Ecologie évolutive des limites de niche : cas de l'adaptation à la salinité de l'artémie

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THÈSE

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Soutenue le 19 juin 2015 devant le jury composé de

Mme Laurence DESPRES, Professeur, Université Joseph Fourier	Rapporteur
M. Adrien FRANTZ, Maitre de Conférences, Université Paris VI	Examinateur
M. Doyle MCKEY, Professeur, Université Montpellier	Examinateur
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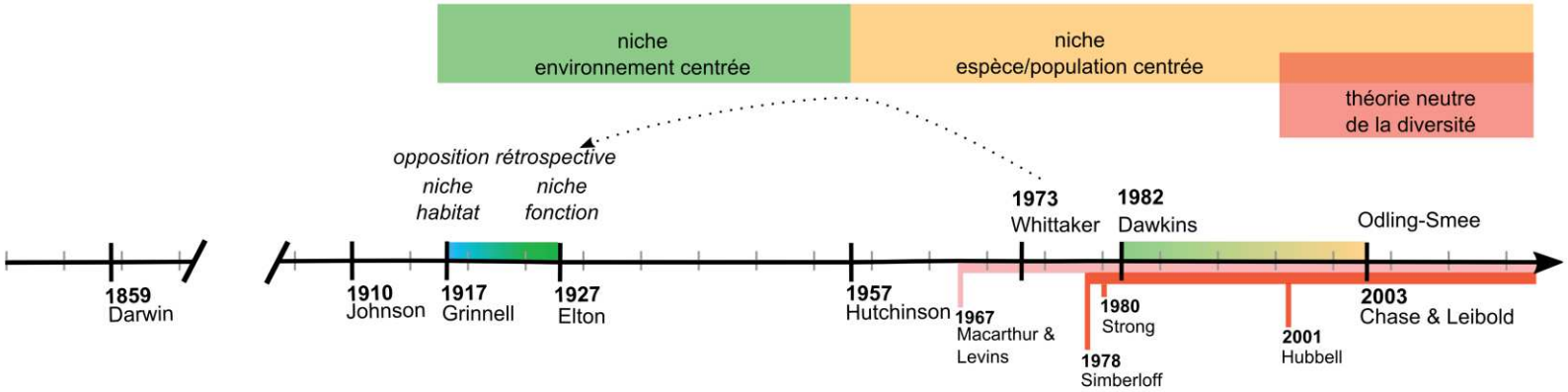
Panel 1. Historique du concept de niche, construit en s'appuyant sur la revue du sujet proposée par Pocheville (2015).



Darwin (1859). Sans utiliser le terme de niche, l'idée que l'évolution et la coexistence des espèces se fasse dans un complex de facteurs environementaux existait.

Johnson (1910). Premier à employer le terme de niche pour définir l'espace dans lequel les espèces coexistent.

Niche en tant qu'habitat (ensemble decaractéristiques environnementales) dans laquelle les espèces vivent :

Grinnell (1917). Niche faisant partie d'une hiérarchie propre à l'écologie parralèlle à la hiérarchie existant en systématique.

Elton (1927). Niche comme position de l'espèce dans la chaine trophique.

En 1973, Whittaker propose une relecture des définitions de la niche données par Grinnell ("niche habitat") et Elton ("niche fonction") en les opposant.

Formalisation du concept de niche comme une propriété des espèces/populations et non de l'environnement :

Hutchinson (1957). Niche comme espace multidimensionnel de variables environnementales (biotique ou abiotique) dont certaines représentent des limites de viabilités pour l'espèce/la population. Distinction entre :

Niche fondamentale: limites où l'environnement permettrait à l'espèce de survivre indéfiniment (niche non locale)

Niche réalisée: espace réellement occupé par l'espèce quand non exclu par des compétiteurs (zone géographique définie)

Macarthur & Levins (1967) proposent de remplacer les variables environnementales, non mesurables facilement, par des distributions d'utilisations des ressources. Ces ressources peuvent correspondre à toute variable relative à l'utilisation de l'environnement (biotique ou abiotique) par les espèces/populations.

Depuis le début des années 1980 (Simberloff, 1978; Strong, 1980), le concept de niche a largement été remis en question. Nombre d'études sur des patrons de compétitions n'incluaient pas d'hypothèse nulle adéquate. Hubbell (2001) propose la théorie neutre de la diversité dont l'hypothèse nulle d'assemblage des communautés est supposé aléatoire. Il conserve le concept de niche fondamentale en supposant qu'elle est équivalente pour toutes les espèces d'une communauté

Chase & Leibold (2003). Niche comme la description des conditions environnementales permettant à une espèce de survivre, associée à l'impact des individus sur ces conditions environnementales.

Une autre opposition existe au sein de la théorie de la niche. D'un côté, les externalistes (Dawkins, 1982) proposent une vision de la niche comme d'un phénotype étendu où les pressions de sélection extérieures ou internes à l'organisme ne sont pas modifiables. De l'autre, la théorie de la construction de niche (Odling-Smee, 2003) propose une vision dynamique de la niche où l'impact des populations modifie la pression de sélection que celle-ci exerce.

Introduction

Le concept de niche permet à la fois de décrire l'impact de l'environnement sur la répartition des espèces, mais aussi d'avoir un cadre prédictif pour comprendre l'influence de l'environnement sur l'évolution. La niche est donc un concept important à la fois en écologie et en évolution. Si l'on prend l'exemple des pinsons de Darwin, leur niche écologique est l'espace occupé par les différentes espèces et les relations qu'elles ont entre elles. D'un point de vue évolutif, certains aspects de leur niche est le résultat d'une adaptation de la taille du bec à la taille des graines.

Au cours de ma thèse, je me suis intéressée à ce concept intégrateur dans le cas de l'adaptation à la salinité de l'artémie. Je commencerai mon introduction par quelques définitions et concepts essentiels (particulièrement ceux de niche écologique et d'adaptation), suivi d'un questionnement sur les limites conceptuelles que présentent ces définitions. Mon travail de recherche m'a amenée à réfléchir à de nouveaux cadres théoriques et expérimentaux pour mieux comprendre les limites conceptuelles de la théorie de la niche et tenter d'y répondre. Je poursuivrai mon introduction en explicitant les raisons qui font du cas de l'adaptation à la salinité de l'artémie un choix intéressant, avant de terminer par un inventaire des questions opérationnelles des recherches que j'ai réalisées.

1.1 Concepts d'adaptation et de niche

1.1.1 La niche écologique

Le concept de niche a subi de nombreuses modifications au cours de son existence. J'ai réalisé une chronologie détaillant les étapes principales de ces modifications dans le Panel 1. Johnson (1910) propose pour la première fois le terme de niche mais l'idée existait déjà chez Darwin (1859). La niche est d'abord définie comme l'ensemble des conditions environnementales (biotiques et abiotiques) occupées par une espèce Cette conception de la niche est environnement-centrée (Grinnell 1917; Elton 1927). Dans la définition proposée par Hutchinson (1957, 1959), le concept de niche devient population-centrée : celle-ci est alors définie comme l'ensemble des conditions environnementales (biotiques et abiotiques) dans lesquelles une

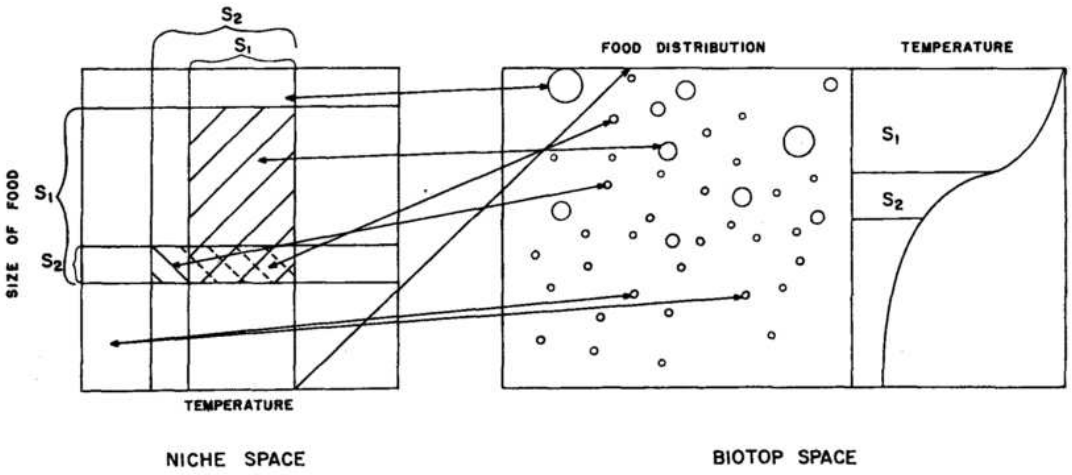


FIGURE 1. Two fundamental niches defined by a pair of variables in a two-dimensional niche space. Only one species is supposed to be able to persist in the intersection subset region. The lines joining equivalent points in the niche space and biotop space indicate the relationship of the two spaces. The distribution of the two species involved is shown on the right hand panel with a temperature depth curve of the kind usual in a lake in summer.

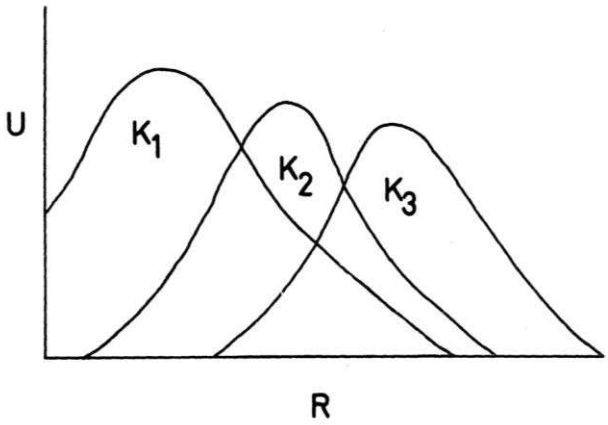


FIG. 1. The form of the niche. For each resource r, U is the probability of its utilization in a unit time by an individual. The area under each curve, therefore, is the total resource utilization K_i for species i.

Figure 1.1 : Représentation de la niche dans (A) Hutchinson, 1957 et (B) MacArthur & Levins, 1967. **A** : "Deux niches fondamentales définies par une paire de variables est un espace de niche à deux dimensions. Seule une espèce doit être capable de persister dans la région d'interaction. Les lignes reliant des points équivalent dans l'espace de la niche et l'espace du biotope met en évidence la relation entre les deux espaces. La distribution des deux espèces impliquées est montrée sur le panneau de droite avec un gradient de température habituel pour un lac en été." **B** : "Pour chaque ressource R, U est sa probabilité d'utilisation par unité de temps et par individu. L'aire sous chaque courbe correspond donc à la quantité totale de ressources K_i utilisée par l'espèce i."

population peut se maintenir. On passe d'une conception de niche habitat où cohabitent des espèces, à des espèces possédant une niche écologique. Cette définition englobe pourtant la précédente puisque Hutchinson propose pour pendant à cette niche fondamentale, la niche réalisée qui correspond à l'ensemble des habitats où l'on trouve effectivement les espèces/populations. Néanmoins, la niche réalisée pose un réel problème en écologie des communautés car ce modèle ne présente pas d'hypothèse nulle. En effet, l'explication des assemblages de communautés passait exclusivement par des rapports d'exclusion ou de partition de niche (Pocheville 2015). Il faut attendre Hubbell (2001) et sa théorie neutre de l'assemblage des communautés pour qu'un modèle propose un assemblage aléatoire comme hypothèse nulle. Le modèle neutre impose d'abandonner la niche réalisée. Toutefois, le concept de **niche fondamentale** comme l'**espace multidimensionnel de variables environnementales où l'espèce survie indéfiniment en l'absence d'interaction,** est maintenu.

1.1.2 Mesurer la niche

Jusqu'en 1967, la niche est représentée comme la gamme d'états de l'environnement permettant l'existence d'une espèce. On suppose que, dans la niche fondamentale, tous les points de la niche correspondent à la même probabilité de persistance de l'espèce, et, les points en dehors de cette niche ont une probabilité nulle de survie de l'espèce (Hutchinson 1957). Cette vision est extrêmement limitante car elle ne peut se référer qu'à des contraintes très fortes (par exemple physiologiques) sur les organismes. Par exemple, un animal aquatique ne peut pas survivre sur la terre ferme, la représentation de sa niche correspond donc sur cet axe environnemental à celle que propose Hutchinson. En revanche, comment représenter un gradient de salinité existant par exemple aux embouchures des fleuves ?

MacArthur et Levins (Macarthur & Levins 1967) proposent de modifier cette représentation par une donnée mesurable : l'utilisation des ressources le long d'une gamme environnementale (Figure 1.1). Les ressources dont parlent les auteurs ne sont pas uniquement métaboliques mais plus généralement n'importe quelle variable du milieu abiotique et biotique. Dans le cas de l'animal aquatique vivant à

l'embouchure d'un fleuve, la salinité pourra être considérée comme la ressource utilisée et on aura un gradient entre le fleuve et l'océan avec un optimum d'utilisation de cette ressource pour une salinité intermédiaire. Ici, le terme de 'ressource' est abstrait et très différent de celui plus classique de substance matérielle (nourriture, énergie, etc.) à la croissance d'un organisme.

Le concept d'utilisation des ressources proposé par MacArthur et Levins est très proche de celui de l'adaptation (Dobzhansky *et al.* 1968; Endler 1986). Lorsque l'on parle d'**adaptation** en écologie et évolution, plusieurs concepts que recouvre ce terme ne sont pas toujours bien distingués. Les définitions ont été largement discutées par le passé (Dobzhansky 1956, 1970; Dobzhansky *et al.* 1968; Endler 1986), j'utiliserai dans la suite de ce mémoire les suivantes: (i) la **capacité d'adaptation** ("*adaptability*"): le degré auquel un organisme, une population ou une espèce peut rester ou devenir adapté à une gamme d'environnements plus larges *via* des modifications génétiques ; (ii) le **degré d'ajustement adaptatif** ("*adaptedness*"): proximité entre le phénotype réalisé et le phénotype optimal; (iii) la **fitness** : le degré auquel un organisme est capable de vivre et se reproduire dans un environnement donné ou dans un ensemble d'environnements.

En écologie, le concept d'adaptation est complémentaire à celui de niche. Ce sont les limites dans la capacité d'adaptation qui contraignent l'étendue de la niche et son évolution. La niche peut être représentée par le degré d'adaptation des organismes à une gamme d'environnements. Actuellement, les **représentations graphiques du degré d'adaptation** (moyenne des contributions absolue d'un phénotype ou d'une classe de phénotypes à la génération suivante) **en fonction d'un gradient environnemental** sont nommées **courbes de tolérance** (Endler 1986; Baker 2009). On pourra alors directement représenter la fitness (contribution moyenne relative d'un phénotype ou d'une classe de phénotype à la génération suivante) en fonction du gradient environnemental (Endler 1986; Baker 2009).

1.1.3 Evolution de la niche

La niche écologique définit le contexte environnemental dans lequel les organismes s'adaptent et évoluent. Ses limites pour un organisme donné résultent des limites à

la sélection naturelle dans cette espèce (Barton & Partridge 2000; Holt 2009). Wright propose l'idée que les populations occupent les sommets d'un paysage adaptatif (Wright 1932). L'évolution de la niche passe par la capacité d'adaptation d'un organisme, une population ou une espèce. Ainsi, une population se déplace dans un paysage adaptatif en perpétuel mouvement (Gavrilets 2004).

L'adaptation au niveau phénotypique résulte principalement du mécanisme darwinien classique, la sélection naturelle favorisant l'augmentation des fréquences alléliques de mutations favorables introduites par mutations ou migration. Cependant la plasticité phénotypique, capacité pour un génotype de produire différents phénotypes selon l'environnement dans lequel il se développe, peut aussi contribuer à produire des phénotypes adaptatifs si elle a elle-même évolué en réponse à la sélection naturelle (Via & Lande 1985; Scheiner 1993).

1.2 Limites d'adaptation et contraintes de niche

1.2.1 Environnement et capacité d'adaptation

1.2.1.1 Echelle de niche : génotype ou espèce ?

Certaines approches envisagent que les gradients environnementaux de la niche varie peu sur des échelles de temps importantes. Sur de nombreux aspects, cette vision est valable. Par exemple, des dizaines de milliers d'espèces de mollusques, crustacés et poissons vivent uniquement dans la mer et les océans. Les limites de tolérance, sur le gradient de salinité, sont donc conservées de génération en génération autour de 40 grammes de sel par litre d'eau (g/L). Dans ce cas, il est question de conservatisme de niche (Peterson *et al.* 1999; Wiens & Graham 2005; Peterson 2011).

Pourtant, cette vision dépend beaucoup de l'échelle de temps et d'espace considéré (Wiens & Graham 2005; Warren *et al.* 2008). En effet, l'environnement varie dans le temps (glaciations, saisons, intempéries, etc). Géographiquement, les conditions environnementales varient selon les latitudes mais aussi les altitudes (Pianka 1966; Haverkort 1990; Thomas *et al.* 1998). La nature des sols (Ettema & Wardle 2002; Berg & Smalla 2009) structure aussi grandement l'environnement dans lequel les

organismes se développent. Enfin, ceux-ci peuvent être à l'origine de modification de leur environnement. Par exemple, les vers de terre qui brassent et structurent les sols localement (Lavelle *et al.* 2006) ou encore l'impact globale de l'anthropisation sur le milieu naturel (Halpern *et al.* 2008).

Lorsque les conditions environnementales de leur habitat varient sur de longues périodes, les organismes peuvent modifier leur répartition géographique de génération en génération (dispersion) pour suivre leur gamme d'environnement favorables (Walther *et al.* 2002; Parmesan & Yohe 2003). Si ces organismes suivent parfaitement les déplacements de leur gamme environnementale, leur niche telle que définie précédemment (voir 1.1.1) n'est pas modifiée. Les variations de l'environnement peuvent aussi arrivé à l'échelle d'une génération. Dans ce cas, les déplacements des organismes sont appelés migration. Les organismes migrent en traversant parfois des habitats pour lesquels ils sont mal adaptés. Un exemple extrême est celui de la sterne arctique (*Sterna paradisaea*). Cet oiseau est adapté aux étés polaires et tous les 6 mois migre d'un pôle à l'autre (Egevang *et al.* 2010). La encore, pour ces organismes migrateurs, la niche ne varie pas. En effet, ces organismes migrent entre habitats parfois séparés par des distances immenses mais qui présentent des environnements similaires. La niche fondamentale de ces organismes n'est donc pas modifiée.

Pour les organismes qui ne peuvent pas migrer ou disperser suffisamment pour suivre les modifications de leur environnement, l'autre réponse possible est l'adaptation aux variations locales de l'environnement. Que la réponse soit génétique ou phénotypique, elle implique une variation de la niche. Là encore, l'échelle choisie modifie grandement les prédictions et observations qui peuvent être faites. Par exemple, le bouleau verruqueux (*Betula pendula*) a une répartition géographique qui s'étend des climats tempérés du centre de la France à la taiga Sibérienne (Atkinson 1992). Ces arbres ne peuvent pas migrer et les populations de Sibérie sont donc adaptées à un environnement radicalement différent de celui présent en France. Le même type d'exemples existe pour illustrer les structurations le long de clines altitudinaux (e.g. Bresson *et al.* 2011; Pitchers *et al.* 2013). Pourtant, il y a des flux géniques entre ces différentes populations. D'autre part, la variation génétique d'une population à l'autre peut énormément varier chez le bouleau dont la reproduction végétative est particulièrement efficace (certaines forêts sont entièrement

constituées de clones d'un même individu ; Atkinson 1992). Considère-t-on le genre *Betula* comme généraliste avec une très grande aire de répartition ? Ou bien, est-il préférable de considérer comme un ensemble de populations spécialisées à différentes conditions environnementales ?

Problème 1 : A quelle échelle (genre, espèce, population, génotype) doit-on définir la niche ?

Ces questions montrent l'existence d'une première limitation du concept de niche qui impose d'adapter le choix de l'échelle utilisée au besoin spécifique de la question de recherche. Cette question se pose d'autant plus dans les populations asexuées où l'absence de recombinaison peut conduire à une vision des populations clonales comme une communauté de 'micro-espèces' (Vrijenhoek & Davis Parker Jr. 2009).

1.2.1.2 Echelle de niche : juvénile et adulte?

Au sein d'une population, l'âge des individus ou leur stade de développement peut influencer quelles variables environnementales contraignent l'étendue de leur niche, et comment : il y a une variation ontogénique de la niche. Par exemple, les juvéniles de la truite arc-en-ciel sont adaptés à l'eau douce, les jeunes adultes vivent dans l'océan Pacifique et les adultes matures remontent de nouveau les rivières vers l'eau douce pour se reproduire (Johnsson & Clarke 1988). De même, chez de nombreux insectes, la métamorphose implique que les juvéniles ont une niche qui diffère de celle des adultes par bien des aspects. Chez les Lépidoptères, les Odonates ou les Culicidés (voir Resh & Cardé 2009), les juvéniles et les adultes présentent des différences de niche en terme de ressources métaboliques (herbivores vs. butineurs pour les Lépidoptères, carnivores vs. herbivores pour les Odonates) et/ou en terme d'environnement abiotique (aquatiques vs aériens pour les Odonates et les Culicidés). Par ailleurs, même pour des organismes qui pourraient à priori occuper la même niche à différents âges, les conditions environnementales rencontrées plus tôt dans la vie peuvent influencer celles tolérées plus tard, du fait de la plasticité phénotypique. Cette idée est sous-jacente à la notion d'acclimatation pour les environnements extrêmes (Angilleta Jr. 2009), mais elle s'étend plus généralement à tout organisme dont les réponses plastiques à un environnement donné peuvent affecter la fitness ultérieure dans différents environnements.

Problème 2 : Comment la niche à un stade de développement donné dépend-elle des environnements rencontrés aux stades précédents ?

Dans ce cas, la limitation au concept de niche ne demande pas d'adapter son choix de l'échelle de niche à la question envisagé. Ici, il s'agit d'appliquer le concept de niche en tenant compte l'ensemble de l'histoire de vie.

1.2.1.3 Niche multidimensionnelle et différences de contraintes.

Le concept de niche est utilisé pour expliquer et prédire les limites de répartition des espèces (Hutchinson 1957; Matesanz & Valladares 2014; Valladares *et al.* 2014). Les gradients environnementaux responsables de ces limites de répartitions sont souvent proposés (e.g. les arbres adaptés aux régions froides ne tolèrent pas l'humidité des régions plus chaudes ; Loehle 1998). En revanche, les contraintes sous-jacentes qui s'exercent aux limites de niche sont rarement explicitées. La connaissance de ces contraintes est pourtant essentielle à la compréhension des contraintes évolutives qui pèse sur la niche. C'est le cas par exemple dans l'étude de Morin *et al* (2007) qui montrent que les limites de répartition des arbres sont contraintes par la date de floraison et la maturation des fruits. Selon les espèces, des combinaisons de floraisons précoces et/ou maturation tardives vont contraindre les limites nord tandis que des floraisons tardives et/ou des maturations précoces vont contraindre les limites sud. Dans l'exemple cité, les mécanismes sont bien connus et explicité car la phénologie des arbres est particulièrement bien connue. Dans de nombreux cas pourtant, il est difficile de déterminer expérimentalement les mécanismes sous-jacents.

Si les limites d'aires de répartitions peuvent être liées à différents axes de la niche fondamentale, un autre aspect du problème est de connaitre les contraintes qui limitent l'extension de la niche fondamentale aux extrémités d'un gradient environnemental. Par exemple, les arbres caducifoliés ne mettent pas en place les mêmes mécanismes de résistance aux températures basses et élevées. Dans le premier cas, on observe des mécanismes de résistance à la formation de cristaux de gel à l'intérieur des tissus. La structure des bourgeons en couches de tissus morts pour protéger le méristème des conditions extérieures, le retrait des sèves lié à la perte du feuillage ou la production de tissus secondaires isolants sont autant d'exemples de la diversité de ces mécanismes de résistance (Parker 1963; Howe *et*

al. 2003). Dans le second cas, la résistance passe par la limitation de l'évapotranspiration. Pour ce faire, d'autres mécanismes entrent en jeu comme l'épaississement de la cuticule ou la réduction de la surface foliaire (e.g. le chêne dans Hamerlynck & Knapp 1994).

Dans les exemples précédents, on ne s'intéresse qu'à un ou deux axes de la niche. Or, la définition de Hutchinson d'un espace à n dimensions est le reflet conceptuel de la réalité où les niches des espèces sont clairement pluridimensionnelles. Si l'on compte que chaque extrémité de ces axes présente des modalités d'adaptation différentes, on multiplie par 2 cette dimensionnalité de la niche. Enfin, il faut aussi ajouter que ces modalités diffèrent d'un axe à l'autre.

Problème 3 : Comment peut-on utiliser le concept de niche multidimensionnelle lorsque les contraintes diffèrent d'un axe à l'autre ainsi qu'aux extrémités de chacun de ces axes ?

C'est un problème qui se pose concrètement, lorsque l'on étudie des populations naturelles ou pour les expériences au laboratoire. Il pose la question du nombre de variables environnementales nécessaires à la capture d'une bonne image de la niche, mais aussi de l'importance relative de ces variables dans l'adaptation à l'environnement ainsi que leur corrélation.

1.2.1.4 Comment les interactions influencent-elles les limites de niche ?

Le concept de niche fondamentale tient compte du rôle fonctionnel de l'espèce dans la communauté, ainsi que des qualités biotiques et abiotiques de l'environnement dans lequel se maintient une espèce. La niche réalisée est la projection de la niche fondamentale de l'espèce en tenant compte des compétitions (Hutchinson 1957). En plus des limites évoquées plus haut (voir 1.1.1), cette définition pose la question des autres interactions écologiques entre organismes. En effet, restreindre les interactions négatives à la seule compétition est un premier problème car de nombreux exemples illustrent l'importance de la prédation et du parasitisme (Bruno *et al.* 2003). De plus, ne considérer que l'impact des interactions négatives, en négligeant l'importance des interactions positive, rend cette vision de la niche réalisée particulièrement limitée.

L'importance des interactions biotiques dans le fonctionnement du vivant est établie depuis longtemps. On peut citer en exemple l'importance des bactéries de la peau dans la création d'odeur utilisées par de nombreuses espèces pour reconnaître leurs congénères et qui pourront avoir un impact fort en sélection sexuelle ou en sélection de parentèle (Wedekind *et al.* 1995; Thierry *et al.* 2011). Un autre exemple est l'importance des microorganismes associés aux plantes, aussi bien dans la phyllosphère que dans la rhizosphère. Par exemple, leur importance, pour l'assimilation de composés azotés et parfois carbonés, est telle chez les orchidées, que les champignons symbiotiques des racines produisent des spores qui sont stockées sous la cuticules et dispersent avec les graines (Rasmussen 1995). Malheureusement, leur impact est trop souvent négligé sur la niche de l'hôte : comment conceptualiser la niche réalisée en présence de mutualistes ?

Des tentatives ont été faites pour intégrer les interactions positives comme la facilitation (Bruno *et al.* 2003) au concept de niche fondamentale. Mais, l'effet de ces interactions positives est souvent résumé en une expansion de la niche par rapport à la niche fondamentale prédite (Bruno *et al.* 2003; Travis *et al.* 2006; Warren & Bradford 2013; Afkhami *et al.* 2014). A l'inverse, l'effet des interactions négatives est globalement vu comme une contrainte qui va réduire la niche fondamentale de l'espèce (Hutchinson 1957; Bell 2008; Holt 2009). Or, l'interacteur possède lui aussi une niche fondamentale multidimensionnelle. Résumer son effet sur la niche d'une espèce focale à une expansion ou contraction sur l'axe de l'interaction entre les différents partenaires est donc nécessairement limité. Et si l'on tient compte de l'aspect multidimensionnel de la niche, là encore, une limitation du concept de niche apparaît.

Problème 4 : Les interactions dites positives/négatives peuvent-elles contraindre ou faciliter l'expansion de la niche de l'hôte ?

1.2.2 Environnement, fitness et traits

La notion de phénotype/trait, sous-jacente à toute cette première partie, est aussi un aspect important de l'adaptation. Le phénotype est défini comme l'ensemble des caractéristiques observables d'un organisme (traits). Les traits désignent aussi bien

des caractéristiques morphologiques que métabolique ou comportementale d'un organisme. La sélection naturelle s'exerce sur les phénotypes et non directement sur les génotypes (Endler 1986; West-Eberhard 2003; Ghalambor *et al.* 2007). En effet, lorsqu'un prédateur attaque préférentiellement une morphologie de proie, la survie et le succès reproducteur du phénotype évité augmente. Par exemple, des prédateurs, comme les oiseaux insectivores, vont attaquer préférentiellement des phalènes du bouleau sombres ou claires en fonction de leur visibilité sur les troncs d'arbres d'environnement plus ou moins pollués (Clarke & Sheppard 1963). La sélection résulte donc de l'adéquation entre un phénotype et son environnement. On observe aussi que dans les forêts d'Amérique du sud, un fort mimétisme entre les espèces de papillons toxiques qui est corrélé à la reconnaissance de patrons d'ornements alaires et la prédation sélective par les oiseaux de différentes localités (Mallet & Joron 1999). Dans ce dernier cas, c'est l'environnement biotique qui influence la persistance des phénotypes.

L'environnement ne joue pas seulement le rôle d'agent de sélection sur les phénotypes des organismes. Lors du développement d'un organisme, deux facteurs interagissent : le génotype et l'environnement (Scheiner 1993). Par exemple, chez *Hydrangea macrophylla* le pH du sol a un effet direct sur la couleur des fleurs (Yoshida *et al.* 2003). Or, la couleur de ces organes reproducteurs est importante dans la reconnaissance par les pollinisateurs et donc dans le succès reproducteur des plantes (Pecetti & Tava 2000; Arista *et al.* 2013). L'environnement n'est donc pas seulement un déterminant du paysage adaptatif. C'est aussi un facteur du développement des phénotypes (Schlichting & Pigliucci 1998). La capacité pour un génotype à exprimer différents phénotypes selon l'environnement dans lequel il se développe est appelée plasticité phénotypique (Scheiner 1998). La plasticité est adaptative lorsque la fitness d'un génotype le long du gradient environnemental est plus forte en présence qu'en absence de plasticité. Nombre d'études s'intéressent à la plasticité adaptative, pour des traits morphologiques (morphologie adaptive à la 'terrestrialisation' chez *Polypterus*; Standen *et al.* 2014), physiologiques (modification de la concentration en transporteur du glucose chez la drosophile lorsque élevée sur du citron ; Garland & Kelly 2006), développementaux (moment de la métamorphose dépendant de la vitesse d'assèchement des étangs chez les amphibiens; Newman 1992) ou encore, comportementaux (phénologie de ponte chez la mésange bleue en

réponse au changement climatique; Charmantier *et al.* 2008). D'autres modélisent son évolution (Via & Lande 1985; Price *et al.* 2003; Lande 2009, 2014), pourtant, les coûts et limites de la plasticité phénotypique restent encore mal étudiés (Dewitt *et al.* 1998).

1.2.2.1 Acclimatation et mauvaise qualité d'habitat.

On appelle acclimatation le phénomène par lequel la fitness (et par extension la performance) d'un organisme dans un environnement stressant est augmentée par une exposition préalable à cet environnement, soit de courte durée, soit à un degré moins stressant (Hoffmann 1995). Cet effet résulte de la plasticité phénotypique adaptative de caractères impliqués dans l'adaptation à l'environnement stressant, et le temps passé au préalable dans cet environnement peut être vu comme une accumulation d'information permettant le développement du phénotype adéquat. Dans cette optique, plus un individu reste longtemps dans un environnement donné, plus le phénotype résultant aura une fitness élevée dans cet environnement. Pourtant, tous les environnements ne sont pas équivalents, et certains ont un effet globalement stressant sur les organismes, tel que la fitness est réduite pour tous les génotypes ou phénotypes possibles. Le temps passé dans ces habitats de mauvaise qualité aura l'effet inverse de réduire la fitness. Un aspect souvent négligé dans l'étude de la plasticité phénotypique est le trade-off entre acclimatation et accumulation du coût de la mauvaise qualité d'habitat. Comment distinguer et mesurer l'impact de ces deux effets antagonistes sur la fitness ?

La qualité d'habitat aura non seulement un impact sur l'étendu de la niche, mais aussi sur son évolution. Une nouvelle limitation du concept de niche apparaît alors.

Problème 5 : Comment tenir compte de la qualité d'habitat dans le concept de niche ?

1.2.2.2 Phénotype comme fonction du génotype et de l'environnement.

Classiquement, le phénotype est considéré comme le résultat d'une interaction entre génotype et environnement. Afin d'étudier l'influence de ces deux paramètres sur l'adaptation, il faut s'attacher à évaluer la fonction liant le phénotype au génotype et à l'environnement. Il s'agit d'un côté d'expliciter la forme de cette fonction (linéaire, quadratique, etc.) pour ensuite en estimer les paramètres.

Or, dans la nature comme au laboratoire, il est uniquement possible de mesurer des phénotypes ponctuels résultant du développement d'un génotype dans un environnement donné. Il faut donc envisager des expériences (e.g. jardins communs) donnant accès à un ensemble de phénotypes résultant de l'interaction de plusieurs génotypes dans une gamme environnementale. On pourra ainsi séparer les effets dépendants (plasticité, qualité d'habitat) et indépendant (génétique) de l'environnement.

Problème 6 : Comment faire le lien entre ces mesures ponctuelles et les paramètres de la fonction liant environnement, trait et fitness ?

Ce problème a déjà été soulevé en évolution expérimentale sur levure (Gonzalez & Bell 2013). Les auteurs s'intéressent à l'impact de l'adaptation à différentes salinités de populations de taille variables, issues de deux espèces (*Saccharomyces cerevisiae* et *Saccharomyces paradoxus*) sur leur capacité de sauvetage évolutif lorsqu'elles sont exposées à des salinités plus élevées. Dans le cas de micro-organismes comme la levure, les temps de génération sont tellement court que l'adaptation est toujours décrite à l'échelle d'une population. Pour séparer l'impact de l'environnement sur l'adaptation et la plasticité, il s'agira d'expérience similaire mais à l'échelle d'une unique génération.

1.3 Cas de l'adaptation à la salinité de l'artémie

« *The power of brine shrimp (Artemia sp.) to survive and even thrive in forbidding environments has long been of interest to biologists.* » (Eads 2004)

Au cours de ma thèse, je me suis intéressée aux différentes thématiques abordées précédemment dans le cas de l'adaptation à la salinité des artémies. Les *Artemia* sont des anostracées (Weekers *et al.* 2002). Ce genre permet en effet de contourner plusieurs contraintes classiques dans l'étude de la niche:

- Dans une niche de dimension n, quelles dimensions environnementales contraignent l'étendue de la niche ?
- A l'échelle d'un écosystème, les interactions biotiques sont extrêmement nombreuses. Comment établir l'influence des unes et des autres sur la niche ?

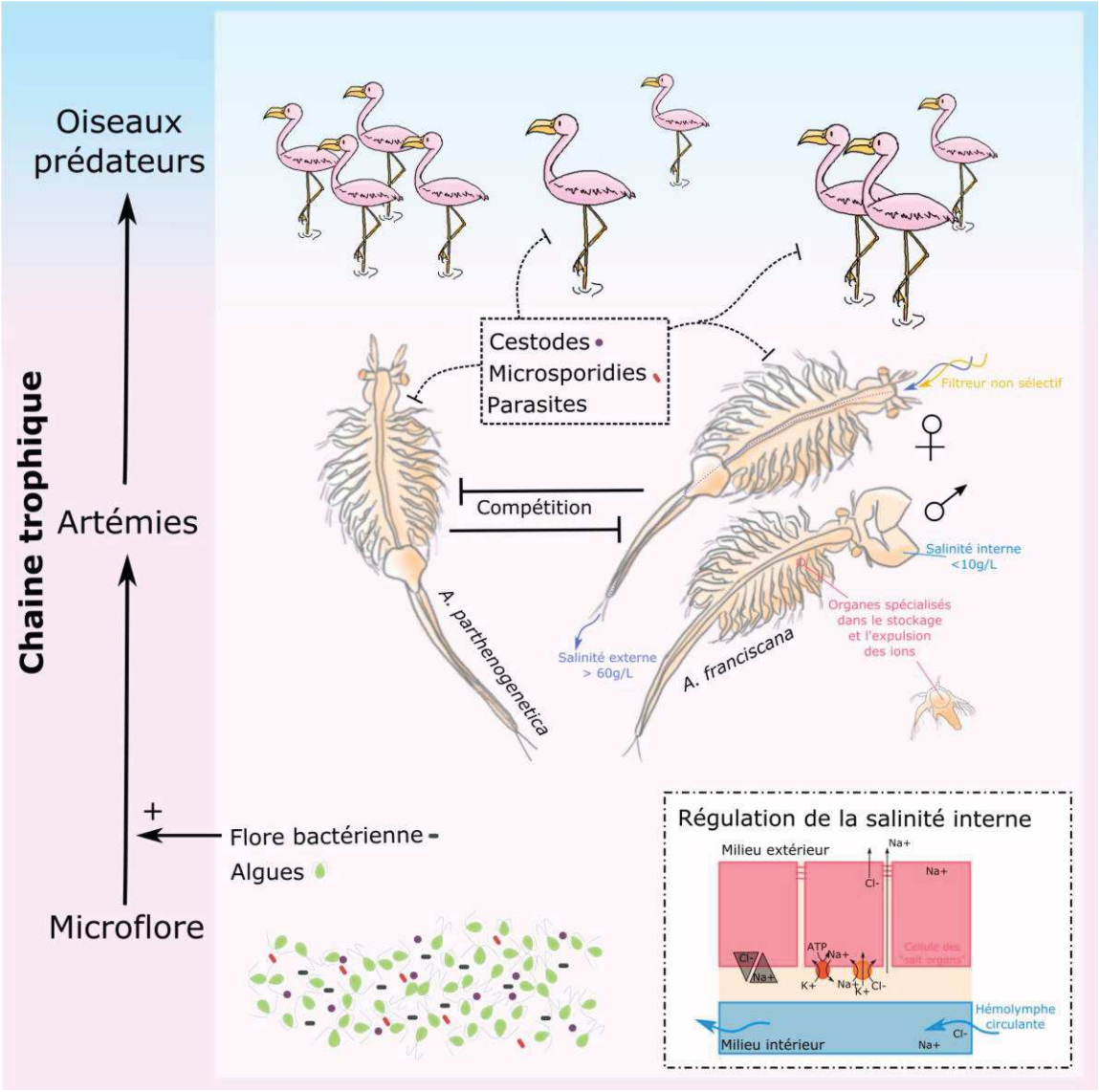


Figure 1.2 : Réseau d'interactions simplifié autour de l'artémie et mécanismes de régulation de la salinité. Dans ce schéma, nous ne présentons que les interactions directement liées à l'artémie. La chaine trophique à laquelle appartient l'artémie dans le salin peut se réduire à trois niveaux : la microflore, les artémies et les oiseaux prédateurs (flamants roses, échassiers, etc.). La microflore est composée essentiellement d'algues, de micro-organismes (bactéries, levures, etc.). On trouve aussi des parasites (microsporidies, cestodes) des artémies et de leurs prédateurs. Dans le salin d'Aigues-Mortes, *A. parthenogenetica* est l'espèce endémique (asexuée), tandis qu'*A. franciscana* (sexuée) est invasive depuis la fin des années 1960 (Rode *et al.* 2013). Encart "*Régulation de la salinité interne*" : fonctionnement des *salt glands* et mécanismes de l'expulsion des ions (adapté de Zadunaisky 1984; Holliday *et al.* 1990).

Les artémies vivent dans des milieux hypersalins (>50g/L de sel, alors que l'eau de mer se trouve à des salinités comprises entre 30 et 50g/L), dans des habitats très divers allant des lagunes de la baie de San Francisco aux lacs salés des hauts plateaux du Tibet (Tanguay *et al.* 2004). Nombre de paramètres environnementaux (oxygénation, température, ressources, etc.) sont corrélés à la salinité (van Stappen 2002). Ceci a pour effet de réduire la dimensionnalité de la niche de l'artémie, et ainsi de la rendre mesurable et surtout d'avoir une idée claire a priori des variables environnementales importantes qui structurent la niche (Problème 3). Ensuite, le nombre d'espèces présentent dans les milieux extrêmes diminue (Pianka 1966; Kristjansson & Hreggvidsson 1995), les écosystèmes sont donc réduit au minimum. Dans le cas de l'artémie, il est possible de construire un écosystème très simplifié (Figure 1.2) où l'on peut décrire l'influence des interactions sur la niche de l'artémie (Problème 4). Cette simplification est cruciale car elle permet plus raisonnablement d'envisager obtenir une vue d'ensemble des interactions tout en étudiant un système naturel (par opposition au modèle théorique ou de laboratoire).

Durant cette thèse, qui s'appuie sur les questions générales introduites plus haut, j'ai utilisé différentes méthodes, du suivi en population naturelle aux expériences en laboratoire. Ceci requiert des qualités particulières de l'espèce choisie. Dans ce chapitre, je m'attacherai à décrire la biologie de l'artémie, sa diversité phylogéographique et son adaptation à la salinité, montrant en quoi (1) elle est un modèle compatible à la fois au suivi de populations naturelles et aux expériences de laboratoire, (2) que sa biologie soulève de nombreuses questions opérationnelles qui permettent d'étudier les questions générales présentées dans cette introduction.

