Barrières au flux génique en Méditerranée Occidentale : étude de la différenciation génétique chez deux mollusques marins, Mytilus galloprovincialis & Stramonita haemastoma,

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THÈSE

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RESUME

La génétique des populations a révélé que la diversité génétique des espèces marines était très souvent distribuée de façon discrète dans l'espace, en mosaïque de patchs populationnels génétiquement homogènes délimités par des discontinuités appelées barrières au flux génique. L'objectif de cette thèse était de contribuer à mieux comprendre les processus expliquant l'origine, le maintien et la position des barrières génétiques au niveau de la zone de transition entre l'Atlantique et la Méditerranée. Dans un premier temps a été étudiée la structure génétique de la moule *Mytilus galloprovincialis*. Contrairement au cline abrupt et étroit reporté en Espagne, nous avons découvert en Algérie une vaste zone hybride mosaïque sur 600 km de côtes à l'Est du front océanique Almeria-Oran. Dans un deuxième temps a été menée une étude de la structure génétique du gastéropode marin *Stramonita haemastoma*. Nous avons découvert deux lignées cryptiques différentiellement fixées pour des haplogroupes mitochondriaux, et différenciées sur 3 marqueurs microsatellites développés dans cette thèse. La distribution spatiale en mosaïque est étonnante avec un patch de la lignée atlantique enclavé au nord de la Méditerranée occidentale et bordé par une zone hybride au sud dans la région de Valence. Ces deux études mettent en avant l'importance de l'isolement reproductif intrinsèque dans l'explication de la distribution mosaïque de la diversité génétique marine. Bien que les frontières entre patchs correspondent à des barrières physiques à la dispersion ou à des écotones, l'hydrographie et l'environnement n'expliquent sans doute que la position des discontinuités génétiques mais ni leur origine ni leur maintien.

Mots-clés: Barrière au flux génique, isolement reproductif, zone d'hybridation, *Mytilus galloprovincialis*, *Stramonita haemastoma*, Front Almeria-Oran.

ABSTRACT

Population genetics has revealed the genetic diversity of marine species is often subdivided into a mosaic of discrete patches, within which populations are genetically homogeneous, delineated by discontinuities called barriers to gene flow. The aim of this thesis was to contribute to better understand the processes explaining the origin, maintenance and location of genetic barriers at the Atlantic/Mediterranean transition zone. First, we studied the genetic structure of the mussel *Mytilus galloprovincialis*. In contrast to the abrupt narrow cline reported in Spain, we discovered along the Algerian coastline a 600 km wide mosaic hybrid zone eastward of the Almeria-Oran oceanic front. Second, we studied the genetic structure of a marine gastropod *Stramonita haemastoma*. We discovered two cryptic lineages differentially fixed for alternative mitochondrial haplogroups, and differentiated at three microsatellite markers developed in this PhD work. Surprisingly, the spatial distribution proved to be an unusual mosaic with a patch of the Atlantic lineage enclaved in the north of the Western Mediterranean Sea, bordered in the South by a hybrid zone in eastern Spain around Valencia. These two studies highlight the importance of intrinsic reproductive isolation in explaining the mosaic distribution of the marine genetic diversity. Although boundaries between patches coincide with physical barriers to dispersal or ecotones, hydrography and environment mainly explain the position of the genetic discontinuities but neither their origin nor their maintenance.

Keywords: Barrier to gene flow, reproductive isolation, hybrid zone, *Mytilus galloprovincialis*, *Stramonita haemastoma*, Almeria-Oran Front.

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SOMMAIRE

1- INTRODUCTION

L'identification et l'étude des barrières au flux génique est un axe de recherche central de la génétique évolutive. Ces barrières conditionnent le niveau d'indépendance évolutive des populations qui les encadrent avec des conséquences sur le maintien de la diversité génétique et les possibilités d'adaptation locale. De plus, elles favorisent l'évolution de mécanismes d'isolement reproductif, comme l'accumulation d'incompatibilités génétiques, autorisant la divergence entre populations pouvant aboutir à la spéciation. La plupart des espèces marines présentent des caractéristiques démographiques extrêmes (très grande fécondité, très grande taille des populations et un haut potentiel de dispersion par le biais d'une phase larvaire planctonique) qui ont des conséquences sur les modalités de rencontre de deux génomes et leurs rapports aux hétérogénéités du milieu, et doivent ainsi influer sur la structure des barrières au flux génique.

La plupart des organismes marins (~70%) ont un cycle de vie complexe incluant une phase larvaire microscopique dans le plancton (Pinet 2009). Inaccessible à l'observation directe, cette phase planctonique demeure encore une indéchiffrable boîte noire de l'océanographie. Elle est cependant la clé de compréhension des patrons de connectivité marine, car les capacités de dispersion diminuent souvent fortement après la phase larvaire en raison du mode de vie sessile ou sédentaire des adultes. D'importants progrès ont été réalisés dans la compréhension des flux larvaires par l'approche génétique (Gagnaire *et al.* 2015; Grosberg & Cunningham 2001; Hellberg *et al.* 2002). Cependant, l'équation flux de gène = flux larvaire est une simplification de la complexité des barrières au flux génique. Elles sont en fait bien souvent la résultante d'une combinaison de facteurs à la fois historiques, écologiques, physiques et génétiques (Bierne *et al.* 2011; Lemaire *et al.* 2005). Il est donc regrettable qu'un seul facteur soit si souvent mis en avant, soit les courants marins (Patarnello *et al.* 2007; Selkoe *et al.* 2010), soit les différences environnementales (Johannesson & Andre 2006). Pourtant il est probable que ces facteurs n'expliquent principalement que la position des barrières mais ni leur origine ni leur maintien sur le long terme (Bierne *et al.* 2011). Pour bien comprendre le fonctionnement des barrières au flux génique il est nécessaire de mener, d'une part, des études fines de la variation génétique au niveau des points chauds de différenciation génétique et, d'autre part, des expériences de terrain et en laboratoire. Dans cette thèse nous nous focalisons sur la première approche.

La Méditerranée Occidentale est une zone géographique pertinente pour l'étude des barrières génétiques en milieu marin car elle est délimitée par deux points chauds de différenciation génétique, le Front Almería-Oran à l'Ouest (Patarnello *et al.* 2007) et le détroit Siculo-Tunisien à l'Est (Bahri-Sfar *et al.* 2000; Borsa *et al.* 1997; Mejri *et al.* 2009; Stefanni & Thorley 2003; Zardoya *et al.* 2004), alors que les exemples de différenciation en son sein sont très rares, du moins chez les espèces à phase larvaire. De façon surprenante, les études de génétique des populations ont, d'une manière générale, sous-échantillonné la rive Sud de la Méditerranée Occidentale négligeant ~1600 km de littoral Algérien qui représente pourtant le meilleur endroit de mélange des eaux atlantique et méditerranéenne (Font *et al.* 1998; Tintore *et al.* 1988). Le but de cette thèse est de dévoiler les secrets de cette zone jamais explorée par les généticiens des populations en étudiant deux mollusques marins: la moule *Mytilus galloprovincialis* et le gastéropode *Stramonita haemastoma*. L'approche comparative permettra de faire la part entre les effets de l'histoire des populations, de l'environnement et des fronts océaniques dans la mise en place et le maintien des barrières au flux génique en milieu marin.

1.1 Les originalités de la génétique des populations marines

Pour la plupart des organismes marins, sédentaires à l'état adulte, la dispersion se fait essentiellement durant la phase larvaire (Bonhomme & Planes 2000; Doherty *et al.* 1995; Hedgecock 1986; Palumbi 2003). Une corrélation significative a été détectée entre la structure génétique (F_{ST}) (Bonhomme & Planes 2000) et la durée de la phase larvaire planctonique (PLD) (Bohonak 1999; Doherty *et al.* 1995; Palumbi 1992). Le potentiel de dispersion de ces espèces est essentiellement lié aux traits de vie tels que:

(1) Le type de fécondation. Le plus rencontré en milieu aquatique est la fécondation externe, les gamètes émis dans la colonne d'eau s'unissent de manière aléatoire au sein de la population (= panmixie).

(2) Le type d'œuf (pélagique ou benthique), le développement larvaire (direct ou pélagique) et la durée de la phase larvaire (Shulman & Bermingham 1995). Pour la plupart des espèces marines le développement embryonnaire donne lieu à une larve planctonique autorisant un taux de migration (*m*) important pour des effectifs des populations (*Ne*) souvent très grands; de ce fait on s'attend à des flux géniques forts qui assurent une homogénéité génétique sur de longues distances en absence de discontinuités.

(3) Les forts taux de fécondité. Une forte fécondité qui pourrait permettre de tolérer des fardeaux de sélection importants, et les grandes tailles des populations permettent une sélection efficace même avec des coefficients faibles, deux raisons d'espérer mieux observer les processus de sélection mis en œuvre (Bierne 2001).

Un tel cycle favorise le brassage génétique inter- et intra-population. Cependant, dans le milieu naturel, ce cycle est soumis à plusieurs facteurs qui peuvent l'affecter de différentes manières. Une forte variance du succès reproducteur chez des organismes très féconds avec de forte mortalité dans les stades précoces peut conduire à des tailles de populations plus faible localement et transitoirement (David *et al.* 1997; Hedgecock 1994; Hedgecock & Pudovkin 2011). De plus, le potentiel de dispersion peut être modifié à cause du changement dans le comportement des larves (Cronin & Forward 1986; Forward & Tankersley 2001). Ces changements peuvent avoir des conséquences sur la structure des populations différentes de l'attendu.

En génétique des populations, on s'attend à un fort niveau de diversité génétique et de faible différentiation génétique chez les espèces marines à larves planctoniques par rapport à celles à développement direct et en absence de barrières physiques (Kyle & Boulding 2000; Nielsen *et al.* 2009). En effet, les espèces à larves très dispersives devraient être panmictiques sur de grandes échelles spatiales. Les espèces à potentiel de dispersion plus limité devraient montrer la panmixie sur des échelles spatiales fines et modérées, et un isolement par la distance à plus grande échelle (Wright 1943) ; et les espèces à larves non dispersives devraient montrer un modèle d'isolement par la distance. Contrairement à l'attendu plusieurs espèces à phase larvaire relativement longue, ont été documentées comme génétiquement très différenciées (Barber *et al.* 2000; Palumbi *et al.* 1997; Taylor & Hellberg 2003), mettant en question l'importance relative entre le potentiel de dispersion et la dispersion réalisée (Bohonak 1999; Cunningham & Collins 1994; Grosberg & Cunningham 2001; Hellberg 1994; Shulman 1998). En effet, la dispersion planctonique peut être modifiée et/ou stoppée par des barrières invisibles telles que: les fronts océaniques, les gyres, les upwellings, les tourbillons et les gradients environnementaux (Palumbi 1994; Patarnello *et al.* 2007; Rougharden *et al.* 1988). Toutefois, les barrières physiques ne sont pas les seules à pouvoir expliquer une structure génétique, souvent elle apparait suite à la mise en place d'un mécanisme d'isolement reproductif créant un autre type de barrière appelé 'barrière génétique' (Bierne *et al.* 2011). Les mécanismes d'isolement reproductif peuvent intervenir avant la fécondation (pré-

zygotique) ou après (post-zygotique), ils sont aussi classés selon leur dépendance à l'environnement (extrinsèque) ou non (intrinsèque).

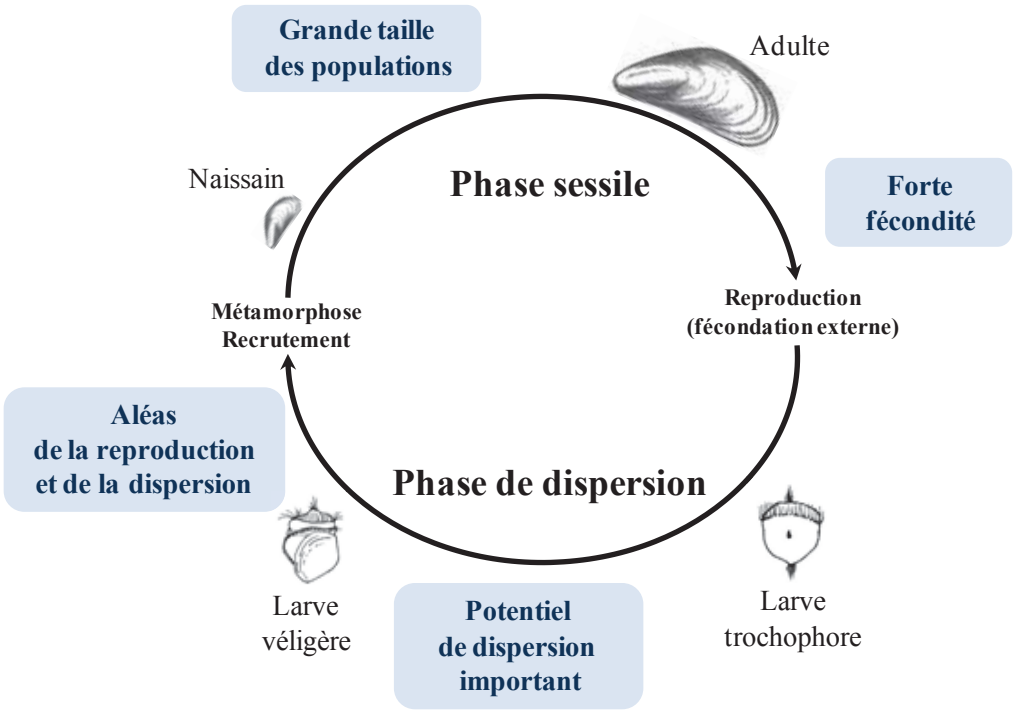

Figure 1: Cycle de vie d'un bivalve marin sessile avec les caractéristiques importantes pour la génétique des populations.

1.2 Les points chauds de différenciation génétique en milieu marin

1.2.1 La diversité génétique marine est souvent distribuée en mosaïque

L'homogénéité apparente du milieu marin aurait pu suggérer l'attendu d'une structure génétique qui augmente graduellement avec la distance (isolement par la distance, IBD) mais un bilan des études génétiques révèle, plutôt, que des régions panmictiques ou avec une différenciation génétique faible sont séparées par des transitions abruptes. Parmi les exemples les plus célèbres, on cite le Front Almeria-Oran. Ce Front constitue la zone de transition entre les eaux Atlantiques de surface moins salées, moins denses et froides et les eaux méditerranéennes profondes, plus salées, plus denses et chaudes. Il en résulte de cette circulation particulière la formation de deux gyres anticycloniques: les gyres d'Alboran Ouest et Est. La limite du gyre d'Alboran Est coïncide avec le Front Almeria-Oran considéré comme un point chaud de différenciation génétique chez plusieurs espèces (Bouchenak-Khelladi *et al.* 2008; Patarnello *et al.* 2007; Reuschel *et al.* 2010). Au sein de la Méditerranée, le détroit

Tableau 1: Les barrières les plus citées dans la littérature ainsi que les potentiels processus impliqués pour expliquer ces dernières.

Barrière au flux de gènes	**Potentiels processus impliqués Vicariance (V), Température (T), Salinité (S), Hydrographie (H)**	**Références**
L'Öresund et le Belt Danois	S	Johannesson & Andre (2006)
Front Almeria-Oran	V, H, S, T	Patarnello *et al.* (2007)
Front Baléares	H	Galarza *et al.* (2009)
Détroit Siculo-Tunisien	V, H, S, T	(Bahri-Sfar *et al.* 2000 ; Mejri *et al.* 2009)
Cap Cod (États-Unis)	V, H	(Kelly *et al.* 2006; Koehn *et al.* 1976 ; McGovern & Hellberg 2003)
Cap Canaveral (Floride)	V, H, T	(Avise *et al.* 1992; Pelc *et al.* 2009; Reeb & Avise 1990)
Cap Mendocino (Californie)	C, H	(Blanchette *et al.* 2008; Sivasundar & Palumbi 2010)
Point Conception (Californie)	V, H	(Burton 1998; Cassone & Boulding 2006) (Dawson 2001; Pelc *et al.* 2009)
Cap Agulhas (Afrique du Sud)	H	(Evans *et al.* 2004; Teske *et al.* 2014)
30°S Chilie	H	Brante *et al.* (2012)

Siculo-Tunisien, séparant le bassin occidental influencé par le courant Atlantique du bassin oriental, a été détecté dans plusieurs études (Bahri-Sfar *et al.* 2000; Mejri *et al.* 2009). Plusieurs autres zones ont été reportées dans la littérature (voir Tableau 1). Pour la plupart, de fort niveaux de structure génétique entre zones biogéographiques ont été observés formant des patchs d'habitats déconnectés par la courantologie et/ou les gradients environnementaux (Cunningham & Collins 1994; Johannesson & Andre 2006; Patarnello *et al.* 2007). Toutefois, les exemples d'espèces qui ont montré peu ou pas de structure génétique existent, comme l'huître plate *Ostrea edulis* (Launey *et al.* 2002), la girelle *Coris julis* (Aurelle *et al.* 2003), l'oblade *Oblada melanura* (Galarza *et al.* 2009) et l'algue rouge *Asparagopsis armata* (Andreakis *et al.* 2004). Ces espèces font partie d'une liste qui ne cesse d'augmenter pour le cas du Front Almeria-Oran. Ces contre-exemples prouvent que ces zones ne s'opposent pas nécessairement à la dispersion et à l'homogénéisation des populations comme prétendu. En effet, il a été suggéré, récemment, que la barrière physique au niveau du Front Almeria Oran devrait être couplée à une barrière génétique afin d'être suffisamment forte pour maintenir la différenciation génétique (Bierne *et al.* 2011; Borsa *et al.* 1997; Gosset & Bierne 2012).

1.2.2 Les différents types de barrières génétiques

Le flux de gènes inter- et/ou intra-spécifique est souvent freiné par deux types de barrières: une barrière à la dispersion et/ou une barrière à la reproduction. Dans le milieu marin, les barrières à la dispersion ne sont pas aussi évidentes qu'en milieu terrestre, même si d'énormes progrès en modélisation océanographique sont en train d'inverser la donnée (Berline *et al.* 2014; Rossi *et al.* 2014). Les barrières marines sont plutôt dues aux interactions entre masses d'eau ayant différentes propriétés physico-chimiques (les fronts océaniques, les zones à fort gradient de température, salinité, densité…). La plupart des océanographes, biologistes et même les généticiens des populations marines, ont tendance à croire que ces barrières à la dispersion ont un effet majeur et les considérer comme unique et importante cause empêchant l'homogénéisation des populations ignorant ainsi l'influence des barrières génétiques (Bierne *et al.* 2011). Pourtant, les recherches sur les processus de spéciation ont dévoilé qu'il existait un continuum de différenciation génomique (Feder *et al.* 2012) et que les premiers stades ne diffèrent pas fondamentalement de la différenciation intra-spécifique (Bierne *et al.* 2011). Dans la compréhension des processus de spéciation, il est important de connaître les parts respectives des différentes barrières dans l'isolement reproductif (Abbott *et al.* 2013; Palumbi 1994). Souvent seulement deux types de barrières sont mis en avant, les barrières reproductives pré-zygotiques et post-zygotiques (Figure 2) (Coyne & Orr 2004; Palumbi 1994). L'identification de ces mécanismes nécessite une meilleure connaissance du cycle de vie de l'espèce et de son écologie. Toutefois, l'étude de l'isolement post-zygotique n'est pas toujours possible vu les difficultés liées à l'expérimentation (Palumbi 1994). Parmis ces difficultés, on cite l'inaccessibilité aux hybrides de la deuxième génération (F2). Chez les Cichlidés qui sont un modèle très bien étudié, les hybrides de la F2 n'ont été obtenus que récemment (Stelkens *et al.* 2015). En effet, quand les substitutions sont récessives, la dépression n'apparaît qu'en F2 (Dobzhansky 1937; Edmands & Burton 1999). Pour *M. galloprovincialis* et *M. edulis* les mécanismes d'isolement reproductif ont été très bien illustrés suite à une série d'expériences (Bierne *et al.* 2006; Bierne *et al.* 2003a; Bierne *et al.* 2002a). Les croisements larvaires entre *M. edulis* et *M. galloprovincialis* ont révélé une hétérosis (vigueur hybride) chez les hybrides F1 et une dépression des hybrides de la F2, ces résultats montrent l'existence d'isolement post-zygotique de type Dobzhansky-Muller (Bierne *et al.* 2006).

L'importance des zones hybrides dans la compréhension des processus impliqués dans la spéciation, et plus généralement dans la divergence des génomes, n'a cessé d'augmenter. Les zones hybrides sont considérées comme 'des laboratoires naturels' (Barton & Hewitt 1989; Hewitt 1988) ou 'des fenêtres sur le processus évolutif' (Harrison 1990). Elles fournissent les exemples de divergence naturelle indispensables à la compréhension des processus évolutifs (Hewitt 1988). La génétique de la spéciation et des zones hybrides sont intéressantes à considérer pour mieux comprendre la génétique des populations. Souvent considérées comme des champs disciplinaires différents, pour les généticiens des populations ce n'est qu'un continuum. Parmi les facteurs recensés de l'isolement reproductif entre *M. galloprovincialis* et *M. edulis*, la spécialisation d'habitat est un facteur exogène très important pour expliquer la structure spatiale. Cependant, son rôle dans le maintien de la différenciation génétique, bien que promu par de nombreux auteurs (Hilbish *et al.* 2002), est plus débattu par d'autres (Bierne *et al.* 2006; Bierne *et al.* 2003b; Bierne *et al.* 2002b). La spécialisation à un habitat est évidente aussi dans plusieurs zones hybrides mosaïques tels que la zone hybride *Bombina, Gryllus,* et *Littorina* (Johannesson *et al.* 2010; Rand & Harrison 1989; Vines *et al.* 2003). Dans la zone hybride des moules, il a été trouvé que les *M. galloprovincialis* étaient favorisés dans les sites battus alors que les *M. edulis* étaient abondants dans les sites abrités (Gardner & Skibinski 1990; Hilbish *et al.* 2002). A la spécialisation d'habitat, s'ajoute d'autres facteurs endogènes tels que: l'isolement gamétique impliquant une fécondation préférentielle entre gamètes issus de parents de la même espèce, l'isolement temporel avec une reproduction asynchrone entre les deux espèces se produisant chez *M. edulis* avant *M. galloprovincialis* (Bierne *et al.* 2003b). A ces derniers s'ajoute un mécanisme d'isolement post-zygotique intrinsèque (Bierne *et al.* 2006). Le couplage entre ces barrières d'isolements endogènes et exogènes explique mieux la structure en mosaïque à grains fins répétée à plusieurs endroits (trois fois en France).

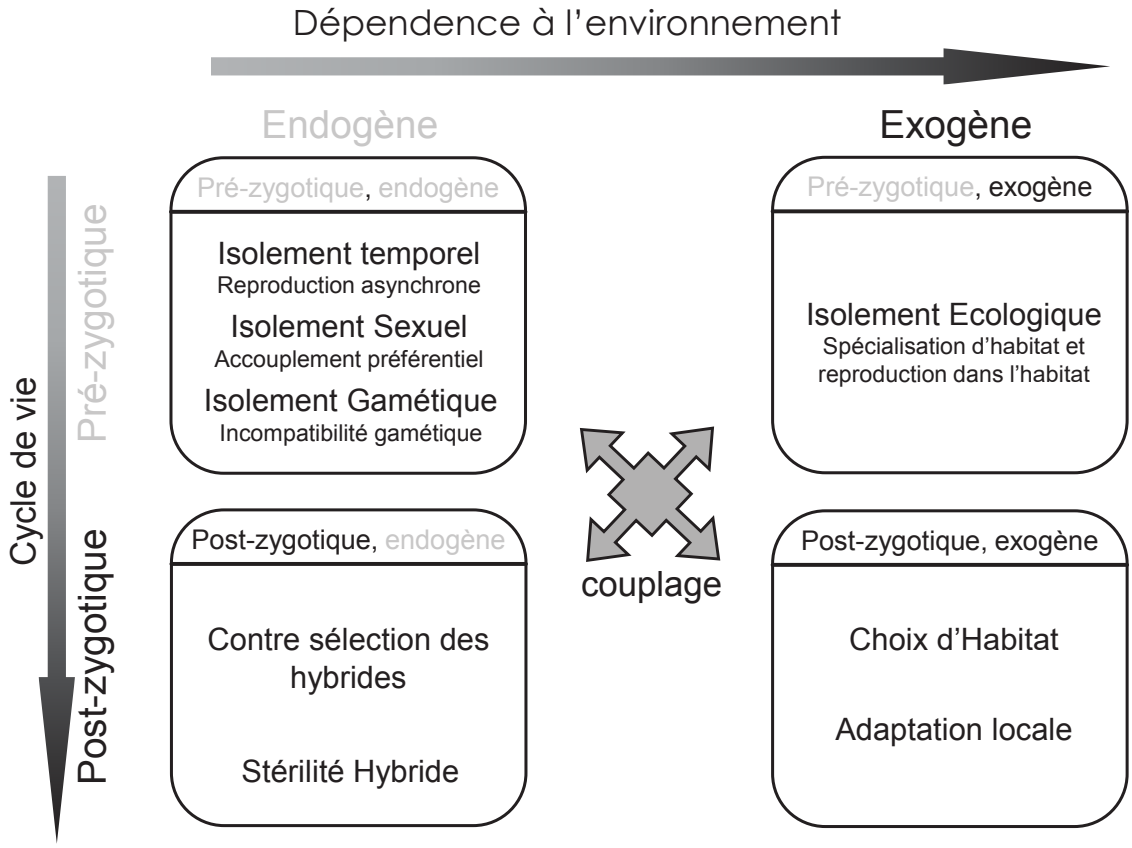

Figure 2: Classification des mécanismes d'isolement reproductif en fonction de leur indépendance à l'environnement et au stade du cycle de vie au moment duquel ils interviennent.

Le couplage entre plusieurs mécanismes d'isolement reproductif a été reconnu depuis longtemps dans la littérature des zones hybrides (Barton & Hewitt 1985; Hewitt 1988) comme un processus essentiel au maintien de l'intégrité des espèces. Parmi les exemples connus, on cite la zone hybride des crapauds du genre *Bombina* (Kruuk *et al.* 1999; MacCallum *et al.* 1998), des gastéropodes du genre *Littorina* (Cruz *et al.* 2004; Hull 1998; Janson & Sundberg 1983), des bivalves du genre *Mercenaria* (Bert & Arnold 1995) et des criquets du genre *Melanoplus* (Orr 1996). La plupart des zones hybrides sont des points de tension entre différents fonds génétiques, appelés aussi 'zones de suture' (Avise 2000; Hewitt 2004; Remington 1968; Swenson & Howard 2005). Barton (1979b) a prédit que ces zones peuvent se déplacer en réponse à des variations des densités des populations et du potentiel de dispersion par exemple mais elles ont tendance à être plutôt piégées par des barrières naturelles à la dispersion. Par ailleurs, Bierne *et al.* (2011) ont montré que les barrières écologiques pouvaient aussi les piéger, ainsi les facteurs exogènes indiquent souvent la position des clines génétiques mais pas leur maintenance qui est alors assurée par les facteurs

endogènes (Bierne *et al.* 2011). Ceci est illustré par des simulations, la figure 3 montre comment un couplage entre un cline exogène et un cline endogène se produit au niveau d'une frontière environnementale. En milieu naturel, parmi les exemples les plus célèbres, on cite la différenciation génétique observée chez *M. galloprovincialis* atlantique et méditerranéenne, cette dernière peut être expliquée par l'existence de deux lignées qui ont divergé en allopatrie avant l'apparition du font Almeria Oran et dont la remise en contact a résulté en une zone de tension qui aurait été secondairement piégée par la barrière naturelle et environnementale au niveau du Front Alméria-Oran (Gosset & Bierne 2012). Un autre exemple très intéressant concerne la zone de tension située entre la Mer Baltique et la Mer du Nord, cette zone a été plutôt piégée par un gradient de salinité à l'entrée de la Baltique (Bierne *et al*. 2011).

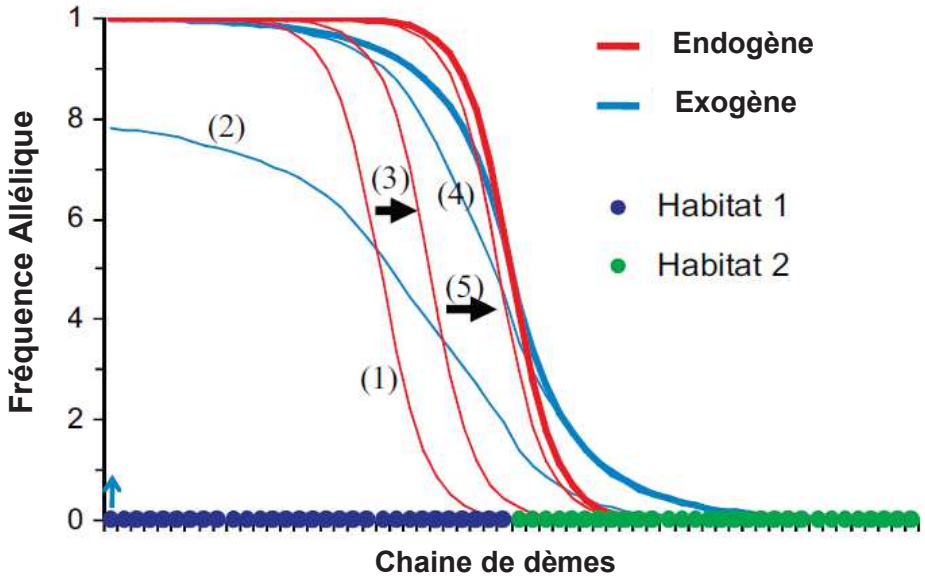

Figure 3: Couplage entre un cline exogène et un cline endogène au niveau d'une frontière environnementale. Une chaîne de 60 dèmes a été divisée en parcelles de 30 dèmes formant l'habitat 1 (points bleus) et l'habitat 2 (points verts). La migration connecte les dèmes séparés par six dèmes au maximum et la sélection est modérée. Le cline endogène (ligne rouge) est initialement positionné au dème 20 de l'habitat 1 (1). Un nouvel allèle exogène apparait dans le premier dème de l'habitat 1 (flèche bleue) et se répand dans tout l'habitat 1, croisant le cline endogène (2), qui se déplace de manière transitoire (3), et forme un cline à la frontière environnementale (4). Comme les clines endogène et exogène se chevauchent, leur couplage se poursuit et le cline endogène se déplace jusqu'à ce qu'il coïncide avec la frontière environnementale (5). Les flèches noires indiquent le mouvement du cline endogène (d'après Bierne *et al.* 2011)

1.3 Modèle d'étude

1.3.1 La Méditerranée occidentale, un bassin homogène bordé de deux barrières au flux génique

La Méditerranée occidentale est une Mer presque fermée, bordée par deux détroits: le détroit de Gibraltar à l'Ouest et le Siculo-Tunisien à l'Est. Les données océanographique et génétique ont montré que la transition entre la Mer Méditerranée et l'Océan Atlantique se fait réellement au niveau du Front Almeria-Oran (Patarnello *et al.* 2007). Le Front Almeria-Oran est situé à environ 400 km à l'Est du détroit de Gibraltar, il forme une frontière semi-permanente, instable et peu forte entre les eaux Atlantique et Méditerranéenne (Galarza *et al.* 2009). C'est un front de densité thermo-haline, confiné à la partie supérieure de la colonne d'eau (~300 m) (Galarza *et al.* 2009). Le détroit Siculo-Tunisien sépare la Méditerranée Ouest de l'Est, il a été reporté comme barrière génétique chez plusieurs espèces (Bahri-Sfar *et al.* 2000; Ben Slimen *et al.* 2004; Gharbi *et al.* 2011; Zitari-Chatti *et al.* 2008). L'histoire géographique de la Méditerranée a largement gouverné la répartition actuelle des espèces (Domingues *et al.* 2005). La dessiccation de cette Mer pendant la Crise de Salinité Messénienne a causée l'extinction de plusieurs espèces à l'exception de celles vivant dans les lagunes hyper-salines et dans les eaux saumâtres. L'ouverture du détroit de Gibraltar à la fin du Miocène (il y a 6 à 5,5 Ma) a permis la colonisation à nouveau de la Mer Méditerranée par de nouvelles espèces d'origines Atlantiques (Figure 3) (Almada *et al.* 2001). Le retour des glaciations au quaternaire (il y a environ 2 Ma) a contribué aux changements des aires de répartitions des espèces marines (Palumbi 1994). Plusieurs espèces se sont concentrées dans des refuges glaciaires (Wilson *et al.* 2009). Actuellement, les expansions postglaciaires ont aussi fortement contribué aux changements des aires de répartitions des espèces en leur permettant des recolonisations d'habitats redevenus favorables. Durant ces expansions les espèces se sont retrouvées face à deux types de barrières; les barrières géographiques et les contacts entre populations issues d'autres refuges créant ainsi des zones hybrides ou points chauds de structure génétique ou encore zones de sutures (Hewitt 1996; Remington 1968). Dans cette thèse on s'intéresse à l'étude du Front Almeria-Oran reporté comme point chaud de différenciation génétique chez 43 espèces sur un total de 61 espèces indiquées par Patarnello *et al.* (2007). Ce nombre n'a cessé d'augmenter avec l'ajout de nouvelles espèces. Parmis ces espèces on cite par exemple: *Cymodocea nodosa* (Alberto *et al.* 2008), *Caulerpa prolifera* (Varela-Álvarez *et al.* 2015), *Diplodus vugaris*, *Mullus surmuletus*, *Seranus cabrilla*, *Tripterygion delaisi*, *Apogon imberbis* et *Symphodus tinca* (Galarza *et al.* 2009).

