MbraAG-P6-6          : ..........     ..........     ..........
MbraAG-P6-7          : ..........     ..........     ....A.....
MbraAG-P6-8          : ..........     ..........     ....A.....
MbraAG-P6-9          : ..........     ..........     ..........
MbraAG-P6-10         : ..........     ..........     ....A.....
MbraAG-P6-11         : ..........     ..........     ....A.....
MbraAG-P6-12         : ..........     ..........     ..........
MbraAG-P6-13         : ..........     ..........     ..........
MbraAG-P6-14         : ..........     ..........     ..........
MbraAG-P6-15         : ..........     ..........     ..........
MbraAG-P6-16         : ..........     ..........     ..........
MbraAG-P7-pleopod    : ....C.....     ..........     ..........
MbraAG-P7-1          : ....C.....     ..........     ..........
MbraAG-P7-2          : ....C.....     ..........     ....A.....
MbraAG-P7-3          : ....C.....     ..........     ..........
MbraAG-P7-4          : ....C.....     ..........     ..........
MbraAG-P7-5          : ....C.....     ..........     ..........
MbraAG-P7-6          : ....C.....     ..........     ..........
MbraAG-P7-7          : ....C.....     ..........     ....A.....
MbraAG-P8-pleopod    : ....C.....     ..........     ....T.....
MbraAG-P8-1          : ....C.....     ..........     ....T.....
MbraAG-P8-2          : ....C.....     ..........     ....T.....
MbraAG-2DN-pleopod   : ....C.....     ....A.....     ....T.....
MbraAG-2DN-1         : ..........     ....A.....     ....T.....
MbraAG-2DN-2         : ....C.....     ....A.....     ....T.....
MbraAG-3IN-pleopod   : ....C.....     ..........     ....T.....
MbraAG-3IN-1         : ....C.....     ..........     ....T.....
MbraAG-3IN-2         : ....C.....     ..........     ....T.....
MbraAG-4DN-pleopod   : ....C.....     ..........     ....T.....
MbraAG-4DN-1         : ....C.....     ..........     ....T.....
MbraAG-4DN-2         : ....C.....     ..........     ....T.....
MbraAG-5IN-pleopod   : ..........     ..........     ....T.....
MbraAG-5IN-1         : ..........     ..........     ....T.....
MbraAG-5IN-2         : ....C.....     ..........     ....T.....
MbraAG-G6-pleopod    : ....C.....     ..........     ....T.....
MbraAG-G6-1          : ....C.....     ..........     ....T.....
MbraAG-G6-2          : ....C.....     ..........     ....T.....
```

Fig. 4.1. Alignment of the sequences of 16S gene around positions 365, 442 and 580. The sequences were named with the following codes: MbraAG-specimen code-tissue, in the case of adults; MbraAG-female code-number, in the case of the eggs. Dots indicate identity with the consensus sequence (on top).

The next step involved to perform a more extensive analysis of the degree of heteroplasmy in Galician populations. In order to achieve that, we used the COI sequences obtained from 50 adult individuals from the Golfo Ártabro and 33 from the Ría de Arousa to establish a consensus sequence, since the level of genetic variation in this mtDNA region is higher than in 16S rDNA. The electropherograms of the 83 studied individuals were checked in search of double peaks (Fig. 4.2). After removing the sequences with ambiguous sites (double peaks), an alignment was performed with the remaining 61 sequences, which allowed to verify the existence of 26 haplotypes (Table 4.3, GenBank accession numbers: MN027519 - MN027544). Thus, the existence of 25 variable sites was tested, 12 of which were singleton variable sites, while 13 were parsimony informative sites. No stop codons were detected in any case, five being the maximum number of synonym differences between two sequences and four being the maximum number of non-synonym differences.

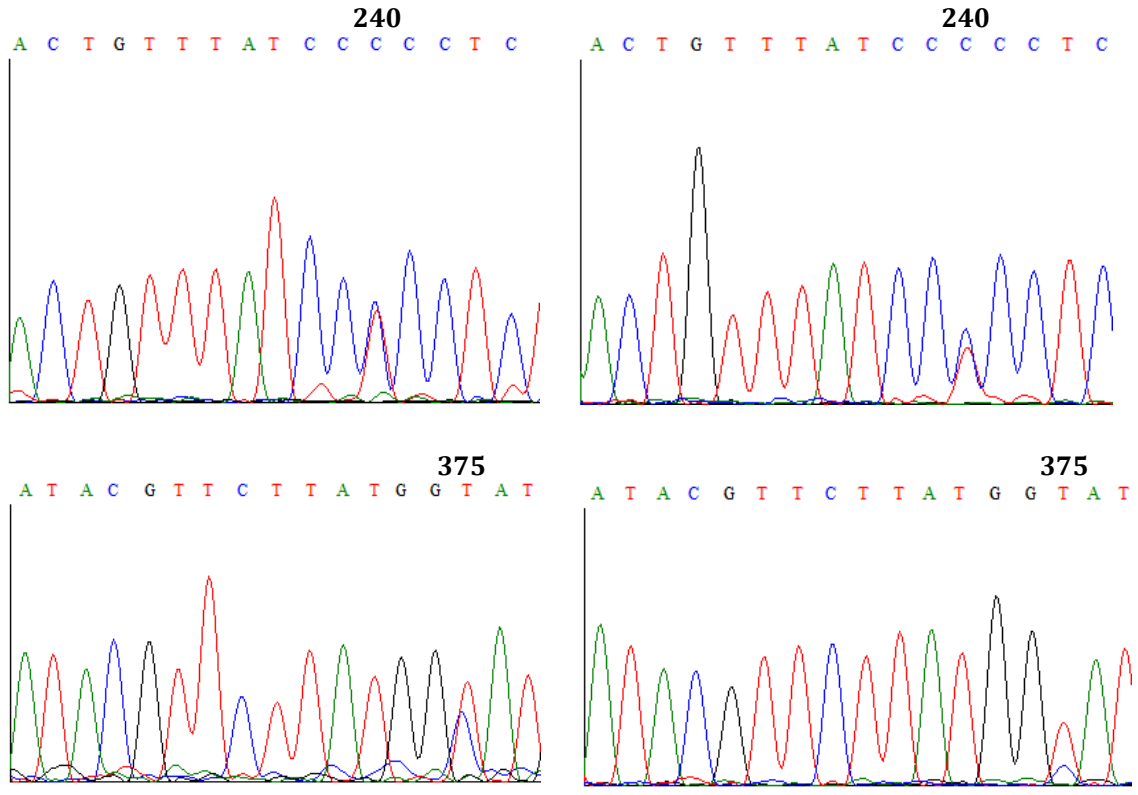


Fig. 4.2. Example of double peaks in the forward (left) and reverse (right) reads of the COI region at positions 240 (top) and 375 (bottom).

Table 4.3. Detected haplotypes for the COI gene and adult individuals in which they were observed.

Haplotype	N	Individual		
Hap-COI-1	2	MbraAG-5TM-muscle	MbraAG-16TM-muscle	
Hap-COI-2	1	MbraAG-13TM-muscle		
Hap-COI-3	7	MbraAG-14H-muscle	MbraAG-15TM-muscle	MbraAG-17H-muscle
		MbraAG-23TM-muscle	MbraRA-G8-gonad	MbraRA-G46-gonad
		MBraRA-G53-gonad		
Hap-COI-4	1	MbraAG-10TM-muscle		
Hap-COI-5	1	MbraAG-18H-muscle		
Hap-COI-6	1	MBraRA-G47-gonad		
Hap-COI-7	1	MbraAG-22TM-muscle		
Hap-COI-8	1	MbraRA-G20-gonad		
Hap-COI-9	2	MbraAG-3TM-muscle	MbraRA-G21-gonad	
Hap-COI-10	1	MbraAG-2DN-pleopod		
Hap-COI-11	3	MbraAG-3H-muscle	MbraAG-20TM-muscle	MbraRA-G30-gonad
Hap-COI-12	2	MbraAG-11H-muscle	MbraAG-13H-muscle	
Hap-COI-13	15	MbraAG-2H-muscle	MbraAG-4H-muscle	MbraAG-6H-muscle
		MbraAG-7H-muscle	MbraAG-9H-muscle	MbraAG-12TM-muscle
		MBraAG-14TM-muscle	MbraAG-19TM-muscle	MbraAG-19H-muscle
		MbraAG-27TM-muscle	MbraRA-G26-gonad	MbraRA-G33-gonad
		MbraRA-G34-gonad	MbraRA-G39-gonad	MbraRA-G50-gonad
Hap-COI-14	1	MbraAG-26TM-muscle		
Hap-COI-15	1	MBraAG-28TM-muscle		
Hap-COI-16	1	MbraAG-4TM-muscle		
Hap-COI-17	3	MbraAG-16H-muscle	MbraRA-G18-gonad	MBraRA-G29-gonad
Hap-COI-18	1	MbraAG-25TM-muscle		
Hap-COI-19	7	MbraAG-6TM-muscle	MbraAG-15H-muscle	MbraRA-G5-gonad
		MbraRA-G9-gonad	MbraRA-G16-gonad	MbraRA-G23-gonad
		MBraRA-G36-gonad		
Hap-COI-20	2	MbraRA-G10-gonad	MbraRA-G35-gonad	
Hap-COI-21	1	MBraAG-18TM-muscle		
Hap-COI-22	2	MbraAG-20H-muscle	MbraRA-G43-gonad	
Hap-COI-23	1	MBraRA-G40-gonad		
Hap-COI-24	1	MbraAG-7TM-muscle		
Hap-COI-25	1	MbraAG-17TM-muscle		
Hap-COI-26	1	MbraAG-24TM-muscle		
Total	61			

N: number of haplotypes. Sequences with double peaks were not included in this analysis. Samples tagged as "pleopod" and "muscle" correspond to pleopod setae and pereiopod muscle, respectively.

The electropherograms of the 13 parsimony informative sites were analysed at four positions (240, 264, 303 and 375) because of their variability in the sequences and the production of synonymous changes. Thus, a total of 22 individuals (four males and seven females from the Golfo Ártabro, 11 females from the Ría de Arousa) showed double peaks at one of these four positions (Table 4.4, Fig. 4.3). After repeating the DNA extraction and COI amplification, double peaks were detected at the same positions. These double peaks were only taken into consideration when the electropherograms were well-defined and

there was a clear difference from the baseline. It should be highlighted that the two reads (forward and reverse) were not always identical in height due to the PCR yield may vary depending on the hybridisation of the primers and the polymerase activity. For the rest of the nine informative positions, no clear double peaks were detected and therefore they were not taken into consideration in the analyses.

Table 4.4. Nucleotidic proportions (%) at positions 240, 264, 303 and 375 of the COI fragment for 16 adult individuals with ambiguous sites.

		Position							
		240		264		303		375	
Sample	Sex	T	C	T	C	T	C	T	C
MbraAG-2TM-muscle	male	15.3	84.7	0	100	20.2	79.8	100	0
MbraAG-8TM-muscle	male	100	0	0	100	100	0	80.9	19.1
MbraAG-10H-muscle	female	26.4	73.6	0	100	31.0	69.0	100	0
MbraAG-29TM-muscle	male	0	100	74	26.3	0	100	77.3	22.7
MbraAG-30TM-muscle	male	27.1	72.9	0	100	31.3	68.7	75.8	24.2
MbraRA-G1-gonad	female	12.6	87.4	0	100	100	0	100	0
MbraRA-G2-gonad	female	100	0	0	100	78.8	21.2	0	100
MbraRA-G4-gonad	female	100	0	13	86.6	0	100	100	0
MbraRA-G22-gonad	female	17.5	82.5	0	100	19.1	80.9	0	100
MbraRA-G24-gonad	female	11.0	89.0	0	100	13.8	86.2	100	0
MbraRA-G25-gonad	female	100	0	0	100	100	0	74.7	25.3
MbraRA-G28-gonad	female	16.8	83.2	0	100	18.4	81.6	100	0
MbraRA-G40-gonad	female	40.5	59.5	0	100	42.0	58.0	100	0
MbraRA-G42-gonad	female	10.7	89.3	0	100	0	100	100	0
MbraRA-G44-gonad	female	84.2	15.8	0	100	100	0	100	0
MbraRA-G45-gonad	female	47.3	52.7	0	100	46.6	53.4	100	0

Proportions were calculated by dividing the height of each peak in the electropherogram by the summation of the height of both peaks. The remaining adult females in which heteroplasmy was detected are shown in Table 4.5.

The sequences of COI obtained from the broods of the females MbraAG-M26, MbraAG-P6 and MbraAG-P7 showed that a great majority of the eggs presented heteroplasmy for this gene (Table 4.5). All the eggs of the females MbraAG-M26 (eight eggs) and MbraAG-P7 (six eggs) presented double peaks at the some positions. In the case of the female MbraAG-P6, 9 of the 11 eggs showed ambiguous sites.

```
                                240          264          303          375
MbraAG-5TM-muscle          : ATCCCCCTCT   CGCACGCAGG   TTCACTTAGC   ATGGYATGAC
MbraAG-6TM-muscle          : ..........   ..........   ....T.....   ..........
MbraAG-16TM-muscle         : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-8TM-muscle          : ....T.....   ..........   ....T.....   ..........
MbraAG-7TM-muscle          : ....T.....   ....Y.....   ....T.....   ....T.....
MbraAG-10H-muscle          : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-14TM-muscle         : ....T.....   ..........   ....Y.....   ....C.....
MbraAG-16TM-muscle         : ..........   ..........   ....Y.....   ....T.....
MbraAG-28TM-muscle         : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-29TM-muscle         : ..........   ....Y.....   ..........   ..........
MbraAG-2DN-pleopod         : ....Y.....   ..........   ....T.....   ....C.....
MbraAG-2TM-muscle          : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-30TM-muscle         : ....Y.....   ..........   ....Y.....   ..........
MbraAG-G1-gonad            : ....Y.....   ..........   ....T.....   ....T.....
MbraAG-G2-gonad            : ....T.....   ..........   ....Y.....   ....C.....
MbraAG-G4-gonad            : ....T.....   ....Y.....   ..........   ....T.....
MbraAG-G22-gonad           : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-G24-gonad           : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-G25-gonad           : ....T.....   ..........   ....T.....   ..........
MbraAG-G28-gonad           : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-G29-gonad           : ....T.....   ....Y.....   ....T.....   ....T.....
MbraAG-G36-gonad           : ....Y.....   ..........   ..........   ....T.....
MbraAG-G40-gonad           : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-G42-gonad           : ....Y.....   ..........   ..........   ....T.....
MbraAG-G44-gonad           : ....Y.....   ..........   ....T.....   ....T.....
MbraAG-G45-gonad           : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-G47-gonad           : ....T.....   ..........   ....Y.....   ....C.....
MbraAG-G53-gonad           : ....Y.....   ..........   ....T.....   ....C.....
MbraAG-M26-pleopod         : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-M26-1               : ..........   ..........   ....Y.....   ....C.....
MbraAG-M26-2               : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-M26-3               : ..........   ..........   ....Y.....   ....C.....
MbraAG-M26-4               : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-M26-5               : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-M26-6               : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-M26-7               : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-M26-8               : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-P6-pleopod          : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-P6-mouth-part       : ..........   ..........   ....Y.....   ....T.....
MbraAG-P6-muscle           : ..........   ..........   ..........   ....T.....
MbraAG-P6-setae            : ....Y.....   ....Y.....   ....Y.....   ....T.....
MbraAG-P6-1                : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-P6-2                : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-P6-3                : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-P6-4                : ..........   ..........   ..........   ....C.....
MbraAG-P6-5                : ..........   ..........   ..........   ....T.....
MbraAG-P6-6                : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-P6-7                : ..........   ..........   ....Y.....   ....T.....
MbraAG-P6-8                : ..........   ..........   ....Y.....   ....T.....
MbraAG-P6-9                : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-P6-14               : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-P6-15               : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-P7-pleopod          : ..........   ..........   ....Y.....   ....T.....
MbraAG-P7-1                : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-P7-3                : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-P7-5                : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-P7-6                : ....Y.....   ....Y.....   ....Y.....   ....C.....
MbraAG-P7-7                : ..........   ..........   ....Y.....   ....C.....
MbraAG-P7-8                : ....Y.....   ..........   ....Y.....   ....C.....
MbraAG-3IMDB-gonad         : ..........   ..........   ....Y.....   ..........
MbraAG-3IMDB-gill          : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-3IMDB-muscle        : ..........   ..........   ..........   ....T.....
MbraAG-4D2V-pleopod        : ..........   ..........   ..........   ....T.....
MbraAG-4D2V-gonad          : ....Y.....   ..........   ....Y.....   ..........
MbraAG-4D2V-gill           : ....Y.....   ....Y.....   ....Y.....   ....T.....
MbraAG-4D2V-muscle         : ..........   ..........   ..........   ....T.....
MbraAG-4D2V-heart          : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-4D2V-stomach        : ..........   ..........   ..........   ....T.....
MbraAG-4D2V-intestine      : ..........   ..........   ....Y.....   ....T.....
MbraAG-4D2V-nerve          : ..........   ..........   ..........   ....T.....
MbraAG-4D2V-eye            : ....Y.....   ..........   ....Y.....   ....T.....
MbraAG-4D2V-integument     : ..........   ..........   ....Y.....   ....T.....
MbraAG-5DV-pleopod         : ....T.....   ..........   ....T.....   ....T.....
MbraAG-5DV-gonad           : ....T.....   ..........   ....T.....   ....T.....
MbraAG-5DV-gill            : ....T.....   ..........   ....T.....   ....T.....
MbraAG-5DV-muscle          : ....T.....   ..........   ....T.....   ....T.....
MbraAG-5DV-heart           : ....T.....   ..........   ....T.....   ....T.....
MbraAG-5DV-stomach         : ....T.....   ..........   ....T.....   ..........
MbraAG-5DV-intestine       : ....T.....   ..........   ....T.....   ....T.....
MbraAG-5DV-nerve           : ....T.....   ..........   ....T.....   ....T.....
MbraAG-5DV-eye             : ....T.....   ..........   ....T.....   ..........
MbraAG-5DV-integument      : ....T.....   ..........   ....T.....   ....T.....
MbraAG-5DV-hepatopancreas: ....T.....   ..........   ....T.....   ....T.....
```

Fig. 4.3. Alignment of the sequences of COI gene around positions 240, 264, 303 and 375. The sequences were named with the following codes: MbraAG-specimen code-tissue, in the case of adults; MbraAG-female code-number, in the case of the eggs. Dots indicate identity with the consensus sequence (on top).

Regarding the sequences of COI from different tissues of four females, electropherogram analyses showed that the presence of ambiguous sites is very variable among different tissues. Thus, while in the female MbraAG-4D2V the heteroplasmy is maintained in most of the tissues (except pleopod setae, pereiopod muscle, stomach and nerve), in the female MbraAG-5DV it is only observed in two of the ten tissues analysed (stomach and eye) (Table 4.5).

Table 4.5. Nucleotidic proportions (%) of the COI fragment in broods and different tissues of several adult females.