1.3.1 Biologie de l'artémie

Le genre *Artemia* est constitué d'espèces bisexuées et de lignées parthénogénétiques (rassemblées sous le nom d'*Artemia parthenogenetica*). Dans tous les cas, les femelles peuvent se reproduire de façon ovipare ou ovovivipare (Figure 1.3). En effet, elles peuvent produire soit des nauplii (juvéniles), soit des œufs de résistances appelés cystes (Jackson & Clegg 1996; Clegg *et al.* 1999; Liang & MacRae 1999). La proportion de nauplii produit par reproduction ovipare ou ovovivipare varie selon les espèces. Les conditions environnementales jouent aussi

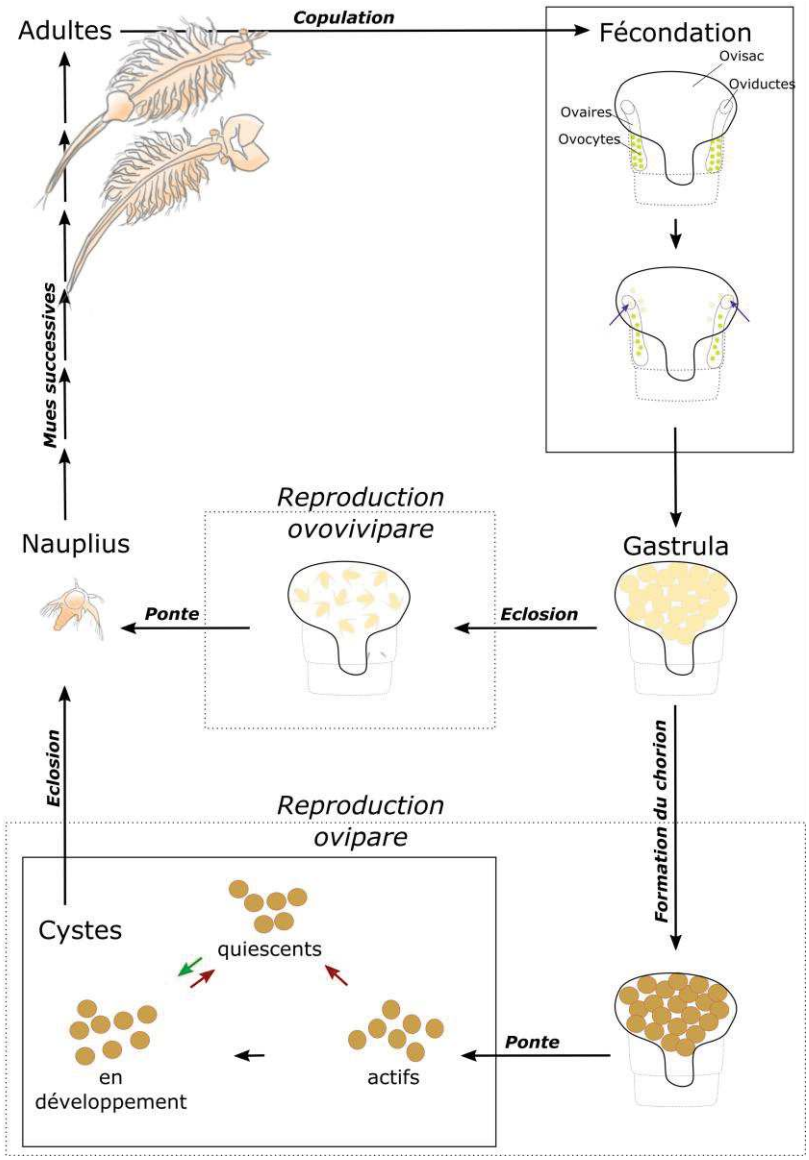


Figure 1.3 : Cycles de vie alternatifs d'artémies bisexuées. Les adultes présentent un dimorphisme, les antennes surdéveloppées chez les mâles qui leur permettent de s'accrocher à l'ovisac des femelles lors de la copulation. Les ovocytes sont fécondés lors de leur entrée dans l'ovisac (flèches bleues). **Reproduction ovovivipare** : le zygote se développe complètement dans l'ovisac maternel, les nauplii éclosent dans l'ovisac avant d'être pondu. **Reproduction ovipare** : le zygote se développe jusqu'au stade gastrula, puis le chorion se forme autour des œufs pour former les cystes. Les cystes sont pondus sous forme active (l'embryon se développe jusqu'à éclosion). Ils entrent en dormance en cas de conditions environnementales défavorables (flèches rouges). Un retour à des conditions favorables lève la dormance (flèche verte). Une fois éclos, une succession de mue est nécessaire pour obtenir des artémies adultes à partir de nauplii.

énormément (Lavens & Sorgeloos 1987; Abatzopoulos *et al.* 2003). Lorsque les cystes sont pondus, si les conditions environnementales sont favorables, ils finissent leur développement et éclosent. Si les conditions environnementales sont mauvaises (e.g. forte salinité, basse température, etc.), les cystes entrent dans un cycle de dormance (diapause) sous une forme déshydratée dont ils sortiront au retour de conditions plus clémentes (Lavens & Sorgeloos 1987).

Le développement post-diapause conduisant à l'éclosion des cystes a été très bien étudié. Le processus est réversiblement stoppé par la dessiccation et l'anoxie (Criel & MacRae 2002) et les mécanismes diffèrent d'une population/espèce à l'autre (Lavens & Sorgeloos 1987; Drinkwater & Clegg 1991). Les cystes sont la forme la plus résistante dans le cycle de vie de l'artémie (Clegg 2005). Ils peuvent donc être conservé au laboratoire durant de longues périodes. On a ainsi une banque de populations qui peut être utilisée dans diverses expériences.

Enfin, la dessiccation et l'anoxie sont des conditions que les cystes peuvent rencontrer dans leur milieu naturel. En effet, des salinités élevées (>100g/L) augmentent la proportion de cystes par ponte et par femelle. Dans une lignée iso-femelle, isolée par Abatzopoulos *et al* (2003) à partir d'une population d'un salin Grec, la proportion de cystes produit par pontes varie de 14,6% à 87,4% lorsque la salinité varie de 50 g/L à 120 g/L. D'autre part, les cystes sont transportés au gré des courants dans les lacs et lagunes (Moscatello & Belmonte 2009; Muñoz *et al.* 2013). Ils finissent par s'accumuler sur les rivages au milieu de débris organiques en décomposition. Eventuellement enfouis, ils vont rencontrer des périodes de sévères hypoxie (faible disponibilité en oxygène) voir d'anoxie complète. On peut imaginer que dans les salins, l'entretien des bassins par labour de la terre va exposer des cystes enfouis depuis plusieurs années. Ils pourront éclore à ce moment-là. Enfin, s'ils sont « stockés » dans les sédiments, les cystes peuvent être vu comme des banques de gènes qui conservent l'histoire génétique de la population (Gajardo & Beardmore 2012). Ce stockage et les collections existantes, permettent d'obtenir des cystes de différentes périodes temporelles (écologie de la résurrection ; Orsini *et al.* 2013). Ce principe a notamment été utilisé sur artémie pour expérimenter au laboratoire l'adaptation à la température (Rode *et al.* 2011).

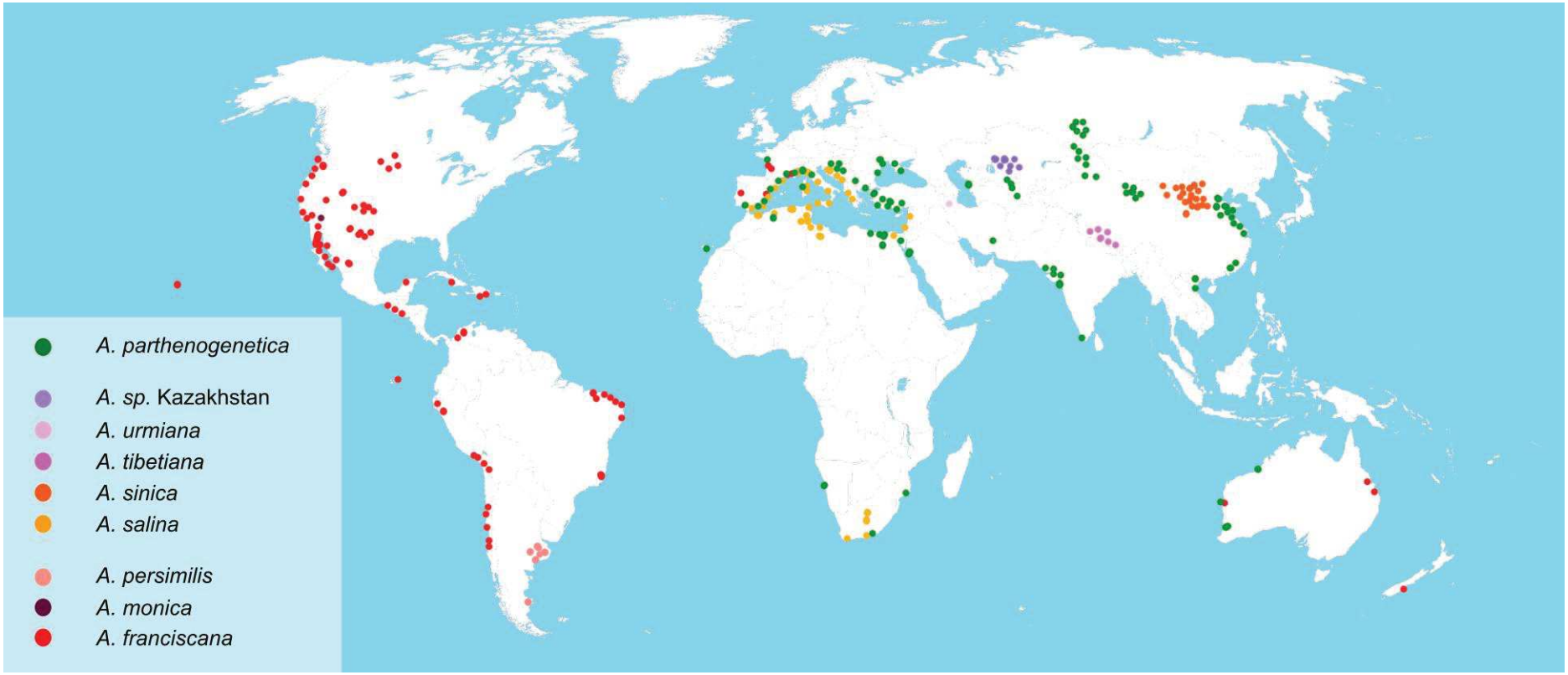


Figure 1.4 : Distribution mondiale des espèces d'artémies. La carte est adaptée des travaux de van Stappen (van Stappen 2002) ainsi que Muñoz *et al.* (2010).

1.3.2 Phylogéographie du genre

« *Artemia is widely distributed on the five continents in many salt lakes, coastal lagoons, and solar saltworks' - this, or a variation on the same theme, is the standard opening phrase in most scientific contributions related to brine shrimp zoogeography.* » (van Stappen 2002)

Au cours du dernier siècle, le nombre de sites où des populations d'artémies ont été répertoriées est passé de 80 à plus de 500 (Vanhaecke *et al.* 1987; Triantaphyllidis *et al.* 1998; van Stappen 2002). En Europe, Asie et Afrique, il y a un mélange de lignées parthénogénétiques et d'espèces sexuées dont elles sont issues (Figure 1.4). Sur ce « vieux » continent, on compte au moins cinq espèces différentes (*A. salina*, *A. tibetiana*, *A. urmiana*, *A.* sp. Kazahkstan, *A. sinica ;* van Stappen 2002). En Australie, les artémies cohabitent avec le genre *Parartemia*, groupe endémique de l'île présentant de fortes différences morphologiques avec les artémies (Geddes 1981). Enfin, sur le continent américain, on observe uniquement des artémies bisexuées dont on dénombre deux espèces (*A. franciscana* et *A. persimillis ;* van Stappen 2002; Munoz *et al.* 2013). A l'échelle locale, les populations sont structurées par la salinité. C'est le cas par exemple dans le lac Urmia, les lignées d'*A. parthenogenetica* peuvent grandir et se reproduire pour des salinités plus basses que *A. urmiana* (Agh *et al.* 2007).

La répartition hétérogène des populations sur le globe est liée aux efforts d'échantillonnage qui suivent les intérêts commerciaux que représentent les artémies. En effet, l'essor de l'aquaculture vient (entre autre) de l'utilisation de nauplii comme nourriture vivante pour les alevins de certains poissons d'importance économique (Sorgeloos 1980a). Les artémies sont commercialisées sous forme de cystes que les éleveurs de poissons font éclore avant de les donner à manger aux alevins.

Depuis les années 1950, les cystes d'artémies commercialisés viennent quasi exclusivement de la baie de San Francisco (SFB, Californie, USA) et du grand lac salé (GSL, Utah, USA) (van Stappen 2002). Ceux-ci sont connus pour la petite taille de leurs œufs et une levée de dormance facile (Vanhaecke & Sorgeloos 1980). Ils ont donc souvent été utilisés pour inoculer des salins exploités (FAO 2015), malgré l'absence de différence nutritive significative par rapport à d'autres espèces

d'artémies (Sorgeloos 1980b). Ces inoculations se sont souvent faites dans des salins (e.g. Aigues-Mortes en France, où nous effectuons nos études, Bohai Bay en Chine) où se trouvaient déjà d'autres espèces d'artémies (respectivement, lignées parthénogénétiques et *A. sinica ;* FAO 2015). Plusieurs études ayant suivi l'arrivée de ces nouvelles espèces ont montré qu'elles étaient souvent plus compétitives et que leur introduction a souvent été au détriment des populations locales (Amat *et al.* 2005, 2007). A l'heure actuelle, A. franciscana est clairement une espèce invasive à l'échelle mondiale et particulièrement autour du bassin méditerranéen.

1.3.3 Adaptation à la salinité

« *The encysted gastrula embryo ('cyst') is arguably the most resistant of all animal life history stages to extremes of environmental stress, while the motile stages are among the best osmoregulators in the animal kingdom.* » (Clegg & Trotman 2002)

L'adaptation à la salinité dans les stades motiles de l'artémie est étudiée depuis de nombreuses années (Croghan 1957, 1958a, 1958b). Contrairement à un grand nombre d'animaux vivant dans des milieux salés et qui adaptent leur salinité à celle de leur environnement, les artémies maintiennent leur hémolymphe à des salinités extrêmement basses par rapport au milieu extérieur (Gajardo & Beardmore 2012). Pour cela, les nauplii comme les adultes ont des organes particuliers nommés glandes à sel (« *salt gland* ») dans lesquels les ions sont accumulés localement afin de créer un gradient qui permettra leur rejet dans le milieu extérieur (Figure 1.2). Chez les nauplii, cet organe prend environ 1/4 du volume de l'individu et se situe dans la nuque de la larve (« *neck organ* ») (Conte *et al.* 1972), tandis que les adultes ont des cellules spécialisées à la base de leurs branchiopodes (Clegg & Conte 1980; Holliday *et al.* 1990).

Les ions sodium (Na^+) et chlorure (Cl^-) (constituants majoritaires du sel marin ; DOE 1997) sont assimilés majoritairement au niveau des branchiopodes et du tube digestif. Ils sont transportés dans l'organisme *via* l'hémolymphe. Arrivés aux *salt glands*, l'action combinée d'un co-transporteur $Na^+/K^+/Cl^-$ et d'une pompe active Na^+/K^+ ATPase permet la création d'un gradient local favorisant le rejet des ions Na^+ et Cl^- dans le milieu extérieur (Figure 1.2). Ces pompes sont au cœur du mécanisme

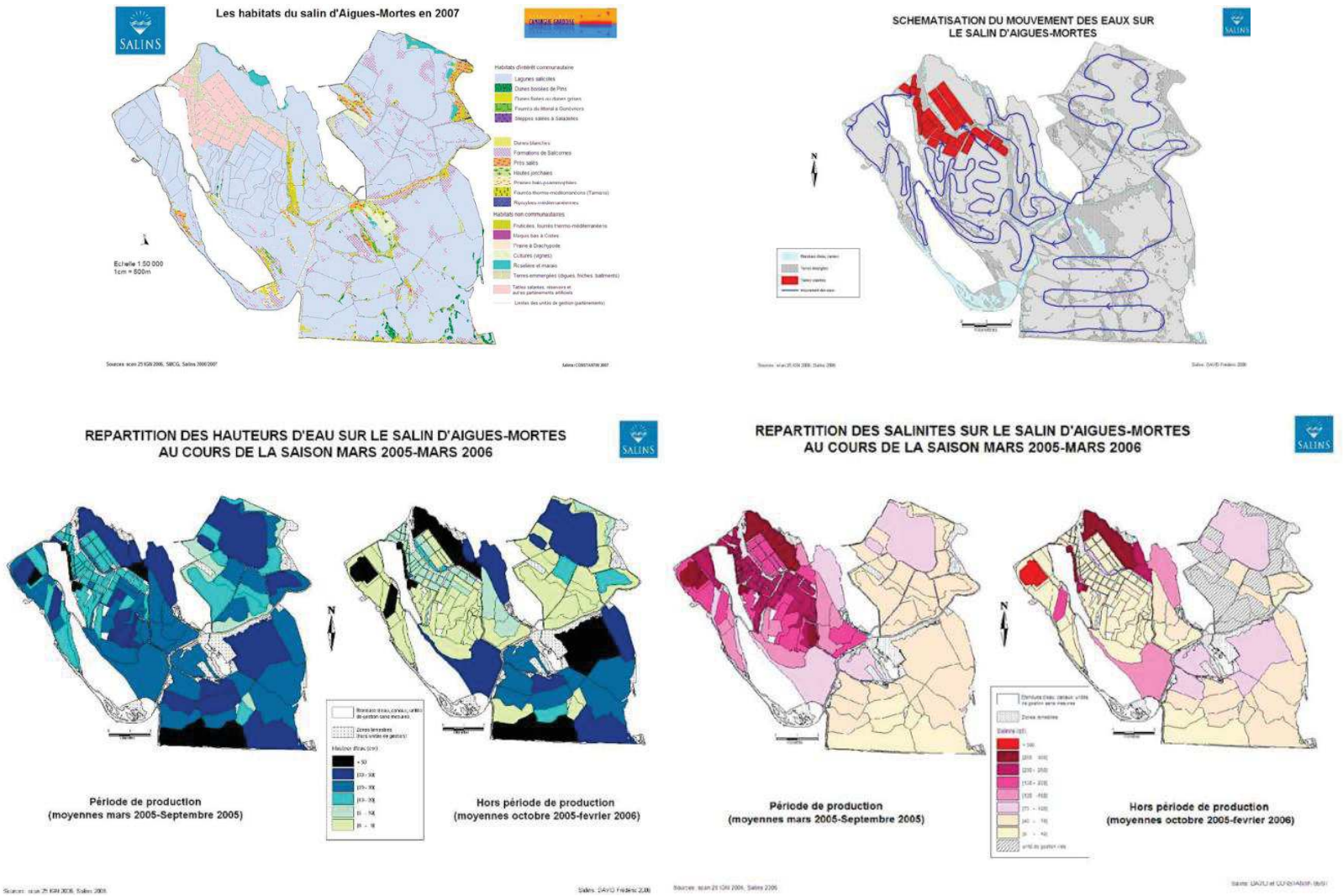


Figure 1.5 : Variations environnementales liée à l'exploitation du salin d'Aigues-Mortes (d'après Constantin & Séjourné 2007).

d'adaptation des artémies à la salinité. L'augmentation de la salinité extérieur a pour effet d'amplifier l'activité des pompes Na^+/K^+ ATPase (Holliday *et al.* 1990).

Un autre mécanisme d'importance est en jeu dans l'adaptation à la salinité des artémies. Lorsque la salinité augmente, la pression en oxygène du milieu extérieur diminue (Dwivepi *et al.* 1987). Chez l'artémie, trois types d'hémoglobines existent, résultant de permutation entre sous-unités dont l'affinité pour l'oxygène varie (Ferry *et al.* 1983; Clegg & Trotman 2002). Elles permettent d'adapter au mieux les réponses physiologiques liées à la disponibilité en oxygène et aux capacités respiratoires qui diffèrent entre nauplii et adultes ainsi qu'entre mâles et femelles (Gilchrist 1954).

1.4 Problématiques

1.4.1 Population du salin d'Aigues-Mortes et suivi à long terme

Le salin d'Aigues-Mortes (Gard, France), abrite une grande population d'*A. parthenogenetica* (Van Stappen 2000). On y trouve aussi *A. franciscana*, introduites dans le salin à partir de la fin des années 1960 à des fins de pêche industrielle (Rode *et al.* 2013). Depuis septembre 2008, un suivi de cette population est effectué par Thomas Lenormand et son équipe. Ce suivi consiste en un échantillonnage d'artémies dans une dizaine de bassins répartis sur l'ensemble des 10 700 ha du salin (Figure 1.5), ainsi que des données environnementales pour chaque relevé (température, salinité, proportion d'*A. franciscana*).

1.4.1.1 Aspects génétiques et environnementaux de la structuration

Le salin d'Aigues-Mortes est exploité par l'homme pour la production de sel. Celle-ci demande la récupération d'eau de mer qui est ensuite concentrée par évaporation de l'eau sous l'action du soleil et du vent dans différents bassins appelés « partènements ». Le chlorure de sodium (NaCl) finit par précipiter lorsque la saumure arrive au niveau des tables salantes. Le salin d'Aigues-Mortes produit en moyenne 430 000 tonnes de sel par an (Constantin & Séjourné 2007).

Cette exploitation implique une forte hétérogénéité environnementale à relativement faible échelle géographique (de l'ordre de 100m-1km) dans le salin (Figure 1.5).

Celle-ci est structurée spatialement par le parcours de l'eau lié à l'activité humaine sur le site (Figure 1.5). On peut donc s'attendre à observer de l'adaptation locale dans les populations, c'est-à-dire une structuration génétique entre populations adaptées à leurs conditions locales et connectées, au moins partiellement, par la migration et les flux de gènes (Lenormand 2002; Kawecki & Ebert 2004). La salinité est un facteur déterminant sur la survie et la reproduction des artémies. Dans le cas d'une adaptation locale, on peut donc s'attendre à observer une forte structuration à l'échelle du salin d'Aigues-Mortes. Or, une étude, sur la population clonale du Salin de Giraud (Browne & Hoopes 1990), très semblable à celle d'Aigues-Mortes, a montré qu'à part pour un bassin, la structuration génétique était quasi inexistante à l'échelle du salin. L'anthropisation de ces milieux, via les flux d'eau, implique de forte migration entre les bassins. Ceci pourrait expliquer l'absence globale de structuration à l'échelle du salin. Néanmoins, cette étude ne prenait en compte qu'un seul relevé alors que la structure du salin varie énormément d'une saison à l'autre (Figure 1.5). On est donc dans un cas de niches structurées dans l'espace mais qui varient aussi dans le temps. Quel impact cela pourrait avoir sur la structuration de la population d'*A. parthenogenetica* du salin d'Aigues-Mortes ?

D'autre part, on est dans le cas d'une population clonale particulièrement importante. Généralement, le maintien du polymorphisme génétique dans des populations asexuées est majoritairement étudié pour des microorganismes comme les levures (Lang *et al.* 2013) ou les bactéries (Le Gac *et al.* 2012). Le cas du salin d'Aigues-Mortes est une occasion particulièrement intéressante d'étudier les scénarios de maintien du polymorphisme en population asexué dans un milieu naturel et pour un organisme pluricellulaire.

Enfin, cette étude nous permet d'adresser concrètement certains des problèmes évoqué plus tôt : (i) celui de l'échelle employé pour définir la niche dans le cas d'une large population asexuée (Problème 1) et (ii) celui de la dimensionnalité de la niche et des axes environnementaux utilisés pour la définir (Problème 3). Concrètement, l'étude du maintien du polymorphisme dans les populations clonale demande de s'interroger sur la définition d'un clone et donc sur le mode de reproduction des *Artemia parthenogenetica*. Le second point est directement lié au caractère extrêmophile de l'artémie (voir 1.3.3).

1.4.1.2 Impact d'une invasion du passé

A l'heure actuelle dans le salin d'Aigues-Mortes, les artémies parthénogénétiques sont majoritaires en été (60,2%) tandis que les *A. franciscana* sexuées sont ultra majoritaires en hiver (97%) (Sánchez *et al.* 2012). Ces dernières ont été massivement introduites à la fin des années 1960 (Rode *et al.* 2013). Les artémies sexuées ont de meilleures capacités démographiques. Dans de nombreux salins de France et d'Espagne, elles ont remplacé les populations clonales (Amat *et al.* 2005, 2007). L'invasion est encore en cours dans le salin d'Aigues-Mortes, il est alors intéressant d'étudier quelle influence elle a eu par le passé sur la structure des populations asexuées.

L'impact d'une espèce invasive sur la population locale peut-être de deux ordres. Soit la population locale n'utilisait pas l'ensemble des habitats disponibles et utilisable par les deux espèces, et l'espèce invasive prend alors une place précédemment libre (Godoy *et al.* 2009). Soit la population locale utilisait l'ensemble des habitats. Dans ce cas, l'impact de l'espèce invasive peut-être de deux ordres : (i) elle remplace tout ou partie de la population locale, (ii) il y a un déplacement de caractères dans la population locale et/ou invasive, de sorte que chacune se spécialise sur un sous-ensemble de sa niche initiale (Brown & Wilson 1956).

Pour répondre à cette question, on utilise la propriété de résistance à l'anoxie et la déshydratation des cystes dormants (voir 1.3.1). En effet, sur le salin d'Aigues-Mortes, les cystes produits chaque années dans les bassins sont transportés par les courants de surfaces et s'accumulent dans des localisations connues. A certains endroits, les cystes sédimentent d'année en année et on a dans les sédiments une série temporelle datant parfois de plusieurs dizaines d'années. Cette banque de cystes peut alors servir pour suivre l'invasion d'*A. franciscana* dans le salin ainsi que son impact sur les populations du passé.

1.4.2 Mécanismes de l'adaptation et niche à la salinité chez l'artémie

L'artémie est adaptée à une large gamme de salinité, de 45g/L à 340g/L (saturation) dans la nature (Post & Youssef 1977; Persoone & Sorgeloos 1980). Je me suis

demandé si les mécanismes sous-jacents à l'adaptation aux deux extrémités de ce gradient sont les mêmes. Nous nous sommes intéressés ici directement à l'impact que ces différences de contraintes soulèvent sur l'utilisation du concept de niche multidimensionnel (Problème 3).

1.4.2.1 Low salinity paradox

L'adaptation aux faibles salinités est souvent rapportée comme contrainte par la présence de prédateurs (poissons). Van Stappen déclare (2002): « The lower salinity limit in nature is basically a function of the presence of predatory animals. Brine shrimp are rarely found in waters with salinity lower than 45 ppt, although physiologically they thrive in seawater and even in brackish waters», en s'appuyant sur une revue de Persoone et Sorgeloos (1980) qui ne démontre pas son propos. Or, l'étude de Castro-Mejia *et al* (2011) montre que les limites basses de tolérance de l'artémie au laboratoire (en absence de potentiel prédateurs) ne sont pas inférieure à des salinités de 40g/L. L'idée que les artémies prospèrent dans les eaux saumâtres et que leur absence dans de tels milieux est lié à la présence de prédateurs est donc erronée.

On se pose ici concrètement la question de la limitation du concept de niche réalisée aux seules interactions négatives (compétiteurs, pathogènes, etc.) sans tenir compte de l'influence des interactions positives (Problème 4). Ceci demande à la fois une réflexion théorique sur l'impact de ces symbioses sur l'ensemble des axes de la niche. Cela demande aussi une réflexion opérationnelle sur la mise en place d'expérience permettant d'étudier la niche de l'artémie sans sa microflore intestinale (conditions axéniques).

1.4.2.2 Evolution de la niche et plasticité phénotypique

Dans la littérature, l'adaptation aux fortes salinités est souvent reliée au fonctionnement des pompes Na^+/K^+ ATPases. Les limites de performance de chaque population étant dues à la présence ou non d'allèles codant pour différentes sous-unités, plus ou moins performantes, de ces pompes. Or, ce trait présente une plasticité phénotypique développementale.

On a donc ici un double impact de l'environnement sur la fitness des artémies. D'un côté la qualité de l'habitat dans lequel se trouve l'artémie va influencer sa survie instantanée. Ceci nous permet d'interroger l'importance de cet effet sur la niche

(Problème 5). D'autre part, on s'attend à ce que la fitness des artémies soit influencée par leur histoire de vie (Problème 2). Ceci impose alors une réflexion concrète de la mise en place d'expériences et d'analyses adaptées à l'estimation de ces paramètres dont les effets sont souvent confondus dans les mesures ponctuelles de fitness des phénotypes réalisés (Problème 6).

1.5 Plan de la thèse

Au cours de cette thèse, je me suis donc concrètement intéressée à trois questions principales permettant d'interroger théoriquement et opérationnellement les limites du concept de niche : Quels sont les mécanismes à l'origine du maintien du polymorphisme génétique dans une large population clonale ? Quel est l'impact de la flore bactérienne intestinale sur l'adaptation des artémies aux faibles salinités ? Comment la plasticité phénotypique et la qualité d'habitat influence l'adaptation de l'artémie aux fortes salinités ?

Pour répondre à ces questions, je m'attacherai à présenter les méthodes employées et les résultats essentiels, issus du travail effectué au cours de ma thèse. Par souci de clarté, je suivrai le plan suivant :

- Chapitre 1 : Déterminants environnementaux structurant la diversité génétique d'une large population clonale,
- Chapitre 2 : Flore intestinale et tolérance aux faibles salinités chez l'artémie,
- Chapitre 3 : Dynamiques des courbes de tolérances et adaptation aux fortes salinités chez l'artémie.

Je terminerai par une généralisation des apports de ce travail au travers d'une partie discussion et perspectives.

Chapitre 1

Déterminants environnementaux structurant la diversité génétique d'une large population clonale

Le suivi à long terme de la population d'artémies du salin d'Aigues-Mortes nous a permis d'éclairer de nombreux aspects de la génétique des populations à la fois de l'espèce asexuée et de l'espèce sexuée invasive. Ce suivi comporte une étude de la structuration spatiale et saisonnière du salin d'Aigues-Mortes dans les populations clonales (Article 1 & Annexe 1). Une étude annexe sur ces données s'intéresse au mode de reproduction des *A. parthenogenetica* (Annexe 2). Enfin, des données préliminaires ont été collectées à partir des cystes des sédiments, en vue d'un suivi à plus long terme de l'invasion du salin d'Aigues-Mortes par *A. franciscana* et son impact sur les populations locales (Annexe 3).

2.1 Analyse spatio-temporelle de la structuration génétique

L'analyse génétique des échantillons issus du suivi à long terme de la population d'artémies du salin d'Aigues-Mortes nous a permis de mieux comprendre l'impact de l'exploitation du salin par l'homme, ainsi que des variations du milieu biotique et abiotique, sur la structuration spatiale et temporelle. Dans cette étude, nous nous sommes intéressés à différentes échelles géographiques de structuration : inter-salins et intra-salin, avec dans ce dernier cas une comparaison inter-bassins sur l'ensemble des dates d'échantillonnage et inter-date d'échantillonnage pour un même bassin.

Afin d'étudier la structuration génétique de la population clonale d'*A. parthenogenetica* du salin d'Aigues-Mortes, de nouveaux marqueurs microsatellites ont été développés au sein de l'équipe (Annexe 1). Préalablement, il n'existait que cinq marqueurs microsatellites pour les lignées diploïdes d'*A. parthenogenetica* (Muñoz *et al.* 2008). Les neufs nouveaux marqueurs polymorphes, développés à partir de trois banques génétiques obtenues par pyroséquençage, nous permettent de distinguer les clones parthénogénétiques des espèces sexuées dont ils sont issus.

Nous nous sommes ensuite intéressés plus directement à la problématique du maintien du polymorphisme génétique dans une grande population clonale naturelle. Nous avons étudié le lien entre ce polymorphisme et différentes variables

environnementales afin de mettre en évidence les potentielles spécialisations écologiques des clones.

Concrètement, nous sommes partis d'une description de la diversité génétique à l'échelle du salin d'Aigues-Mortes, via une ACP sur les données microsatellites d'échantillons provenant de différents basins (sites), nous avons pu mettre en évidence plusieurs choses. Tout d'abord, qu'il y a un fort impact de l'activité humaine sur la structure génétique. En effet, la majorité des sites échantillonnés font partie du circuit qu'emprunte l'eau dans la production de sel. Ces bassins sont fortement connectés entre eux et on n'observe pas de structuration dans la diversité des clones observés. A l'inverse, quelques-uns des sites étudiés n'étant jamais (ou très rarement) connectés au réseau général, ils avaient des diversités génétiques plus ou moins fortes, mais avec une composition bien distincte de celle des bassins connectés. Il est aussi intéressant de noter que pour l'un de ces sites (Site 9), la diversité à l'une des dates échantillonnées est la même que pour le reste des bassins connectés. Ceci semble être une illustration d'une connexion de ce bassin au reste du réseau. Au final, il semble donc que la forte hétérogénéité environnementale dans le salin ne parvient pas à créer une forte structuration génétique géographique du fait des flux d'eau liés à l'activité humaine de production du sel.

Malgré ce fort impact de l'activité humaine sur la structure spatiale et temporelle, nous avons cherché à voir si une part de la structure génétique dans les bassins les plus isolés pouvait être expliquée par les conditions environnementales du salin, indiquant ainsi une spécialisation écologique des clones. Nous nous sommes donc intéressés à l'impact de ces variations temporelles de l'environnement sur la structuration génétique. Pour cela, nous avons utilisé des techniques d'interpolation cartographique en utilisant les coordonnées de l'ACP et les valeurs environnementales pour l'altitude. Dans les sites isolés, cette technique nous a permis d'isoler des clusters d'haplotypes dont certains semblaient adaptés aux faibles valeurs de salinités et de température, et d'autres à des fortes valeurs de ces variables environnementales. Ces clones ont par la suite pu être utilisés dans nos études sur la plasticité au laboratoire (voir Chapitre 3).

Les études précédentes s'intéressant à l'impact de l'environnement sur la structuration génétique qui traitent ces corrélations par locus. Par exemple, Hancock et al (2011), ont comparé les enrichissements relatifs en SNPs non-synonymes par rapports aux synonymes et aux inter-géniques pour des locus corrélant avec des variables environnementales. Ils ont ainsi montré que chez l'arabette (*Arabidopsis thaliana*), les locus correspondant à des changements d'acides-aminés étaient significativement supérieurs dans 12 des 13 variables environnementales testées. Notre méthode d'analyse s'appuie sur les génotypes multi-locus des individus. Cela permet de prendre en compte les effets de proximité génétique entre individus pour d'un côté prendre en compte le mode de reproduction d'*A. parthenogenetica* (voir 2.2.1) mais aussi de lisser les erreurs de mesures possibles, notamment celles liées à la stochasticité démographique.

Finalement, l'étude du polymorphisme dans la population clonale ainsi que la meilleure compréhension du régime de reproduction des artémies parthéno-génétiques diploïdes (voir 2.2.1) a permis de montrer que dans le cas étudié, on pouvait envisager la niche à l'échelle d'un nuage d'individu tous issu d'un même clone (Problème 1).

Article 1
Environmental determinants structuring the genetic diversity of a large clonal population

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In preparation

Abstract

While genetic polymorphism in sexual species is well studied, much less is known and developed for asexual populations. In absence of recombination/segregation and to avoid the impact of drift, large asexual are required to study selective processes occurring within populations. Here, we investigate genetic polymorphism in *Artemia*, to better understand the cause of genetic diversity in large asexual population. We monitored the Aigues-Mortes saltern population during three years and found cyclical occurrence of clones and a maintenance of the polymorphism observed through time. We tested several environmental variables to link this frequency dependent selection regime with niches. We were able to find clusters of clones adapted to high and low values of environmental variables in one of the isolated sites sampled.

Key words: Genetic polymorphism, Artemia parthenogenetica, asexual reproduction, Aigues-Mortes saltern

Introduction

In sexual populations, most molecular polymorphism is thought to be neutral, and can be used to investigate current and historical patterns of gene flow and drift. Statistically, each locus can be treated as being independent, and many methods have been developed to analyze population structure, inbreeding or isolation by distance, among others (Allen *et al.* 2010; Hancock *et al.* 2011; Muñoz *et al.* 2014). Selection can detected when some loci exhibit patterns of polymorphism or geographic structure that are strongly discordant with neutral expectations or the rest of the genome (e.g. Fst outliers; Barreiro *et al.* 2008; Coop *et al.* 2009). Alternatively, signatures of selection can be searched at candidate genes that are expected to be adaptive (Linnen *et al.* 2009).

Comparatively, much less is known and developed to analyze and understand genetic polymorphism in large asexual populations. In asexuals, the absence of recombination/segregation generates strong genetic associations among loci, so that none can be considered 'independent' from the rest of the genome. Genetic drift caused by wide-ranging hitchhiking effects implies that no locus can be considered neutral in asexuals (Gillespie 2001). Furthermore, for asexuals that have large population sizes (such as many microbes, or small invertebrates as studied here), genetic drift is weak, such that most observed frequency changes are likely to be due to selection. For these reasons, asexual populations can be useful 'windows' on selective processes occurring within populations. Yet, analyzing, interpreting and understanding genetic polymorphism in asexual population can be particularly difficult.

Polymorhism at selected loci (or loci tightly linked to them) can occur for two broad classes of reasons. On the one hand, genetic polymorphism may transient. In asexuals, adaptation proceeds by the occurrence of competing beneficial mutations (some of the competing lineages may occur by migration). Only the best genetic background will eventually fix, but at any time many lineages coexist. In this regime of 'clonal interference' (Gerrish & Lenski 1998), the rise and fall of particular lineages is contingent on the occurrence (or absence) of better backgrounds. A distinctive pattern of clonal turnover is expected. This regime has been observed in experimental

evolution in large bacteria population in homogeneous and stable environments (Lang *et al.* 2013).

On the other hand, genetic polymorphism can maintained stably by selection. Selection can favor polymorphism in different ways, notably by frequency dependence (when the rare type is selectively favored, due e.g. to interactions with parasites), or by spatially variable selection (different types being favored in different habitats). In the latter case, polymorphism is maintained because different lineages have different niches (sensu Hutchinson 1957), justifying to consider each asexual lineages as 'micro-species' (Vrijenhoek & Davis Parker Jr. 2009). We would thus expect some clones to be associated with specific environmental conditions. Such a mechanism for the maintenance of polymorphism is more likely in environments that are highly structured and heterogeneous, but has also been observed in partly simple 'test-tube' environment in the lab (Le Gac *et al.* 2012; Plucain *et al.* 2014; Ribeck & Lenski 2015).

In this paper, we investigate genetic polymorphism in *Artemia,* in order to better understand the cause and extent of genetic diversity in large asexual population. *Artemia* is a genus of fairy shrimps (*Anostraca*) living in highly salted environments such as salt lakes and lagoons (van Stappen 2002; Gajardo & Beardmore 2012). The genus comprises seven bisexual species, and asexual clones with diverse ploidy levels collectively referred to as *Artemia parthenogenetica* (Amat *et al.* 2007; Rode 2012). In the Mediterranean basin, the historical sexual species *A. salina* co-exists with *A. parthenogenetica* (Amat 1983; Lenz & Browne 1991; Hontoria & Amat 1992; Barata *et al.* 1995; van Stappen 2002) as well as an American sexual species (*A. franciscana*), now invasive in many sites (Amat *et al.* 2005; Ben Naceur *et al.* 2010; Rode *et al.* 2013; Muñoz *et al.* 2014). Large populations of endemic parthenogenetic *Artemia* can be found in commercial coast salt ponds (Van Stappen 2000). This gives an opportunity to study the impact of the environment (both abiotic and biotic factors) on the adaptation of large clonal population in the wild. It has been shown that clonal populations of *A. parthenogenetica* from various geographical origins have different tolerances to environmental variables such as temperature and salinity in the laboratory

(Vanhaecke *et al.* 1984), so it is likely that their niches also vary in their natural habitats.

Browne and Hoopes (1990) studied the Salin de Giraud population of *Artemia*, which is comparable to the Aigues-Mortes saltern, and found that it was highly polyclonal (63 allozymatically unique genotypes identified). They discussed that the observed diversity level might depend on the structuration and fluctuations of environmental conditions. However, they found no geographic structure in the clonal repartition in the saltern. The salt marsh environment is geographically structured, but even more strongly timely structured. Indeed, it takes all summer for the salt contained in the water pumped from the Mediterranean Sea to crystallize on the salt pans. By only analyzing data from one sampling date, Browne & Hoopes (1990) only get a snapshot in the time structuration that might influence genetic structuration in the saltern.

We monitored the Aigues-Mortes population of *Artemia* during three years (2010 to 2013). There, a large population of both *A. parthenogenetica* and *A. franciscana* has been observed. Like in Salin de Giraud, the Aigues-Mortes saltern present structured habitats (e.g. salinity gradient, food availability, temperature…) that fluctuate through seasons and years, giving a good opportunity to study the influence of the environment (abiotic and biotic) on the structuration of a large parthenogenetic population.

Methods

Artemia sampling

Mediterranean salterns sampling

This study focuses mainly on the Aigues-Mortes meta-population, but also includes samples from other salterns (Odiel and Salin de Giraud). Each meta-population refers to a saltern. It includes many sites that are more or less connected through the water network.

Salin de Giraud (Salins du Midi, Arles, France; here after SG) covers an area of 11 000 ha and produces about 10^6 tons of salt per year, while Odiel (Huelva, Spain; hereafter OD) covers 7 185 ha and is no longer used for salt production. Aigues-Mortes saltern (Salins du Midi, Aigues-Mortes, France hereafter AM) is more similar to Salin de Giraud in terms of size (10 000 ha),

location (Camargue in the south of France), and management practices (the same company runs them both).

The production of salt in the Salin de Giraud and Aigues-Mortes salterns involves a complex water network (Figure S1). Indeed, seawater is pumped and transported throughout the saltern and then, serially distributed in several sites where salts will concentrate in the water until it arrives in the salt pans where water will evaporate and salts will be harvested. This structuration result in a heterogeneous habitat with salinity gradient and water movement following the water network.

Aigues-Mortes field survey. From 2010 to 2013, a survey of the *Artemia* populations in Aigues-Mortes saltern has been conducted. A total of 48 samples were collected over 10 sites and 20 sampling dates. In Aigues-Mortes, the water network (white line in Figure S1) is divided into two main branches, with the salinity increasing along each branch. We selected collecting sites on both branches at different positions along the water network (see Figure S1). We also selected a few sites that were not part of the general network: Site 9 and Saint-Louis are both used to recoil surface rain waters from the salt pans, and Puits-Romain site is independent from the water network. At each collection date (approximately every 3 months), samples originating from these sites were collected and conserved in 96% ethanol, and the salinity level of the site was also recorded. In addition, samples from several sites (Site 1, Site 9 and Saint-Louis) were sent every two weeks by the company Camargue pêche S.A.R.L. (Aigues-Mortes, France), but we could not retrieve salinity for these samples.