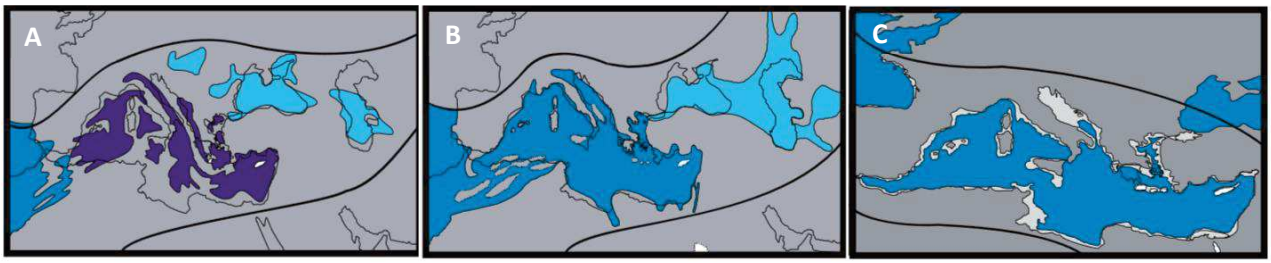

Figure 3: Paléogéographie de la Mer Méditerranéenne. (A, B et C) Séparation de la Mer Méditerranéenne de l'océan Atlantique et sa fragmentation en plusieurs basins après la Crise de Salinité Messénienne (5.6 – 5.3 Ma) (d'après Patarnello *et al.* 2007).

1.3.2 Un modèle emblématique, la moule méditerranéenne *Mytilus galloprovincialis*

Mytilus galloprovincialis est un bivalve du genre *Mytilus*. Ce genre comporte quatre espèces: *Mytilus californianus*, *M. trossulus*, *M. edulis* et *M. galloprovincialis*. La première espèce est complètement isolée, du point de vue reproductif, des trois autres espèces. Parmis ces dernières, *M. trossulus* semble être la forme ancestrale à partir de laquelle *M. galloprovincialis* et *M. edulis* ont divergé (Seed 1992; Stanley 1972). La divergence entre *M. edulis* et *M. galloprovincialis* a été estimée à environ 2.5 Ma, suivie d'une période de contact secondaire il y a environ 0.7 Ma (Roux *et al.* 2014). Durant environ 1.8 Ma les deux lignées allopatriques ont accumulé des incompatibilités génétiques. L'ancêtre de *M. edulis* et *M. galloprovincialis* aurait divergé de *M. trossulus* il y a plus longtemps, probablement à l'occasion de l'ouverture du détroit de Béring qui a permis la colonisation de l'Atlantique (Rawson & Hilbish 1995). Précédemment daté à 3.5 Ma (Vermeij 1989), l'ouverture du détroit de Béring pourrait être plus ancienne, entre 4.8 et 7.5 Ma (Marincovich & Gladenkov 1999). Le flux de gènes entre les espèces du complexe *M. trossulus*, *M. edulis* et *M. galloprovincialis* est désormais encore possible. Les chevauchements dans les aires de répartition entre les espèces du complexe, correspondent à des zones d'hybridation. Entre la Mer du Nord et la Mer Baltique une zone hybride clinale a été signalée entre *M. edulis* et *M. trossulus* (Gardner 1994; Gosling 1992; Väinölä & Strelkov 2011; Zbawicka *et al.* 2014). En Atlantique, *M. edulis* et *M. galloprovincialis* s'hybrident en mosaïque le long des côtes françaises (Bierne *et al.* 2003c), la mosaïque étant formée par trois zones de contact indépendantes (Bierne *et al.* 2003c; Hilbish *et al.* 2012); la première zone hybride (ZH_1) résulte de l'hybridation entre *M. galloprovincialis* (Péninsule Ibérique) et *M. edulis* (Golfe de

Gascogne), la deuxième (ZH_2) entre *M. edulis* (Golfe de Gascogne) et *M. galloprovincialis* (Bretagne) et la troisième (ZH_3) entre *M. galloprovincialis* (Bretagne) et *M. edulis* (Mer du Nord). En Méditerranée, une autre zone de contact a été détectée à l'Est du Front Almeria-Oran en Espagne (ZH_{AOF}) entre *M. galloprovincialis* Méditerranéennes et Atlantiques (Figure 4) (Daguin & Borsa 1999; Quesada *et al.* 1995a). Cette dernière zone serait la conséquence d'un contact secondaire entre populations allopatriques antérieures à l'origine du Front Almeria-Oran (Quesada *et al.* 1995b), la différenciation étant maintenue par l'addition d'un autre type de barrière dite barrière génétique renforçant la barrière naturelle et environnementale au niveau du Front Almeria-Oran (Gosset & Bierne 2012).

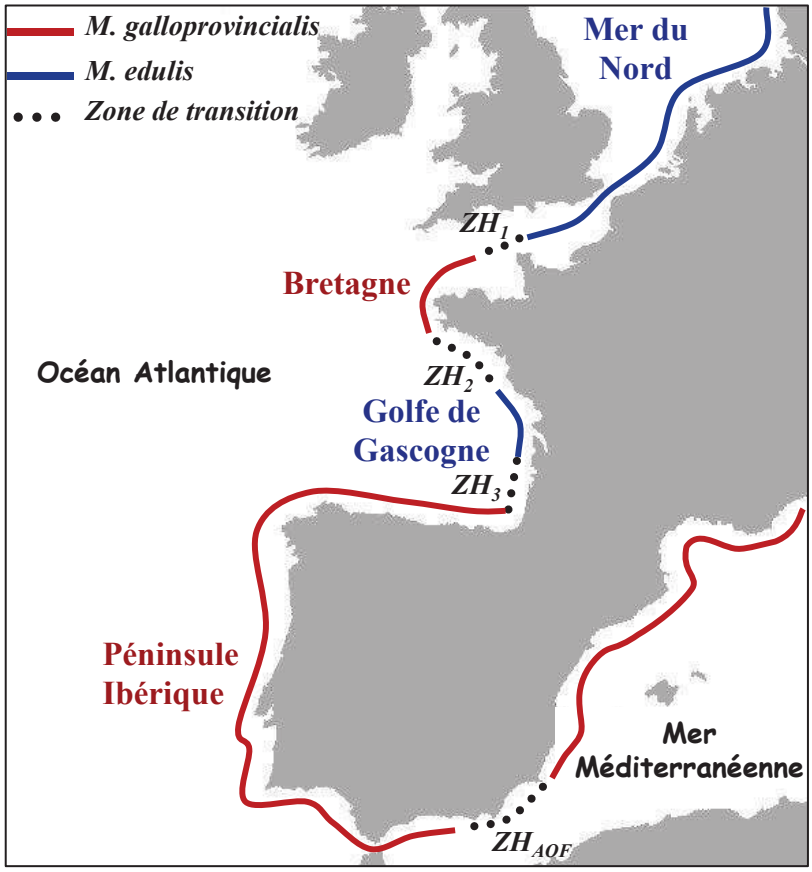

Figure 4: La zone hybride Mosaïque en Europe entre *Mytilus edulis* et *M. galloprovincialis*. Les zones de transition sont représentées en pointillés (ZH_1, ZH_2, ZH_3 pour les zones d'hybridation et ZH_{AOF} pour la zone de transition au niveau du Front Almeria-Oran (d'après Gosset & Bierne 2012).

La barrière s'est révélée être semi-perméable. Bien que ce concept ne soit pas nouveau (Barton & Hewitt 1985), ce n'est seulement qu'avec les travaux de Gosset & Bierne (2012) puis Fraïsse *et al.* (2015) qu'il a été montré que la nature de cette zone était sans-doute semi-perméable. Certains gènes contribuent à empêcher l'homogénéisation entre lignées génétiques différenciées appelées aussi gènes « verrous », soit parce qu'ils participent à l'adaptation aux conditions environnementales locales, soit parce qu'ils sont incompatibles avec les gènes d'autres lignées. D'autres parties du génome sont soit neutres, soit soumises à une sélection qui tend à homogénéiser les différentes lignées entre elles (Figure 5).

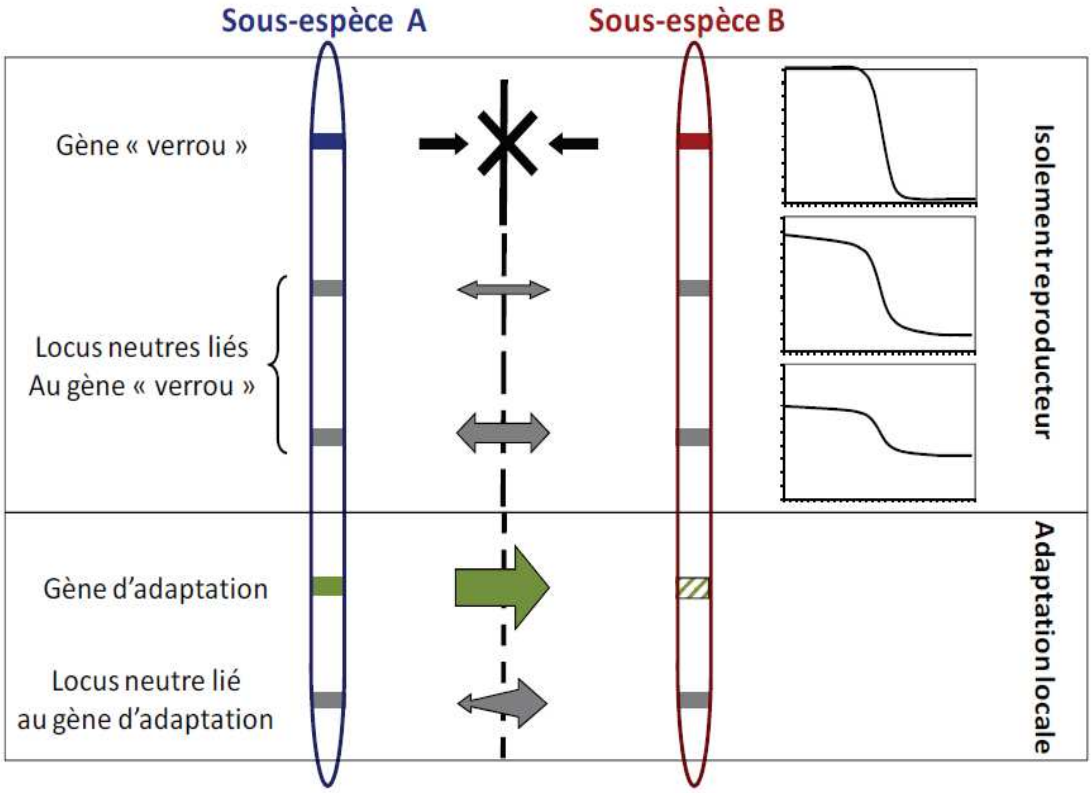

Figure 5: Schématisation de deux backgrounds génétiques **(**représentés chacun par un chromosome) séparés par une barrière semi-perméable au flux génique.

La division entre *M. galloprovincialis* Atlantique et Méditerranéenne a été mise en évidence grâce aux allozymes en particulier le locus *ODH* (Quesada *et al.* 1995a), les séquences RFLP (Sanjuan *et al.* 1996), le marqueur nucléaire *mac-1* (Daguin *et al.* 2001), 3 marqueurs nucléaires (*Me 15/16*, *EFbis* et *ITS*) et un marqueur mitochondrial (*ND2-COIII*) (Kijewski *et al.* 2011). Récemment, un scan génomique de 31 marqueurs nucléaires codominants dont 14 allozymes à partir de Quesada *et al.* (1995a), 6 microsatellites de Dizand Presa (2008) et 11 polymorphismes de longueur de Daguin et al. (2001), Bierne *et al.* (2002b), Boon *et al.*

(2009) et 357 marqueurs AFLP, Gosset & Bierne (2012) ont révélé une différentiation moyenne faible mais significative (F_{st} = 0.03) (Gosset & Bierne 2012). Les scans génomiques permettent de détecter les locus sous sélection dits « locus outliers ». Sept locus outliers (*EF2*, *EFbis*, *ODH*, *mt*, *AFLP_1-187*, *AFLP_3-363*, *AFLP_1-111*) ont été identifiés par trois méthodes (Beaumont & Nichols 1996; Foll & Gaggiotti 2008; Vitalis *et al.* 2003), seuls deux locus (*EFbis* et *AFLP_3-363*) ont été détectés comme anormalement différenciés par les trois méthodes à la fois (Figure 6) (Gosset & Bierne 2012). La différenciation entre les populations Méditerranéenne et Atlantique est accentuée par l'introgression du background Méditerranéen par des allèles de l'espèce *M. edulis*. Parmis les sept locus cinq étaient aussi outliers entre *M. galloprovincialis* Atlantiques et *M. edulis* (Gosset & Bierne 2012). La différenciation intra-spécifique détectée, chez *M. galloprovincialis*, n'est pas la seule au sein du complexe *Mytilus*, chez *M. edulis* elle a été aussi observée entre les populations Américaine et Européenne (Riginos *et al.* 2004).

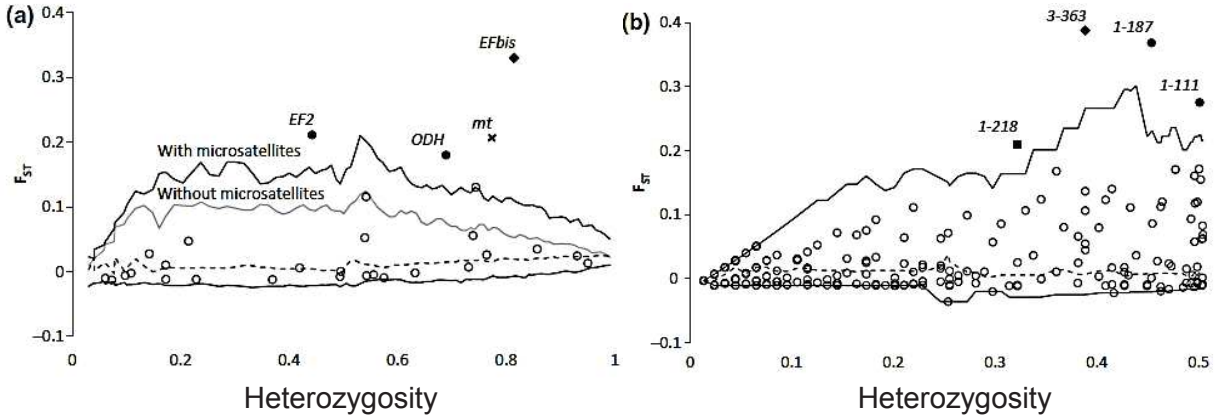

Figure 6: Scan génomique d'outliers de la différenciation chez *Mytilus galloprovincialis* entre la Mer Méditerranéenne et l'Océan Atlantique (Péninsule Ibérique). (a) 31 marqueurs nucléaires codominants (14 allozymes, 6 microsatellites et 11 polymorphismes de longueur). (b) 357 marqueurs AFLPs. Moyenne (ligne pointillée) et enveloppe de confiance à 95% (lignes en gras, la limite supérieure pour les données hors locus microsatellites est représentée en gris) sont des résultats à partir de simulations réalisées avec le programme FDIST2 (a) et le programme de DFDIST (b). Les locus aberrants sont annotés et représentés par des points noirs (lorsqu'ils sont identifiés par une seule méthode), carré (lorsqu'ils sont identifiés par deux méthodes) et les diamants (lorsqu'ils sont identifiés par trois méthodes, y compris BAYESCAN) (d'après Gosset & Bierne 2012).

Des progrès dans la compréhension de l'histoire de la spéciation et de l'adaptation au sein du complexe *Mytilus* ont été réalisés. Fraïsse *et al.* (2014a) ont pu montrer, grâce à une méthode statistique d'inférence de scénarios de spéciation 'Approximate Bayesian Computation (ABC)', que les moules Européennes ont connu une histoire complexe de divergence stricte suivie d'une période de connectivité périodique. En accord avec le concept de barrière semi-perméable au flux génique, il a été montré que les taux d'introgression sont hétérogènes le long du génome.

Des scans génomiques de la différenciation ont été menés entre populations du complexe d'espèces. Ces derniers ont permis d'identifier des régions du génome avec des taux inhabituels d'introgression. En effet, les régions à fort niveau de différenciation pourraient être des obstacles au flux génique, tandis que les régions à faible niveau de différenciation pourraient indiquer une introgression adaptative (Fraïsse *et al.* 2014b; Piálek & Barton 1997). Le locus *mac-1* est le seul outlier identifié par toutes les méthodes (six méthodes). Ce locus montre une empreinte caractéristique d'une introgression locale et une fréquence anormalement élevée d'allèles chez *M. edulis* introgressés dans les populations de *M. galloprovincialis* enclavées en Bretagne (Figure 7A, B).

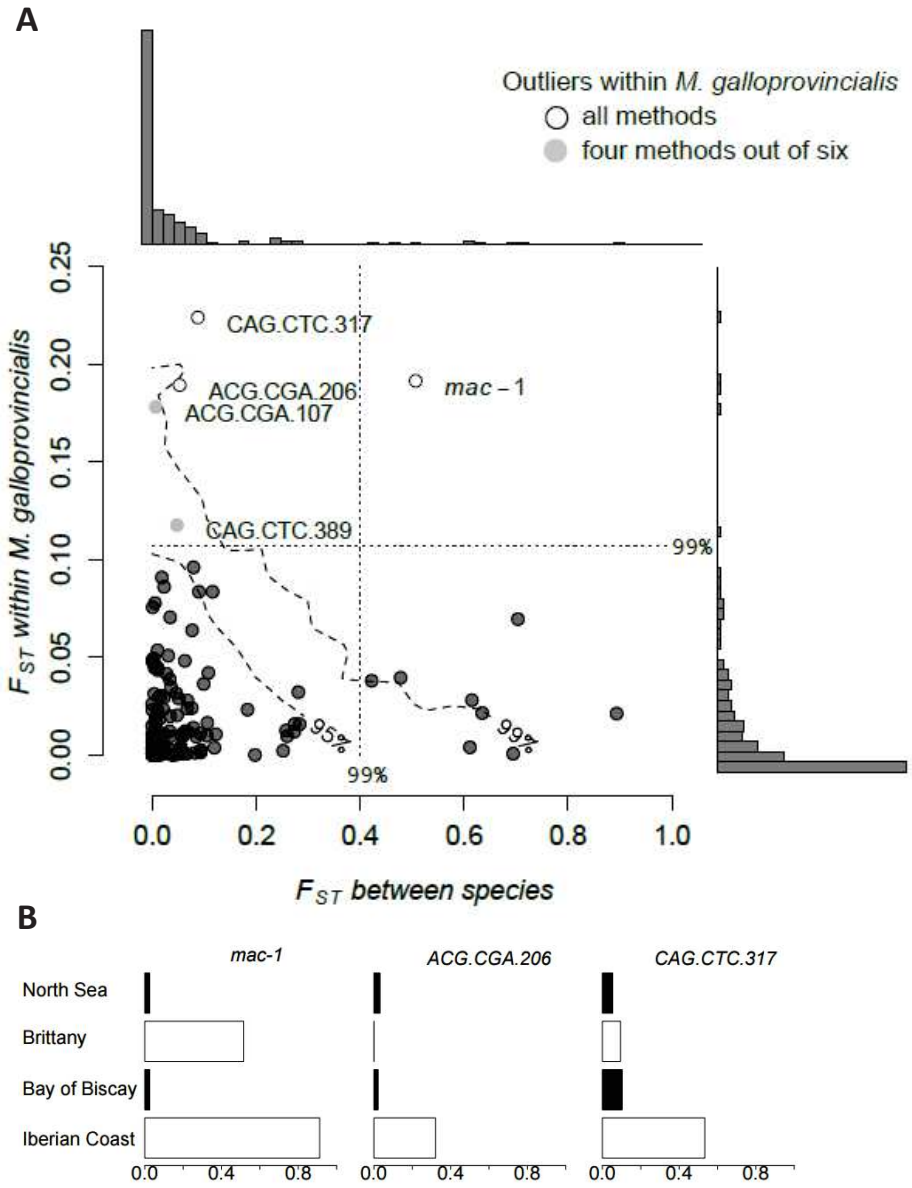

Figure 7: Scan génomique inter- et intra-spécifiques basée sur 440 loci (422 AFLP et 18 marqueurs nucléaires codominants). (A) Les valeurs du F_{ST} entre espèces contre celles au sein de *M. galloprovincialis* (côtes ibériques vs. Bretagne). Les lignes en pointillées représentent le seuil à 99% du test de Lewontin et Krakauer pour les deux comparaisons. Les lignes en pointillées délimitent les quantiles à 95% et 99% de l'enveloppe neutre. Les locus ouliers détectés au sein de *M. galloprovincialis* par toutes les méthodes sont représentés par des cercles blancs alors que ceux obtenus par seulement quatre méthodes sont représentés par des cercles gris. (B) Fréquence de l'allèle *galloprovincialis* pour les trois locus ouliers.

L'analyse de la variation génétique et des généalogies d'allèles, sur une échelle chromosomique localisée, a permis de reconstituer l'histoire évolutive de plus de 1000 régions du génome des moules. Cette analyse a révélé qu'une cause majeure, mais insoupçonnée, de la différenciation génétique intraspécifique est l'introgression différentielle d'allèles hétérospécifiques (Fraïsse *et al.* 2015).

1.3.3 Un nouveau modèle, la pourpre bouche de sang *Stramonita haemastoma*

Stramonita haemastoma (Linnaeus, 1767), communément appelé "pourpre bouche de sang", est un gastéropode de la famille des Muricidae. Il vit sur les fonds rocheux à proximité des côtes souvent exposés aux vagues dans les eaux tempérées à chaudes et parfois enfoncé dans la vase (Rilov *et al.* 2001). Pendant la saison estivale, les individus matures regagnent la zone intertidale pour s'accoupler et émettre des capsules, environ 20 à 86 capsules, contenant entre 1724 et 6956 œufs/capsule (El Ayari *et al.* 2015; Lahbib *et al.* 2011). L'éclosion donne naissance à des larves planctoniques pélagiques (Spence *et al.* 1990). La durée de la phase larvaire est relativement longue, elle a été estimée à 2-3 mois (Claremont *et al.* 2011; Lahbib *et al.* 2011; Scheltema 1977). Il constitue de ce fait un bon modèle puisqu'il présente tous les paramètres démographiques qui pourraient intéresser un généticien des populations marines. En plus, une forte variabilité morphologique a été observée entre populations géographiquement proches (Figure 8) (Claremont *et al.* 2011, observation personnelle).

La répartition géographique ainsi que la nomenclature, au sein du genre *Stramonita* sont sujet à débat, tout comme l'identification basée sur la morphologie de la coquille. Ceci s'est avéré insuffisant pour la distinction entre espèces et sous-espèces (Butler 1954; Claremont *et al.* 2011; Gunter 1979; Liu *et al.* 1991; Walker 1982).

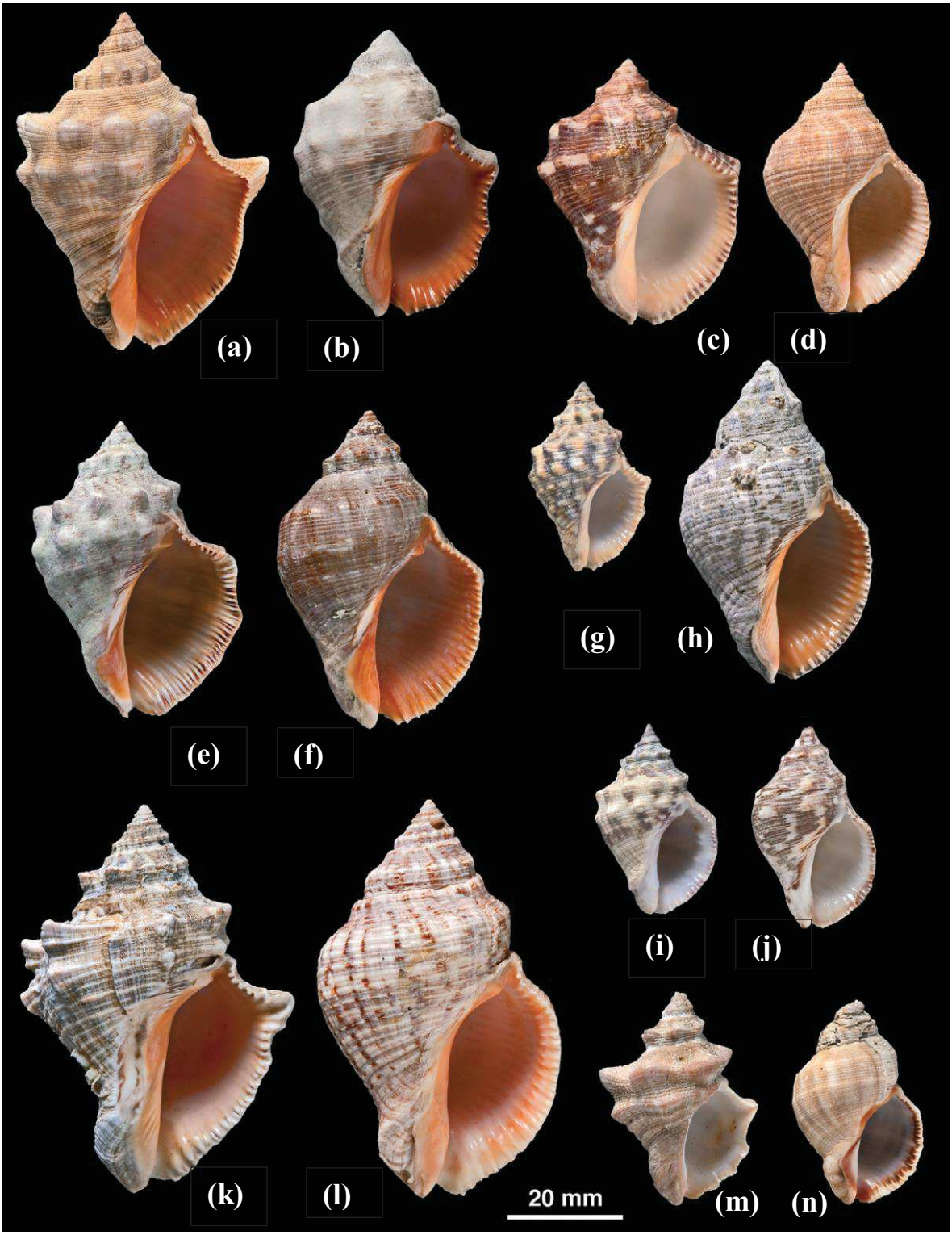

Figure 8: Variations morphologiques de la coquille au sein du genre *Stramonita*, (à gauche) sculptés et (à droite) lisses. a, b, *S. haemastoma* (a, b, Espagne); c, d, *S. biserialis* (c, Mexique, d, Panama) e, f, *S. brasiliensis* (e, Tobago, f, Brésil); g, h, *S. floridana* (g, h, USA); i, j, *S. rustica* (i, Bermudes, j, Brésil); k, i, *S. canaliculata* (k, i, USA) m, n, *S. delessertiana* (m, n, Chilie) d'après Claremont *et al.* (2011).

Dans la littérature, les études portant sur la génétique du complexe *Stramonita* en général et sur l'espèce *S. haemastoma* en particulier sont rares. Dans une étude sur les allozymes, Liu *et al.* (1991) ont montré, la présence de deux sous-espèces *S. haemastoma floridana* et *S. haemastoma canaliculata* au Sud-Est des Etats Unis, alors que ces populations étaient considérées pendant longtemps comme faisant partie d'une seule espèce, *Stramonita floridana*. Les deux sous-espèces sont considérées sympatriques avec un faible taux d'hybrides détecté (1.2%). Ces résultats ont aussi été confirmés avec un marqueur mitochondrial (*COI*) (Harding & Harasewych 2007). A ces deux études, s'ajoutent l'étude de Claremont *et al.* (2011). qui ont combiné à la fois des données morphologiques et moléculaires pour délimiter les espèces du genre *Stramonita* et tracer les aires de répartition ainsi que la phylogénie entre les espèces. Il résulte que le genre *Stramonita* comporte Sept espèces: *Stramonita haemastoma*, *S. delessertiana*, *S. rustica*, *S. floridana*, *S. canaliculata*, *S. biseralis* et *S. brasiliensis*. Toutes les espèces sont allopatriques ; cependant trois cas de sympatries ont été reportés entre *S. haemastoma*, *S. brasiliensis* et *S. rustica* au Venezuela, *S. floridana* et *S. canaliculata* dans le golfe du Mexique et *S. rustica* et *S. brasiliensis* au Brésil. L'âge du genre *Stramonita* a été estimé à 28 Ma (Claremont *et al.* 2011) et la présence de *S. haemastoma* en Europe à seulement 5 Ma (début du Pliocène) (Landau *et al.* 2007). *S. haemastoma* est une espèce qui se caractérise par une répartition géographique particulièrement large, elle a été signalée en Méditerranée, Atlantique Est et Ouest, Pacifique Est et à l'Ouest de l'océan Indien (Figure 8) (Abbott 1972 ; Claremont *et al.* 2011; Clench 1947).