		Position							
		240		264		303		375	
Sample	Sex	T	C	T	C	T	C	T	C
MbraAG-M26-pleopod	female	23.3	76.7	26.4	73.6	27.6	72.4	0	100
MbraAG-M26-1	egg	0	100	0	100	25.4	74.6	0	100
MbraAG-M26-2	egg	20.8	79.2	12.0	88.0	28.6	71.4	0	100
MbraAG-M26-3	egg	0	100	0	100	29.1	70.9	0	100
MbraAG-M26-4	egg	20.4	79.6	15.4	84.6	26.4	73.6	0	100
MbraAG-M26-5	egg	24.4	75.6	10.1	89.9	27.8	72.2	0	100
MbraAG-M26-6	egg	29.1	70.9	20.7	79.3	34.7	65.3	0	100
MbraAG-M26-7	egg	27.9	72.1	0	100	29.6	70.4	0	100
MbraAG-M26-8	egg	25.3	74.7	0	100	30.2	69.8	0	100
MbraAG-P6-pleopod	female	28.7	71.3	0	100	30.5	69.5	100	0
MbraAG-P6-mouth-part	female	0	100	0	100	21.6	78.4	100	0
MbraAG-P6-muscle	female	0	100	0	100	0	100	100	0
MbraAG-P6-setae	female	32.5	67.5	26.1	73.9	30.1	69.9	100	0
MbraAG-P6-1	egg	25.1	74.9	0	100	28.8	71.2	0	100
MbraAG-P6-2	egg	27.6	72.4	30.5	69.5	34.0	66.0	0	100
MbraAG-P6-3	egg	19.8	80.2	0	100	24.8	75.2	0	100
MbraAG-P6-4	egg	0	100	0	100	0	100	0	100
MbraAG-P6-5	egg	0	100	0	100	0	100	100	0
MbraAG-P6-6	egg	18.2	81.8	0	100	23.6	76.4	0	100
MbraAG-P6-7	egg	0	100	0	100	33.7	66.3	100	0
MbraAG-P6-8	egg	0	100	0	100	22.8	77.2	100	0
MbraAG-P6-9	egg	43.1	56.9	0	100	48.6	51.4	0	100
MbraAG-P6-14	egg	23.8	76.2	0	100	39.3	60.7	0	100
MbraAG-P6-15	egg	22.5	77.5	0	100	25.5	74.5	0	100
MbraAG-P7-pleopod	female	0	100	0	100	22.6	77.4	100	0
MbraAG-P7-1	egg	34.7	65.3	24.7	75.3	39.0	61.0	0	100
MbraAG-P7-3	egg	48.7	51.3	42.8	57.2	64.9	35.1	0	100
MbraAG-P7-5	egg	37.6	62.4	46.7	53.3	49.8	50.2	0	100
MbraAG-P7-6	egg	32.2	67.8	18.9	81.1	64.6	35.4	0	100
MbraAG-P7-7	egg	0	100	0	100	26.0	74.0	0	100
MbraAG-P7-8	egg	44.5	55.5	0	100	60.9	39.1	0	100
MbraAG-3IMDB-gonad	female	0	100	0	100	16.2	83.8	79.8	20.2
MbraAG-3IMDB-gill	female	18.6	81.4	0	100	18.5	81.5	100	0
MbraAG-3IMDB-muscle	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-pleopod	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-gonad	female	6.7	93.3	0	100	15.4	84.6	82.7	17.3
MbraAG-4D2V-gill	female	57.2	42.8	50.0	50.0	66.3	33.7	100	0
MbraAG-4D2V-muscle	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-heart	female	34.3	65.7	0	100	41.8	58.2	100	0
MbraAG-4D2V-stomach	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-intestine	female	0	100	0	100	28.6	71.4	100	0
MbraAG-4D2V-nerve	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-eye	female	82.5	17.5	0	100	71.3	28.7	100	0
MbraAG-4D2V-integument	female	0	100	0	100	32.3	67.7	100	0

Table 4.5 (Cont.). Nucleotidic proportions (%) of the COI fragment in broods and different tissues of several adult females.

		Position							
		240		264		303		375	
Sample	Sex	T	C	T	C	T	C	T	C
MbraAG-5DV-pleopod	female	100	0	0	100	100	0	100	0
MbraAG-5DV-gonad	female	100	0	0	100	100	0	100	0
MbraAG-5DV-gill	female	100	0	0	100	100	0	100	0
MbraAG-5DV-muscle	female	100	0	0	100	100	0	100	0
MbraAG-5DV-heart	female	100	0	0	100	100	0	100	0
MbraAG-5DV-stomach	female	100	0	0	100	100	0	68.0	32.0
MbraAG-5DV-intestine	female	100	0	0	100	100	0	100	0
MbraAG-5DV-nerve	female	100	0	0	100	100	0	100	0
MbraAG-5DV-eye	female	100	0	0	100	100	0	62.5	37.5
MbraAG-5DV-integument	female	100	0	0	100	100	0	100	0
MbraAG-5DV-hepatopancreas	female	100	0	0	100	100	0	100	0

Proportions were calculated by dividing the height of each peak in the electropherogram by the summation of the height of both peaks. Samples tagged as "pleopod", "mouth-part" and "muscle" correspond to pleopod setae, endites and muscle from pereiopod, respectively.

For the female P6, the muscle is homoplasmic for the 16S gene and the COI gene and the pleopod and pereiopod setae are heteroplasmic for both genes. Regarding the mouth-parts, heteroplasmy was detected only for the mtDNA of the COI gene. *A priori*, it would be expected that the samples of pleopods and mouth-parts are mostly composed of muscular tissue. However, when sampling the pleopods, we collected only the setae that cover these structures. For this reason, it makes sense that the result obtained for the pleopods would be the same as for the pereiopod setae. On the other hand, the endite is a multi-tissular structure, so, after homogenisation, a mixture of mtDNA from different tissues (homo or heteroplasmic in the case of the COI gene) could be present in the sample.

Discussion

Until now, a low incidence of heteroplasmy was recorded in animals except for mussels. However, Williams *et al.* (2017) had detected high levels of heteroplasmy in the blue crab *Callinectes sapidus*, identifying a high number of haplotypes never before found in metazoans (Williams *et al.*, 2017). In that work, the researchers propose several explanations to this phenomenon (Nuclear Mitochondrial DNA segments or NUMTS, paternal leakage, accumulated mutations and replication errors) without opting for one. In our study, the analysis of the mtDNA of a large offspring for seven spider crab females (Williams *et al.* [2017] only analysed one male, one female and one megalopa) allows us to discard several hypotheses of the origin of heteroplasmy in *M. brachydactyla,* until reduce them to one option. In the case of this species, the high number of heteroplasmic cells per

individual/tissue makes it possible to detect this phenomenon by conventional PCR. However, new techniques such as qPCR (quantitative Polymerase Chain Reaction) and ARMS-qPCR (Amplification Refractory Mutation System-quantitative PCR) are now available to detect very low levels of heteroplasmic cells (Burgstaller *et al.*, 2018; Mitrofanov *et al.*, 2018).

Electropherogram reads are conditioned by the quality and efficiency of the PCR. This is associated with the hybridisation primer/target and the efficiency of Taq polymerase among other circumstances. Contamination of samples or PCR products is a factor to have into consideration. For this reason, DNA extraction was repeated in many cases, particularly in those where double peak was detected, as it was mentioned in materials and methods and results sections. However, no different results were obtained. It should be noted that double peaks only affect two specific positions in 16S rDNA and four in COI and that none of these COI positions are involved in stop codons. The similarity between the sequences obtained and the sequences available on databases indicates that it is *M. brachydactyla* DNA in all cases.

Once the contamination has been discarded as the cause of the double peaks, there are two different possibilities that can lead to an erroneous interpretation of the electropherograms. Firstly, the existence of NUMTs. In this sense, there are references to the presence of COI-like sequences in many crustaceans, for instance in krill, crabs, amphipods, crayfish, squat lobsters, shrimps, isopods, barnacles and copepods (Buhay, 2009; Gíslason *et al.*, 2013). Assuming that they were, these nuclear sequences would have Mendelian inheritance, which would imply that all the cells of an individual should have them. This hypothesis would not explain why some of the analysed tissues have a duplicate COI sequence and why others do not. For example, for COI, the female MbraAG-4D2V does not present double peaks in the pleopod setae, pereiopod muscle, stomach and nerve tissue, while they are present in gonad, gill, heart, intestine, eye and integument (Table 4.5). In addition, each individual could be homozygous or heterozygous for the presence of such NUMT. In this case, the female MbraAG-M26 for 16S rDNA has three combinations of sequences between positions 365 and 580 (T-A, C-T, T-T). If one of these sequences is mitochondrial, the other two would be nuclear, therefore, all her eggs should carry two combinations (one mitochondrial and one maternal NUMT) and this is not detected (Table 4.2). If a female were homozygous for the NUMT all descendants would have to present double peak (one mitochondrial and one NUMT) which does not happen in many cases (16S rDNA for females MbraAG-M26, MbraAG-P7 and MbraAG-5IN, and COI for the female MbraAG-P6). The second possibility is that there would have been a duplication in the mitochondrial genome of these sequences.

This would not make sense because then all descendants of a female with a double peak should also have a double peak. In addition, we would have mitochondria in which there have been two independent duplications of the 16S rDNA and the COI gene (for example, the females MbraAG-M26, MbraAG-P6 or MbraAG-P7) and mitochondria in which the duplication only occurred in the 16S rDNA (MbraAG-10H or MbraAG-2DN).

Once these assumptions are discarded as explanations for the double peaks detected, we can conclude that we detected a true heteroplasmy in 22 adult individuals of a total of 83 studied (incidence of 27%). Koolkarnkhai *et al.* (2019) had previously reported an incidence of heteroplasmy of 8% in *Portunus pelagicus*.

Heteroplasmy has been extensively studied at individual level in terms of the number of involved cells or tissues. However, it also has great importance at evolutionary level, since it affects Muller's ratchet. This theory holds that uniparental inheritance (homoplasmy) creates non-combined asexual lineages, which accumulate deleterious mutations more rapidly than their sexual counterparts (Muller, 1964; Felsenstein, 1974; Gordo and Charlesworth, 2000). Several mechanisms have been proposed to explain how mtDNA can overcome this evolutionary limitation such as genetic bottleneck, compensatory mutations, back mutations, mitochondrial DNA copy recruitment from the nucleus, purifying selection or recombination (Loewe, 2006; Stewart *et al.*, 2008), this last one being the main accepted mechanism to eliminate Muller's ratchet. In a population of identical mitochondrial DNA molecules, recombination generates molecules that are identical to themselves and to parent molecules. Thus, mutation accumulation in the mtDNA molecule would be inevitable. The leakage of paternal mitochondrial DNA could have evolved to provide a means to overcome this limitation.

Due to the low frequency of recombination and *de novo* mutations in mitochondrial genome (Kajander *et al.*, 2001; Xu *et al.*, 2012), in our opinion, the main cause of heteroplasmy in *Maja brachydactyla* are failures in the elimination of the male mitochondria. This fact could have two main explanations. Firstly, a special type of DUI-type inheritance as in molluscs (Zouros *et al.*, 1992; Breton *et al.*, 2007; Theologidis *et al.*, 2007; White *et al.*, 2008; Zouros, 2013). However, this can be discarded because in our species heteroplasmy is indistinctly detected in both males and females. Secondly, that there has been a generalised failure on the mechanisms of elimination of paternal mitochondria (Rokas *et al.*, 2003). Until now, true biparental transmission of mitochondrial genomes from both parents (or from two distinct mating types) to zygotes as part of normal reproductive processes within a species and their persistence throughout development has not been demonstrated (Breton and Stewart;

2015). Nevertheless, sporadic biparental inheritance events have been documented in mammals, birds, reptiles, fish, molluscs, nematodes or arthropods (Breton *et al.*, 2007).

One possible explanation for the retaining of male mitochondria in the offspring in *M. brachydactyla* is that these individuals come from interspecies or interpopulation hybridisations. In these cases, a breakdown of mechanisms to recognise and remove paternal mtDNA may occur (Kaneda *et al.*, 1995; Shitara *et al.*, 1998; Sutovsky *et al.*, 2000; Breton *et al.*, 2007; Wolff *et al.*, 2016). Several researches have been based on analysing the mtDNA of the offspring of intra- and interspecific crosses. Kondo *et al.* (1990) performed crosses with different species of *Drosophila* during 10 consecutive generations (140 intraspecific and 191 interspecific crosses). The results of this study showed the presence of maternal mtDNA in the offspring of intraspecific crosses, while some individuals from interspecific crosses showed paternal leakage (three homoplasmic and one heteroplasmic individuals). Dokianakis and Ladoukakis (2014) also analysed the mtDNA of the offspring of crosses between seven *Drosophila* species (31 interspecific crosses, 4 intraspecific crosses). They only detected paternal leakage in some of the hybrids, while in the descendants of intraspecific crosses the mitochondrial inheritance was strictly maternal. Kaneda *et al.* (1995) conducted a similar study in mice. In crosses between individuals of the species *Mus musculus* the paternal mtDNA was detected only through the early pronucleus stage while in crosses between *M. musculus* and *M. spretus* the paternal mtDNA was detected throughout development from pronucleus stage to neonates. In other vertebrates, paternal leakage has been detected in natural hybrids of fish (Morgan *et al.*, 2013), birds (Gandolfi *et al.*, 2017) or amphibians (Vershinin *et al.*, 2019).

The studies of Clark *et al.* (2001) based on biometric characters suggested the lack of complete separation of the Atlantic and Mediterranean populations of *Carcinus*, with the potential existence of a hybrid zone between *C. maenas* (Atlantic) and *C. aestuarii* (Mediterranean). Something similar happens in the case of *Maja brachydactyla* and *M. squinado,* species very close morphological and genetically (Neumann, 1998; Sotelo *et al.*, 2008b; De Grave *et al.*, 2009; Guerao *et al.*, 2011). Although the distribution of *M. brachydactyla* is usually associated to the northeast Atlantic and the distribution of *M. squinado* to the Mediterranean (Neumann, 1996; Neumann, 1998; Sotelo *et al.*, 2008b), Abelló *et al.* (2014) reported specimens identified as *M. brachydactyla* in the Alboran Sea (western Mediterranean Sea). Our results suggest that this gene flow could occur bi-directionally across the Strait of Gibraltar, the only area of direct contact between both species. The complex life cycles of the planktonic larval stages and benthic post-larvae determine the distribution and population dynamics of spiny spider crabs. During the

planktonic larval phase, the individuals are drifted by the action of the latitudinal marine currents that are active mainly during the months of April to October. This fact could facilitate the transferring of individuals, extending the overlapping zones to northern regions. In addition to this, considerable displacements have also been described in adult stages, reaching 250 km (Corgos *et al.*, 2011). If this fact is one of the causes involved in the appearance of heteroplasmy, its incidence should be higher in regions to the south of the Iberian Peninsula than in more northerly regions, such as the French coasts or the British Islands. It should be noted that the differences in the COI mitochondrial sequences of *M. brachydactyla* and *M. squinado* range from 5 to 10 synonymal changes, so that, *a priori*, there should not be a problem of cytoplasmic inheritance and intragenomic conflict between the nucleus and the cytoplasm.

As for the distribution of the different mtDNA types in the embryo during development, the results obtained do not show a pattern that would allow conclusions to be drawn. Tissues that show the same mtDNA do not necessarily come from the same embryonic layer. For example, in the case of the female Mbra-AG-4D2V, tissues formed from mesoderm show differences in their COI mitochondrial sequences: muscular tissue has no ambiguous sites, while gonadal tissue shows three heteroplasmic positions and cardiac tissue, two. Differences in mtDNA between tissues of the same individual had already been detected in mice by Shitara *et al.* (1998). They analysed 12 different tissues from 38 F1 hybrids. They detected paternal leakage in 17 of the individuals analysed, but in most of them, the paternal mtDNA was limited to between one and three tissues, which varied from one mouse to another. Regarding ovarian tissue, only 6.6% of F1 hybrid females reported paternal leakage, so they concluded paternal mitochondrial DNA does not propagated stably to future generations.

If the male contribution is a sporadic and punctual phenomenon restricted to certain contact zones between congeneric species, meiotic cell drift and what happened during embryonic phases would be the cause of the differences between tissues. Mitochondrial DNA molecules pass through a genetic bottleneck process, this is, the decreasing of the number of copies, both during oogenesis and early development (Bergtrom and Pritchard, 1998). This process is apparently due to random partitioning of organelles containing only one or a very few mtDNA molecules and lead to rapid segregation of polymorphic mtDNA species in the progeny (Hauswirth and Laipis, 1985). Its effect varies according to taxa, ranging from 1 to 349 in humans (Howell *et al.*, 1992; Bendall *et al.*, 1996; Jenuth *et al.*, 1996), 200 in mice (Cree *et al.*, 2008) and from 370 to 740 in fruit fly (Solignac *et al.*, 1984). It should also be noted that PCR amplifications are not 100% effective. This implies that by

chance when any combination is in low proportion may not be detected by conventional PCR due to its low amplification. These facts could explain why in the sample MbraAG-2DN-pleopod we did not detect heteroplasmy but we detected it in one of its eggs.

To summarise, a considerable frequency of heteroplasmy were detected in the spider crab *Maja brachydactyla*, possibly associated to hybridisation of congeneric species. Future studies in other populations of *M. brachydactyla* as well as the analysis of the offspring from crosses in captivity would be necessary to confirm the true origin and dimension of this finding. Nevertheless, heteroplasmy is an important aspect to take into account in studies of population management, especially in those under commercial exploitation, since effective population size could be overestimated. In species with this mitochondrial particularity, it is recommendable to use nuclear markers in molecular genetics studies. If mitochondrial markers are anyway chosen, heteroplasmy will have to be taken into consideration when the results are interpreted in order to avoid erroneous conclusions.

5

General discussion

Importance of obtaining high-quality DNA for molecular studies

Increasingly, genetic studies are becoming a fundamental tool for the knowledge of wildlife, providing information of great interest for its management and conservation. These studies, based on the analysis of molecular markers, require high-quality DNA for their development. For this reason, the establishment of an adequate starting tissue, a correct conservation of the samples and an efficient extraction method are aspects to consider in order to maximise the quantity and quality of the DNA for the subsequent analysis of molecular markers.

DNA extraction from eggs provides a number of advantages as compared to adult tissues: (a) eggs facilitate a big sample size, (b) embryos contain all the DNA sequences of the individual, (c) obtaining DNA not only from adults and larvae but also eggs, allows to study the effect of natural selection throughout the development, (d) extracting DNA from eggs allows to perform studies on paternity and linkage analysis. However, as they are related individuals, the use of eggs from the same brood to extend the sample size is not suitable for all types of studies (e.g. estimation of effective population size), as the results would be biased and unreliable. Sometimes the DNA extraction methodology used on adult tissues is not effective for eggs and larvae. For this reason, many methodological studies have focused on obtaining quality DNA from eggs of small crustaceans (Moorad *et al.*, 1997; Reid *et al.*, 2002; Duff *et al.*, 2007; Montero-Pau *et al.*, 2008; Xu *et al.*, 2011; Ishida *et al.*, 2012).