We focused on three environmental factors that may explain cause genetic structure in this population, beyond geography: salinity, monthly temperature and the proportion of *A. franciscana*. The temporal variation in salinity of several sites of the Aigues-Mortes saltern is presented in Figure 1B. Sites differ in mean salinity, as well as in the magnitude of fluctuations in salinity. Monthly temperature (see Figure S1) was obtained from a climatological station based in le Grau-du-Roi (Quillé 2000). Monthly temperature provides a proxy for the variations of water temperature, evaporation, O_2 availability as well as salinity. Finally, the proportion of the introduced (and potentially

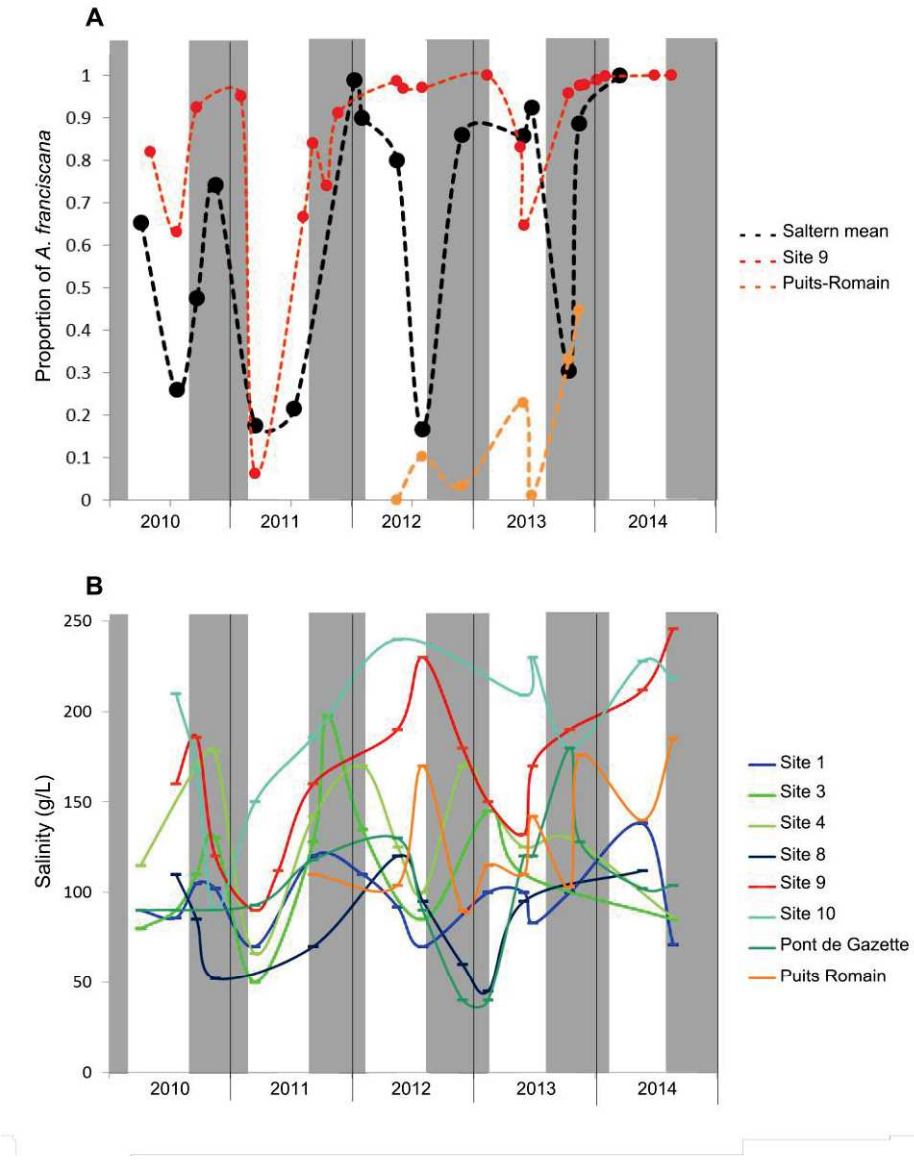

Figure 1. Variation in (A) the proportion of *A. franciscana* and (B) salinity throughout seasons in the Aigues-Mortes saltern. Seasons are highlighted by the shaded zones (white: spring and summer; shaded: autumn and winter). *A. franciscana* proportion is calculated on adult females, and mean is calculated on connected site (ponds connected to the water network at least once a year; see Figure 2). See map in Figure S1 for sites location and water network in Aigues-Mortes saltern.

competitor) species *A. franciscana* (Figure 1A) was estimated for each sampling date (see below 'Native vs. invasive *Artemia*').

Salin de Giraud and Odiel saltern sampling. A total of 13 parthenogenetic female *Artemia* of the Salin de Giraud were sampled from four different sites in October 2011. Likewise, a total of 70 parthenogenetic female *Artemia* of the Odiel saltern were sampled from five different sites in September 2013.

Native vs. invasive *Artemia*

In each ethanol sample from the Aigues-Mortes field survey, the proportion of *A. franciscana* was estimated by distinguishing between sexual and parthenogenetic adult female *Artemia*. This distinction was made according to two morphological criteria: the length of antennae and the ratio of abdomen over total body size (both antennae length and body ratio are greater in *A. parthenogenetica*), following Hontoria and Amat's (1992) measurement protocol. Samples containing mostly juveniles, or with too high salinities, made these morphological criteria useless. Indeed, *Artemia*'s morphology responds plastically to salinity, and this plastic response is not the same in parthenogenetic and sexual species (Triantaphyllidis *et al.* 1995; Abatzopoulos *et al.* 2003). When morphological distinction was not possible, 40 to 100 individuals (juveniles or adult females) were randomly selected, and were discriminated using a molecular approach (detailed below; Muñoz et al. 2008).

DNA extraction. PCR plates were filled with one individual per well, with 15 µL of E buffer (HotSHOT, Sigma-Aldrich). Plates were placed in a thermocycler and warmed at 95°C for 10 min, then at 20°C for another 10 min. Finally, 25 µL of sterile water were added in each well to elute the DNA extract.

Microsatellite amplification. To discriminate between *A. parthenogenetica* and *A. franciscana,* Ap02 and Af03 microsatellite markers (Muñoz *et al.* 2008) were respectively used with the following protocol: 9µL of a PCR mix - containing 5 µL of Multiplex buffer, 1 µL of both forward and reverse primers [2 µM] and 2 µL of sterile water - was added to 1 µL of DNA extract; thermocycler program was as follow: initial denaturation ran for 15 min at 95°C, 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1min30, and extension at 72°C for 1 min and, a final extension ran for 30 sec at 60°C. Ap02 and Af03 PCRs were conducted in parallel for each individual.

Tables

Table 1. Genetic diversity at each microsatellite locus comparing three Mediterranean salterns. AM: Aigues-mortes; OD: Odiel; SG: Salin de Giraud. Population sizes are indicated in parenthesis. *NA*: number of alleles; Mon.: monomorphic locus; *He*: expected heterozygosity; *Ho*: observed heterozygosity; *: non-biased estimator following Nei and Chesser (1983); *PNL*: percentage of double null allele at the locus.

	AM (420)					OD (70)					SG (13)				
	NA	**Size range**	***He****	***Ho***	***PNL***	***NA***	**Size range**	***He****	***Ho***	***PNL***	***NA***	**Size range**	***He****	***Ho***	***PNL***
Apcpm1	*3*	*104 - 110*	0.65	0.78	-	*3*	*104 - 110*	0.53	0.99	-	*3*	*104 - 110*	0.67	0.85	-
Aupm5	*3*	*161 - 173*	0.12	0.09	-	*1*	*161*	Mon.	Mon.	-	*1*	*161*	Mon.	Mon.	-
Aupm7	*1*	*124*	0.00	0.00	0.24%	*1*	*124*	0.03	0.00	1.43%	*1*	*124*	Mon.	Mon.	-
Appm26	*5*	*170 - 190*	0.53	0.58	1.43%	*2*	*178 - 190*	0.50	0.94	-	*2*	*178 - 190*	0.52	0.62	-
Aupm15	*3*	*89 - 95*	0.25	0.03	0.48%	*2*	*93 - 95*	0.11	0.03	1.43%	*2*	*93 - 95*	0.15	0.00	-
Aupm16	*8*	*120 - 160*	0.81	0.91	0.48%	*3*	*128 - 132*	0.52	0.99	-	*6*	*120 - 156*	0.83	1.00	-
Aupm21	*4*	*108 - 117*	0.46	0.47	1.90%	*2*	*108 - 113*	0.50	0.93	-	*2*	*108 - 113*	0.22	0.08	7.69%
Appm20	*6*	*104 - 114*	0.67	0.88	0.24%	*3*	*106 - 112*	0.68	0.66	12.86%	*6*	*104 - 114*	0.81	0.85	15.38%
Appm4	*2*	*83 - 87*	0.49	0.16	-	*2*	*83 - 87*	0.43	0.11	-	*2*	*83 - 87*	0.51	0.08	-
Ap01	*15*	*157 - 195*	0.82	0.30	1.43%	*2*	*167 - 169*	0.03	0.03	-	*5*	*157 - 173*	0.82	0.15	7.69%
Ap02	*10*	*221 - 257*	0.85	0.65	2.14%	*5*	*191 - 242*	0.54	0.87	-	*6*	*224 - 245*	0.61	0.31	-
Ap03	*9*	*194 - 218*	0.70	0.37	2.62%	*2*	*206 - 212*	0.50	0.94	-	*4*	*194 - 216*	0.58	0.69	-

PCR amplification migrated on 2% agarose gel for 30 min for visual reading of the result.

Clone characterization

Samples from all salterns, genetically and morphologically identified as *A. parthenogenetica*, were characterized using 12 microsatellites markers arranged in four multiplexes (Table 1; Muñoz et al. 2008; Nougué et al. 2015). For each multiplex, we used the same DNA extraction and microsatellite amplification procedure as above. Individuals were genotyped by assessing allele size on an ABI 3130xl Genetic Analyzer (Plateforme de Génomique Environnementale/Labex). Allele scoring was carried out using GeneMapper 3.7 software (Applied Biosystems) and was individually reported into a clone characterization file.

Salterns structuration

Diversity and genetic structure of Mediterranean salterns

We used several population genetic methods to estimate clonal genetic diversity in the different salterns and the structuration among salterns.

First, Principal Component Analysis (hereafter PCA) was performed on individual allele frequencies, using the adegenet 1.4-1 package (Jombart 2008) in R. Essentially, this method treats the frequencies of all microsatellite alleles in each individual (with values 0, 0.5 for heterozygotes and 1 for homozygotes) as a multivariate dataset with dimensionality equal to the number of alleles. The correlation between alleles (due to linkage disequilibrium) is then used to reduce dimensionality, by finding combinations of alleles that most explain how genetic variance is structured.

In order to assess to what extent our samples were representative of the clonal diversity in the population, we used a rarefaction analysis. In this analysis, we were quite stringent and defined clones as individuals with strictly the same scoring at all alleles of microsatellite loci. We estimated clone rarefaction in the samples following the species rarefaction function (Gotelli & Colwell 2001),

$$E(K) = \sum_{i=1}^{K} \left(1 - \left[\frac{\binom{N - N_i}{n}}{\binom{N}{n}} \right] \right), \quad (1)$$

with N the total number of individual in the sample, K the number of clones present in the sample, N_i the number of individual of the i^{th} clone (i = 1,…,K) and n the resampling size. In order to characterize genetic variation using information on genetic distance contained in the PCA cloud in each sample, we used an inertia analysis on all PCA axes. We estimated the centroid on all PCA axes for each population, and calculated the inertia as

$$I_T = \frac{1}{n}\sum_{i=1}^{n}\sum_{j=1}^{p}\left(x_{ij} - x_{bj}\right)^2, \quad (2)$$

with p the number of PCA axes and x_{bj} the centroid coordinates on the j^{th} axis of the PCA. We implemented these in Mathematica 10 (Wolfram Research) as functions of the resampling size (n).

Finally, we computed observed heterozygosity (hereafter H_o), and the expected heterozygosity at Hardy Weinberg equilibrium,

$$H_e = 1 - \sum_{i=1}^{m}(q_i)^2, \quad (3)$$

where q_i is the frequency of ith allele of m alleles at a microsatellite locus.

Aigues-Mortes geographical and seasonal structuration

To test whether the high genetic diversity in Aigues-Mortes saltern is structured, we performed a PCA between sites (pooling for all sampling dates), as described above. In order to follow the frequency variations of sub-population of genetically similar clones, we defined 10 clusters based on PCA coordinates for all individuals in the Aigues-Mortes saltern, using the clustering function in Mathematica 10. Then, for two connected populations (S1 and S3) and two isolated populations (S9 and PR), we estimated the rarefaction function and the PCA cloud's inertia following the equations (1) and (2).

To assess the impact of the environment on the genetic structuration estimated through PCA analysis, we used cartography interpolation techniques on the dataset that associates environmental values (as z) to the coordinates along the first two axes of the PCA (as x and y). The smoothing function interpolated values of the environmental variable (z^*) at any (x^*, y^*) coordinates in the PCA plan, by averaging values from each individual in the sample weighted by their distance, using a Gaussian Kernel. This led to

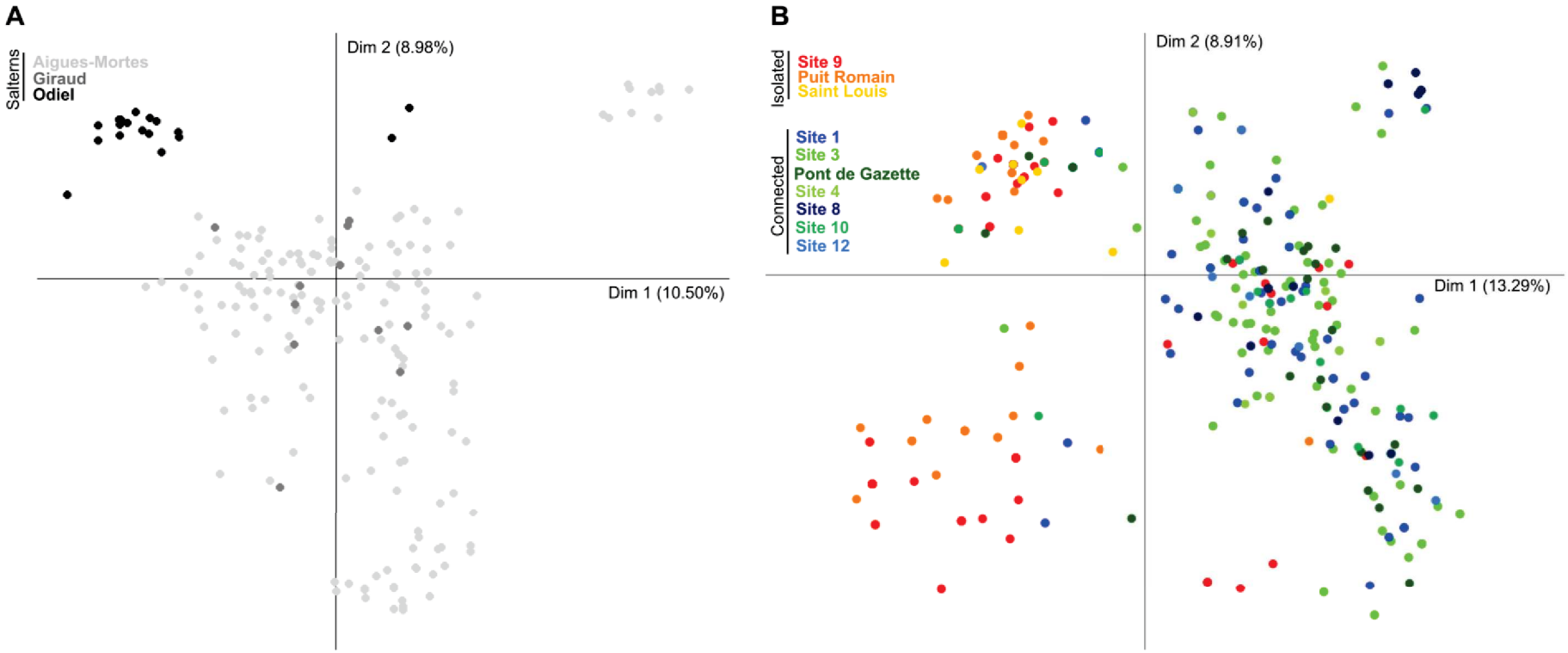

Figure 2. Principal component analysis comparing (A) three Mediterranean salterns and (B) local populations from different sites in the Aigues-Mortes saltern. In B, 'isolated' refers to sites connected to the water network less than once a year, and 'connected' refers to sites connected to the water network at least once a year. See map in Figure S1 for sites location and water network in Aigues-Mortes saltern.

$$z^*(x^*, y^*) = \frac{\sum_{i=1}^{N}\left[\frac{z_i}{{D_i}^2} e^{-\frac{1}{2}\left(\frac{(x^*-x_i)^2+(y^*-y_i)^2}{(D_i h)^2}\right)}\right]}{\sum_{i=1}^{N}\left[\frac{1}{{D_i}^2} e^{-\frac{1}{2}\left(\frac{(x^*-x_i)^2+(y^*-y_i)^2}{(D_i h)^2}\right)}\right]} \quad (3)$$

where *N* is the total number of individuals in the sample, z_i is the value of the environmental variable for the i^{th} individual, and h is a smoothing parameter controlling the width of the kernel. The smoothing parameter h equaled 1 for all sites (S1, S3 and S9) except for PR ($h = 2$). To account for the variable density of individuals across the (x, y) plan, we also scaled the width of the kernel for each individual to the Euclidian distance D_i of this individual to the closest different genotype. We performed resampling test for each environmental variable (z) and delimited the contour lines corresponding to the 5%, 25% and 50% of the z^* distribution. We implemented this analysis in Mathematica 10 (Wolfram Research).

Results

Clonal diversity in Mediterranean salterns

The Aigues-Mortes saltern presented more alleles at all loci than Salin de Giraud or Odiel (Table 1). On the other hand, the mean heterozygosity in Aigues-Mortes (He = 0.53; Ho = 0.44) is similar to that in Salin de Giraud (He = 0.57; Ho = 0.46), while Odiel differed (He = 0.40; Ho = 0.59). This result is consistent with the PCA analysis (Figure 2A), where the Salin de Giraud sample looks like a sub-sample of Aigues-Mortes, while Odiel represents a non-overlapping group. However, the rarefaction and inertia curves display the same slope for AM and OD, while SG as smaller amount of clonal lineages (Figure 3A). The diversity seems to be similar between AM and OD, and even though the sample size are different, the distance between genotypes also looked similar. However, the small sample size for SG (13 individuals collected at one date in one site) might explain the difference we found in diversity between this saltern and AM even though they are very similar in ecology. Overall, the clonal diversity was extremely high, with saturation curves showing minimal diminishing return with sample size. This is likely the cause of a reproductive mode which is not apomictic and involves

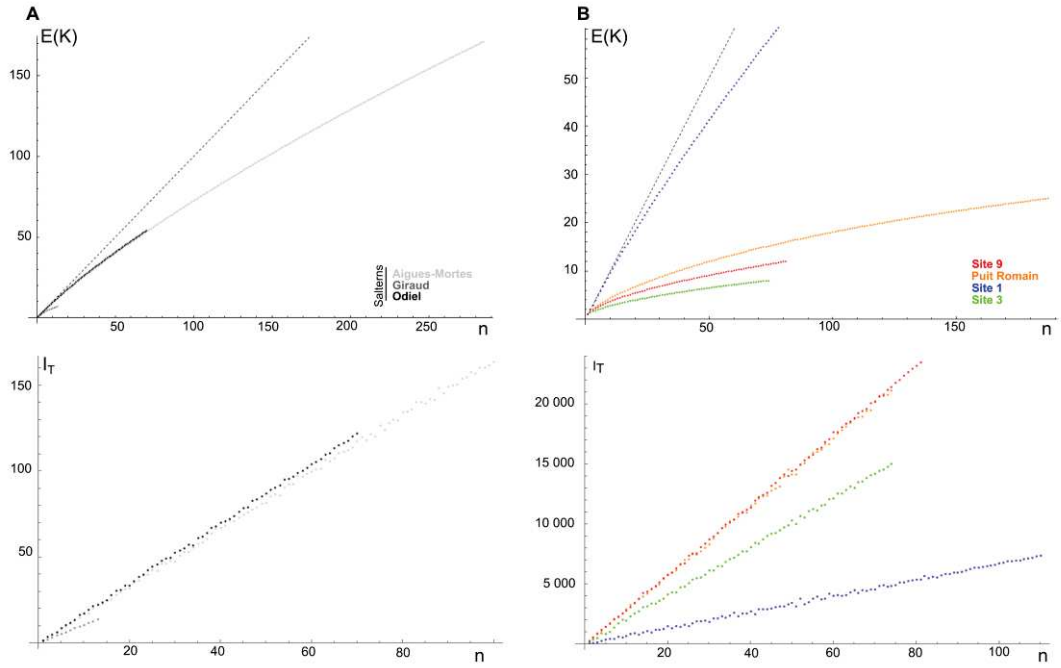

Figure 3. Diversity estimates comparing (A) three Mediterranean salterns and (B) four sites' populations in the Aigues-Mortes saltern. Rarefaction E(K) is presented as a function of the subsample size n. The dashed black line corresponds to E(K) = n. PCA cloud inertia I_T is presented as a function of the subsample size n. See map in Figure S1 for sites location and water network in Aigues-Mortes saltern.

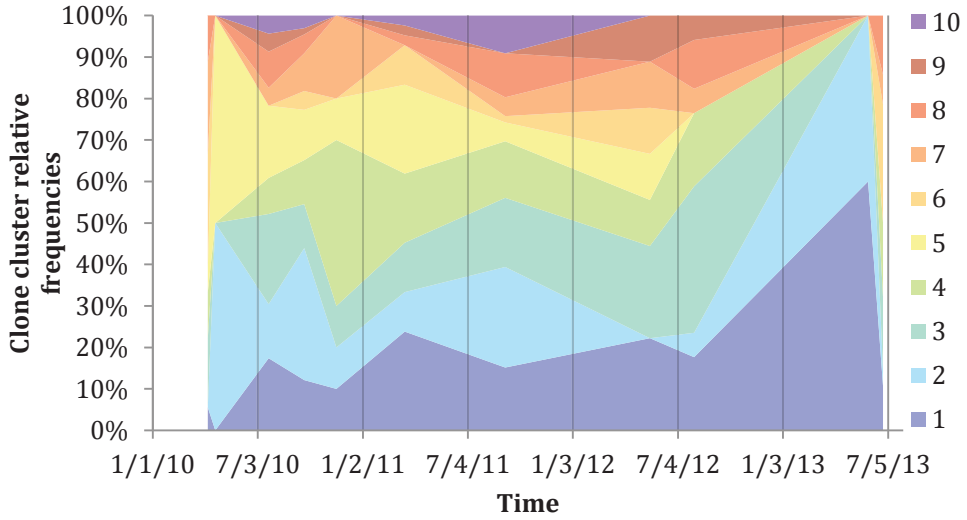

Figure 4. Relative frequencies of 10 clonal clusters from the connected sites of the Aigues-Mortes saltern, along sampling times. Clusters 1 to 10, identified using the PCA coordinates from all sites, are ordered using their total frequency in the population.

some segregation (Nougué *et al.* in prep), resulting in regular production of new clones.

Diversity and structure in the clonal population of Aigues-Mortes

Seasonal variations of environmental conditions

In Figure 1A, the proportion of *A. franciscana* in the samples is plotted along sampling time. The mean for all connected sites (in black) displays cyclical variations throughout seasons. The proportion of *A. franciscana* rose during autumn/winter (grey shaded area) and fell during spring/summer (white shaded area). This cyclicality was also present in the isolated site 9 (red line in Figure 1A) at the beginning of our monitoring, but *A. franciscana* seems to have overrun *A. parthenogenetica* in this particular site since the end of 2012. On the opposite, we only began to detect the invasive species in the isolated site PR (orange line in Figure 1A) in august 2012. In this site, we also observe cyclicality in the proportion of the sexual species, which never exceeded 50% of the samples.

In Figure 1B, we plotted the salinity variations for all sites monitored in Aigues-Mortes. There are two types of variations between sites: (i) variation in mean salinity (e.g. site 1 always presents lower salinity than site 9); (ii) variation in the seasonal pattern (cyclical increase and decrease of salinity) between sites (e.g. larger amplitude of salinity variations in site 3 than in site 1).

Geographic structuration

To measure the geographic structure from Aigues-Mortes population, we first pooled for each site all the samples collected at different dates. As seen on Figure 2B, the first axis of the PCA mostly distinguishes three sites (S9, PR and SL) from the rest of the saltern. These sites are noteworthy because of their isolation from the saltern water network. Indeed, PR is not connected at all with the network, while both S9 and SL are recoil channels only used occasionally to drain rainwater out of the saltpans back to the sea. When looking at diversity estimators (Figure 3B), the rarefaction curves of connected sites (S1 and S3) present variable slopes. S1 present a very high diversity while S3 has the lowest diversity. On the other hand, the inertia for these two sites shows that the genetic distance between individual is lower in S1 than

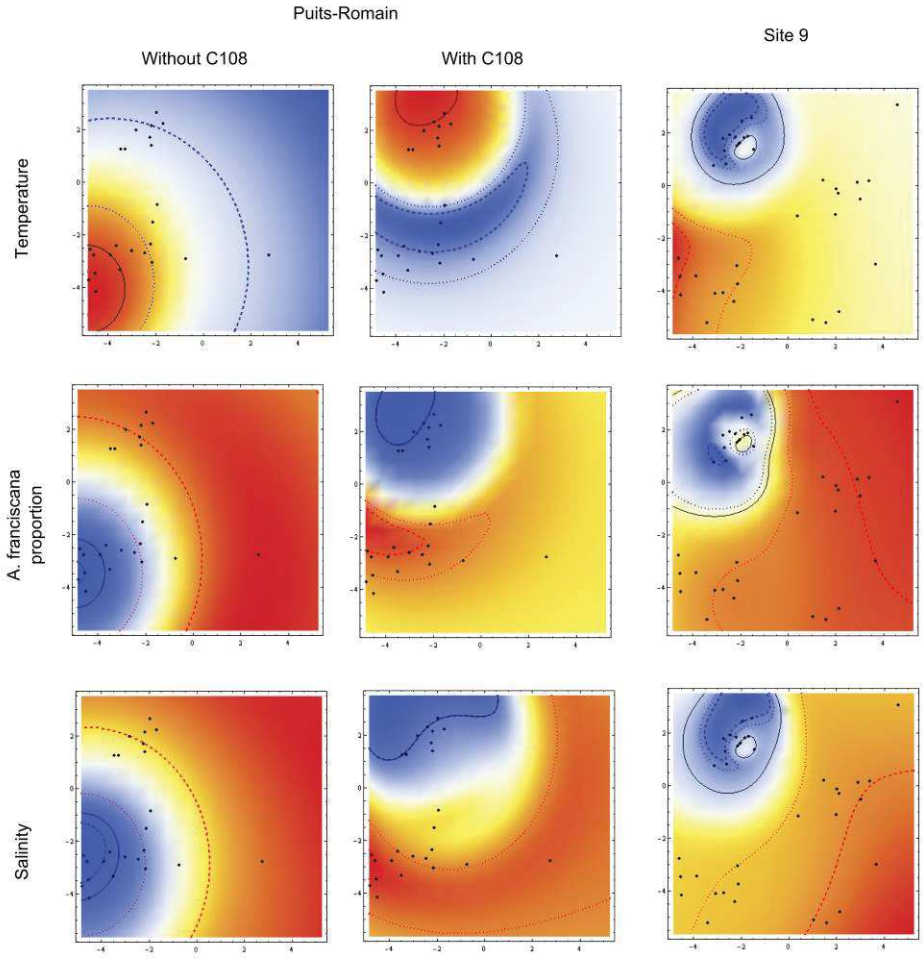

Figure 5. Interpolation maps of environmental factors against genetic PCA coordinates, for two isolated sites of Aigues-Mortes. Columns correspond to sites, lines correspond to environmental factors. In PR, 108 individuals had the same coordinates; we grouped them under the name C108. High values of the environmental factor are presented in red, and low values in blue. Contour lines represent the 5% lowest (dashed blue), 25% lowest (dotted blue), 50% (continuous black), 25% highest (dotted red) and 5% highest (dashed red) of the distribution.

S3. The isolated sites (S9 and PR) present similar slopes when comparing their rarefaction curves as well as the inertia of their point cloud. They present intermediate rarefaction slope but the highest inertia values which can be interpreted as an intermediate number of clones but that are genetically more distant than in connected sites.

Temporal structuration

We then looked at the temporal structure, by comparing the distribution of different sampling date on the axes of the PCA. No structure was found for connected ponds, while the isolated sites S9 and PR present a structuration along the second PCA axis (Figure 2B). S9 had several individuals whose PCA coordinates were similar to those of samples from the connected sites In (Figure 2B). These individuals all originated from a single sampling date (May 2010), which might correspond to an event of connection of S9 to the rest of the saltern (by collecting rainwater out of the saltpans).

We were able to create interpolated maps of temperature, salinity and proportion of *A. franciscana* over the PCA coordinates on the first two axes. For the connected sites, there was no correlation between environmental factors, as the highest and lowest values differed in each map (see Figure S2). In Figure 4, we plotted the 10 clusters along sampling times for all the connected sites. We showed that clusters proportion raised and fell several times throughout time. This was particularly visible for clusters with low frequencies (e.g. 2, 5, 7, 9 and 10).

On the other hand for the isolated sites, interpolation maps for different environmental variables overlapped (Figure 5). In S9, we isolated groups of clones corresponding to a combination of low temperature, low proportion of *A. franciscana* and low salinity (blue area in top left of all S9 graphs in Figure 5). As this group of clones consisted of genetically similar individuals (they covered a small PCA area), we used the 50% contour line of the interpolated environmental surfaces to delimit them into a cluster. For the reciprocal group of high values of the three environmental variables, we used the 25% contour line of the interpolated environmental surfaces for the temperature only, because we had more information on this abiotic peculiar variable, enabling us to build a more accurate interpolation map. This allowed us to exclude the sampling from May 2010, which was genetically similar to the connected sites

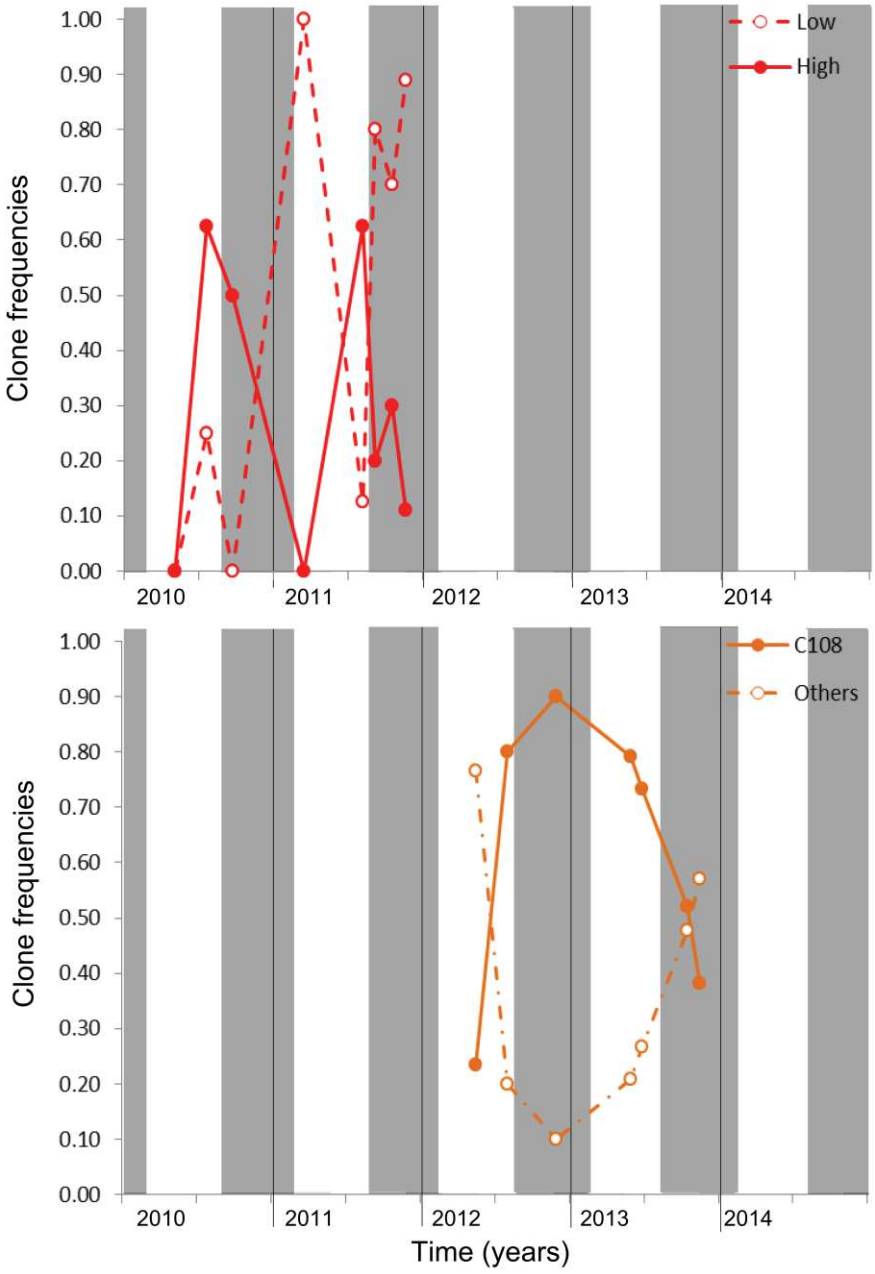

Figure 6. Frequency variations of clone groups over the seasons. Site 9 is in red (top panel) and PR in orange (bottom panel). Continuous lines correspond to the group of clones with low or high temperature respectively in S9 or PR ($x_{coord.} < 0$ and $y_{coord.} > 0$ in Figure 4). Dashed lines correspond to the group of clone with high and low temperature respectively in S9 or PR ($x_{coord.} < 0$ and $y_{coord.} < 0$ in Figure 4). Seasons are highlighted by the variation of grey (white: spring and summer; grey: autumn and winter).

from further analysis. Figure 5 shows the frequencies of clone clusters from high (continuous line) and low (dashed line) values of environmental variables, along sampling times. Even though we had information from spring 2010 to summer 2013, we restricted this plot to before 2012, due to the invasion of the site by *A. franciscana*. Indeed, starting in 2012, the proportion of *A. franciscana* is almost constantly 100% (Figure 1A), so the sample size of *A. parthenogenetica* during this period was too small. During the 2010-2012 period, frequencies of the clone groups isolated in S9 (Figure 6) present cyclical variations. We observed that the clone cluster from the 'high' environments had higher frequencies in summer (end of the white shaded area) or in autumn (beginning of the grey shaded area). On the other hand, the clone cluster friom the 'low' environments had higher frequencies in winter and spring (end and beginning of respectively grey and white areas). Highest and lowest frequencies therefore matched with the corresponding season.

In PR, we showed in Figure 5 that 108 individuals corresponded to a single clone (here after C108) that was present in all collected samples. When the frequency of this clone was plotted against the others (Figure 6), it did not correlate with seasonal variations.

Discussion

Clonal diversity in Mediterranean salterns

The structuration observed between the Odiel saltern population and the two French saltern populations can be linked with the distance that separates them: Odiel is in southern Spain (near from the strait of Gibraltar), while Aigues-Mortes and Salin de Giraud are in the Camargue delta in southern France. Most of *Artemia*'s long-distance dispersal is due to cyst transportation by birds (Munoz *et al.* 2013), and it has been shown that bird migration does not have the same intensity in every direction between Mediterranean salterns. Indeed, the bird exchange is much higher from Camargue to Fuente de Piedra (close to Odiel) than in the opposite direction (Nager *et al.* 1996; Balkiz *et al.* 2010). This potential asymmetrical gene flow between populations may explain the structuration observed between these two locations. It is interesting to note that the structuration is maintained even

though there is no structuration between flamingo populations (Geraci *et al.* 2012).

Diversity and structure in the clonal population of Aigues-Mortes

Geographical structuration

The lack of structuration between most sites of the saltern, regardless of the season, and in spite of strong gradients of salinity, indicates that there is a huge impact of human activity. The modern usage of pumps to move the water from one part of the saltern to another is probably the main reason for this lack of structuration. Some sites from our samples were isolated from the general water circulation, and showed genetic differentiation. These results are similar to previous study on Salin de Giraud (Browne & Hoopes 1990), where no was found structuration between sites of the saltern, except for one isolated site. This site had high salinity and its location indicated that it was probably used to drain surface rain water from the salt pans (like S9 and Saint-Louis in Aigues-Mortes).

It would be interesting to further monitor the Odiel saltern which is no longer exploited, to compare its geographical structuration with the result found in Aigues-Mortes. Indeed, if the lack of structuration in the two exploited saltern is caused by human activity, we would predict a greater structuration between sites in the Odiel saltern, provided the environment is also highly heterogeneous in space. Our present genetic data (70 individuals spread over 5 sites) and the lack of any environmental data do not allow us to further probe this hypothesis.

Temporal structuration

In the connected sites, we found the same lack of temporal structuration as for geographical structuration (Figure S2). In Figure 4, we observed cyclical rise and fall of the clusters' frequencies. Same clones were maintained in the saltern thus, the genetic polymorphism is maintained in the Aigues-Mortes saltern. This matches well with the second regime described in the introduction, where polymorphism is maintained due to differences in the niches of the lineages. In this regime, we expected clones to be associated with specific conditions. In the case of the connected sites, the cyclicality observed in cluster frequency variations had larger period than the seasonal

variations in environmental variables. Some other environmental variations (e.g. prevalence in parasites) must be monitored to better understand this pattern.

In PR, this pattern was mostly driven by the presence of a very frequent clone (C108), whose frequency increased and then decreased from spring 2012 to summer 2013. However, this did not match with the variations in the environmental factors we tested. This temporary invasion of the PR pound by C108 might be linked to another environmental factor. For example, parasites infections (like microsporidia; Rode *et al.* 2013) also present variations in prevalence. It would be interesting to test whether C108 is sensitive or not to such parasites and if its predominance in the PR pound correlates with the prevalence of these parasites.

In S9, we were able to isolate groups of clones whose frequencies variation in the population correlate with seasonal variations in temperature, salinity and proportion of *A. franciscana* (Figure 6). These pattern was observed for two consecutive seasons before the pound was overcome by *A. franciscana*. In the lab, iso-clonal lineages of both groups of clones have been isolated (low temperature named PAM5 and PAM10; high temperature PAM6 and PAM7). Further lab experimentations on these isolated candidates would help us better characterize the niche limits of those clones.

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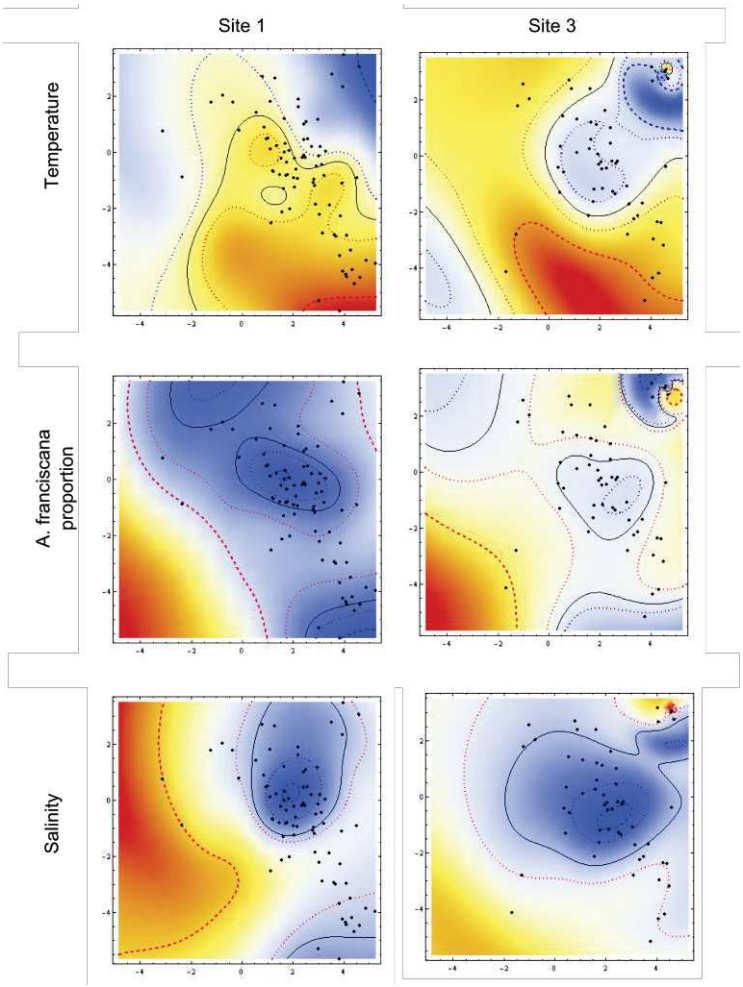
Supplementary Material

Figure S1 - Sites location on a Google map of the Aigues-Mortes saltern.

The main water network is depicted with white lines. The water flows from the sea (bottom right of the map) to the salt pans (pink squares in the middle top part of the map). The salinity gradient is also visible as ponds with low salinities present blue/green waters while ponds with high salinities present orange/pink waters.

Site 1: 43°29'53"N, 4°14'23.1"E; Site 3: 43°31'2.6"N, 4°14'29.5"E; Site 4: 43°32'24.6"N, 4°13'25.9"E; Puits Romain: 43°30'17.8"N, 4°13'27.2"E; Pont de Gazette: 43°31'4.6"N, 4°10'48.6"E; Site 8: 43°31'37.2"N, 4°10'37.8"E; Site 9: 43°32'40.3"N, 4°09'16.6"E; Site 10: 43°32'40.1"N, 4°09'17.2"E; Site 12: 43°31'55.7"N, 4°10'23.9"E; Saint Louis: 43°32'56.8"N, 4°10'8"E.