Figure 8: Répartition géographique de l'ensemble des espèces du complexe *Stramonita* dans le monde, les symboles remplis représentent les spécimens BMNH (Natural History Museum, Londres), les symboles vides correspondant aux spécimens détectés dans la littérature (d'après Claremont *et al.* 2011).

Dans cette thèse on s'intéresse à l'étude d'une seule espèce du complexe '*Stramonita haemastoma' sensu stricto.* Dans les données de Claremont *et al.* (2011) il est possible de distinguer deux clades mitochondriaux (Figure 7), un résultat sur lequel les auteurs ne se sont pas attardés, sans doute parce que la divergence est faible et qu'un clade, qu'on nommera par

la suite A regroupe des individus provenant des tropiques (Sénégal et Venezuela) et l'autre clade, qu'on nommera par la suite B, rassemble des individus européens (Murcie, île d'Alboran et Macaronésie (Açores, Madère et îles Canaries)) (Claremont *et al.* 2011). De ce fait, la divergence pourrait simplement s'expliquer par un isolement géographique. Une inspection minutieuse révèle cependant que deux individus de Croatie appartiennent au clade A.

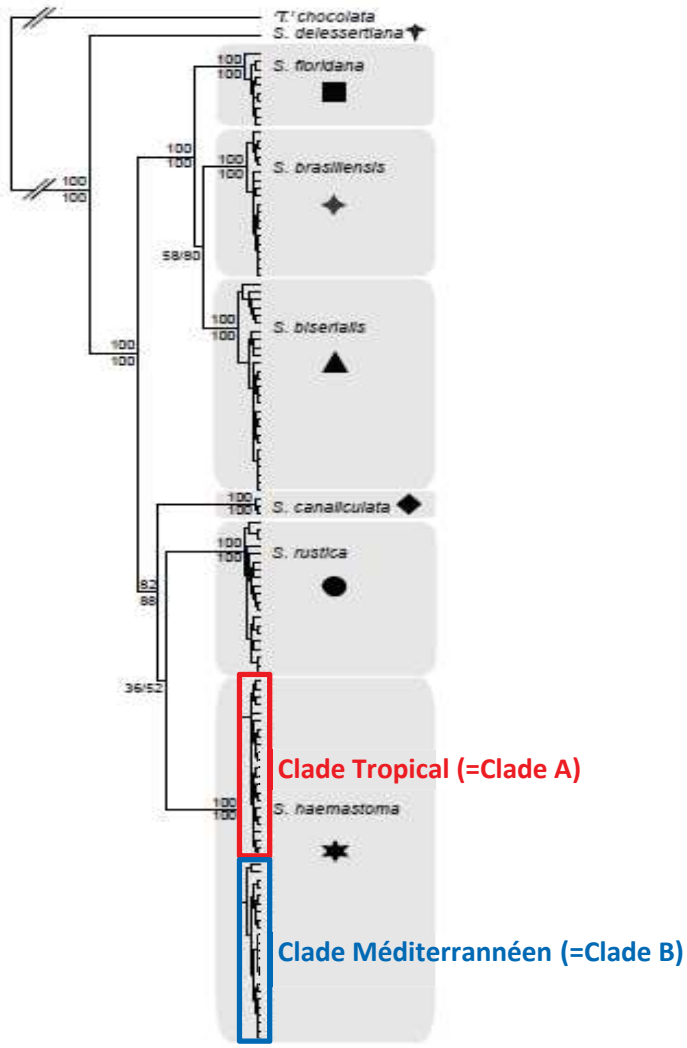

Figure 9: Phylogénie moléculaire du complexe *Stramonita haemastoma* basée sur l'analyse combinée du *28S*, *12S* et COI par approche bayésiennne (BEAST). Les cases grises indiquent les clades sélectionnés par l'analyse GMYC. Le rectangle rouge montre le clade tropical ou clade A, le rectangle bleu regroupe les individus du clade Méditérrannéen ou clade B (d'après Claremont *et al.* 2011).

2- OBJECTIFS DE LA THESE

L'objectif de cette thèse est d'étudier la structure spatiale fine des barrières génétiques au flux génique en Méditerranée occidentale chez deux mollusques marins: la moule *Mytilus galloprovincialis* et le gastéropode *Stramonita haemastoma sensu stricto*. L'échantillonnage intensif des deux espèces, à partir de la Méditerranée occidentale et en particulier de la rive Sud, permettra (i) d'obtenir une description fine de la structure génétique au niveau d'une barrière génétique marine ' le Front Almeria-Oran'. (ii) Produire des descriptions fines de clines de fréquences alléliques chez deux espèces marines: *M. galloprovincialis* et *S. haemastoma* à phase larvaire planctonique et d'intérêt économique. *M. galloprovincialis* a été analysé avec des marqueurs choisis à partir d'études antérieures comme étant les plus structurés au niveau du Front Almeria-Oran. *S. haemastoma* a été étudié grâce à de nouveaux marqueurs développés dans cette thèse.

3- ARTICLES

ARTICLE I

La face cachée de la zone de transition Atlantique-Méditerranée: Une zone hybride mosaïque de 600 km de large à l'Est d'Oran révèle l'interaction complexe entre l'hydrographie et l'isolement reproducteur chez les moules

I.1- Résumé

Des décennies de recherche en génétique des populations marines ont révélé de nombreux hotspots de différenciation génétique. Le Front Almeria-Oran (FAO) qui concentre de nombreuses discontinuités génétiques entre les populations Atlantique et Méditerranéenne est l'un des plus célèbres. Si la position du hotspot est bien expliquée par la barrière physique à la dispersion imposée par le FAO, l'origine et le maintien de la structure génétique restent imparfaitement compris. Dans la plupart des études, le grain d'échantillonnage est souvent insuffisant pour bien localiser la position du cline génétique, et la rive sud en Algérie est systématiquement oubliée. Nous avons utilisé des marqueurs semi-diagnostiques identifiés à partir d'un scan génomique pour étudier la structure génétique fine le long du littoral Algérien et Espagnol chez la moule *Mytilus galloprovincialis*. L'analyse des échantillons Algériens a révélé une vaste zone hybride mosaïque de 600 km de large à l'Est d'Oran. La composition génétique des populations dans lesquelles les deux lignées coexistent suggère que des mécanismes d'isolement reproductif intrinsèques contribuent au maintien de la différenciation génétique. Bien que la structure de la zone hybride soit une mosaïque, la lignée Atlantique a tendance à augmenter en fréquence en allant vers l'Est le long de la zone. Celle-ci se termine par un changement génétique abrupt au niveau du golfe de Bejaia, le lieu probable d'une barrière courantologique à la dispersion. La simulation d'une zone hybride dans un habitat linéaire en forme de Y démontre que deux zones de tension sœurs auraient pu être différentiellement piégées, l'une au niveau du FAO, au Nord, et l'autre au niveau du golfe de Bejaia, au sud. Un flux de gènes unidirectionnel du Nord vers le Sud suffit alors à maintenir un patch de la lignée Méditerranéenne au sud et à générer la mosaïque observée. Nos résultats fournissent un soutien supplémentaire à l'hypothèse du couplage qui propose que les facteurs physiques et environnementaux expliquent principalement la position des discontinuités génétiques alors que le maintien de la différenciation est mieux expliquée par l'existence d'un isolement reproductif intrinsèque.

A mosaic hybrid zone at the Atlantic-Mediterranean divide highlights the unappreciated interaction between the seascape and reproductive isolation

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ABSTRACT

Decades of research in marine population genetics have revealed many hotspots of genetic differentiation in the sea, among which the Almeria-Oran Front (AOF) is one emblematic example. The AOF is both a physical barrier to larval dispersal and an ecological boundary, and this may well explain the position of genetic discontinuities. However, the origin and maintenance of the genetic differentiation remains unsatisfactorily understood. A general drawback of previous studies is an insufficient sampling grain at the transition zone, with a conspicuous lack of samples from the southern side. We analysed the fine-scale genetic structure of *Mytilus galloprovincialis* populations along the Algerian and Spanish coastlines. Unexpectedly, analyses of Algerian samples revealed a 600 km wide mosaic hybrid zone eastward of Oran. This mosaic zone completely modifies our understanding of the Atlantic/Mediterranean transition because (i) the Atlantic lineage is naturally present eastward of the AOF within the Mediterranean Sea and (ii) the two lineages can leave in sympatry with ample opportunities to interbreed, implying intrinsic reproductive isolation must exist to maintain the genetic differentiation. The mosaic zone ends with an abrupt genetic shift in the Gulf of Bejaia, the likely place of a barrier to dispersal. Simulations of a Y-shape stepping stone model support the hypothesis that sister tension zones could have been differentially trapped at two alternative barriers to larval dispersal, at Almeria in the north and Bejaia in the south. A preponderantly unidirectional north-south gene flow next to the AOF is then sufficient to explain the maintenance of the Mediterranean lineage in southern coasts. Our results support the differentiation is primarily maintained by intrinsic reproductive isolation while hydrography mostly explains the position of genetic breaks, not their maintenance.

KEYWORDS: Barrier to gene flow, mosaic hybrid zone, reproductive isolation, marine connectivity, Almeria-Oran Front, *Mytilus galloprovincialis*.

1. INTRODUCTION

Natural populations are often subdivided into discrete patches within which apparent panmixia is observed or a gentle increase in genetic differentiation with geographic distance (Charlesworth *et al.* 2003). These roughly homogeneous genetic patches are bordered by abrupt genetic discontinuities generically called "barriers to gene flow" (Arnold 2006 ; Slatkin 1987). Barriers to gene flow allow divergence between populations and possibly the evolution of reproductive isolation and speciation. They also guarantee the maintenance of the beta genetic and specific diversity. Barriers to gene flow are therefore crucial in promoting and protecting biodiversity and understanding their functioning is a central issue in evolutionary biology and ecology.

Barriers to gene flow are sometimes shared among different species that are then thought to react to the same factors, for instance because they share a common history (e.g. privileged zones of secondary contact sometimes called suture zones (Hewitt 2000)), a comparable mode of dispersal (e.g. planktonic dispersers should be similarly affected by marine connectivity (Pelc *et al.* 2009)) or a similar ecological niche (e.g. adaptation to the same habitat such as brackish water in the Baltic Sea (Johannesson & Andre 2006)). In the marine realm, decades of research in population genetics have allowed to identify now well described hotspots of genetic differentiation (Hellberg 2009; Riginos *et al.* 2011). Probably because many marine species have large population sizes and important dispersal potential via the larvae phase, genetic homogeneity is near to be the rule within patches delineated by these barriers and the concentration of genetic breaks at privileged positions is ostensible in the sea (Gagnaire *et al.* 2015; Hellberg 2009; Riginos *et al.* 2011). Localisations of marine barriers to gene flow are characterized by strong environmental gradients or oceanographic currents and upwelling cells preventing dispersal along shores (Galarza *et al.* 2009). Many factors can be involved: physical (oceanic fronts, local and global oceanic currents (Patarnello *et al.* 2007)), ecological (temperature, salinity, tide level (Johannesson & Andre 2006; Johannesson *et al.* 2010)), historical (secondary contact (Avise 2000; Quesada *et al.* 1995), or genetical (intrinsic reproductive isolation (Bierne *et al.* 2011)). While barriers to gene flow may more probably result from a combination of factors acting synergistically (Barton & Hewitt 1985; Bierne *et al.* 2011; Cordero *et al.* 2014), a single factor is often emphasised to explain the genetic structure. In addition, the questions of the origin, localisation and maintenance of the genetic differentiation are often addressed simultaneously while these are different questions. To better understand the maintenance of genetic breaks, the simple genetic analysis of distant

samples well aside the transition zone is hardly sufficient, be it with high-throughput genomic data, one needs to fine-grained sample the transition zone in order to well characterise its fine-scale geographic structure and analyse how genetic lineages have the opportunity to interbreed at the boundary. Ultimately one needs to conduct experiments in the lab and in the field (Harrison 1993).

One of the most emblematic marine barriers to gene flow is the Almeria-Oran Front (AOF), the zone of genetic transition between populations of the Atlantic Ocean and the Mediterranean Sea (Patarnello *et al.* 2007; Quesada *et al.* 1995a; Tine *et al.* 2014). The front is generated by the convergence between Mediterranean and Atlantic waters. Atlantic waters enter into the Alboran Sea through the strait of Gibraltar and produce anticyclonic gyres westward of the AOF, the Western and Eastern Alboran Gyres, and a north-south unidirectional current eastside of the AOF (see Figure 1). In addition, part of the flow of the Eastern Alboran Gyres is trapped at the AOF to contribute to the Algerian current that flow eastward of Oran along the Algerian coastline (Viúdez & Tintoré 1995).

Among examples of genetic break at the AOF an iconic one is found in the marine mussel *Mytilus galloprovincialis* (Quesada *et al.* 1995; Quesada *et al.* 1995a). Since the early reports the origin of the genetic break has been thought to be a secondary contact between two lineages isolated in a Mediterranean and an Atlantic glacial refugia (Quesada *et al.* 1995), as for other species such as sea bass (Tine *et al.* 2014). The genetic differentiation between the two lineages proved to be highly heterogeneous among loci (Gosset & Bierne 2012). Such a semi-permeability is expected for a genetic barrier maintained by partial reproductive isolation (Barton & Hewitt 1985; Harrison 1993). To date, however, only the northern coasts neighbouring the AOF in Spain have been intensively sampled and revealed an abrupt shift in allele frequency each side of a 290 km wide no-mussel zone between Almeria and Alicante (Quesada *et al.* 1995a). On the other hand, the geographic structure of the transition zone in the southern coast was unknown. This is an important sampling gap to fill; notwithstanding Atlantic waters enter the Mediterranean Sea through the South by the Algerian current described above. This sampling gap is indeed a general lacuna in the study of the Atlantic-Mediterranean genetic divide. More generally the recent population genomics literature has tended to focus on the sampling of the genome too much while neglecting geographic sampling (Meirmans 2015).

In the present study we aimed to progress in our understanding of the marine transition zone at the AOF by analysing at fine sampling grain one mitochondrial and three nuclear semi-diagnostic molecular markers between the Atlantic and Mediterranean lineages previously identified by a genome scan (Fraïsse *et al.* 2015; Gosset & Bierne 2012). By sake of precaution we re-analysed the genetic shift of South-Eastern Spain that was reported twenty years ago. As could have been expected it proved to have remained nearly unchanged. We analysed new samples at a fine-scale along Algerian coasts between Oran and Bizerta, over a distance of 1330 km. We report a 600 km wide mosaic hybrid zone with samples of mixed ancestry that definitively reject low larval dispersal at the AOF as the principal factor of the genetic differentiation between the Atlantic and Mediterranean lineages but point to partial intrinsic reproductive isolation as the preponderant factor. Connectivity is nonetheless a major factor explaining the position of tension zones and we explore simple stepping-stone simulation models which show that the Y-shape unidimensional structure of the coastlines together with two alternative barriers to larval dispersal, one at the AOF and one in the Gulf of Bejaia, and a preponderantly unidirectional north-south gene flow at the AOF, can generate the mosaic structure observed.

2. MATERIALS AND METHODS

(a) *Sampling and molecular markers*

Mytilus galloprovincialis (Lamarck, 1819) samples were collected from 13 locations in the southern coast of the Alboran and Mediterranean Sea from Nador (Morocco) to Bizerte (Tunisia) and in 7 locations in the Northern coast from Manilva (Spain) to Peniscola (Table S1, Figure 1). We also analysed reference samples already reported in previous studies (Gosset & Bierne 2012) in Faro (Portugal) and Sète (France). DNA was extracted from gills using the QIAGEN DNeasy Blood & Tissue Kit following the instructions of the manufacturer. DNA concentration was measured for each sample using a NanoDrop8000 Spectrophotometer (Thermo Scientific) and standardised to a DNA concentration of 100 ng µL-1.

A 350-bp fragment of the cytochrome oxidase subunit III was PCR amplified and sequenced with primers *FOR1* (5'-TATGTACCAGGTCCAAGTCCGTG-3') and *REV1* (5'-TGCTCTTCTTGAATATAA GCGTA-3') (Zouros *et al.* 1994). Sequence reactions were precipitated using a standard EDTA/ethanol protocol, suspended in 15µl Hi-Di formamide and sequenced on an ABI 3130XL automated sequencer.

Three semi-diagnostic nuclear markers were chosen from previous genome scans for their ease of analysis. They are all three producing length-polymorphism of PCR products. *EFbis* is a now widely used marker in mussels that is amplified with the following two primers: *EFbis-F* (5'-ACAAGATGGACAATACCGAACCACC-3') and *EFbis-R* (5'-CTCAAT CATGTTGTCTCCATGCC-3') (Bierne *et al.* 2002a). *EF2* has been described in (Gosset & Bierne 2012) and is amplified with the following two primers: *EF2-F* (5'-GGAAATCCCATGGGTGATTTAGCGG-3') and *EF2-R* (5'-GTCAAATAAATACTGAAACACAGTGACTTC-3'). A contig containing the *Precollagen-D* gene was identified from a genome scan of 1269 contigs as the most differentiated contig between Atlantic and Mediterranean *M. galloprovincialis* populations (Fraïsse *et al.* 2015). Using publicly available sequences we designed primers to amplify the fifth intron of the gene with the 3' end of each primers positioned at the exon-intron junction, which allowed avoiding amplification of paralogous sequences of the gene family. The new locus was called *Precol-D* and the primers used to amplify it were: *Precol-D-F* (5'-GACAAGGACCAGCAGGTACCATATT-3') and *Precol-D-R* (5'-GAGTGGTCCGGCTGGTCCTAAGAA-3'). Indel polymorphisms in the intron produced nine length alleles in our samples with strong allele frequency differences between Atlantic and Mediterranean samples. Standard PCR protocols were used with annealing temperatures set at 54°C for *EFbis* and *EF2*, and 49°C for *Precol-D*. We pooled 2µl of PCR products and a mix of Hi-Di formamide/ROX 500 size standard (12.8µl formamide and 02 µl ROX), and the mixtures were then loaded on an ABI 3130XL capillary automated sequencer. GeneMapper® v4.5 software (Applied Biosystems) was used to read the resulting chromatograms.

(b) *Data analysis*

COIII sequences were aligned using ClustalW multiple alignment in BIOEDIT V7.1.3.0 and revised manually. Aligned sequences were then used to construct a Neigbour-Joining tree with MEGA software v6. Allelic and genotyping frequencies were obtained using Genetix software v4.0.5.2 (Belkhir *et al.* 2002). Number of haplotypes (h), nucleotide diversity (π), average number of nucleotide substitutions per site between populations (Dxy) and net number of nucleotide substitutions per site between populations (Da) were calculated using DnaSP V5.10.1 (Librado & Rozas 2009). The genetic structure was examined by two methods: a Bayesian clustering method implemented in STRUCTURE 2.3.4 (Falush *et al.* 2003) and a discriminant analysis of principal components (DAPC) implemented in the adegenet package in R software (Jombart & Ahmed 2011). STRUCTURE was run for k = 2,

with a burn in period of 50.000 steps and a run length of 100.000 iterations. Analysis with Structure was conducted under the admixture model, assuming that the allele frequencies are correlated across populations (Falush *et al.* 2003). To depict allele frequency clines, all four loci were transformed into bi-allelic loci by pooling alleles according to their frequencies in reference samples. The average pairwise linkage disequilibrium across all loci, *LD*, was estimated using the variance in the hybrid index as described in (Barton & Gale 1993).

(c) ***Simulations***

We used a model of evolution in a metapopulation of 2 x 60 demes arranged in two parallel linear stepping-stones of *n* demes each (Bierne *et al.* 2013). At generation zero, two partially reproductively isolated backgrounds meet and start to exchange genes. The auto-recruitment rate was 1 - 2*m*, and migration to adjacent demes was *m* (when not otherwise stated $m = 0.3$). Two weak barriers to gene flow were set between demes 25 and 26 in the northern chain (with $m = mbar$, when not otherwise stated $mbar = m/6 = 0.05$), and optionally in the southern chain, which is aimed to simulate the AOF, and between demes 40 and 41 in the southern chain of demes, which is aimed to simulate the Gulf of Bejaia Barrier (GBB). Bidirectional gene flow was possible between the northern and the southern chain for the first 25 demes (left to the first barrier to gene flow, i.e. in the Alboran Sea). For a few more demes (from 1 to 10) bidirectional or unidirectional north-south gene flow was possible between the north and the south (with the same migration rate *m* as between adjacent demes) and then stopped for the remaining demes. The aim of the parameterization was to account that the Spanish and the Algerian coasts become too far away eastward of the AOF to be connected by a single generation of larval dispersal. Selection ($s = 0.2$) acted against recombinant genotypes (intrinsic post-zygotic selection) at two freely recombining reproductive isolation loci (Bierne *et al.* 2011), one of which was linked to a neutral marker located at recombination map distance of 1cM. Random drift was simulated by multinomial sampling of genotypes within each deme at each generation ($N = 200$ per deme).

3. RESULTS

(a) *Analysis of the genetic diversity*

The analysis of 568 *COIII* sequences has revealed the existence of 150 haplotypes and a high nucleotide diversity (π = 0.023). Average and net nucleotide divergence measures between Atlantic and Mediterranean populations were also high (Dxy = 0.029, Da = 0.011). The NJ-tree is presented in the supplementary Figure S1. Two clades of haplotypes can be defined according to their phylogenetic relationships and their frequency in Atlantic and Mediterranean samples (Figure S1). The clade nearly fixed in Atlantic samples is known to be the consequence of introgression of alleles from the sister species *M. edulis* (Quesada *et al.* 1998). We used the phylogenetic relationship to define the Mediterranean and the Atlantic haplogroups. Nine size-alleles were detected at the new locus *Precol-D* and allele frequencies are presented in Table S1. *EF2* was found bi-allelic as in (Gosset & Bierne 2012). The use of capillary electrophoresis allowed us to reveal *EFbis* size-alleles with 1bp differences that were not detected previously with acrylamide gels. We observed 22 alleles and the correspondence with previous allele names is given in Table S1. Clustering analysis was performed with multi-allelic data. In order to represent allele frequency clines we transformed multi-allelic loci into bi-allelic loci by pooling alleles according to their frequency in reference samples (see Table S1).

(b) *Spatial genetic structure*

The frequencies of the Mediterranean allele at the mitochondrial *COIII* locus and the three nuclear markers (*Precol*-D, *EFbis* and *EF2*) in each sample are presented in Figure 2A and 2B. As already reported 20 years ago (Quesada *et al.* 1995a) the northern transect along Spanish coasts revealed an abrupt change in allele frequencies in the zone of transition between Almeria and Cartagena with all four loci. The three samples collected in Atlantic waters (1-Faro, N2-Manilva and N3-Almeria) have high frequencies of the Atlantic alleles (Figure 2A), uniformly high membership probabilities to the Atlantic cluster in the DAPC analysis (Figure 1) as well as a high proportion of Atlantic ancestry in the STRUCTURE analysis (Figure 2E). Conversely samples from Mediterranean waters (N6-Tabarca, N7-Nules, N8-Peñíscola del Pinatar, and N9-Sète) have high frequencies of the Mediterranean alleles (Figure 2B), uniformly high membership probabilities to the Mediterranean cluster in the DAPC analysis (Figure 1) as well as a high proportion of Mediterranean ancestry in the STRUCTURE analysis (Figure 2F). Two samples (N4-Cartagena and N5-San Pedro) were

obtained from the region reported to be devoid of mussels 20 years ago. These samples were predominantly of Mediterranean ancestry, but some individuals were assigned to the Atlantic genetic cluster with high confidence and a few mussels with a genome of mixed ancestry were also present (Figure 1 and 2E). Linkage disequilibrium was maximal in these intermediate samples as expected from hybrid zone theory (Barton & Gale 1993). These samples are crucial for our understanding of the functioning of the AOF barrier to gene flow as they show Atlantic mussels are found in observable numbers eastward of the AOF within the Mediterranean Sea. One can notice that the *EFbis* cline is not perfectly coincident with the other three clines. Its centre is slightly shifted to the east between San Pedro and Tabarca (Figure 2A). The southern transect along Algerian coasts revealed a much wider and complex structure with a mosaic pattern of alternation between predominantly Atlantic and intermediate samples (Figure 1 and 2). Individuals assigned to the Atlantic cluster predominated in the sample from S2-Nador (Morocco) situated in the Alboran Sea (Atlantic water) (Figure 1 and 2). The Mediterranean cluster predominated in three samples from eastern Algeria (S11-Zaima Mansouriah, S12-Collo and S13-Skikda) and Tunisia (S14-Bizerta) (Figure 1 and 2). From S3-Oran to S10-Bejaia, a mosaic hybrid zone was observed with a tendency for the Mediterranean ancestry to decrease eastward along the zone. The zone ends with an abrupt genetic shift in the Gulf of Bejaia (Gulf of Bejaia Barrier, GBB), east of which is only observed the Mediterranean cluster. Within the mosaic zone, samples were found to be a mix of individuals belonging to both clusters and only a few hybrids (Figure 1 and 2F). Again strong linkage disequilibrium (Figure 2D) attests for a strong deficit of hybrids relative to random mating. The discordance between *EFbis* and the other three loci is not as neat in this mosaic zone as it is in the northern clinal zone but in the first samples eastward of the AOF (S3-Oran) and eastward of GBB (S12-Collo) the frequency of the Atlantic allele was higher at *EFbis* than at the other three loci.

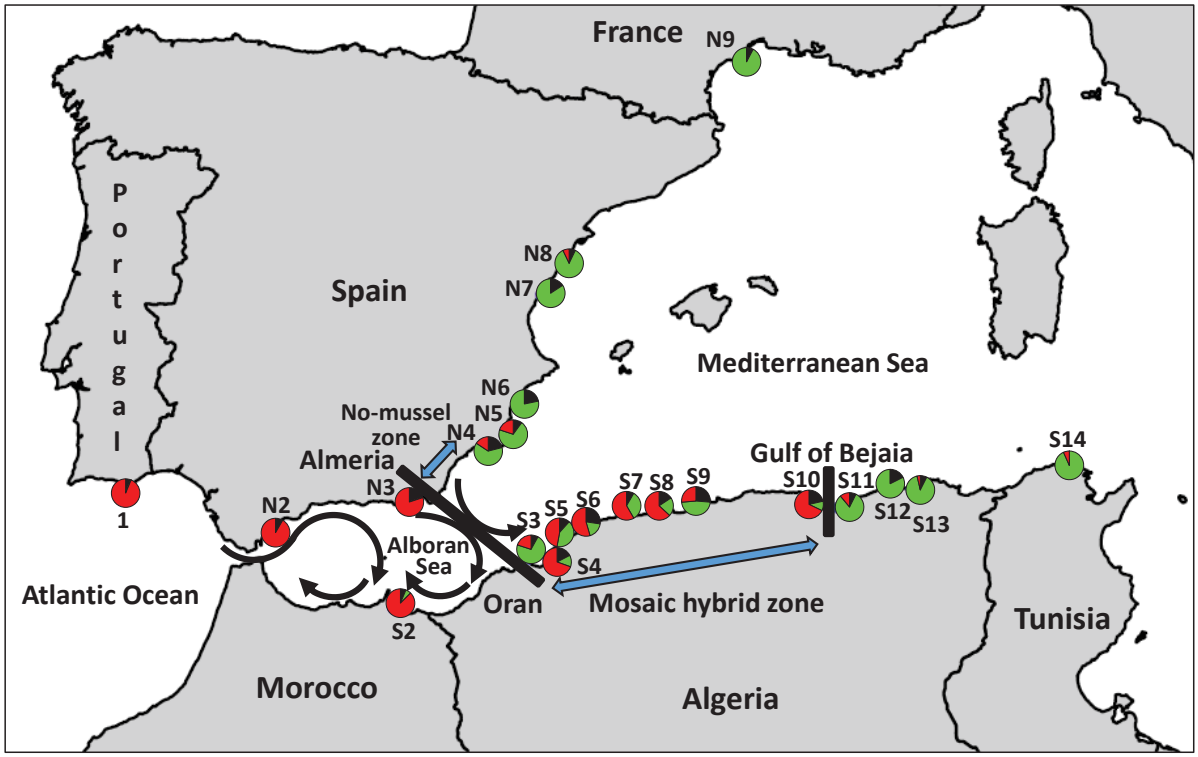

Figure 1. Sampling localities of *Mytilus galloprovincialis* samples in the north-eastern Atlantic and the Mediterranean Sea. Proportion of individuals assigned to the Atlantic cluster (in red), the Mediterranean cluster (in green) and unassigned (in black), based on a DAPC analysis with the four loci (*COIII*, *Precol-D*, *EFbis and EF2*). Sample names and their GPS positions are given in the supplementary Table S1.