Several paternity studies in decapods have highlighted the importance of obtaining sufficient quantities of high-quality DNA from eggs or larvae (McKeown *et al.*, 2008; Pardo *et al.*, 2016). For a paternity study in *Callinectes sapidus* (Hill *et al.*, 2017), samples of eggs (stage of development not specified) preserved at -20 °C were used for DNA extraction, without success. However, extraction from fresh juvenile samples allowed for DNA extraction and subsequent genotyping of the offspring. This fact highlights the importance of both the developmental stage of the offspring at which the sample is collected and the choice of an appropriate conservation method.

Pooling analysis of eggs or larvae is a commonly used technique to overcome the problem of the small amount of DNA obtained from a single embryo in paternity analyses. Although this method also allows for the analysis of a larger number of offspring (Urbani *et al.*, 1998; Gosselin *et al.*, 2005), it is less accurate than the analysis of individual eggs. Our results and those presented by previous studies (McKeown *et al.*, 2008) indicate that the pooling strategy must not replace the analysis of individual eggs, but are complementary. In our

case, the use of a commercial kit based on silica columns allowed us to obtain considerable amounts of DNA of sufficient quality to amplify the target genes from a single *Maja brachydactyla* egg.

Regarding adult tissues, the most commonly used for DNA extraction in decapods is muscle (Toonen, 2004; Gosselin *et al.*, 2005; Baggio *et al.*, 2011; Jensen and Bentzen, 2012). If the animal is not slaughtered, muscle is commonly obtained from pereiopods or pleopods. Despite the fact that this implies a sampling without death, the removal of these appendages or part of them causes injuries to the specimens, that can lead to a reduction in fitness by limiting their movements or reproductive success. In addition, animal suffering is a problem of increasing concern even for invertebrates, traditionally considered as inferior organisms in this respect. In other cases, slaughtering the animals is not an option, either because they are needed alive to continue research activities or because they belong to endangered species.

For these reasons, there has been increased interest in finding tissues for sampling that are minimally detrimental to the study specimens. Our results show that setae are a good alternative tissue, as they provide good quality DNA in sufficient quantity to be used in molecular studies, and their sampling produces hardly any alteration in the normal life of the specimens.

The methodological improvements described in Chapter 2 of this thesis have not only been fundamental for performing the molecular analyses of the subsequent chapters, but are also very useful for conducting genetic studies in other crustacean species, especially those that are threatened.

Benefits and costs of multiple mating and multiple paternity

Multiple mating have been reported in a large number of terrestrial and aquatic organisms (Neff and Svensoon, 2013; Mclay and Dennenmoser, 2020). This reproductive trait carries a number of benefits and costs, both at the individual and population level. In the case of males, the benefits of multiple mating clearly outweigh the costs, especially in species that lack parental care and mate guarding by males. Although, *a priori*, the advantages for females may not be as clear (Hosken, 1999; Birkhead, 2000; Jennions and Petrie, 2000), it has been suggested that they obtain a number of both direct (non-genetic) and indirect (genetic) benefits from multiple mating.

Direct benefits include obtaining a sufficient amount of sperm for fertilisation of broods (Anderson, 1974; Walker, 1980; Pitnick, 1993; Pitnick and Markow, 1994; Levitan and Petersen, 1995; Evans and Marshall, 2005; Pardo *et al.*, 2016), as well as buffering the effects of mating with a sterile male (Sheldon, 1994; but see Olsson and Shine, 1997). Along with sperm, males provide the females with nutritional and antimicrobial components that increase the survival of gametes in the seminal receptacles (Thornhill and Alcock, 1983; Subramoniam, 1993). At other times, the female is given substances that promote oocyte maturation and oviposition (Thornhill and Alcock, 1983; Subramoniam, 1993), or more obvious benefits such as nuptial gifts or breeding resources (Thornhill and Alcock, 1983; Wedell, 1997; McLain, 1998; Arnqvist and Nilsson, 2000). In species with mate guarding or parental care, females that mate several times get more protection for both themselves (Davies, 1992) and their offspring (Nakamura, 1998). In some taxa, multiple mating leads to the formation of social coalitions that are beneficial to females (Smuts, 1985).

Even when females seem to get no benefits, multiple mating may be the least costly option by avoiding punishment by the male (Clutton-Brock and Parker, 1995) or the energy cost of fighting the male when it exceeds that of allowing for copulation (Smuts and Smuts, 1993).

In addition to these direct benefits, other genetic benefits that are not so obvious have to be added (Jennions and Petrie, 2000; Neff and Pitcher, 2005). Polyandry allows females to select a suitable partner, both in terms of genetic compatibility (Zeh and Zeh, 1997; Tregenza and Wedell, 2000; Zeh and Zeh, 2003; Marshall and Evans, 2005) and in terms of obtaining quality genes (Trivers, 1972; Zeh and Zeh, 1996, 2001; Kokko *et al.,* 2006). Moreover, multiple paternity increases genetic variability within offspring (McLeod and Marshall, 2009), which increases the probability of the progeny to survive in changing environments (Forsman *et al.,* 2008). On the other hand, multiple mating helps to avoid fertilisation with genetically damaged sperm stored in the seminal receptacles for a long time (Halliday and Arnold, 1987).

At population level, it has been suggested that multiple paternity influences effective population size (Sugg and Chesser, 1994; Martínez *et al.*, 2000; Pearse and Anderson, 2009), genetic diversity (Morán and García-Vázquez, 1998; Jennions and Petrie 2000) and inbreeding (Stockley *et al.*, 1993; Yasui 1998; Foerster *et al.*, 2003; Mokhtar-Jamaï *et al.*, 2013).

However, multiple mating is not exempt from costs. In addition to energetic costs, during mating the risk of infection, predation or injury increases (Herre, 1993; Koga *et al.*, 1998; Watson *et al.*, 1998; Holland and Rice, 1999; Jennions and Petrie, 2000). In *Drosophila*, it

was observed that exposure to seminal products that reduce the receptivity of females to new matings results in an increase in the mortality rate (Chapman *et al.*, 1995).

On the other hand, clades with polyandry may be more specious than those with monandry, as gene flow is limited and reproductive isolation from part of the population may occur (Jennions and Petrie, 2000). This is not the case for *Maja brachydactyla*, as intense gene flow has been reported among its populations (Sotelo *et al.*, 2008a; Corgos *et al.*, 2011).

The spiny spider crab lacks courtship, mate guarding and parental care from males. Therefore, multiple mating will not result in more nuptial gifts or more protection for the females and their offspring. One of the main benefits of multiple mating for *M. brachydactyla* females is likely to be greater success in brood fertilisation. Mating with several males provides females with large amounts of sperm and minimises the fertilisation of eggs with damaged sperm.

Furthermore, spider crab females may obtain a number of genetic benefits from multiple paternity. On the one hand, the broods fertilised by multiple males allow the females to avoid genetic incompatibilities and increase the variability of their progeny, increasing the probability of at least part of their offspring to reach sexual maturity. On the other hand, multiple paternity may help to maintain genetic diversity and effective population size, as well as reduce inbreeding. This effect is especially noticeable in intensely exploited populations, as the Galician population of *M. brachydactyla*.

Mitochondrial heteroplasmy and hybridisation in *Maja brachydactyla*

Haploidy forces many asexual organisms to accept a large number of deleterious mutations for lack of an additional DNA copy to allow for recombination (Gabriel *et al.*, 1993). Prokaryotic organisms have mechanisms to repair single-strand damage (Szostack *et al.*, 1983), but mitochondria have so reduced their genome that they lack genes for this purpose. In addition, the metabolic properties of these organelles make them highly mutagenic environments, promoting the genomic decay due to Muller's ratchet (Rand, 2001). This theory holds that uniparental inheritance (homoplasmy) creates non-combined asexual lineages, which accumulate deleterious mutations more rapidly than their sexual counterparts (Muller, 1964; Felsenstein, 1974; Gordo and Charlesworth, 2000). Individuals carrying a greater number of these mutations will have a low fitness, will produce fewer offspring and their genes will eventually disappear from the population. The same is

applicable at the level of the "organelle population" within a cell or individual. Purifying selection is an important mechanism for eliminating these deleterious mutations, especially in the germline cells (Palozzi *et al.*, 2018).

Traditionally, mitochondria have been considered to be transmitted only by maternal via. However, currently many exceptions to this general rule have been found (Gyllensten *et al.*, 1991; White *et al.*, 2008; Nunes *et al.*, 2013; Williams *et al.*, 2017). The coexistence of several DNA molecules within a mitochondrion or cell (heteroplasmy), added to the probability of recombination, is another way to escape from Muller's ratchet (Loewe, 2006).

For the spider crab, we detected heteroplasmy in both sexes and different tissues. Heteroplasmy for the COI gene was found in 36% of female gonadal tissues. In addition, part of the descendants from heteroplasmic females showed the same mitochondrial sequences as their mother. Considering these data, it is possible that heteroplasmy is inherited by maternal via, although bottlenecks during gametogenesis and embryogenesis and genetic drift play an important role in selecting the mitochondrial sequences that persist in the offspring (Hauswirth and Laipis, 1985; Bergstron and Pritchard, 1998). Despite this fact, our data and those from previous studies support the hypothesis of paternal leakage as the main cause of the coexistence of several mtDNA molecules in individuals of *M. brachydactyla*.

Multiple investigations showed that the mechanisms of elimination of paternal mitochondria can fail in hybrids from interspecific crosses, leading to paternal leakage (Kondo *et al.*, 1990; Kaneda *et al.*, 1995; Shitara *et al.*, 1998; Sherengul *et al.*, 2006; Dokianakis and Ladoukakis, 2014). In hybrids of periodical cicadas it was reported that paternal mitochondria are not only not eliminated, but also capable of proliferating throughout embryonic development to reach large numbers in the most advanced stages (Fontaine *et al.*, 2007).

On the other hand, several eastern Atlantic species have been recorded in the Alboran Sea (western Mediterranean Sea). This has been related to the entry of less dense Atlantic surface waters into the Mediterranean Sea through the Strait of Gibraltar (Abelló *et al.*, 2002; García-Muñoz *et al.*, 2008; García Raso *et al.*, 2014). This contact area between these two seas is a potential region for hybridisation between phylogenetically close species (Clark *et al.*, 2001).

The classification of *Maja squinado* and *Maja brachydactyla* as distinct species was recently accepted due to morphological and molecular studies that support it (Neumann, 1996, 1998; Sotelo *et al.*, 2008b). However, as they are anatomically very similar species that

recently diverged (in the late Miocene; Sotelo *et al.*, 2009), and whose distribution ranges overlap in the area of the Strait of Gibraltar and the Alboran Sea (Abelló *et al.*, 2014), the hypothesis of hybridisations between these two spider crabs seems very plausible. Previously, the possibility of hybrids between *M. brachydactyla* and its congeners *M. squinado*, in the Alboran Sea (Abelló *et al.*, 2014), and *M. cornuta* (previously *M. capensis*), on the South African coast (Sotelo *et al.*, 2009), had already been suggested.

Although the overlapping zone between *M. brachydactyla* and *M. squinado* is restricted to the south of the Iberian Peninsula, our results show that the influence of the heteroplasmy reaches the Galician coasts. Larval dispersal is the main way in which gene flow occurs in this species, since the numerous spider crab larvae spend 2-3 weeks in the water column being drifted by marine currents (Iglesias *et al.*, 2002; Guerao *et al.,* 2008). In particular, the west coast of the Iberian Peninsula is under the influence of the Iberian Poleward Current (Frouin *et al.*, 1990; Haynes and Barton, 1990), which flows northwards along about 1500 km of the coasts of the Iberian Peninsula and southeastern France. This current favours the genetic flow between the southern and northern spider crab populations of the Atlantic coast of the Iberian Peninsula. In addition, adult individuals are capable of moving up to 250 km along the coast (Corgos *et al.*, 2011).

Anthropic activity is another factor that may promote the transit of individuals between populations. Many shellfish farms import individuals from other regions and their larvae are released into the natural environment with water renewals (Sotelo *et al.,* 2008a). However, Galician shellfish farms usually import spider crabs from countries further north (France, Ireland, etc.), so the arrival of mtDNA of *M. squinado* to Galician coasts due to human activity is unlikely. Even assuming that exports from the south of the Iberian Peninsula or the Mediterranean occur, the proportion of the released larvae that would reach the adult stage would be very low, so the detection of this effect in the mitochondrial sequences of Galician spider crabs seems improbable.

Despite the high connectivity of the *M. brachydactyla* populations (Sotelo *et al.*, 2008a, Corgos *et al.*, 2011), the probability that a hybrid spider crab from the area of the Strait of Gibraltar reaches the Galician coast in a single generation is still remote. Therefore, the most probable hypothesis is that the hybrids resulting from mating between *M. squinado* and *M. brachydactyla* are viable, and that the mitochondrial sequences typical of the Mediterranean spider crab reached the Galician coasts after several generations.

If this hypothesis is correct, it would be expected a gradient in the incidence of heteroplasmy along the distribution range of *M. brachydactyla*, whose effect would be diluted as moving

away from the Strait of Gibraltar. The analysis of mitochondrial sequences of Irish spider crabs also supports this hypothesis. A much lower frequency of heteroplasmy was found in Ireland (5.4%) than in Galicia (26.5%).

Paternal leakage challenges some of the assumptions made when using mtDNA as a molecular or forensic marker (Budowle *et al.*, 2003; Williams *et al.*, 2017). Biparental mitochondrial inheritance, followed by recombination, can complicate phylogenetic reconstruction and molecular dating (Posada and Crandall, 2002; Piganeau *et al.*, 2004). Rubinoff *et al.* (2006) suggested that heteroplasmy and recombination in mtDNA limit the usefulness of mitochondrial markers in DNA barcoding studies, and that, in most cases, the use of these markers as the only resource for species determination and identification is not reliable. Although in some cases the assumption of haploidy for the mitochondrial genome may not interfere with mitochondrial marker analyses, this will depend on the degree of heteroplasmy in the study organism.

A study in *Portunus pelagicus* using mitochondrial markers showed that the presence of heteroplasmy did not affect the identification of families and species (Koolkarnkhai *et al.*, 2019). Based on this information and on several morphometric studies, the molecular separation between *M. brachydactyla* and *M. squinado* using the COI gene (Sotelo *et al.*, 2008b) is probably correct. However, heteroplasmy in *M. brachydactyla* mitochondria could complicate analyses or lead to misinterpretation of results in many other studies using mitochondrial markers.

Implications of multiple paternity and heteroplasmy on effective population size

The effective population size (N_e) is an important concept for the conservation and management of exploited stocks. The effective population size is defined as the size of an ideal population whose genetic composition is influenced by random processes in the same way as the real population (Wright, 1931; Lande and Barrowclough, 1987). Multipaternity and heteroplasmy are two genetic aspects closely related to this parameter.

The effect of multiple paternity on effective population size is not without controversy. Numerous theoretical and empirical studies argue that multiple paternity has a positive effect on the N_e (Murray, 1964; Sugg and Chesser, 1994; Morán and García-Vázquez, 1998; Martínez *et al.*, 2000; Pearse and Anderson, 2009; Rafajlović *et al.*, 2013; Vulstek *et al.*, 2013; Perrier *et al.*, 2014), while others state that the effect is the opposite (Waite and Parker,

1997; Karl, 2008) or that it is variable (Lotterhos, 2011). These discrepancies lie in the effect that multiple paternity has on the variance in male reproductive success, a factor inversely related to N_e.

Nunney (1993) reported that effective population size is strongly influenced by the generation time, the mating system and the sex ratio of the population. In populations where the sex ratio is 1:1, the variance in female reproductive success is 0 and there is perfect monogamy (each male mates with only one female and vice versa), the variance in male reproductive success is also 0 (Pearse and Anderson, 2009). However, these conditions do not usually occur in natural populations.

According to Lotterhos (2011), the effect of multiple paternity on N_e depends on how the generation time, the mean and variance in offspring production, the frequency of multiple matings and the distribution of paternity within a brood affect variance in the male reproductive success. It has been suggested that, when the contribution of different sires to brood fertilisation is similar, multiple paternity is likely to increase or help to maintain N_e (Balloux and Lehmann 2003; Zbinden *et al.*, 2007). Conversely, competition between males can lead to varying male reproductive success (Lotterhos, 2011), with some males contributing many offspring to the next generation and others contributing little or none. Therefore, the more pronounced the competition, the more the variance in male reproductive success will increase and the more N_e will decrease.

For females, multiple paternity results in increased reproductive success (Snyder and Gowaty, 2007; Rodríguez-Muñoz *et al.*, 2010), especially in populations susceptible to sperm limitation. In addition, females with sperm from several males in the seminal receptacles or with multiple-sired broods help increase the N_e after a bottleneck, since they represent the genes of more than one individual (Murray, 1964).

In natural populations of *Maja brachydactyla*, the sizes of the adult individuals fit to a Poisson distribution, so the extreme sizes, less abundant, are the most susceptible to disappear due to overfishing. Mature individuals of small sizes are protected by the minimum legal landing size, but large individuals, which are primarily males, are removed in great amounts from stocks. This reduction in the number of large males may have several consequences: an imbalance in the sex ratio, a decrease in the average size of the population and a narrowing of the size range, a reduction in the competition between males and a reduction in the reproductive success of females. Furthermore, our results show that in spider crab broods fertilised by multiple males, the contribution of the different sires is usually not too far from equality. Under these circumstances, it is probable that multiple

paternity emerge in populations with selective fishing as a mechanism to increase genetic diversity and N_e, as well as to avoid sperm limitation.

Mitochondrial heteroplasmy can also influence the effective population size when estimates are made from mtDNA (Williams *et al.*, 2017). Compared with nuclear markers, a mtDNA marker has smaller effective population size (a quarter of that of the bisexually inherited diploid nuclear genome if the sex ratio is balanced). That makes mtDNA more responsive to reductions in genetic variability due to bottlenecks (Birky *et al.,* 1983).

However, when uniparental inheritance of mitogenomes is assumed in species with heteroplasmy, N_e is overestimated. In presence of paternal leakage, the use of nuclear markers facilitates a correct estimation of the effective population size. Within nuclear markers, microsatellites are considered a better choice than SNPs for detecting recent demographic events (Haasl and Payseur, 2011), such as reductions in effective population size, recent dispersal events, and parentage (Luikart and England, 1999), due to their higher mutation rate. The microsatellite markers developed in this paper, together with those previously designed by Sotelo *et al.* (2007), are a useful tool for conducting this type of studies.

Sotelo *et al.* (2008a) provided approximate values of N_e for NE Atlantic spider crab populations using both mitochondrial and nuclear (microsatellites) markers. Mitochondrial markers provided N_e values of around 150,000 individuals, while the estimate from nuclear markers was around 2,000 individuals. For the Golfo Ártabro (A Coruña, Galicia), N_e values were 252,500 from mtDNA and 2,320 using microsatellite markers.

Sotelo *et al.* (2008a) highlight that these estimates are very rough, due to the lack of temporal samples and the low number of individuals sampled in some of the populations. Moreover, the weak genetic structure found in the spider crab populations means that the estimates represent more like the N_e of the metapopulation (hence the similarities found between the N_e of the different populations). The fact that the estimates using mtDNA are based on a single locus and a single summary statistic may also make them not completely reliable. However, the great difference between the values obtained using mitochondrial and nuclear markers could be due to the existence of heteroplasmy in the mitochondria of *M. brachydactyla*, which causes N_e to be overestimated.