Figure S2 - Interpolation maps of environmental factors varying along genetic coordinates of individuals from two connected sites. Columns correspond to sites, lines corresponds to environmental factors. In the colors gradient, high values of the environmental factor are presented in red while blue corresponds to low values. Contour lines represent the 5% lowest (dashed blue), 25% lowest (dotted blue), 50% (continuous black), 25% highest (dotted red) and 5% highest (dashed red) of the distribution.



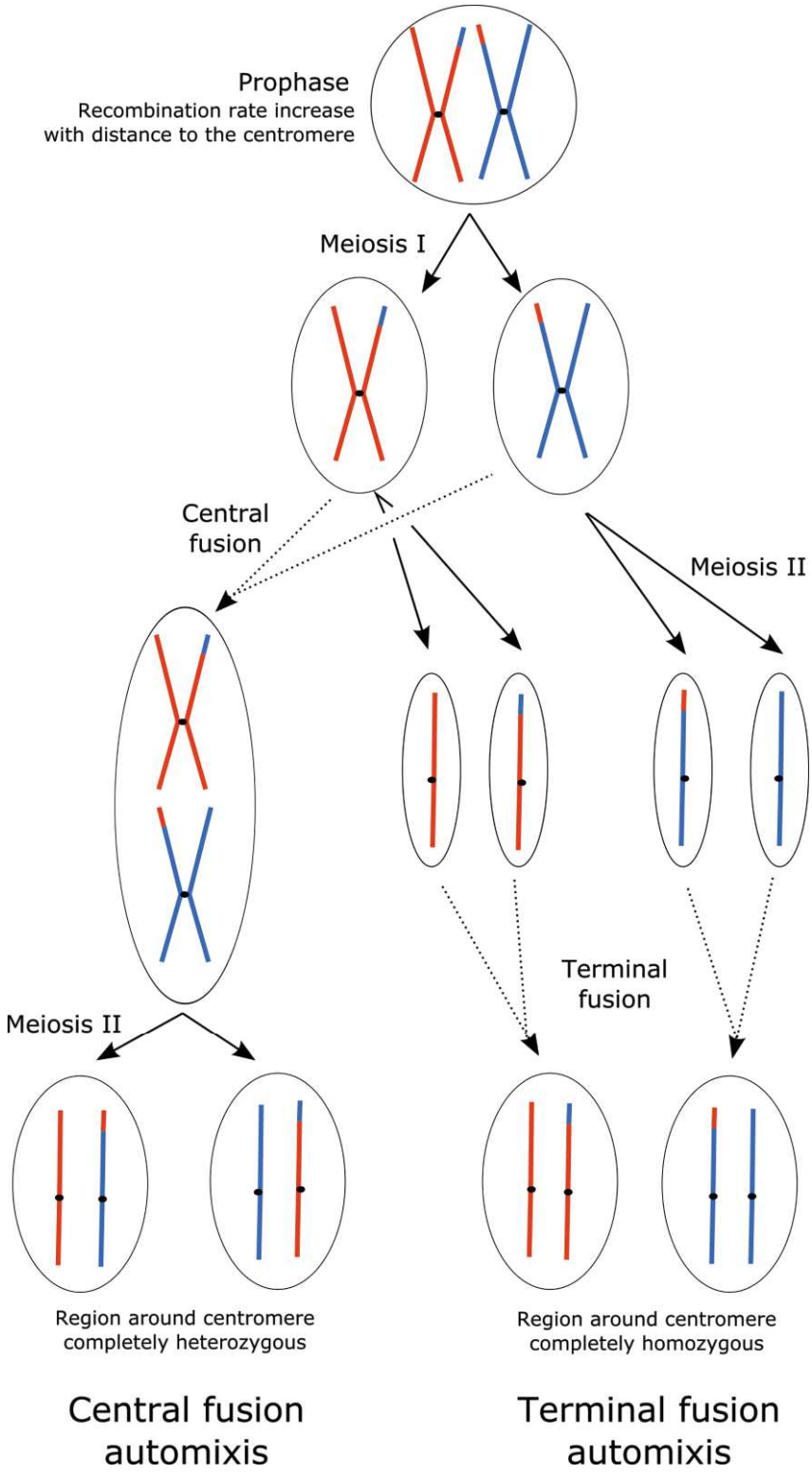


Figure 2.1 : Schéma des méioses modifiées pour l'automixie avec fusion centrale ou terminale (adapté d'après Stenberg & Saura 2009)**.** Dans le cas de fusion centrale, on attend 100% d'hétérozygotie aux centromères et 66% d'hétérozygotie aux télomères. Dans le cas d'une fusion terminale, on attend 0% d'hétérozygotie aux centromères et 66% d'hétérozygotie aux télomères.

2.2 Résultats complémentaires

2.2.1 Reproduction d'Artemia parthenogenetica

L'analyse de la structure génétique d'*A. parthenogenetica* nous a amenés à nous pencher sur l'hétérozygotie de nos marqueurs microsatellites, qui bien entendu dépend fortement du système de reproduction. Mais il se trouve que depuis plus d'un siècle, le mode de reproduction des artémies asexuées diploïdes fait débat. Les données cytologiques n'ont pas réussi à départager différents régimes d'automixie (reproduction avec méiose modifiée). Or pour chacun de ces régimes, il est possible de faire des prédictions sur l'excès ou le déficit en hétérozygote. La comparaison du Fis (l'écart de fréquence d'hétérozygotes par rapport aux attendus d'Hardy-Weinberg) entre locus microsatellites d'une part, et entre deux salins de l'ouest méditerranéen (Odiel et Aigues-Mortes) d'autre part, nous a permis d'apporter des éléments de réponse importants à cette question (Annexe 2). En effet, les valeurs de Fis obtenues sont très fortement hétérogènes entre locus, mais corrélées entre salins. Ce parton particulier nous a permis de conclure que le régime de reproduction le plus probable des *A. parthenogenetica* diploïdes est de l'automixie avec fusion centrale (fusion de produits issus de la première division de méiose) avec un faible taux de recombinaison, qui préserve 100% de l'hétérozygotie au niveau du centromère, et réduit l'hétérozygotie vers les télomères (Figure 2.1).

2.2.2 Impact d'une invasion sur la structuration passée

Afin d'étudier la structuration génétique passée de la population d'Aigues-Mortes, ainsi que l'impact de l'invasion par *A. franciscana* du salin, nous nous sommes appuyés sur la propriété des cystes de résistance à la conservation dans les sédiments (voir 1.3.1). Il s'agissait de récupérer des cystes du passé dans les sédiments, puis de les caractériser génétiquement afin de suivre les variations des fréquences alléliques au cours du temps. Ceci requiert de lever deux verrous techniques (Annexe 3) :

- Récolter et analyser les sédiments dans lesquels les cystes sont contenus,
- Développer de nouveaux marqueurs permettant la caractérisation génétique des populations du passé malgré la dégradation de leur ADN.

La récolte et la caractérisation temporelle des sédiments nous ont demandé une collaboration étroite avec des géologues. Nous avons pu ainsi combiner des techniques de datation relatives et absolues. Les premières nous ont permis de mettre en évidence une cyclicité qui distingue des périodes d'immersions et d'émersions de la zone prélevée, dont trois couches d'ensablement rapide qui nous ont permis de corréler les différents prélèvements sédimentaires, mais aussi de placer des points temporels absolu grâce aux relevés météorologiques. Les secondes techniques nous ont permis de montrer que les sédiments prélevés remontaient jusqu'avant l'introduction d'*A. franciscana* ce qui nous permet d'envisager l'étude complète de cette invasion sur la structuration des populations locales. Au total, nous avons montré que les sédiments prélevés s'étalent sur une durée entre 20 et 30 ans du début des années 1960 aux années 1980/1990.

Les données biologiques, outres de répondre à nos questions concernant l'invasion du salin par A. franciscana, permettront de compléter cette échelle temporelle. Notamment, la date de première introduction d'A. franciscana dans le salin que nous connaissons par ailleurs (Rode *et al.* 2013). Les premiers relevés que nous avons faits ont néanmoins montré une forte dégradation de l'ADN dans les cystes enfouis. Nous avons donc cherché à développer de nouveaux marqueurs adaptés à cette problématique.

Ces nouveaux marqueurs SNPs nous permettront de suivre par une technique de séquençage massif l'invasion des sédiments par les cystes d'*A. franciscana* ainsi que les variations en fréquences de loci diagnostiques des différents clones d'*A. parthenogenetica.* Nous espérons ainsi pouvoir mieux caractériser l'impact de l'invasion de l'espèce sexuée sur les lignées locales. D'autre part, les cystes introduits dans le salin ont deux origines : la baie de San Francisco (Californie) et le grand lac salé (Utah). Ces deux populations sont divergentes génétiquement (Muñoz *et al.* 2010; Annexe 2). Il serait donc intéressant d'observer l'impact de leur mélange au sein de la population d'Aigues-Mortes.

Chapitre 2

Flore intestinale et tolérance aux faibles salinités chez l'artémie

Nous avons ensuite cherché à caractériser les mécanismes qui limitent la niche écologique. Pour commencer, nous nous sommes intéressés au "low salinity paradox" (voir 1.4.2.1) que l'on peut résumer ici par l'incapacité des artémies à survivre dans des eaux saumâtres ou douces. Sur le plan physiologique pourtant, les artémies semblent capables de vivre aux faibles salinités (voir 1.3.3).Comme nous le décrivions en introduction (Figure 1.2), les artémies se nourrissent majoritairement d'algues unicellulaires dans le salin, mais aussi au laboratoire (où, même en l'absence de prédateurs, les artémies ne peuvent survivre dans des saumures à moins de 40g/L ; Castro-Mejía *et al.* 2011). A l'instar de l'ensemble des herbivores, les artémies ont donc très probablement associées à une flore intestinale, leur permettant de digérer ces algues. D'autre part, le seul article traitant du sujet indique que la composition de la microflore intestinale des artémies varie avec la salinité (Tkavc *et al.* 2011).

Pour étudier l'impact de la microflore sur l'adaptation des artémies, un verrou technique essentiel a dû être levé. En effet, pour étudier les interactions entre organismes, une méthode classiquement employée est de comparer les niches jointes et disjointes de ces organismes. Dans le cas d'interaction avec des microorganismes symbiotiques est difficile pour deux raisons principales. Tout d'abord, le caractère plus ou moins obligatoire de la symbiose peut rendre extrêmement difficile la séparation des interacteurs. Ensuite, il a été montré que seule une faible partie des bactéries présentes dans l'environnement peuvent pousser sur les milieux utilisés en laboratoire. Ceci rend particulièrement difficile l'étude de la niche de ces micro-organismes. Dans le cas de l'artémie, nous sommes parvenu à produire des nauplii axéniques (stériles et donc sans micro-organismes). En effet, bien que produisant des cysts au stade gastrula, il n'y a pas de transmission verticale (des mères aux petits) de la flore intestinale. L'éclosion et le maintien de nauplii dans des conditions stérile permet donc d'étudier la niche de l'artémie en absence de leur flore intestinale. Nous avons alors pu réaliser une expérience réalisée au laboratoire nous a permis de comparer la survie de nauplii axéniques et non-stériles, nourris avec des algues ou de la levure, dans de l'eau très faiblement salée (5g/L) et de la saumure (80g/L). Pour l'étude la niche de leur flore intestinale, nous avons utilisé les croissances bactériennes en milieu liquide (moins sélectif que les milieux solides), en parallèle pour la communauté intestinale et pour une bactérie

candidate qu'il est possible d'étudier au laboratoire. L'ensemble des résultats obtenus nous a permis de montrer que le "low salinity paradox" est lié à une incapacité de la microflore intestinale à survivre pour des salinités plus faibles que 40g/L.

Cette découverte nous a amené à réfléchir sur l'impact des interactions biotiques sur la niche de leur hôte. En effet, dans le cas des artémies, leur microflore intestinale augmente le nombre de ressources qu'elles sont capable de digérer, mais limite leur adaptation aux faibles salinités. Nous nous sommes alors engagés dans une revue plus large du problème de l'impact des symbiontes sur la niche de leur hôte. Plus précisément sur les effets parfois opposés que la symbiose a, sur différents axes de la niche de l'hôte. A notre connaissance, cette idée n'avait jamais été explicitement exposée et discuté auparavant dans la littérature, même dans la seule étude exposant un patron similaire dans le cas d'une interaction obligatoire (Dunbar *et al.* 2007). Nous avons ainsi proposé une nouvelle manière d'appréhender l'effet des interactions positives sur la niche de l'hôte (Problème 4).

Article 2
Niche limits of symbiotic gut microbiota constrain the salinity tolerance of brine shrimp

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Abstract

Symbiosis generally causes an expansion of the niche of each partner along the axis for which a service is mutually provided. However for other axes, the niche can be restricted to the intersection of each partner's niche, and can thus be constrained rather than expanded by mutualism. We explore this phenomenon using *Artemia* as a model system. This crustacean is able to survive at very high salinities, but not at low salinities, although its hemolymph's salinity is close to freshwater. We hypothesized that this "low salinity paradox" results from poor performance of its associated microbiota at low salinity. We showed that in sterile conditions, *Artemia* had low survival at all salinities when algae were the only source of carbon. In contrast, survival was high at all salinities when fed with yeast. We also demonstrated that bacteria isolated from *Artemia*'s gut reached higher densities at high than at low salinities, including when grown on algae. Taken together, our results show that *Artemia* can survive at low salinities, but their gut microbiota, required for algae digestion, have reduced fitness. Widespread facultative symbiosis may thus be an important determinant of niche limits along axes not specific to the mutualistic interaction.

Key words: Symbiosis, mutualism, *Artemia franciscana*, adaptation, salinity.

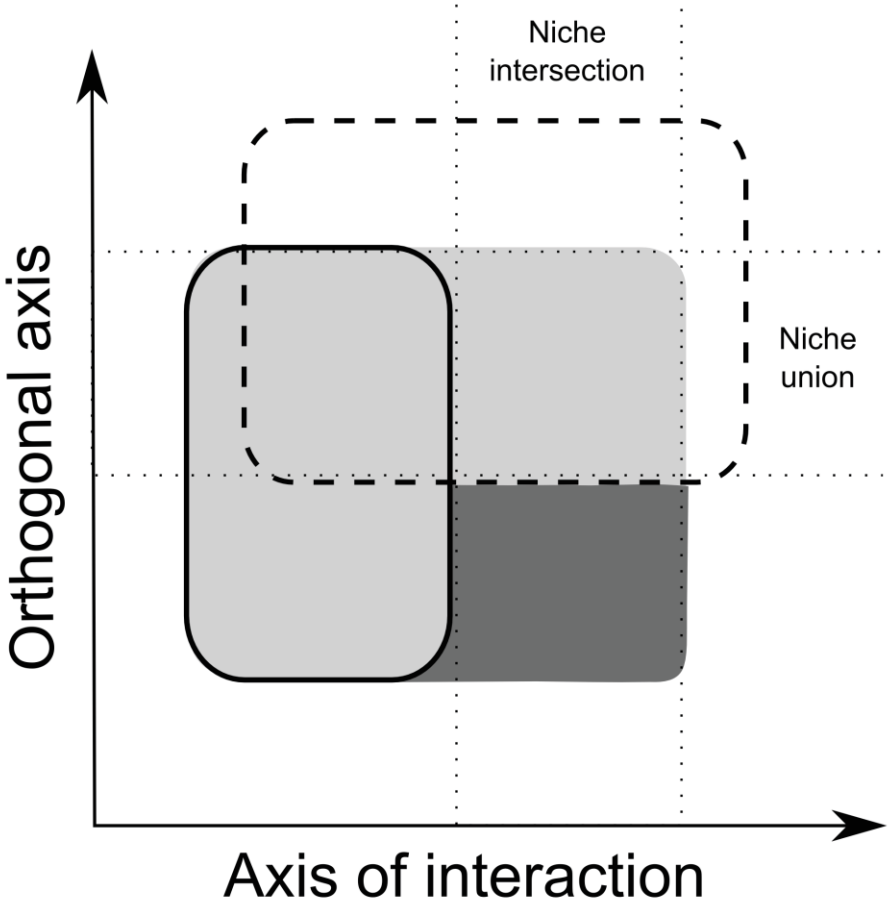

Figure 1: Effect of mutualism on niche limits. The niche of a focal species is represented in presence (light shaded area) or absence (continuous line) of its mutualistic interactor, whose niche is delimited by a dashed line. The X-axis represents environments where the focal species can benefit from a service traded in the mutualism (the service is useless on the left, but becomes increasingly beneficial on the right). In our study system, the focal specie is the host (*Artemia*). The environments on the left are environments with a type of food which can be digested by *Artemia* in absence of microbiota (yeast), while environment on the left represent environments where the available food requires the presence of microbiota to be digested (algae). More generally, services traded can be as diverse as defense against predators/pathogens, provision of nutrients or improved tolerance to some abiotic factors, among others (see introduction). In general, this service differs among interactors (for instance the reciprocal service from *Artemia* to microbiota is probably not related to algae digestion). In environments tolerated well by both partners, the mutualism causes a niche expansion along the axis of interaction (the light shaded area expands to the right), resulting in niche union. The Y-axis represents environmental variables that are not directly related to the service traded in the mutualism for the focal species ("orthogonal axis"). In our case, it represents the salinity gradient (low salinity at the bottom). Along these orthogonal axes, the mutualism can cause a constrain on niche extension (dark shaded area), in environments where the host needs the service traded by the mutualist. In these environments, the niche of the symbiont and the host cannot exceed the intersection of each of their niches when isolated.

Introduction

The fundamental niche of a genotype results from its adaptation to its environment, meaning that natural selection has optimized a suite of traits so as to favor increased reproductive abilities in this environment (Antonovics 1976). Implicit, but central to this view, is the idea that this process occurred by genetic changes (beneficial mutations or combination thereof) in the line of descent to this genotype (Antonovics 1976; Barton and Partridge 2000; Holt 2009). However, this view of niche evolution becomes necessarily limited when dealing with organisms that have a mutualistic partner. The niche of an organism then is not only the product of its own genes, but also an extended phenotype of the genes of its partner (Dawkins 1983), such that the (co)evolution of each partner will affect the other partner's niche (Saffo 1992; Case et al. 2005). This mutual functional and evolutionary dependence further entails that mutualistic partners not only expand each other's niches through provision of specific services, but also share each other's niche limitations along niche axes unrelated to their interaction. On these axes, the resulting niche is the intersection of the niches of each partner, rather than their union as for the axis of the interaction (see fig.1). Here, the words 'union' and 'intersection' are used as in set theory (for two sets A and B, intersection $A \cap B$ versus union $A \cup B$), consistent with their use in the classical fitness set theory (Levins 1962). This phenomenon may often go unnoticed, as it involves dimensions of the niche that are not directly related to the mutualistic partnership, but it may be an important determinant of the niche of a broad variety of organisms. In particular, it is important to understand whether it is restricted to the somewhat specific case of obligate mutualisms, or whether it also matters for the much broader class of non-obligate mutualisms, such as that between animals and their gut microbiota.

It is well known that symbiotic associations impact the ecological niche. Positive biotic interactions are often thought to mostly cause an extension of the niche (Bruno et al. 2003; Afkhami et al. 2014), afforded by the services traded by mutualistic partners, such as: provision of nutrients (Paul et al. 2007; Ley et al. 2008; Akman Gündüz and Douglas 2009), resistance to the abiotic and biotic (pathogens/predators) environment (Mueller et al. 2011; Koch and Schmid-Hempel 2012), among others. Research on mutualism even led to the idea of a holobiont, an emergent supra-organism encompassing a host and its community, as opposed to

the focus on individual “focal species” (Margulis 1998; Feldhaar 2011; Hansen and Moran 2013).

Extension of the niche along the interaction axis is sometimes demonstrated by a reduction of the corresponding environmental range after removal of the symbiont (Boettcher et al. 2000; Zimmer et al. 2002; Pike and Kingcombe 2009; Rosengaus et al. 2011). But in fact, there are also costs to mutualistic interactions, as revealed by the loss of symbionts when their benefit decreases (Thrall et al. 2008). These costs include producing one good to trade for another (e.g. nectar production for pollen transportation, Bronstein 2001), or susceptibility to cheaters (e.g. opportunists using goods without reciprocity, Case et al. 2005), among others. One such cost, on which we focus here, concerns adaptation on niche axes not directly involved in the interaction. Being dependent on a symbiont may entail a constraint on niche extension along orthogonal axes, in conditions not tolerated by the symbiont. A general illustration of this phenomenon is given in fig.1, where the niche of a focal species is represented in presence (light shaded area) or absence (continuous line) of its mutualist interactor, whose niche is also delimited by a dashed line. Along the vertical axis (not directly involved in the interaction), the niche of an organism cannot exceed the intersection between its niche in isolation and that of its partner. This can cause a constraint on niche extension along this axis (dark shaded zone in fig.1). Overall, the mutualism effect thus combines an extension along the niche axis of the traded services, and a constraint on niche extension along other axes.

Although similar ideas have been discussed in the literature (Bronstein 2001; Case et al. 2005; Hansen and Moran 2013), the conceptualization of the ecological impact of mutualisms as a combination of niche union (along the axis that corresponds to the mutual service) and intersection (along other axes) has rarely been articulated explicitly. Furthermore, empirical studies addressing related questions rarely provide a comprehensive view of the process based on manipulative evidence. For instance, climate change or biological invasions cause range mismatches between mutualistic partners (Parker 2001; Stanton-Geddes and Anderson 2011; Jevanandam et al. 2013; Warren and Bradford 2013), leading several authors to recently call for more systematic integration of biotic interactions in the study of niche limits under climate change (Case et al. 2005; Travis et al. 2006; Sexton et al. 2009; Gilman et al. 2010). However, most studies of range mismatches rest on correlative evidence (Montllor et

al. 2002; Ness et al. 2004; Ferrari et al. 2012; Hansen and Moran 2013), and geographic ranges can only inform us on ecological niches for species that are at equilibrium with respect to climate (Araujo and Pearson 2005). In other words, a crucial step that is generally lacking in such studies is the demonstration that the absence of the mutualist results from its maladaptation to the local environment (rather than historical or anthropic contingency e.g. pollinator in Kjellberg and Valdeyron 1990). More direct evidence comes from studies showing in the laboratory that the environment can destabilize a symbiotic interaction, causing a decrease in host fitness. Several studies investigate the effect of symbiont removal on host survival, under different types of environmental stresses (e.g. oil pollution: Newton and McKenzie 1995; antibiotics: Rosengaus et al. 2011; Willing et al. 2011; or high temperature: Rosenberg et al. 2007; Rosenberg et al. 2009; Prado et al. 2010; Wernegreen 2012). These studies clearly point out that environmental stresses have the potential to disrupt mutualistic interactions, with large phenotypic impact on the host. However, they do not partition the effects of such stresses on both the symbionts and the hosts. To our knowledge, Dunbar et al (2007) is the only study that fully demonstrated constraints on the niche of a host (for dimensions other than the mutualistic service) caused by lack of adaptation of its mutualistic symbiont. The authors showed, by switching obligate symbionts (*Buchnera*) in aphid hosts, that a point mutation in the symbiont's genome reduced the thermal tolerance of their host. But in general, even though niche intersection effects are likely to occur in many systems, they remain poorly documented: studies on facultative symbionts usually lack a partition of environmental effects on each of the partners, while in obligatory symbiosis the niches of each partner are by definition difficult to study independently.

In this paper, we investigate the influence of symbiotic microbes on niche limitations, using the brine shrimp (*Artemia*) as a model system. *Artemia* are Branchiopoda that live in continental hypersaline environments, from salt marshes to lakes, where their abiotic niche is strongly structured by salt concentration (van Stappen 2002). This predominant axis of their niche offers a puzzling and unexplained situation, which can be labeled the "low salinity paradox". Brine shrimps are not found *in natura* at salinities below 40g/L (Lenz and Browne 1991). This distribution is often explained by the presence of fish predators at low, but not high salinities (Camargo 2002; Litvinenko et al. 2007). While this is certainly an important factor in the wild,

experiments also show that *Artemia* have strongly reduced juvenile survival at low salinities in the laboratory without predators, which probably contributes significantly to their niche limits (Abatzopoulos et al. 2003; Baxevanis and Abatzopoulos 2004; Castro-Mejía et al. 2011). The underlying mechanism could be that brine shrimps have a low physiological tolerance to low salinity, like most marine organisms. However, *Artemia* conserved their ancestral near-freshwater hemolymph thanks to very efficient Na/K-ATPase pumps that regulate inner medium in the face of salinities up to 140g/L (Holliday et al. 1990; Weekers et al. 2002). Hence, we do not expect a physiological cost at low salinities for the brine shrimp. On the contrary, low salinity induces lower salt excretion, and thus lower ATP consumption, which should cause less metabolic cost relative to high salinity. From the physiological standpoint, there is thus a paradox. Given the biology and evolutionary history of brine shrimps, what causes *Artemia* to not tolerate well low salinities?

Brine shrimps feed mostly on unicellular algae, such as *Dunaliella salina*, found in salt marshes and lakes (Lenz and Browne 1991). Most animals are unable to feed on such algae without a specialized microbiota that provide them with essential nutrients (vitamin or amino acid), help them digest complex molecules (e.g. long carbohydrates such as cellulose), or eliminate toxins (Burroughs et al. 1950; Karley et al. 2002; Brune and Ohkuma 2011). Salinity in brine shrimp guts is the same as in the external environment (Geddes 1975; Holliday et al. 1990), and can thus directly affect growth and survival of microbiota. Here, we hypothesize and investigate whether poor adaptation of their gut microbiota causes the poor performance of brine shrimp at low salinity; in other words, whether the fundamental and abiotic niche of *Artemia* results primarily from the process of adaptation of its gut microbiota, rather than of its own genome.

Material and methods

Artemia survival experiment

Artemia franciscana is a sexual species of brine shrimp from North America. Like all species of this genus, it can produce both active larvae (nauplii) and encysted diapause eggs (cysts), depending on environmental conditions. Cysts are produced as resistant forms, and can hatch into nauplii larvae after a dormancy period ranging from few days to several years. In our experimentations, we used *A. franciscana*

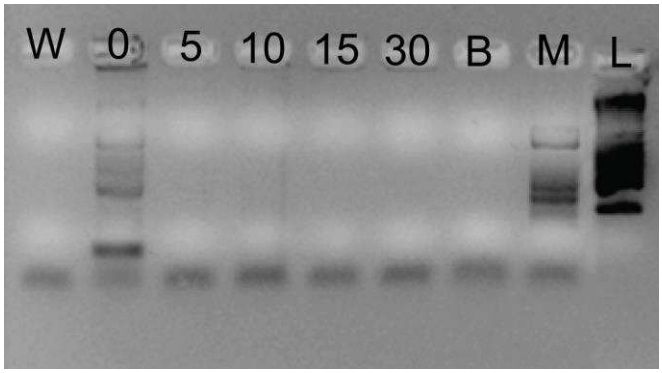

Figure 2: Test for vertical transmission in cysts. **W** and **B** denote negative control with sterile water and brine, respectively, while **M** indicates the positive control of the non-sterile hatching media. Numbers (**0**, **5**, **10**, **15** and **30**) indicate the duration of the bleach treatment on cysts in minutes. **L** is for the ladder. Our negative controls showed no amplification, confirming the absence of contaminant DNA in our PCR mix. Our positive control displayed several fragments of different sizes, demonstrating the efficiency of our primers.

cysts sampled in 2007 from the Great Salt Lake (Utah, USA; hereafter GSL07). Great Salt Lake *Artemia* were massively introduced in Aigues-Mortes salt ponds (Languedoc-Roussillon, France) in 1979 (Rode et al. 2013b) and are now coexisting in the salt ponds with parthenogenetic endemic populations. These cysts were provided by the Artemia Reference Center (reference ARC1710).

Axenic culture and microbiota transmission. We first determined whether bacteria could be vertically transmitted through cysts. As cysts are at the gastrula development stage, it is indeed possible that vertical transmission of gut microbiota occurs from the mother *Artemia* directly into the gastrula. To test this, we removed the chorion layer that acts like a shell and protects the cysts. This operation, called decapsulation, consists in soaking cysts in bleach, and is often used to improve hatchability in brine shrimp cultures (Clegg 1986), but here our aim was to eliminate bacteria that would be on the surface of the chorion. We performed this experiment for 0 to 30 minutes, by increments of 5 min until all cysts took a light orange color. In order to evaluate whether some bacteria were still present after decapsulation, we performed PCR tests using universal bacterial primers (63f: 5'-CAG GCC TAA CAC ATG CAA GTC-3', Marchesi et al. 1998; B6r: 5'-TTG CGG GAC TTA ACC CAA CAT-3', Manceau and Horvais 1997) on crushed decapsulated cysts (30µL Phusion mix, 6µL sterile water, 1µL of each primer and 2µL of crushed decapsulated cysts; 94°C for 3min, 35 cycles of 94°C for 30s, 48°C for 45s and 72°C for 1.5min, finally 72°C for 7min; fig.2).

Survival experiment. To determine the influence of microbiota on the salinity tolerance of *Artemia*, we first measured juvenile survival in an experiment involving four treatments, at both high and low salinities. Previous studies indicated that juvenile survival is a strong determinant of variation in overall fitness. For instance, using a full demographic projection model, Sukumaran and Grant (2013) demonstrated that the elasticity of population growth rate (Caswell 2001) is strongest with respect to juvenile survival in *A. franciscana*. More specifically, *A. franciscana*'s low performance at low salinity was shown to be due mostly to high juvenile mortality (Castro-Mejía et al. 2011). Technically, investigating niche limits would require determining environmental conditions where the population growth rate becomes negative. This is not possible under laboratory conditions that do not include the effects of density dependence and resource fluctuation, of predator or of parasites,

which all largely reduce demographic performances. However, if large differences in juvenile survival are found in the laboratory, they are likely to also have a large demographic impact in the field. Furthermore, the functional importance of microbiota on juveniles is expected to carry over on adults that have similar diet, thus also affecting survival also in later ages.

We did a full factorial experiment with conditions being axeny (sterile or non-sterile), diet (algae or yeast) and salinity (low or high). When brine shrimps were grown with autoclaved algae as a food source, in standard non-axenic conditions, we expected to observe the low salinity paradox, i.e. that brine shrimps survive less at low salinity under “natural” conditions. When brine shrimps were grown with the same food source, but this time in axenic conditions, we expect very poor performance at either low or high salinity if gut microbiota are important for algae digestion. Finally, processed yeasts are easily digestible, and in particular do not require gut microbiota to be assimilated (Coutteau et al. 1990). If gut microbiota cause the low salinity paradox, we expect high survival at both salinities with yeast food source, regardless of sterility condition. However, as yeast is not a natural food source of *Artemia*, and can also be used as a food source by bacteria, we do not have strong expectation regarding the relative performance of axenic vs. non-axenic conditions with yeast as a food source (yeast may either interact positively or negatively with microbiota in non-axenic conditions).

GSL07 cysts were hatched in sterile conditions (Makridis et al. 2000; Dhont and Sorgeloos 2002). Cysts were hydrated for 1h in sterile water, and decapsulated using bleach for 10 min (*i.e.* all chorion layer were removed). They were then rinsed in sterile water, and placed in a 400 mL autoclaved solution of brine (5g/L) containing a small amount of *Dunaliella salina* algae. Cysts were left to hatch in the sealed bottle for 3 days at ambient temperature under continuous light. Hatched nauplii were then placed in sterile and non-sterile brine solutions and fed with sterilized food source: *Dunaliella salina* or *Saccharomyces cerevisiae* (Superlevure, Gayelord Hauser). Brine solutions at 5g/L and 80g/L were obtained by diluting saturated brine (250g/L) collected directly in the Aigues-Mortes salt ponds with osmosed water. In the sterile treatments, these brine solutions were autoclaved and checked for salinity before and after sterilization. Algae and yeast food solutions were autoclaved independently; 300mL of those food solutions were added for 1L of sterile or non-sterile brine

Table 1: Generalized Linear Model selection for *Artemia* survival experiment. *Saturated* model and *AIC best model* helped estimating the over-dispersion parameter value. **K** is the number of parameters for each model, **logLik** the log likelihood, **AIC** the Akaike information criterion, **QAIC** the quasi-likelihood AIC corrected for over-dispersion (where the over-dispersion estimate ĉ = 2.23), **ΔQAIC** the QAIC differences, w_i the Akaike weights. **Parameters** of models are Salinity (high or low), Food (algae or yeast), Axeny (sterile or non-sterile), Date (experimentation date), Light (five levels of light condition), Plate (position of the plate in the experiment) and Tube (tube number in the experimentation).

Model	K	logLik	AIC	QAIC	ΔQAIC	w_i	Parameters
Best QAIC model	18	-703.50	1443.00	666.63	0.00	0.58	Axeny x Salinity + Axeny x Food + Plate + Date
Double interactions	19	-703.36	1444.73	668.50	1.87	0.23	Axeny x Salinity + Axeny x Food + Food x Salinity + Plate + Date
Full interaction + Plate + Date	20	-703.10	1446.20	670.27	3.64	0.09	Axeny x Food x Salinity + Plate + Date
Best AIC model	25	-693.99	1437.97	672.10	5.48	0.04	Axeny x Food + Axeny x Salinity + Date x Plate
Double interactions + Plate x Date	26	-693.83	1439.67	673.96	7.33	0.01	Axeny x Food + Axeny x Salinity + Food x Salinity + Date x Plate
Best QAIC model + Light	22	-702.85	1449.70	674.05	7.42	0.01	Axeny x Salinity + Axeny x Food + Plate + Date + Light
Full interaction + Plate x Date	27	-693.52	1441.03	675.68	9.05	0.01	Axeny x Food x Salinity + Date x Plate
Full interaction	8	-790.25	1596.50	724.39	57.76	0.00	Axeny x Food x Salinity
Independent effects	4	-824.52	1657.04	747.11	80.48	0.00	Axeny + Food + Salinity
Axeny	2	-836.35	1676.71	753.72	87.09	0.00	Axeny
Food	2	-1012.26	2028.52	911.41	244.78	0.00	Food
Salinity	2	-1017.86	2039.72	916.43	249.80	0.00	Salinity
Saturated	400	-275.65	1351.31	1040.54	387.00	0.00	Date x Tube

solutions. This ensured that the sterile and non-sterile conditions did not differ in terms of possible denaturation of food source by the autoclave. For each treatment, 6 nauplii per tube were transferred in 25 sterile 50 mL Falcon tubes containing 40 mL of brine solutions. This transfer was carried out under a laminar flow hood to preserve sterile conditions. The tubes were closed to maintain sterility, leaving enough air to allow respiration for the duration of the experiment. The full experiment was carried out twice at two different date to ensure reproducibility. In each experimental repeat, a total of 900 nauplii were evenly distributed among the nutrition treatments (non-sterile algae, sterile algae and sterile yeast) and salinities (5g/L or 80g/L). Tubes were placed vertically and randomly, using a Latin square design (Saville and Wood 1991), and incubated for four days at 25°C and 12-hour daylight. At the end of this period, tubes were emptied in a net (120-µm mesh), and surviving nauplii were counted under a binocular.

Statistical analysis on nauplii survival. Data from the two replicated experiments were analyzed jointly. We used generalized linear models (glm) with binomial error distributions to test our hypothesis about the influence of axeny, diet and salinity on the survival of nauplii 8 days after hatching. Several possibly confounding factors might have an influence on our results, and were included in our model selection. First, there may be an effect of experimentation date or plate position (one parameter per stove level). We also controlled for an effect of light, with five levels depending on the distance of tubes to the light source. Model selection was performed in R (R2.14.2, www.r-project.org/) using the MuMIn package (Barton 2013), and was based on quasi Akaike information criterion, QAIC (Akaike 1974; Peng et al. 2006). QAIC corrects for potential overdispersion in the data. Overdispersion estimate (ĉ) was calculated from the residual deviance between the best model (based on AIC score) and a saturated model where one survival probability is estimated per tube. Results from this model selection are presented in table 1 and fig.3. To determine the significant differences in survival among conditions in the best model, we performed pairwise post-hoc comparisons based on two-tailed Z-tests (see Supplement 1), penalizing for multiple testing (Bonferroni correction). This analysis indicated which combinations of axeny, diet and salinity were significantly different from each other.

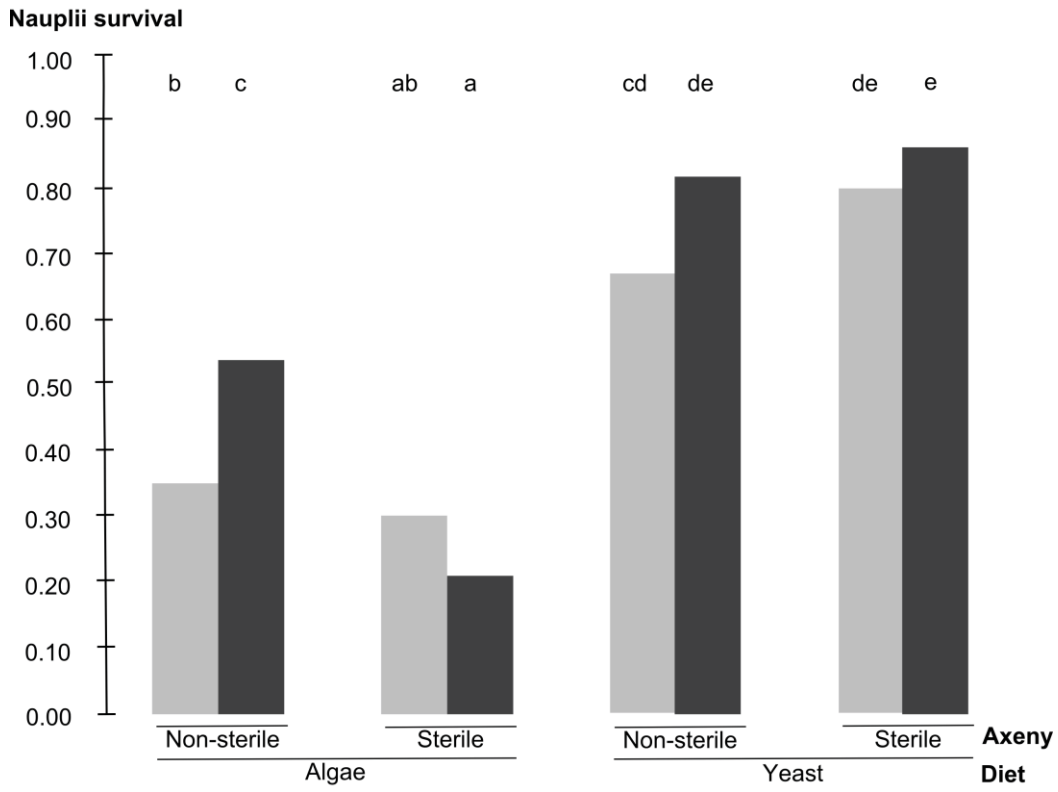

Figure 3: Survival of brine shrimp juveniles at low and high salinities, in different food and axeny conditions. The bars represent nauplii survival estimates from the generalized linear model termed *QAIC best model* in table 1. The color distinguishes low salinity (5g/L) in light grey and high salinity (80g/L) in dark grey. Axeny (sterile or non-sterile) and Diet (algae or yeast) correspond to the combination of treatments used to raise nauplii. Letters represent categories corresponding to post-hoc analysis (see Supplement 1). Estimates sharing the same letter are not significantly different.

Microbiota growth experiment

To determine whether *Artemia*'s gut microbiota were able to digest algae and to grow at low salinities, we isolated microbiota from *A. franciscana* guts, and measured their population growth capacity in different media. We used a full-factorial design with two treatments: diet (rich media with a combination of sources of carbon, versus poor media with autoclaved algae as sole source of carbon) and a gradient of salinity (from freshwater to saturation). As the composition of the rich media is more diverse and easily accessible, we expected that bacteria would grow better than on the poor media. The poor media was also used as a test for algae digestion by gut microbiota. We expected a lower growth rate in the poor media than in the rich media, regardless of salinity, and a screening of bacteria specialized in algae digestion. However, if gut microbiota are constraining the niche of *Artemia*, we also expected that bacteria growth would be inhibited at low salinities in both media.

For the extraction of gut microbiota, adult *A. franciscana* from the Aigues-Mortes salt ponds (sampling in the Fangouze pond: 43.504455, 4.224652; France, salinity 170g/L, 13/02/2013) were washed using ethanol to eliminate external bacteria, and then rinsed with sterile water. They were then crushed, and the homogenate was filtered using a vacuum pump and a 1-µm filter to concentrate the microbiota inoculum. This bacterial community was grown in marine medium (25g/L, Marine Broth 2216, Difco, Fisher) for one night at 30°C, and conserved in a 15% glycerol solution at -80°C. The rich media contained 0.5% yeast extract, 0.5% casamino acid, 0.5% $MgSO_4$ $7H_2O$, 0.3% tri-sodium citrate and 5% sodium chloride (Wang et al. 2009). The poor medium was composed with 1 000 cell/mL of algae. We used a low density of food in the poor media to prevent the senescence of algae (degradation of chlorophyll to non-fluorescent catabolites; Hörtensteiner 2004) from modifying optical density, thus interfering with the increased optical density caused by bacterial growth. We used a TECAN Infinite 200 microtiter plate reader to measure growth curves at different salinities (0, 20, 40, 60, 80, 100, 140, 180, 220 and 250g/L). To quantify bacterial growth along the salinity gradient, we used carrying capacity (maximal optical density after 24h) for 3 replicates per treatment.

Re-inoculated Artemia survival experiment

To identify bacteria involved in algae digestion in *Artemia*, we used a candidate species approach. A recent study found that *Salinivibrio* was prevalent in guts of nauplii and adults brine shrimp sampled in ponds with minimal salinity of 71g/L and 89 g/L respectively in an Israeli salt ponds (Tkavc et al. 2011). We thus expect that the inoculation of axenic nauplii with isolates of *Salinivibrio* from the gut of wild *Artemia* might help restore, at least partially, the "wild-type" phenotype (that is, higher survival in hypersaline environments, 80g/L in our conditions). To ascertain that *Salinivibrio* bacteria were not used as food resource by *Artemia* nauplii, which are non-selective filterer, we exposed nauplii to gut bacteria for a short inoculation period (only 2 hours), and then grew them in sterile medium.