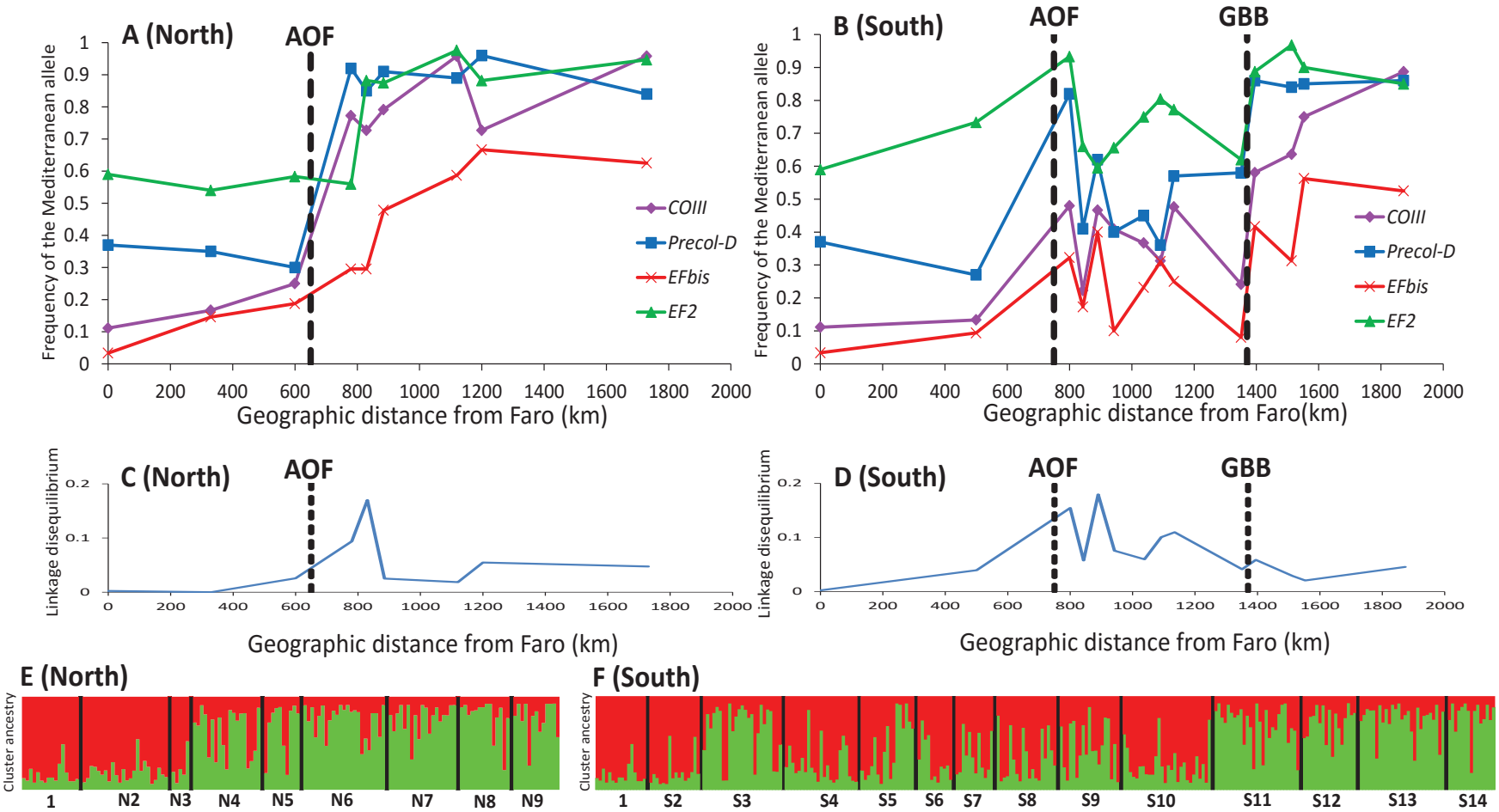

Figure 2. *Mytilus galloprovincialis* Mediterranean allele frequencies at the four semi-diagnostic loci analysed (*COIII*, *Precol-D*, *EFbis* and *EF2*) for the 9 samples of the northern coastline (A) and the 14 samples of the southern coastline (B). AOF: Almeria Oran Front, GBB: Gulf of Bejaïa Barrier. Average multilocus linkage disequilibrium in samples of the northern coastline (C) and samples of the southern coastline (D). Bar plot of the estimated cluster membership fraction (Q) estimated by STRUCTURE for the samples of the northern coastline (E) and southern coastline

(c) *Modeling a Y-shape stepping stone that account for the geographical context and the recognized hydrographic features of the AOF transition zone*

Our simple tension zone model in a Y-shape stepping stone allowed us to obtain useful indications on how the principal features of the seascape around the AOF area could produce the mosaic structure observed. It is important to remind that in the tension zone model selection against hybrids maintains the cline structure but the position of the cline is not stabilized by selection. Theory predicts that tension zones should be trapped by and coincides with natural barriers to dispersal, zones of low population density such as mountains, rivers and oceanic fronts (Barton 1979a). Stochastic factors, such as random genetic drift or temporal variations in population density or dispersal rates, can sometimes allow the cline to escape the barrier trap and the cline move again more or less haphazardly until it becomes trapped again by the same or another barrier. One expects tension zones of restricted dispersers to be trapped by the first minor barrier encountered near a point of contact, while those of high dispersers could potentially escape such minor barrier and be durably trapped only when reaching a strong barrier. However, we also expect that the point of contact coincide with a barrier to dispersal so it is not clear how far a tension zone can stabilized from its original zone of contact. In our simulations for example, if the initial condition is a contact at the AOF (between demes 25 and 26), the barrier is strong (low *mbar*) and drift is slow, the cline is indefinitely trapped at the AOF. Note that even when the barrier is simulated only in the northern chain (migration between demes 25 and 26 is fixed at *mbar* in the northern chain and at *m* in the southern chain), this is sufficient to stabilize the cline on both side, even in the south, providing the two chains are connected by migration somewhere. To obtain a discordance between the two chains and observe the northern cline trapped at the AOF and the southern cline trapped at the GBB (between demes 40 and 41 in the southern chain) we need either (i) to simulate a moderate barrier at the AOF and strong drift in order to allow the southern cline to escape the AOF and reach the GBB or (ii) to simulate the initial contact inside the Mediterranean Sea (somewhere between demes 26 and 40) so that the northern cline is trapped by the only one barrier simulated in the northern chain, at the AOF, and the southern cline can be trapped by at the AOF or the GBB and more frequently to the closest barrier to the point of contact (e.g. if we simulate the contact at the GBB the southern cline is always trapped by the GBB). Sister tension zones can therefore be differentially trapped at two alternative barriers to larval dispersal, at Almeria in the north and Bejaia in the south.

However, this does not produce a mosaic zone in the south (see the insert in Figure 3A). Only when we simulate a preponderantly asymmetrical north-south migration rate east of the AOF can we obtain a "pocket" of the Mediterranean background formed eastward of Oran. Figure 3A show one simulation output that illustrates the structure obtained with this model, and which resembles quite well the observed structure (Figure 3B).

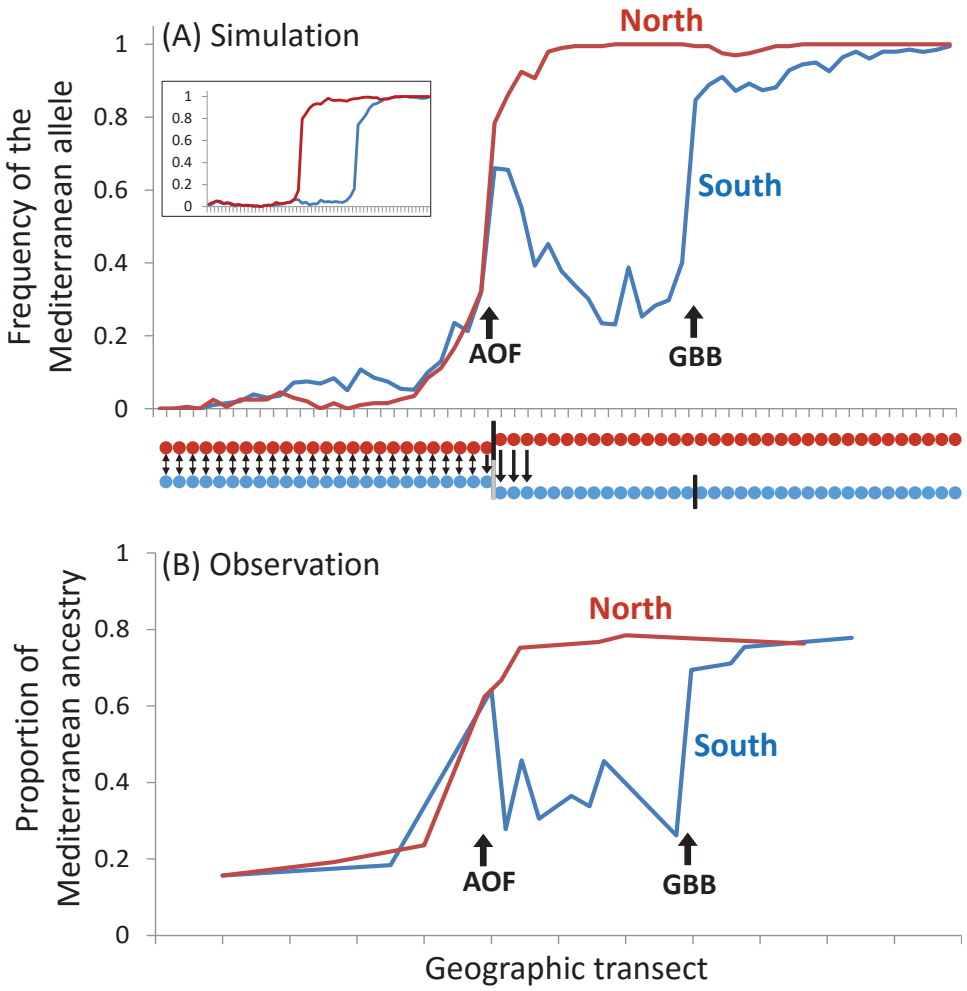

Figure 3. (A) A simulation output obtained with the Y-shape stepping stone model, including two weak barriers to gene flow between demes 25 and 26 (AOF) in the northern chain and between demes 40 and 41 (GBB) in the southern chain of demes and unidirectional migration right to the AOF. The insert shows an output when migration was bidirectional between the two chains. (B) Proportion of Mediterranean ancestry under an admixture model (STRUCTURE Q values). Geographic positions are rescaled so that Almeria superposes to Oran.

4. DISCUSSION

The Almeria-Oran Front (AOF) is arguably one of the most famous hotspots of genetic differentiation in the sea (Patarnello *et al.* 2007); and *Mytilus galloprovincialis* one of the iconic species exhibiting a pronounced genetic break at the AOF (Quesada *et al.* 1995a). Although we had good presumptions that the genetic break originated from a secondary contact between a Mediterranean and an Atlantic refugia and indirect evidence that reproductive isolation needed to occur in order to maintain the genetic differentiation and to explain the semi-permeable nature of the barrier to gene flow, the story could not be definitively settled without more direct evidences that interbreeding opportunities exist at the transition zone, even if rare. Maybe for this lack of compelling evidence, the idea that the Atlantic-Mediterranean divide could simply be explained either by the AOF itself acting as a barrier to larval dispersal or by the ecological differences between Atlantic and Mediterranean waters remains strongly anchored in the marine literature. In addition, the description of the southern side of the transition zone remained a strong lacuna that deserved to be filled as we knew Atlantic waters enter the Mediterranean Sea through the south by the Algerian current. Although a promising project, sampling the southern coast unrevealed far more than one could have expected. The unexplored side of the barrier proved to hide a 600 km wide mosaic hybrid zone. We will here argue that the existence of this large mosaic hybrid zone refutes the hypothesis that a natural barrier to larval dispersal or/and adaptation to different environments could be the main factors explaining the maintenance of the genetic differentiation. On the other hand it provides support for the existence of partial intrinsic reproductive isolation which needs to prevent gene flow when the two lineages live in sympatry/syntopy and would have all the opportunity to interbreed and exchange genes in the absence of such a reproductive isolation. The coupling hypothesis provides a fuller explanation to why the hybrid zone coincides with the AOF (Bierne *et al.* 2011), and also explains the mistaken belief that the AOF itself could be responsible of the genetic differentiation, while it rather explains the position of the genetic break but not its maintenance. Although high population size high gene flow marine species in the generally well connected marine environment are good systems to exacerbate the concentration of genetic differentiation in well recognised hotspots and thus to provide support for the coupling hypothesis, there is no reason for it to be restrained to the sea. The literature on terrestrial species that has also strongly emphasised the importance of the landscape in explaining genetic structure (Manel & Holderegger 2013)

could well considers coupling as being similarly and more broadly acting in the terrestrial realm.

The AOF is both a barrier to larval dispersal and an environmental boundary. However, at a finer scale it is also an area of mixing between the inflow of surface Atlantic waters into the Western Mediterranean basin and the outflow of deeper Mediterranean waters (Tintore *et al.* 1988). The front is marked by a strong gradient of temperature ($\Delta T=2°C$), salinity ($\Delta S=2ppm$) and density ($\Delta s=1.0$ kg m^{-3}) (Oguz *et al.* 2014). The Western Alboran gyre is mainly fuelled by Atlantic waters and the Eastern anticyclonic gyre was found to be a mix of Atlantic waters and surface waters of the Alboran Sea called 'Modified Atlantic water' (MAW) (Font *et al.* 1998). MAW leaves the Alboran Sea split into two veins; one feeds current reaching Spanish coast, the other goes to the east forming the Algerian Current that flows eastward along North African coast (Font *et al.* 1998).

The absence of mussels between Almeria and Alicante (Quesada *et al.* 1995a) in the Spanish coast suggested that there was no strong opportunity for interbreeding between the Atlantic and the Mediterranean linages, and that the AOF could be an efficient barrier to gene flow. However, we obtained two samples south to Alicante in Cartagena and San Pedro (N4 and N5 in Figure 1) with a low but non-negligible proportion of individuals assigned to the Atlantic genetic cluster. This is sufficient proportion of migrant mussels to refute the barrier to dispersal as sufficiently strong to explain the maintenance of the genetic structure. In any case, the hydrographic characteristics suggested that the southern coast should be more pertinent to better understand the contact between the two lineages. With the result of the southern coast, there remains no ambiguity that the Atlantic lineage enters the Mediterranean Sea. Note that the existence of species without genetic break at the AOF while sharing similar biological and ecological characteristics (e.g. the European flat oyster *Ostrea edulis* (Launey *et al.* 2002), the Mediterranean rainbow wrasse *Coris julis* (Aurelle *et al.* 2003), or the saddled seabream *Oblada melanura* (Galarza *et al.* 2009)) was already a strong argument against the hypothesis of the barrier to dispersal.

We now turn to the more complex issue of adaptation to the macroscopic environment (e.g. salinity, temperature, pH, wave action etc…). There is little doubt that differential adaptation exists between the two lineages that inhabit such contrasted habitats as are the Mediterranean Sea and the Atlantic Ocean. The very question is its actual role in the maintenance of the genetic differentiation knowing that each lineage survives well in the alternative environment

and reproduces sympatrically with the other lineage in Algeria. Again the northern coast was not ideal to address the question. We can well imagine that the migrants sampled in Cartagena and San Pedro were sufficiently maladapted to contribute little to the next generations, generating an efficient genome-wide barrier to gene flow (Marshall *et al.* 2010; Nosil *et al.* 2005). However, when considering the 600 km wide Algerian mixing zone, the hypothesis becomes much less parsimonious. Although one may argue that Algerian waters might well be ecologically intermediate, this rather results in the co-existence of the two lineages in sympatry in a large area, allowing the potential for widespread interbreeding and gene exchange. Therefore the ecological contrast required to explain the barrier to gene flow would need to be more fine-grained within the mosaic zone. In any case, the mere existence of sites where the two lineages coexist at similar frequencies, suggests that habitat specialisation alone is not sufficient to maintain the differentiation between genomes. Such populations represent bridges between specialised lineages that are likely sufficient to homogenise genomes in the absence of additional mechanisms of reproductive isolation. Indeed, local selection needs to be extremely strong to generate an efficient barrier to gene flow (Barton & Bengtsson 1986; Feder & Nosil 2010; Slatkin 1973), producing a strong segregation of each ecotype in its own favoured habitat, certainly not the coexistence in balanced proportions. Although mosaic hybrid zones are usually associated with a patchy fine-grained environment which explains the mosaic structure (Rand & Harrison 1989), they are often multifactorial and maintained by both exogenous and endogenous isolating mechanisms (Barton & Hewitt 1985; Bierne *et al.* 2011). For instance this is the case of another mussel hybrid zone between *Mytilus galloprovincialis* and *M. edulis* in southern Brittany, where the two backgrounds segregate spatially according to wave action but for which intrinsic post-zygotic isolation distributed genome-wide on many Dobzhansky-Muller incompatibilities is the main guarantee of the genetic differentiation (Bierne *et al.* 2006). In addition, habitat heterogeneity was far from evident between our sampling sites in Algeria as we have sampled very similar habitats: high-shore rocks protected from wave action by artificial port breakwaters and with no evident influence of freshwater from nearby estuaries.

Overall our results provide additional support for the coupling hypothesis that proposes genetic breaks are often secondary contact semipermeable tension zones trapped by physical barriers to dispersal or environmental boundaries, and that physical and environmental factors mostly explain the position of the breaks but the maintenance of the genome-wide genetic differentiation is best explained by partial intrinsic reproductive isolation (Bierne *et al.* 2011).

To sustain our argumentation we used a simple simulation model that proved able to produce the mosaic structure with purely intrinsic post-zygotic selection (Figure 3). Long distance dispersal during colonization can result in a mosaic hybrid zone (Le Corre *et al.* 1997) that can be maintained by intrinsic isolation only (M'Gonigle & FitzJohn 2010). However, it is unclear how stable they can be. Our model show that a more complex spatial structure than the standard 1D or 2D stepping stone model can generate a stable mosaic structure. It implies the differential coupling of sister tension zones at two different geographic positions, at the AOF in the North and at the GBB in the south. The abrupt genetic shift at the GBB is a new observation in an understudied area. However it fits well with the position of a barrier to dispersal identified with oceanographic modelling in two recent analyses (Berline *et al.* 2014). Differential coupling of sister tension zones can well occur in coastal or river species leaving in unidimensional linear landscapes with tree-like reticulation. For instance, some hybrid zones secondarily trapped at the entrance of the Baltic Sea are likely to have produced sister hybrid zones which have moved further north along the Norwegian coast to be trapped somewhere in northern Scandinavia (e.g. in *Mytilus trossulus*, *Macoma balthica*, *Gadus morhua*, *Platichthys flesus* and *Gammarus zaddachi*; reviewed in (Bierne *et al.* 2011)). The unidirectional north-south migration at the AOF is an additional essential hydrographic feature required to explain the presence of a patch of the Mediterranean lineage eastward of Oran. Finally, we also have noticed a more subtle result in our genetic data that could well sustain the tension zone model, which is the slight but visible discordance between *EFbis* and the other three loci in the northern cline (Figure 2A) and also slightly in the mosaic zone and at the GBB (Figure 2B). Stochastic drift would have play on all four loci not just one against the other three. Exogenous selection generates discordance when loci react to different ecological variables that vary differently in space (Slatkin 1975) but it is difficult to imagine an ecotone just a few kilometers of the other in the sea. On the other hand, discordant clines are expected as a consequence of negative epistatic interactions among genetic incompatibilities (Gavrilets 1997).

To conclude, the mosaic hybrid zone between Oran and Bejaia observed in *M. galloprovincialis* mussels contributes to definitively refute the hypothesis that the AOF itself generate a sufficiently strong barrier to dispersal to maintain the genetic differentiation and to cast serious doubt against differential adaptation to Atlantic and Mediterranean waters as being the principal factor to reduce effective gene flow. On the other hand it provides new evidence in favour of the coupling hypothesis that will need to be supplemented by lab

experiments (hybrid crosses) and field studies (settlement and reproduction). Our results also call for new genetic studies along Algerian coasts in other marine species. Finally they reveal how complex can be the interplay between hydrography and reproductive isolation, and, without minimising the importance of hydrography, suggest an excessive focus on oceanographic features in the interpretation of the genetic structure in the sea.

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ANNEXE ARTICLE I

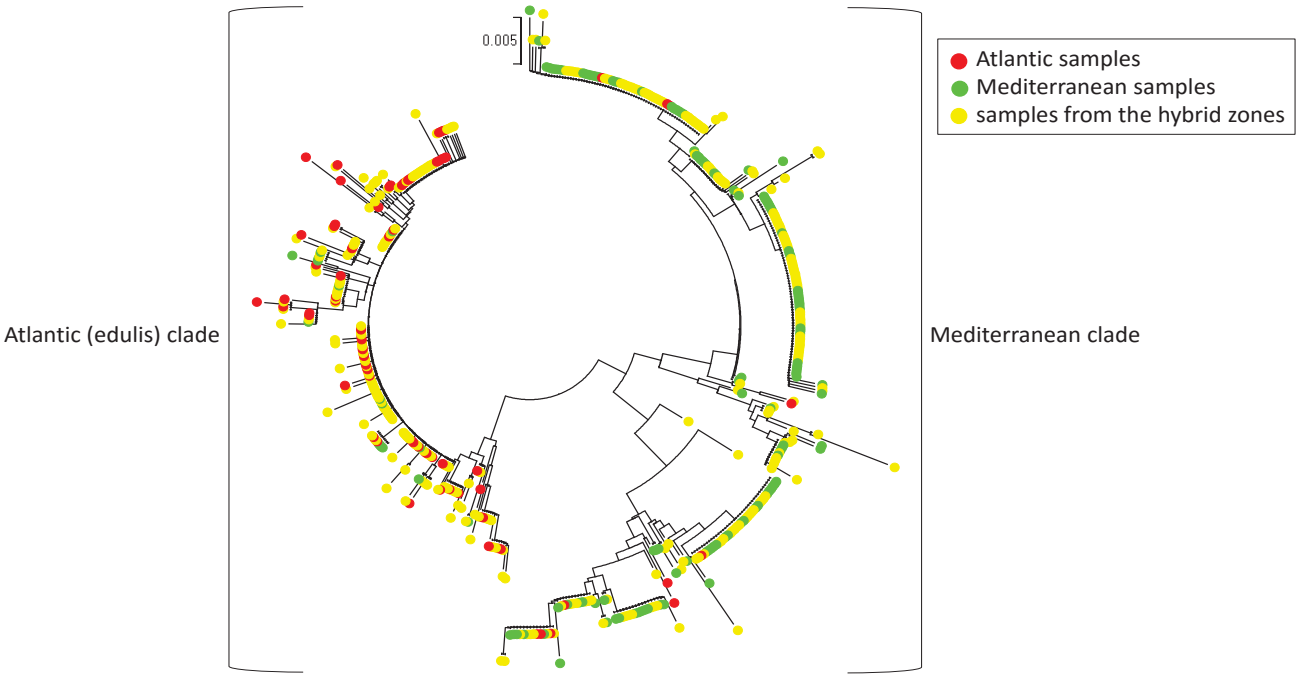

Figure S1. Neighbor-joining tree of of mtDNA *COIII* sequences from *Mytilus galloprovincialis* samples

Table S1. *Mytilus galloprovincialis* sample location.

		GPS coordinates	
Sample code	**Sample name**	**Latitude**	**Longitude**
1	Faro	36°961635 N	-7°926326 E
N2	Manilva	36°358011 N	-5°227035 E
N3	Alméria	36°826029 N	-2°463499 E
N4	Cartagena	37°580509 N	-0°987318 E
N5	San Pedro	37°797880 N	-0°787735 E
N6	Tabarca	38°183158 N	-0°529731 E
N7	Nules	39°878883 N	-0°148893 E
N8	Peñíscola del Pinatar	40°354345 N	0°401050 E
N9	Sète	43°393343 N	3°702986 E
S2	Nador	35°166107 N	-2°908631 E
S3	Oran	35°901409 N	-0°331933 E
S4	Moustaganem	35°55234 N	0°03.505 E
S5	Sidi Lakdher	36°12731 N	0°23.932 E
S6	El Marsa	36°24113 N	0°54.732 E
S7	Ghouraya	36°34488 N	1°54.139 E
S8	Tipaza	36°35661 N	2°26.982 E
S9	Algiers	36°45873 N	2°50.838 E
S10	Bejaia	36°721214 N	5°077531 E
S11	Zaima Mansouriah	36°681967 N	5°480515 E
S12	Collo	37°005711 N	6°560856 E
S13	Skikda	36°912062 N	6°899974 E
S14	Bizerta	37°268824 N	9°879091 E

Table S2. Allelic frequencies at the *COIII*, *Precol-D*, *EFbis* and *EF2* loci. allele absent in sample (0.00).

		1	N2	N3	N4	N5	N6	N7	N8	N9	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
COIII																							
A_1	MED	0.11	0.16	0.25	0.77	0.73	0.79	0.96	0.73	0.96	0.14	0.48	0.22	0.47	0.41	0.37	0.32	0.47	0.24	0.58	0.64	0.75	0.88
A_2	ATL	0.89	0.83	0.75	0.23	0.27	0.21	0.04	0.27	0.04	0.86	0.52	0.78	0.53	0.59	0.64	0.68	0.53	0.76	0.42	0.36	0.25	0.11
Precol-D																							
237	ATL	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.02	0.03	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
238	ATL	0.00	0.19	0.00	0.05	0.00	0.02	0.00	0.00	0.04	0.09	0.00	0.13	0.06	0.23	0.08	0.03	0.08	0.07	0.02	0.03	0.02	0.07
239	ATL	0.53	0.45	0.70	0.02	0.15	0.06	0.10	0.03	0.07	0.56	0.16	0.39	0.26	0.32	0.41	0.55	0.29	0.32	0.11	0.12	0.13	0.06
240	ATL	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.04	0.03	0.04	0.04	0.00	0.05	0.02	0.00	0.00	0.00	0.00
241	MED	0.25	0.23	0.00	0.23	0.10	0.41	0.39	0.14	0.00	0.12	0.16	0.08	0.09	0.13	0.08	0.10	0.10	0.30	0.07	0.09	0.22	0.06
242	MED	0.12	0.12	0.30	0.68	0.65	0.50	0.50	0.82	0.84	0.15	0.66	0.32	0.53	0.27	0.37	0.26	0.47	0.28	0.79	0.76	0.63	0.80
263	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00
266	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
440	ATL	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EFbis																							
403	ATL	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
406	ATL	0.03	0.06	0.00	0.03	0.00	0.06	0.03	0.00	0.00	0.00	0.00	0.02	0.10	0.00	0.00	0.03	0.00	0.02	0.02	0.00	0.02	0.00
422	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
423	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.04	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00
425	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
426	ATL	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
427	ATL	0.00	0.00	0.00	0.03	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
428	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.02	0.00	0.00	0.04	0.02	0.00	0.00	0.03	0.00	0.02	0.00
429	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.10	0.04	0.00	0.09	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00
430	ATL	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.06	0.03	0.00	0.00	0.08	0.02	0.16	0.13	0.00	0.05	0.07
431	ATL	0.81	0.33	0.30	0.10	0.00	0.04	0.16	0.18	0.00	0.06	0.16	0.28	0.20	0.41	0.50	0.34	0.13	0.40	0.18	0.14	0.09	0.13

432	ATL	0.06	0.27	0.40	0.18	0.20	0.11	0.05	0.07	0.27	0.69	0.12	0.15	0.12	0.18	0.04	0.00	0.29	0.21	0.02	0.29	0.07	0.06
433	ATL	0.00	0.06	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
434	ATL	0.00	0.06	0.00	0.05	0.00	0.02	0.05	0.00	0.00	0.00	0.00	0.04	0.00	0.04	0.00	0.00	0.08	0.00	0.00	0.00	0.02	0.03
435	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
436	ATL	0.00	0.02	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.03	0.02	0.00	0.00	0.02	0.03
437	ATL	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
438	MED	0.00	0.04	0.00	0.08	0.00	0.00	0.00	0.28	0.15	0.06	0.06	0.11	0.23	0.00	0.17	0.10	0.05	0.05	0.15	0.03	0.20	0.13
439	MED	0.00	0.06	0.10	0.37	0.45	0.45	0.63	0.39	0.38	0.03	0.28	0.15	0.15	0.09	0.17	0.16	0.10	0.10	0.39	0.41	0.42	0.36
440	MED	0.03	0.04	0.00	0.03	0.00	0.09	0.00	0.07	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.08	0.02	0.07
444	MED	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
455	MED	0.06	0.02	0.10	0.13	0.25	0.13	0.03	0.00	0.11	0.15	0.08	0.09	0.17	0.04	0.08	0.15	0.10	0.02	0.04	0. 03	0.05	0.10
EF2																							
420	MED	0.59	0.54	0.58	0.56	0.88	0.87	0.98	0.88	0.95	0.73	0.93	0.66	0.60	0.66	0.75	0.80	0.77	0.62	0.89	0.97	0.90	0.85
485	ATL	0.41	0.46	0.42	0.44	0.12	0.13	0.02	0.12	0.05	0.27	0.07	0.34	0.40	0.34	0.25	0. 20	0.23	0.38	0.11	0.03	0.10	0.15

ARTICLE II

Répartition en mosaïque et distribution géographique inattendue de deux lignées cryptiques du gastéropode marin *Stramonita haemastoma*, s'hybridant en Espagne

II.1- Résumé

Les approches moléculaires ont prouvé leur efficacité dans l'identification des lignées cryptiques au sein des entités taxonomiques, et parfois l'invasion cryptique de certaines d'entre-elles. La structure génétique du gastéropode marin *Stramonita haemastoma sensu stricto* a été examinée dans les populations Ouest Méditerranéenne et Nord-Est Atlantique par barcoding grâce à un marqueur mitochondrial, le *Cytochrome Oxydase I,* et trois marqueurs nucléaires nouvellement développés. Nous avons identifié deux lignées cryptiques, différentiellement fixées pour les haplogroupes ADNmt (*COI*) et nettement différenciées pour les microsatellites. La répartition géographique des deux lignées est inhabituelle pour un invertébré marin à phase larvaire planctonique longue (trois mois) et à préférendum thermique de tempéré à chaud. Les deux lignées se répartissent en mosaïque avec un patch de la lignée Atlantique enclavé au nord de la mer Méditerranée Occidentale entre l'Est de l'Espagne et la Côte d'Azur, et la lignée méditerranéenne trouvée dans les îles Macaronésiennes. Bien qu'un déséquilibre nucléo-cytoplasmiques soit maintenu, une introgression asymétrique se produit dans les populations sympatriques de la zone hybride Espagnole. Une première interprétation de nos résultats est la discordance mito-nucléaire dans une zone hybride postglaciaire stable. Sous cette hypothèse, l'emplacement des discontinuités génétiques serait inhabituel parmi les organismes planctoniques. Une autre interprétation est que la lignée Atlantique, également trouvée au Sénégal et au Venezuela, a été introduite par les activités humaines, se propage, et introgresse des allèles méditerranéens lors de son expansion, en accord avec la théorie. Cette seconde hypothèse permet d'ajouter un exemple supplémentaire à la liste croissante des invasions cryptiques non révélées par des études moléculaires.

Mosaic distribution with unusual geographic delineations of two hybridizing cryptic lineages in the teleplanic disperser snail *Stramonita haemastoma*

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ABSTRACT

Molecular approaches have proven efficient to identify cryptic lineages within single taxonomic entities, and sometimes cryptic invasion of some of them. The genetic structure of the marine gastropod *Stramonita haemastoma sensu stricto* has been examined in the Western Mediterranean and North-Eastern Atlantic populations with mitochondrial *COI* barcodes and three newly developed nuclear microsatellite markers. We identified two cryptic lineages, differentially fixed for alternative mtDNA *COI* haplogroups and significantly differentiated at microsatellite loci. The geographic distribution of the two lineages is unusual for a marine invertebrate with a three-month long teleplanic larvae stage and a warm temperate thermal preferendum. It is a mosaic with a patch of the Atlantic lineage enclaved in the north of the Western Mediterranean Sea between eastern Spain and the French Riviera, and the Mediterranean lineage found in Macronesian Islands. Although cyto-nuclear disequilibrium is maintained, asymmetric introgression occurs in sympatric populations of the Spanish hybrid zone. A first interpretation of our results is mito-nuclear discordance in a stable post-glacial hybrid zone. Under this hypothesis, though, the location of genetic discontinuities would be unusual among planktonic dispersers. An alternative interpretation is that the Atlantic lineage, also found in Senegal and Venezuela, has been introduced by human activities, is spreading, and is introgressing Mediterranean alleles during its propagation as theoretically expected. This second hypothesis would add an additional example to the growing list of cryptic invasions unrevealed by molecular studies.

KEYWORDS: Cryptic species, hybrid zone, biological invasion, introgression, *Stramonita haemastoma*, Western Mediterranean Sea.