Contributions of this thesis to the management of the exploited stocks of *Maja brachydactyla* in Galicia

On the Galician coasts, where the exploitation of the spider crab is very intense, the prohibition on landing ovigerous females means that fishing is focused on males for much of the year. Faced with this situation, the females may present problems to fertilising completely their broods. However, it has been observed that Galician females from wild are capable of fertilising several broods without the need of new copulations, which rule out the existence of sperm limitation in these populations. This fact could be explained by the high prevalence of genetic polyandry found in Galicia.

Another fact that contrasts with the intense selective fishing practised on the Galician coast are the high levels of genetic diversity found in its stocks. Sotelo *et al.* (2008a) analysed nuclear and molecular markers in samples from A Coruña (Galicia) and they recorded high levels of genetic diversity. In the present study, we have reached similar conclusions analysing other microsatellite markers. The low FIS values obtained for the Golfo Ártabro suggest that there are no inbreeding in this population. Furthermore, we do not find great differences in terms of allelic richness between the Golfo Ártabro, a highly exploited population, and Carna, a population hardly exploited. This high genetic diversity, both mitochondrial and nuclear, in individuals from the NW of the Iberian Peninsula could be favoured by mitochondrial heteroplasmy and genetic polyandry, respectively.

Another mechanism that helps to conserve the genetic diversity of spider crab populations is the high connectivity that exists between them (Corgos *et al.*, 2011). Sotelo *et al.* (2008a) reported that spider crab stocks are interconnected forming a large metapopulation along the NE Atlantic coast. Our data on heteroplasmy, which suggest that the genetic influence of hybrids from the area of the Strait of Gibraltar reaches the north of the Iberian Peninsula, also support this hypothesis of a high population connectivity. The contribution of individuals from less exploited areas, both in the form of larval dispersal and adult migrations, could be compensating for the bottleneck caused by overexploitation.

These three mechanisms are probably responsible for the effects of overfishing in terms of sperm limitation and genetic diversity are still not detectable in the Galician spider crab populations. Multiple paternity would introduce genetic variability at nuclear level and heteroplasmy at mitochondrial level, increasing the individual fitness, the N_e and, ultimately, the health of the stocks.

However, if intense selective fishing continues, it will lead to the gradual reduction of stocks and increasingly evident imbalances in their size structure and sex ratio, reaching a bottleneck that would be unbridgeable even for these compensation mechanisms. A too low proportion of males would limit polyandry, resulting in a population where some females would not mate, while others would carry in their seminal receptacles insufficient sperm amount to fertilise all their broods.

For these reasons, it is urgent to review the regulation that prohibits fishing for ovigerous females in Galicia, as its long-term effects could lead to the collapse of populations. The metapopulation condition already described by Sotelo *et al.* (2008a) for the *Maja brachydactyla* stocks is another factor that should be considered for the management of this species.

6

Conclusions and future perspectives

Conclusions

Main conclusions of Chapter 2: Optimisation of DNA extraction in the spiny spider crab *Maja brachydactyla*

- The use of commercial kits of silica columns is the only method with a high success rate for DNA extraction from individual eggs of *M. brachydactyla* among all the tested methods.

- The best embryonic developmental stage for obtaining quality DNA from spider crab eggs is the stage B. There are no significant differences between the amount of DNA obtained from lyophilised eggs and those preserved in ethanol, but the use of the second method is recommended because of its greater immediacy and simplicity.

- Both pleopod and pereiopod setae provide a good alternative for DNA extraction without slaughtering or damaging the specimens, being especially interesting for endangered decapod species.

Main conclusions of Chapter 3: Differences in prevalence of multiple paternity between two populations of *Maja brachydactyla* with different exploitation levels and management measures

- Multiple paternity has been detected for the first time in *M. brachydactyla*, with an average prevalence of 43.5%, similar to that of other brachyuran species.

- Maternal size does not affect the presence or absence of multiple paternity in spider crab broods (however, this lack of correlation could be due to an insufficient sample size).

- The contribution of the different sires to the fertilisation of the broods showed a moderate skew (mean contribution of the single male = 66%) compared to that of other brachyuran species.

- There are not an organised patron of the paternal alleles in the multiple-sired broods of *M. brachydactyla*, which indicates that there are sperm mixing within the seminal receptacles, egg mixing during the oviposition or both of them.

- The prevalence of multiple paternity varies strongly between populations, being almost seven times higher in the Golfo Ártabro (Galicia, NW Spain), a highly exploited area, than in Carna (Co. Galway, western Ireland), a hardly exploited area.

Main conclusions of Chapter 4: High incidence of heteroplasmy in the mtDNA of a natural population of the spider crab *Maja brachydactyla*

- Mitochondrial heteroplasmy in spider crabs from the NW Iberian Peninsula was detected. This fact questions the reliability of using mtDNA as a molecular marker in phylogenetic and population dynamics studies.

Future perspectives

In summary, this thesis provides valuable information on crucial genetic aspects to the management of the spider crab *Maja brachydactyla* in Galicia and other areas where male-selective fishing occurs. As a result of the findings presented here, new questions have arisen and further research along several aspects would be useful:

- To determine if maternal size has an effect on the number of sires fertilising a brood, by expanding the sample size covering the entire size range of the female population.

- To establish the possible effect of paternal size and order of mating on the paternity of the broods, by genotyping sperm masses and eggs from matings in captivity.

- To identify the cause of the differences in paternity found between the Golfo Ártabro (Galicia, NW Spain) and Carna (Co. Galway, western Ireland) by analysing broods from the Magharees area (SW Ireland). This region share characteristics with the Golfo Ártabro (strong selective fishing) and Carna (one brood per annual cycle), so the results will reveal if the high prevalence of multiple paternity in Galician spider crabs is due to the removal of males from the population or to higher sperm requirements of females from low latitudes.

- To study the strength of natural selection throughout embryonic and larval development by performing paternity tests on eggs, zoeas and megalopes.

- To check if the cause of the heteroplasmy found in *M. brachydactyla* is the paternal leakage due to hybridisation with *M. squinado* in the contact zone of both species, by analysing the possible gradient of the incidence of heteroplasmy along the European coast.

- To check the mating compatibility between *M. brachydactyla* and *M. squinado* and the viability of the hybrids, by conducting interespecific matings in captivity and culturing the resulting larvae, if applicable.

7

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A

Research articles

Chapter 2

Optimization of DNA extraction in the spiny spider crab *Maja brachydactyla*: determining the best extraction method, sample conservation and starting tissue

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TECHNICAL NOTE

Optimization of DNA extraction in the spiny spider crab *Maja brachydactyla*: determining the best extraction method, sample conservation and starting tissue

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Abstract Methodological studies are an important previous step in molecular research. We optimize a method for DNA extraction from eggs and a noninvasive extraction method for adult tissues of Maja brachydactyla. Our results show that lyophilized eggs in B developmental stage are the most suitable for DNA extraction. Regarding adult tissues, we propose setae as an alternative tissue for DNA extraction, since prevents mutilation of specimens, which avoids infections and fitness reduction. That is especially relevant in studies with endangered species of crustaceans.

Keyword Crab · Maja · DNA extraction method · Egg developmental stage · Sample conservation · Sampling without death

Methological studies focused on improving DNA extraction and sample storage (Bitencourt et al. 2007; Palero et al. 2010) are an important previous step in multiple molecular studies. In the present study we optimized the DNA extraction process from embryonic and adult tissues of *Maja brachydactyla* (Balss 1922), a decapod crustacean present in the northeastern Atlantic (Neumann 1998; Sotelo et al. 2009). We analyzed different extraction methods, sample conservation, type of adult tissue, and the developmental stage of the eggs. We consider the three macroscopically distinguishable stages described by González-Gurriarán et al. (1998): A (orange eggs, much yolk), B (brown eggs, visible eyes) and C (dark eggs, many cromatophores, hardly yolk).

We tested several DNA extraction methodologies in fresh and stored in ethanol eggs of ten females from Golfo Ártabro and Ría de Arousa (Galicia, NW Spain). These methods included incubation in 10% bleach, heat shock, mechanical methods (cutting, puncture or homogenization), extraction with Chelex 100 (Bio-Rad), hypotonic shock and extraction with the NucleoSpin Tissue kit (Macherey–Nagel). DNA was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and we amplified the 16S gene to check its quality. We used the primers 16L29-F and 16HLeu-R (Schubart 2009), and the PCR was performed with the NZYTaq 2× Green Master Mix kit, separate $MgCl_2$ (Real Laboratory) in a volume of 25 µL. Each reaction contained 25 ng of DNA, 12.5 µL of Green Master Mix, 4 mM of $MgCl_2$ and a concentration of 0.5 µM for every primer. The PCR consisted in a denaturalization of 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 53.1 °C and 45 s at 72 °C, and a final extension of 5 min at 72 °C. The amplification success was tested by electrophoresis and a gel of genomic DNA was performed to corroborate the data obtained by spectrophotometry. We only achieve to amplify the target gene with the DNA obtained by mechanical homogenization and using the commercial kit, as well as with some combinations of these methods with the others. However, the kit had the highest success rate.

We also tested the best developmental stage of eggs and sampled conservation method for DNA extraction in six *M. brachydactyla* females from the Ría de Arousa. We took two ovigerous females for each egg stage and, for each

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spawn, we analyzed two replicas of frozen eggs, two of lyophilized eggs and two of eggs conserved in absolute ethanol. The DNA of these eggs was extracted using the NucleoSpin Tissue kit. Concentration and quality were tested by spectrophotometry and electrophoresis. The statistical analysis showed that B stage eggs provide the higher DNA concentration. However, the ANOVA only detected statistically significant differences between the amount of DNA obtained from lyophilized and frozen eggs, being lyophilized eggs which provided more DNA.

We also took samples of pleopod setae, pereiopod setae and an endite of nine females from Golfo Ártabro. The DNA of these samples was extracted using the NucleoSpin Tissue kit, and the DNA concentration and quality was checked as in the test of the conservation method. The ANOVA showed that endites and pleopod setae provide a higher DNA concentration than pereiopod setae. However, we achieved to amplify the target gene from the three tissues.

Although we expected C stage to be the best developmental stage for DNA extraction, we found B stage to provide more DNA. A possible reason is the presence of substances that interfere in the extraction or hard structures that obstruct the access to the DNA in C stage eggs.

We expected freezing to be the most suitable conservation method for the posterior DNA extraction. Nevertheless, we got a higher quantity of DNA from lyophilized eggs and eggs in ethanol. One possible reason is that part of the DNA was lost during egg handling. Lyophilization and conservation in ethanol induce tissue hardening which facilitate handling. Another advantage is that storage in ethanol does not require special equipment, and DNA quality is similar that in the case of lyophilized eggs.

The most used tissue for DNA extraction in crustaceans is the muscle (McKeown and Shaw 2008; Baggio et al. 2011). However, this study aims to find alternative tissues which avoid the specimen death. This enables to continue researching with the same specimens or even the specimens' liberation after the sampling. Moreover, DNA extraction from structures that prevent the specimens' mutilation, reduces the probability of infections caused by the sampling and avoid fitness decrease. Therefore, we propose setae as an alternative tissue for DNA extraction. In females, the best tissue is indisputably the pleopod setae. Nevertheless, males pleopods are transformed into copulatory appendages, so DNA cannot be extracted from pleopod setae. Furthermore, the extraction of endites from a live specimen is complicated. So, pereiopod setae seem to be a good alternative for DNA extraction in males. From these structures we obtained enough DNA to amplify and sequence the 16S gene of *M. brachydactyla*, which indicates a good quality of the DNA. These setae have a sensorial function (Laverack 1988) and play a role in the camouflage (Fürböck and Patzner 2005). Nevertheless, sampling would involve such a small area that hardly affects crabs life. Thereby, the utilization of setae for DNA extraction enables to return the specimens to the marine environment. This is especially relevant for sampling in endangered species of crustaceans, since it allows obtaining genetic information without affecting natural populations.

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Chapter 4

High incidence of heteroplasmy in the mtDNA of a natural population of the spider crab *Maja brachydactyla*

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RESEARCH ARTICLE

High incidence of heteroplasmy in the mtDNA of a natural population of the spider crab *Maja brachydactyla*

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Abstract

Mitochondria are mostly inherited by maternal via, that is, only mitochondria from eggs are retained in the embryos. However, this general assumption of uniparentally transmitted, homoplasmic and non-recombining mitochondrial genomes is becoming more and more controversial. The presence of different sequences of mtDNA within a cell or individual, known as heteroplasmy, is increasingly reported in several taxon of animals, such as molluscs, arthropods and vertebrates. In this work, a considerable frequency of heteroplasmy were detected in the COI and 16S genes of the spider crab *Maja brachydactyla*, possibly associated to hybridisation with the congeneric species *Maja squinado*. This finding is a fact to keep in mind before addressing molecular analyses based on mitochondrial markers, since the assumption of maternal inheritance could lead to erroneous results. As *M. brachydactyla* is a commercial species, heteroplasmy is an important aspect to take into account for the fisheries management of this resource, since effective population size could be overestimated.

Introduction

Mitochondria are mostly inherited by maternal via, that is, only mitochondria from eggs are retained in the embryos. Uniparental transmission of cytoplasmic genomes has had multiple and independent origins and it must involve a strong evolutionary advantage over biparental transmission [1, 2]. However, this general assumption of uniparentally transmitted, homoplasmic and non-recombining mitochondrial genomes is becoming more and more controversial [3–5]. Until now, the presence of different sequences of mtDNA within a cell or individual was a rare phenomenon in animals, but new detection methods based on NGS (Next-Generation Sequencing) or qPCR (quantitative Polymerase Chain Reaction) are allowing their detection [6–9]. Nowadays, heteroplasmy has been reported in many organisms such as insects [10, 11], crustaceans [12, 13], molluscs [14], fishes [15, 16], frogs [17] birds [18, 19], mice [20] and humans [21, 22], being the paternal leakage the primary cause of it. The existence of heteroplasmy in the studied organisms can provide erroneous results in researches that use mitochondrial markers. This factor must be taken into consideration before interpreting the obtained results. For example, the assumption of maternal mitochondrial inheritance in

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species with multiple mtDNAs per individual can result in an estimation of the effective population size larger than it really is as well as create false negatives in lineage determination.

There may be five main reasons for the presence of heteroplasmy [23]:

i. occurrence of a *de novo* mutation in somatic line or germ line cells. In the first case, heteroplasmy is not transmitted to the offspring, while in the second case, it is. Studies in the crustacean *Daphnia pulex* estimate the frequency of *de novo* mutations at 1.63×10^{-7} / site / generation [24].

ii. recombination events. However, despite the high number of mitochondrial molecules that exist in an individual, the possibility of being able to detect this type of heteroplasmy is limited since its frequency is usually very low. In humans, recombination intermediates were detected in the mtDNA, varying their abundance between tissues (high in heart, intermediate in skeletal muscle or placenta, but low or absent in cultured cells) [25].

iii. paternal leakage as it has been reported in fruit fly [11] and hen [26]. There are several mechanisms for preventing the transmission of paternal mtDNA to offspring in animals: (a) degradation of paternal mtDNA before or after fertilisation, (b) blocking paternal mitochondria from entering the oocyte, (c) elimination of paternal mitochondria by autophagy and/or ubiquitin-proteasome systems and (d) uneven distribution of paternal mitochondria with remaining paternal mtDNA during embryogenesis [27]. In the nematode *Caenorhabditis elegans*, paternal mitochondria and their mtDNA degenerate almost immediately after fertilisation and are selectively degraded by 'allophagy' (allogeneic [non-self] organelle autophagy) [28]. In the fruit fly *Drosophila melanogaster*, paternal mtDNA is largely eliminated by an endonuclease G-mediated mechanism. Paternal mitochondria are subsequently removed by endocytic and autophagic pathways after fertilisation [29]. The reason of the existence of mechanisms to prevent paternal mtDNA transmission is not well understood yet. The most plausible explanation is that the retaining of two different, but individually fully functional, mtDNAs within a cell can cause mitochondrial dysfunction due to a potentially lethal genome conflict [30, 31]. Nevertheless, it has also been argued as an adaptation to anisogamy, which prevents sperm mtDNA, damaged from intense respiration activity, entering the egg [32]. Nevertheless, in some cases, a breakdown of mechanisms to recognise and remove paternal mtDNA may occur resulting in paternal leakage.

iv. biparental inheritance. The concepts of parental leakage and biparental inheritance may not be clearly delimited, depending on the definition of the second one. While some authors consider incidental paternal leakage constitutes a form of biparental mtDNA transmission, Breton and Stewart [23] define "true" biparental transmission as the systematic transfer of mitochondrial genomes from both parents (or two different mating types) to zygotes as part of normal reproductive processes within a species, followed by the persistence of both parental mitochondrial types throughout development. So far, following this narrow definition, it has not been reported any organism that present this mode of biparental mtDNA inheritance.

v. doubly uniparental inheritance (DUI), typically observed in some bivalvian molluscs [2, 33–36]. Many bivalves have a sperm-transmitted mitochondrial genome (M), along with the standard egg-transmitted one (F). During embryonic development of the mussel *Mytilus*, sperm mitochondria disperse randomly among blastomeres in females, but form an aggregate in the same blastomere in males. Consequently, in adults, somatic tissues of both

sexes are dominated by the F mitochondrial genome, sperm contains only the M genome, and eggs contain the F genome (and perhaps traces of M).

In addition, events of duplications of some mitochondrial genes [33] or the formation of nuclear-encoded, mitochondrial pseudogenes (NUMTs) [33, 37] may also be interpreted erroneously as heteroplasmy. NUMTs are described as a transposition of mitochondrial DNA into the nuclear genome that can retain close homology to the original mitochondrial genes [38].

The spiny spider crab *Maja brachydactyla* (Balss 1922) [39] is a decapod crustacean of the Majidae family [40,41] very common on the European Atlantic coast. This species was initially classified within the species *Maja squinado* (Herbst 1788) [42], but in recent years morphological [43, 44] and molecular [45] differences between the Atlantic and Mediterranean populations have confirmed that it is a different species. After this separation, its distribution is considered to be limited to the Eastern Atlantic, from the South of the North Sea to South Africa, including the islands of Madeira, Azores, Canary Islands and Cape Verde [44, 46–49].

This species presents a complex life cycle, with planktonic larval and benthic post-larvae phases, which determines that its distribution and population dynamics are closely related to larval dispersion processes, mediated by physical factors, as well as habitat selection, linked to movements and migrations in post-larval stages [50, 51]. The life cycle of *M. brachydactyla* consists of three main phases: larval phase, juvenile or growth phase, and adult or reproductive phase. The planktonic larval phase lasts from two to three weeks and consists of two zoeal stages (zoea I and II) and one megalopa stage [52, 53]. The juvenile phase lasts from two to three years [54], inhabiting shallow bottoms (<15m) where they perform limited, non-directional, small-scale movements of less than 10 m per day [47, 55, 56]. Their growth occurs through successive moults and, after terminal moult, which takes place in spring and summer, individuals reach sexual maturity and begin the adult phase.