Salinivibrio isolation and inoculation protocol. We first isolated colonies of *Salinivibrio* by spreading some *A. franciscana* homogenate from Aigues-Mortes (Fangouze pond) on a solid marine medium (Marine Broth 2216, Difco, Fisher) with 5% of Agar. Single bacterial colonies were picked out for conservation and characterization. Sampled colonies were grown in liquid marine medium for one night at 30°C, and conserved in a 15% glycerol solution at -80°C. The same colonies were used for genus identification, running PCRs using universal bacterial primers (63f: Marchesi et al. 1998; B6r: Manceau and Horvais 1997; see above paragraph "Axenic culture and microbiota transmission"). PCR products were sequenced (GenoScreen, Lille, France) and the sequences were blasted against the NCBI prokaryote database to identify the isolates corresponding to *Salinivibrio*.

Artemia cysts (GSL07) were hatched in sterile conditions (see above paragraph "*Artemia* survival experiment" for details). After 3 days under continuous light and ambient temperature, hatched nauplii were transferred for 2 hours in three types of inoculation solutions: (1) a non-sterile brine adjusted to 80g/L by diluting saturated brine (250g/L) collected directly in the Aigues-Mortes salt ponds with osmosed water; (2) a sterile brine (solution 1 autoclaved), and (3) a re-inoculated brine obtained by adding overnight fresh culture of one *Salinivibrio* strain to solution 2. The latter was done independently for six Salinivibrio strains. For each inoculation treatment, 25 replicated groups of 6 nauplii were transferred, in sterile conditions, in Falcon tubes containing 40 mL of brine solutions and sterilized algae as food source (*D. salina*;

Table 2: Generalized Linear Model selection for re-inoculation *Artemia* survival experiment. *Best AIC model* and *Saturated* models helped estimating the over-dispersion parameter value (ĉ = 0.97). **K** is the number of parameters for each model, **logLik** the log likelihood, **AIC** the Akaike information criterion, **ΔAIC** the AIC differences, w_i the Akaike weights. **Parameters** of models are Condition (Sterile, Non-sterile and 6 levels of Salinivibrio strains), Date (experimentation date), Plate (position of the plate in the experiment) and Tube (tube number in the experimentation).

Model	K	logLik	AIC	ΔAIC	w_i	Parameters
Best AIC model	9	-209.51	437.02	0.00	0.74	Condition + Date
Best AIC model + Plate	16	-204.08	440.16	3.14	0.15	Condition + Date + Plate
Condition + Plate	15	-206.01	442.02	5.00	0.06	Condition + Plate
Condition	8	-213.61	443.22	6.20	0.03	Condition
Condition + Date x Plate	21	-201.85	445.7	8.68	0.01	Condition + Date x Plate
Saturated	264	-85.93	699.87	262.85	0.00	Tube x Date

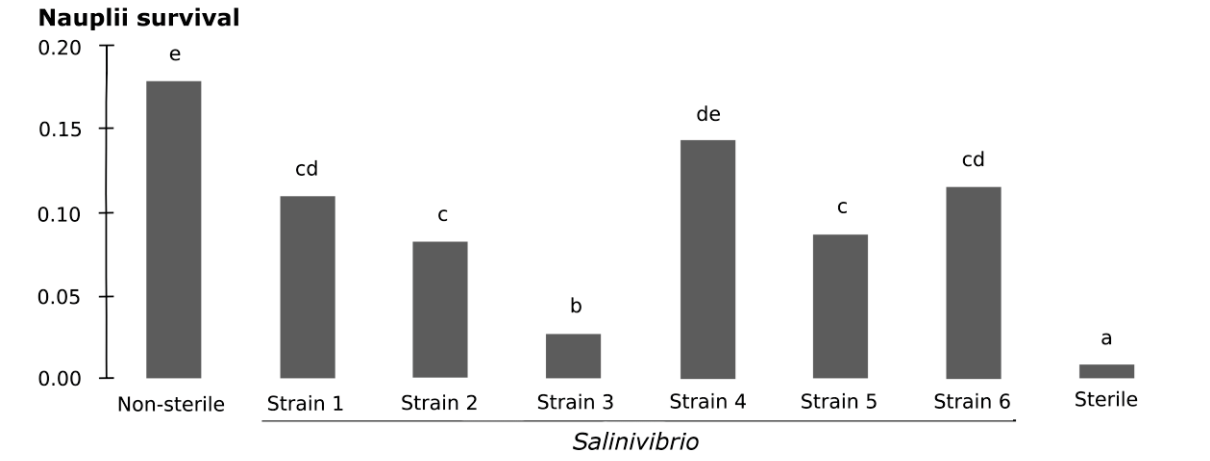

Figure 5: Survival of brine shrimp when inoculated with different *Salinivibrio* strains. Nauplii survival estimates calculated from the effect sizes in the generalized linear model termed *AIC best model* in table 2. **Sterile** and **Non-sterile** conditions correspond to experimental controls. ***Salinivibrio* strains (1 to 6)** correspond to nauplii inoculated with various strains of *Salinivibrio* isolates. Letters represent categories corresponding to post-hoc analysis (Table 3). Survival rate estimations sharing the same letter are not significantly different.

see above paragraph "*Artemia* survival experiment", for details). The experiment was repeated twice at two different dates, with three different strains of *Salinivibrio* used in both repetitions, plus three other strains of *Salinivibrio* used only in the second repetition. In each experimental repeat, a total of 750 (respectively 720) nauplii were evenly distributed among the treatments. Tubes were placed horizontally to eliminate light effect (see Supplement 2), randomized using a Latin square design (Saville and Wood 1991), and incubated for four days at 25°C and 12-hour daylight. At the end of this period, tubes were emptied in a net (120-µm mesh), and the number of surviving nauplii per tube was counted under a binocular.

Re-inoculation experiment statistical analysis. Data from the two replicated experiments were analyzed jointly. We used glm with binomial error distributions to test our hypothesis about the influence of *Salinivibrio* inoculation on the survival of nauplii 8 days after hatching. As there may be an effect of experimentation date or plate position (one parameter per stove level), we included these confounding factors in our model selection. Model selection was performed in R using the MuMIn package (Barton 2013), and was based on AIC (Akaike 1974). We used AIC based selection, as data were not overdispersed ($\hat{c} = 0.97$). Overdispersion was calculated from the residual deviance between the best model (based on AIC score) and a saturated model where one survival probability is estimated per tube. Results from the model selection are presented in table 2 and fig.5. To determine the significant differences in survival within and among conditions, we used a post-hoc AIC based analysis. This analysis helped us determine which combinations of treatments were significantly different. Combinations tested are displayed in table 3.

Results

Artemia survival experiment

Axenic culture and microbiota transmission. For our axenic treatments to be valid, we first needed to rule out the possibility that bacteria were vertically transmitted through cysts. We detected the presence of bacteria by amplification only for homogenate of cysts that were not bleach-decapsulated (fig.2). The size of the amplified fragments for the homogenate of non-decapsulated cyst was different from that for the positive control consisting in non-sterile brine. This could be explained by a differential composition of bacterial communities between the growing medium and the lumen of

Table 3: Post-hoc AIC based analysis for re-inoculation *Artemia* survival experiment. To determine which treatments were significantly different, we tested several models (*Grouping A to I*) where treatments with the same letter are set to have the same nauplii survival value. **K** is the number of parameters for each model, **logLik** the log likelihood, **AIC** the Akaike information criterion, **ΔAIC** the AIC differences and w_i the Akaike weights. The best grouping is the model with the highest w_i.

Post hoc model	Non-sterile	Salinivibrio strains						Sterile	K	logLik	AIC	ΔAIC	w_i
		S1	S2	S3	S4	S5	S6						
Grouping F	d	c	c	b	d	c	c	a	5	-210.38	430.75	0.00	0.33
Grouping E	e	d	c	b	e	c	d	a	6	-209.75	431.49	0.74	0.23
Grouping G	d	c	c	b	c	c	c	a	5	-210.86	431.72	0.97	0.20
Grouping D	f	d	c	b	e	c	d	a	7	-209.55	433.11	2.36	0.10
Grouping C	e	c	b	a	d	b	c	a	6	-211.05	434.10	3.35	0.06
Grouping A	g	e	c	b	f	d	e	a	8	-209.54	435.07	4.32	0.04
Grouping B	f	d	b	a	e	c	d	a	7	-211.04	436.07	5.32	0.02
Best AIC model	g	f	c	b	g	d	e	a	9	-209.51	437.02	6.27	0.01
Grouping H	c	c	c	b	c	c	c	a	4	-215.47	438.95	8.20	0.01
Grouping I	c	b	b	b	b	b	b	a	4	-219.75	447.51	16.76	0.00

the brine shrimps' gut, or by a difference in origin of the brine (Aigues-Mortes, France) and the cysts (Great Salt Lake, Utah, USA). Non-amplification on cysts that were bleach-decapsulated from 5 to 30 min (fig.2) indicated that bacteria on or under the surface of the cyst shell were entirely removed. Cysts were still able to hatch after 30 min in bleach (result not shown), which indicates that bleach does not enter the embryo. Hence, bacteria are not present inside of the embryo but only on the cyst's chorion, and cysts are therefore sterile after decapsulation.

Influence of microbiota on Artemia juvenile survival. We measured juvenile survival in an experiment involving four treatments, at both high and low salinities. The survival analysis indicated a moderate overdispersion ($\hat{c}$ = 2.23) requiring a model selection based on QAIC to correct for the variability in our dataset. The models that best describe the data on *Artemia* survival include a large and significant axeny x salinity and axeny x diet interactions (table 1). These effects were consistent across the two replicated experiments (the term interaction with date is absent from all best models). The best model included also a plate effect and experimental date effect. We will come back on these effects below, but overall these results demonstrate that the global interaction between the four treatments and salinity pattern is the same for all replicates. Parameter estimations and post-hoc analyses indicated that our results were consistent with the hypothesis that microbiota are responsible for the reduced survival of *Artemia* at low salinity on algae diet. This pattern is presented in fig.3 (for observed data results see Supplement 3). In treatments mimicking natural conditions, with standard (non-axenic) culture and an algae diet, *Artemia* survive significantly less at low salinity (35% survival) than at high salinity (54% survival). Under axenic conditions with an algae diet, *Artemia* displayed similarly low survival at either low or high salinity (<30% survival). With a yeast diet, *Artemia* displayed high (>80% survival) and comparable survival at both low and high salinities, in both sterile and non-sterile treatments.

As mentioned above, the best model included two other effects than the axeny x salinity and axeny x diet interactions. The plate and date effects are significant in our best QAIC model. We hypothesized that this results from the vertical position of the tubes and the uneven plate repartition in the stove, causing unequal light and temperature exposure (light effect might also explain overdispersion in our

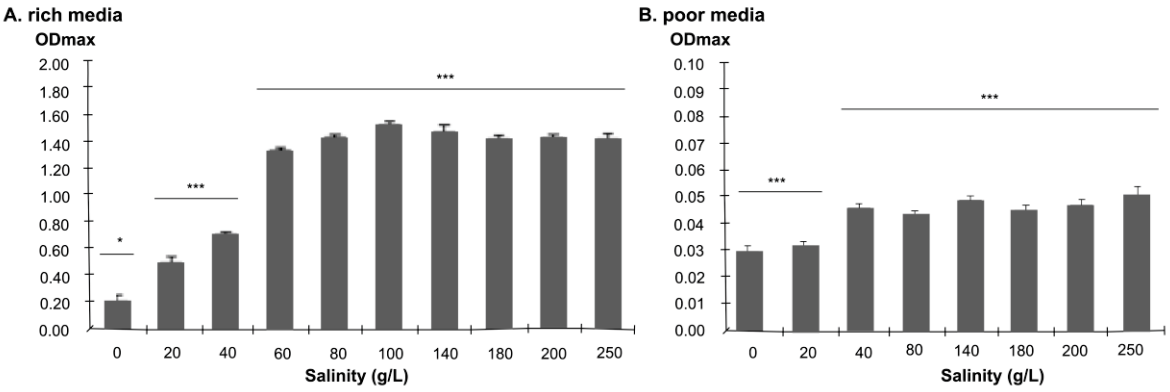

Figure 4: Salinity niche of bacteria community from *Artemia* gut. Graphs display maximal absorbance (OD_{max}) recorded, at each salinity, for communities grown in (A) a rich medium with yeast extract as main metabolic resource and (B) a poor medium containing only algae for metabolic use. Error bars represent standard deviation. Lines represent categories determined by ANOVA tests (rich medium with three salinity categories: F=167.9, df=2, *P* –value=1.22 10^{-6}; poor medium with two salinity categories: F=51.05, df=1, *P*-value<10^{-3}).

replicates). To test these hypotheses, a third experimentation (see Supplement 2 and 3) was performed aimed at entirely removing light effects. In this experiment, tubes were placed horizontally (instead of vertically), with equal exposure to light, and therefore same influence of light and temperature. As expected, the best model had axeny x salinity and axeny x diet interactions alone, and was significantly better than the same model with a plate effect. Moreover, there was no light effect in this experimentation and over-dispersion was strongly reduced compared to the two other experiments (ĉ = 0.75). Other results were entirely consistent with previous findings.

Microbiota growth experiment

The microbiota associated with *Artemia* was able to grow on both the rich and the poor media. This indicates that at least some of the bacteria are able to digest algae. The maximal optical density (OD_{max}) of the cultures, in the two growing media, is depicted in fig.4, from null to saturation salinity. As expected, the mean OD_{max} was globally higher on the rich (fig.4A) than on the poor medium (fig.4B). The carrying capacity was substantially reduced at low salinities, in both the rich and the poor media. The carrying capacity in the rich media plateaued around OD_{max} = 1.44 for high salinities (between 80g/L to 250g/L; fig.4A), but significantly decreased to 0.71 and 0.21 for salinities of 40-20g/L, and 0g/L, respectively (ANOVA with three salinity categories: F = 167.9, df = 2, *P*-value < 10^{-5}). In the poor medium, carrying capacity remained around OD_{max} = 0.047 for salinities above 40g/L (fig.4B), but switched to lower OD_{max} values around 0.031 for salinities under 20g/L (ANOVA with two salinity categories: F = 51.05, df = 1, *P*-value < 10^{-3}). Hence regardless of the media, bacteria isolated from *Artemia*'s gut grew less well at low salinities (below 40g/L), where brine shrimp tend to have reduced survival.

These results involved bacteria retrieved from brine shrimps living in high salinity brine in the field (170g/L). Tkavc and collaborators (2011) showed that salinity has an impact on the composition of the bacteria retrieved from *Artemia*'s gut. We thus also sampled gut bacteria from *Artemia* living at low and medium salinities (40 and 80g/L). These samples were isolated in different salinity conditions (0, 40 and 80g/L), and their carrying capacity (OD_{max}) was similarly measured along a salinity gradient. For all samples, we found that bacteria grew better at high than low salinities. The salinity of origin (from where *Artemia* were sampled in the field) only affected importance of

the difference between OD_{max} at low and high salinity, while the salinity-of-isolation increased or decreased the height of the plateau in OD_{max} (Supplement 4).

Re-inoculated Artemia survival experiment

To identify bacteria involved in algae digestion in *Artemia*, we used *Salinivibrio* as a candidate species. We isolated strains of this bacterial genus from guts of wild *Artemia*. We found that the growth of those strains presented the same pattern across salinities than the overall bacterial gut community (see Supplement 5). We used those strains to perform the re-inoculation experiments. We found that nauplii inoculated with *Salinivibrio* strains survived significantly better than nauplii in sterile condition, which supported *Salinivibrio* as a good candidate to explain the "low salinity paradox" in *Artemia*. This recovery was only partial compared to non-sterile conditions, suggesting that other interactions (involving different *Salinivibrio* or other bacteria) might be involved in algae digestion. The recovery was also variable among different *Salinivibrio* (from 3% to 14% nauplii survival depending on strains). These patterns were consistent across replicates within experiments, and between the two replicated experiments (we did not detect an interaction between conditions and experiment date in the glm). Note that these re-inoculation experiments consistently showed higher mortality than our other experiments following nauplii survival. The likely reason is that (1) in all treatments, brine shrimps were exposed to a very limited amount of microbiota (two hours of inoculation only), which probably limited their digestion, and (2) they were manipulated and transferred a larger number of time.

Discussion

Microbiota and adaptation to salinity in Artemia

We used the brine shrimp and its gut microbiota as a model system to investigate how interspecific interaction with a symbiotic partner results in union of the partners' niches along the axis of the interaction, but intersection of their niches along orthogonal axes. This is illustrated in fig.1. The horizontal axis in this graph corresponds to the service traded by the symbiont to its host. In our case, this service is the ability to digest algae, but it could be protection against pathogens, or provision of water and nutrients, for instance. Clearly, a mutualism causes an expansion of the niche along this axis, and conversely removing the symbiont experimentally will result

in niche contraction, as discussed (and graphically illustrated) recently by e.g. Afkhami et al (2014). In contrast, the vertical axis in fig. 1 is seldom discussed in the literature on mutualism. It represents other niche dimensions, not related to the particular service traded by the symbiont (here, tolerance to salinity). Along these dimensions, the range of environments accessible to the host is necessarily reduced by intersection with the range of environments that its symbiont can tolerate. In other words, in conditions that require the service provided by the symbiont, there is necessarily a constraint on niche extension along axes orthogonal to this service (dark shaded zone in fig.1); at best, the niche along these axes is unchanged if the symbiont's tolerance limits are broader than its host's.

We conducted a set of experiments that jointly provide support for such a combination of niche union along the axis of the service traded, but niche intersection along other axes, for the brine shrimp and its microbiota. In the absence of the symbiotic partner (sterile treatments), *Artemia* has *high* survival at high and low salinities when fed on yeast, but *low* survival at both salinities when fed on algae. This corresponds to the area delimited by a continuous line on fig.1 (niche in the absence of interaction). With the symbiont (non-sterile condition in our experiments), *Artemia* can survive on algae, but juvenile survival is severely reduced at low salinity. Hence as compared to the niche without interaction, the niche with the interaction (shaded area in fig.1) expands towards algae along the horizontal axis (light shaded area in fig.1). Along the other axis, the niche with the interaction is constrained by the very poor performance of the gut microbiota to survive at low salinities (dark shaded area in fig.1). Our experiments on bacterial growth (fig.4) show that this is caused by the low tolerance of the microbiota to low salinity (niche limited by dashed line on the fig.1), some of which are likely to digest algae (fig.4B). Our re-inoculation experiments with *Salinivibrio* strains isolated from adult wild *Artemia* identifies these strains as good candidates mediating these effects, as they partially restore survival when re-inoculated in axenic nauplii. These bacterial strains also exhibit very limited growth below salinity of 40g/l, consistent with the prediction that their niche constrains the niche of the host. Overall, in natural conditions, where algae are the prominent food source, niche limit of *Artemia* at low salinity is thus probably largely set by their microbiota.

While we do not evaluate fitness in the field, our results do provide a functional basis for understanding constraints on *Artemia*'s niche at low salinity. The niche of *Artemia* is known to exclude low salinity (<40g/L), even in absence of predators, but this niche limit remains poorly understood, notably because of the absence of clear physiological constraints for *Artemia* at low salinity. We report a substantial effect of salinity on the proportion of surviving juveniles over only 4 days of experiment (42, 31 and 52% proportional decrease in survival in experiments 1, 2 and 3, respectively, see fig.3 and Supplement 3). Extrapolating these results to a full demographical impact in the field (or even just in the laboratory) is beyond reach with our data, but juvenile survival is known to be a prominent determinant of *Artemia*'s demographic performance (Sukumaran and Grant 2013). In the non-sterile treatment, our results on juvenile survival are consistent with earlier studies on the low salinity paradox (McCarraher 1970; Castro-Mejía et al. 2011), so we are confident that our experiments reveal important factors that constrain *Artemia*'s niche at low salinity.

Niche limit are generally thought to result from ecological trade-offs, whereby mutations conferring adaptation to some conditions exhibit antagonistic pleiotropic effects in other conditions (Antonovics 1976; Barton and Partridge 2000). Here the antagonism underlying the food-salinity trade-off is not set by *Artemia*'s genes, but by their interaction with the genes of its microbiota. This is particularly remarkable given that salinity is a prominent axis of *Artemia*'s niche (van Stappen 2002), overriding other abiotic parameters such as ionic composition or temperature (Bowen et al. 1985; Vanhaecke et al. 1987; Browne et al. 1988), or biotic parameters such as fish and bird predators or parasites (van Stappen 2002; Rode et al. 2013a). Thus, in this case study, the symbiont plays a prominent ecological role as it constrain a major axis of *Artemia*'s niche.

Several features of *Artemia* and its microbiota remain to be fully elucidated, which are also relevant to the more general issue of symbiont-mediated constraints on the niche. First, the candidate bacteria that we reported (*Salinivibrio*) may only be a part of the microbial community involved in the mutualism. Second, it remains to be shown whether there is strong specificity between *Artemia* species or genotypes and their gut symbionts. Third, since host-microbiota specificity generally depends on environmental factors (Spor et al. 2011), it would be important to investigate whether *Artemia* can switch their symbionts under different environmental conditions, as done

by corals with their zooxanthellae (Berkelmans and Van Oppen 2006; Reshef et al. 2006), or leafcutter ants with their mutualistic fungi (Mueller et al. 2011). In more complex symbiotic communities (e.g. plant ectomycorrhizal networks, Selosse et al. 2006), symbiont switching can even lead to the establishment of symbiont "market" mediated by the niche limits of all possible partners (Noë and Hammerstein 1995; Cowden and Peterson 2009; Bever et al. 2010; Kiers et al. 2011). In *Artemia*, the overall gut bacterial community does change across salinities (Tkavc et al. 2011), but this may not directly concern the mutualistic community. If brine shrimp could switch their symbionts depending on salinity, the low salinity paradox could be overcome, in a form of symbiont-mediated phenotypic plasticity. Nevertheless in our case, there is very limited evidence in favor of this symbiont-switching hypothesis. As we showed (Supplement 4), bacteria from the gut of *Artemia* collected at low or high salinity did not grow differently along the salinity gradient (and both grew very poorly at low salinities). Furthermore, the low salinity paradox, commonly reported in *Artemia* research (see in van Stappen 2002; Castro-Mejía et al. 2011), is at odds with this symbiont switching idea. However, it would be worthwhile inspecting this hypothesis in more detail, and with a more functional approach, to understand whether acclimation to a changing salinity can be partly attributed to a switching of facultative symbionts. This phenomenon may be important in the first stages of invasions involving large shifts in salinity, in euryhaline organisms for which genetic changes in the physiological determinants of the tolerance curve have already been demonstrated (Lee 1999; Lee et al. 2011; Lee et al. 2012; Kozak et al. 2013). Fourth, the possibility to acquire or change microbiota may be age or development-dependent. *Artemia*'s diet switches from stored maternal resources to an algal diet during their larval development. Whether early-established microbiota community changes later in life or remains constant is open to further investigations, but may shed a new light on the underlying mechanisms of larval versus adult survival under different environmental conditions.

Empirical and Conceptual Implications

Beyond their conceptual importance for evolutionary and ecological thinking, these findings have direct relevance to the study of traits within species. Interactions with the microbiota may contribute to generating large experimental error in the laboratory, if they are not controlled for (which they rarely are). This is especially true

in experiments that study variation (heritability, plasticity…) of highly integrated traits (e.g. fitness components), in organisms that acquire their microbiota from their environment rather than from direct maternal transmission.

Regarding the underpinnings of environmental tolerance curves, a key tool in the context of species persistence in the face of climate change (Deutsch et al. 2008; Chevin et al. 2010; Lande 2014), our findings fit within the broad $G_1 \times G_2 \times E$ niche concept (Vale et al. 2008): the range of environments (E) suitable for a focal species (G1) depends not only on its adaptation to the environment ($G_1 \times E$), but also on its interactions with symbionts ($G_1 \times G_2$), adaptation of these symbionts to the environment ($G_2 \times E$), or even interactions with symbionts that depend on the environment ($G_1 \times G_2 \times E$).

For mutualistic interactions, this $G_1 \times G_2 \times E$ takes the form of niche expansion along the axis of the interaction, combined with niche intersection on other axes (fig.1). This effect of biotic interaction on the niche adds to the more commonly acknowledged exclusion effect between antagonists, which is central to the concepts of realized versus fundamental niche. Combining intersection-union with exclusion provides an integrated understanding of how biotic interactions transform the abiotic niche in more complex ecosystems involving more actors. For instance, if a pathogen's niche envelope was added to fig.1, the interplay of these simple rules (union-intersection, exclusion) would define a clearer set of possible effects of any interaction on the niche of the focal species.

Conclusion

Almost every living macro-organism has symbiotic interactions with micro-organisms, whose influence on the adaptation of the host is only starting to be elucidated (Feldhaar 2011; Hansen and Moran 2013). Our case study of a non-obligatory association between *Artemia* and its gut microbiota show that the niche limit of brine shrimp at low salinities might not directly be caused by its history of adaptation or by mutations in the *Artemia* genome from its freshwater ancestor. Instead, it probably results from its dependence, for algae digestion, on bacterial microbiota that do not tolerate low salinity. That symbionts and their hosts have to share niche limits along all axes, not just those directly involved in their interaction, is probably a major cost of

symbiotic mutualism, which opens a rich array of interesting questions in evolutionary ecology.

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Online enhancement

Supplement 1 - Pairwise post-hoc tests on mean differences among treatments.

Supplement 2 - Nauplii survival experimentation with control for light condition.

Supplement 3 - Nauplii survival data for the three experiments.

Supplement 4 - Influence of Artemia origin and isolation salinity on microbiota community growth.

Supplement 5- *Salinivibrio* as a good candidate for re-inoculation experimentation.

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Supplement 1

Table S1: *P*-values of post-hoc tests on mean differences among treatments. Pairwise comparisons were based on two-tailed Z-test given the large sample sizes. Standard errors of treatment means were recomputed from the var-covariance matrix of estimated effects in the generalized linear model. Significance was assessed using Bonferonni correction for multiple tests (28 tests, α = 0.0018). Non-significant (*n.s.*) comparisons are labelled with the same letter on fig.3 in the main text.

P-values		Diet	algae				yeast			
		Axeny	non-sterile		sterile		non-sterile		sterile	
Diet	Axeny	Salinity (g/L)	5	80	5	80	5	80	5	80
algae	non-sterile	5								
		80	$1.8\ 10^{-3}$							
	sterile	5	*n.s.*	$4.6\ 10^{-5}$						
		80	$5.3\ 10^{-3}$	$3.0\ 10^{-9}$	*n.s.*					
yeast	non-sterile	5	$8.2\ 10^{-8}$	*n.s.*	$2.4\ 10^{-10}$	$4.4\ 10^{-16}$				
		80	$2.2\ 10^{-16}$	$1.2\ 10^{-7}$	0	0	*n.s.*			
	sterile	5	$8.9\ 10^{-16}$	$5.0\ 10^{-7}$	0	0	$6.1\ 10^{-3}$	*n.s.*		
		80	0	$1.5\ 10^{-11}$	0	0	$8.6\ 10^{-6}$	*n.s.*	*n.s.*	

Supplement 2

Model selection in our study displayed an effect of light that might increase data's over-dispersion and a plate x date effect caused by non-totally random plate distribution of plates in the stove. To test these hypotheses, we raised nauplii in algae sterile and non-sterile treatments at low and high salinities, while controlling for light and plate position. In practice, we hatched GSL07 cysts in sterile conditions. For each condition (algae in sterile and non-sterile medium, two salinities), 3 nauplii were transferred in 25 sterile 50 mL Falcon tubes containing 40 mL of brine solutions. A total of 300 nauplii were transferred. Tubes were placed in the stove horizontally (instead of vertically in the previous two experimentations) and randomly, using a Latin square design (controlling for the distance of plates to the stove walls). Then, tubes were incubated for four days at 25°C and 12-hour daylight. At the end of this period, tubes were emptied in a net (120-µm mesh), and surviving nauplii were counted under a binocular.

The statistical analysis was performed using the same protocol as in the previous experimentations. Results of the model selection following AIC are presented in the table S2.

Table S2: Generalized Linear Model selection for survival analysis. *Saturated* and *Interaction* models helped estimating the over-dispersion parameter value (ĉ = 0.75). **K** is the number of parameters for each model, **logLik** the log likelihood, **AIC** the Akaike information criterion, **ΔAIC** the AIC differences, $\mathbf{w_i}$ the Akaike weights. **Parameters** of models are Salinity (high or low), Axeny (sterile or non-sterile), Plate (position of the plate in the experiment) and Tube (tube number in the experimentation).

Model	K	logLik	AIC	ΔAIC	wi	Parameters
Interaction	4	-73.28	154.57	0.00	0.69	Salinity x Axeny
Addition	3	-75.24	156.49	1.92	0.26	Salinity + Axeny
Interaction + Plate	10	-70.28	160.56	5.99	0.03	Salinity x Axeny + Plate
Saturated	100	-36.49	272.98	118.41	0.00	Tube

Best model clearly was *Interaction* indicating a salinity x axeny effect. The comparison of this model with the saturated model helped us calculate an estimate of

over-dispersion in our data. The over-dispersion estimate (ĉ) equaled 0.75 in this experiment where light was controlled while ĉ equaled 1.92 in the pooled experimentations with not controlled light effect. This result demonstrates that light exposition might increase over-dispersion rate in a dataset. Finally, *Interaction + Plate* model was significantly poorer than the *Interaction* model. This indicated that in addition to reducing over-dispersion, controlling for light might also have reduced the temperature/light heterogeneity in the stove.

Other results were entirely consistent with previous findings (see Supplement 3). These results further support our conclusions that the intolerance of *Artemia* to low salinities is due to dependence on bacterial microbiota, for algae digestion.

Supplement 3

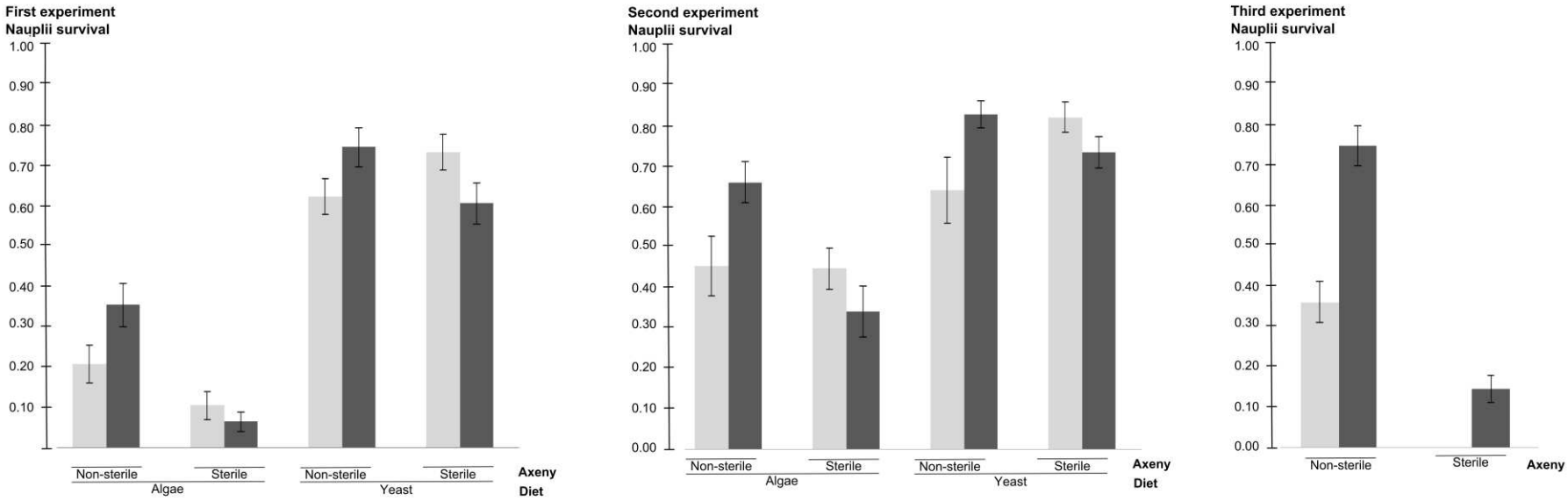

Figure S3: Survival of brine shrimp juveniles at low and high salinities. Nauplii survival means from all experimental replicates. The color distinguishes low salinity (5g/L) in light grey and high salinity (80g/L) in dark grey. Axeny (sterile or non-sterile) and Diet (algae or yeast) correspond to the treatments used to raise nauplii. Error bars represent standard errors.

Supplement 4

Our results on nauplii bacteria community growth involved bacteria retrieved from *Artemia* living in high salinity brine (170g/L). Tkavc and collaborators (2011) showed that salinity has an impact on the composition of the bacteria retrieved from *Artemia*'s gut. We therefore inspected whether the salinity of the *Artemia* from which the gut bacteria were retrieved could impact our results on microbiota community capacity to grow at different salinity levels. To do so, we extracted gut bacteria from *Artemia* at low and high salinity, and observed the growth of their communities along a salinity gradient. The extraction and conservation of gut microbiota required an enrichment process. This enrichment was realized in three different salinity conditions. In the hypothesis that Artemia can easily change their microbiota depending on salinity, we expect to see a better growth at low salinity of microbiota community isolated from Artemia living at low salinity.

Table S4: ANOVA model selection. **Parameters** are: two salinities of the ponds where *Artemia* were retrieved (SA), three salinities used to enrich the bacterial communities (S1) and, three salinities where community growths were followed (S2). **Simplification** exhibits the nesting model minus retrieved parameters or interactions. **df** gives the degrees of freedom when comparing nesting with simplified model. Significant **p-value** indicated that nesting model was significantly better than simplified model.

Model	Parameters	Simplification	df	p-value	
m0	S1 + S2 + SA + S1:S2 + S1:SA + SA:S2 + S1:S2:SA				
m1	S1 + S2 + SA + S1:S2 + S1:SA + SA:S2	m0 - S1:S2:SA	2	0.106	
m2	S1 + S2 + SA + S1:S2 + S1:SA	m1 - SA:S2	1	**0.030**	*
m3	S1 + S2 + SA + S1:S2 + SA:S2	m1 - S1:SA	2	0.619	
m4	S1 + S2 + SA + S1:SA + SA:S2	m1 - S1:S2	2	0.310	
m5	S1 + S2 + SA + S1:S2	m3 - SA:S2	1	**0.029**	*
m6	S1 + S2 + SA + SA:S2	m3 - S1:S2	2	0.305	
m7	S1 + S2 + SA	m6 - SA:S2	1	**0.030**	*
m8	S2 + SA + SA:S2	m6 - S1	2	**1.27E-09**	***

The experiment was conducted with *Artemia* originating from a brackish pond (43.540993, 4.146470; France, salinity 40g/L, 29/01/2014) and a salted pond (43.541211, 4.146764; France, salinity 80g/L, 27/06/2013) in Aigues-Mortes. The bacterial communities were grown in the rich media at three different salinities (0, 40, 80g/L) for one night at 30°C. This enrichment process enabled us to conserve in a

15% glycerol solution at -80°C referenced microbiota community. We used a TECAN Infinite 200 microtiter plate reader to measure growth curves for each isolate at different salinities (0, 40, 80g/L). To quantify bacterial growth along the salinity gradient, we used carrying capacity (maximal optical density after 24h) for 4 replicates per treatment. Data analysis was conducted using ANOVA. Parameters tested as factors were: the two salinities of the ponds where *Artemia* were retrieved (SA), the three salinities used to enrich the bacterial communities (S1) and, the three salinities where community growths were followed (S2). From a full interaction model (*m0*), we compared several nested simplifications (*m1* to *m8*). Results of model selection are presented in the table S4.

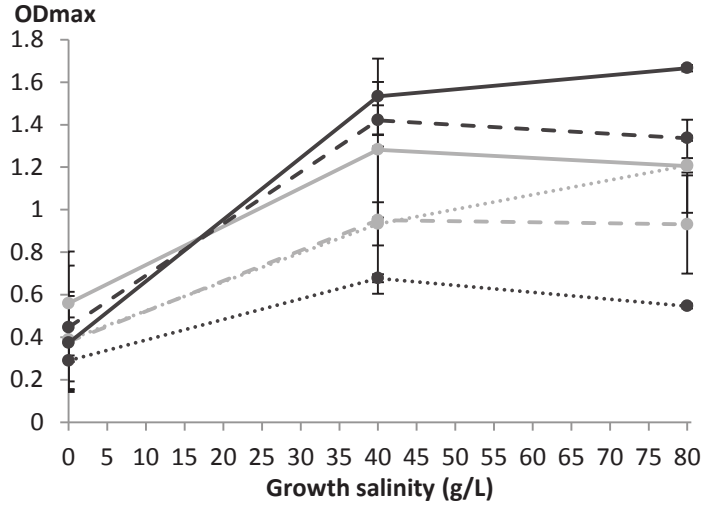

Figure S4: Influence of Artemia gut salinity on the niche of microbiota community. Graph displays maximal absorbance (ODmax) recorded, for three growth salinities, for communities retrieved from Artemia living at low (40g/L; grey) and high (80g/L; black) salinities. Enrichment salinity is represented with doted (0g/L), dashed (40g/L) and solid (80g/L) lines. Error bars represent standard error.

Microbiota isolated from both *Artemia* was able to grow regardless of salinity (see figure S4). OD_{max} was significantly higher at medium or high salinities (40 or 80g/L) in comparison with low salinity (0g/L). This difference was greater for *Artemia* retrieved at 80g/L than 40g/L, *i.e.* there is an interaction between salinity of the pond where *Artemia* were retrieved (SA) and the growth salinity (S2). The enrichment salinity (S1) has an important impact on the global growth of bacterial communities, with OD_{max} significantly higher for communities enriched at medium or high salinities (40 or 80g/L) in comparison with low salinity (0g/L). No significant interaction was detected

with SA and S2, meaning that S1 only influenced survival of the community at all salinities.

To conclude, our results show that no matter the salinity of the pond was the *Artemia* was retrieved or the isolation process salinity, microbiota community always display greater OD_{max} at high than low salinity. This suggests that *Artemia* are not able to recruit microbiota adapted to low salinity. Our results on nauplii survival should then have been the same if conducted with lower salinity bacteria communities.

Supplement 5

To identify bacteria involved in algae digestion in *Artemia*, we used a candidate species approach. A recent study found that *Salinivibrio* was prevalent in guts of nauplii and adults brine shrimp sampled in ponds with minimal salinity of 71g/L, and 89 g/L respectively, in an Israeli saltern (Tkavc et al. 2011). After isolating six *Salinivibrio* strains from the gut of wild *Artemia*, we wanted to check if they would be as good candidates. To do so, we observed their growth capacity along salinity gradient and their ability to digest algae. To follow the growth of the different strains along salinity gradient (from 0 to 160g/L) we used the protocol described in paragraph "*Microbiota growth experiment*" (restricted to the rich media).

We found that all six *Salinivibrio* strains tested grew better at high than low salinities (Figure S5). This matched the pattern we observed for total gut microbiota growth capacity (see "*Microbiota growth experiment*"). In total, our results on the rich media support our choice to use such strains of Salinivibrio as candidates to identify bacteria involved in algae digestion in *Artemia*.

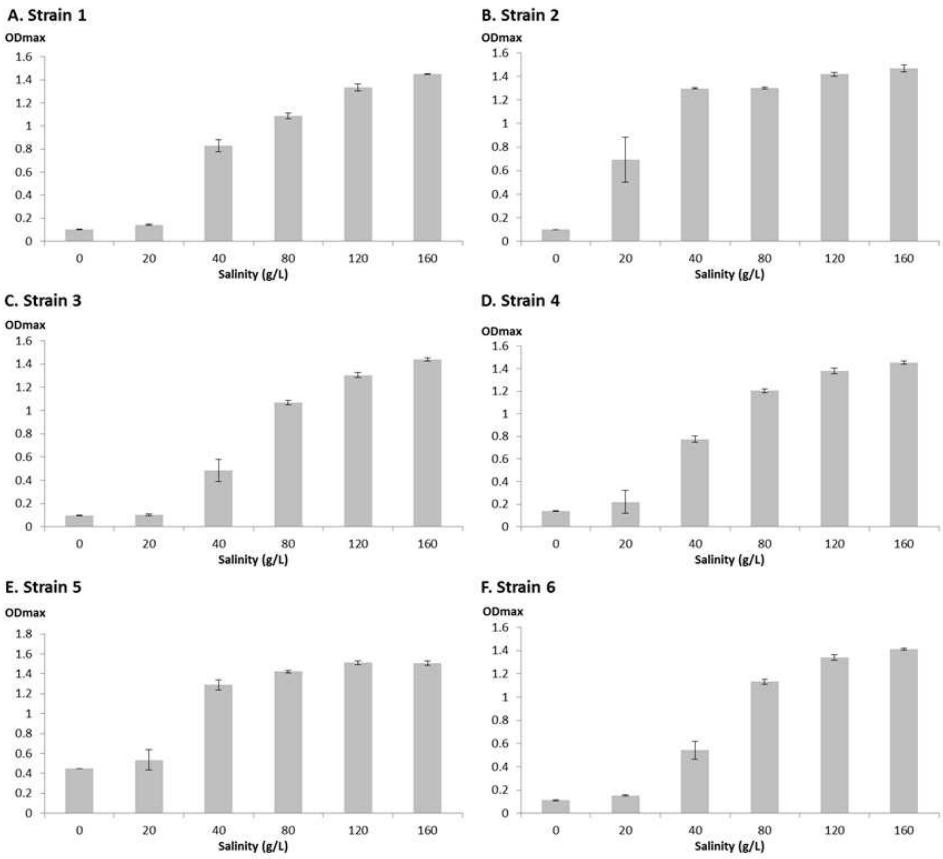

Figure S5: Salinity niche of six *Salinivibrio* strains (A to F) isolated from *Artemia* gut. Graphs display maximal absorbance (OD_{max}) recorded, at each salinity, for bacteria grown in a rich medium with yeast extract as main metabolic resource. Error bars represent standard deviation.

Literature Cited

Tkavc, R., L. Ausec, A. Oren, and N. Gunde-Cimerman. 2011. Bacteria associated with *Artemia* spp. along the salinity gradient of the solar salterns at Eilat (Israel). FEMS microbiology ecology 77:310–321.