INTRODUCTION

Patterns of genetic structure in marine systems have been shown to be mainly related to two main factors: dispersal ability and biogeographic barriers (Riginos *et al.* 2011), to which we should now also append recent human-related introductions of differentiated lineages by anthropogenic vectors (Rius *et al.* 2015). It has long been expected that species with a dispersive larval stage should be less differentiated than species with direct development (Hellberg 1996; Johnson & Seger 2001; Palumbi & Baker 1994), and this has been corroborated by meta-analyses of the marine population genetics literature (Kelly & Palumbi 2009; Selkoe & Toonen 2011). However, planktonic dispersers have also long revealed pronounced genetic breaks between populations of what is recognized as a single species, and these often collocate with well recognised biogeographic boundaries (Gagnaire *et al.* 2015; Pelc *et al.* 2009; Riginos *et al.* 2011). Marine biogeographic boundaries often coincide with oceanic fronts thought to impose a physical barrier to dispersal and with strong environmental gradients. As a consequence reduced connectivity and selection to different environmental conditions are often advocated explaining the genetic differentiation observed at these places (Selkoe *et al.* 2008). For instance, the Almeria-Oran front that delimits the Mediterranean and Atlantic biogeographic provinces is a hotspot of genetic differentiation in planktonic dispersing species (Patarnello *et al.* 2007) and this is interpreted as a consequence of an efficient barrier to larval dispersal (Galarza *et al.* 2009). Conversely, the strong salinity gradient observed at the entrance of the Baltic Sea and adaptation to brackish waters have been proposed to explain the genetic differentiation observed in many marine species with high dispersal capabilities between the North Sea and the Baltic Sea (Johannesson & Andre 2006; Lamichhaney *et al.* 2012). However, increasing evidences are accumulating suggesting populations each side of these boundaries could more likely be considered cryptic semi-differentiated lineages that evolved reproductive isolation mechanisms. Reproductive isolation might better explain the maintenance of the genetic differentiation, while reduced connectivity and ecotones better explain the position of the genetic breaks rather than their maintenance (Bierne *et al.* 2011; El Ayari *et al.* soubmitted; Tine *et al.* 2014). Finally, planktonic dispersers can display pronounced genetic differentiation in non-equilibrium scenarios of recent contact between a native and an introduced lineage by human activities (Heath *et al.* 1995). We are probably at the dawn to uncover many such cases thanks to the increasing democratization of molecular approaches, and the persistent propagule pressure imposed by unaltered anthropogenic fluxes.

We studied the genetic structure of the red-mouthed rock shell *Stramonita haemastoma* (Linnaeus, 1767) in the Western Mediterranean Sea and North-Eastern Atlantic Ocean. *S. haemastoma* is a rocky shore gastropod belonging to the family of muricidae usually found in warm-temperate waters (Barash & Danin 1992; Butler 1985). *S. haemastoma* is gonochoristic. Female brood 20-86 egg capsules each containing 1724-6956 eggs (Lahbib *et al.* 2011). Larvae pelagic duration was estimated to last between 2 and 3 months (Claremont *et al.* 2011; El Ayari *et al.* 2015; Lahbib *et al.* 2011). Adults are benthic and have low dispersal capabilities. *S. haemastoma* is a complex of species with widespread distribution in the Atlantic Ocean and Eastern Pacific Ocean (Abbott 1972 ; Claremont *et al.* 2011; Clench 1947). Claremont *et al.* (2011) conducted a study on the genus *Stramonita* which identified, in addition to the previously recognized outgroup Pacific species *S. delessertiana,* six members in the *S. haemastoma* complex, *S. biserialis* in the South-Eastern Pacific Ocean, *S. floridana* and *S. canaliculata* in the North-Western Atlantic Ocean, *S. rustica* and *S. brasiliensis* in the South-Western Atlantic Ocean, and *S. haemastoma sensu stricto* is the single representative of the species complex in the Eastern Atlantic and Mediterranean Sea although molecular data are mostly lacking for Mediterranean samples. The two North American Atlantic species *S. canaliculata* and *S. floridana* were previously described with allozymes and mitochondrial markers (Harding & Harasewych 2007; Liu *et al.* 1991) and have been showed to hybridize, suggesting that reproductive isolation cannot be assumed complete between members of the complex.

In this study we developed three microsatellite loci and used them in addition with mtDNA *COI* barcodes to analyze a wide sample of *S. haemastoma* snails in North Africa, Spain and France. We identified two cryptic lineages with a mosaic distribution and a hybrid zone in Spanish coasts.

MATERIALS AND METHODS

Sampling and molecular markers

Individuals of *Stramonita haemastoma* were collected by SCUBA diving in 18 localities; 5 from the North-Eastern Atlantic Ocean and 13 from the Mediterranean Sea (Table S1, Figure 1). DNA was extracted from foot tissue using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following the instructions of the manufacturer. DNA concentration was measured for each sample using a NanoDrop8000 Spectrophotometer (Thermo Scientific) and standardised to a DNA concentration of 50 ng µL-1. A 586-bp fragment of the cytochrome oxidase subunit I was amplified with a cocktail (*COI-2*) of four forward and four reverse primers (Table S2) in the following ratio; 10 pmol/µL, *VF1_t1*: *VF1d_t1*: *LepF1_t1*: *VF1i_t1* (1:1:1:3) or *VR1_t1*: *VR1d_t1*: *LepRI_t1*: *VR1i_t1* (1:1:1:3) and sequenced with *M13* (Ivanova *et al.* 2007). PCR reactions with an initial denaturation at 95 °C for 5 min followed by 35 cycles (94 °C 30 s; 42 °C 30 s, 72 °C 45 s) followed by a final extension at 72 °C for 7 min were performed in 15 µl reaction volume consisting of Dream Buffer 10X (1.5 µl), 5mM DNTPs (0.6 µl), each primer (0.24 µl), DreamTaq Fermentas (0.06 µl), double distilled autoclaved water (10.36 µl) and 2 µl template DNA. Sequence reactions were precipitated using a standard EDTA/ethanol protocol, suspended in 15µl Hi-Di formamide 0.2 µl ROX and sequenced on an ABI 3130XL automated sequencer. As for the microsatellite loci, three loci were selected among eighteen tested. The sequence motifs and primers are provided in Table S3. PCR products were diluted at 1:50 double distilled autoclaved water, (2 µl) were the pooled in a mix of Hi-Di formamide/ROX 500 size standard (12.8µl formamide and 0.2 µl ROX); lastly the mixtures were loaded on an ABI 3130XL capillary automated sequencer. GeneMapper® v4.5 software (Applied Biosystems) was used to read the resulting chromatograms.

Data analysis

BIOEDIT V7.1.3.0 was used for sequences alignments. We added in the analysis sequences retrieved from Genbank corresponding to 7 geographic samples reported in Claremont *et al.* (2011) (Table S1). Aligned sequences were then used to construct a Neigbour-Joining tree with MEGA software v6. Allelic, genotyping frequencies and average number of alleles/locus were obtained using Genetix software v4.0.5.2 (Belkhir *et al.* 2002). The DnaSP V5.10.1 software (Librado & Rozas 2009) was used to calculate the number of haplotypes (h), haplotypes diversity (hd), nucleotide diversity (π), average number of nucleotide *substitutions*

per site between clades (Dxy) and net number of nucleotide *substitutions* per site between clades (Da). The genetic structure was examined using STRUCTURE 2.3.4 (Falush *et al.* 2003), with 100.000 burn-in iterations and 50.000 iterations. We used the admixture model with sampling as prior for the microsatellite data set with k = 2 and 3.

RESULTS

a- Genetic variability

Two mitochondrial clades were observed in the Neighbor-Joining tree (Figure 2). Clade A and B were defined according to their phylogenetic relationships, and also to their frequency in the samples (Figure 1). We also defined two haplogroups within clade A: haplogroup A' and A" (Figure 2). The analysis of the mitochondrial diversity has revealed the existence of 167 haplotypes, high haplotypes diversity (hd = 0.976) and a high nucleotide diversity (π = 0.016). Average and net nucleotide divergence measures between Atlantic populations fixed for the A haplogroup and Mediterranean populations fixed for the B haplogroup were high: Dxy = 0.022, Da = 0.012. A total of 62 alleles were identified using three microsatellites (22 size-alleles at the locus *Strhae3*, 19 at *Strhae8* and 21 at *Strhae9*). Allele frequencies are presented in Table S4. Because of unevenness of sample sizes, samples were pooled according to their geographic proximity as described with the ellipses in Figure 1. The average number of microsatelitte alleles for each sample is represented together with the mtDNA *COI* haplotype diversity in Figure 3. In Figure 3 is also reported the haplotype diversity of the two samples from Senegal and Venezuela in Claremont *et al.* (2011). Finally, microsatellite alleles were also pooled to construct bi-allelic loci according to their frequencies in Atlantic populations fixed for the A haplogroup and Mediterranean populations fixed for the B haplogroup. The frequency of the compound lineage A allele and compound lineage B allele is reported below Figure 1.

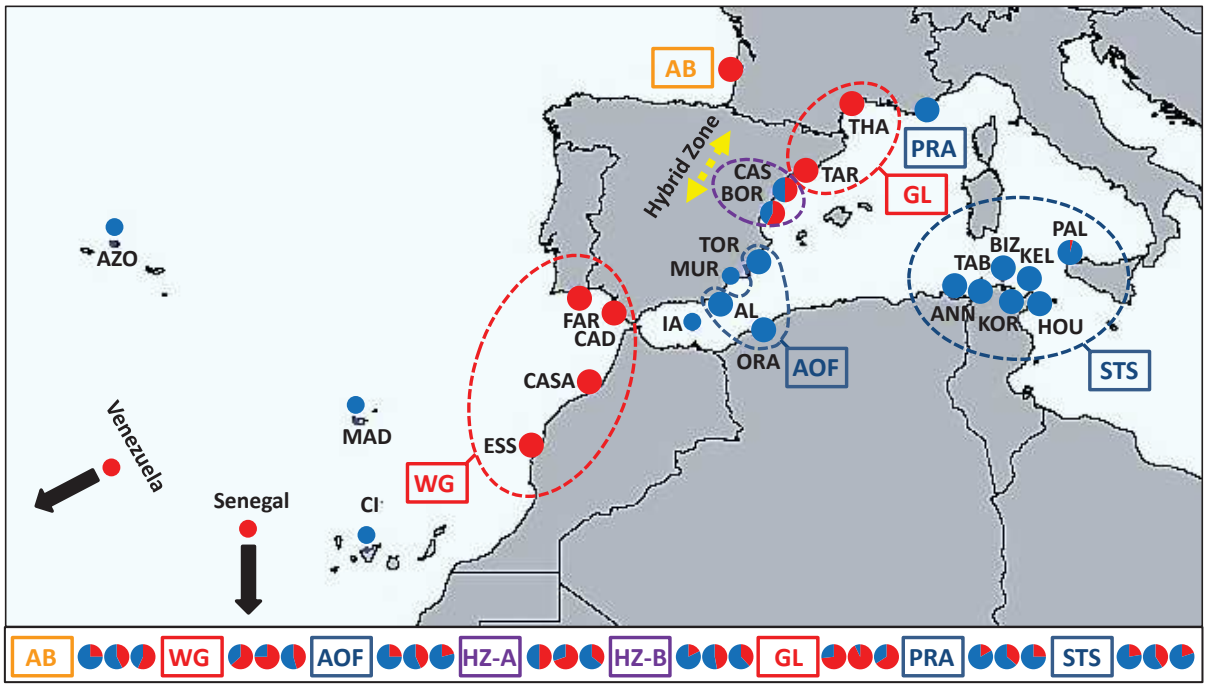

Figure 1: Sampling localities of *Stramonita haemastoma*. Proportion of individuals assigned to the mitochondrial haplogroup A (in red), and B (in blue). Pie charts below the map represent the frequency of the two compound alleles at the three nuclear loci (*Strhae3*, *Strhae8* and *Strhae9* given from left to right respectively) for each study location. Sample names and their GPS positions are given in the supplementary Table S1.

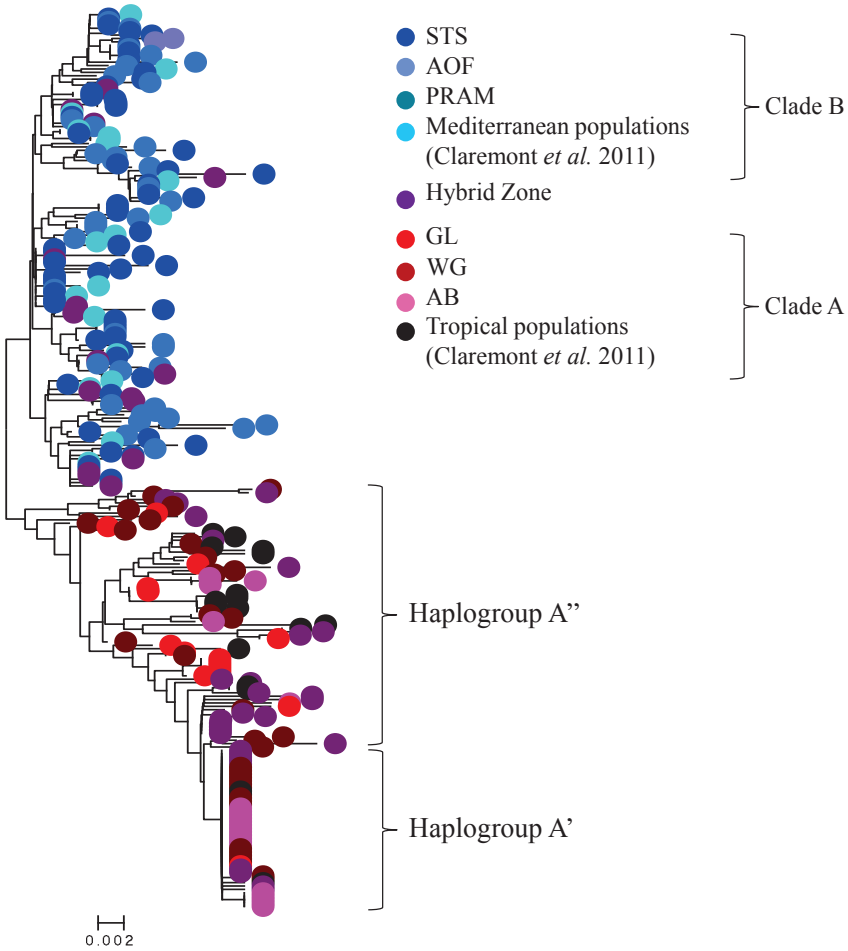

Figure 2: Neighbor-joining tree of mtDNA *COI* sequences from *Stramonita haemastoma* samples. AB (Arcachon Bay), WG (Casablanca, Essaouira, Cadiz, Faro); AOF (Oran, Almeria, Torreveija), HZA (*Stramonita haemastoma* Clade A from Castellon and Borriana), HZB (*S. haemastoma* Clade B from Castellon and Borriana), GL (Thau and Tarragona) PRAM (Pramousquier), STS (Annaba, Tabarca, Bizerte, Kourbous, Kelibia, Houaria and Palermo), see Figure 1.

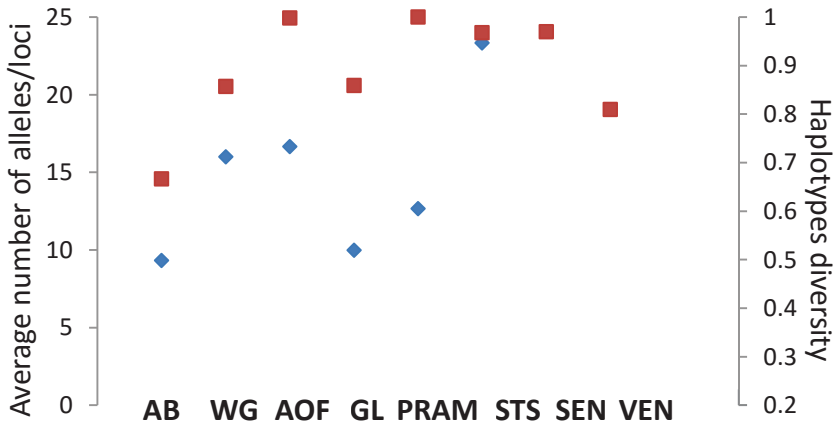

Figure 3: Average number of alleles at microsatellites loci (blue diamonds) and haplotype diversity (red square) in *Stramonita haemastoma* samples. Senegal (SEN) and Venezuela (VEN) data from Claremont *et al.* (2011).

a- Genetic structure of Stramonita haemastoma populations

The group of samples from the area around the Siculo-Tunisian Strait (STS), including samples from Tabarca, Bizerte, Kourbous, Kelibia and Houaria (Tunisia), Annaba (Algeria) and Palermo (Italy) were nearly exclusively composed of clade B haplotypes at the mitochondrial locus, with the exception of two individuals found in Palermo. Samples from the area around the Almeria-Oran Front (AOF) including Torreveija and Almeria (Spain), and Oran (Algeria), were fixed for the B haplogroup. Finally, our sample from the French Riviera in Pramousquier (France) was also composed of clade B haplotypes. In addition, data from Claremont *et al.* (2011) show that the B haplogroup is also found in Murcia, Alborán Island and Macaronesian islands (Figure 1). The group of samples from the area westward to Gibraltar (WG), including Casablanca and Essaouira (Morocco), Cadiz (Spain) and Faro (Portugal) were fixed for the A haplogroup. Similarly, our sample from the Atlantic coast of France in Arcachon Bay was also entirely composed of clade A haplotypes, although preponderantly the star-shaped haplogroup A'. Surprisingly, clade A haplotypes were also found at high frequency in North-Western Mediterranean samples and fixed in Thau (France) and Tarragona (Spain). Again data from Claremont *et al.* (2011) were used and the A haplogroup is also found in tropical waters in Senegal and in Venezuela (Figure 1). Finally, and importantly, two samples from the Valencia area along Eastern Spanish coasts (Castellon and Borriana) were found to be a balanced mixture of both mitochondrial clades.

The analysis of the three nuclear microsatellite loci revealed a mostly concordant pattern with the mtDNA data (see the STRUCTURE outputs in Figure 4) with populations fixed for the B haplogroup assigned to a genetic cluster (blue cluster in Figure 4), that we accordingly called cluster B, and populations fixed for the A haplogroup assigned to another genetic cluster (red cluster in Figure 3), accordingly called cluster A. However, the genetic differentiation was much lower at nuclear loci than at the mtDNA locus. The average *Fst* between samples fixed for the mtDNA haplogroup A and samples fixed for the mtDNA haplogroup B was 0.065 (permutation tests $P<0.001$), while no significant genetic differentiation was found within lineages with the single exception of the Arcachon Bay sample in lineage A (*Fst*=0.02, $P<0.001$) we will come back on below. The structure observed at nuclear loci can also be viewed with the frequency of the compound lineage-specific alleles reported in Figure 1. The frequency of the compound A-allele is higher in samples fixed for the A haplogroup, and the frequency of the compound B-allele higher in samples fixed for the B haplogroup. The two admixed samples in Castellon and Borriana have intermediate allele frequencies. In order to highlight the mito-nuclear disequilibrium maintained in hybrid populations, individuals have been sorted according to their mitochondrial genotype in the STRUCTURE analysis (Figure 4) and in pie charts of microsatellite compound lineage-specific allele frequencies of Figure 1. Individuals with a B mtDNA haplotype have a homogeneously high proportion of cluster B ancestry, while individuals with a A mtDNA haplotype overall have a higher proportion of cluster A ancestry but with a strong variance as some individuals have a higher proportion of cluster B ancestry than others (Figure 4). Interestingly the two individuals with a mtDNA A haplotype sampled in Palermo in the STS area also have a high proportion of cluster A ancestry (see stars in Figure 4), showing that the mito-nuclear disequilibrium extends to this population. Finally some individuals of the western Gibraltar strait area also have a higher proportion of cluster B ancestry than the others while all carrying the mtDNA A haplotype.

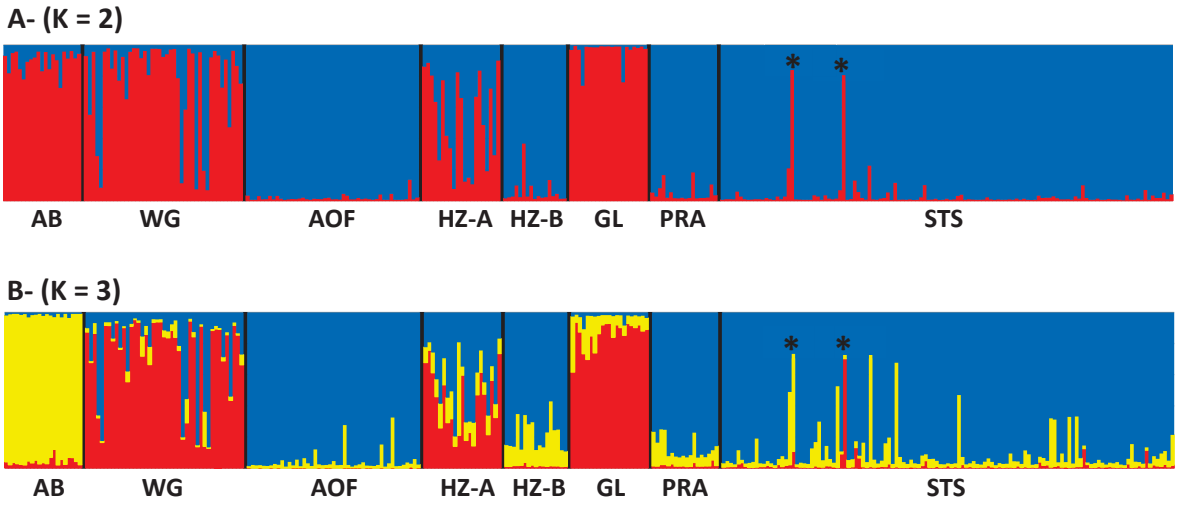

Figure 4: Bar plot of the estimated cluster membership fraction (Q) estimated by STRUCTURE. k = 2 (A) and K = 3 (B). (*) individuals carrying a clade A mtDNA haplotype in sample from Palermo.

In order to illustrate the genetic compositions of allopatric and sympatric samples, we have plotted in Figure 5 the distribution of the hybrid index, the number of A alleles per individual at the mtDNA and three microsatellite loci. Figure 5 shows that the two distributions overlap little in allopatric populations. The overlapping is due to shared ancestral polymorphisms and/or introgression at nuclear loci, and possibly to a few hybrids (eg in the WG area were some A mtDNA haplotype carrying individuals have a high proportion of B ancestry at microsatellite markers. In the populations of the Spanish hybrid zone the overlapping is much stronger, highlighting hybridization and local introgression, which were already visible in the STRUCTUR plot in Figure 4.

To finish with, we found the Arcachon Bay sample to be differentiated from the other populations of the A lineage both at the mitochondrial locus, with the Arcachon Bay sample having a higher frequency of the A' haplogroup, and at nuclear microsatellite loci as is made clear the STRUCTURE analysis with K=3 (Figure 4B). The lower diversity observed at both type of markers (Figure 2) suggest a population bottleneck could explain this observation.

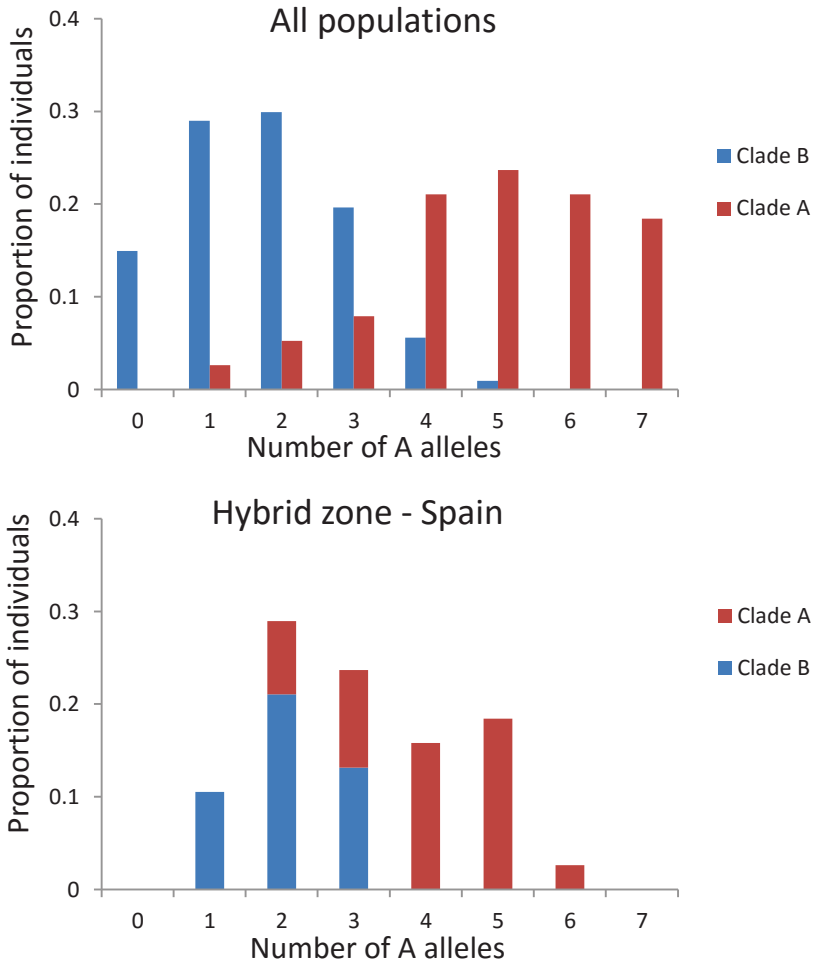

Figure 5: Distribution of the hybrid index, the number of lineage A alleles per individual, in (A) allopatric populations and (B) populations of the Spanish hybrid zone.

DISCUSSION

Our genetic survey of the teleplanic disperser snail, *Stramonita haemastoma*, in western Mediterranean Sea and Eastern Atlantic Ocean has revealed (i) two cryptic lineages, (ii) that hybridize when in sympatry, and (iii) which display a mosaic distribution with a surprising patch of the Atlantic lineage in the North-Western part of the Occidental basin of the Mediterranean Sea. There is little need to discuss at length the discovery of cryptic lineages with molecular markers as pervasive this observation is in the Sea (Pante *et al.* 2015; Suzuki *et al.* 2005; Zhan *et al.* 2010). However, the geographic distribution of molecularly identified cryptic lineages is usually compatible with vicariant divergence between well recognised biogeographic regions, especially when the divergence is recent and hybridization happens in contact zone. Here, the two discovered semi-isolated lineages have an unusual distribution and meet in a hybrid zone which position is novel for a planktonic dispersing marine species. We will here discuss the two possible scenarios that can explain the mosaic distribution and genetic composition of hybrid zone samples: (i) a stable post-glacial distribution with mito-nuclear discordance or (ii) a recent invasion of the A lineage.

Hypothesis 1: A stable post-glacial hybrid zone with mito-nuclear discordance, and a patch of cold-water adapted Atlantic-derived populations blocked in the North-Western Occidental Mediterranean Sea

The geography of the Mediterranean Sea, perpendicular to north-south population displacements due to glacial oscillations, has been prone to trap cold-adapted species in pockets of cold waters in the north, in the Golf of Lion in the Occidental basin, in the northern Adriatic Sea and the Black Sea. The flounder *Plathichthys flesus* (Borsa *et al.* 1997), the sprat *Sprattus sprattus* (Debes *et al.* 2008), or also the planktonic chaetognath *Sagitta setosa* (Peijnenburg *et al.* 2004), are examples of species with a discontinuous geographic distribution in the Northern Mediterranean Sea and which have revealed genetic divergence between Atlantic and Mediterranean populations and also between Northern Mediterranean pockets. These observations are compatible with isolation provoked by post-glacial warming. However, we are not aware of a species with this distribution that would have remained in contact with a sister hybridizing lineage distributed elsewhere in the Mediterranean Sea, as we observed in *S. haemastoma* with the B lineage. Furthermore, in each of these other examples the distribution in the Atlantic Ocean is preponderantly north to the Cantabrian Sea, in accordance with a temperate water preferendum. On the contrary, the distribution of the A

lineage of *S. haemastoma* corresponds to warm temperate preferendum. It is south to the Cantabrian Sea (Claremont *et al.* 2011), with the A lineage found in abundance in Portugal and Morocco, and according to the data of Claremont *et al.* (2011) found in tropical waters in Senegal and Venezuela. The genetic differentiation associated with a decrease of diversity in the Arcachon Bay is not expected in the middle of the range of a planktonic dispersing marine invertebrate and this is in better accordance with a population at the margin of the distribution, or to a demographic bottleneck after introduction by anthropogenic vectors. It is nonetheless difficult to refute that the mosaic distribution observed in *S. haemastoma* is natural. Even the absence of genetic differentiation between Atlantic and North-Western Mediterranean populations is not unexpected with only a few thousand years of isolation (Faure *et al.* 2008). We can also imagine that the 3 month long teleplanic larvae phase could have permitted the implantation of the A lineage in the Mediterranean Sea. If the Spanish hybrid zone is a stable post-glacial contact zone, then the observation that A mtDNA haplogroup carrying individuals tend to sometimes have a high proportion of B lineage ancestry at the nuclear loci would be well explained by the widespread observation of mito-nuclear discordance in hybrid zones (Toews & Brelsford 2012). A finer sampling grain along the coast would be required to better describe this phenomenon. The two sexes dispersing similarly in a planktonic disperser, and assuming the zone did not move recently under this hypothesis of a post-glacial hybrid zone, the best explanation would be the existence of cyto-nuclear incompatibilities (Burton *et al.* 2013; Chou & Leu 2015). Finally, a last surprising observation would be the position of the hybrid zone itself. Post-glacial secondary contact zones between Atlantic and Mediterranean lineages have to date always been detected at the Almeria-Oran front in planktonic dispersing marine species (Patarnello *et al.* 2007). One explanation is that the barrier to larval dispersal and/or the environmental boundary at the Almeria-Oran front have acted as a trap for hybrid zones (Bierne *et al.* 2011, El Ayari *et al.* submitted). The Spanish hybrid zone of *S. haemastoma* is therefore at an unusual place. In addition the genetic break between the Gulf of Lion and the French Riviera, while reported in restricted dispersers (Boissin *et al.* 2008; Rastorgueff *et al.* 2014), would also be an unusual result in a planktonic disperser. We would emphasise that while unrevealing genetic breaks at unusual geographic locations, our samples from Almeria, together with the data of Claremont *et al.* (2011) from the Island of Alboran, suggest that the Almeria-Oran front has no effect on the genetic structure of lineage B.