M. brachydactyla is a species of high commercial interest in several countries, especially in Spain. Galicia (a region of NW Spain), with around 300 vessels devoted to spider crab fishing, represents the main exploitation region of the country, both in terms of production (73%) and incomes (more than 80%). In 2018, the captures of *M. brachydactyla* reached the 525934 kg in Galician coast, which represented around 4640000 euros in sales [57].

During the course of a study with mitochondrial molecular markers in *M. brachydactyla*, double peaks were detected in some of the electropherograms obtained. The studies by Abelló *et al.* [58] already suggested the possible existence of heteroplasmy in this species. The present work confirms the existence of heteroplasmy in the spiny spider crab and delves into this aspect to determine the magnitude and possible origin of this phenomenon.

Material and methods

Specimens of *M. brachydactyla* were captured in two locations of the Galician coast, NW Spain: Golfo Ártabro (43˚25'N 8˚21'W) and Ría de Arousa (42˚33'N 8˚54'W). Most of the spider crabs were captured by the scuba divers of the Aquarium Finisterrae of A Coruña (Spain) with a permit emitted by the Consellería do Medio Rural e do Mar of the Xunta de Galicia (regional government, resolution of 18/02/2014). The rest of the specimens were directly bought at the fish market. Since *M. brachydactyla* is a commercial species, no protected species were sampled during this study, and sample collection has not been conducted in a protected or privately owned area. All the specimens were anesthetized at -20˚C for 10 min before being slaughtered. Regarding the Golfo Ártabro, pereiopod muscle from 27 males and pleopod setae from 23 females were collected. Fertilised eggs from 11 of these females were also sampled (2–17 eggs depending on the female). Furthermore, different tissues and organs from four of these females from Golfo Ártabro (pleopod setae, integument, eye, pereiopod muscle, gonad,

heart, nervous tissue, hepatopancreas, stomach, gill, intestine, pereiopod setae and mouthparts) were collected. In addition to this, gonadal tissue were sampled from 33 females from Ría de Arousa. All samples were preserved in absolute ethanol until the time of DNA extraction. The sampling of individuals of both sexes and broods of known females was performed in order to determine the origin and the inheritance mechanism of the heteroplasmy in *M. brachydactyla*. The analysis of different tissues of the same individual provides information about the distribution of the mtDNA sequences in early development stages and the extension of the heteroplasmy in the different tissues in adults.

DNA extraction was conducted using the NZY Tissue gDNA Isolation kit (nzytech, Portugal). All the laboratory material used in this process (scissors, tweezers, pestles) was washed in absolute ethanol between consecutive samples. Two different mitochondrial DNA gene fragments were amplified by PCR: the mitochondrial 16S rDNA and the cytochrome c oxidase subunit I (COI). While COI was analysed in all the samples, 16S was only sequenced for the 11 ovigerous females from Golfo Ártabro and their eggs. The PCR conditions for the amplification of 16S rDNA were described in Rodríguez-Pena *et al*. [59]. COI fragment was amplified using the primers COIMaja_F 5´-gaatggccggaacatcttta-3´, and COIMaja_R 5´-ccaccagctggatcaaagaa-3´ and the NZYTaq 2xGreen Master Mix separate $MgCl_2$ kit (NZYTech) according to the manufacturer´s protocol in a final concentration of 1.5 mM of $MgCl_2$ and 0.5μM of each primer. The PCR consisted in a denaturalization step of 5 min at 94˚C, 35 cycles of 30 s at 94˚C, 30 s at 48.5˚C and 60 s at 72˚C, and a final extension of 5 min at 72˚C. All PCRs yielded single-band patterns, so that the resultant amplicons were directly sequenced in both directions.

Some of the PCR products of 16S rDNA were cloned using StrataClone PCR Cloning Kit according to the manufacturer´s protocol in order to determine the number of nucleotide combinations present in heteroplasmic samples. Cells were spread on an LB-ampicillin plate and let it grow overnight at 37˚C. Transformant colonies were selected, and insert size was checked by PCR. Plasmids were purified using the NZYMiniprep kit (nzytech, Portugal), and they were sequenced using the M13 forward and reverse primers. The PCR consisted in a denaturalization step of 5 min at 94˚C, 35 cycles of 60 s at 94˚C, 60 s at 55˚C, 60 s at 72˚C, and a final extension of 5 min at 72˚C.

The sequencing profiles were examined using BioEdit 7.0.9.0 [60]. In order to detect the presence of double peaks in the PCR products, reads in both directions were checked. We repeated the DNA extractions, amplifications and sequencing to verify these double peaks were not due to errors in the PCRs. The frequency of each nucleotide was estimated as the average of the peaks of the two reads. From the unambiguous sequences, a consensus sequence was established. Next, all the amplicons were aligned using the tool Muscle [61] of MEGA 6.06 [62]. The variable sites, the haplotypes and their frequencies were calculated using the DnaSP 6.10.04 [63] and the mitochondrial genetic code of *Drosophila*.

Results

The sequence alignments of 16S mtDNA allowed us to detect the existence of three variable sites: 365, 442 and 580. Two of them are parsimony informative (365 and 580), while 442 is a singleton. Table 1 (GenBank accession numbers: MN006155—MN006157) shows the three different haplotypes detected. We identified double peaks in the electropherograms of 16S mtDNA of the following adult females: MbraAG-M26 (at positions 365 and 580), MbraAG-P6 and MbraAG-5IN (at position 365), and MbraAG-P7 (at position 580) (Table 2 and Fig 1). After repeating DNA extractions, PCRs and sequencing, we detected again these ambiguous sites at the same positions of the same samples.

Table 1. Detected haplotypes for the 16S gene and adult individuals in which they were observed.

Haplotype	N	Individual			
Hap-16S-1	8	MbraAG-M2-pleopod	MbraAG-M3-pleopod	MbraAG-P7-pleopod	MbraAG-P8-pleopod
		MbraAG-3IN-pleopod	MbraAG-4DN-pleopod	MbraAG-5IN-pleopod	MbraAG-G6-pleopod
Hap-16S-2	1	MbraAG-2DN-pleopod			
Hap-16S-3	2	MbraAG-M26-pleopod	MbraAG-P6-pleopod		
Total	11				

N: number of haplotypes. Sequences with double peaks were not included in this analysis.

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Regarding the fertilised eggs of these 11 ovigerous females, we detected heteroplasmy in all the eggs of MbraAG-P6, in five out of seven of MbraAG-P7 and in one out of two of MbraAG-5IN (Table 2) at least at one of the two positions (365 and/or 580). In addition to this, a double peak was also detected at position 365 of the MbraAG-2DN-1 egg, in whose mother no type of polymorphism were previously detected.

Due to the existence of double peaks at positions 365 and 580, two to four combinations of different mitochondrial molecules may be present. To clarify this aspect, the PCR products of the pleopod setae of the female MbraAG-M26, in which the double peaks were well-defined, were cloned and sequenced. In this case, it was detected that at least three different mitochondrial combinations are present in the pleopod setae of this female (Table 2).

The next step involved to perform a more extensive analysis of the degree of heteroplasmy in Galician populations. In order to achieve that, we used the COI sequences obtained from 50 adult individuals from Golfo Ártabro and 33 from Ría de Arousa to establish a consensus sequence, since the level of genetic variation in this mtDNA region is higher than in 16S rDNA. The electropherograms of the 83 studied individuals were checked in search of double peaks (Fig 2). After removing the sequences with ambiguous sites (double peaks), an alignment was performed with the remaining 61 sequences, which allowed to verify the existence of 26 haplotypes (Table 3, GenBank accession numbers: MN027519—MN027544). Thus, the existence of 25 variable sites was tested, of which 12 were singleton variable sites, while 13 were parsimony informative sites. No stop codons were detected in any case, being five the maximum number of synonym differences between two sequences and being four the maximum number of non-synonym differences.

The electropherograms of the 13 parsimony informative sites were analysed at four positions (240, 264, 303 and 375) because of their variability in the sequences and the production of synonymous changes. Thus, a total of 22 individuals (four males and seven females from the Golfo Ártabro, 11 females from the Ría de Arousa) showed double peaks at one of these four positions (Table 4 and Fig 3). After repeating the DNA extraction and COI amplification, double peaks were detected at the same positions. These double peaks were only taken into consideration when the electropherograms were well-defined and there was a clear difference from the baseline. It should be highlighted that the two reads (forward and reverse) were not always identical in height due to the PCR yield may vary depending on the hybridisation of the primers and the polymerase activity. For the rest of the nine informative positions, no clear double peaks were detected and therefore they were not taken into consideration in the analyses.

The sequences of COI obtained from the broods of the females MbraAG-M26, MbraAG-P6 and MbraAG-P7 showed that a great majority of the eggs presented heteroplasmy for this gene (Table 5). All the eggs of the females MbraAG-M26 (eight eggs) and MbraAG-P7 (six eggs) presented double peaks at the some positions. In the case of the female MbraAG-P6, 9 of the 11 eggs showed ambiguous sites.

Table 2. Nucleotidic proportions (%) at positions 365 and 580 of the amplified mitochondrial 16S rDNA fragment for all the samples analysed.

Position		365		580	
Sample	Origin	T	C	T	A
MbraAG-M2-pleopod	female	0	100	100	0
MbraAG-M2-1	egg	0	100	100	0
MbraAG-M2-2	egg	0	100	100	0
MbraAG-M3-pleopod	female	0	100	100	0
MbraAG-M3-1	egg	0	100	100	0
MbraAG-M3-2	egg	0	100	100	0
MbraAG-M26-pleopod	**female**	**78.4**	**21.6**	**19.4**	**80.6**
MbraAG-M26-1	egg	0	100	100	0
MbraAG-M26-2	egg	0	100	100	0
MbraAG-M26-3	egg	0	100	100	0
MbraAG-M26-4	egg	0	100	100	0
MbraAG-M26-5	egg	0	100	100	0
MbraAG-M26-6	egg	0	100	100	0
MbraAG-M26-7	egg	0	100	100	0
MbraAG-M26-8	egg	0	100	100	0
MbraAG-M26-pleopod-C1	clone female	100	0	0	100
MbraAG-M26-pleopod-C2	clone female	0	100	100	0
MbraAG-M26-pleopod-C3	clone female	100	0	100	0
MbraAG-M26-pleopod-C4	clone female	100	0	0	100
MbraAG-M26-pleopod-C5	clone female	100	0	0	100
MbraAG-M26-pleopod-C6	clone female	100	0	0	100
MbraAG-M26-pleopod-C7	clone female	100	0	0	100
MbraAG-M26-pleopod-C8	clone female	100	0	0	100
MbraAG-M26-pleopod-C9	clone female	100	0	0	100
MbraAG-P6-pleopod	**female**	**93.5**	**6.5**	**0**	**100**
MbraAG-P6-mouth-part	female	100	0	0	100
MbraAG-P6-muscle	female	100	0	0	100
MbraAG-P6-setae	**female**	**12.2**	**87.8**	**80.6**	**19.4**
MbraAG-P6-1	egg	87.3	12.7	15.5	84.5
MbraAG-P6-2	egg	65.9	34.1	26.1	73.9
MbraAG-P6-3	egg	85.0	15.0	11.1	88.9
MbraAG-P6-4	egg	68.0	32.0	26.3	73.7
MbraAG-P6-5	egg	68.7	31.3	29.1	70.9
MbraAG-P6-6	egg	74.1	25.9	19.9	80.1
MbraAG-P6-7	egg	91.4	8.6	0	100
MbraAG-P6-8	egg	90.2	9.8	0	100
MbraAG-P6-9	egg	57.6	42.4	83.6	16.4
MbraAG-P6-10	egg	92.9	7.1	0	100
MbraAG-P6-11	egg	90.6	9.4	0	100
MbraAG-P6-12	egg	87.2	12.8	9.8	90.2
MbraAG-P6-13	egg	75.8	24.2	21.8	78.2
MbraAG-P6-14	egg	72.9	27.1	23.9	76.1
MbraAG-P6-15	egg	83.7	16.3	12.5	87.5
MbraAG-P6-16	egg	75.1	24.9	22.2	77.8
MbraAG-P7-pleopod	**female**	**0**	**100**	**43.5**	**56.5**
MbraAG-P7-1	egg	0	100	28.0	72.0

(*Continued*)

Table 2. (Continued)

Position		365		580	
Sample	**Origin**	**T**	**C**	**T**	**A**
MbraAG-P7-2	egg	0	100	0	100
MbraAG-P7-3	egg	0	100	57.9	42.1
MbraAG-P7-4	egg	0	100	64.5	35.5
MbraAG-P7-5	egg	0	100	35.7	64.3
MbraAG-P7-6	egg	0	100	19.8	80.2
MbraAG-P7-7	egg	0	100	0	100
MbraAG-P8-pleopod	female	0	100	100	0
MbraAG-P8-1	egg	0	100	100	0
MbraAG-2DN-pleopod	female	0	100	100	0
MbraAG-2DN-1	egg	16.7	83.3	100	0
MbraAG-2DN-2	egg	0	100	100	0
MbraAG-3IN-pleopod	female	0	100	100	0
MbraAG-3IN-1	egg	0	100	100	0
MbraAG-3IN-2	egg	0	100	100	0
MbraAG-4DN-pleopod	female	0	100	100	0
MbraAG-4DN-1	egg	0	100	100	0
MbraAG-4DN-2	egg	0	100	100	0
MbraAG-5IN-pleopod	**female**	**19.2**	**80.8**	**100**	**0**
MbraAG-5IN-1	egg	10.3	89.7	100	0
MbraAG-5IN-2	egg	0	100	100	0
MbraAG-G6-pleopod	female	0	100	100	0
MbraAG-G6-1	egg	0	100	100	0
MbraAG-G6-2	egg	0	100	100	0

Proportions were calculated by dividing the height of each peak in the electropherogram by the summation of the height of both peak. The eggs and the clones correspond to the female that precedes them in the list. All samples tagged as "pleopod" correspond to pleopod setae from adult females. The samples MbraAG-P6-pleopod, MbraAG-P6-mouth-part, MbraAG-P6-muscle and MbraAG-P6-setae, correspond respectively to pleopod setae, one endite, pereiopod muscle and pereiopod setae of the same female. Adult heteroplasmic individuals are indicated in bold.

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Regarding the amplifications of COI gene from different tissues of four females, electropherogram analyses showed that the presence of ambiguous sites is very variable among different tissues. Thus, while in the female MbraAG-4D2V the heteroplasmy is maintained in most of the tissues (except pleopod setae, pereiopod muscle, stomach and nerve), in the female MbraAG-5DV it is only observed in two of the ten tissues analysed (stomach and eye) (Table 5).

For the female P6, the muscle is homoplasmic for the 16S gene and the COI gene and the pleopod and periopod setae are heteroplasmic for both genes. Regarding the mouth-parts, heteroplasmy was detected only for the mtDNA of the COI gene. *A priori*, one would expect that the samples of pleopods and mouth-parts are mostly composed of muscular tissue. However, when sampling the pleopods, we collected only the setae that cover these structures. For this reason, it makes sense that the result obtained for the pleopods would be the same as for the pereiopod setae. On the other hand, the endite is a multi-tissular structure, so, after homogenization, a mixture of mtDNA from different tissues (homo or heteroplasmic in the case of the COI gene) could be present in sample.

```
                                365         442         580
MbraAG-M26-pleopod    : ATTTYTTTTG  CTAAGAGAGA  GATTWACCAG
MbraAG-M26-1          : ....C.....  ..........  ....T.....
MbraAG-M26-2          : ....C.....  ..........  ....T.....
MbraAG-M26-3          : ....C.....  ..........  ....T.....
MbraAG-M26-4          : ....C.....  ..........  ....T.....
MbraAG-M26-5          : ....C.....  ..........  ....T.....
MbraAG-M26-6          : ....C.....  ..........  ....T.....
MbraAG-M26-7          : ....C.....  ..........  ....T.....
MbraAG-M26-8          : ....C.....  ..........  ....T.....
MbraAG-M26-pleopod-C1: ....T.....  ..........  ....A.....
MbraAG-M26-pleopod-C2: ....C.....  ..........  ....T.....
MbraAG-M26-pleopod-C3: ....T.....  ..........  ....T.....
MbraAG-M26-pleopod-C4: ....T.....  ..........  ....A.....
MbraAG-M26-pleopod-C5: ....T.....  ..........  ....A.....
MbraAG-M26-pleopod-C6: ....T.....  ..........  ....A.....
MbraAG-M26-pleopod-C7: ....T.....  ..........  ....A.....
MbraAG-M26-pleopod-C8: ....T.....  ..........  ....A.....
MbraAG-M26-pleopod-C9: ....T.....  ..........  ....A.....
MbraAG-M2-pleopod     : ....C.....  ..........  ....T.....
MbraAG-M2-1           : ....C.....  ..........  ....T.....
MbraAG-M2-2           : ....C.....  ..........  ....T.....
MbraAG-M3-pleopod     : ....C.....  ..........  ....T.....
MbraAG-M3-1           : ....C.....  ..........  ....T.....
MbraAG-M3-2           : ....C.....  ..........  ....T.....
MbraAG-P6-pleopod     : ..........  ..........  ....A.....
MbraAG-P6-mouth-part  : ....T.....  ..........  ....A.....
MbraAG-P6-muscle      : ....T.....  ..........  ....A.....
MbraAG-P6-setae       : ..........  ..........  ..........
MbraAG-P6-1           : ..........  ..........  ..........
MbraAG-P6-2           : ..........  ..........  ..........
MbraAG-P6-3           : ..........  ..........  ..........
MbraAG-P6-4           : ..........  ..........  ..........
MbraAG-P6-5           : ..........  ..........  ..........
MbraAG-P6-6           : ..........  ..........  ..........
MbraAG-P6-7           : ..........  ..........  ....A.....
MbraAG-P6-8           : ..........  ..........  ....A.....
MbraAG-P6-9           : ..........  ..........  ..........
MbraAG-P6-10          : ..........  ..........  ....A.....
MbraAG-P6-11          : ..........  ..........  ....A.....
MbraAG-P6-12          : ..........  ..........  ..........
MbraAG-P6-13          : ..........  ..........  ..........
MbraAG-P6-14          : ..........  ..........  ..........
MbraAG-P6-15          : ..........  ..........  ..........
MbraAG-P6-16          : ..........  ..........  ..........
MbraAG-P7-pleopod     : ....C.....  ..........  ..........
MbraAG-P7-1           : ....C.....  ..........  ..........
MbraAG-P7-2           : ....C.....  ..........  ....A.....
MbraAG-P7-3           : ....C.....  ..........  ..........
MbraAG-P7-4           : ....C.....  ..........  ..........
MbraAG-P7-5           : ....C.....  ..........  ..........
MbraAG-P7-6           : ....C.....  ..........  ..........
MbraAG-P7-7           : ....C.....  ..........  ....A.....
MbraAG-P8-pleopod     : ....C.....  ..........  ....T.....
MbraAG-P8-1           : ....C.....  ..........  ....T.....