Chapitre 3

Dynamiques des courbes de tolérances et adaptation aux fortes salinités chez l'artémie

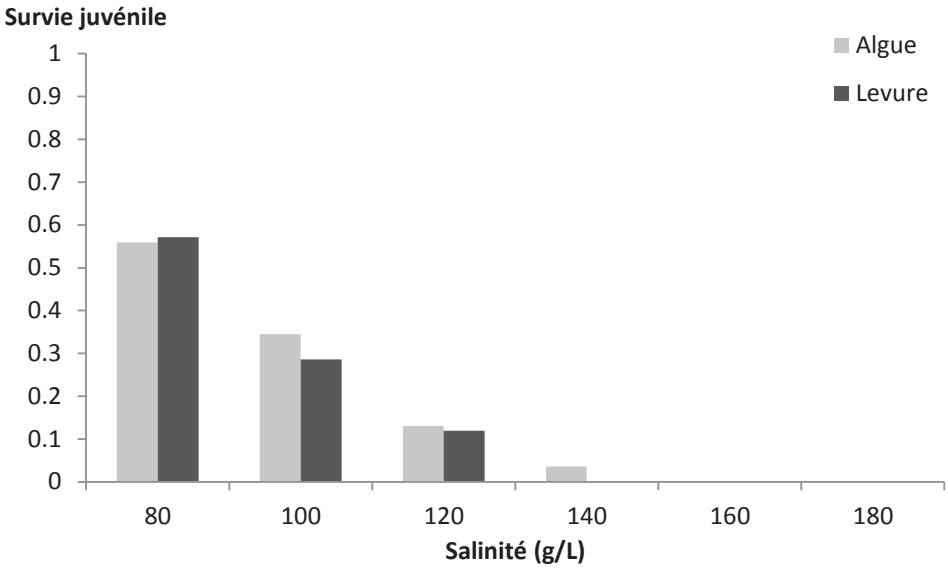

Figure 4.1 : Survie juvénile le long d'un gradient de salinité pour des artémies nourries avec des algues ou des levures.

Nous nous sommes ensuite intéressés à l'autre « côté » de la niche, c'est-à-dire à la survie aux fortes salinités. Nils Svendsen (étudiant de master 1 que j'ai co-encadré) a mis en évidence que le régime alimentaire (et donc la flore intestinale) ne jouait pas sur la survie aux fortes salinités (Figure 4.1). En effet, les artémies nourries avec des levures (nourriture qui ne nécessite pas de microflore associée pour être digéré) ne survivent pas mieux aux fortes salinités que celles nourries avec des algues. Pour ces fortes salinités, nous avons donc étudié l'impact des interactions entre qualité d'habitat et plasticité phénotypique sur la survie de différentes lignées d'*A. parthenogenetica* (voir 1.2.2.1). Notre étude propose une nouvelle méthodologie expérimentale et analytique pour répondre à cette problématique.

L'expérience consiste à élever différents génotypes d'*A. parthenogenetica* dans un gradient de salinité. Nous avons choisi d'utiliser un clone issu d'un salin espagnol (La Mata) et deux clones d'Aigues-Mortes correspondant aux clusters de clones adaptés respectivement aux hautes et basses température/salinité (voir 2.1).

L'influence des environnements d'acclimatation sur la forme des courbes de tolérance a récemment été explorée dans le cadre d'études sur la tolérance à la chaleur (Deere & Chown 2006; Cooper *et al.* 2010). Ces expériences suivent un protocole similaire au notre avec première phase d'acclimatation et une phase de mesure. Dans les deux cas, ils montrent que l'environnement de phase 1 a un effet sur la performance en phase 2. Néanmoins, ils ne peuvent distinguer l'impact de la plasticité et de la qualité d'habitat car ils ne s'intéressent qu'à un temps donné dans la seconde phase. De plus, il ne mesure pas une composante directe de la fitness mais simplement un caractère leur permettant de mesurer la performance. Ce sont les deux nouveautés de notre étude.

Nous avons mesuré la survie (composante de la fitness) tout au long des histoires de vie individuelles. Puis, nous avons développé une analyse de survie permettant d'estimer des taux de mortalité instantanée différents entre les deux phases, ainsi que différents effets de l'environnement sur ces taux. Ceci nous a permis de mettre en évidence et de manière indépendante l'effet de la qualité d'habitat des effets de la 'mémoire' des environnements passé, mais aussi de tester différentes hypothèses distinguant la plasticité phénotypique adaptative et la condition générale (résultat des qualités d'habitat passé).

En suivant les survies instantanées tout au long des histoires de vie des artémies, nous avons apporté une solution opérationnelle au problème qui se posait sur la mesure de l'impact de la qualité d'habitat sur la niche (Problème 5). La réalisation d'une expérience en deux phases ainsi que le développement d'une analyse de survie explicite ont en sus permis d'expliciter l'influence de l'histoire de vie sur la fitness des artémies dans la phase 2 (Problème 2), tout en proposant des tests permettant d'évaluer séparément les effets dépendants et indépendant de l'environnement (Problème 6).

Article 3

Dynamic tolerance curves: Habitat quality, condition, and adaptive plasticity in acclimation to a stressful environment

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In preparation

Abstract

Survival in a stressful environment depends both on direct effects of current stress on instantaneous mortality, and on indirect effects of past environments on current mortality, mediated by phenotypic plasticity (adaptive or not). Both types of effects are cumulative in time, so the probability to survive to a given age is a function of age, and of the temporal sequence of environments experienced by an individual. However, such a dynamical perspective is rarely applied in practice, preventing an understanding of the life-history components of environmental tolerance. We performed an experiment to address this question, on salinity tolerance in the brine shrimp *Artemia*. Individuals from three different clones of *A. parthenogenetica* were placed over a range of salinities during a week, before being transferred to a possibly different salinity for the rest of their lives, and individual life histories were monitored throughout. We used a modified survival analysis to partition effects of salinity on instantaneous mortality in each phase (habitat quality effects), from impacts of past salinity on future survival. We also attempted to distinguish effects of condition from those of adaptive plasticity. We showed clear effects of early salinity (in interaction with late salinity) on late survival. Our data was consistent both with adaptive plasticity under asymmetrical selection, or condition impacting fitness more strongly in more stressful

environments. Analysis of the dynamics of the tolerance curve through life showed that acclimation affects different parts of the curve at different ages. Such a dynamical view of the tolerance curve (and its potential underlying phenotypically plastic traits) should prove useful for understanding fitness and adaptation in temporally changing environments.

Keywords: Environmental tolerance; Adaptive plasticity; Habitat quality; Condition; Changing environment; Hazard rate.

Introduction

Environmental stress is a broad concept that has been used to describe different phenomena and processes depending on the context (physiological, ecological, or evolutionary). Stress can be generally defined as extreme environmental conditions that are adverse to an organism, and may elicit defense mechanisms from this organism (Hoffmann & Parsons 1991; Bijlsma & Loeschcke 2005). This is however a somewhat vague delimitation – What does adverse mean? What are extreme environments? Over what taxonomic range is a given environment stressful? What distinguishes stress response from others? –, and different aspects of stress have been emphasized in different fields of biology. Physiologists and cell biologists are mostly interested in response mechanisms at the individual level (i.e., phenotypic plasticity), notably stereotyped stress-response pathways that are induced systematically by different forms of adverse conditions (e.g. SOS response in bacteria, heat-shock proteins). In this context, stress is often used to describe the response itself (e.g. adrenaline pulse in animals), rather than the environment that induces it. In evolutionary biology, environmental change is considered stressful if it causes a strong reduction in fitness, generally thought to also cause strong selection (Hoffmann & Hercus 2000; Agrawal & Whitlock 2010). Hence stress is seen as a consequence of maladaptation, a genotype-by-environment ($G \times E$) effect on fitness caused for instance by a mismatch between the current phenotype and the optimum phenotype in the environment where selection operates. On the other hand, some environments are inherently less favorable than others, regardless of genotype, and for a wide range of taxa, which is described in the ecological literature as low habitat quality. This may occur for instance when some habitats offer more resources, more stable conditions, or fewer/less intense antagonistic interactions (competition, predation or parasitism). A well-studied example is temperature. Studies of thermal tolerance have shown that the upper thermal limit, beyond which performance and fitness vanish, is generally steep for a broad variety of organisms (insects: Deutsch *et al.* 2008,

lizards: Sinervo *et al.* 2010; viruses : Knies *et al.* 2006, etc, reviewed in Angilleta Jr. 2009), and has little genetic variance within species and thus responds little to selection (Gilchrist 1996; Kellermann *et al.* 2012; Klepsatel *et al.* 2013). In this paper, our aim is to experimentally disentangle these different aspects of stress: overall reduction of fitness, maladaptation, and individual responses.

The most direct effect of exposure to stress in the form of low-quality environments is an instantaneous increase in the mortality risk (hazard rate in survival analysis). This implies that the expected life span is all the more reduced when spending more time in the stressful environment. But of course this cumulative effect only can be assessed as an average over a group of individuals (e.g., a cohort). On the other hand, the time spent in a stressful environment also has cumulative effects at the individual level. Indeed, the environment influences the development and expression of phenotypes for a given genotype through phenotypic plasticity (Clausen *et al.* 1947; Schlichting & Pigliucci 1998; Pigliucci 2001; West-Eberhard 2003; Dewitt & Scheiner 2004). Adaptive plasticity, such that the fitness of a genotype across environments is higher with than without plasticity, is generally thought to have evolved under natural selection (Via & Lande 1985; Scheiner 1993; Agrawal 2001; Price *et al.* 2003; Dewitt & Scheiner 2004; de Jong 2005; Ghalambor *et al.* 2007). With such adaptive plasticity, the environment acts as a cue, such that spending more time in a stressful environment allows accumulating more information about the future selective conditions, producing a phenotypes that matches this selective pressure, and ultimately increasing survival later in life. Conversely, the environment may induce plastic responses that decrease fitness later in life in all environments (maladaptive plasticity). Importantly, these phenotypic effects at the individual level constitute a memory of the environment, and thus influence the vital rates later in life.

The life histories of individuals in natural conditions combine all the effects above, but they are often not distinguished empirically, preventing an understanding of the processes underlying fitness variation in heterogeneous

environments. For instance, local adaptation is generally investigated using reciprocal transplant experiments, where individuals from different locations are translocated from their birth, and their fitness is monitored (reviewed in Kawecki and Ebert 2004; Hereford 2009; Blanquart et al. 2013). This provides a measurement for the relative fitnesses of genotypes from different locations, assuming individuals spent all their lives in the same environmental conditions, which applies best to sessile organisms in temporally stable environments. However some organisms cannot be relocated at birth (e.g. birds or mammals), such that their life histories in reciprocal transplants necessarily combine influences from different environments. Perhaps more importantly, many organisms experience different environments throughout their lives under natural conditions, so it is important to know how different sequences of environments influence overall fitness.

Assessing the fitness of individuals experiencing different environments along their lives is particularly relevant to understand the fitness effects of phenotypic plasticity. Theory has shown that the predictability of the environment of selection at the moment of development - resulting from temporal autocorrelation of the environment or from the reliability of indirect cues used to predict selection - is a key driver of the evolution of phenotypic plasticity, with higher plasticity evolving under higher environmental predictability (Moran 1992; Gavrilets & Scheiner 1993; De Jong 1999; Tufto 2000; Lande 2009). This suggests that spending more time in a given environment may allow integrating environmental information over a longer duration, thus producing a phenotype that more accurately matches the selective pressure, and increasing the corresponding component of survival. In experimental biology, this line of thinking underlies the concept of acclimation, whereby individuals placed in a sub-lethal environment are later able to tolerate stronger environmental stresses (reviewed in Hoffmann *et al.* 2003; Angilleta Jr. 2009; see also hardening or hormesis for shorter exposures, Gems & Partridge 2008). However, this argument neglects the fact that if environmental stress partly results from low habitat quality, then the time spent in a stressful environment also reduces life expectancy through the

cumulative effect of stress-induced increase in mortality risk, which can contribute to costs of acclimation (Hoffmann 1995; Hoffmann *et al.* 2003). Distinguishing these effects requires an experiment where the environment is varied along life, and individual life histories are monitored at each stage of the experimentation. We have performed such an experiment on salinity tolerance in the brine shrimp *Artemia*. We have monitored the survival of individuals that experience different salinities during two successive phases of their lives, and analyzed their life histories using a modified survival-analysis model. This allowed us to partition the effects of environmental quality on fitness for each phase from the impact of past environments on future survival, conditionally on surviving in the earlier phases.

Methods

Clonal lineage construction and nauplii acquisition

Artemia parthenogenetica clonal lineages can easily be bred in the lab through parthenogenetic reproduction (Baxevanis *et al.* 2006). Indeed, parthenogenetic females can produce up to 100 individual per brood, and juveniles can be raised in tanks in the lab in order for them to face the same environmental conditions (Browne *et al.* 1988). For a few generations, mutation rate is too low to induce genotypic variation so monoclonal lines can be acclimatized to lab conditions and suppress maternal effects while keeping a single genotype.

In practice, three monoclonal lines of *A. parthenogenetica* were maintained in the lab for at least five generations. One line was isolated from the La Mata pond population in Spain (here after LM7 as it is the seventh monoclonal lineage isolated in our lab) and the other two originated from the Aigues-Mortes saltern population in France (here after PAM7 and PAM10 as they are the seventh and tenth monoclonal lineage isolated in our lab). To avoid maternal effects, each line was propagated for at least two generations before the experimentation, under 80g/L salinity and algae food.

Ten adult females from PAM7 and PAM10 and seven females from LM7 were put in individual cups in a 80g/L salt solution and fed every two days with 5 mL of an algae and yeast mix (75% of a 500 000 cells/mL algae solution +

25% of a 1 000 cells/mL yeast solution). For two weeks, we monitored the cups every day for new born nauplii (apart from nauplii produced during weekends that were not used in the experimentation). These were immediately placed in individual tubes to run the experimentation. After two weeks, we had a total of 1826 nauplii (744 LM7; 748 PAM7; 333 PAM10) divided in 9 cohorts.

Experimental rearing

The experimentation protocol included two phases (P_1 and P_2), separated by a transfer of nauplii across salinities. In practice, during P_1, nauplii were placed in individual tubes and evenly dispatched into three salinities (40, 80 and 120 g/L) for 7 days. Then individual tubes of each clonal lineage from each salinity were randomly dispatched into four salinities (40, 80, 120 and 180 g/L) for the second phase P_2, which lasted at least four weeks and up to seven weeks. The highest salinity of P_2 (180 g/L) was not used in P_1, because preliminary work and published literature suggested juvenile mortality would be too high at such salinity, thus impairing our experiment (Triantaphyllidis *et al.* 1995; Saygi 2004). During these two phases, nauplii were fed every day with 1 mL of the same algae and yeast mix as adult females. Survival was checked twice a week in both P_1 and P_2.

Survival analysis

We developed a two-phase survival analysis model, where survival in the second phase is conditional on survival in the first phase. This allowed us to split overall survival into instantaneous effects of environmental quality in each phase, and "individual memory" effects of P_1 on P_2 caused by phenotypic plasticity and condition.

General survival model. We ended our experimentation before the death of all individuals. Therefore, we implemented a censored survival analysis model (see Supplementary material 3). Hazard functions were supposed exponential, with a mortality risk λ that does not change in time within each phase, such that the survival function $S(t)$ followed

$$\begin{cases} S(t) = e^{-\lambda_1 t} & t < T_{P1} \\ S(t) = e^{-(\lambda_1 T_{P1} + \lambda_T (t - T_{P1}))} & T_{P1} \le t < T_{P1} + T_{P2T} \\ S(t) = e^{-(\lambda_1 T_{P1} + \lambda_T T_{P2T} + \lambda_2 (t - T_{P2T} - T_{P1}))} & t \ge T_{P1} + T_{P2T} \end{cases}$$

where T_{P1} is the time spent in P_1 before transfer (7 days), and T_{P2T} corresponds to the first three days spent in P_2 after transfer. The parameters λ_1 and λ_2 are the hazard rates in P_1 and P_2, respectively. Note that the hazard rate may change between P_1 and P_2 even for individuals that stay at the same salinity, simply because survival depends on age (this is treated in our model as an 'age' effect). We also introduced an additional hazard rate λ_T that applies only to the first 3 days in P_2, to test whether mortality was transiently increased following the transfer. Several generalized linear models were implemented using the λs as the linear variable, with the factors being age, environmental quality (3 and 4 salinity levels in P_1 and P_2, respectively), genotype (clonal lineages LM7, PAM7 and PAM10), and their possible interactions. Survival analysis was implemented using Mathematica program (Wolfram Research 2008). Likelihood was estimated for each model and best model selection followed Akaike information criterion (hereafter AIC; Akaike 1974).

We tested several models to discriminate between hypotheses presented in the introduction. First, we wanted to determine the impact of environmental quality on instantaneous survival. We thus tested if hazard rates in P_1 and/or P_2 were influenced by the environment (here, salinity) in the corresponding phase (denoted as $SP_1 \rightarrow \lambda_1$ and $SP_2 \rightarrow \lambda_2$, respectively). We expect higher hazard rates in environment of poor quality. Second, we investigated effects of past environments on hazard rates, caused by individual memory of the environment through phenotypic plasticity (both adaptive and not). This was quantified as an effect of the environment faced in P_1 on the hazard rate in P_2 (denoted as $SP_1 \rightarrow \lambda_2$). A pure $SP_1 \rightarrow \lambda_2$ effect (with no interaction) indicates that P_1 salinity affects P_2 survival regardless of SP_2, consistent with an effect of early habitat quality on condition that carious over later in life, as opposed to adaptive plastic responses (see below). For each of these hypotheses, we

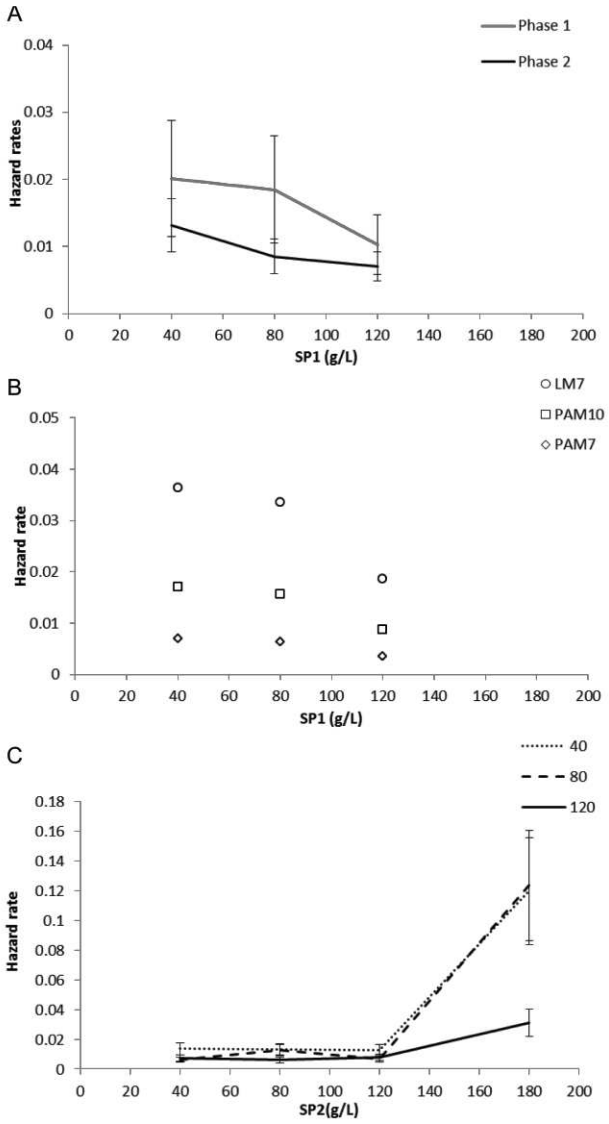

Figure 1: Effects of salinities in phases 1 and 2 on instantaneous mortality. A: hazard rates in phase 1 (λ_1: grey line) and phase 2 (λ_2: black line) are plotted against salinity in phase 1 (SP_1). The mean λ_2 is averaged only over the SP_1 (40, 80, and 120g/L), for consistency. **B:** λ_1 is plotted against $SP_1$for each clone (LM7: circle; PAM10: square; PAM7: diamond); **C:** λ_2 is plotted against salinity in phase 2 (SP_2), with the line format corresponding to different SP_1 (continuous: 120g/L; dashed: 80g/L; dotted: 40g/L). Error bars correspond to the standard deviation caused by clone and SP_1 in A, and only clone in C.

also looked for interactions between environment and clone, to test if the response to stress differs among genotypes.

We also investigated possible interactions between effects of environments in P_1 and P_2 on λ_2, meaning that individual responses to salinity in P_1 have different effects depending on the salinity in P_2. We tested several simplified models regarding these interactions. First, we tested a model where salinity in P_1 only affects survival in P_2 for some values of salinity in P_2, consistent with effects on condition that affect fitness only under stress. Next, we searched for an effect of adaptive plasticity on survival, by testing whether individuals that developed in a given environment would survive better in more similar environments. To do so, we implemented a linear function increasing with the distances between salinities faced in P_1 and P_2. We also tested combinations of these models.

Results

All best models included an 'age' effect in both λ_T and λ_2 (see m4 to m7 in Table S1 for comparison to models that removed this effect), indicating that regardless of salinity or transfer, the instantaneous mortality rate was higher in phase 1 (P_1) than in phase 2 (P_1) because mortality is higher in juveniles in this species.

Environmental quality

The best model describing *Artemia* survival (Table 1) included, during the first phase, significant effects of salinity in phase 1 (SP_1) and clone lineage (in Table 1, the model with no clone effect increased AIC by 275.86), with no interaction of clone and S_1 effects on λ_1 (see m3, m28 and m30 in Table S1). Hazard rates decreased from 40 to 80g/L and 120g/L (Figure 1A), indicating that 120g/L was the most favorable environment in P_1 in our experiment, while 40g/L was a relatively low quality environments. There was also a significant effect of environmental quality in phase 2 (P_2): the best model includes an $SP_2 \rightarrow \lambda_2$ effect, a clone effect, and an interaction between these two factors. In P_2, hazard rates were much higher at 180g/L than at any other salinity (Figure 1C; see also Figure S2).

Table 1 - Model selection for the survival analysis. The properties of a selection of models for the survival of brine shrimps in the different phases of our experiment are shown (the complete set of tested models is referenced in Table S1). **K** is the number of parameters for each model, **logLik** the log likelihood, **AIC** the Akaike information criterion, **ΔAIC** the AIC differences, w_i the Akaike weights. All models include as main effects, in phase 1 (P_1): clone (LM7, PAM7 or PAM10) and salinity in phase 1 (SP_1 = 40, 80 or 120g/L); in phase 2 (P_2): clone, age (hazard rates changing relative to P_1, even when remaining at the same salinity), SP_1 and SP_2 (40, 80, 120 or 180g/L). Parameters that varied between models (including interactions) are listed in the table below (where "–" denotes a main effect that was removed from the model). Modifications concerning clone effects applied to all phases, those concerning $Clone \times SP_2$ applied to P_2, while modifications of $SP_1 \times SP_2$ applied to P_2 but past the first 3 days following the nauplii's transfer (transition phase). In the *Adaptive plasticity* model, *a* and *b* are respectively the intercept and slope of the linear function relating hazard rate in P_2 to the difference in salinity between P_1 and P_2.

	K	logLik	AIC	ΔAIC	w_i	Modified effects
Best model	27	-4597.43	9248.86	0.00	0.75	$+ \boldsymbol{Clone} \times \boldsymbol{SP_2} + \boldsymbol{SP_1} \times \boldsymbol{SP_2}$
No clone effect	13	-4749.36	9524.72	275.86	0.00	$- \boldsymbol{Clone} + \boldsymbol{SP_1} \times \boldsymbol{SP_2}$
Triple interaction	44	-4585.35	9258.70	9.84	0.01	$+ \boldsymbol{Clone} \times \boldsymbol{SP_1} \times \boldsymbol{SP_2}$
No interaction	8	-4669.48	9354.96	106.10	0.00	
Stress limited history effect	24	-4604.21	9256.42	7.56	0.02	$+ \boldsymbol{Clone} \times \boldsymbol{SP_2} + \begin{cases} \boldsymbol{if\ SP_2 < 180g/L} \\ \boldsymbol{if\ SP_2 = 180g/L} \end{cases} \boldsymbol{SP_1} \times \boldsymbol{SP_2}$
Adaptive plasticity	10	-4919.04	9858.08	609.22	0.00	$+ \boldsymbol{Clone} \times \boldsymbol{SP_2} + \boldsymbol{a} + \boldsymbol{b} \times (\boldsymbol{SP_2} - \boldsymbol{SP_1})$
Stress-limited adaptive plasticity	26	-4599.72	9251.44	2.58	0.21	$+ \boldsymbol{Clone} \times \boldsymbol{SP_2} + \begin{cases} \boldsymbol{if\ SP_2 < 180g/L} \\ \boldsymbol{if\ SP_2 = 180g/L} \end{cases} \boldsymbol{a} + \boldsymbol{b}\ (\boldsymbol{SP_2} - \boldsymbol{SP_1})$

In both P_1 and P_2, the LM7 clone presented a lower survival than PAM7 and PAM10. For instance in phase 1, $\lambda_1 < 0.02$ at all salinities for PAM7 and PAM10, while $\lambda_1 > 0.02$ at all salinities for LM7. This may be indicative of a survival fecundity trade-off, as LM7 tends to produce more nauplii per brood than PAM7 and PAM10 (pers. obs.). Regarding the two clones from Aigues-Mortes, we observed higher hazard rate for PAM10 than PAM7 at 180g/L in P_2, but not at lower salinities. PAM7 was isolated from a high salinity, high temperature cluster of clones while PAM10 was isolated from a low salinity, low temperature cluster of clones from Aigues-Mortes (Nougué *et al.* in prep). The experimental result therefore agrees with our population genetic study, confirming the environmental specialization of these clones.

Memory of past environments

The model that best describes the survival of *Artemia* (Table 1) also included an $SP_1 \rightarrow \lambda_2$ effect, indicating an effect of past environments of future survival. In Figure 1A, this is illustrated by the fact that the hazard rates in P_2 are higher when low-quality environment was faced in P_1: groups of individual raised at 40 or 80 g/L in P_1 had higher hazard rates on average than group of individuals raised at 120g/L. Interestingly, the effect of the environment of acclimation on survival ($SP_1 \rightarrow \lambda_2$) is in the same direction as the direct effect of environmental quality in phase 1 ($SP_1 \rightarrow \lambda_1$), as shown in Figure 1A. They are also in the same direction as the effect of environmental quality in phase 2 ($SP_2 \rightarrow \lambda_2$), over the same range of environments (40-120g/L), even though mortality is much higher at 180g/L (Figure 1B).

The best models also included large and significant $SP_1 \times SP_2$ and $Clone \times SP_2$ interactions (the model without interaction increased AIC by 106.1, while the model with the triple interaction $Clone \times SP_1 \times SP_2$ increased AIC by 9.84). The $SP_1 \times SP_2$ interaction implies that the effect of past environment is not only a result of low condition that is similar across environments in P_2, but also depends to some extent on the salinity in P_2. This can be seen in Figure 1C, where λ_2 remained low (around 1.53%) and varied little with SP_1 for all SP_2, except for SP_2 = 180g/L, where hazard rate was always significantly higher (thus defining a more stressful environment), and varied strongly across

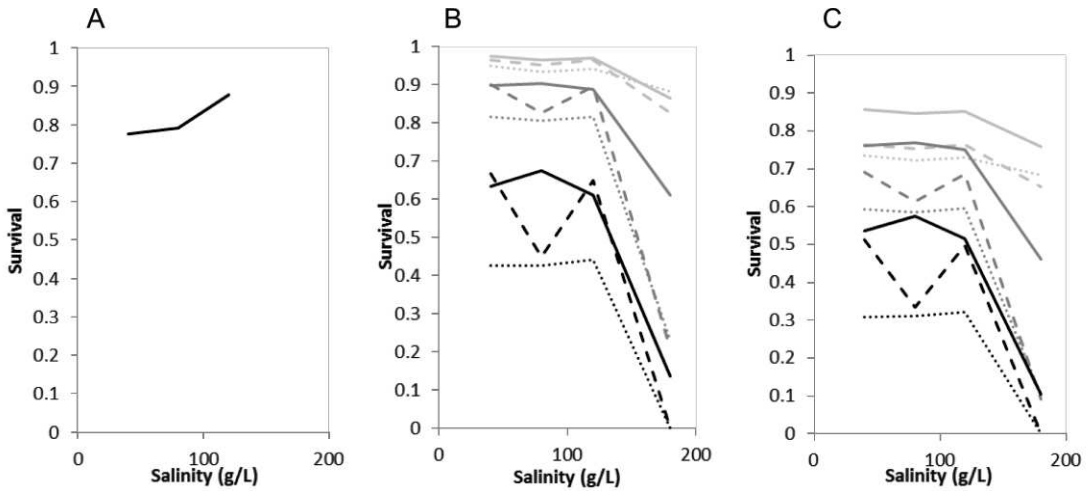

Figure 2: Estimated survival at different times for the LM7 clone. A: survival at the end of phase 1 (P_1) along salinity gradient; **B:** survival at different times in phase 2 (P_2) for individuals who survived at the end of P_1; **C:** survival at different times in $P_2$for all individuals. In B and C colours correspond to different timings (light grey: 3 days in P_2; grey: 10 days in P_2; black: 40 days in P_2), and the line format correspond to the salinity faced in phase 1 (dotted: 40g/L; dashed: 80g/L; continuous: 120g/L).

acclimation salinities. However, a model where a $SP_1 \times SP_2$ interaction only exists for SP_2 = 180g/L, while the response to SP_1 is the same for all other values of SP_2, increased AIC by 7.56 relative to the best model ('Stress-limited history effect' in Table 1), and thus was not selected.

A $SP_1 \times SP_2$ interaction would be indicative of adaptive phenotypic plasticity if survival is higher for individuals that developed in P_1 in environments closer to their environment in P_2. However, models addressing this hypothesis did not improve AIC ('Adaptive plasticity' model in Table 1 increased AIC by 609.22; see also m16, m19 and m22 in Table S1). We also tested models where the benefit of adaptive plasticity is higher in more stressful environments ('Stress-limited adaptive plasticity' in Table 1), but this did not improve AIC either relative to the model with a full interaction.

Dynamics of the tolerance curve along life

Our method decomposes survival into instantaneous mortality risks over different phases, where an organism experiences different environments. The estimated model can then be used to predict the proportion of survivors (survivorship) at different points in time, thus showing temporal changes in the environmental tolerance curve. Applying this approach to our experiment shows important aspects of both our approach and our results, as illustrated in Figure 2 for a single clone (LM7).

Figure 2A shows the cumulated survival at the end of P_1 for LM7. Even though this clone has the highest hazard rate in P_1 (Figure 1B and above), the model predicts that over 77.5% of individuals still survive at all salinities at the end of P_1. The reason is that mortality risk cannot accumulate much during this short phase. However, an effect of salinity can already be observed on the tolerance curve at the end of P_1 (Figure 2A), consistent with the fact that the effect of salinity on the hazard rate ($SP_1 \rightarrow \lambda_1$) is relatively strong for LM7 (Figure 1B).

Figure 2B shows the survival probability in P_2, conditional on having survived P_1 - which we call the conditional tolerance curve -, for different times in P_2. The shape of the tolerance curve, and its dependence on the salinity in the

acclimation phase (SP_1), both change along life. Three days after the transfer to the new environment (end of the transfer phase), survival is already slightly lower at 180g/L, but still around 90%, and the effect SP_1 on the conditional tolerance curve is barely noticeable, because the effect of past environment on death risk has not accumulated enough. After 10 days, the shape of the conditional tolerance curve becomes more concave, with substantially reduced survival at 180g/L as compared to other salinities. More importantly, much stronger differences in survival between acclimation treatments are found at 180g/L than at other salinities, because of the stronger $SP_1 \rightarrow \lambda_2$ effect at 180g/L. In contrast, 40 days after the transfer, the effect of the acclimation treatment on survival is more pronounced at 40-120g/L than at 180g/L. The reason is that most individuals are already dead at the highest (and most stressful) salinity, so there is little variance in survivorship. Figure 2B thus illustrates that the qualitative and quantitative influence of the acclimation treatment on the conditional tolerance curve changes with the timing of the observation, which can have important consequences on the biological interpretation of such experiments.

The same applies to the unconditional tolerance curve, which shows the overall survivorship resulting from the cumulative mortality risks over P_1 and P_2 (Figure 2C). This curve looks somewhat similar to the conditional curve in Figure 2B, because the shorter duration of P_2 in our experiment means that this phase contributes less to the cumulative mortality risk. However this needs not be true in general, and the relative contributions to overall survival of different environments encountered at different ages depends on the duration of exposure to each environment, and the instantaneous mortality risk at different ages. The main differences between Figure 2B and 2C are that in Figure 2C (unconditional tolerance curve): (i) survival is lower at any time; and (ii) the influence of the salinity in P_1 is already non-negligible 3 days after the transfer to the new environment. It is important to note that studies of environmental tolerance usually focus on the unconditional tolerance curve (Figure 2C), while studies of acclimation sometimes focus on the conditional curve (Figure 2B), showing survival of individuals that survived prior exposure

to stress. But in general, richer information is contained in the full survival model.

Discussion

From an eco-evolutionary perspective, environmental stress can be defined through its effect on reduced fitness (Hoffmann & Parsons 1991; Agrawal & Whitlock 2011) Reduced fitness in a given environment can be due to maladaptation, when a genotype does not express the right phenotype in this environment, or to habitat quality effects that are independent of the phenotype and genotype. For a given set of genotypes, any effect of current environment on fitness that applies identically to all the genotypes can be described as an environmental quality effect. In contrast, an effect of past environments on the present fitness of a given genotype can have several origins. On the one hand, the past environment may have induced the development/expression of specific values for plastic traits, causing fitness to be higher in environments where these trait values are favored (adaptive plasticity hypothesis). Assuming that the environmental variable has strong positive autocorrelation between development and selection in nature, adaptive plasticity will cause higher fitness for organisms that develop closer to their environment of selection (Gavrilets & Scheiner 1993; Lande 2009). On the other hand, habitat quality, beyond just causing increased instantaneous mortality risk, may also affect the condition of individuals, thus increasing their death risk later in life.

When fitness (or a component thereof) is the only measured trait, these alternatives might be separated by comparing how changes in fitness late in life in response to the ‘early’ environment depend on the ‘late’ environment. A fitness response to the early environment that is independent of the late environment is strongly suggestive of an effect of condition (memory of past habitat quality). In contrast, a response to the early environment that changes sign depending on the late environment is strongly suggestive of adaptive plasticity. More intermediate situations, with a generic interaction between the early and late environment, are more difficult to interpret. For instance, the situation where the early environment affects fitness more strongly for some

values of the late environment is compatible both with an interaction of condition with current environmental quality (such that individuals in bad shape survive less well in more stressful environments), and with adaptive phenotypic plasticity of traits selected with different strength in different environments. As an example of the latter alternative, think of inducible defenses to predators (Hammill *et al.* 2008): this adaptively plastic trait is under asymmetric selection, since not having a predator defense is strongly counter-selected in the presence of predators, but having this defense is weakly counter-selected in the absence of predators.

We conducted an experiment on acclimation to salinity with clonal lineages of *Artemia*, and implemented a statistical procedure for dissecting the effects of habitat quality, adaptive plasticity, and condition, on temporal changes of the salinity tolerance curve. First, we found that all genotypes of *Artemia* (LM7, PAM7 and PAM10) survived better at high (120g/L), indicating a difference in habitat quality for this species. *Artemia* cannot survive in fresh and brackish water (< 40g/L), partly because of their microbiota maladaptation (Nougué *et al.* 2015). We therefore chose to test only salinities ≥ 40g/L, and added yeast to the algae food to compensate with food not requiring gut microbiota to be digested. In phase 2, survival was substantially reduced at 180g/L, and 120g/L thus appeared as an optimum salinity on our measurement scale. Previous studies described that the optimum salinities for many species of *Artemia* was comprised between 60g/L and 100g/L (Triantaphyllidis *et al.* 1995; van Stappen 2002; Castro-Mejía *et al.* 2011). However, this was shown to change with other environmental conditions such as temperature (Vanhaecke *et al.* 1984). A salinity of 120g/L still appear pretty close from the optimum but the few studies who tested survivorship of *Artemia* above such salinity (Triantaphyllidis *et al.* 1995; Browne & Wanigasekera 2000), clearly depict a dropout in survivorship above this salinity.

Second, we detected an effect of memory of past environments, in that survival in phase 2 (P_2) depended on the environment in phase 1 (P_1). Furthermore, this effect was not homogeneous across environments in P_2, as attested by the $SP_1 \times SP_2$ effect in the best model (Table 1). In particular, we

found that the instantaneous mortality risk in P_2 is reduced for individuals that developed at 120g/L in P_1, relative to those that developed at 40 or 80g/L, and that this effect is most marked at 180g/L in P_2 (Figure 1B). Unfortunately, we were not able to distinguish between two competing hypotheses to account for this result: adaptive plasticity (causing higher survival of individuals developing in similar environments in the two phases) versus an effect of past habitat quality on condition (causing reduced survival in P_2 for individuals that experienced low quality habitat in P_1). These hypotheses cannot be distinguished in our dataset because (i) the effect of environment in P_1 is mostly observed at a single salinity in P_2 (180g/L); (ii) the lowest mortality risk in P_2 is found for individuals that were exposed to 120g/L in P_1, which is the closest to 180g/L in P_2, consistent with adaptive plasticity; but (iii) 120g/L is also the salinity at which survival is highest in P_1 and P_2 because of direct habitat quality effects, so the 'memory effect' is also consistent with an effect of past habitat quality on condition. We attempted to further discriminate between these hypotheses by studying the phenotypic plasticity of the abdomen-to-total-size ratio for a morphological trait that was shown to respond plastically to salinity (Triantaphyllidis *et al.* 1995; Saygi 2004), and impact survival, namely (see Supplementary material 4). However, our results indicated that most of the plastic response of that trait was caused by the second phase, probably because of its longer duration – while the acclimation phase represented approximately 1/3 of *Artemia*'s expected development time. Similarly, we were not able to discriminate if this trait had a significant effect on post-measurement survival (Supplementary material 4).

Acclimation, whereby fitness (or performance as a surrogate) in the face of environmental stress is increased by prior exposure to this stress, is a well know phenomenon, which has been described in a wide variety of organisms (reviewed for temperature by Hoffmann 1995; Hoffmann *et al.* 2003; Angilleta Jr. 2009). Recently, acclimation has attracted considerable attention in the context of climate change (Calosi *et al.* 2008; Palumbi *et al.* 2014). The influence of variation of the acclimation environment on the shape of tolerance curves has been investigated in recent studies of thermal tolerance. For

example, Deere and Chown (2006) used an experiment similar to ours, where 5 species of marine to terrestrial mites were acclimated to different temperatures in a first phase, and their final performance was measured in the same range of environments. They clearly showed an effect of P_1 on the performance in P_2. Cooper et al. (2010) investigated the acclimation of *Drosophila melanogaster* to stable or stochastic environments, and also found an effect of the acclimation phase on the tolerance curve later in life. However, these authors used performance measured at one point in life to measure environmental tolerance. In contrast, we focused on a direct component of fitness, and used a survival analysis that allows the acclimation phase and the later phase to have different hazard rates (instantaneous mortality risks), and different dependence of these rates on salinity. This allowed us to distinguish direct effects of habitat quality on instantaneous survival from memory effects of past environments (Figure 1). Regarding the latter effects, our approach also allowed us to test alternative hypotheses, namely adaptive plasticity of an underlying trait, versus general condition resulting from past habitat quality (even though our data does not allow distinguishing these hypotheses).

Our approach based on survival analysis accounts for the dynamic nature of environmental tolerance curves, and of acclimation effects on these curves, by building survivorship through time from instantaneous mortality rates (Figure 2). In our experiment, this allowed us to distinguish the contributions of survival during acclimation (Figure 2A) and survival post-acclimation (conditional tolerance curve, Figure 2B) to the overall tolerance curve (Figure 2C). Our analysis also highlighted that acclimation affected the shape of tolerance curves differently at different times, such that 'static' measurements of the tolerance curve would lead to different interpretations depending on when these curves are measured. More generally, analyzing the potential for temporal changes in tolerance curves should be useful for understanding and predicting fitness in temporally changing environments.

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Supplementary material

Supplement 1. Table S1: Model comparison results.