Hypothesis 2: recent invasion of the A lineage

This number of surprising observations suggests that as an alternative to natural distribution, the mosaic structure observed in *S. haemastoma* could result from a biological invasion of the A lineage. Cases of biological introduction by anthropogenic vectors have increased (Chiesa *et al.* 2014; Heath *et al.* 1995; Riquet *et al.* 2013; Suchanek *et al.* 1997). The Thau lagoon in the Gulf of Lion and the Arcachon Bay are recognised hotspots of marine invasion. Species were introduced either for aquaculture purpose like the Indo-pacific clam *Ruditapes philippinarium* (Chiesa *et al.* 2014; Gosling 2003) or the Japanese oyster *Crassostrea gigas* (Grizel & Héral 1991; Ruesink *et al.* 2005) or, for most of them, accidentally with shellfish stocks, like *Grandidierella japonica* introduced in the Arcachon Bay with oysters (Lavesque *et al.* 2014) or *Sargassum miticum* (Critchley et al. 1990). But identified exotic species might be the tip of the iceberg and an increasing number of studies in marine invasion genetics emphasize cryptic invasions, the invasion of a cryptic lineage, often at the intra-specific level, previously undistinguishable from the native lineage (Geller *et al.* 2010). In order to identify a cryptic invasion, one ideally needs to identify the source of the invasion, and in order to do so the geographic gap between the source and the invasion area needs to be obviously too far from possible naturally. However, in the case of *S. haemastoma* the source is very difficult to identify. Such a possible source could be (i) the Atlantic coasts, but as explained above, it is difficult to refute a natural post-glacial redistribution of vicariant lineages, (ii) Western Africa, but again natural spreading is difficult to refute, (iii) America, as Claremont *et al.* (2011) found the A lineage in Venezuela, but the widespread distribution in Eastern Atlantic when oppose to the very limited distribution in America, and the reduced genetic diversity in Venezuela rather suggest an East to West migration by the South Equatorial Current (Boehm *et al.* 2013; Claremont *et al.* 2011; Lapègue *et al.* 2002), and (iv) South-West Africa, as *S. haemastoma* has been reported in this area (Clench 1947 ; Penrith & Kensley 1970), although it is far from clear if the equatorial region represents a barrier in this species that seems to tolerate tropical waters quite well. The genetic arguments for a cryptic invasion are (i) an asymmetric pattern of introgression that resemble well the theoretical prediction of native alleles introgression into the invading background (Currat *et al.* 2008) and (ii) the presence of two lineage A individual, with strong mito-nuclear and intra-nuclear disequilibrium (Figure 4) in Palermo, an area dominated by the B lineage, suggesting a very recent arrival of the A lineage in Sicily. None of these arguments however allowed to clearly concluding whether the mosaic distribution is due to human-mediated introductions. New genetic data can help,

however, whether increasing the number of loci analysed by taking advantage of new high-throughput sequencing method will help to better understand the genetic differentiation in this system, broadening the geographic sampling of this species (in contact zones, in South-West Africa and Central America etc.) is certainly the most important issue.

CONCLUSION

We have discovered two cryptic lineages in North-Eastern Atlantic and Mediterranean populations of the teleplanic dispersing marine snail *Stramonita haemastoma*, with an unusual mosaic distribution. Although this mosaic distribution could result from human-mediated introduction and the asymmetric introgression pattern support the hypothesis that one lineage is invading, we were not able to reject the alternative hypothesis that the mosaic distribution result from post-glacial population displacement and the asymmetry of introgression in the hybrid zone a consequence of mito-nuclear discordance in a stable hybrid zone. A broader geographic and genomic sampling together with a monitoring of the distribution could allow disentangling the two hypotheses. In any case, *Stramonita haemastoma* proves to be an interesting model species to better understand the connectivity of planktonic dispersers in the Occidental basin of the Mediterranean Sea.

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ANNEXE ARTICLE II

Table S1. *Stramonita haemastoma* sample location.

			GPS coordinates	
Abbreviation	**Sample name**	**Reference**	**Latitude**	**Longitude**
AB	Arcachon Bay	Present study	44°592602 N	-1°214601 E
FAR	Faro	Present study	36°988717 N	-7°983696 E
CAD	Cádiz	Present study	36°349616 N	-6°261922 E
CASA	Casablanca	Present study	33°810509 N	-7°185520 E
ESS	Essaouira	Present study	31°692465 N	-9°815664 E
PRA	Pramousquier	Present study	43°152917 N	6°448441 E
THA	Thau	Present study	43°393343 N	3°702986 E
TAR	Tarragona	Present study	41°047535 N	1°254303 E
CAS	Castellon	Present study	39°903025 N	0°020299 E
BOR	Borriana	Present study	39°818293 N	-0°077588 E
TOR	Torreveija	Present study	37°939670 N	-0°685915 E
ALM	Almeria	Present study	36°829075 N	2°456408 E
ORA	Oran	Present study	35°901409 N	-0°331933 E
ANN	Annaba	Present study	36°898241 N	7°771432 E
TAB	Tabarca	Present study	37°514243 N	9°869430 E
BIZ	Bizerta	Present study	36°836030 N	11°101338 E
KOR	Korbous	Present study	36°816849 N	10°566951 E
KEL	Kélibia	Present study	38°126280 N	12°793723 E
HOU	Houaria	Present study	37.033306 N	11°059370 E
PAL	Palermo	Present study	43°332742 N	3°612416 E
SEN	Senegal	Claremont *et al.* (2011)	14°289621 N	-16°950554 E
VEN	Venezuela	Claremont *et al.* (2011)	10°548262 N	-66°294977 E
IA	Isla de Alborán	Claremont *et al.* (2011)	35°879294 N	-3°099339 E
MUR	Murcia	Claremont *et al.* (2011)	37°847526 N	-0°744507 E
AZO	Azores	Claremont *et al.* (2011)	38°652175 N	-27°228235 E
MAD	Madeira	Claremont *et al.* (2011)	32°672642 N	-17°065984 E
CI	Canary Islands	Claremont *et al.* (2011)	28°378507 N	-16°683835 E

Table S2. Sequences (5'–3′) of primers used for the Cytochrome Oxydase subunit I.

COI-2	Primer sequence (5'–3')
LepF1_t1	*TGTAAAACGACGGCCAGTATTCAACCAATCATAAAGATATTGG*
VF1_t1	*TGTAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG*
VF1d_t1	*TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYGG*
VF1i_t1	*TGTAAAACGACGGCCAGTTCTCAACCAACCAIAAIGAIATIGG*
LepRI_t1	*CAGGAAACAGCTATGACTAAACTTCTGGATGTCCAAAAAATCA*
VR1d_t1	*CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA*
VR1_t1	*CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAGAATCA*
VR1i_t1	*CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAAICA*

Table S3. Sequences (5'–3′) of primers used for microsatellites loci.

Locus	Repeat motif	Dye color	Primer sequence (5′–3′)	Annealing temperature (°C)
Strhae3	(*CCTA*)	FAM	*ACCCACCTCTACCTAACTGC*	60°C
Strhae8	(*CTGT*)	NED	*CTTCCTACACCACCGGAGAG*	58°C
Strhae9	(*ACTT*)	HEX	*TTCTGGCACTCCCCCATTTC*	54°C

Table S4. Allelic frequencies at the *COI-2*, *Strhae3*, *Strhae8* and *Strhae9*.

	AB	Faro	CAD	CASA	ESS	THA	TAR	CAS	BOR	TOR	ALM	ORA	ANN	TAB	BIZ	KOR	KEL	HOU	PAL	PRA
COI-2																				
100	1	1	1	1	1	1	1	0.6	0.6333	–	–	–	–	–	–	–	–	–	0.0769	–
200	–	–	–	–	–	–	–	0.4	0.3667	1	1	1	1	1	1	1	1	1	0.9231	1
Strhae3																				
80	–	–	–	–	–	–	–	0.0333	0.0167	0.0385	–	–	–	0.05	0.0208	–	–	–	0.0167	–
84	–	0.0833	–	–	–	0.0192	–	0.1	–	0.1923	–	–	–	0.25	0.0417	–	0.4333	–	–	–
86	0.0147	–	0.0333	0.0333	0.0625	–	0.25	–	0.25	0.0385	0.5	0.1333	0.0357	–	0.0208	0.3182	–	0.22	0.1333	0.3333
90	0.4412	–	–	0.0333	0.125	0.0192	–	0.0333	0.1	0.0385	0.2	0.1	0.5	–	0.1458	–	0.1	0.14	0.1667	0.0714
94	0.2059	–	0.0667	0.1	0.0313	0.0769	–	0.0667	0.0333	–	–	0.0167	–	0.15	–	–	–	–	–	0.0476
98	–	0.0833	0.0167	0.0333	0.0625	0.0385	–	–	0.05	0.0385	–	0.05	0.0357	–	0.125	0.1364	0.0333	0.1	0.3833	0.1429
100	–	–	–	–	–	–	–	–	–	–	–	–	0.0357	–	–	–	–	–	–	–
102	–	–	0.0167	–	0.0313	–	–	–	0.0333	–	–	0.1	0.0357	–	0.0208	0.1364	0.0667	0.08	0.0667	0.0238
106	0.0147	0.5	0.3333	0.3333	0.3438	0.4808	0.5	0.2667	0.15	0.3462	0.2	0.0667	0.1429	0.2	0.0833	0.0455	0.2	0.16	0.0333	0.1429
110	–	–	0.0667	0.0333	0.0313	–	–	0.1333	0.0833	–	–	0.1333	–	0.05	0.1875	0.0909	0.0667	0.08	0.0333	–
114	0.0588	0.0833	0.0667	0.1667	0.0625	0.0577	–	0.1667	0.05	0.0385	–	0.15	–	0.05	0.125	0.0455	0.0333	0.06	0.0667	0.119
118	–	–	0.0167	–	0.0938	–	–	–	–	0.0385	–	0.0333	–	–	0.0208	0.0455	0.0333	–	–	–
122	–	–	–	–	–	–	–	–	0.0167	0.0385	–	0.05	–	–	0.0417	–	–	0.04	–	0.0238
126	0.0441	–	–	0.0333	–	0.0577	–	–	0.0333	0.1154	–	0.0333	0.0357	–	0.0208	–	–	0.02	0.0167	0.0476
130	–	–	–	–	–	–	–	–	–	0.0385	–	0.0167	–	0.1	–	–	–	–	0.0167	0.0238
134	–	0.0833	0.1833	0.0333	0.0625	0.0577	0.25	0.0333	0.05	0.0385	–	0.0333	–	–	–	0.0455	–	–	0.05	0.0238
138	–	0.0833	0.1167	0.1667	0.0313	0.1154	–	0.0333	0.0333	–	0.1	0.05	0.0714	–	0.0208	0.0909	–	0.02	0.0167	–
142	–	–	–	–	–	–	–	0.0333	–	–	–	–	0.0714	0.05	0.0417	–	–	0.02	–	–
146	0.2059	0.0833	0.0333	0.0333	0.0625	0.0577	–	0.0667	0.0833	–	–	0.0333	0.0357	0.1	0.0833	–	–	0.06	–	–
154	0.0147	–	0.0333	–	–	–	–	0.0333	–	–	–	–	–	–	–	–	0.0333	–	–	–
162	–	–	0.0167	–	–	0.0192	–	–	–	–	–	–	–	–	–	0.0455	–	–	–	–
166	–	–	–	–	–	–	–	–	0.0167	–	–	–	–	–	–	–	–	–	–	–
Strhae8																				
128	–	–	–	–	0.0263	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
134	0.0147	–	0.25	0.0667	0.0263	0.0769	–	0.0667	0.0167	–	–	–	–	0.05	–	–	–	–	–	–
138	–	–	0.1	0.0333	0.0526	0.0769	–	0.2	0.05	0.0294	–	0.0667	0.125	0.1	0.0909	0.125	–	0.0192	0.0167	0.0682
139	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.0227
142	–	0.0833	0.0833	0.0667	0.1053	0.0577	–	–	0.1	–	–	0.0167	0.05	–	0.0114	0.025	0.1	0.0385	0.0167	0.0682
146	0.3382	0.3333	0.1333	0.3667	0.3158	0.3269	–	0.3333	0.2167	0.1176	0.6	0.25	0.15	0.1	0.2273	0.35	0.1	0.1923	0.1667	0.1136

150	0.0147	0.0833	0.0667	0.0333	0.0263	–	–	0.1	0.0333	0.1765	–	0.0833	0.075	0.05	0.0795	0.05	0.1	0.1154	0.0667	0.0455
154	0.0294	–	0.0167	–	–	0.0577	–	–	0.0167	0.0588	–	0.0167	0.025	0.05	–	0.025	0.0333	–	–	0.0455
158	0.0294	0.1667	0.1167	0.1667	0.1316	0.2115	0.5	0.0333	0.05	–	–	–	0.025	0.05	–	0.025	–	–	–	–
162	–	–	0.0167	–	0.0526	0.1346	0.25	–	0.0333	–	–	–	0.025	–	0.0114	–	–	–	0.0333	–
166	0.0735	0.1667	0.1	0.1	–	–	0.25	0.1333	0.05	0.2353	–	0.0667	0.05	0.1	0.0455	0.075	0.1	0.1154	0.0167	0.0682
170	–	–	0.0333	–	–	–	–	0.1	0.05	0.0588	0.1	0.2	0.1	0.05	0.1023	0.05	0.1	0.0962	0.1	0.1364
174	0.4118	–	0.0333	0.0333	0.0263	0.0192	–	–	0.1	0.0588	0.1	0.1167	0.175	0.15	0.2159	0.125	0.1667	0.1538	0.1	0.1591
178	–	–	–	–	0.0263	–	–	–	0.0333	–	–	–	0.025	0.05	0.0455	–	0.0667	0.0962	0.0667	0.0682
182	–	–	0.0167	0.0333	0.1053	–	–	0.0333	0.15	0.1176	0.2	0.1167	0.1	0.15	0.1136	0.125	0.1	0.1538	0.3167	0.1818
186	–	–	–	–	0.0526	–	–	–	0.0167	0.1176	–	0.0667	0.05	0.1	0.0568	0.025	0.1333	0.0192	0.1	0.0227
195	–	–	–	–	–	–	–	–	0.0333	–	–	–	–	–	–	–	–	–	–	–
198	0.0882	0.1667	0.0333	0.1	0.0263	0.0385	–	–	0.0333	0.0294	–	–	0.025	–	–	–	–	–	–	–
208	–	–	–	–	0.0263	–	–	–	0.0167	–	–	–	–	–	–	–	–	–	–	–
Strhae9																				
130	–	–	–	–	–	–	–	–	–	–	–	0.0167	–	–	–	–	–	–	–	–
138	0.0455	–	–	0.1	0.0455	0.025	–	–	0.0192	–	–	–	0.0294	–	–	0.0357	–	–	–	0.0556
141	–	0.5	0.1429	0.2	0.2273	0.125	–	0.0417	0.0192	–	–	–	–	–	–	–	–	–	–	–
144	–	–	0.0714	–	–	0.025	–	0.0417	0.0192	0.0667	0.1	0.0167	0.1471	0.0556	0.0444	–	–	0.06	0.0333	0.0833
148	0.1136	0.3	0.1786	0.2333	0.0455	0.05	–	0.2083	0.0577	0.0333	–	0.1	0.0588	0.0556	0.1111	0.1429	0.1667	0.02	0.05	0.1389
152	0.0227	–	0.1429	0.0333	–	0.2	–	0.0417	0.0192	0.1333	–	0.0333	0.1765	0.2778	0.1111	0.1071	0.2	0.14	0.15	0.0556
155	–	–	–	–	–	–	–	–	0.0192	–	–	–	–	–	–	–	–	–	–	–
156	0.1364	–	–	0.0333	0.1818	–	–	–	0.2885	0.0667	0.2	0.1667	0.0882	0.0556	0.1333	0.1786	0.0333	0.2	0.0833	0.1944
160	0.0682	0.1	0.0714	0.0333	0.0455	0.05	–	0.1667	0.1154	0.1333	0.1	0.2333	0.1471	0.1667	0.1333	0.0357	0.2	0.12	0.1333	0.0833
162	–	–	–	–	0.1364	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
164	0.25	–	0.0357	0.1333	0.0455	0.275	–	0.125	0.1346	0.1667	0.2	0.05	0.0294	0.2222	0.1	0.1071	–	0.06	0.1333	0.0556
165	–	–	–	–	–	–	–	–	0.0192	–	–	–	–	–	–	–	–	–	–	–
168	0.2727	–	0.2143	0.0667	–	0.2	1	0.1667	0.1923	0.1	0.1	0.1167	0.0882	0.0556	0.1	0.1786	–	0.1	0.15	0.1389
172	0.0455	–	–	–	0.0909	–	–	–	0.0962	0.1333	0.1	0.0667	0.1176	0.0556	0.1	0.1071	0.1	0.06	0.1333	–
176	0.0227	0.1	–	0.0333	0.0909	0.05	–	0.0833	–	0.0667	0.1	0.1	0.0588	–	0.0778	0.0357	0.1333	0.1	0.0667	0.0556
180	–	–	–	0.0667	0.0909	–	–	0.0417	–	0.0333	0.1	0.0333	0.0588	0.0556	0.0333	–	0.0333	0.06	0.05	0.0556
184	–	–	0.1429	–	–	–	–	0.0417	–	0.0667	–	0.0333	–	–	0.0333	0.0714	0.0333	0.02	0.0167	0.0278
188	0.0227	–	–	0.0667	–	–	–	–	–	–	–	0.0333	–	–	0.0222	–	0.1	–	–	0.0556
192	–	–	–	–	–	–	–	0.0417	–	–	–	–	–	–	–	–	–	0.02	–	–
200	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.02	–	–
206	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.02	–	–

4- CONCLUSIONS ET PERSPECTIVES

4.1 Synthèse des résultats

Bien qu'il soit couramment admis que la divergence entre populations se fasse souvent dans un contexte géographique disjoint (allopatrie ou parapatrie), l'origine et le fonctionnement des barrières au flux génique, qui délimitent des populations discrètes au sein des espèces, est encore mal compris. La question de leur origine, leur maintien ainsi que de leur localisation et structure spatiale sont souvent adressées simultanément en cherchant une réponse unique, alors que des processus différents peuvent y contribuer. En milieu marin des décennies de recherche en génétique des populations ont dévoilé de nombreux point-chauds de différenciation génétique, partagés par de nombreuses espèces, et cette distribution discrète, en mosaïque, de la diversité génétique est très prononcée. Dans cette thèse nous nous sommes principalement intéressés à décrire la structure génétique fine au niveau de barrières marines au flux génique.

Dans le premier chapitre, l'étude de la structure génétique de la moule méditerranéenne *Mytilus galloprovincialis* au niveau de la zone de transition Atlantique-Méditerranée a montré que contrairement aux discontinuités abruptes et étroites reportées le long des côtes espagnoles chez de nombreuses espèces, dont la moule, nous avons découvert en Algérie une vaste zone hybride mosaïque de 600 km de large à l'Est du front océanique Almeria-Oran. L'existence de cette vaste zone de coexistence démontre que la différenciation génétique est principalement maintenue par des mécanismes d'isolement reproductif intrinsèques. L'hydrographie explique la localisation et la structure de la zone de transition, mais elle n'explique ni son origine ni son maintien.

Dans le deuxième chapitre, l'étude de la structure génétique d'un gastéropode marin *Stramonita haemastoma,* qui avait été peu étudié jusqu'alors, a montré l'existence de deux lignées cryptiques, réciproquement monophylétiques sur le marqueur mitochondrial *COI*, et significativement différenciées sur trois marqueurs microsatellites développés pour ce travail de thèse. La distribution spatiale des deux lignées est étonnante, en mosaïque. La lignée appelée A a été échantillonnée sur les côtes atlantiques mais aussi dans un patch au nord de la Méditerranée occidentale entre Valence et Marseille, alors que la lignée appelée B a été échantillonnée dans le reste de la Méditerranée occidentale, y compris sur les côtes varoises à l'est de Marseille et au sud de Valence jusqu'en Mer d'Alboran. Les deux lignées sont trouvées en sympatrie, et s'hybrident et s'introgressent, dans une zone hybride au niveau de la région de Valence en Espagne. Bien qu'un déséquilibre nucléo-cytoplasmique fort subsiste,

l'introgression résulte en un remaniement asymétrique des génomes créant des individus principalement B sur le nucléaire avec une mitochondrie A mais jamais l'inverse. Deux scénarios peuvent expliquer ce résultat, soit (i) la zone hybride est naturelle et stable, et il existe une discordance spatiale des clines mitochondriaux et nucléaires, observation courante dans les zones hybrides, soit (ii) la lignée A a été introduite récemment par des activités anthropiques et elle étend son aire de répartition au dépend de la lignée B. Ces deux études mettent en avant l'importance de l'isolement reproductif intrinsèque dans l'explication de la distribution mosaïque de la diversité génétique marine. Bien que les frontières entre patchs correspondent à des barrières physiques à la dispersion ou à des écotones, l'hydrographie et l'environnement n'expliquent que la position des discontinuités génétiques mais ni leur origine ni leur maintien.

4.2 Perspectives

Les résultats de ce travail montrent l'importance de l'échantillonnage, comme le dit *Nicolas Bierne* 'en génétique des populations, *l'échantillonnage c'est le nerf de la guerre'*, en effet pour réussir cette guerre, un échantillonnage à échelle très fine comme on l'a réalisé pour cette thèse est indispensable et doit être même complété pour les deux espèces.

L'étude de la structure génétique de la moule méditerranéenne *Mytilus galloprovincialis* au niveau de la zone de transition Atlantique-Méditerranée grâce à des échantillons de la rive Sud méditerranéenne nous a permis de détecter pour la première fois une barrière au niveau du golf de Bejaïa, il sera intéressant d'étudier l'effet de cette barrière sur d'autres organismes marins.

Les zones hybrides sont considérées comme des *laboratoires* pour l'étude de la spéciation (Hewitt 1988), en effet, celles-ci aident à la compréhension des processus impliqués dans la spéciation. Malgré leur utilité, les conclusions sur la spéciation et les définitions d'espèces restent limitées, car pour la plupart la divergence n'a pas abouti à la spéciation. Dans la zone hybride *Bombina* par exemple, la divergence a eu lieu il y a 3-4 Ma sans atteindre la spéciation. En effet, plusieurs zones hybrides ont été trouvées stables sur de très longues périodes avec une production abondante d'hybrides (Barton & Hewitt 1989; Endler 1977; Harrison 1993; Hewitt 1989), y compris la zone hybride *Mytilus* au Sud-Est de l'Espagne (El Ayari, soumis). D'autres zones se sont par contre déplacées, le suivi temporel de trois zones hybrides *Pecia*, *Swallowtail* et *chickadee* a permis de détecter des changements dans la

position associée aux changements climatiques anthropiques (Taylor *et al.* 2015). De la même manière, il a été trouvé que la zone hybride *Anartia* s'est déplacée vers l'Est à une vitesse moyenne de 2,5 km/an au cours des 20 dernières années à cause de la déforestation (Dasmahapatra *et al.* 2002). Pour le cas de la zone hybride *Mytilus*, seule la troisième zone hybride a été repérée avec un décalage de 100 km à l'Est de la Manche (Hilbish *et al.* 2012). L'échelle spatiale est une question importante quand on regarde les structures des zones hybrides car les patrons de variation n'apparaîtront qu'à des échelles spatiales particulières. Différents modèles peuvent être révélés par échantillonnage à différentes échelles spatiales, et certains peuvent être manqués parce qu'ils existent à une échelle non résolue par l'échantillonnage (Bridle & Butlin 2002). La zone hybride *Gryllus* échantillonnée à une échelle spatiale plus fine s'est avérée être une zone mosaïque, et non clinale (Harrison 1986; Harrison & Arnold 1982; Ross & Harrison 2002). Une perspective de ce travail sera de re-échantillonner la rive Sud de la Méditerranée afin de suivre l'évolution de la zone hybride mosaïque de moules.

En ce qui concerne le modèle *Stramonita haemastoma*, il serait intéressant d'analyser des échantillons des deux lignées cryptiques avec les nouvelles techniques de séquençage haut débit. Ceci nous permettrait de mieux comprendre les flux de gènes nucléaires, notamment grâce à des marqueurs diagnostiques et de mieux détecter des sous-structures génétiques et d'inférer les histoires démographiques des patchs à l'intérieure des lignées, notamment de la lignée A. D'autre part, nous suspectons l'existence d'incompatibilités mito-nucléaires et il serait intéressant d'étudier les gènes nucléaires codant pour des protéines mitochondriales, ce qui est dorénavant accessible avec le séquençage massif de transcrits (Pante *et al.* 2012). Cependant, il est crucial de poursuivre en parallèle les efforts d'échantillonnages spatiaux, à la fois dans les zones de transitions identifiées en Europe et en Afrique du Nord, et dans d'autres régions de l'Atlantique comme en Amérique centrale et surtout dans l'Atlantique du Sud-Est en Namibie et en Angola. Enfin, un échantillonnage temporel de la zone hybride espagnole pourrait donner des indices sur sa stabilité et éventuellement suivre dans le temps le déplacement de la vague d'invasion, si cette hypothèse se révèle être la bonne.

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ANNEXES

(Articles publiés sur des sujets différents de la problématique principle de la thèse)

Article 1 : The effect of size and epibiotic barnacles on imposex in Stramonita haemastoma collected from the northern coast of Tunisia.

Article 2: Associated fauna and effects of epibiotic barnacles on the relative growth and reproductive indices of *Stramonita haemastoma* (Gastropoda: Muricidae).

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ORIGINAL ARTICLE

The effect of size and epibiotic barnacles on imposex in Stramonita haemastoma collected from the northern coast of Tunisia

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Abstract

Imposex was quantified in *Stramonita haemastoma* from the Bizerta Channel during a one-year study period and the effect of gastropod size and shell fouling by epibiotic barnacles investigated. Butyltin concentrations (tributyltin (TBT) and its derivatives di- and mono-butyltin) were also investigated in the soft tissue of the gastropods to depict temporal trends in pollution. Kelibia beach was chosen as a reference site (not polluted by organotin) to depict whether barnacle fouling could intervene in imposex development. Imposex incidence in the Bizerta Channel was greater in females fouled by epibiotic barnacles compared to non-fouled females. Considering the size of gastropods, large females (fouled and non-fouled) always exhibit higher imposex indices than smaller females. The concentrations of organotin compounds were found to be similar in the studied species, independent of sex or fouling condition. For Kelibia beach, no imposex was recorded in fouled and non-fouled snails, while butyltins were below the limit of detection. Results gathered in the present study confirm the effect of size and epibiotic barnacles as factors affecting imposex quantification in gastropods. The absence of imposex for Kelibia beach suggest that barnacles do not promote the development of imposex, but could be a secondary factor amplifying the effect of imposex. Furthermore, this investigation showed a significant decrease in imposex and TBT levels in Bizerta Channel as compared to previous work reflecting the effectiveness of a TBT ban on a global scale.

Key words: *Imposex, shell fouling,* Stramonita haemastoma*, tributyltin compounds (TBT), Tunisia*

Introduction

Genital abnormalities observed in gonochoristic gastropods are mainly due to the presence of organotin compounds such as tributyltin (TBT) and, in some species, to triphenyltin (TPT) in the aquatic environment (Gibbs et al. 1987). The presence of the active biocide is predominantly the result of the use of antifouling paints covering ship hulls and immersed structures. Abnormality consists of the imposition in female marine and freshwater gastropods of male sexual organs (penis and/or sperm-duct; Schulte-Oehlmann et al. 1995). Currently, approximately 260 gastropod species are known to be affected by imposex wordwide (Sternberg et al. 2010; Titley-O'Neal et al. 2011), largely dominated by species belonging to muricidae (76 species; Shi et al. 2005). This list has increased with further investigations and has recently been recorded for *Nassarius mutabilis* (Linnaeus, 1758) (Lahbib et al. 2013a).

In the Mediterranean Sea, there are many imposex studies for *Hexaplex trunculus* (Linnaeus, 1758) (Martoja & Bouquegneau 1988; Axiak et al. 1995, 2003; El Hamdani et al. 1998; Terlizzi et al. 1998; Rilov et al. 2000; Chiavarini et al. 2003; Pellizzato et al. 2004; Prime et al. 2006; Lahbib et al. 2007, 2013b), but such studies are scarce for *Bolinus brandaris* (Linnaeus, 1758) (Ramon & Amor 2001; Abidli et al. 2009, 2010) and there are few studies on *Stramonita haemastoma* (Linnaeus, 1767) (Rilov et al. 2000, 2001; Chiavarini et al. 2003; Lemghich & Benajiba 2007; Lahbib et al. 2010). *Stramonita*

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haemastoma is a widespread muricid found in Mediterranean, Atlantic and Pacific waters (Harding & Harasewych 2007), living in rocky habitats exposed to waves in temperate and tropical waters and occasionally in sandy habitats (Rilov et al. 2000). Imposex was observed in *S. haemastoma* for the first time on the Azores Islands (Spence et al. 1990). After a series of contamination experiments performed under laboratory conditions, *S. haemastoma* was considered as an adequate bioindicator of organotin contamination (Limaverde et al. 2007).

Imposex quantification can be affected by a variety of factors including variations in male penis length at different reproductive stages or shell size, which affect the calculation of relative penis length (Vasconcelos et al. 2011; Lahbib et al. 2013b). Occurrence of shell fouling by epibionts is widespread in molluscs. In basibiont species, epibiont fouling can have both advantages and disadvantages (Buschbaum & Reise 1999; Chan & Chan 2005; Vasconcelos et al. 2007). The occurrence of fouling with heavy colonization could protect the animal against desiccation, predation and cannibalism in some gastropods (Buschbaum & Reise 1999; Vasconcelos et al. 2007). In other cases, as with boring epibionts, fouling affects shell structure and growth, which makes it fragile and vulnerable to attacks (Lleonart et al. 2003; Vasconcelos et al. 2007). Furthermore, heavily affected shells can be a disadvantage to the mobility of the basibiont (Buschbaum & Reise 1999; Chan & Chan 2005).

In Tunisia, imposex levels and concentrations of organotin compounds were compared in *S. haemastoma* and *H. trunculus*. Both species are considered good bioindicators for TBT pollution (Lahbib et al. 2010). However, *S. haemastoma* and *H. trunculus* follow different imposex paththways with *H. trunculus* exhibiting the 'c'pathway (aphalic females from VDS1 to VDS4) while in *S. haemastoma*, imposex is developed following the 'a'pathway, i.e. the penis development occurs before the vas deferens. Imposex in *S. haemastoma* is therefore easier to identify and their abundance and ease of collection makes them an ideal bioindicator species. In the present study, a more advanced investigation of imposex in *S. haemastoma* was conducted in order to depict seasonal variability, influence of size and shell fouling. Butyltin burden was also investigated to show temporal trends of TBT pollution in the Bizerta Channel, which is known as a TBT hot spot in Tunisia.