MbraAG-P8-2           : ....C.....  ..........  ....T.....
MbraAG-2DN-pleopod    : ....C.....  ....A.....  ....T.....
MbraAG-2DN-1          : ..........  ....A.....  ....T.....
MbraAG-2DN-2          : ....C.....  ....A.....  ....T.....
MbraAG-3IN-pleopod    : ....C.....  ..........  ....T.....
MbraAG-3IN-1          : ....C.....  ..........  ....T.....
MbraAG-3IN-2          : ....C.....  ..........  ....T.....
MbraAG-4DN-pleopod    : ....C.....  ..........  ....T.....
MbraAG-4DN-1          : ....C.....  ..........  ....T.....
MbraAG-4DN-2          : ....C.....  ..........  ....T.....
MbraAG-5IN-pleopod    : ..........  ..........  ....T.....
MbraAG-5IN-1          : ..........  ..........  ....T.....
MbraAG-5IN-2          : ....C.....  ..........  ....T.....
MbraAG-G6-pleopod     : ....C.....  ..........  ....T.....
MbraAG-G6-1           : ....C.....  ..........  ....T.....
MbraAG-G6-2           : ....C.....  ..........  ....T.....
```

Fig 1. Alignment of the obtained sequences of 16S gene around positions 365, 442 and 580. The sequences were named with the following codes: MbraAG-specimen code-tissue, in the case of adults; MbraAG-female code-number, in the case of the eggs. Dots indicate identity with the consensus sequence (on top).

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Discussion

Until now, a low incidence of heteroplasmy was recorded in animals except for mussels. However, Williams *et al.* [13] had detected high levels of heteroplasmy in the blue crab *Callinectes sapidus*, identifying a high number of haplotypes never before found in metazoans [13]. In that work, the researchers propose several explanations to this phenomenon (Nuclear

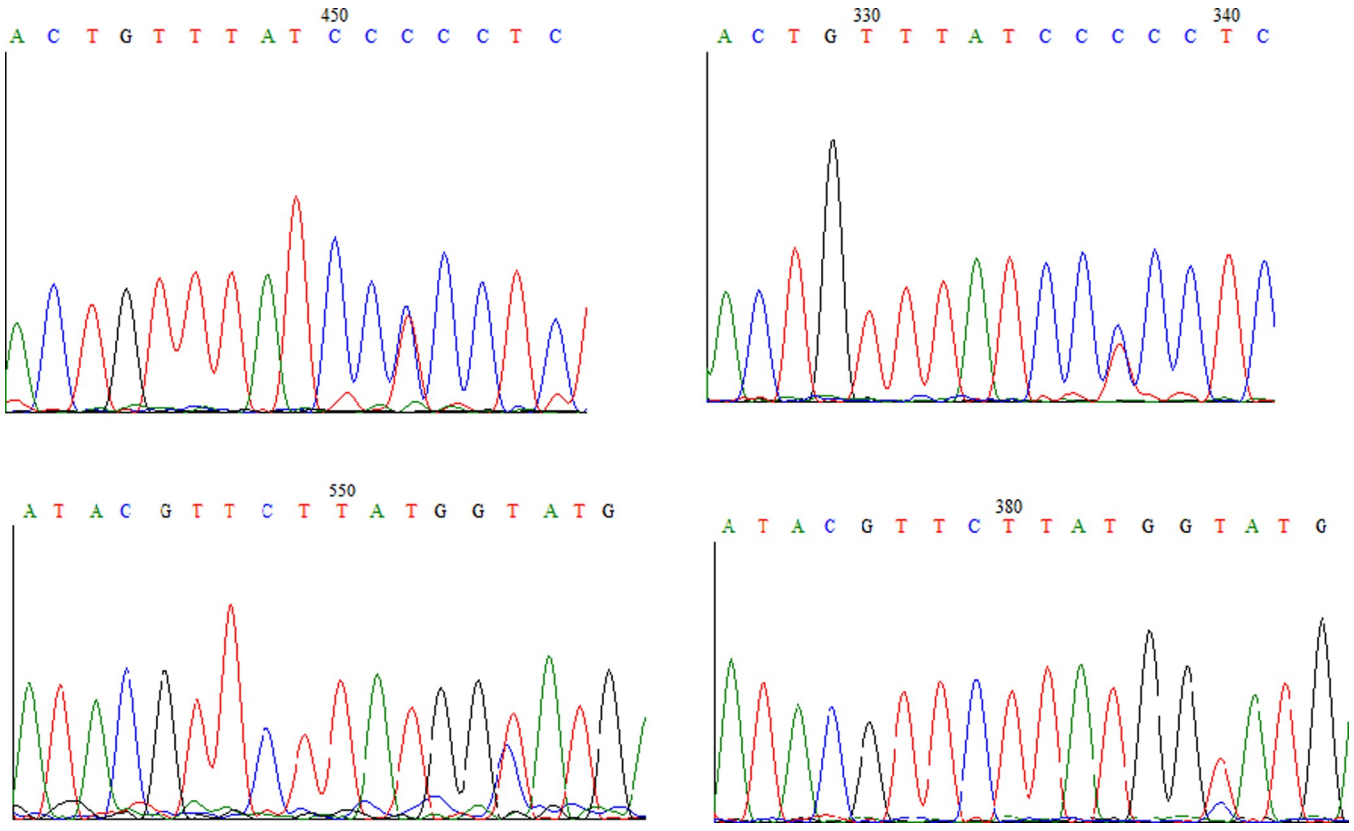

Fig 2. Example of double peaks in the forward (left) and reverse (right) reads of the COI region at positions 240 (top) and 375 (bottom).

https://doi.org/10.1371/journal.pone.0230243.g002

Mitochondrial DNA segments or NUMTS, paternal leakage, accumulated mutations and replication errors) without opting for one. In our study, the analysis of the mtDNA of a large offspring for seven spider crab females (Williams *et al.* [13] only analysed one male, one female and one megalopa) allows us to discard several hypotheses of the origin of heteroplasmy in *M. brachydactyla*, until reduce them to one option. In the case of this species, the high number of heteroplasmic cells per individual/tissue makes it possible to detect this phenomenon by conventional PCR. However, new techniques such as qPCR (quantitative Polymerase Chain Reaction) and ARMS-qPCR (Amplification Refractory Mutation System-quantitative PCR) are now available to detect very low levels of heteroplasmic cells [7, 8].

Electropherogram reads are conditioned by the quality and efficiency of the PCR. This is associated with the hybridisation primer/target and the efficiency of Taq polymerase among other circumstances. Contamination of samples or PCR products is a factor to have into consideration. For this reason, DNA extraction was repeated in many cases, particularly in those where double peak was detected, as it was mentioned in material and methods and results sections. However, no different results were obtained. It should be noted that double peaks only affect two specific positions in 16S rDNA and four in COI and that none of these COI positions are involved in stop codons. The similarity between the sequences obtained and the sequences available on databases indicates that it is *M. brachydactyla* DNA in all cases.

Once the contamination has been discarded as the cause of the double peaks, there are two different possibilities that can lead to an erroneous interpretation of the electropherograms. Firstly, the existence of NUMTs. In this sense, there are references to the presence of COI-like sequences in many crustaceans, for instance in krill, crabs, amphipods, crayfish, squat lobsters,

Table 3. Detected haplotypes for the COI gene and adult individuals in which they were observed.

Haplotype	N	Individual		
Hap-COI-1	2	MbraAG-5TM-muscle	MbraAG-16TM-muscle	
Hap-COI-2	1	MbraAG-13TM-muscle		
Hap-COI-3	7	MbraAG-14H-muscle	MbraAG-15TM-muscle	MbraAG-17H-muscle
		MbraAG-23TM-muscle	MbraRA-G8-gonad	MbraRA-G46-gonad
		MBraRA-G53-gonad		
Hap-COI-4	1	MbraAG-10TM-muscle		
Hap-COI-5	1	MbraAG-18H-muscle		
Hap-COI-6	1	MBraRA-G47-gonad		
Hap-COI-7	1	MbraAG-22TM-muscle		
Hap-COI-8	1	MbraRA-G20-gonad		
Hap-COI-9	2	MbraAG-3TM-muscle	MbraRA-G21-gonad	
Hap-COI-10	1	MbraAG-2DN-pleopod		
Hap-COI-11	3	MbraAG-3H-muscle	MbraAG-20TM-muscle	MbraRA-G30-gonad
Hap-COI-12	2	MbraAG-11H-muscle	MbraAG-13H-muscle	
Hap-COI-13	15	MbraAG-2H-muscle	MbraAG-4H-muscle	MbraAG-6H-muscle
		MbraAG-7H-muscle	MbraAG-9H-muscle	MbraAG-12TM-muscle
		MBraAG-14TM-muscle	MbraAG-19TM-muscle	MbraAG-19H-muscle
		MbraAG-27TM-muscle	MbraRA-G26-gonad	MbraRA-G33-gonad
		MbraRA-G34-gonad	MbraRA-G39-gonad	MbraRA-G50-gonad
Hap-COI-14	1	MbraAG-26TM-muscle		
Hap-COI-15	1	MBraAG-28TM-muscle		
Hap-COI-16	1	MbraAG-4TM-muscle		
Hap-COI-17	3	MbraAG-16H-muscle	MbraRA-G18-gonad	MBraRA-G29-gonad
Hap-COI-18	1	MbraAG-25TM-muscle		
Hap-COI-19	7	MbraAG-6TM-muscle	MbraAG-15H-muscle	MbraRA-G5-gonad
		MbraRA-G9-gonad	MbraRA-G16-gonad	MbraRA-G23-gonad
		MBraRA-G36-gonad		
Hap-COI-20	2	MbraRA-G10-gonad	MbraRA-G35-gonad	
Hap-COI-21	1	MBraAG-18TM-muscle		
Hap-COI-22	2	MbraAG-20H-muscle	MbraRA-G43-gonad	
Hap-COI-23	1	MBraRA-G40-gonad		
Hap-COI-24	1	MbraAG-7TM-muscle		
Hap-COI-25	1	MbraAG-17TM-muscle		
Hap-COI-26	1	MbraAG-24TM-muscle		
Total	61			

N: number of haplotypes. Sequences with double peaks were not included in this analysis. Samples tagged as "pleopod" and "muscle" correspond to pleopod setae and muscle from pereiopod, respectively.

https://doi.org/10.1371/journal.pone.0230243.t003

shrimps, isopods, barnacles and copepods [64, 65]. Assuming that they were, these nuclear sequences would have Mendelian inheritance, which would imply that all the cells of an individual should have them. This hypothesis would not explain why some of the analysed tissues have a duplicate COI sequence and why others do not. For example, for COI, the female MbraAG-4D2V does not present double peaks in the pleopod setae, pereiopod muscle, stomach and nerve tissue, while they are present in gonad, gill, heart, intestine, eye and integument (Table 5). In addition, each individual could be homozygous or heterozygous for the presence of such NUMT. In this case, the female MbraAG-M26 for 16S rDNA has three combinations

Table 4. Nucleotidic proportions (%) at positions 240, 264, 303 and 375 of the amplified COI fragment for 16 adult individuals with ambiguous sites were detected.

Position		240		264		303		375	
Sample	Sex	T	C	T	C	T	C	T	C
MbraAG-2TM-muscle	male	15.3	84.7	0	100	20.2	79.8	100	0
MbraAG-8TM-muscle	male	100	0	0	100	100	0	80.9	19.1
MbraAG-10H-muscle	female	26.4	73.6	0	100	31.0	69.0	100	0
MbraAG-29TM-muscle	male	0	100	74	26.3	0	100	77.3	22.7
MbraAG-30TM-muscle	male	27.1	72.9	0	100	31.3	68.7	75.8	24.2
MbraRA-G1-gonad	female	12.6	87.4	0	100	100	0	100	0
MbraRA-G2-gonad	female	100	0	0	100	78.8	21.2	0	100
MbraRA-G4-gonad	female	100	0	13	86.6	0	100	100	0
MbraRA-G22-gonad	female	17.5	82.5	0	100	19.1	80.9	0	100
MbraRA-G24-gonad	female	11.0	89.0	0	100	13.8	86.2	100	0
MbraRA-G25-gonad	female	100	0	0	100	100	0	74.7	25.3
MbraRA-G28-gonad	female	16.8	83.2	0	100	18.4	81.6	100	0
MbraRA-G40-gonad	female	40.5	59.5	0	100	42.0	58.0	100	0
MbraRA-G42-gonad	female	10.7	89.3	0	100	0	100	100	0
MbraRA-G44-gonad	female	84.2	15.8	0	100	100	0	100	0
MbraRA-G45-gonad	female	47.3	52.7	0	100	46.6	53.4	100	0

Proportions were calculated by dividing the height of each peak in the electropherogram by the summation of the height of both peaks.

https://doi.org/10.1371/journal.pone.0230243.t004

of sequences between positions 365 and 580 (T-A, C-T, T-T). If one of these sequences is mitochondrial, the other two would be nuclear, therefore, all her eggs should carry two combinations (one mitochondrial and one maternal NUMT) and this is not detected (Table 2). If a female were homozygous for the NUMT all descendants would have to present double peak (one mitochondrial and one NUMT) which does not happen in many cases (16S rDNA for females MbraAG-M26, MbraAG-P7 and MbraAG-5IN, and COI for the female MbraAG-P6). The second possibility is that there would have been a duplication in the mitochondrial genome of these sequences. This would not make sense because then all descendants of a female with a double peak should also have a double peak. In addition, we would have mitochondria in which there have been two independent duplications of the 16S rDNA gene and the COI gene (for example, the females MbraAG-M26, MbraAG-P6 or MbraAG-P7) and mitochondria in which the duplication only occurred in the 16S rDNA gene (MbraAG-10H or MbraAG-2DN).

Once these assumptions are discarded as explanations for the double peaks detected, we can conclude that we detected a true heteroplasmy in 22 adult individuals of a total of 83 studied. Heteroplasmy has been extensively studied at individual level in terms of the number of involved cells or tissues. However, it also has great importance at evolutionary level, since it affects Muller's ratchet. This theory holds that uniparental inheritance (homoplasmy) creates non-combined asexual lineages, which accumulate deleterious mutations more rapidly than their sexual counterparts [66–68]. Several mechanisms have been proposed to explain how mtDNA can overcome this evolutionary limitation such as genetic bottleneck, compensatory mutations, back mutations, mitochondrial DNA copy recruitment from the nucleus, purifying selection or recombination [69–70], being this last one the main accepted mechanism to eliminate Muller's ratchet. In a population of identical mitochondrial DNA molecules, recombination generates molecules that are identical to themselves and to parent molecules. Thus,

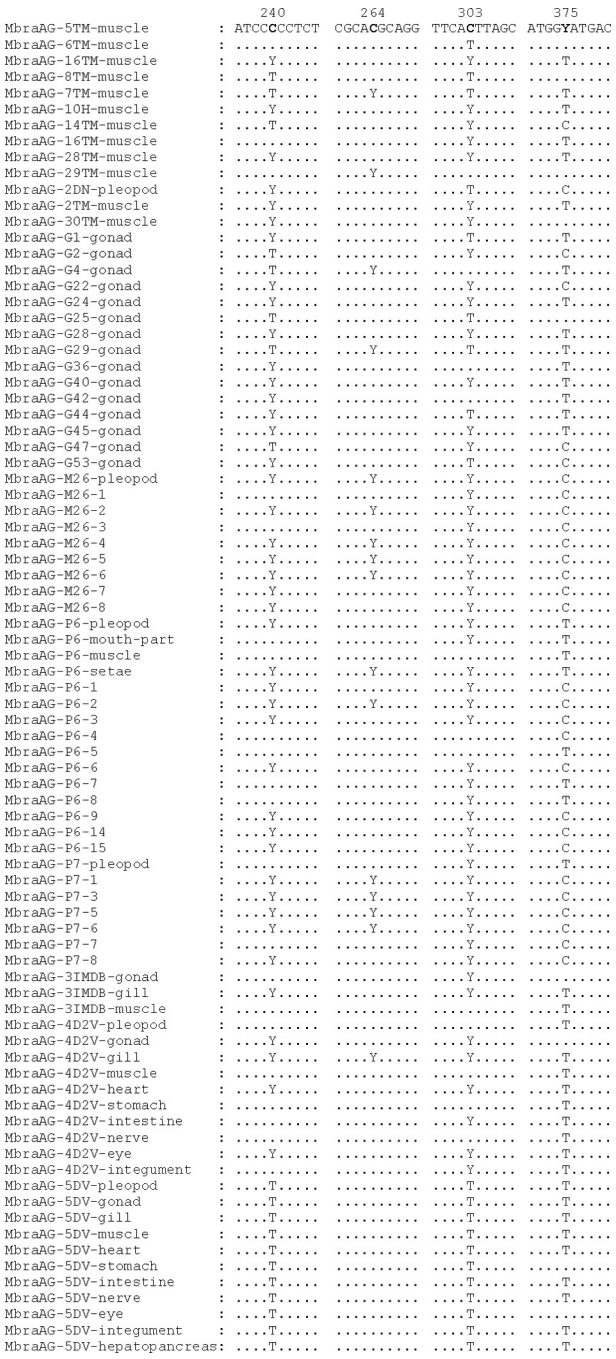

Fig 3. Alignment of the obtained sequences of COI gene around positions 240, 264, 303 and 375. The sequences were named with the following codes: MbraAG-specimen code-tissue, in the case of adults; MbraAG-female code-number, in the case of the eggs. Dots indicate identity with the consensus sequence (on top).

https://doi.org/10.1371/journal.pone.0230243.g003

mutation accumulation of the mtDNA molecule would be inevitable. The leakage of paternal mitochondrial DNA could have evolved to provide a means to overcome this limitation.

Due to the low frequency of recombination and *de novo* mutations in mitochondrial genome [24, 25], in our opinion, the main cause of heteroplasmy in *M. brachydactyla* are failures in the elimination of the male mitochondria. This fact could have two main explanations.

Table 5. Nucleotidic proportions (%) of the amplified COI fragment in broods and different tissues of several adult females.