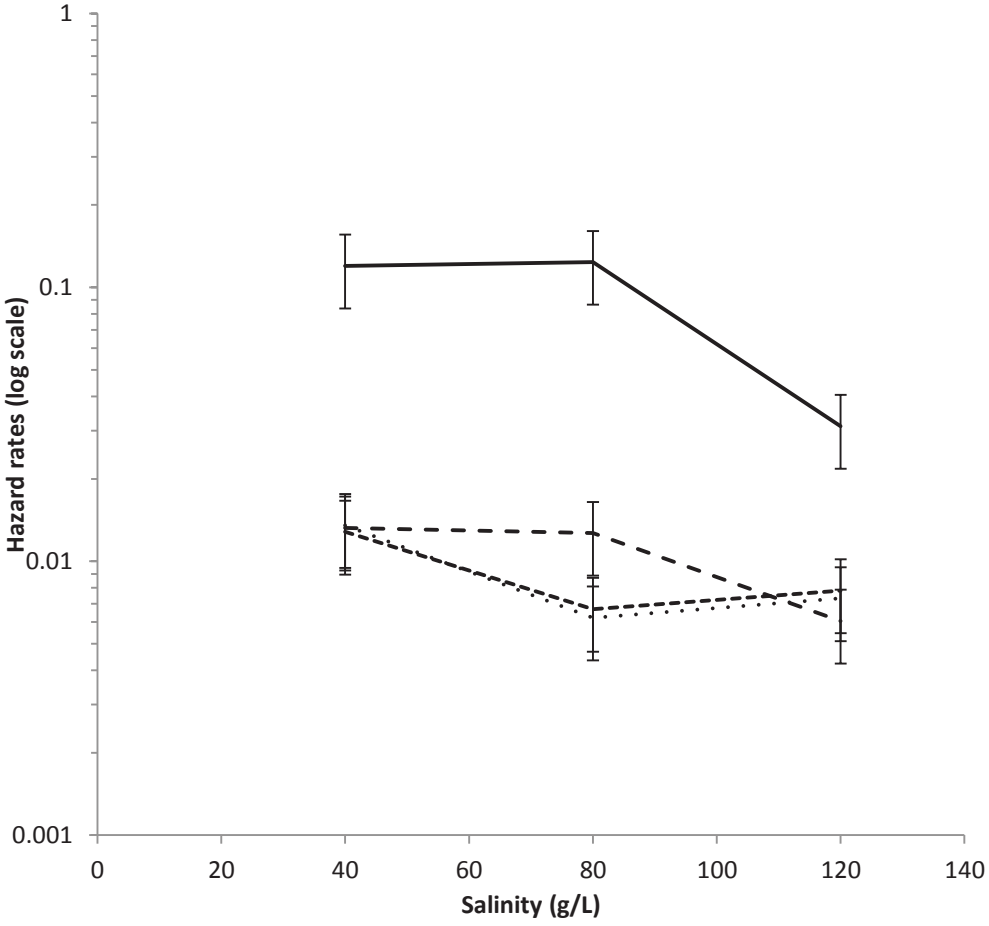
K is the number of parameters for each model, **logLik** the log likelihood, **AIC** the Akaike information criterion, **ΔAIC** the AIC differences, w_i the Akaike weights. **Parameters** are: C corresponds to the three clones categories (LM7, PAM7 and PAM10); S1 is the three salinity levels of phase 1 (40, 80 and 120g/L); S2 is the four salinity levels of phase 2 (40, 80, 120 and 180g/L); A is the aging parameter (AT is the aging parameter specific to the acclimation phase).

Models	K	logLik	AIC	ΔAIC	w_i	Parameters in Phase 1	Transition phase	Phase 2
m31	27	-4597.43	9248.86	0.00	0.75	C S1	C S1 S2 CxS2 A AT	C S1 S2 CxS2 A S1xS2
m37	26	-4599.72	9251.44	2.58	0.21	C S1	C S1 S2 CxS2 A AT	C S1 S2 CxS2 A If S2<180 - If S2=180 (a+b(S2-S1))
m32	24	-4604.21	9256.42	7.56	0.02	C S1	C S1 S2 CxS2 A AT	C S1 S2 CxS2 A If S2<180 - If S2=180 S1xS2
m10	15	-4613.53	9257.06	8.20	0.01	C S1	C S1 S2 A AT	C S1 S2 A S1xS2
m13	11	-4617.70	9257.40	8.54	0.01	C S1	C S1 S2 A AT	C S1 S2 A If S2<180 - If S2=180 S1xS2
m40	44	-4585.35	9258.70	9.84	0.01	C S1	C S1 S2 CxS1xS2 A AT	C S1 CxS1xS2 S2 A
m36	27	-4605.04	9264.08	15.22	0.00	C S1	C S1 S2 A AT	C S1 S2 CxS2 A S1xS2
m34	27	-4611.53	9277.06	28.20	0.00	C S1	C S1 S2 A S1xS2 AT	C S1 S2 A S1xS2
m9	15	-4624.28	9278.56	29.70	0.00	C S1	C S1 S2 A S1xS2 AT	C S1 S2 A S1xS2
m41	56	-4585.54	9283.08	34.22	0.00	C S1	C S1 S2 CxS1xS2 A S1xS2 AT	C S1 CxS1xS2 S2 A S1xS2
m8	15	-4630.15	9290.30	41.44	0.00	C S1	C S2 A S1xS2 AT	C S2 A S1xS2
m32	24	-4621.76	9291.52	42.66	0.00	C S1	C S1 S2 CxS2 A S1xS2 AT	C S1 S2 CxS2 A If S2<180 - If S2=180 S1xS2
m12	11	-4636.55	9295.10	46.24	0.00	C S1	C S1 S2 A If S2<180 - If S2=180 S1xS2 AT	C S1 S2 A If S2<180 - If S2=180 S1xS2
m33	24	-4636.06	9320.12	71.26	0.00	C S1	C S1 S2 CxS2 A S1xS2 AT	C S1 S2 CxS2 A If S2<180 - If S2=180 S1xS2
m35	27	-4639.07	9332.14	83.28	0.00	C S2	C S1 S2 A S1xS2 AT	C S1 S2 A
m15	11	-4657.26	9336.52	87.66	0.00	C S1	C S1 S2 A If S2<180 - If S2=180 S1xS2 AT	C S1 S2 A
m39	42	-4626.63	9337.26	88.40	0.00	C S1	C S1 S2 CxS1xS2	C S1 CxS1xS2 S2
m11	15	-4656.40	9342.80	93.94	0.00	C S1	C S1 S2 A S1xS2 AT	C S1 S2 A
m19	10	-4663.64	9347.28	98.42	0.00	C S1	C S2 A If S2<180 - AT	C S2 A If S2<180 -

														If S2=180 (a+b(S2-S1))									If S2=180 (a+b(S2-S1))
m22	10	-4664.92	9349.84	100.98	0.00	C	S1		C		S2		A	If S2-S1>0 (a+b(S2-S1)) If S2-S1<0 -	AT	C			S2		A	If S2-S1>0 (a+b(S2-S1)) If S2-S1<0 -
m7	8	-4669.48	9354.96	106.10	0.00	C	S1		C	S1	S2		A		AT	C	S1		S2		A	
m16	10	-4668.33	9356.66	107.80	0.00	C	S1		C		S2		A	(a+b(S2-S1))	AT	C			S2		A	(a+b(S2-S1))
m6	7	-4689.00	9392.00	143.14	0.00	C	S1		C	S1	S2		A			C	S1		S2		A	
m25	10	-4688.62	9397.24	148.38	0.00	C	S1		C		S2		A	If S2-S1>0 - If S2-S1<0 (a+b(S2-S1))	AT	C			S2		A	If S2-S1>0 - If S2-S1<0 (a+b(S2-S1))
m29	18	-4710.61	9457.22	208.36	0.00	C	S1		C		S2	CxS2				C			S2	CxS2		
m3	6	-4747.28	9506.56	257.70	0.00	C	S1		C		S2					C			S2			
m28	18	-4738.56	9513.12	264.26	0.00	C	S1	CxS1	C		S2	CxS2				C			S2	CxS2		
m14	11	-4749.58	9521.16	272.30	0.00	C	S1		C		S2		A		AT	C			S2		A	If S2<180 - If S2=180 S1xS2
m42	13	-4749.36	9524.72	275.86	0.00		S1			S1	S2		A		AT		S1		S2		A	S1xS2
m38	42	-4746.09	9576.18	327.32	0.00	C	S1		C	S1	S2					C	S1	CxS1xS2	S2			
m24	10	-4872.11	9764.22	515.36	0.00	C	S1		C		S2		A	If S2-S1>0 (a+b(S2-S1)) If S2-S1<0 -	AT	C			S2		A	
m21	10	-4872.19	9764.38	515.52	0.00	C	S1		C		S2		A	If S2<180 - If S2=180 (a+b(S2-S1))	AT	C			S2		A	
m27	10	-4872.57	9765.14	516.28	0.00	C	S1		C		S2		A	If S2-S1>0 - If S2-S1<0 (a+b(S2-S1))	AT	C			S2		A	
m5	7	-4878.83	9771.66	522.80	0.00	C	S1		C		S2		A			C			S2		A	
m26	10	-4914.06	9848.12	599.26	0.00	C	S1		C		S2		A		AT	C			S2		A	If S2-S1>0 - If S2-S1<0 (a+b(S2-S1))
m30	15	-4912.77	9855.54	606.68	0.00	C	S1	CxS1	C		S2					C			S2			
m17	10	-4919.04	9858.08	609.22	0.00	C	S1		C		S2		A		AT	C			S2		A	(a+b(S2-S1))
m23	10	-4921.51	9863.02	614.16	0.00	C	S1		C		S2		A		AT	C			S2		A	If S2-S1>0 (a+b(S2-S1)) If S2-S1<0 -
m20	10	-4921.83	9863.66	614.80	0.00	C	S1		C		S2		A		AT	C			S2		A	If S2<180 - If S2=180 (a+b(S2-S1))
m2	4	-4997.01	10002.02	753.16	0.00		S1				S2								S2			
m4	6	-4997.74	10007.48	758.62	0.00	C	S1		C	S1	S2					C	S1		S2			
m28	11	-5002.47	10026.94	778.08	0.00	C	S1		C	S1	S2		A		AT	C	If S2<180 S1 If S2=180 -		S2		A	If S2<180 - If S2=180 S1xS2
m18	10	-7287.63	14595.26	5346.40	0.00	C	S1		C	S1	S2		A		AT	C	S1		S2		A	(a+b(S2-S1))
m1	2	-25802.60	51609.20	42360.34	0.00	C			C							C						

Supplement 2.

Figure S2: Hazard rates in phase 2 along salinity gradient in phase 1 for each salinity faced in phase 2 (180g/L: solid line; 120g/L: dashed line; 80g/L: loose dashed line; 40g/L: dotted line). Error bars correspond to the standard deviation caused by clone effect.



Supplement 3. Survival analysis

Survival Analysis script

Data Imput

Data structure is as follow: {C, SP1, SP2, A, D}
where C is the clonal line of the individual (LM7 = 1, PAM10 = 2 and PAM7 = 3); SP1 and SP2 are the respective salinities during phase 1 and 2 of the experimentation (40, 80, 120 or 180g/L); A is the last day the individual was seen alive; D is the first day the individual was seen dead [D = 10 000 for censored individuals].

```
data = Import["Path too\\data.txt", "Table"];
group[Clone_, selI_, selII_] :=
  Select[data, #[[1]] == Clone && #[[2]] == selI && #[[3]] == selII &];
S1 = {40, 80, 120, 180};
S2 = {40, 80, 120};
Clones = {1, 2, 3};
```

```
group[3,120,180]//MatrixForm
```

$$\begin{pmatrix} 3 & 120 & 180 & 10 & 11 \\ 3 & 120 & 180 & 55 & 10\,000 \\ 3 & 120 & 180 & 30 & 31 \\ 3 & 120 & 180 & 55 & 10\,000 \\ 3 & 120 & 180 & 55 & 10\,000 \\ 3 & 120 & 180 & 9 & 10 \\ 3 & 120 & 180 & 23 & 24 \\ 3 & 120 & 180 & 23 & 24 \\ 3 & 120 & 180 & 55 & 10\,000 \\ 3 & 120 & 180 & 23 & 24 \\ 3 & 120 & 180 & 12 & 13 \end{pmatrix}$$

The two time limits of the experimentation are set for the rest of the script.

TP1: the time spent in phase 1 before transfer

TP2: TP1 + the first three days spent in phase 2

```
TP1 = 7; TP2 = 10;
```

Survival function S(t) and log Likelihood

```
S[t_, a_, b_, c_] := Exp[
   -Piecewise[{{a t, t < TP1}, {7 a + b t, TP1 ≤ t < TP2}, {7 a + 3 b + c t, TP2 ≤ t}}]];

ll[Clone_, selI_, selII_] := (dat = group[Clone, selI, selII];

   size := Length[group[Clone, selI, selII]];

    (Sum[Log[S[t1_k, λ1{Clone,selI}, λT{Clone,selI,selII}, λ2{Clone,selI,selII}] -
          S[t2_k, λ1{Clone,selI}, λT{Clone,selI,selII}, λ2{Clone,selI,selII}]], {k, 1, size}]

     /. t1_k_ :> dat[[k, 4]] /. t2_k_ :> dat[[k, 5]]);

lltot :=
  Sum[Sum[Sum[ll[listclone[[h]], listsel[[i]], listsel[[j]]], {j, 1, Length[S1]}], {i, 1, Length[S2]}], {h, 1, Length[Clones]}];
```

No interactions models

In these models, the mortality risk in phase 1 and phase 2 only depends on the salinity of there own phase.

```
NI1 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
       λT{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift + λTransfert],
       λ2{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift]};

NI2 = {λ1 → 0};
λS1 = Flatten[Table[{{λS1[[i]], 0, -10, 10}}, {i, 1, Length[S1]}], 1];
λclone = Flatten[Table[{{λClones[[i]], 0, -10, 10}}, {i, 2, Length[Clones]}], 1];
allλ = Join[λS1, λclone, {{λshift, 0, -10, 10}}, {{λTransfert, 0, -10, 10}}];

Result = FindMaximum[lltot /. NI1 /. NI2, allλ]
```

```
{-4707.31, {λ40 → -3.36463, λ80 → -3.36514, λ120 → -3.79539, λ180 → -2.02088,
  λ2 → -0.916909, λ3 → -0.771497, λshift → -0.554492, λTransfert → -0.299274}}
```

Interactions models

In these models, we test all possible interactions between SP1 and SP2 that only depends on the relative distance between environments.

```
I1 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift + λTransfert],
          λ2{Clone_,selI_,selII_} →
    Exp[λClone + λselII + λshift + Piecewise[{{λinter1, selII - selI == -40},
          {λinter2, selII - selI == 40},
      {λinter3, selII - selI == 60}, {λinter4, selII - selI == -80},
          {λinter5, selII - selI == 80}, {λinter6, selII - selI == 100},
      {λinter7, selII - selI == 140}}]]};
I2 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} → Exp[
     λClone + λselII + λshift + λTransfert + Piecewise[{{λinter1, selII - selI == -40},
          {λinter2, selII - selI == 40}, {λinter3, selII - selI == 60},
      {λinter4, selII - selI == -80},
          {λinter5, selII - selI == 80}, {λinter6, selII - selI == 100},
      {λinter7, selII - selI == 140}}]],
          λ2{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift]};
I3 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} → Exp[
     λClone + λselII + λshift + λTransfert + Piecewise[{{λinter1, selII - selI == -40},
          {λinter2, selII - selI == 40}, {λinter3, selII - selI == 60},
      {λinter4, selII - selI == -80},
          {λinter5, selII - selI == 80}, {λinter6, selII - selI == 100},
      {λinter7, selII - selI == 140}}]],
          λ2{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift +
       Piecewise[{{λinter1, selII - selI == -40},
          {λinter2, selII - selI == 40}, {λinter3, selII - selI == 60},
      {λinter4, selII - selI == -80},
          {λinter5, selII - selI == 80}, {λinter6, selII - selI == 100},
      {λinter7, selII - selI == 140}}]]};

I4 = {λ1 → 0};
listinter = {1, 2, 3, 4, 5, 6, 7};
λS1 = Flatten[Table[{{λS1[[i]], -.5, -4, 1}}, {i, 1, Length[S1]}], 1];
λclone = Flatten[Table[{{λClones[[i]], -.5, -4, 1}}, {i, 2, Length[Clones]}], 1];
allλinter =
  Flatten[Table[{{λinterlistinter[[i]], -.5, -4, 1}}, {i, 1, Length[listinter]}], 1];
allλ = Join[λsel, λclone, {{λshift, -.5, -3, 1}},
   {{λTransfert, 0, -10, 10}}, allλinter];
```

```
ResultsI1 = FindMaximum[lltot /. I1 /. I4, allλ]
ResultsI2 = FindMaximum[lltot /. I2 /. I4, allλ]
ResultsI3 = FindMaximum[lltot /. I3 /. I4, allλ]
```

$$\{-4734.82,\ \{\lambda_{40} \to -2.65816,\ \lambda_{80} \to -2.61283,\ \lambda_{120} \to -3.06408,\ \lambda_{180} \to -0.682389,\ \lambda_{2} \to -1.17461,\ \lambda_{3} \to -1.16074,\ \lambda\text{shift} \to -1.13585,\ \lambda\text{Transfert} \to -0.624284,\ \lambda\text{inter}_{1} \to -0.578204,\ \lambda\text{inter}_{2} \to -0.326256,\ \lambda\text{inter}_{3} \to -0.946859,\ \lambda\text{inter}_{4} \to -0.48071,\ \lambda\text{inter}_{5} \to -0.389344,\ \lambda\text{inter}_{6} \to -0.036901,\ \lambda\text{inter}_{7} \to 0.0415099\}\}$$

$$\{-4695.13,\ \{\lambda_{40} \to -3.36592,\ \lambda_{80} \to -3.37022,\ \lambda_{120} \to -3.79823,\ \lambda_{180} \to -1.92815,\ \lambda_{2} \to -0.922882,\ \lambda_{3} \to -0.773263,\ \lambda\text{shift} \to -0.558185,\ \lambda\text{Transfert} \to -0.118901,\ \lambda\text{inter}_{1} \to -0.273333,\ \lambda\text{inter}_{2} \to -0.0498084,\ \lambda\text{inter}_{3} \to -1.547,\ \lambda\text{inter}_{4} \to -0.665638,\ \lambda\text{inter}_{5} \to -0.122966,\ \lambda\text{inter}_{6} \to -0.373231,\ \lambda\text{inter}_{7} \to -0.590519\}\}$$

$$\{-4630.15,\ \{\lambda_{40} \to -3.34005,\ \lambda_{80} \to -3.35326,\ \lambda_{120} \to -3.89607,\ \lambda_{180} \to -1.22602,\ \lambda_{2} \to -0.877098,\ \lambda_{3} \to -0.778584,\ \lambda\text{shift} \to -0.445615,\ \lambda\text{Transfert} \to -0.399401,\ \lambda\text{inter}_{1} \to -0.979005,\ \lambda\text{inter}_{2} \to -0.123755,\ \lambda\text{inter}_{3} \to -1.92512,\ \lambda\text{inter}_{4} \to -0.758541,\ \lambda\text{inter}_{5} \to 0.228187,\ \lambda\text{inter}_{6} \to -0.147085,\ \lambda\text{inter}_{7} \to -0.153822\}\}$$

Acclimation models

In these models, we tested for acclimation to stressful environments through adaptive plasticity.

```
A1 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift + λTransfert],
        λ2{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift +
      Piecewise[{{λinter1, selII == 40}, {λinter2, selII == 80},
        {λinter3, selII == 120}, {λinter4, selII - selI == 60},
        {λinter6, selII - selI == 100}, {λinter7, selII - selI == 140}}]]};
A2 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift + λTransfert +
      Piecewise[{{λinter1, selII == 40}, {λinter2, selII == 80},
        {λinter3, selII == 120}, {λinter4, selII - selI == 60},
        {λinter6, selII - selI == 100}, {λinter7, selII - selI == 140}}]],
        λ2{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift]};
A3 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift + λTransfert +
      Piecewise[{{λinter1, selII == 40}, {λinter2, selII == 80},
        {λinter3, selII == 120}, {λinter4, selII - selI == 60},
        {λinter6, selII - selI == 100}, {λinter7, selII - selI == 140}}]],
        λ2{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift +
      Piecewise[{{λinter1, selII == 40}, {λinter2, selII == 80},
        {λinter3, selII == 120}, {λinter4, selII - selI == 60},
        {λinter6, selII - selI == 100}, {λinter7, selII - selI == 140}}]]};

A4 = {λ1 → 0};
listinterA = {1, 2, 3, 4, 6, 7};
λS1 = Flatten[Table[{{λS1[[i]], 0, -10, 10}}, {i, 1, Length[S1]}], 1];
λclone = Flatten[Table[{{λClones[[i]], 0, -10, 10}}, {i, 2, Length[Clones]}], 1];
allλinterA = Flatten[
   Table[{{λinterlistinterA[[i]], 0, -10, 10}}, {i, 1, Length[listinterA]}], 1];
allλ = Join[λsel, λclone, {{λshift, 0, -10, 10}},
   {{λTransfert, 0, -10, 10}}, allλinter];

ResultsA1 = FindMaximum[lltot /. A1 /. A4, allλ]
ResultsA2 = FindMaximum[lltot /. A2 /. A4, allλ]
ResultsA3 = FindMaximum[lltot /. A3 /. A4, allλ]
```

$$\{-4945.07, \{\lambda_{40} \to -2.46805, \lambda_{80} \to -2.36916, \lambda_{120} \to -2.64128, \lambda_{180} \to -0.0176845, \lambda_2 \to -1.55802, \lambda_3 \to -1.26884, \lambda\text{shift} \to -1.37045, \lambda\text{Transfert} \to -1.20549, \lambda\text{inter}_1 \to -0.0723567, \lambda\text{inter}_2 \to 0.0949367, \lambda\text{inter}_3 \to -0.282167, \lambda\text{inter}_4 \to -0.224333, \lambda\text{inter}_6 \to 0.140429, \lambda\text{inter}_7 \to 0.178537\}\}$$

$$\{-4694.92, \{\lambda_{40} \to -3.3576, \lambda_{80} \to -3.37915, \lambda_{120} \to -3.80131, \lambda_{180} \to -1.93607, \lambda_2 \to -0.925676, \lambda_3 \to -0.777155, \lambda\text{shift} \to -0.554476, \lambda\text{Transfert} \to -0.461757, \lambda\text{inter}_1 \to 0.181575, \lambda\text{inter}_2 \to 0.404942, \lambda\text{inter}_3 \to 0.32093, \lambda\text{inter}_4 \to -1.12198, \lambda\text{inter}_6 \to -0.0265492, \lambda\text{inter}_7 \to -0.220677\}\}$$

$$\{-4643.33, \{\lambda_{40} \to -3.19684, \lambda_{80} \to -3.65849, \lambda_{120} \to -3.72158, \lambda_{180} \to -1.2627, \lambda_2 \to -0.867783, \lambda_3 \to -0.774432, \lambda\text{shift} \to -0.734982, \lambda\text{Transfert} \to -0.395154, \lambda\text{inter}_1 \to -0.0925359, \lambda\text{inter}_2 \to 0.554835, \lambda\text{inter}_3 \to 0.0654162, \lambda\text{inter}_4 \to -1.60562, \lambda\text{inter}_6 \to 0.174357, \lambda\text{inter}_7 \to 0.168567\}\}$$

Low quality habitat models

In these models, we tested for low quality habitat accumulation.

```
LH1 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift + λTransfert],

    λ2{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift + a + b × (selII - selI)]};
LH2 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} →
     Exp[λClone + λselII + λshift + λTransfert + a + b × (selII - selI)],
            λ2{Clone_,selI_,selII_} → Exp[λClone + λselII + λshift]};
LH3 = {λ1{Clone_,selI_} → Exp[λClone + λselI],
        λT{Clone_,selI_,selII_} →
     Exp[λClone + λselII + λshift + λTransfert + a + b × (selII - selI)],
            λ2{Clone_,selI_,selII_} →
     Exp[λClone + λselII + λshift + a + b × (selII - selI)]};

LH4 = {λ1 → 0};
λS1 = Flatten[Table[{{λS1[[i]], -4, -10, 0}}, {i, 1, Length[S1]}], 1];
λclone = Flatten[Table[{{λClones[[i]], -0.05, -1, 1}}, {i, 2, Length[Clones]}], 1];
allλ1 = Join[λS1, λclone, {{a, -0.04, -10, 10}}, {{b, 0, -0.5, 5}},
    {{λshift, -0.4, -0.45, 0.5}}, {{λTransfert, -0.1, -10, 0}}];

ResultsLH1 = FindMaximum[lltot /. LH1 /. LH4, allλ]
ResultsLH2 = FindMaximum[lltot /. LH2 /. LH4, allλ]
ResultsLH3 = FindMaximum[lltot /. LH3 /. LH4, allλ]
```

```
{-4919.04, {λ40 → -4., λ80 → -4., λ120 → -4., λ180 → -4., λ2 → -0.05,
  λ3 → -0.05, a → -0.04, b → 0., λshift → -0.4, λTransfert → -0.1}}
```

```
{-4871.64, {λ40 → -4., λ80 → -4., λ120 → -4., λ180 → -4.,
  λ2 → -0.5, λ3 → -0.5, a → -0.01, b → 0., λshift → 0., λTransfert → 0.}}
```

```
{-4668.33, {λ40 → -3.28291, λ80 → -3.39253, λ120 → -3.93133,
  λ180 → -3.03436, λ2 → -0.884075, λ3 → -0.753894, a → -0.302055,
  b → 0.0114359, λshift → -0.262055, λTransfert → -0.346375}}
```

Supplement 4. Plasticity of the *Artemia* morphology with salinity

One of the major goals when studying adaptive phenotypic plasticity is to find the trait that adaptively varies along the environmental gradient. In our study, we measured a morphological trait of *Artemia* that was previously described as plastic along the salinity gradient (Triantaphyllidis *et al.* 1995; Saygi 2004).

Methods

Size measurement

We used total length (TL) and abdominal length (AL). These morphological characters, were first proposed by Hontoria & Amat (1992) to compare strains of *Artemia* from different Mediterranean origins. They were later found to vary with salinity (Triantaphyllidis *et al.* 1995). Indeed, *Artemia* grown at high salinities (180g/L) were stockier (smaller AT/TL ratio) than *Artemia* grown in medium salinities (35g/L). It has not been shown that this plasticity is adaptive, although this character might be involved in the resistance of *Artemia* to high salinities, as stocky *Artemia* have lower exchange surface (in particular along the gut track), which might cause reduced ion intake. Indeed, ionic regulation is a key process for saline extremophiles, as efficient osmolarity regulation system will enable the organism to survive and reproduce at higher salinity levels.

In practice, TL and AL were measured once at the age of four weeks (7 days P1 + 21 days P2) for every surviving individual. The four weeks corresponded to the delay for nauplii to turn into adult *Artemia* (pers. obs.).

Morphological variations in Artemia

Variations in *Artemia* body proportion was a good candidate as an adaptive plastic trait to salinity (Triantaphyllidis *et al.* 1995). We run our statistical analysis on the ratio of AL to TL that corresponds to the stockiness of the *Artemia*. We expect such ratio to decrease with salinity for the more stocky *Artemia* are, the less contact surface they expose to high salinities.

We used a generalized linear model (glm) with Gaussian distribution to test the influence of salinity in P1 and P2 (as fixed factors) on the size ratio of adult *Artemia*. This analysis was corrected for random effects such as place of the individual in the

plate, position of the plate in the room or grand-maternal clonal line. All statistical analysis were performed in R (www.r-project.org/) and best model choice followed Akaike information criterion (Akaike 1974) with correction for small samples size.

Adaptive morphological plasticity

To determine if the morphological plastic variations found in *Artemia* are adaptive, it is necessary to link those variations to survival. If the morphological plasticity is adaptive, we expect stocky morphology to present better survival at high salinities and conversely, depending on the type of morphology that is better adapted to an environment.

To link morphological plasticity to *Artemia* survival, we ran a survival analysis on the subset of nauplii that were measured in phase 2. We tested several factorial effects (C, SP1 and SP2) and continuous factors regarding morphology (TL, AL and AL/TL) as well as quadratic effects (TL^2, AL^2 and AL/TL^2). All statistical analysis were performed in R (www.r-project.org/) and best model choice followed Akaike information criterion (Akaike 1974).

Results & Discussion

A plastic morphology

The ratio of *Artemia* (AL/TL) linearly decreased along the salinity gradient in phase 2 for every C tested (Figure S4). The two best models selected in the glm analysis (Table S4.1) included significant effects of the Salinity 2 x C interaction. At 40 g/L, the two Aigues-Mortes Cs presented similar ratios (AL/TL = 0.6) while the LM7 C present a higher ratio (AL/TL = 0.63). On the other extreme of 180 g/L, the LM7 and PAM10 C present similar ratios (AL/TL = 0.54) while the PAM7 has a higher ratio (AL/TL = 0.57).

Is the plasticity adaptive?

The survival analysis of nauplii measured at four weeks (Table S4.2) presented a large and significant effect of salinity in phase 2 and total body length (TL). Both these effects as well as a quadratic effect of the total length (TL^2) were present in all four best models (parted from less than 2 AIC points). The ratio (AL/TL) was present in the second and fourth best models but absent from the third, while a quadratic effect of this ratio (AL/TL^2) was present in the first best model.

Finally, the abdomen length (AL) was present in the third and fourth best models and as a quadratic effect (AL^2) in the first three models.

Stockiness (AL/TL) is therefore present in three of the four best models and seems to represent an adaptation to high salinities. In such case, given the large range of AL/TL ratio that LM7 C present, it is a potential generalist. On the other hand, given the low ratio of PAM7 and PAM10 at low salinity, these Cs might both be specialized to higher salinities. PAM7 being possibly specialized in even higher salinities than PAM10, given is lower ratio at high salinities.

Conclusion

We were able to describe a plastic trait that modifies the morphology of *Artemia* along the salinity gradient. However, the continuous development of this organism probably prevented us from detecting an interaction between the two phases of the experimentation on the morphology. Similarly, we were not able to discriminate if this trait had a significant effect on post-measurement survival.

References

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4. Triantaphyllidis, G. V, Poulopoulou, K., Abatzopoulos, T.J., Antonio, C., Perez, P. & Sorgeloos, P. (1995). International study on *Artemia* XLIX . Salinity effects on survival, maturity, growth, biometrics, reproductive and lifespan characteristics of a bisexual and a parthenogenetic population of *Artemia*. *Hydrobiologia*, 302, 215–227.

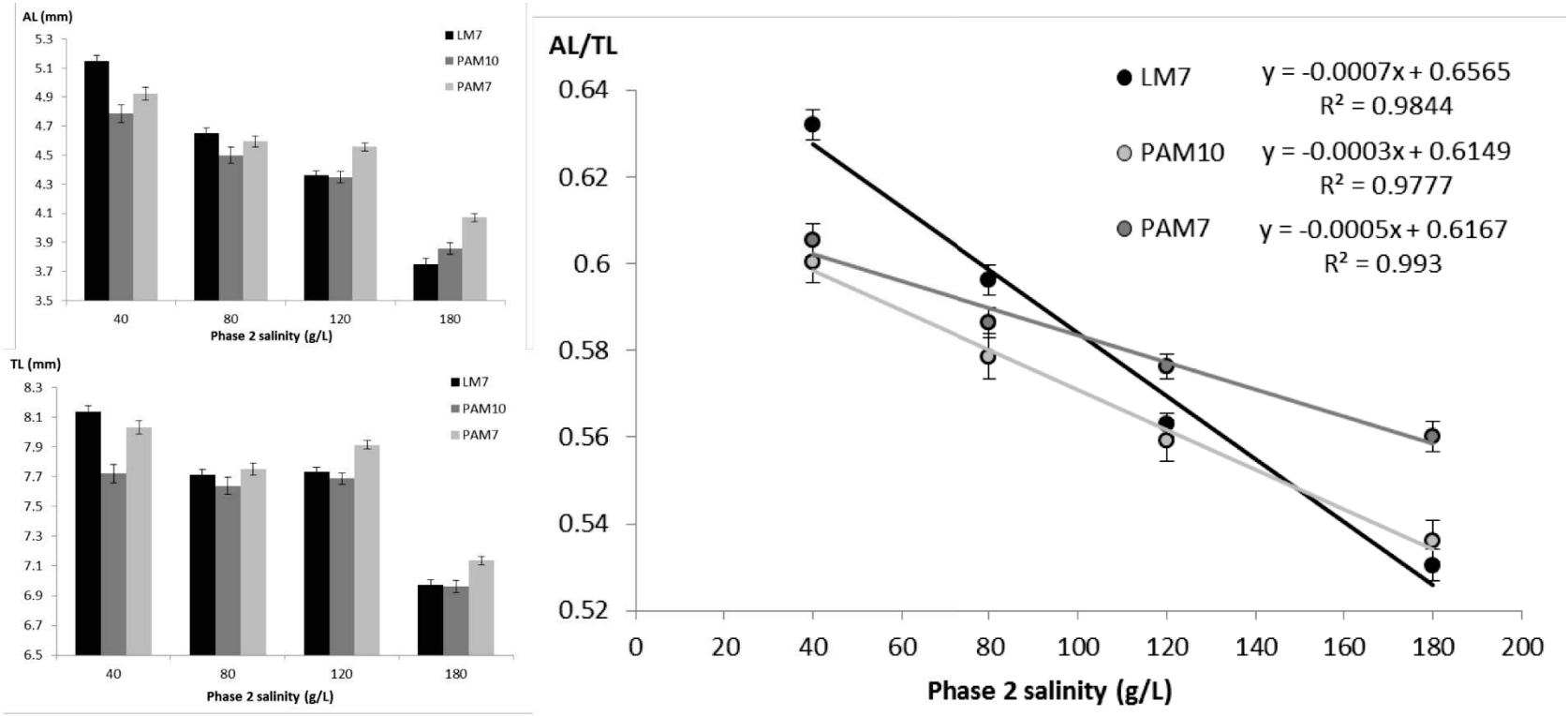

Figure S4: Length and retraction estimates in phase 2. Graph **A** represents the means of abdominal length (AL), **B** the means of total length (TL) and **C** the ratio of AL/TL at four weeks (7 days P1 + 21 days P2) along P2 salinity gradient. Colours represent the different Cs: LM7 in black, PAM10 in grey and PAM7 in light grey). Error bars represent standard error. Lines in graph C represent linear regression between stockiness and salinity in phase 2 ($R^2 > 0.95$).

Table S4.1 - Size generalized linear model selection. K is the number of parameters for each model, **logLik** the log likelihood, **AIC** the Akaike information criterion, **AICc** the second order AIC corrected for sample size, **ΔAICc** the AICc differences, w_i the Akaike weights. **Parameters** of models are Salinity 1 (40, 80 or 120g/L), Salinity 2 (40, 80, 120 or 180g/L), C (LM7, PAM7 or PAM10), Lineage (A or B for each C), Female (female in the clonal lineage from which nauplii were isolated), TP1 (position of the tube in plate of phase 1), TP2 (position of the tube in plate of phase 2), PP1 (position of the plate during phase 1) and PP2 (position of the plate during phase 2).

Model	**K**	**logLik**	**AIC**	**AICc**	**ΔAICc**	**exp**	**w_i**	**Parameters**
C interaction	27	-3205,5	-3151,5	-3150,7	0,00	1,00	0,51	Salinity 1 + Salinity 2 x C + Female + AB
C interaction – Salinity 1	24	-3199,3	-3151	-3150,6	0,03	0,95	0,49	Salinity 2 x C + Female + AB
Full interaction	48	-3238,6	-3142,6	-3140,0	10,71	0,004	0,0024	Salinity 1 x Salinity 2 x C + Female + AB
C interaction – variation parameters	15	-3169	-3139,0	-3138,7	11,92	0,003	0,0013	Salinity 1 + Salinity 2 x C
Salinity interaction	27	-3193,2	-3139,2	-3138,4	12,30	0,002	0,0011	Salinity 1 x Salinity 2 + C + Female + AB
Sum	22	-3177,4	-3133,4	-3132,8	17,82	0	0,00	Salinity 1 + Salinity 2 + C + Female + AB
All parameters	204	-3393,3	-2985,3	-2933,7	216,99	0	0,00	Salinity 1 + Salinity 2 + C + Female + AB + TP1 + TP2 + PP1 + PP2

Table S4.2 – Survival analysis after four weeks model selection. K is the number of parameters for each model, **logLik** the log likelihood, **AIC** the Akaike information criterion, **AICc** the second order AIC corrected for sample size (n=921), **ΔAICc** the AICc differences, w_i the Akaike weights. **Parameters** of models are C (LM7, PAM7 or PAM10), Salinity 1 (40, 80 or 120g/L) and Salinity 2 (40, 80, 120 or 180g/L) as factors and, Total Length (TL), Abdomen Length (AL), ratio (AL/TL) and quadratic effects (TL^2 and AL^2) as continuous effects.

Model	K	LogLik	AIC	AICc	ΔAICc	wi	Parameters
m6	9	-1025.25	2068.50	2068.70	0.00	0.32	Salinity 2 + TL + AL + AL/TL + TL^2
m10	7	-1027.91	2069.83	2069.95	1.25	0.17	Salinity 2 + TL + TL^2
m3	10	-1025.01	2070.02	2070.27	1.57	0.15	Salinity 2 + TL + AL + AL/TL + TL^2 + AL^2
m9	8	-1027.31	2070.61	2070.77	2.07	0.11	Salinity 2 + TL + AL + TL^2
m8	8	-1027.51	2071.03	2071.18	2.49	0.09	Salinity 2 + TL + AL/TL + TL^2
m2	12	-1023.48	2070.97	2071.31	2.61	0.09	Salinity 1 + Salinity 2 + TL + AL + AL/TL + TL^2 + AL^2
m1	14	-1021.72	2071.43	2071.90	3.20	0.06	C + Salinity 1 + Salinity 2 + TL + AL + AL/TL + TL^2 + AL^2
m5	9	-1030.05	2078.09	2078.29	9.60	0.00	Salinity 2 + TL + AL + AL/TL + AL^2
m11	6	-1039.76	2091.52	2091.61	22.92	0.00	Salinity 2 + TL^2
m4	7	-1039.52	2093.04	2093.16	24.47	0.00	TL + AL + AL/TL + TL^2 + AL^2
m7	8	-1038.81	2093.62	2093.78	25.09	0.00	Salinity 2 + AL + AL/TL + TL^2

5 Discussion et perspectives

5.1 Apports théoriques de la thèse

La thèse demande un large travail de maitrise des concepts, d'analyse de leurs limites et de détections de celles qui n'ont pas encore été explorées. Ce travail est bien plus complexe que la réalisation des expériences et des analyses qui pourront nous apporter les réponses aux questions posées. C'est le travail que j'ai taché d'effectuer dans la longue introduction de ce manuscrit. Je suis arrivée à un total de 6 limites encore mal étudiée concernant le concept de niche. Il s'agit maintenant d'y apporter quelques éléments de réponse.

Problème 1 : A quelle échelle (genre, espèce, population, génotype) doit-on définir la niche ?

Bien entendu, cette question pourrait avoir pour réponse évidente : "ça dépend des cas". Ici, nous nous sommes intéressé au cas plus particulier des populations asexuées que nous avons évoqué cette limite. L'étude du maintien du polymorphisme dans une large population naturelle d'artémies parthénogénétique (voir Chapitre 1) nous a permis de montrer qu'une lignée clonale pouvait s'appréhender comme un nuage de points d'individu génétiquement proches. La vision d'une niche à l'échelle du génotype pour les populations clonales ne s'applique donc pas ici.

Problème 2 : Comment la niche à un stade de développement donné dépend-elle des environnements rencontrés aux stades précédents ? Problème 5 : Comment tenir compte de la qualité d'habitat dans le concept de niche ? Problème 6 : Comment faire le lien entre ces mesures ponctuelles et les paramètres de la fonction liant environnement, trait et fitness ?

Ces trois questions sont complémentaires, je ferais donc une réponse globale. C'est dans le contexte de l'adaptation de l'artémie aux fortes salinités (voir Chapitre 3) que nous avons apporté des réponses à ces questions opérationnelles. En effet, notre étude s'intéressait aux effets de la qualité d'habitat, de l'histoire de vie et de l'acclimatation sur la fitness. Nous avons amélioré des méthodes expérimentales existantes, mais surtout développé une méthode d'analyse de survie explicite

permettant d'appréhender la niche tout au long de l'histoire de vie des individus et d'y inclure l'impact de la qualité d'habitat. Cette méthode permet, en outre, à partir de mesures ponctuelles d'estimer la forme et les paramètres de la fonction liant l'environnement et la fitness.

Problème 3 : Comment peut-on utiliser le concept de niche multidimensionnelle lorsque les contraintes diffèrent d'un axe à l'autre ainsi qu'aux extrémités de chacun de ces axes ?
Cette réponse n'est pas lié à une étude particulière mais plutôt au modèle utilisé. En effet, le caractère extrêmophile de l'artémie est particulièrement bienvenu quand il s'agit de comprendre sa niche. L'artémie a comme n'importe quel organisme une niche avec un grand nombre de dimensions. A la différence des autres espèces, les extrêmophiles présentent un très petit nombre de paramètres environnementaux extrêmes qui ont une importance majeure dans l'adaptation à l'environnement de l'espèce en question. Si l'on se demande où chercher des artémies, la forte salinité fera automatiquement partie de la réponse. Donc pour ces espèces, même si les contraintes diffèrent aux extrémités des axes de la niche (ce que nous avons montré pour l'artémie dans les Chapitres 2 et 3), la réduction de dimensionnalité de la niche permet d'étudier ces contraintes et d'avoir une bonne appréhension de leur impact sur les limites de niche.

Problème 4 : Les interactions dites positives/négatives peuvent-elles contraindre ou faciliter l'expansion de la niche de l'hôte ?
Ici, on soulève une question liée au problème évoqué précédemment. En effet, en plus des différentes contraintes pouvant s'exercer aux extrémités des axes de la niche, il faut pouvoir aussi envisager des interactions entre les axes. C'est ce qu'illustre notre exemple des effets différents que peuvent avoir les mêmes interacteurs sur différents axes de la niche de l'hôte (voir Chapitre 2). En plus de montrer expérimentalement les impacts variables des symbiontes sur différents axes de la niche de leur hôte, notre étude propose un cadre théorique pour intégrer ces interaction dans le concept de niche.

Au final, que les réponses soient conceptuelles ou opérationnelles, elles sont des petites pierres qui rendent un peu plus solide le concept de niche et permettent de s'appuyer dessus avec une confiance certaine pour les perspectives à envisager.

5.2 Niche et répartition des espèces

Le suivi saisonnier à long terme de la population d'artémies du salin Aigues-Mortes (voir Article 1) ainsi que la comparaison entre différents salins de France et d'Espagne est une illustration de l'importance de l'échelle utilisée pour étudier la niche. En effet, selon l'échelle géographique et temporelle à laquelle une population est définie, l'impact de l'environnement biotique et abiotique varie. D'autre part, les deux études réalisées au laboratoire (voir Article 2 & 3) montrent que les contraintes s'exerçant aux extrémités des gradients environnementaux, et par conséquent aux limites de la niche, peuvent varier d'un extrême à l'autre (rôle du microbiota et donc de la digestion à un extrême, probablement des contraintes physiologiques à l'autre). Les limites de répartitions des espèces peuvent donc dépendre de différents axes de leur niche, mais aussi être liés aux différences de contraintes aux extrémités d'un des axes de la niche.