Material and methods

Studied site and sampling

The Bizerta channel was the site chosen for this investigation (Figure 1). The Bizerta channel links

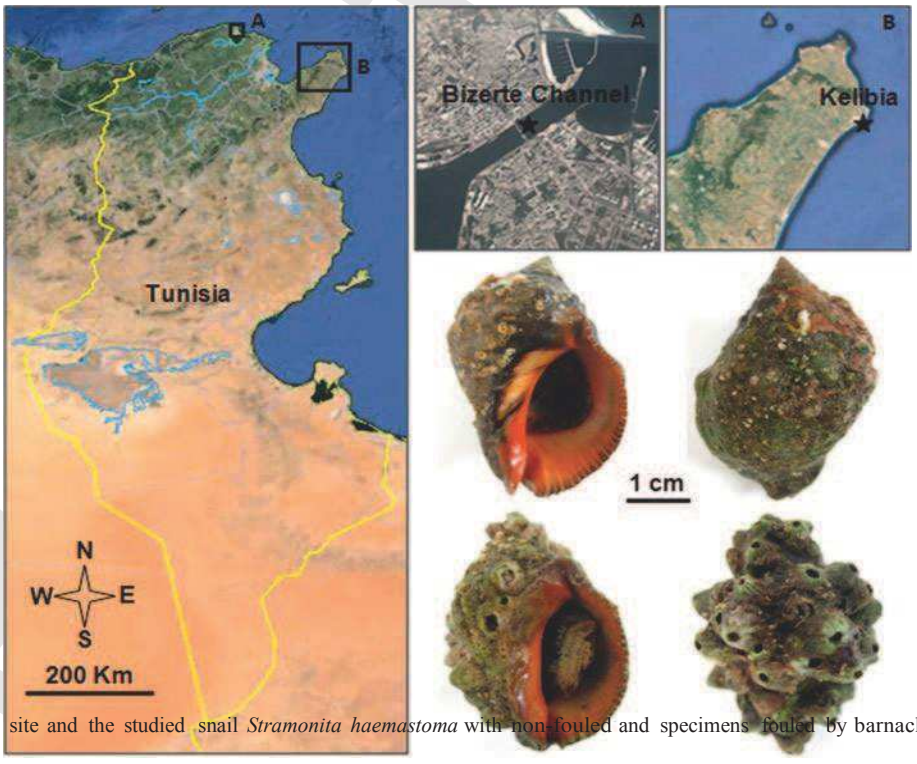

Figure 1. Sampling site and the studied snail *Stramonita haemastoma* with non-fouled and specimens fouled by barnacles.

the Bizerta lagoon to the Mediterranean and has very high levels of boating traffic, exceeding 1000 boats per year, including fishing boats, oil tankers, gas tankers, passenger ships, container ships and naval boats. This site has been considered in previous investigations as a TBT hot spot in Tunisia where sterile females of *Hexaplex trunculus* were recorded (Lahbib et al. 2008, 2011). Specimens of *Stramonita haemastoma* (N = 100–150) with a shell length ranging from 20 to 80 mm were collected monthly from a rocky site from June 2009 to May 2010 by SCUBA diving at about 11 m depth. A reference site with no TBT pollution was chosen as Kelibia beach where no boating traffic or anti-fouling coatings are recorded (Figure 1). From this site, 60 *S. haemastoma* were collected on May 2010 and subjected to imposex evaluation and chemical analyses.

Biological analysis

In the laboratory, specimens were washed using seawater from Bizerta channel in order to identify the fauna living on the external shell surface. The epifauna mainly consisted of crustaceans, polychaetes, molluscs, echinoderms, Ascidiacea, sponges, bryozoans, sipunculids and green and brown algae (Najoua Trigui-El Menif, unpublished data). *Stramonita haemastoma* were frozen at −20°C. After thawing, specimens were divided into two groups; non-fouled and fouled by epibiotic barnacles. For fouled individuals, barnacles were removed using a scalpel and the position and number of barnacles per individual recorded. Shell length (SL) was measured using a vernier calliper to the nearest 0.1 mm. The soft body was extracted after breaking the shell using a bench vice and the sex determined under a binocular microscope based on the presence of a capsule gland and a vagina in females and the presence of a large penis in males. Gonad colour was also used to identify sex; in this case, the gonad is yellowish in females and beige in males. For each mollusc, the presence or absence of a penis was recorded. For those with a penis, the length of this organ was measured under a dissecting microscope using a calibrated eyepiece. The assessment of the imposex development was made using the following indices:

- imposex incidence or frequency (I%) = percentage of imposex-affected females compared to the total number of females in the sample;
- vas deferens sequence index (VDSI) = sum of imposex stages of all females/total number of females, following Gibbs et al. (1987). VDS stages were determined according to the general scheme proposed by Stroben et al. (1992), as partially modified for *Hexaplex trunculus* (Axiak et al. 1995; Lahbib et al. 2007) and recently for *S. haemastoma* by Toste et al. (2013). The VDSI varies from 0 to 4, with stage 0 corresponding to healthy females; stage 1 to the first sign of imposex development and this could be the appearance of a bud penis or a first stage in the sequence of the development of the vas deferens; stage 2, a small penis with a penis duct; stage 3, the growth in the vas deference length but still not reaching the spawning orifice; stage 4, the simultaneous presence of penis and vas deferens reaching the spawning orifice (Lahbib et al. 2007; Toste et al 2013);
- relative penis length index (RPLI) = average length of female penises in the sample including aphallic females × 100/average length of male penises in the sample (Lahbib et al. 2008). All imposex indices were calculated for two size-classes, from 20 to 50 mm and from 50 to 80 mm.

Butyltin analysis

Five males and five females were selected from the sample collected in May 2010 for each site. The operculum was removed and the soft body was finely ground in glass bottles using a T 18 basic Ultra-Turrax® disperser at 6000 rotations min^{-1}. Thereafter, tissue was freeze-dried, weighed and maintained at −20°C in the dark until analysis. Approximately 150 mg of each sample (two replicates) were spiked with a diluted solution of the ^{119}Sn-enriched spike of MBT, DBT and TBT and immediately 4 ml of a mixture of acetic acid and methanol (3:1) (g/g) were added in 7 ml glass vials with screw cap (Supelco, Bellefonte, PA). The vials were placed in a thermostatic bath at 37°C for at least 2 h with mechanical shaking. During the derivization, 2 ml of acetate buffer to adjust the pH to 5.4, 2 ml of hexane and 300 µl of a 2% w/v sodium tetraethyl borate in 0.2 M NaOH were added to 1 ml of the extract for ethylation of the organotin compounds. The vials were centrifuged at 3000 rpm for 5 min to allow for a better phase separation. The organic layer was transferred to a 2 ml chromatographic vial with a Pasteur pipette. The hexane phase was evaporated under a gentle stream of argon until almost dry (a few microlitres). Finally, 2 µl of this final volume was injected into GC-MS system using an Agilent Model 6890N gas chromatograph (Agilent Technologies, Waldbronn, Germany) fitted with a splitness injector and a HP-5MS column (30 mm × 250 mm i.d. 0.25 mm) and equipped with an Agilent Model 5973 Network MSD mass spectrometric detector (Agilent Technologies, Tokyo, Japan). The detection limits in solid environmental samples

(sediment and lyophilized biota, 3 replicates) were 0.09 ng Sn/g for MBT, 0.03 ng Sn/g for DBT and 0.06 ng Sn/g for TBT. Analysis of a certified reference material (mussel tissue BCR 477, 3 replicates) using this procedure resulted in the following recoveries: 116.17 ± 2.65 for MBT, 98.13 ± 1.76 for DBT and 91.43 ± 1.57 for TBT, being in agreement with the certified range.

The butyltin degradation index (BDI) was calculated according to the following equations (BDI = [MBT] + [DBT] / [TBT]) in order to evaluate whether contamination in the Bizerta channel is recent or not (Díez et al. 2002).

Statistical analysis

The Chi-square test was used to compare the frequency of imposex and VDSI stages, while the Mann–Whitney rank sum test was used for pairwise comparison of VDSI. Paired *t*-tests were used for RPLI and organotin burden comparison. Statistical tests were initially applied for making seasonal pairwise comparisons for the total of the population. Then, the same comparison was applied to fouled and non-fouled snails taking into account the class-size, thus small snails were compared to large snails (seasonal pairwise comparison). The effect of fouling was studied separately for each size class by making seasonal pairwise comparisons between fouled and non-fouled individuals. Statistical tests were performed using the software Sigmastat® 3.5 for Windows.

Results

Imposex assessment

Imposex was recorded in most of the females collected in the Bizerta channel (Table I). Four of the six known stages of imposex were recorded in this study. All stages follow the a-pathway (stage 1a, 2a, 3a and 4). No sterile females were found in the samples. Imposex incidence varied from 80% to 84%, VDSI from 1 to 2 and RPLI from 2 to 9 without showing any significant seasonal differences (ANOVA, $P > 0.05$).

Taking into account the size of females in the imposex analysis, results showed that larger females exhibited a higher incidence and degree of imposex (Table I). This finding was recorded in both fouled and non-fouled females by epibiont barnacles. In non-fouled females, differences in imposex indices

Table I. Imposex data recorded in *Stramonita haemastoma* according to the size of gastropods and shell fouling by barnacles.

	Season	*N*	Imposex (%)	VDSI	RPLI
Non-fouled snails					
Size-class (20–50 mm)	Summer	31	68	1	2
	Autumn	28	71	1	3
	Winter	20	70	1	2
	Spring	41	66	1	2
	Total/Average	120	69	1	3
Size-class (50–80 mm)	Summer	45	82	2	4
	Autumn	22	91	2	7
	Winter	15	87	2	7
	Spring	13	92	2	6
	Total/average	95	88	2	6
Fouled snails					
Size-class (20–50 mm)	Summer	24	79	1	4
	Autumn	30	83	2	5
	Winter	24	75	2	5
	Spring	31	84	1	2
	Total/average	109	80	1	4
Size-class (50–80 mm)	Summer	58	91	2	9
	Autumn	52	92	2	7
	Winter	26	96	2	8
	Spring	22	95	1	3
	Total/average	158	94	2	7
All snails					
	Summer	158	80	1	5
	Autumn	132	84	2	6
	Winter	85	82	1	6
	Spring	107	84	1	3
	Total/average	482	83	1	5

(I%, VDSI, and RPLI) were statistically significant between the two size-classes. However, in fouled snails significant differences were only recorded for the VDSI (ANOVA, $P < 0.05$).

Shell fouling in the population of *Stramonita* colonization rate varying from 44% to 55% of the population in small females and from 56% to 70% in large females. The number of barnacles recorded per gastropod varied between 4 and 76 (average: 21.34 ± 11.82). Such a level of biofouling allows an assessment of its effects on imposex development. For the same class-size, imposex indices were always higher in fouled snails than in non-fouled snails, whatever the season. Statistical analysis revealed that imposex indices were significantly different only for the small class-size (ANOVA, $P < 0.05$; Table I). To depict the effect of shell fouling on imposex degree, the imposex stages incidence was calculated for each population and class-size (Table II). Results showed that advanced imposex stages (VDS 2 and VDS 3) were recorded at higher rates in fouled females as compared to non-fouled ones (chi-square test, $P < 0.05$), while stages 1 and 4 were recorded at similar rates in the two populations (chi-square test, $P > 0.05$; Table II).

Imposex was not recorded in fouled and non-fouled *S. haemastoma* collected from Kelibia.

Table II. Percentage of imposex stages in *Stramonita haemastoma*.

VDSI%	Fouled snails	Non-fouled snails	All snails
N (20–50 mm)	109	120	229
VDS1	54	54	54
VDS2	8	2	5
VDS3	15	11	13
VDS4	4	4	4
N (50–80 mm)	158	95	253
VDS0	7	14	10
VDS1	44	51	46
VDS2	23	7	17
VDS3	10	10	10
VDS4	16	18	17
Total (20–80 mm)	267	215	482
VDS0	13	21	17
VDS1	49	52	50
VDS2	16	5	11
VDS3	12	11	11
VDS4	10	11	11

N, number of females.

Butyltin burden

Chemical data were in concordance with biological data (imposex) from both sites. TBT and its derivatives were only detected in the flesh of gastropods collected from the Bizerta channel. Higher butyltin accumulation was recorded in females than in males and in fouled snails than in non-fouled snails, being statistically similar for all snails (ANOVA, $P > 0.05$). Also, all butyltin compounds were detected at a similar scale range (4–8 ng Sn g^{-1} dw, Table III). In *Stramonita haemastoma* collected from Kelibia all butytins were below the limit of detection. The butyltin degradation index was higher than the unity (BDI ≥ 1) in all analysed snails from the Bizerta channel, which reveals an historical contamination of the studied site.

Discussion

The present study reported recent and detailed data on imposex in 482 females of *Stramonita haemastoma* from the Bizerta channel taking into consideration the size of the gastropods and shell fouling. An imposex incidence of 83% was revealed in this study, a value lower than that reported 3 years ago from the same location using a sample size of 40 females (95%, Lahbib et al. 2010). This decrease in imposex incidence was reported while the number of boats entering Bizerta channel has increased each year from 1062 in 2004 to 1397 in 2012. This finding could be explained by the fact that Tunisian paint factories are aware of the effect of TBT on aquatic organisms and prohibit its use in paint formulation.

Table III. Butyltin analysis in the flesh of *Stramonita haemastoma* in May 2010 (concentrations are given as ng/g dry weight; BDI, butyltin degradation index).

		TBT	DBT	MBT	BuTs	BDI
Bizerta channel			Non-fouled snails			
	Males	6.35 ± 0.21	4.63 ± 0.32	4.68 ± 0.46	15.65 ± 0.35	1.46
	Females	7.20 ± 0.28	5.58 ± 0.39	4.48 ± 0.39	17.25 ± 0.28	1.40
			Fouled snails			
	Males	7.23 ± 0.46	5.90 ± 1.13	4.25 ± 0.71	17.38 ± 1.38	1.40
	Females	7.73 ± 0.11	6.53 ± 0.04	5.83 ± 0.32	20.08 ± 0.25	1.60
	All snails	7.13 ± 0.57	5.66 ± 0.79	4.81 ± 0.70	17.59 ± 1.83	1.47

In *Bolinus brandaris* (Linnaeus, 1758) collected from the Bizerta lagoon in 2007 and 2010, a slight decrease in imposex incidence was also reported (Abidli et al. 2010, 2013). In *Hexaplex trunculus* tissues collected from the Bizerta channel, TBT concentration also decreased from 2002 to 2010 (Lahbib et al. 2011). In Moroccan and Sicilian populations of *S. haemastoma*, imposex was recorded in all the females (100%; Chiavarini et al. 2003; Lemghich & Benajiba 2007). The values of VDSI and RPLI obtained for the present study were lower than those recorded in *S. haemastoma* from other Mediterranean locations (Morocco, Italy, Palestine) and Atlantic Ocean (Portugal, Brazil) (Spence et al. 1990; Rilov et al. 2000; Chiavarini et al. 2003; Fernandez et al. 2005; Lemghich & Benajiba 2007). Imposex indices did not exhibit significant seasonal variability, suggesting that TBT leaching is constant throughout the year. This could not be attributed to imposex irreversibility because imposex indices did not reach saturation in the present study. RPLI values were lower in spring, suggesting that the effect of penis length variability in males varies according to the reproductive cycle. Similar observations were also reported in the muricids *B. brandaris* and *H. trunculus* (Ramon & Amor 2001; Trigui El-Menif et al. 2006; Lahbib et al. 2013b).

Imposex was found to be higher in larger females, suggesting that gastropod size should be considered in imposex analysis to achieve more reliable quantification (Vasconcelos et al. 2011; Lahbib et al. 2013b). This finding can be explained by the exposure duration to TBT, which is longer in older females. In this case, larger females could only reflect old contamination because of the irreversibility of imposex. Therefore, analysis of juvenile females is more reliable for assessing the recent input of TBT. Indeed, juveniles that have recently been exposed to TBT (1–3 years old) are known to be more sensitive to TBT than older snails. Another factor implicated in imposex quantification in the present study is shell fouling by epibiotic barnacles. Imposex was more pronounced in fouled females with a clear effect in small gastropods. It appeared that younger females fouled by epibiotic barnacles are more vulnerable to imposex development than non-fouled females. The link between imposex and barnacle fouling has not been previously recorded in gastropods, but it seems that by reducing the mobility of the basibiont, barnacles expose the basibiont to local environmental conditions and TBT leaching (Buschbaum & Reise 1999; Chan & Chan 2005). Another explanation is the stress that could be generated by epibionts on the basibiont shell growth could allow imposex induction by making gastropods more sensitive to TBT pollution. Indeed, in the gastropod *H. trunculus*, imposex was induced in the laboratory in the absence of any contamination by stressing the animals (Garaventa et al. 2008), while in the gastropod *Haustrum vinosum* (Lamarck, 1822), others factors such as copper exposure, exposure to the paint matrix and environmental stress may also induce imposex (Nias et al. 1993). However, in this investigation, barnacle fouling was found to act as secondary factor amplifying imposex development because fouled females from Kelibia did not exhibit imposex. In this case, banacles could not promote imposex by simply stressing the animal, but TBT was presumably the underlying cause.

Several studies, including those of Terlizzi et al. (1998) and Toste et al. (2013), revealed a positive correlation between imposex and organotin concentrations in seawater. Butyltin concentrations in the whole body of *S. haemastoma* recorded in the present study in 2010 (17.59 ng Sn g^{-1} dw) were lower than those recorded at the same site in July 2007 (29.1 ng Sn g^{-1} dw, Lahbib et al. 2010). This finding was also reported in other species like *H. trunculus*, *Conus ventricosus* Gmelin, 1791, *Cyclope neritea* (Linnaeus, 1758), *Nassarius mutabilis* and *B. brandaris* collected from Bizerta in 2007 (Lahbib et al. 2010). Decreases in butyltin levels agreed with the imposex reduction recorded in the present study and further suggests the effectiveness of a TBT ban by IMO. Indeed, more than 70% of the Tunisian fleet comes from neighbouring countries already contracted to the AFS convention. Locally, the introduction in Tunisian markets of a new generation of antifouling paints which are TBT-free will certainly have a positive impact on the reduction of organotin pollution in Tunisian waters. Butyltin levels recorded in the present work were also lower than those reported in other Mediterranean localities (32.6–1640.9 ng Sn g^{-1} dw, Rilov et al. 2000). In Kelibia, concentrations of TBT were below detection limits. This finding was also recorded in other Tunisian sites such as Tunis North Lake and the Zarat Sea (Lahbib et al. 2009; Abidli et al. 2013).

The values of BDI calculated in *S. haemastoma* collected in 2010 from the Bizerta channel were higher than 1, suggesting old contamination. Thus, available TBT is most probably the result of release from contaminated sediment. The same observation was also reported in 2004 and 2007 for the Bizerta channel (Lahbib et al. 2009; Abidli et al. 2010) and recently in 2010 by calculating this index for sediment collected at two stations from the Bizerta lagoon–channel complex; in Menzel Abderrahmane (3.87) and in Carrier Bay (1.35) (Abidli et al. 2013).No data are available for the TBT half-life in *S. haemastoma*; however, further investigations are needed to clarify

how TBT is metabolized by *S. haemastoma* and thus to depict the exact age of exposure. The half-life of TBT in molluscs varies with species. In *Thais clavigera* (Küster, 1858), TBT half-life has been shown to be 22 days (Horiguchi et al. 1995), 11–36 days in the clam *Lajonkaira lajonkairii* (Payraudeau, 1826) (Gomez-Ariza et al. 1999), 26 days in the bivalve *Dreissena polymorpha* (Pallas, 1771) (Van Slooten & Tarradellas 1994) and 2–3 months in the gastropod *Nassarius reticulatus* (Linnaeus, 1758) (Sousa et al. 2005). In the sediment, the half-life of TBT is higher, varying from 6 months to 8.7 years according to the sediment characteristic and water temperature (Maguire & Katz 1985; Stang & Seligman 1986; Smith 1996). Therefore, in this study it appears that TBT pollution is historical and that small recent TBT inputs that are present in the field are probably coming from sediment stocks.

Investigations of detailed reports on imposex in *S. haematoma* in a site known as a hotspot of TBT leaching in Tunisia showed that imposex is reducing, but the remaining high level reflects that many years are needed for a complete recovery of the local population. This work suggested assessing imposex in younger females to depict reliable quantification because imposex is irreversible. However, special care should be considered in analysing fouled gastropods because it could affect imposex analysis.

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Associated fauna and effects of epibiotic barnacles on the relative growth and reproductive indices of *Stramonita haemastoma* (Gastropoda: Muricidae)

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Summary: To better understand the impacts of biofouling on the biological processes of the basibiont, the effects of epibiotic barnacles on the relative growth and reproductive indices of *Stramonita haemastoma* (Linnaeus, 1767) were assessed. A total of 1035 specimens were collected monthly for one year from Bizerta Channel (northern Tunisia). Endobiotic species comprised the lithophagous bivalves *Lithophaga aristata* and *Rocellaria dubia* of different sizes, communicating with the outside through tiny perforations. Intra-shell tunnels and galleries also sheltered annelids and sipunculids. Epibiotic species comprised algae and highly diversified invertebrates represented by crustaceans, polychaetes, molluscs, echinoderms, ascidians, sponges, bryozoans and sipunculids, with barnacles being the most common group. Comparison of growth features between non-fouled and fouled *S. haemastoma* revealed higher growth in non-fouled specimens. Differences in reproductive condition indices were detected in few months, being mostly higher in non-fouled snails, but showed no asynchrony in the spawning period for either fouled or non-fouled gastropods hosts.

Keywords: *Stramonita haemastoma*; biofouling; reproductive indices; relative growth; Bizerta Channel.

Fauna asociada y efecto de los balanos epibiontes al crecimiento relativo e índices reproductivos de *Stramonita haemastoma* (Gasterópoda: Muricidae)

Resumen: Para mejorar la compresión de los impactos del biofouling en los procesos biológicos de los basibiontes, se ha evaluado los efectos de los balanos epibiontes en el crecimiento relativo y en los índices reproductivos de *Stramonita haemastoma* (Linnaeus, 1767). Se recogieron un total de 1032 especímenes mensualmente, durante un año, en el canal de Bizerta (norte de Túnez). Las especies endobióticas estaban compuestas por los bibalvos litófagos *Lithophaga aristata* y *Rocellaria dubia,* de diferentes tamaños, que se comunicaban con el exterior a través de pequeñas perforaciones. Los túneles y galerías del interior de la concha también albergaban anélidos y sipuncúlidos, siendo los balanos el grupo más común. La comparación del crecimiento entre los gasterópodos con y sin fouling mostró un mayor crecimiento en los *S. haemastoma* sin fouling. Las diferencias en los índices reproductivos se detectaron en pocos meses, siendo mayor en los caracoles no invadidos por el fouling, pero ninguno de los gasterópodos hospedadores mostró asincronía en el periodo de desove.

Palabras clave: *Stramonita haemastoma*; biofouling; índices reproductivos; crecimiento relativo; canal de Bizerta.

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INTRODUCTION

The mollusc shell is a suitable biotope for the settlement and development of several groups of invertebrates. In molluscs, biofouling has mainly been described in bivalves such as *Pinna bicolor* (Keough 1984), *Chlamys opercularis* (Ward and Thorpe 1991), *Crassostrea gigas* (Duault et al. 2001), *Venus verrucosa* (Trigui El Menif et al. 2005), *Flexopecten felipponei* (Schejter and Bremec 2006), *Lithophaga lithophaga* (Trigui El Menif et al. 2007) and *Pinna nobilis* (Rabaoui et al. 2009). The associated fauna highlighted in these bivalve species belongs to several zoological groups: bryozoans, annelids, serpulid polychaetes, tu-

nicates, sponges, crustaceans, ascidians, cnidarians and echinoderms.

Few studies have as yet dealt with shell fouling and its effects in gastropods. These include the study by Buschbaum and Reise (1999) reporting the effects of barnacle colonization on the shell of *Littorina littorea* and those of Bick (2006) and Vasconcelos et al. (2007) on polychaete fixation in the shells of *Stramonita haemastoma* and *Hexaplex trunculus*, respectively.

Few studies of the biology of *S. haemastoma* have been published, and they deal mainly with reproduction, spawning and intracapsular development (Beliste and Stickle 1978, Lahbib et al. 2011). The most studied aspect in *S. haemastoma* is the imposex phenomenon (Spence et al. 1990, Rilov 1999, Lemghich and Benajiba 2007, Lahbib et al. 2010, El Ayari et al. 2015), the sexual deformity associated with marine pollution by organotin compounds such as tributyltin (TBT) and its derivatives (Terlizzi et al. 2001). Recently, the effect of epibiotic barnacles on imposex in *S. haemastoma* sampled from Tunisian coasts was investigated (El Ayari et al. 2015).

Taking into consideration the well-known effects of biofouling that could possibly affect the biology of the basibiont, the present study aimed to identify species associated with the shell of *S. haemastoma* from Tunisian waters and to investigate whether this fouling could affect the relative growth and reproductive indices of this locally abundant gastropod species.

MATERIALS AND METHODS

Specimens of *S. haemastoma* with a shell length of 20-80 mm were collected monthly (N=80-120) on a rocky bottom from June 2009 to May 2010. Sampling was performed by scuba diving at 11 m depth at a station located in the artificial channel linking the Bizerta lagoon with the Mediterranean Sea (Fig. 1). Seawater temperature and salinity were measured using a multiparameter sounder. In the laboratory, fauna living on the shell external surface was collected by washing the shells using seawater from the same sampling station.

Comparison of relative growth and reproductive indices in *S. haemastoma* was investigated monthly in two groups: 40 to 60 gastropods non-fouled by barnacles (GnFB) and 40 to 60 gastropods fouled by barnacles (GFB). The position and number of barnacles per individual were determined after removing them from the shells of the hosts using a scalpel. Gastropods were then sacrificed by freezing at –20°C; shell length (SL), shell diameter (SD), and penis length (PL) were measured to the nearest 0.1 mm using a digital caliper. After thawing, the shell was broken using a bench vice and the soft part of the organism was carefully removed. Endobiotic fauna was collected after shell breakage, identified following macroscopic observation or under a binocular microscope, and then preserved in 70% alcohol.

In *S. haemastoma,* three gonad maturation stages were detected following macroscopic observations of the gonads in both sexes and of the capsule gland in females, following Ramón and Amor (2002) in the sympatric muricid *Bolinus brandaris*. Stage I (immature) corresponds to undifferentiated gonads from the underlying capsule gland in both sexes and females having an inconspicuous capsule gland; stage II (intermediate) gonads in both sexes are more developed and correspond approximately to one-third of the area of the digestive gland; and stage III (mature) males show a well-developed, light brown testicle corresponding to more than half of the area of the digestive gland, whereas females have a voluminous yellowish ovary and a large yellowish capsule gland.

The flesh wet weight (FwW) was recorded after removing the operculum. The female capsule gland was separated and weighed (CGwW). A cross-section was made on the coiled part of the organism, directly under the stomach, and was photographed to measure the gonad area (GA) and the area of the digestive gland–gonad complex (DGGA) using the software Image J 1.38 x. The shell dry weight (SdW) and the flesh dry weight (FdW) were recorded after drying them at 60°C for 3 days.

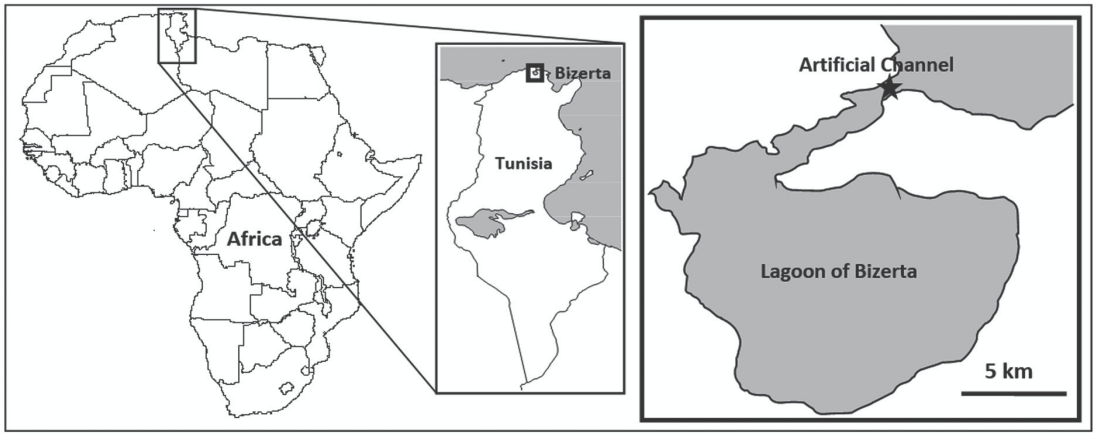

Fig. 1. – Map of Tunisia and sampling site of *Stramonita haemastoma* in the channel of Bizerta.

Table 1. – Monthly seawater temperature and salinity values recorded in Bizerta Channel during the study period (June 2009 - May 2010).

	J	J	A	S	O	N	D	J	F	M	A	M
Temperature (°C)	24.5	25.7	26.7	24.4	20.3	14.0	16.0	15.2	14.0	13.3	17.0	23.9
Salinity	35.8	36.7	37.2	37.9	36.7	27.6	23.1	23.9	28.5	29.3	28.0	31.1

The reproductive condition of *S. haemastoma* was assessed using the following indices, expressed as percentages:

- Gonadosomatic index (GSI) = digestive gland-gonad complex dry weight/SdW (Lucas and Beninger 1985);
- Capsule gland index (CGI) = CGwW/FwW (Giménez and Penchaszadeh 2003);
- Gonad area index (GAI) = GA/DGGA (Poore 1973);
- Penial index (PI) = PL/SL (Vasconcelos et al. 2011).

Relationships between morphometric and ponderal variables (SL, SD, SdW and FdW) were established through regression analysis ($Y=aX^b$). Relative growth between variables was determined by comparing the regression slopes (b) using the Student t-test (Mayrat 1959). A chi-square test was employed to verify a balanced proportion (1M:1F) in the sex ratio of the samples. Comparisons between fouled and non-fouled gastropods were made through analysis of covariance (ANCOVA - model of homogeneity of slopes) using the software STATISTICA 10, with the covariable being always SL. Post-hoc pairwise comparisons were made using the Tukey test. Monthly variations in bio-physiological indices were analysed by one-way ANOVA (Kruskal-Wallis test). In all statistical analysis, significance level was considered for $P<0.05$.

RESULTS

Seawater temperature and salinity

The average seawater temperature measured in Bizerta Channel during the study period (June 2009 to May 2010) was 19.6°C. The lowest temperature (13.3°C) was recorded in March and the highest (26.7°C) in August (Table 1). The salinity reached a maximum (37.9) in September and a minimum (23.1) in December (Table 1), corresponding to an average value of 31.3.

Population sex ratio

The sex ratio of the studied population showed a higher abundance of males in both fouled and non-fouled gastropods. Unbalanced sex ratios were only statistically significant in the monthly samples of April for GFB ($\chi^2=4.07$, $P<0.05$) and of December for GnFB ($\chi^2=4.57$, $P<0.05$).

Associated fauna

Among a total of 1035 individuals of *S. haemastoma* with an average length of 53.4±6.7 mm, an abundant and very diversified associated fauna and flora was identified, composed mainly of crustaceans, polychaetes, molluscs, echinoderms, ascidians, sponges,

Table 2. – Fauna associated with *Stramonita haemastoma* collected from Bizerta Channel (northern Tunisia).

	Epibiotic species	Endobiotic species		Epibiotic species	Endobiotic species
Mollusca	GASTROPODA	BIVALVIA	Errant	SYLLIDAE	
	Diodora graeca	*Lithophaga aristata*	Polychaetes	*Syllis amica*	
	Diodora sp.	*Rocellaria dubia*		*Streptoyllis* sp.	
	Patella sp.			EUNICIDAE	
	Acmaea insessa			*Eunice vitatta*	
	BIVALVIA			*Lysidice ninetta*	
	Anomia ephippium			*Hyalinoecia bilineata*	
	Grassostrea gigas			NEREIDAE	
	Ostrea edulis			*Perinereis cullrifera*	
Crustacea	SESSILIA	Acrothoracica		*Nereis rava*	
	Balanus amphitrite	Unidentified		PHYLLODOCIDAE	
	Balanus perforatus			*Eullalia* sp.	
	AMPHIPODA			CHRYSOPETALIDAE	
	Gammarus aequicauda			*Chrysopetalum debile*	
	Gammarus marinus			SPHAERODARIDAE	
	Gammarus insensibilis			*Sphaerosyllis pirifera*	
	Gammarus pulex		Sedentary	SERPULIDAE	CIRRATULIDAE
	Gammarus chevreuxi		Polychaetes	*Pomatoceros triqueter*	*Dodecaceria concharum*
	Gammarus olivii			*Hydroides uncinata*	*Audonia tentaculata*
	Gammarus locusta			*Hydroides diramphus*	*Heterocirrus* sp.
	Elasmopus rapax			*Serpula concharum*	SPIONIDAE
	DECAPODA			*Serpula vermicularis*	*Pygospio elegens*
	Achaeus cranchi			SABELLIDAE	*Polydora* sp.
	Acanthonyx lunulatus			*Sabella* sp.	LUMBRINERIDAE
	Xantho poressa				*Lumbrinereis* sp.
	ISOPODA		Sipunculoidea		PHASCOLOSOMATIDAE
	Cymodoce sp.				*Phascolosoma stephensoni*
	PYCNOGONIDA				ASPIDOSIPHONIDAE
	Achelia sp.				*Aspidosiphon muelleri*
					Phascolosoma sp.
			Echinodermata	*Amphipholis squamata*	
				Ophiopsila aranea	
				Ophiura sp.	