Position		240		264		303		375	
Sample	Origin	T	C	T	C	T	C	T	C
MbraAG-M26-pleopod	female	23.3	76.7	26.4	73.6	27.6	72.4	0	100
MbraAG-M26-1	egg	0	100	0	100	25.4	74.6	0	100
MbraAG-M26-2	egg	20.8	79.2	12.0	88.0	28.6	71.4	0	100
MbraAG-M26-3	egg	0	100	0	100	29.1	70.9	0	100
MbraAG-M26-4	egg	20.4	79.6	15.4	84.6	26.4	73.6	0	100
MbraAG-M26-5	egg	24.4	75.6	10.1	89.9	27.8	72.2	0	100
MbraAG-M26-6	egg	29.1	70.9	20.7	79.3	34.7	65.3	0	100
MbraAG-M26-7	egg	27.9	72.1	0	100	29.6	70.4	0	100
MbraAG-M26-8	egg	25.3	74.7	0	100	30.2	69.8	0	100
MbraAG-P6-pleopod	female	28.7	71.3	0	100	30.5	69.5	100	0
MbraAG-P6-mouth-part	female	0	100	0	100	21.6	78.4	100	0
MbraAG-P6-muscle	female	0	100	0	100	0	100	100	0
MbraAG-P6-setae	female	32.5	67.5	26.1	73.9	30.1	69.9	100	0
MbraAG-P6-1	egg	25.1	74.9	0	100	28.8	71.2	0	100
MbraAG-P6-2	egg	27.6	72.4	30.5	69.5	34.0	66.0	0	100
MbraAG-P6-3	egg	19.8	80.2	0	100	24.8	75.2	0	100
MbraAG-P6-4	egg	0	100	0	100	0	100	0	100
MbraAG-P6-5	egg	0	100	0	100	0	100	100	0
MbraAG-P6-6	egg	18.2	81.8	0	100	23.6	76.4	0	100
MbraAG-P6-7	egg	0	100	0	100	33.7	66.3	100	0
MbraAG-P6-8	egg	0	100	0	100	22.8	77.2	100	0
MbraAG-P6-9	egg	43.1	56.9	0	100	48.6	51.4	0	100
MbraAG-P6-14	egg	23.8	76.2	0	100	39.3	60.7	0	100
MbraAG-P6-15	egg	22.5	77.5	0	100	25.5	74.5	0	100
MbraAG-P7-pleopod	female	0	100	0	100	22.6	77.4	100	0
MbraAG-P7-1	egg	34.7	65.3	24.7	75.3	39.0	61.0	0	100
MbraAG-P7-3	egg	48.7	51.3	42.8	57.2	64.9	35.1	0	100
MbraAG-P7-5	egg	37.6	62.4	46.7	53.3	49.8	50.2	0	100
MbraAG-P7-6	egg	32.2	67.8	18.9	81.1	64.6	35.4	0	100
MbraAG-P7-7	egg	0	100	0	100	26.0	74.0	0	100
MbraAG-P7-8	egg	44.5	55.5	0	100	60.9	39.1	0	100
MbraAG-3IMDB-gonad	female	0	100	0	100	16.2	83.8	79.8	20.2
MbraAG-3IMDB-gill	female	18.6	81.4	0	100	18.5	81.5	100	0
MbraAG-3IMDB-muscle	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-pleopod	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-gonad	female	6.7	93.3	0	100	15.4	84.6	82.7	17.3
MbraAG-4D2V-gill	female	57.2	42.8	50.0	50.0	66.3	33.7	100	0
MbraAG-4D2V-muscle	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-heart	female	34.3	65.7	0	100	41.8	58.2	100	0
MbraAG-4D2V-stomach	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-intestine	female	0	100	0	100	28.6	71.4	100	0
MbraAG-4D2V-nerve	female	0	100	0	100	0	100	100	0
MbraAG-4D2V-eye	female	82.5	17.5	0	100	71.3	28.7	100	0
MbraAG-4D2V-integument	female	0	100	0	100	32.3	67.7	100	0
MbraAG-5DV-pleopod	female	100	0	0	100	100	0	100	0
MbraAG-5DV-gonad	female	100	0	0	100	100	0	100	0

(*Continued*)

Table 5. (Continued)

Position		240		264		303		375	
Sample	**Origin**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**
MbraAG-5DV-gill	female	100	0	0	100	100	0	100	0
MbraAG-5DV-muscle	female	100	0	0	100	100	0	100	0
MbraAG-5DV-heart	female	100	0	0	100	100	0	100	0
MbraAG-5DV-stomach	female	100	0	0	100	100	0	68.0	32.0
MbraAG-5DV-intestine	female	100	0	0	100	100	0	100	0
MbraAG-5DV-nerve	female	100	0	0	100	100	0	100	0
MbraAG-5DV-eye	female	100	0	0	100	100	0	62.5	37.5
MbraAG-5DV-integument	female	100	0	0	100	100	0	100	0
MbraAG-5DV-hepatopancreas	female	100	0	0	100	100	0	100	0

Proportions were calculated by dividing the height of each peak in the electropherogram by the summation of the height of both peaks. Samples tagged as "pleopod", "mouth-part" and "muscle" correspond to pleopod setae, endites and muscle from pereiopod, respectively.

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Firstly, a special type of DUI-type inheritance as in molluscs [2, 33–36]. However, this can be discarded because in our species heteroplasmy is indistinctly detected in both males and females. Secondly, that there has been a generalized failure on the mechanisms of elimination of paternal mitochondria [3]. Until now, true biparental transmission of mitochondrial genomes from both parents (or from two distinct mating types) to zygotes as part of normal reproductive processes within a species and their persistence throughout development has not been demonstrated [23]. Nevertheless, sporadic biparental inheritance events have been documented in mammals, birds, reptiles, fish, molluscs, nematodes or arthropods [36].

One possible explanation for the retaining of male mitochondria in the offspring in *M. brachydactyla* is that these individuals come from interspecies or interpopulation hybridisations. In these cases, a breakdown of mechanisms to recognise and remove paternal mtDNA may occur [36, 71–74]. Several researches have been based on analysing the mtDNA of the offspring of intra- and interspecific crosses. Kondo *et al.* [75] performed crosses with different species of *Drosophila* during 10 consecutive generations (140 intraspecific and 191 interspecific crosses). The results of this study showed the presence of maternal mtDNA in the offspring of intraspecific crosses, while some individuals from interspecific crosses showed paternal leakage (three homoplasmic and one heteroplasmic individuals). Dokianakis and Ladoukakis [76] also analysed the mtDNA of the offspring of crosses between seven *Drosophila* species (31 interspecific crosses, 4 intraspecific crosses). They only detected paternal leakage in some of the hybrids, while in the descendants of intraspecific crosses the mitochondrial inheritance was strictly maternal. Kaneda *et al.* [71] conducted a similar study in mice. In crosses between individuals of the species *Mus musculus* the paternal mtDNA was detected only through the early pronucleus stage while in crosses between *M. musculus* and *M. spretus* the paternal mtDNA was detected throughout development from pronucleus stage to neonates. In other vertebrates, paternal leakage has been detected in natural hybrids of fish [77], birds [78] or amphibians [79].

The studies of Clark *et al.* [80] based on biometric characters suggested the lack of complete separation of the Atlantic and Mediterranean populations of *Carcinus*, with the potential existence of a hybrid zone between *C. maenas* (Atlantic) and *C. aestuarii* (Mediterranean). Something similar happens in the case of *M. brachydactyla* and *M. squinado*, species very close morphological and genetically [43–45, 81]. Although the distribution of *M. brachydactyla* is usually associated to the northeast Atlantic and the distribution of *M. squinado* to the

Mediterranean [43–45], Abelló *et al.* [58] reported two specimens identified as *M. brachydactyla* in the Alborán Sea (Mediterranean Sea). Our results suggest that this gene flow could occur bi-directionally across the Strait of Gibraltar, the only area of direct contact between both species. The complex life cycles of the planktonic larval stages and benthic post-larvae determine the distribution and population dynamics of spiny spider crabs. During the planktonic larval phase, the individuals are drifted by the action of the latitudinal marine currents that are active mainly during the months of April to October. This fact could facilitate the transferring of individuals, extending the overlapping zones to northern regions. In addition to this, considerable displacements have also been described in adult states, reaching 180 km [82]. If this fact is one of the causes involved in the appearance of heteroplasmy, its incidence should be higher in regions to the south of the Iberian Peninsula than in more northerly regions, such as the French coasts or the British Islands. It should be noted that the differences in the COI mitochondrial sequences of *M. brachydactyla* and *M. squinado* range from 5 to 10 synonymal changes, so that, a priori, there should not be a problem of cytoplasmic inheritance and intragenomic conflict between the nucleus and the cytoplasm.

As for the distribution of the different mtDNA types in the embryo during development, the results obtained do not show a pattern that would allow conclusions to be drawn. Tissues that show the same mtDNA do not necessarily come from the same embryonic layer. For example, in the case of the female Mbra-AG-4D2V, tissues formed from the mesoderm show differences in their COI mitochondrial sequences: muscular tissue has no ambiguous sites, while gonadal tissue shows three heteroplasmic positions and cardiac tissue, two. Differences in mtDNA between tissues of the same individual had already been detected in mice by Shitara *et al.* [72]. They analysed 12 different tissues from 38 F1 hybrids in mouse. They detected paternal leakage in 17 of the individuals analysed, but in most of them, the paternal mtDNA was limited to one to three tissues, which varied from one mouse to another. Regarding ovarian tissue, only 6.6% of F1 hybrid females reported paternal leakage, so they concluded paternal mitochondrial DNA does not propagated stably to future generations.

If the male contribution is a sporadic and punctual phenomenon restricted to certain contact zones between congeneric species, meiotic cell drift and what happened during embryonic phases would be the cause of the differences between tissues. Mitochondrial DNA molecules pass through a genetic bottleneck process, this is, the decreasing of the number of copies, both during oogenesis and early development [83]. This process is apparently due to random partitioning of organelles containing only one or a very few mtDNA molecules and lead to rapid segregation of polymorphic mtDNA species in the progeny [84]. Its effect varies according to taxa, ranging from 1 to 349 in humans [85–87], 200 in mice [88] and from 370 to 740 in fruit fly [89]. It should also be noted that PCR amplifications are not 100% effective. This implies that by chance when any combination is in low proportion may not be detected by conventional PCR due to its low amplification. These facts could explain why in the sample MbraAG-2DN-pleopod we did not detect heteroplasmy but we detected it in one of its eggs.

To summarise, a considerable frequency of heteroplasmy were detected in the spider crab *M. brachydactyla*, possibly associated to hybridisation of congeneric species. Future studies in other populations of *M. brachydactyla* as well as the analysis of the offspring from crosses in captivity would be necessary to confirm the true origin and dimension of this finding. Nevertheless, heteroplasmy is an important aspect to take into account in studies of population management, especially in those under commercial exploitation, since effective population size could be overestimated. In species with this mitochondrial particularity, it is recommendable to use nuclear markers in molecular genetics studies. If mitochondrial markers are anyway chosen, heteroplasmy will have to be taken into consideration when the results are interpreted in order to avoid erroneous conclusions.

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B

Extended abstract

Resumen extendido

1. Introducción

La centolla *Maja brachydactyla* Balss, 1922, es un crustáceo decápodo de la familia Majidae (De Grave *et al.*, 2009; Ng and Richer de Forges, 2015) que habita en el Atlántico nororiental, desde las Islas Británicas hasta Senegal, incluyendo las Azores, Madeira, las Islas Canarias y Cabo Verde (Neumann, 1998; Udekem d'Acoz, 1999; Sotelo *et al.*, 2008b).

El ciclo de vida de la centolla comprende tres fases: la fase larvaria, la fase juvenil o de crecimiento y la fase adulta o de reproducción. La fase larvaria planctónica dura de dos a tres semanas, dependiendo de la temperatura del agua, y consta de dos estados zoea y un estado megalopa (Iglesias *et al.*, 2002; Guerao *et al.*, 2008). El comienzo de la fase juvenil está marcado por el reclutamiento postlarvario y el crecimiento de los juveniles se produce mediante mudas sucesivas (Gónzalez-Gurriarán *et al.*, 1995; Guerao y Rotllant, 2009). Durante esta fase, los juveniles habitan en fondos poco profundos (> 15 m) donde realizan movimientos limitados (Gónzalez-Gurriarán y Freire, 1994; Hines *et al.*, 1995), alcanzando la fase adulta después de 2-3 años (Le Foll, 1993; Meyer, 1993).

El final de la fase juvenil está marcado por la muda terminal, tras la cual los individuos dejan de crecer y comienza la fase adulta (Hartnoll, 1963; Sampedro *et al.*, 1999; Corgos y Freire, 2006). Los adultos migran a aguas profundas (30-100 m) en otoño (Latrouite y Le Foll, 1989; Le Foll, 1993; González-Gurriarán y Freire, 1994; Hines *et al.*, 1995; Freire y González-Gurriarán, 1998; González-Gurriarán *et al.*, 2002), donde se produce la cópula entre individuos con el caparazón duro, sin cortejo ni guardia pre- ni postcopulatoria (González-Gurriarán *et al.*, 1998). Después de llenar sus receptáculos seminales, las hembras realizan una migración de retorno a aguas poco profundas para la incubación de la primera puesta del ciclo anual (Kergariou, 1971; González-Gurriarán *et al.*, 1993; Le Foll, 1993).

La fecundación es interna y se produce simultáneamente a la extrusión de los huevos a la cavidad abdominal de la hembra. Durante la incubación de la puesta, los huevos pasan por tres estados de desarrollo macroscópicamente distinguibles (González-Gurriarán *et al.*, 1993): el estado A, con coloración naranja y gran cantidad de vitelo; el estado B, con coloración marrón y esbozo del ojo; y el estado C, con coloración gris oscura, presencia de cromatóforos, ojos bien desarrollados y apenas vitelo.

El período de incubación de la puesta oscila entre 30 y 74 días, dependiendo de la temperatura del agua (Kergariou, 1975; Brosnan, 1981; Kergariou, 1984; Rodhouse, 1984; González-Gurriarán *et al.*, 1998; Iglesias *et al.*, 2002). Por este motivo, el número de puestas

que una hembra es capaz de incubar en cada ciclo anual varía en función de la latitud: hasta tres puestas en Galicia (González-Gurriarán *et al.*, 1993, 1998), hasta dos en la costa francesa (Kergariou, 1975, 1984; Martin, 1983) y sólo una en Irlanda (Brosnan, 1981; Rodhouse, 1984; Fahy, 2001).

Maja brachydactyla es una especie de gran interés comercial en muchos países, entre ellos Reino Unido, Irlanda, Francia, España, Portugal y Marruecos (Kergariou, 1984; Le Foll, 1993; González-Gurriarán *et al.*, 1998). En España, se desarrolla una importante actividad socioeconómica sobre esta especie, siendo Galicia la región con una mayor explotación. En las costas gallegas, existe una veda para la centolla que abarca los meses de junio a noviembre, la pesca de hembras ovadas está prohibida y existe una talla mínima legal de pesca de 120 mm de longitud de caparazón (Resolución DOG Nº133, 2019). Sin embargo, se ha observado que las regulaciones que limitan la pesca de uno de los sexos pueden producir desequilibrios en el *sex ratio*, provocando alteraciones en los sistemas de apareamiento y en la dinámica poblacional (Sato y Goshima, 2006; Fenberg y Roy, 2008; Alborés *et al.*, 2019).

Debido a su gran valor comercial, en las últimas décadas se han realizado numerosos estudios sobre la biología de *M. brachydactyla* en diferentes puntos de su distribución (Brosnan, 1981; Kergariou, 1984; Le Foll, 1993; González-Gurriarán *et al.*, 1998; Bernárdez *et al.*, 2005; Corgos *et al.*, 2007; Corgos *et al.*, 2011; Thatje y Robinson, 2011; Verísimo *et al.*, 2011). Sin embargo, los estudios genéticos sobre esta especie siguen siendo escasos.

Sotelo *et al.* (2008a, 2008b, 2009) fueron los primeros en proporcionar datos genéticos sobre *M. brachydactyla*, centrándose en aspectos filogeográficos, taxonómicos y de dinámica poblacional. Los análisis del gen mitocondrial COI respaldaron la distinción ya sugerida por Neumann (1998), basada en caracteres morfológicos, entre la centolla del Mediterráneo *Maja squinado* (Herbst, 1788) y la centolla del Atlántico *M. brachydactyla* (Sotelo *et al.*, 2008b). Además, Sotelo *et al.* (2007, 2008a) demostraron mediante el análisis tanto de marcadores mitocondriales como nucleares (microsatélites) que los *stocks* de centolla poseen una alta diversidad genética y una débil estructura poblacional. Basándose en esta información, concluyeron que las poblaciones de centolla se encuentran interconectadas formando una gran metapoblación. Por último, Sotelo *et al.* (2009) establecieron las relaciones filogenéticas entre *M. brachydactyla* y sus especies congéneres del Atlántico nororiental mediante el análisis de ADNmt. En este estudio se encontraron dos haplotipos pertenecientes a *M. brachydactyla* en la costa sudafricana, poniendo en duda la validez de la especie *M. cornuta* (anteriormente *M. capensis*), que habita en las costas de África

meridional. No obstante, otra posible explicación es la formación de híbridos entre *M. brachydactyla* y *M. cornuta*.

Unos años más tarde, Guerao *et al.* (2011) describieron un método sencillo para diferenciar las cuatro especies europeas del género *Maja* (incluida la actual *Neomaja goltziana*), mediante un índice morfométrico y un protocolo basado en PCR-RFLP. Utilizando este método junto con caracteres morfológicos (Neumann, 1998), Abelló *et al.* (2014) informaron de la presencia de individuos de *M. brachydactyla* en el Mar de Alborán (Mediterráneo occidental). Además, la presencia de fragmentos de restricción propios de *M. squinado* en uno de esos individuos, sugirió la posible formación de híbridos entre *M. brachydactyla* y *M. squinado* en la zona de solapamiento entre ambas especies.

A pesar de los esfuerzos realizados en los últimos años para determinar la estructura de población de *M. brachydactyla* y su relación con las especies congéneres, algunos aspectos siguen rodeados de incertidumbre. Las posibles hibridaciones entre especies simpátricas de centolla y la viabilidad de su progenie son cuestiones aún por resolver. Además, aspectos reproductivos de gran interés para la gestión de la centolla, como la posible existencia de paternidad múltiple, todavía no han sido abordados.

Por estas razones, el objetivo principal de esta tesis es estudiar en profundidad algunos de estos aspectos genéticos de la biología de *Maja brachydactyla* que proporcionen información de utilidad para la explotación sostenible de sus *stocks*.

Para alcanzar este objetivo global, se han abordado los siguientes objetivos específicos:

- Optimizar el proceso de extracción de ADN a partir de huevos de centolla para la obtención de ADN de alta calidad. Selección del método de extracción más eficaz y determinación de la etapa de desarrollo más adecuada para la toma de muestras y el mejor método para conservarlas.

- Encontrar un tejido no invasivo para la extracción de ADN de calidad en centollas adultas, que sea extrapolable a otras especies de crustáceos.

- Analizar la paternidad en puestas de centolla procedentes de cópulas en el medio natural empleando marcadores microsatélites. Algunas de las características reproductivas de *M. brachydactyla*, como la presencia de receptáculos seminales y la ausencia de guardia postcopulatoria, podrían favorecer la poliandria genética.

- Comparar la prevalencia de paternidad múltiple entre dos poblaciones de centolla con diferentes grados de explotación y normativas de regulación.

- Determinar si existe un efecto de la talla materna sobre la paternidad de las puestas de *M. brachydactyla*.

- Analizar la variabilidad mitocondrial y detección de la posible existencia de heteroplasmia en *M. brachydactyla*.

2. Optimización del proceso de extracción de ADN en la centolla *Maja brachydactyla*

Los estudios metodológicos centrados en la mejora de la extracción de ADN y la conservación de las muestras (Moorad *et al.*, 1997; Steele *et al.*, 2009; Palero *et al.*, 2010; Moreira *et al.*, 2013) son un paso previo importante para la realización de estudios moleculares (Schubart *et al.*, 2000; Fratini y Vannini, 2002; Mckeown y Shaw, 2008; Yue *et al.*, 2010; Bailie *et al.*, 2011; Pardo *et al.*, 2016; Hill *et al.*, 2017). Por esta razón, el primero de los objetivos de esta tesis doctoral se centró en optimizar el proceso de extracción de ADN, desde la elección de un tejido inicial adecuado hasta la selección del protocolo más eficaz, tanto para adultos como huevos de centolla.