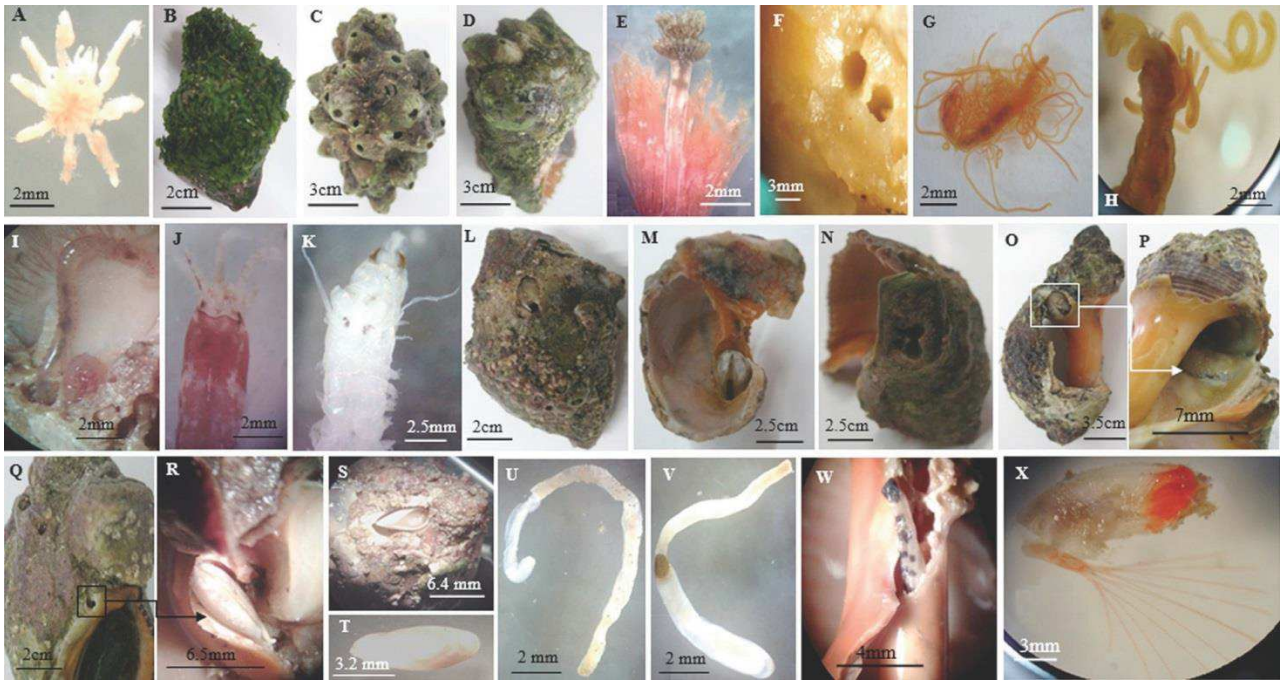

Fig. 2. – Fauna associated with *S. haemastoma*. A, *Achelia* sp. found in the external shell surface of *S. haemastoma*; B, shell of *S. haemastoma* covered by green algae; C, barnacles entirely covering the gastropod shell; D, oysters randomly distributed on the gastropod shell; E, *Hydroides diramphus* removed from white tube in the shell of *S. haemastoma*; F, shell perforation caused by annelids; G, unidentified cirratulid; H, *Dodecaceria concharum* removed from intra-shell gallery; I, J, K, errant polychaetes (*Aponuphis bilineata* and *Perinereis cullrifera*) removed from tunnels in the shell of *S. haemastoma*; L, *Lithophaga aristata* in gallery on the dorsal side of the shell; M, *L. aristata* in gallery located in the direction "apical axis-siphonal canal"; N, eight-shaped hole showing the penetration of *L. aristata*; O, P, Q, penetration of *L. aristata* in perpendicular direction to the apical axis; R, *L. aristata* reached the visceral mass; S, T, *Rocellaria dubia* removed from breach dug in the last spire of the shell; U, *Phascolosoma* sp. found in gallery in the dorsal surface near the last spire of the host shell; V, *Aspidosiphon muelleri muelleri* removed from a gallery in the dorsal surface; W, *Phascolosoma stephensoni* removed from a gallery located on the edge of the columella; X, unidentified crustacean (*Acrothoracica* sp.) removed from intra-shell cavity with 3 mm.

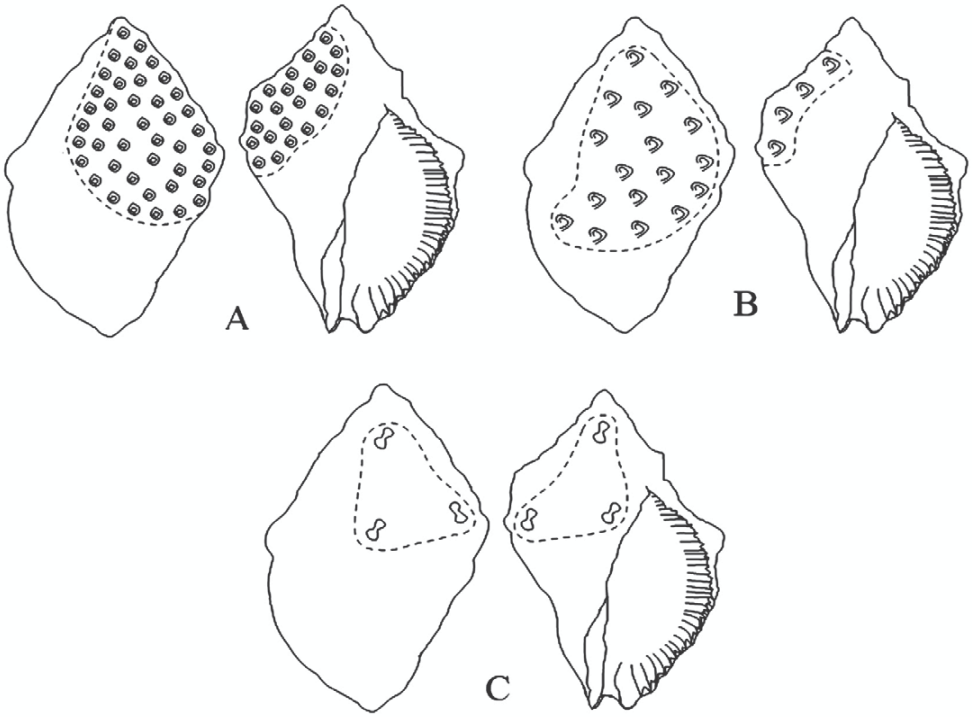

Fig. 3. – Preferential shell colonization zone/location of epibiotic species observed in the shells of heavily fouled *S. haemastoma*; (A) barnacles; (B) polychaete tubes; (C) perforations caused by *Lithophaga aristata*.

Table 3. – Relative growth of non-fouled and fouled *S. haemastoma* (sexes confounded and separate sexes). SL, shell length; SD, shell diameter; SdW, Shell dry weight; FdW, Flesh dry weight; N, number of individuals; M, males; F, females; r, correlation coefficient; t, Student t-test.

Biometric variables	Gastropod non-fouled by barnacles (both sexes) (N=439)		Gastropod fouled by barnacles (both sexes) (N=596)	
SD/SL	$SD=0.58SL^{1.02}$ r=0.95 t=2.48		$SD=0.75SL^{0.95}$ r=0.94 t=4.63	
SdW/SL	$SdW=5E\text{-}03SL^{2.63}$ r=0.93 t=22.20		$SdW=5E\text{-}05SL^{2.55}$ r=0.76 t=19.81	
FdW/SL	$FdW=9E\text{-}06SL^{3.07}$ r=0.88 t=24.04		$FdW=6E\text{-}05SL^{2.60}$ r=0.78 t=157.23	
Sexes	M (N=241)	F (N=198)	M (N=331)	F (N=265)
SD/SL	$SD=0.59SL^{1.01}$ r=0.95 t=1.50	$SD=0.56SL^{1.03}$ r=0.95 t=0.19	$SD=0.76SL^{0.95}$ r=0.95 t=3.78	$SD=0.74SL^{0.96}$ r=0.93 t=2.60
SdW/SL	$SdW=6E\text{-}03SL^{2.57}$ r=0.94 t=13.45	$SdW=3E\text{-}03SL^{2.73}$ r=0.93 t=1.55	$SdW=1E\text{-}02SL^{2.60}$ r=0.93 t=31.96	$SdW=5E\text{-}03SL^{2.42}$ r=0.91 t=20.02
FdW/SL	$FdW\text{-}f=1E\text{-}05SL^{2.96}$ r=0.87 t=7.80	$FdW\text{-}f=5E\text{-}06SL^{3.22}$ r=0.88 t=13.09	$FdW\text{-}f=8E\text{-}05SL^{2.52}$ r=0.77 t=117.62	$FdW\text{-}f=3E\text{-}05SL^{2.73}$ r=0.79 t=64.42

bryozoans, sipunculids, and green and brown algae (Table 2).

Epibiotic species associated with S. haemastoma

The associated community removed from the external shell surface of the basibiont is compiled in Table 2, and some photos of the epibiotic species are depicted in Figure 2 (A - E). Among the epibiotic taxa, barnacles were the most represented group (Fig. 2C) and were fixed mainly on the dorsal left face of the apical part of the shell (Fig. 3A). The number of barnacles per host was 23.2±16.5, against 2.3±2.8 for the oysters, which were randomly distributed on the gastropod shell (Fig. 2D). The shell surface also showed the presence of scattered U-shapped tubes that sheltered sedentary polychaetes (Fig. 3B), with Serpulidae and Sabellidae being the most abundant polychaete families (Table 2). Regarding errant polychaetes, the gastropod shell was generally colonized by the families Syllidae, Eunicidae, Nereidae, Phyllodocidae, Chrysopetalidae and Sphaerodaridae (Table 2).

Endobiotic species associated with S. haemastoma

Following shell breakage, a rich endofauna was detected living in breaches, tunnels or galleries dug by annelids, lithophagous bivalves, sipunculids and crustaceans. The last two groups (sipunculids and crustaceans) were less represented than annelids (sedentary and errant polychaetes), which were found in 65.6% of the gastropods, with an average of 3.4±2.7 annelids per host. Polychaetes burrowing the shell of *S. haemastoma* at different positions are shown in Figure 2 (F-H). Tunnels perforated by these polychaetes sheltered errant annelids such as *Aponuphis bilineata* (Fig. 2I-J) and *Perinereis cullrifera* (Fig. 2K). The lithophagous bivalves *Lithophaga aristata* and *Rocellaria dubia* were extracted from breaches having the same size and form as the endobiont. *L. aristata* (4 to 17.5 mm in length) was found in 10.1% of the gastropods examined and occupied galleries situated mainly at the dorsal side of the shell (Fig. 2L), in the direction "apical axis-siphonal canal" (Fig. 2M, N) or in perpendicular direction (Fig. 2O-R). The preferential colonization zone of *L. aristata* in the shell of *S. haemastoma* is schematically illustrated in Figure 3C. In contrast, *R. dubia* (6.6 mm in length) occurred at a very low rate (0.1%) and was removed from breaches dug in the last spire of the host shell (Fig. 2S, T). Five specimens of sipunculids were found in tunnels and galleries belonging to the families Phascolosomatidae (three *Phascolosoma stephensoni* of 10, 13 and 16 mm length and one *Phascolosoma* sp. of 13.5 mm length) (Fig. 2U) and Aspidosiphonidae (*Aspidosiphon muelleri muelleri* of 11 mm long) (Fig. 2V). One specimen of *P. stephensoni* was found in a gallery located on the edge of the columella (Fig. 2W), while all other individuals were removed from tubes dug in the dorsal face nearby the apex of the shell. An unidentified crustacean (*Acrothoracica* sp.) was found in an intra-shell cavity of 3 mm (Fig. 2X).

Relative growth

Stramonita haemastoma fouled by epibiotic barnacles (GFB) displayed negative allometry for the relationships SD/SL, SdW/SL and FdW/SL, indicating that in both sexes SL grows at a faster rate than SD, SdW and FdW. On the other hand, *S. haemastoma* non-fouled by barnacles (GnFB) showed a higher growth of SD and FdW than of SL (Table 3). The ANCOVA test detected significant differences between GFB and GnFB for the relationships SD/SL and FdW/SL (Table 4).

Biometric variables	SD/SL		SdW/SL		FdW/SL	
	F	P	F	P	F	P
Total GnFB vs GFB	1.51	0.21	5.19	0.02	40.65	<0.01
GnFB: Males vs Females	0.36	0.54	4.74	0.03	7.13	<0.01
GFB: Males vs Females	0.24	0.62	2.22	0.13	46.24	<0.01

Table 4. – Results of the ANCOVA performed with data on relative growth of *S. haemastoma*. SL, shell length; SD, shell diameter; SdW, Shell dry weight; FdW, Flesh dry weight: GFB, gastropods fouled by barnacles; GnFB, gastropods non-fouled by barnacles.

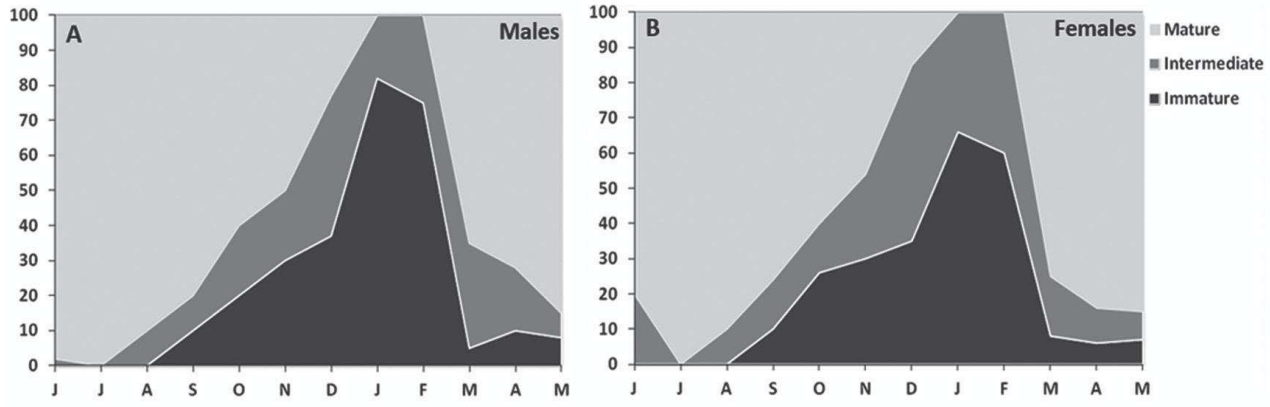

Fig. 4. – Monthly variation of gonad maturation stages in males (A) and females (B) of *S. haemastoma*, following macroscopic observation of the reproductive organs.

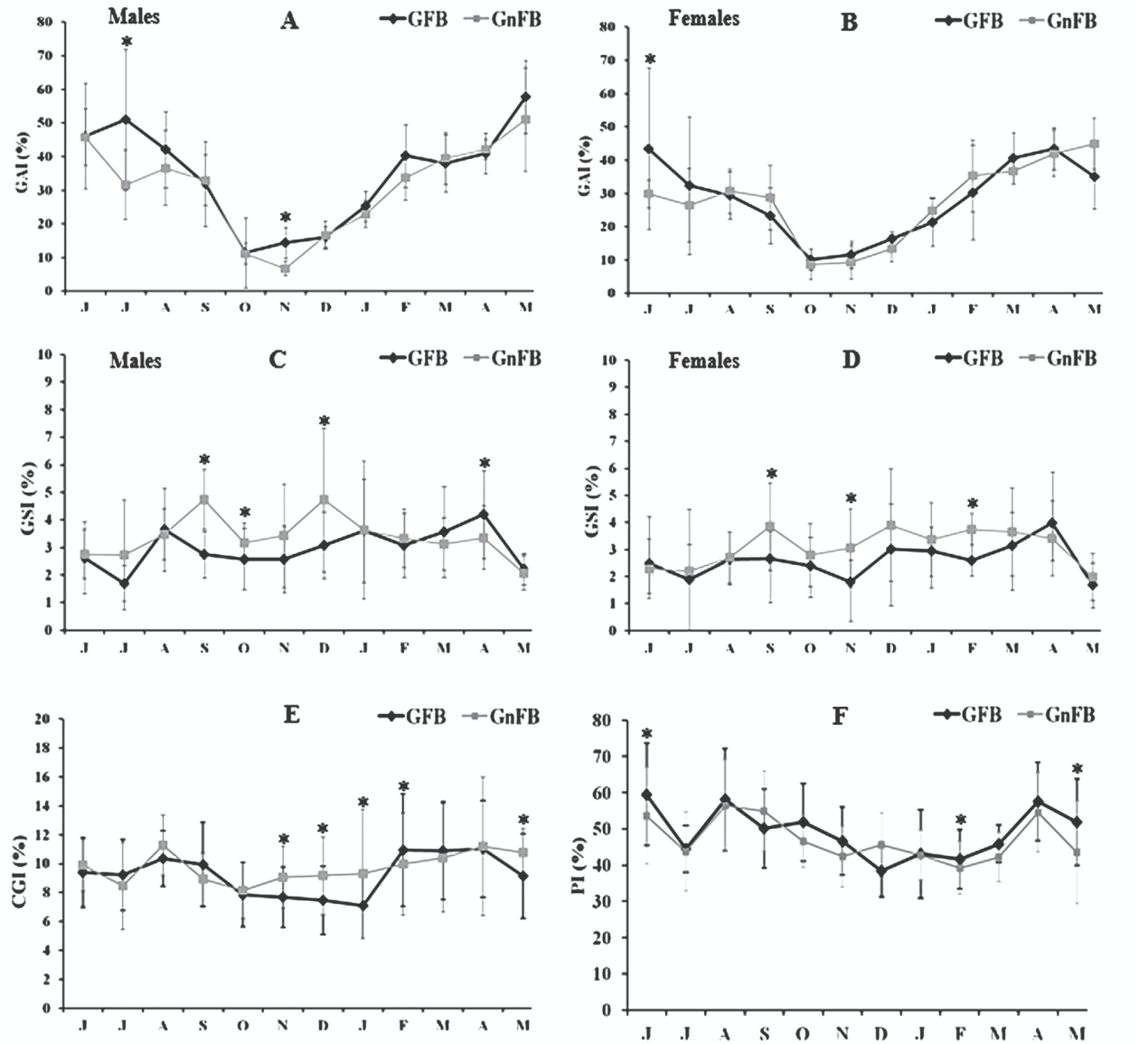

Fig. 5. – Comparison of the monthly variation of the gonad area index (GAI) in males (A) and females (B); gonadosomatic index (GSI) in males (C) and females (D); capsule gland index (CGI) (E) and penial index (PI) (F) in *S. haemastoma* non-fouled (GnFB) and fouled (GFB) by epibiotic barnacles (June 2009 - May 2010). (*) Statistically significant differences GnFB and GFB in each month.

Taking into consideration the sex, both males and females fouled by epibiotic barnacles showed negative allometries in the relationships SD/SL, SdW/SL and FdW/SL, indicating that shell grows faster in length than in diameter, SdW and FdW. This type of allometry was also recorded in non-fouled males for the relationships SdW/SL and FdW/SL. For the relationship SD/SL isometry was recorded in both sexes for the relationship SdW/SL it was recorded in non-fouled females. As for the relationship FdW/SL, positive allometry was detected, indicating higher growth in flesh weight than in shell length (Table. 3). Statistically significant differences between males and females occurred in the relationships FdW/SL for GFB and SdW/SL and FdW/SL for GnFB (Table 4).

Reproductive cycle

Macroscopic observations of the gonads of *S. haemastoma* showed a great similarity in the rhythm of gonad maturation between males and females (Fig. 4A, B), with mature males and females being present throughout the year, except in January and February, when only immature and intermediate individuals were recorded (Fig. 4A, B). Gonadal maturation occurred from May-June to October. The highest percentages of ripe gonads were recorded in July (100%) in both males and females (Fig. 4A, B), with a period of gametogenesis extending from December to April-May (Fig. 4A, B). Once gametes were mature, copulation and capsule deposition occurred about seven days later. All three gonad maturation stages were found almost year-round, except for the absence of stage I (immature) in June, July and August, stage II (intermediate) in July and stage III (mature) in January and February (Fig. 4).

Monthly variations in the reproductive condition indices of *S. haemastoma* during the study period are presented in the Figure 5. All indices showed similar trends in males and females, with slight variations in some months. The GAI showed that gametogenesis occurred from November to April-May. After that, spawning took place between June-July and October. In fouled snails, spawning was significant during this period, while it occurred mainly in September in non-fouled snails. Significant differences between lots were detected during June in males, and during July and November in females, when the highest values of GAI were recorded in GFB (males: $H=0.12$, $P<0.05$; females: $H=0.16$, $P<0.05$) (Fig. 5A, B).

GSI values displayed more fluctuations than GAI values and were more regular in fouled snails. GnFB displayed three decreasing periods in GSI, with two periods (from September to October and from April to July) probably being associated with spawning, and the third one, from December to February, which was due to another unknown activity. Decreases of the index were displayed in GFB from August to September (in males) and October-November (in females) and from April to July in both sexes (Fig. 5C, D). Significant differences were recorded between lots in September, October, December and April for males and in November, February and April for females (males: $H=18.23$, $P<0.05$; females: $H=30.13$, $P<0.05$).

The monthly variation in the CGI showed that hypertrophy of glands was reached in February and continued until August in both female lots. Atrophied capsule glands were observed from October to January, indicating that spawning occurred between August and October. The values of CGI showed that the capsule gland in GnFB was bigger than that in GFB in August, November, December, January and May ($H=7.38$, $P=0.006$) (Fig. 5E).

The PI showed that penis hypertrophy is reached in April, while it becomes atrophied in November-December. Copulation occurred between June and July and between August-September (in GFB) and September-November (in GnFB). The Kruskal-Wallis test ($H=4.98$, $P=0.025$) showed significant differences in May and June in favour of GFB and in December in favour of GnFB (Fig. 5F).

DISCUSSION

The present study has shown that the shell of live *S. haemastoma* collected from Bizerta Channel is a suitable biotope for several invertebrate species. The most abundant epibiotic species were barnacles that totally covered the shell of several snails. Sedentary polychaetes and lithophagous bivalves were the most abundant endobiotic species. For polychaetes, a prevalence of cirratulids especially represented by *Dodecaceria concharum* was recorded. In *S. haemastoma* from the Spanish coast, the shell of live individuals sheltered, in addition to *D. concharum*, three other species, namely *Dipolydora armata, Capitella minima* and *Spirobranchus polytrema* (Bick 2006). Seven families of polychaetes found in the present study (Spionidae, Ciratulidae, Syllidae, Nereidae, Sabellidae, Sabellaridae and Serpulidae) were also reported in *H. trunculus* from Portuguese waters by Vasconcelos et al. (2007). These authors showed that the dorsal surface of the shell, mainly in the proximity of the apex, was generally more fouled by epibiotic polychaetes than the ventral surface, a pattern of colonization which is in agreement with the present results and that is probably related to the mode of locomotion of this snail. Indeed, it has been shown that *S. haemastoma* moves by crawling on hard substrate and by burrowing on soft substrate when the rocks become exposed during low tides (Papp and Duarte 2001). In both cases, the continuous abrasion of the ventral surface leads to an erosion of the settled epibionts, with the same finding being also recorded in *H. trunculus* (Vasconcelos et al. 2007).
Fouling is likely to be a function of time, food availability, seawater salinity and temperature (Wahl 1989, Sahu et al. 2013). In fact, it has been shown that salinity influences all physical-chemical variables of seawater and controls the larval dispersal and recruitment patterns of epi- and endobiotic species (Nair 1965), while seawater temperature influences chemical andbiological interaction and regulates the growth of ben- thic organisms (Sahu et al. 2013). Consequently, the shells of smaller gastropods are usually less

colonized by epifauna than those of larger gastropods, which also agrees with our observations. Abundant colonization could have serious and deleterious consequences on the basibiont. Indeed, Thieltges and Buschbaum (2007) and Buschbaum et al. (2007) showed that the presence of the worm *Polydora ciliata* on the shell of *Littorina littorea* facilitates barnacle fixation, leads to a gradual destruction of the shell, reduces the resistance against predators and reduces the fecundity and the growth of the gastropods. According to Lleonart et al. (2003), an infestation of 30% in the gastropod *Haliotis* spp. from the Australian coast by two spionid species (*Boccardia knoxi* and *Polydora hoplura*) leads to the mortality of half of the affected population.

For endofauna, we recorded the presence of *L. aristata* and *R. rubia*, with infestation rates of 10.1% and 0.1%, respectively. Trigui El Menif et al. (2007) isolated those two boring mytilids from the rock hosting *L. lithophaga* collected in the Bay of Bizerta. *Rocellaria dubia* was also found by the same authors in breaches dug in the shell of live *Venus verrucosa*. Tebble (1976) reported the presence of *R. dubia* in various substrates (sand, limestone, sandstone and dead mollusc shells) but not in live specimens. This bivalve, as well as the other lithophagous species, has a pair of pallial glands that secrete a calcium-binding mucoprotein for boring into calcareous organisms (Jaccarini et al. 1968). The continuous growth of the endobiont involves a growth in the volume of the breach causing a progressive perforation of the shell of the basibiont, which in some cases leads to its death. This finding was recorded by Simone and Gonçalves (2006) and by Trigui El Menif et al. (2006), respectively in *Nodipecten nodosus* infested by *L. aristata* and in *V. verrucosa* infested by *Rocellaria dubia*. With regard to our results, the infestation of *S. haemastoma* by *L. aristata* does not seem to act negatively on the gastropod at this level, because the extracted endobiont had a size of 17 mm in length and 6 mm in thickness, which does not exceed either the length or the thickness of the gastropod columella. Negative effects in the basibiont could probably occur with the progressive growth of the endobiont, since *L. aristata* can attain a length of 52 mm (Turner and Boss 1962). According to Simone and Gonçalves (2006), the geographical distribution of the *L. aristata* is probably limited to the Atlantic coasts. Moreover, Ávila et al. (2009) discovered *L. aristata* as a fossil form on the island of Santa Maria (Azores). The presence of this species in Tunisian coasts, in the same habitat as *L. lithophaga* (Trigui El Menif et al. 2006) and in the shell of *S. haemastoma*, lets us suppose that it is an invasive species coming from the Strait of Gibraltar.

A male-biased sex ratio was found in *S. haemasto- ma* from Bizerta Channel, which agrees with previous studies on this species that concluded that this could be due to either female mortality or imposex in some females (Rilov 1999). Usually, differences in growth between populations collected from different sites are associated with many factors, including environmental conditions, TBT pollution, prey type and parasitism (Crothers 1985). Another factor that seems to affect the growth of gastropods is biofouling, since non-fouled and fouled gastropods were collected at the same sampling site. Indeed, the annual growths in terms of SD and FdW were higher in non-fouled gastropods. In fact, biofouling is time-dependent (Wahl 1989), which means that the growth of *S. haemastoma* is also accompanied by an increase in number and size of epibiotic barnacles. In some cases the weight of the barnacles per gastropod host is equal to its own weight; this most probably has negative effects on locomotion of snails, which must move between different substrates in order to feed, avoid unfavourable environmental conditions and escape predation. Consequently, gastropods fouled by epibiotic barnacles spend more energy during their movements at the expense of their growth than non-fouled gastropods. Indeed, it has been demonstrated that epibiotic barnacles in *L. littorea* decrease the locomotion and consequently the growth of gastropods (Buschbaum and Reise 1999, Thieltges and Buschbaum 2007). Thieltges and Buschbaum (2007) also showed that epibiotic *Crepidula fornicata* reduces the growth and survival of blue mussels (*Mytilus edulis*). Recently, Lacoste et al. (2014) recorded that biofouling reduces the growth of pearl oysters (*Pinctada margaritifera*) in French Polynesia. Our results showed that non-fouled females of *S. haeamstoma* have heavier shells and flesh. In contrast, Lahbib (2004) found that males of *Hexaplex trunculus* have heavier shells and flesh than females, considering this as sexual dimorphism characterizing specimens collected from Menzel Jemil (northern Tunisia).

The study of the reproductive cycle of *S. haemastoma* from the Tunisian coast showed an acceptable agreement between the macroscopic classification of gonad maturation stages and three of the bio-physiological indices used (GAI, CGI and PI). In contrast, the GSI showed significant fluctuations that could not be directly related to the reproductive cycle of *S. haemastoma*. This index is probably influenced by other factors, such as variations in the weight of the digestive gland after food ingestion and also SdW, because it is sometimes difficult to entirely remove all epibionts from the basibiont shell. Therefore, based on this result it would be more suitable to follow the reproductive activity of *S. haemastoma* using macroscopic observations together with GAI, CGI and PI. The simultaneous increase in GAI and decrease in CGI indicate gonad maturation and the beginning of spawning in May, which were probably triggered by an abrupt increase in seawater temperature, from 17°C in April to 23.9°C in May, with a similar finding being recorded in *B. brandaris* from Tunisian waters (Abidli el al. 2012). Vasconcelos et al. (2008) and Elhasni et al. (2010) recommended the use of two indices (GAI and CGI) and considered them simple, practical and efficient for the routine assessment of reproductive activity in a sympatric muricid (*H. trunculus*). Abidli et al. (2012), Vasconcelos et al. (2012) and Elhasni et al. (2013) confirmed these findings in analogous studies with another sympatric muricid (*B. brandaris*). Usually, differences in the reproductive cycle are explained by variation in seawater temperature (Lahbib et al. 2009, Abidli et al. 2012). Another factore that seems to affect the reproductive activity of gastropods is imposex (Lahbib et al. 2009). Results gathered in this study further suggest that the reproductive cycle of *S. haemastoma* is also affected by biofouling. Furthermore, Vasconcelos et al. (2011) established the PI for the study of the reproductive activity of male *H. trunculus*. This index was also practical for the study of the reproductive activity of male *S. haemastoma,* but must be used cautiously at sites highly affected

by TBT pollution, such as Bizerta Channel (Lahbib et al. 2011), because it has been shown that TBT contamination might increase male PL (Castro et al. 2007, Abidli et al. 2013).

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