Primero, se probaron diferentes protocolos de extracción de ADN en huevos individuales, tanto frescos como congelados: incubación en lejía al 10%, choque térmico, métodos mecánicos, resina Chelex 100, choque hipotónico y el kit comercial NucleoSpin Tissue. La cantidad de ADN fue cuantificada mediante espectrofotometría y, para comprobar la calidad, se amplificó el gen mitocondrial 16S mediante PCR. El kit comercial fue indiscutiblemente el método que presentó una mayor tasa de éxito, por lo que se empleó para la extracción de ADN a partir de *pools* de huevos en los tres estados de desarrollo (A, B y C) conservados de tres maneras diferentes (congelación, liofilización y conservación en etanol). Los huevos en estado B fueron los que proporcionaron mayores cantidades de ADN de alta calidad. En cuanto a los métodos de conservación, sólo se detectaron diferencias significativas en cuanto a la cantidad de ADN extraída a partir de huevos congelados y liofilizados, siendo mayor a partir de los segundos. Puesto que no se pudieron establecer diferencias significativas entre los huevos liofilizados y los conservados en etanol, es preferible el uso de etanol debido a su mayor simplicidad y a que no es necesario equipamiento específico.

Por otro lado, el kit comercial también se empleó para la extracción de ADN a partir de tejidos adultos. Se tomaron muestras de enditos, sedas de pleópodo y sedas de pereiópodo de individuos adultos, que fueron conservadas en etanol hasta el momento de su procesado. Aunque los enditos y las sedas de pleópodo proporcionaron cantidades de ADN significativamente mayores, las sedas de pereiópodo también proporcionaron suficiente ADN como para amplificar el gen diana. Por esta razón, las sedas constituyen un buen tejido alternativo al músculo para la obtención de ADN de individuos adultos sin que sea necesario sacrificar ni causar lesiones a los ejemplares de estudio. Esto resulta especialmente interesante en el caso de especies de cangrejos amenazadas, en las que la retirada de individuos del medio natural no es viable.

3. Diferencias en la prevalencia de la paternidad múltiple entre dos poblaciones de *Maja brachydactyla* con diferentes niveles de explotación y normativas de gestión

En las especies marinas, la mortalidad selectiva causada por la pesca produce perturbaciones en los sistemas de apareamiento (Hankin, 1997; Gosselin *et al.*, 2005; Sato *et al.*, 2010; Robertson y Butler, 2013; Rains *et al.*, 2016; Pardo *et al.*, 2017). Cuando las medidas de gestión están dirigidas a proteger unas tallas particulares o un sexo específico, estas perturbaciones se acentúan y puede producirse una disminución del potencial reproductivo y una alteración de la estructura de la población. En poblaciones en las que predominan las hembras, las reservas de esperma de los machos podrían verse afectadas (Kendall y Wolcott, 1999; Sato *et al.*, 2005; Sainte-Marie, 2007; Pardo *et al.*, 2015) y, como consecuencia, también la cantidad de esperma que aportan a cada hembra (Rondeau y Sainte-Marie, 2001; Pardo *et al.*, 2015). Además, la eliminación de machos grandes obliga a muchas hembras a copular con machos más pequeños, incapaces de llenar sus receptáculos seminales. Esta situación provoca limitación de esperma, lo que promueve la promiscuidad de las hembras y la consiguiente paternidad múltiple de sus puestas (Kendall *et al.*, 2002; Gosselin *et al.*, 2003; Hines *et al.*, 2003; Gosselin *et al.*, 2005; Hill *et al.*, 2017). Además, se ha sugerido que las poblaciones con intensa explotación selectiva utilizan la paternidad múltiple como mecanismo de compensación para mantener la diversidad genética (Morán y García-Vázquez, 1998; Jennions y Petrie, 2000) y el tamaño efectivo de población (Sugg y Chesser, 1994; Martínez *et al.*, 2000; Pearse y Anderson, 2009), así como para reducir la endogamia (Stockley *et al.*, 1993; Yasui, 1998).

Para comprobar si las actuales medidas de regulación están afectando a la diversidad genética y el sistema de apareamiento de las poblaciones naturales de centolla, se ha analizado la paternidad en puestas de dos poblaciones con características muy diferentes. Por un lado se han recogido muestras de hembras del Golfo Ártabro (Galicia, NO de España), una población con una intensa pesca selectiva fomentada por la prohibición de pescar hembras ovadas y, en la cual, las hembras son capaces de poner hasta tres puestas por ciclo anual. Por otro lado, se tomaron muestras de hembras de Carna (Co. Galway, Irlanda), donde la pesca de la centolla es accesoria, la captura de hembras ovadas no está prohibida y las hembras sólo ponen una puesta al año.

El análisis de nueve loci microsatélites específicamente diseñados mostró una riqueza alélica alta y coeficientes de endogamia similares para ambas poblaciones. Para los análisis de paternidad se seleccionaron tres de estos loci. De las 23 puestas analizadas se detectó paternidad múltiple en 10 de ellas. Analizando la distribución de los alelos paternos a lo largo de estas 10 puestas, no se encontró un patrón ordenado de los huevos fecundados por diferentes machos. Esto parece indicar que en esta especie la fecundación no ocurre de forma secuencial, es decir, no se favorece la paternidad del último macho en copular.

A pesar de que el nivel de prevalencia de paternidad múltiple en *Maja brachydactyla* (43,5%) resultó ser similar al encontrado en otros braquiuros (Baggio *et al.*, 2011; Jensen y Bentzen, 2012; Reaney *et al.*, 2012), el porcentaje de puesta fecundada por el macho principal fue bajo en comparación con otras especies del infraorden (Koga *et al.*, 1993; Jensen y Bentzen, 2012; Reaney *et al.*, 2012; Josart *et al.*, 2014).

No se observó un efecto significativo de la talla materna en la paternidad de las puestas. En cambio, en el Golfo Ártabro la proporción de puestas fecundadas por más de un macho fue casi siete veces mayor que en Carna. Estas diferencias entre poblaciones podrían deberse a:

a) Diferencias en la densidad de población. La baja densidad de ejemplares en la población irlandesa podría limitar la disponibilidad de parejas sexuales (Jensen *et al.*, 2006) y, por lo tanto, el nivel de poligamia. Sin embargo, esta hipótesis parece poco probable, ya que varios estudios han sugerido la presencia de una densa población de centollas en la costa occidental de Irlanda (Bates, 1981; Rodhouse, 1984; Kelly *et al.*, 2003).
b) Diferencias en la fecundidad de las hembras. Dado que la fecundidad anual de las hembras de centolla gallegas es mayor que la de las irlandesas, su demanda de esperma también debería ser mayor. Por este motivo, es de esperar que las hembras gallegas requieran un mayor número de cópulas para fecundar todos sus huevos.

c) Diferentes niveles de explotación selectiva. Las medidas de gestión que prohíben la pesca de hembras ovadas promueven una explotación centrada en los machos, especialmente los de mayor tamaño con mayor valor comercial (Fahy y Carroll, 2009). Además, los individuos de tallas extremas, más escasos, son los más susceptibles de desaparecer de la población debido a la sobreexplotación. Varios estudios han aportado datos sobre la alteración del *sex ratio* y la estructura de tallas de las poblaciones causada por la pesca selectiva (Rodhouse, 1984; Fahy, 2001; Kelly *et al.*, 2003; Corgos *et al.*, 2006; Fahy y Carroll, 2009). Esta alteración de la estructura de la población conduce a situaciones de limitación de esperma (Hines *et al.*, 2003, Sato *et al.*, 2007, 2010; Pardo *et al.*, 2015), lo que promueve el apareamiento múltiple para asegurar la fecundación de las puestas. Teniendo en cuenta esto, la elevada prevalencia de paternidad múltiple en el Golfo Ártabro podría ser un indicador de que la población gallega se está autorregulando en respuesta a una sobreexplotación selectiva.

Una manera de comprobar cuál de estas hipótesis es la correcta, sería analizar puestas de centolla de la región de Magharees (Irlanda). Esta población está sometida a una intensa explotación selectiva (Fahy y Carroll, 2009; Rodhouse, 1984), pero se encuentra en una latitud en la que las hembras sólo ponen una puesta al año. Una baja prevalencia de paternidad múltiple, similar a la encontrada en Carna, indicaría que la fecundidad anual de las hembras es un factor que puede influir en el nivel de poligamia de las poblaciones. Sin embargo, una alta frecuencia de paternidad múltiple en esta región confirmaría que se trata de un mecanismo de compensación frente a situaciones de limitación de esperma causadas por la pesca selectiva de machos grandes.

4. Elevada incidencia de heteroplasmia en el ADN mitocondrial de una población natural de la centolla *Maja brachydactyla*

La suposición general de que los genomas mitocondriales se transmiten únicamente por vía materna y son homoplásmicos y no recombinantes es cada vez más controvertida (Rokas *et al.*, 2003; Tsaousis *et al.*, 2004; Barr *et al.*, 2005). En la actualidad, se ha notificado la existencia de heteroplasmia en una gran variedad de organismos (Fisher y Skibinski, 1990; Gyllensten *et al.*, 1991; Magoulas y Zouros, 1993; Crochet y Desmarais, 2000; Kvist *et al.*, 2003; Sherengul *et al.*, 2006; Doublet *et al.*, 2008; Nunes *et al.*, 2013; Payne *et al.*, 2013; Radojičic y *et al.*, 2015; Williams *et al.*, 2017).

En estudios realizados por Abelló *et al.* (2014) se había sugerido la posible existencia de heteroplasmia en *Maja brachydactyla*. Para confirmar esta hipótesis, se analizaron fragmentos de los genes mitocondriales COI y 16S en centollas adultas de ambos sexos y huevos procedentes de Galicia (NO de España).

Los resultados mostraron la coexistencia de varias secuencias mitocondriales en el 27% de los individuos adultos. Se observó también que algunos de los huevos presentaban varias secuencias mitocondriales que ya estaban presentes en la madre. En cuanto a los diferentes tejidos analizados, la heteroplasmia fue muy variable dentro de un mismo individuo, y los tejidos que la mostraban no procedían necesariamente de una misma capa germinal.

Tras descartar el resto de hipótesis que podrían explicar la detección de heteroplasmia en *M. brachydactyla* (mutaciones *de novo*, recombinación, herencia uniparental doble, duplicaciones, NUMTs, contaminación de las muestras), se achacó este fenómeno a un fallo en los mecanismos de eliminación de las mitocondrias paternas. Estos fallos son comunes en híbridos interespecíficos (Kondo *et al.*, 1990; Kaneda *et al.*, 1995; Shitara *et al.*, 1998; Sherengul *et al.*, 2006; Dokianakis, y Ladoukakis, 2014). Teniendo en cuenta esto, la hipótesis más probable para explicar la coexistencia de diferentes mitogenomas en una misma centolla es la formación de híbridos entre la centolla mediterránea *M. squinado* y la atlántica *M. brachydactyla* en la zona de contacto entre ambas especies.

5. Discusión general

Cada vez más, los estudios genéticos se están convirtiendo en una herramienta fundamental para el conocimiento de la vida silvestre, proporcionando información de gran interés para su gestión y conservación. Estos estudios, basados en el análisis de marcadores moleculares, requieren de ADN de alta calidad para su desarrollo. Por ello, el establecimiento de un tejido inicial adecuado, una correcta conservación de las muestras y un método de extracción eficiente son aspectos a tener en cuenta para maximizar la cantidad y calidad del ADN para el posterior análisis de marcadores moleculares.

Varios estudios de paternidad realizados en decápodos han puesto de manifiesto la importancia de obtener cantidades suficientes de ADN de alta calidad a partir de huevos o larvas (McKeown *et al.*, 2008; Pardo *et al.*, 2016). La obtención de ADN a partir de huevos individuales permite obtener resultados más precisos. Por otro lado, la búsqueda de tejidos que no supongan la muerte ni provoquen el sufrimiento de los animales durante la toma de muestras se está convirtiendo en una preocupación creciente. En el Capítulo 2 de esta tesis

se describe como se consiguió extraer ADN a partir huevos individuales de centolla y se proponen las sedas como un buen tejido alternativo para la obtención de ADN en adultos. Estos estudios metodológicos previos permitieron analizar marcadores nucleares (Capítulo 3) y mitocondriales (Capítulo 4) en centolla, revelando multipaternidad y heteroplasmia en esta especie.

La detección de heteroplasmia en el genoma mitocondrial de centollas gallegas sugiere que los híbridos entre *Maja brachydactyla* y *Maja squinado* del sur de la Península Ibérica son viables. A pesar de la deriva larvaria y los largos desplazamientos registrados en los individuos adultos (Corgos *et al.*, 2011), la probabilidad de que un individuo híbrido alcance las costas gallegas en una sola generación es baja.

Se ha sugerido que la coexistencia de varias moléculas de ADN dentro de una mitocondria o célula, sumada a la posibilidad de recombinación, puede ser una forma de evitar el trinquete de Muller (Loewe, 2006). Esta teoría sostiene que la herencia uniparental (homoplasmia) crea linajes asexuales no combinados, que acumulan mutaciones deletéreas más rápidamente que sus homólogos sexuales (Muller, 1964; Felsenstein, 1974; Gordo y Charlesworth, 2000).

Aunque la heteroplasmia puede resultar en ocasiones beneficiosa para el individuo portador, ésta podría conducir a resultados erróneos al utilizar el ADNmt como marcador molecular o forense (Budowle *et al.*, 2003; Williams *et al.*, 2017). Se ha visto que la herencia mitocondrial biparental, seguida de recombinación, puede complicar la reconstrucción filogenética y la datación molecular (Posada y Crandall, 2002; Piganeau *et al.*, 2004).

Las estimas del tamaño efectivo de población (N_e) también pueden complicarse cuando coexisten diferentes mitogenomas en un mismo individuo. Sotelo *et al.* (2008a) proporcionaron valores aproximados de N_e para las poblaciones de centolla del Atlántico nororiental utilizando marcadores mitocondriales y nucleares (microsatélites). Los marcadores mitocondriales proporcionaron valores de alrededor de 150.000 individuos, mientras que la estimación a partir de los marcadores nucleares fue de alrededor de 2.000 individuos. Esta gran diferencia probablemente se deba a la existencia de heteroplasmia en las mitocondrias de *M. brachydactyla* que conduce a una sobrestimación del N_e empleando marcadores mitocondriales.

La multipaternidad es otro factor estrechamente relacionado con el N_e, aunque existen discrepancias en cuanto a si esta relación es positiva o negativa (Murray, 1964; Sugg y Chesser, 1994; Morán y García-Vázquez, 1998; Martínez *et al.*, 2000; Karl, 2008; Pearse y Anderson, 2009; Rafajlović *et al.*, 2013; Perrier *et al.*, 2014; Lotterhos, 2011). En las

poblaciones de *Maja brachydactyla*, la pesca selectiva de los machos puede dar lugar a situaciones de limitación de esperma, que disminuyen el éxito reproductivo de las hembras. Por otra parte, en las poblaciones naturales, los individuos de tallas extremas, menos abundantes, son los más susceptibles de desaparecer por sobrepesca. Los individuos maduros de pequeño tamaño están protegidos por una talla mínima legal de desembarque, pero los individuos grandes, que son principalmente machos, son retirados en grandes cantidades del medio marino. Esta reducción del número de machos grandes puede tener varias consecuencias: el desequilibrio en la proporción de sexos, la disminución del tamaño medio de la población y un estrechamiento del rango de tallas, el descenso de la competencia entre los machos y una reducción del éxito reproductivo de las hembras. Además, nuestros resultados muestran que en las puestas de centolla fecundadas por múltiples machos, no hay un claro dominio de uno de ellos. Bajo estas circunstancias, es probable que la paternidad múltiple surja en las poblaciones con pesca selectiva como un mecanismo para aumentar la diversidad genética y el N_e, así como para evitar la limitación del esperma.

Teniendo en cuenta esto, la paternidad múltiple, la heteroplasmia y la elevada conectividad entre poblaciones (Sotelo *et al.*, 2008a; Corgos *et al.*, 2011) son probablemente los mecanismos responsables de que los efectos de la sobrepesca en cuanto a limitación del esperma y diversidad genética todavía no sean detectables en las poblaciones de centolla de Galicia. La paternidad múltiple introduciría variabilidad genética a nivel nuclear y la heteroplastia a nivel mitocondrial, aumentando la fitness individual, el N_e y, en última instancia, la salud de los *stocks*.

Sin embargo, si la intensa pesca selectiva continúa, el tamaño poblacional se irá reduciendo gradualmente y los desequilibrios en la estructura de tallas y la proporción de sexos serán cada vez más evidentes, alcanzándose un cuello de botella que sería insalvable incluso para estos mecanismos de compensación. Por estas razones, es urgente revisar la normativa que prohíbe la pesca de hembras ovadas en Galicia, ya que sus efectos a largo plazo podrían provocar el colapso de las poblaciones.

6. Conclusiones

- El uso de kits comerciales de columnas de sílice es el único método con una alta tasa de éxito para la extracción de ADN de calidad a partir de huevos individuales de centolla de todos los métodos testados.

- El mejor estado de desarrollo embrionario para obtener ADN de calidad a partir de huevos de centolla es el estado B. No hay diferencias significativas entre la cantidad de ADN obtenido a partir de huevos liofilizados y conservados en etanol, pero se recomienda el uso del segundo método por su mayor inmediatez y simplicidad.

- Tanto las sedas de pleópodos como de pereiópodos constituyen una buena alternativa para la extracción de ADN en decápodos sin necesidad de sacrificar ni dañar los ejemplares.

- Se ha detectado paternidad múltiple por primera vez en *Maja brachydactyla*, con una prevalencia media del 43,5%.

- La talla materna no afecta a la presencia o ausencia de paternidad múltiple en las puestas de centolla (aunque esta falta de correlación podría deberse a un tamaño de la muestra insuficiente).

- La contribución de los diferentes machos de centolla a la fecundación de las puestas mostró un sesgo moderado (contribución media del macho principal = 66%) en comparación con otras especies de braquiuros.

- No existe un patrón organizado de los alelos paternos en las puestas de *M. brachydactyla* fecundadas por más de un macho, lo que indica la mezcla del esperma dentro de los receptáculos seminales, la mezcla de los huevos durante la ovoposición o ambas.

- La prevalencia de paternidad múltiple varió enormemente entre poblaciones, siendo casi siete veces mayor en el Golfo Ártabro (Galicia, oeste de España), una zona muy explotada, que en Carna (Galway, oeste de Irlanda), una zona apenas explotada.

- Se detectó heteroplasmia mitocondrial en centollas del noroeste de la Península Ibérica. Este hecho pone en duda la fiabilidad del uso de ADNmt como marcador molecular en estudios filogenéticos y de dinámica de poblaciones en *M. brachydactyla.*