Dans l'optique d'une prédiction du déplacement des espèces en lien avec les changements globaux, l'échelle d'étude des populations ainsi que les différences de contraintes aux limites des gradients environnementaux sont une source supplémentaire de variation dans les prédictions. Certains modèles de prédiction prennent en compte la plasticité des organismes (Morin *et al.* 2007) et même les variations de réponses au sein d'une population (Valladares *et al.* 2014). Pourtant, les contraintes liées aux interactions entre organismes au sein d'une communauté ne sont pas prises en compte (Matesanz & Valladares 2014). Particulièrement dans le cas des symbiontes, où il a été montré à de nombreuses reprises que l'incapacité pour certains organismes à disperser est intimement lié à l'absence ou l'incapacité de leur symbiontes à survivre dans le nouvel environnement (Kjellberg & Valdeyron 1990; Bronstein 2001).

5.3 Niche et interactions

La première étude en laboratoire nous a permis de mettre clairement en évidence le rôle dualiste des interactions biotiques dans les limites de niche (voir Article 2). Ces effets complémentaires aux services rendus par les micro-organismes associés commencent a être bien caractérisés en médecine humaine. La densité de la flore intestinale peut aller jusqu'à 10^{12} bactéries par gramme d'intestin (Gibson & Roberfroid 1995). La fermentation qu'elles réalisent dans l'intestin a des effets

positifs sur la digestion et l'apport d'éléments (minéraux, vitamines) difficiles à assimiler pour l'hôte. Par exemple, la production d'acides gras à chaines courtes permet à l'hôte de réduire son métabolisme de dégradation de glucides complexes (Cummings 1981; Cummings & Macfarlane 1991). Cette digestion pourrait représenter entre 10 et 30% des besoins métaboliques de l'hôte (Rerat *et al.* 1986). En plus du service rendu pour la digestion, de nombreuses études rapportent que la microflore a un rôle important dans la lutte contre les pathogènes (Buffie & Pamer 2013). Par exemple, un traitement antibiotique inhibant les bactéries anaérobiques peut conduire à une prolifération d'*Enterococcus* résistantes dans les intestins et entrainer une infection sanguine (Ubeda *et al.* 2010). Ces rôles annexes des symbiontes sont de plus en plus pris en compte dans le traitement des individus. A l'inverse, de nombreuses études ont montré que la flore intestinale avait un rôle significatif dans le développement de l'obésité (voir revue de Shen *et al.* 2013). La microflore associée à tous les êtres vivants peut donc rendre de multiples services mais aussi avoir des effets antagonistes sur différents axes de la niche de l'hôte.

Ces aspects sont encore trop souvent négligé en écologie, même si de nombreuses études commencent à s'y intéresser (Selosse *et al.* 2006; Dunbar *et al.* 2007; Moran & Yun 2014). Notre étude permet de clarifier les effets antagonistes que peuvent avoir les différentes interactions sur les limites de niche (Article 2). En effet, nous avons montré que la microflore intestinale associée à la digestion d'algue chez l'artémie à un effet antagoniste sur la survie des artémies aux faibles salinités. L'utilisation d'une autre source de nourriture lève complètement cet effet (même en l'absence des bactéries).

Tout d'abord, un axe intéressant à développer serait la meilleure caractérisation de la spécificité de cette interaction. En effet, nous avons montré qu'il n'y avait pas de transfert vertical de la microflore chez l'artémie. D'autre part, des organismes comme les copépodes vivent aussi dans les salins mais dans des bassins saumâtres (< 35g/L), mais leur gamme de tolérance à la salinité se recouvre partiellement. Or, on ne trouve jamais les deux espèces dans le même bassin. Une expérimentation possible serait d'inoculer des artémies axéniques avec une flore bactérienne de copépode et de tester leur fitness sur une gamme de salinités faibles. Si l'association entre l'artémie et sa flore est spécifique, on ne s'attend pas à observer une meilleure fitness des artémies en présence de la flore de copépode aux faibles salinités. A

l'inverse, si l'artémie est simplement associée à des bactéries commensales (qui s'implante dans l'intestin car la concentration de nourriture y est forte et qui pré-digèrent une partie de cette nourriture), on s'attend à ce qu'elle puisse utiliser une partie de la flore intestinale des copépodes (elle aussi commensale). Dans ce cas, la fitness des artémies inoculées avec la flore de copépode sera meilleure aux faibles salinités.

Ensuite, si l'interaction est spécifique, le développement d'outils de génomique serait intéressant. En effet, pour étudier la coévolution entre les génomes de l'artémie et de la communauté bactérienne associée la génomique est un outil efficace (voir 5.4). Notre étude s'inscrit dans le développement d'un nouveau domaine d'étude où la niche ne serait plus seulement définie pour une espèce, mais à l'échelle d'une communauté d'interacteurs. On peut définir une communauté comme l'ensemble des espèces qui interagissent dans un environnement (Giller 2012). Whittaker (1973) insiste sur l'utilisation du terme de niche uniquement dans le cas du rôle de l'espèce à l'intérieur de la communauté. Pourtant, lorsqu'il y a une différence d'échelle aussi importante que dans le cas des interactions avec les microorganismes, la question de maintenir cette distinction se pose. Ici, l'hôte et ses interacteurs forment un système vivant distinct par sa composition, sa structure, ses relations environnementales, son développement et sa fonction.

5.4 Développer la génomique d'un extrêmophile

Comme décrit dans l'introduction, l'étude de la niche est facilitée dans le cas de l'artémie car cet organisme est extrêmophile. Ceci implique une corrélation entre de nombreux axes environnementaux réduisant la dimensionnalité de la niche, mais aussi une limitation du nombre d'espèces interagissant dans l'écosystème (Figure 1.2). D'autre part, il existe de nombreuses espèces sexuées et de lignées parthénogénétiques dans le genre *Artemia* (van Stappen 2002) ce qui en fait un modèle intéressant pour étudier l'asexualité. Néanmoins, la génomique du modèle artémie est pour ainsi dire inexistante, exceptées quelques études développant des marqueurs chez *A. franciscana* (espèce d'importance commerciale ; Valenzuela-Miranda *et al.* 2014). Par exemple, si le mode de reproduction des artémies asexuées est sujet de débat depuis plus d'un siècle (Brauer 1894), aucune réponse claire n'a pu être apportée par la cytologie (Narbel-Hofstetter 1964; White 1973; Bell

1982; Cuellar & Moens 1987). Notre étude s'appuyant sur les taux d'hétérozygotie pour des marqueurs neutres a apporté des éléments de réponse décisifs sur cette question (Annexe 2).

Pourtant, toutes les problématiques ne pourront pas être résolues avec 12 locus microsatellites. L'artémie est un système où le développement de marqueurs génétique est particulièrement difficile, la caractérisation de neuf nouveaux marqueurs microsatellites à partir de trois banques obtenues par pyroséquençage le montre bien (Annexe 1). Or, si l'on souhaite étudier des cystes enfouis dans les sédiments dont l'ADN est dégradé, l'on doit utiliser d'un grand nombre de marqueurs de moins de 100pb (Annexe 3). Le développement de marqueurs SNPs pour *A. parthenogenetica*, couplé à l'utilisation de techniques de séquençage massif nous semble le plus prometteur pour répondre à ces problématiques.

Un autre exemple montrant l'importance du développement de la génomique pour le modèle artémie est lié aux interactions avec sa flore intestinale. Afin de mieux comprendre leurs mécanismes sous-jacents, une possibilité est de s'intéresser à la coévolution des génomes en interactions (Thompson & Burdon 1992). Ces techniques sont déjà utilisées pour caractériser l'impact de la microflore sur la santé humaine (Woolhouse *et al.* 2002; Nicholson *et al.* 2012), ainsi que le rôle de cette microflore pour les herbivores (Ley *et al.* 2008a, 2008b; Hansen & Moran 2013). Or, ces études montrent le nombre important d'intéracteurs jouant dans ces coévolutions (jusqu'à 13 phyla dans l'estomac humain, Andersson *et al.* 2008; 17 phyla détecté dans l'intestin des mammifères, Ley *et al.* 2008a). Dans le cas d'un extrêmophile comme l'artémie, le nombre d'intéracteur jouant dans cette coévolution semble diminué (4 phyla détecté par Tkavc *et al.* 2011). L'artémie semble donc un modèle intéressant pour mieux comprendre les interactions complexes entre une communauté bactérienne et son hôte.

Enfin, pour mieux comprendre les mécanismes sous-jacents à l'adaptation à la salinité chez l'artémie, l'utilisation de la génomique serait intéressante. En effet, la connaissance de plusieurs gènes candidats (gènes codant pour les pompes Na/K ATPase impliqué dans la régulation de la salinité interne, Holliday *et al.* 1990 ; pour différentes hémoglobines permettant d'assimiler plus facilement l'oxygène dont la disponibilité diminue avec l'augmentation de la salinité, Gilchrist 1954 ; etc.) permet

déjà la détection de patrons de signature de sélection (Nielsen 2005). Il serait aussi intéressant d'utiliser des techniques de scan génomique (Nielsen *et al.* 2005) pour détecter de nouveaux gènes candidats. Bien que cette recherche semble plus facile pour les espèces sexuées dont la recombinaison simplifie la localisation de gènes sous sélection, la reproduction automictique avec fusion centrale chez les populations d'*A. parthenogenetica* diploïdes (voir Annexe 2) nous permet d'envisager d'étudier ces signatures de sélection pour les gènes en positions distales sur les chromosomes.

5.5 Intégrer et adapter les méthodes

Pour finir, l'un des points communs à l'ensemble de ce travail de thèse est l'intégration de nombreuses méthodes, mais aussi le développement de nouvelles méthodologies expérimentales et analytiques.

Dans certains domaines d'étude, cette transversalité des méthodes est nécessaire. C'est le cas par exemple des études sur les propagules dormants où l'association de techniques de génomique et de géologie sont indispensables (voir revue par Orsini *et al.* 2013). Ce sont ces méthodes que nous avons mises en place pour dater les sédiments de la carotte prélevé à Aigues-Mortes (voir Annexe 3). Nous avons utilisé des techniques de géologie, combinant sédimentologie, spectrométrie aux rayons X et comptage de la vitesse de décomposition radioactive d'éléments lourd (Plomb et Cesium), très éloignées de la biologie. Cette collaboration entre différentes spécialités était essentielle pour tenter de répondre à la question de l'impact d'une invasion passée sur une population. Elle demande d'avoir une capacité d'adaptation et un recul sur la question posé afin de pouvoir comprendre et se faire comprendre de ses interlocuteurs. Un grand nombre d'outils développé pour un domaine de recherche scientifique ont leur utilité dans d'autres domaines. Par exemple, le développement de programme et de logiciel est un des outils de la recherche en informatique. Actuellement, ces outils sont nécessaires à l'ensemble des autres domaines de la science. La mise en place de plateformes transversales et d'unités mixtes de recherches sont des premiers pas vers une simplification de ce type de collaborations.

Par ailleurs, nous avons dû développer de nouvelles méthodologies expérimentales et analytiques pour l'ensemble des travaux de cette thèse. Lors de notre étude sur

l'impact de l'environnement sur la structure des populations (voir Article 1), nous avons utilisés des outils de cartographie géographique qui nous ont permis d'identifier des groupes de clones associés à différentes conditions environnementales. Lors de notre étude sur l'influence de la microflore sur l'adaptation de l'artémies aux faibles températures (voir Article 2), nous avons été amenés à développer des cultures d'artémies axéniques afin de mettre en évidence la niche à la salinité de l'artémie en l'absence de microflore. Enfin, lors de notre étude de l'impact de la plasticité phénotypique et de la qualité d'habitat sur la fitness des artémies aux fortes salinités (voir Article 3), nous avons développé une méthode d'analyse de survie explicite afin de répondre au mieux à nos questions. Là encore, cela demande une capacité d'adaptation et de recul sur les problématiques abordés ainsi qu'un regard critique certain sur les méthodologies employées.

En conclusion, ce travail de thèse a permi d'apporter des éléments de réponses sur l'influence de l'environnement sur la structuration génétique d'une large population clonale, sur l'impact de la microflore sur l'incapacité des artémies à survivre aux faibles salinités et sur les rôles joints de la plasticité et de la qualité d'habitat sur l'adaptation aux fortes salinités de ce crustacé extrêmophile. Ces réponses nous ont permis de questionner le concept de niche sur son échelle d'application (temporelle, spatial), sur l'impact réel des interactions positives sur la niche de l'hôte et enfin sur l'impact de la plasticité sur les limites de niches. Ce travail a demandé une large gamme de ressources et la collaboration entre un grand nombre de spécialités. Il s'inscrit dans une tendance générale de la recherche actuelle de collaboration transversale et d'intégration des techniques. Dans un contexte où les questions posées en écologie évolutive sont de plus en plus larges (prédictions de la répartition des espèces présente et future face aux changements globaux, conservation des ressources agricoles et du patrimoine mondiale…), ce type d'approche généraliste est nécessaire. Survient alors une lutte pour pallier à l'adage qui veut que "Jack of all trades is master of none" et parvenir en interagissant avec des spécialistes à élargir la niche des savoirs.

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Glossaire

Niche écologique : espace multidimensionnel de variables environnementales (biotiques et abiotiques), où l'espèce survie indéfiniment en l'absence d'interaction.

Capacité d'adaptation ("*adaptability*") : le degré auquel un organisme, une population ou une espèce peut rester ou devenir adapté à une gamme d'environnements plus larges *via* des moyens génétiques.

Degré d'ajustement adaptatif ("*adaptedness*") : proximité entre le phénotype réalisé et le phénotype optimal.

Fitness : le degré auquel un organisme est capable de vivre et se reproduire dans un environnement donné ou dans un ensemble d'environnements.

Courbes de tolérance : graphique représentant la fitness d'un génotype, d'une population ou d'une espèce en fonction de l'environnement.

Plasticité phénotypique : capacité d'un génotype à produire différents phénotypes selon l'environnement dans lequel il se développe.

Oviparité : stratégie de reproduction où les femelles pondent des œufs dont la croissance embryonnaire s'effectue à l'extérieure de l'organisme maternel.

Ovoviviparité : stratégie de reproduction où les œufs produits par la femelles sont incubés et éclosent à l'intérieur de l'organisme maternel.

Gastrula : stade du développement embryonnaire où l'invagination à l'origine du tractus intestinal est formée.

Diapause : phase de dormance des cystes montrant une diminution des activités métaboliques.

Hémolymphe : liquide circulatoire des arthropodes dont le rôle est analogue au sang et au liquide interstitiel des vertébrés.

Annexes

Annexe 1

Characterization of nine new polymorphic microsatellite markers in *Artemia parthenogenetica*

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Abstract

The brine shrimp *Artemia* is a diverse genus of anostracan containing sexual species and parthenogenetic lineages. Parthenogenetic *Artemia* lineages, related to Asian sexual species, occur only in the Old World. Cysts from a sexual specie native from America (*Artemia franciscana*) are used as a food source in aquaculture. It has therefore become an invasive in many Mediterranean hypersaline ecosystems. Nine microsatellite markers were developed to better characterize Mediterranean diploid parthenogenetic *Artemia* populations and their related Asian sexual species as well as the impact of A. franciscana invasion on their diversity. The number of alleles ranged from 2 to 8 per locus and the levels of heterozygosity from 0.000 to 0.933 in parthenogenetic *Artemia*. The isolation of these nine new markers required intensive investigation suggesting Artemia to be alike some lepidopteran whose genome contains very few microsatellites despite having a large haploid genome size.

Keywords: asexuality, parthenogenesis, clone, microsatellites, multiplex PCR, polymorphism, repeat number

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1. Introduction

Artemia are fairy shrimps (Anostraca) adapted to hypersaline environments, distributed in inland salt lakes, solar salterns and lagoons around the world. Six sexual species and several diploid and polyploid parthenogenetic lineages have been described (Bowen and Sterling, 1978; Abeu-Grobois and Beardmore, 1982; Cai, 1989; Abatzopoulos et al., 1998; Maniatsi et al., 2011), the latter being related to Asian sexual species (*Artemia urmiana* and a yet undescribed species from Kazakhstan, Hou et al., 2006; Maccari et al., 2013). Diapausing eggs (cysts), harvested from natural populations, are a major food source in aquaculture and cysts from the American sexual species (*Artemia franciscana*) are exported across the world (Van Stappen, 2000). Cysts have been introduced to the Old World where *A. franciscana* is now invasive. This invasion has led to a serious decline of native *Artemia* species (Amat et al., 2007; Sivagnanam et al., 2011; Vikas et al., 2012; Muñoz et al., 2014). *Artemia parthenogenetica* has the largest distribution among native species (Muñoz and Pacios, 2010) and increasing the number of genetic markers available for this species would allow a large scale quantification of the diversity loss caused by *A. franciscana* invasion. A fine scale characterization of genetic diversity of parthenogenetic *Artemia* would also allow identifying clones that might be suitable for aquaculture purposes (e.g. Abatzopoulos et al., 2003; Baxevanis and Abatzopoulos, 2004), would help tracking the origin of commercial *A.*

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Table 1
Samples used for microsatellite markers selection.

Species	Sample size	Location	Cyst collection	Year of collection
A. parthenogenetica	1	Bameng area, China	ARC 1317	1995
A. parthenogenetica	1	Molentargius, Italy	IATS	2004
A. parthenogenetica	1	La Mata, Alicante, Spain	IATS	1988
A. parthenogenetica	1	Odiel, Huelva, Spain	IATS	1987
A. parthenogenetica	1	La Palme, France	Pers. Col.	2002
A. parthenogenetica	1	Salin-de-Giraud, France	Pers. Col.	2002
A. parthenogenetica	8	Aigues-Mortes, France	Pers. Col.	2010
A. sinica	1	Dongjiagou, Liaoning Province, China	ARC 1216	1991
A. sinica	12	Yimeng area, China	ARC 1188	1991
A. sp. Kazakhstan	11	Unknown location, Kazakhstan	ARC 1039	1988
A. tibetiana	21	Lagkor Co, China	ARC 1347	1997
A. urmiana	20	Lake Urmia, Iran	ARC 1542	2000

Sample size, number of individual *Artemia* used in each sample; *A. parthenogenetica* samples correspond to parthenogenetic lineages, other samples correspond to related *Artemia* sexual species from Asia used to select the nine microsatellite markers; **IATS**, Instituto de Acuicultura de Torre de la Sal, CSIC, Castellón, Spain; **ARC**, Artemia Reference Center, Ghent, Belgium.

parthenogenetica cysts (Campos-Ramos et al., 2003) and provide a better understanding of the relationship between asexual and sexual lineages (Maniatsi et al., 2011; Maccari et al., 2013).

To date, only five polymorphic microsatellites are available for diploid parthenogenetic *Artemia*, two of which are often monomorphic in natural populations (Muñoz et al., 2008). In addition, multiple peak profiles (stutter artifacts) and null alleles can complicate data interpretation from some of these markers, especially when cross-amplification in related sexual or polyploid lineages is required (Maniatsi et al., 2011; Maccari et al., 2013, 2014). The 10 polymorphic microsatellite loci developed in the distantly related *A. franciscana* are not cross-amplifiable in parthenogenetic *Artemia* (E. Flaven, pers. obs.). In this study, we describe the isolation of nine new polymorphic microsatellite loci for diploid parthenogenetic *Artemia*. We also developed three multiplex kits (made of two, three and four markers respectively) for a fast and cost-effective genotyping. We describe the complete procedure used to design the three multiplex kits, paying attention to quality controls and potential transferability to related *Artemia* species.

2. Methods

Three libraries of respectively 1261, 3070 and 4846 microsatellite markers were produced (Genoscreen, Lille, France) by coupling multiplex microsatellite enrichment isolation techniques with the 454 GS-FLX Titanium pyrosequencing (Malausa et al., 2011). For each library, genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) from 40 mg of cysts from either of three populations: La Mata saltern, Spain (diploid parthenogenetic *Artemia*); Dongjiagou, Liaoning Province, China (ARC 1216, diploid and tetraploid parthenogenetic *Artemia*, and distantly related sexual *Artemia sinica*) and Lake Urmia, Iran (ARC 1542, closely related sexual *A. urmiana*). Hereafter, markers belonging to the libraries will be respectively designated with Appm, Apcpm and Aupm.

Respectively, 28 Appm, 29 Apcpm and 21 Aupm polymerase chain reaction (PCR) primer pairs were designed with the open access program QDD (Meglécz et al., 2010), and tested on a sample of 14 parthenogenetic *Artemia* individuals from diverse origins: China, Italy, Spain and France (see Table 1 for details). Cross-amplification in one *A. sinica* individual (Table 1) was also verified. DNA was extracted from whole adults using the DNeasy Blood and Tissue Kit (Qiagen, Germany).

Of the 78 microsatellites markers tested, 25 gave a positive signal on electrophoretic gels. Nine of which were finally retained for further tests based on the proportion of perfect repeats, amplification success and polymorphism level. We searched GenBank database for *A. parthenogenetica* existing microsatellite markers and only found the five markers developed by Muñoz et al., in (2008). We aligned our nine new microsatellite sequences to these and found no significant similarities. Therefore, marker sequences were deposited in GenBank (Number KM489512-KM489520). PCRs were carried out separately for each locus and then multiplexed in three subsets of loci (Kit-1, Kit-2, Kit-3; see Table 2) with a final volume of 10 μl that included 0.2 μM of each primer, 2X Multiplex PCR Master Mix and 1 μl of genomic DNA (multiplex PCR kit, Qiagen, Germany). In each multiplex, the primers were directly labeled using different fluorescent dyes (with 6-FAM, NED, PET or VIC, Applied Biosystems; see Table 2). PCRs were conducted under the following conditions: an initial denaturation step at 95 °C for 15 min, 30 cycles consisting of 30 s at 95 °C, 90 s at 60 °C, and 60 s at 72 °C, finally, a supplementary extension step of 30 min at 60 °C. Three microliters of diluted PCR products (1/100) were pooled in 15 μl of HI-DI TM formamide (Applied Biosystems) and 0.2 μl of GeneScan-500 LIZ size standard, and analyzed on an ABI PRISM 3130xL DNA Analyzer (Applied Biosystems) at the

Table 2
Characteristics of the three multiplex kits (Kit-1, Kit-2, Kit-3) developed for genetic analyses of *Artemia parthenogenetica*.

Locus	MK	Primer sequences (5′–3′)	Repeat sequence	*NA*	Size range (bp)	H_O	H_E	*P*
Appm4	Kit-1	F: **VIC** – CAGGAGTTAAGCAGGGATGTG	$CCCCTTTT(ATAG)_5$	2	83–87	0.133	0.543	<0.001
		R: TGTTTGTGTATGGCCTAGCG						
Appm20		F: **6-FAM** – TCCTCAGAACTCCCTCAGGAT	$(AC)_{12}$	6	104–114	0.667	0.692	0.039
		R: TCCTTTTCGCCTATTTTCTCA						
Appm26	Kit-2	F: **NED** – CATCAATACACAACACCATCCC	$(CA)_8$	2	178–190	0.600	0.481	0.577
		R: GGCGTTTATTTGGAAGTTTATGTC						
Apcpm1		F: **6-FAM** – TCCGTGCTAGTAAAGTAGTTGCAG	$(CTA)_8$	3	104–110	0.867	0.683	0.188
		R: TCAACTGACCAAAGGCAATG						
Aupm5		F: **PET** – TCAGCCGGTTGTCAATATCA	$(CAA)_8$	3	161–173	0.133	0.131	1
		R: AGCAGGGCTACAGCATCACT						
Aupm7		F: **NED** – CTGAATGTATTGGGATGGAG	$(AGT)_{10}$	2	115–124	0.000	0.129	0.033
	Kit-3	R: AGACAACGAAAAGACACAA						
Aupm15		F: **PET** – TTCCAATGCAGCTTTCTCG	$(AG)_9A(TG)_2G(GAA)_2$	3	89–93	0.067	0.131	0.036
		R: AGTCACAATGGGGCTAGACG						
Aupm16		F: **6-FAM** – ATTCGAAATTTGAACTCGTG	$(AC)_3(ATAC)_5AT(ATAC)_4AC(ATAC)_{15}$	8	102–156	0.933	0.823	0.548
		R: TTTGACTCTCAAAAGTGTAATGTG						
Aupm21		F: **VIC** – GCTGTTGTTCTTGTATTATTTG	$(GTA)_3AG(AGT)_2AGC(AGT)_9$	2	108–117	0.267	0.349	0.055
		R: GCACAAGCTAAGGGTATTGA						

MK, Multiplex kit; ***NA***, number of alleles for each locus; $\boldsymbol{H_O}$, observed heterozygosity; $\boldsymbol{H_E}$, expected heterozygosity under Hardy–Weinberg equilibrium; ***P***, *P-value* of an exact test using Markov chains with a confidence interval of 95%. The fluorescent dye of each primer is written in front of each primer sequence.

LabEx CeMEB sequencing platform (Montpellier, France). Fragment analysis and scoring were carried out using GeneMapper v. 3.7 (Soft Genetics). The DNA fragment sizes measured from the peaks were converted into discrete alleles by comparison with reference lists of allele sizes. The results from the analyses of genetic diversity are summarized in Table 2. The results from cross-amplification tests of the nine microsatellites markers in related Artemia sexual species from Asia (see Table 1) are summarized in Table 3.

3. Results and discussion

The number of alleles detected at each locus ranged from 2 to 8 in parthenogenetic *Artemia* samples. Observed and expected heterozygosities, deviations from Hardy–Weinberg equilibrium (HWE) expectations were calculated using Arlequin version 3.1 (Excoffier et al., 2005). The observed and expected loci heterozygosities (H_O, H_E) ranged respectively, from 0.000 to 0.933 and 0.129 to 0.823 in parthenogenetic *Artemia* (see Table 1). Appm4 was the only locus showing significant departure from HWE, but as these loci are sampled from asexual populations/species, there is no expectation that they should be in HWE.

For sexual species (Table 3), the nine microsatellite markers presented a large range of allele numbers (from 3 to 27). Some markers failed to cross-amplify in sexual species (Appm20 in *A. sinica* and Aupm21 in *A. sinica* and *Artemia tibetiana*). Some markers were monomorphic in some species (Appm4 in all species but *A. sinica*, Aupm5 in *A.* sp. Kazakstan and *A. tibetiana*, and Aupm15 in *A. sinica*). The observed and expected heterozygosities (H_O, H_E) ranged from 0.00 to 1.00 and 0.05 to 0.92 (see Table 2). In *A.* sp. Kazakstan, *A. sinica*, *A. tibetiana* and *A. urmiana*, respectively 2, 3, 0 and 3 loci presented significant departure from the HWE. This result is likely due to the presence of null alleles at those loci (over 9% in Table 3).

Finally, we assessed the overall genetic differentiation between all Asian sexual species and the parthenogenetic samples by performing a principal component analysis of the nine loci (PCA; see Fig 1) using the R Adegenet package (Jombart, 2008). The microsatellite markers described here are powerful tools to discriminate both parthenogenetic *Artemia* and related sexual species (although some of the markers with null alleles should be used cautiously in sexual species).

These new microsatellite markers will provide useful tools to better characterize the relationship between parthenogenetic *Artemia* lineages and their related Asian sexual species and assess the genetic diversity as well as the spatial and temporal structures of parthenogenetic populations. They should also help quantifying the influence of *A. franciscana* invasion on the structuration of these populations. Such results could assist in better understanding the coexistence dynamics between sexual and asexual species as well as between invasive and native *Artemia*.

It should be noted that despite our intensive screening effort, we recovered relatively few polymorphic microsatellite markers. Hence, despite having a large haploid genome size (~1 Gb; De Vos et al., 2013), *Artemia* species seem to have relatively few microsatellite loci. *Artemia* appears to resemble well known microsatellite poor taxa such as mites and Lepidoptera (Schlötterer and Pemberton, 1998).

Table 3
Cross-amplification of microsatellite markers in related sexual *Artemia* species from Asia.

Locus	*A.* sp. Kazakhstan ($n = 11$)					*A. sinica* ($n = 12$)					*A. tibetiana* ($n = 21$)					*A. urmiana* ($n = 20$)				
	NA	Size range (bp)	H_O	H_E	*P* [PNL]	*NA*	Size range (bp)	H_O	H_E	*P* [PNL]	*NA*	Size range (bp)	H_O	H_E	*P* [PNL]	*NA*	Size range (bp)	H_O	H_E	*P* [PNL]
Appm4	1	83	Mon.	Mon.	–	2	83–87	0.00	0.57	<0.001 [33%]	1	83	Mon.	Mon.	–	1	83	Mon.	Mon.	–
Appm20	4	108–114	1.00	0.68	0.02	–	–	Null	Null	–	5	110–118	0.62	0.71	0.18 [5%]	7	100–118	0.25	0.77	<0.001 [40%]
Appm26	2	178–190	0.00	0.45	<0.001 [9%]	3	170–190	0.08	0.59	<0.001 [58%]	4	178–190	0.38	0.52	0.04	3	178–194	0.20	0.19	1.00
Apcpm1	2	107–110	0.64	0.57	1.00	3	104–11	0.08	0.16	0.05	2	104–107	0.38	0.37	1.00	2	98–101	0.05	0.05	1.00
Aupm5	1	161	Mon.	Mon.	–	2	155–161	0.50	0.51	1.00	1	161	Mon.	Mon.	–	7	158–179	0.75	0.77	0.17
Aupm7	2	124–127	0.00	0.66	<0.001 [45%]	2	115–118	0.00	0.16	0.04	2	124–127	0.38	0.50	0.38	5	121–133	0.45	0.75	0.01
Aupm15	3	93–97	0.18	0.45	0.06	1	89	Mon.	Mon.	–	3	89–101	0.14	0.14	1.00	6	87–103	0.45	0.55	0.06
Aupm16	5	124–136	0.55	0.58	0.73	3	102–118	0.25	0.61	<0.001 [25%]	10	116–152	0.67	0.61	0.86	14	126–176	0.70	0.92	<0.001 [10%]
Aupm21	3	108–117	0.36	0.32	1.00	–	–	Null	Null	–	–	–	Null	Null	–	7	93–120	0.35	0.70	<0.001 [20%]

NA, number of alleles for each locus; $\boldsymbol{H_O}$, observed heterozygosity; $\boldsymbol{H_E}$, expected heterozygosity under Hardy–Weinberg equilibrium; ***P***, *P-value* of an exact test using Markov chains with a confidence interval of 95%; ***PNL***, percentage of double null allele at the locus; **Mon.**, monomorphic locus; **Null**, null locus.

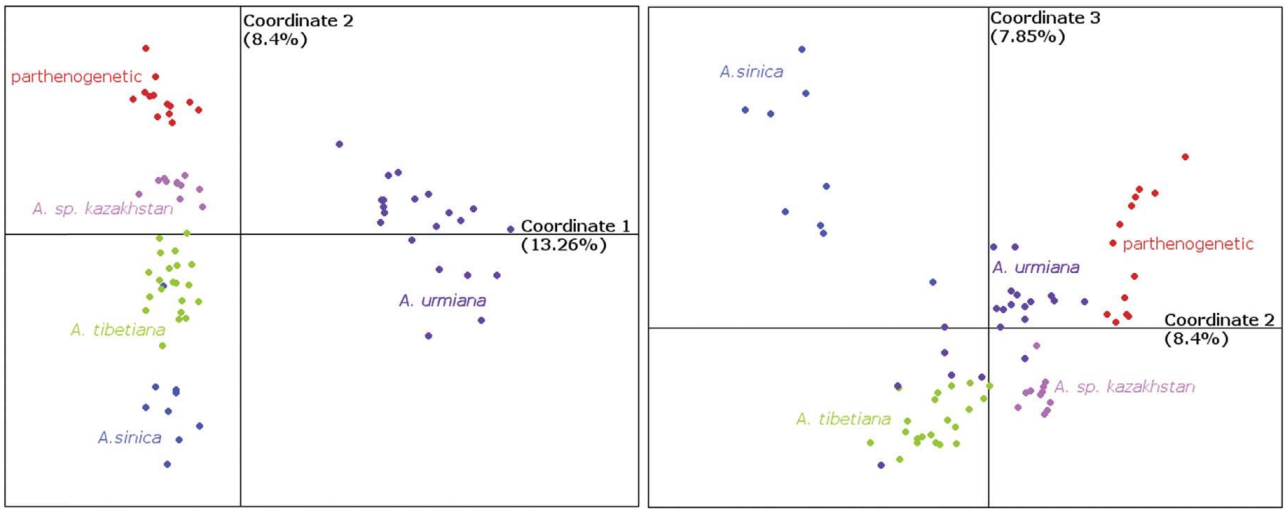

Fig. 1. PCA of parthenogenetic and sexual Artemia species using the nine microsatellite markers described. The percentage of variance explained by each axis is written between parentheses.

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Annexe 2:
Automixis in Artemia: solving a century-old problem

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Abstract

The term “asexuality” summarizes a large diversity of reproductive modes. In particular, parthenogenesis (asexual reproduction through unfertilized eggs) can be achieved through mitosis-based cloning (apomixis) or through various types of modified meioses (automixis). These different forms of automixis can have very different genetic and evolutionary consequences but can be particularly difficult to tease apart. In this paper, we propose a new method to discriminate different types of automixis from population-level genetic data. We apply this method to asexual *Artemia parthenogenetica*, a crustacean whose reproductive mode remains controversial despite a century of intensive cytogenetic observations. We focus on *A. parthenogenetica* from two western Mediterranean populations. We show that they are diploid, and that markers remain heterozygous in cultures maintained up to ~36 generations in the laboratory. Moreover, patterns of population-wide heterozygosity levels strongly support the conclusion that diploid *A. parthenogenetica* reproduce by automictic parthenogenesis with central fusion and low, but non-zero recombination. This settles a century-old controversy on *Artemia*, and, more generally, shows that many automictic organisms can be understood as showing within-genome gradients between clonality and a mild form of self-fertilization. This offers a new avenue for investigating the genomic consequences of asexuality and inbreeding.

Key words: parthenogenesis, central fusion, asexuality, genetic, breeding systems, recombination.

Introduction

Most eukaryotes have an unknown breeding system. Yet, this major feature of the life cycle has profound impacts on the distribution of genetic diversity both within and among individuals. Breeding systems therefore play a central role in many evolutionary and ecological processes (e.g. the rate of adaptation, genetic load or inbreeding depression Hedrick & Kalinowski, 2000; Haag & Roze, 2007; Glémin & Ronfort, 2013; Park & Krug, 2013). The breeding system, *sensu lato,* includes the details of meiosis (number and distribution of crossing-overs) and syngamy (mate choice, level of inbreeding), and encompasses a wide array of variants (from modified meiosis in parthenogens to self-fertilization in hermaphrodites).

Asexual reproduction is often equated with clonality (also called apomixis), under which offspring are produced that are genetically identical to their mothers except for mutation. However, as shown in Table 1, asexual reproduction may take many different forms, each with its own consequences for genetic variation (Asher, 1970; Cuellar, 1987; Suomalainen & Lokki, 1987; Schön *et al.*, eds, 2009; Neiman *et al.*, 2014). Depending on the taxon studied, breeding system can be investigated directly or inferred indirectly, using various techniques. For instance, sexual reproduction can simply be inferred from the presence of males (although the presence of males does not imply obligate sexuality, and neither does it inform on the actual occurrence of recombination). Conversely, parthenogenesis or self-fertilization can be inferred from the occurrence of reproduction of isolated, virgin individuals. However, directly discriminating among the different parthenogenetic breeding systems listed in Table 1 is much more difficult as it requires precise cytological or genetic analyses. For instance inferring genetic consequences of automixis from microscopic observations of meiotic stages, chromosomes pairing and crossing over is often difficult (Narbel-Hofstetter, 1964). Directly observing the pattern of inheritance of genetic markers from parents to offspring, may overcome this problem (Asher, 1970; Stenberg & Saura, 2009). However, both cytological and classical genetics approaches can fail at uncovering rare events. This problem is particularly acute when characterizing the breeding system of species where sex or recombination, if present at all, is rare or cryptic. Yet, there is a large qualitative evolutionary difference between breeding systems lacking entirely sex and recombination, and breeding systems exhibiting very low degrees of sex and recombination. Indeed many

processes, such as Hill-Robertson effects on beneficial or deleterious mutations, are largely modified by a small amount of sex (Charlesworth *et al.*, 1993; Otto & Barton, 1997).

Population genetic analyses represent a powerful alternative to detect cryptic or rare sex / recombination, as different forms of breeding systems give rise to different genetic patterns at the population level. By sampling natural populations at several loci, one can use different measures of genetic associations within or across loci (e.g. linkage disequilibrium, heterozygosity, etc) to infer the breeding system of a species (Halkett *et al.*, 2005). This approach has been widely used to investigate levels of self-fertilization in hermaphrodites (David *et al.*, 2007), but has also been used to discriminate between different kinds of parthenogenesis, or to detect low rates of sexual reproduction (Asher, 1970; Burt *et al.*, 1996; Tsai *et al.*, 2008; Saleh *et al.*, 2012; Flot *et al.*, 2013; Tucker *et al.*, 2013). The rationale behind these approaches is that breeding systems may have dramatically different effects on genetic associations, such that even slight differences in these systems (e.g. rare sex versus apomixis) can have strong impacts on the patterns of molecular variation, as the effects of these processes cumulate over many generations. In some asexual systems (Table 1, cases 1-2), heterozygosity is preserved at all loci across generations (except for gene conversion during DNA double strand break repair). In other systems, heterozygosity is largely reduced at all loci across generations (Table 1, cases 8-9) or can even lost totally within a single generation (Table 1, case 4). Finally in several cases (Table 1, cases 5-7), the reduction in heterozygosity depends on marker position along the chromosome (proximal or distal relative to the centromere), and the rate of recombination. Linkage disequilibrium patterns are less informative, as they depend quantitatively (and not only qualitatively) upon the presence of meiosis and the amount of recombination (number and distribution of crossing-overs per megabases).

Table 1. Description of the different breading systems which allow a single individual to reproduce without any mate. Apomixis (1) does not involve meoisis. Automixis (2-8) involves the production of an offspring by fusion of the two products from a single meiosis (unlike self-fertilization where offspring are produced by fusion of two products from two independent meioses, in the male and the female gametes, respectively). Central and terminal fusions are usually distinguished. Central (resp. terminal) fusion corresponds to the fusion of meiotic products derived from the first (resp. second) meiotic division. Central fusion retains heterozygosity at centromere positions while terminal

fusion leads to the loss of heterozygosity at centromere positions. In both cases heterozygosity is reduced by two thirds at position far away from the centromeres. Thus, automixis through central fusion combined with very low recombination rates leaves a genetic signature very similar to that of apomixis (with maintenance of high level of heterozygosity). In contrast, central, terminal and mixed fusions combined with very high recombination rates leaves a genetic signature very similar to self-fertilization (with nearly complete loss of heterozygosity). Different mechanisms have been described in *Artemia* based on cytological observations: Ref 1: (Barigozzi, 1944); 2: (Artom, 1931); 3: (Brauer, 1894); 4: (Gross, 1932); (Stefani, 1960), critically reviewed in (Narbel-Hofstetter, 1964) .

N°	Reproductive mode	# meiosis	Details	% of heterozygosity retention	Described in *Artemia*
1	Apomixis	0	Mitosis	100%	Yes in polyploids ref 1
2	Gonoid thelytoky	(1)	Abnormal meiosis with inversion of meiosis I and II (first, separation of chromatids, then homolog pairing)	100%	No
3	Premeiotic doubling	1	Duplication of chromosomes before meiosis, identical chromosomes resulting from duplication pair during meosis I	100%	No
4	Postmeiotic doubling	1	Endomitosis of the meiotic product	0%	Yes, ref 2, 4
5	Central fusion	1	Central fusion in ordered tetrads or suppression of meiosis I	From 100% at centromere to 66% at large genetic distances (in Morgan) from centromere	Yes, ref 2, 5
6	Terminal fusion	1	Terminal fusion in ordered tetrads or suppression of meiosis II	From 0% at centromere to 66% at large genetic distances (in Morgan) from centromere	Yes, ref 1, 2, 3, 4
7	Mixed fusion	1	Random fusion in tetrads = mixed fusion with terminal and central fusion in proportion 2/3 and 1/3, respectively	Intermediate between central and terminal fusion	Yes, ref 2
8	'Single step' meiosis	(1)	Meiosis I and II occur at the same time with the random assortment of chromatids, independently for each homolog pair (Asher's mechanism 5)	66%	No
9	Self-fertilization	2	Syngamy of a male and a female gamete from two independent meioses within the same individual	50%	No

For many automictic species, the details of meiosis is mostly known from cytological observations (Narbel-Hofstetter, 1964). However, as mentioned above, these observations are difficult to undertake and interpret, and many uncertainties remain even in intensely studied systems. Such issues are epitomized by the debate

over the reproduction mode of *Artemia parthenogenetica*. For over a century, the details of automixis in this clade has been controversial. As stated by White (1973): "the most famous instance of thelytoky in the Crustacea, and the one that has been most extensively studied, is that of the branchiopod 'Brine shrimp' *Artemia*. However, in spite of the large number of cytologists who have worked on this 'super-species' (…) some uncertainties remain". More than a century after the first description of the reproductive system of parthenogenetic *Artemia* (Brauer, 1894), this controversy is still unsettled (Narbel-Hofstetter, 1964; White, 1973; Bell, 1982; Cuellar, 1987).

Artemia parthenogenetica is an asexual crustacean (Crustacea, Branchiopoda, Anostraca) that was once listed among the famous 'asexual scandals' (Judson & Normark, 1996), as its asexuality was assumed to be very ancient. *Artemia* are extremophiles living in hypersaline environments (Browne, 1992; Abatzopoulos *et al.*, 2002). They belongs to a genus with at least six gonochoric sexual species, which are all diploid (2n = 42, except *A. persimilis* with 2n=44) (Abatzopoulos *et al.*, 2002). Parthenogenetic *Artemia* exhibit different ploidy levels (2n to 5n, Abreu-Grobois & Beardmore, 1982; Maniatsi *et al.*, 2011). Asexual lineages of different origins are grouped together under the common binomen *Artemia parthenogenetica* (Bowen & Sterling, 1978; Baxevanis *et al.*, 2006; Maccari *et al.*, 2013a). The origin(s) and age(s) of these different parthenogenetic lineages have been debated (in the range 10^4-10^7 years, Perez *et al.*, 1994; Muñoz *et al.*, 2010), owing to the limited genetic information available to date. Parthenogenetic diploids (hereafter *Ap*2*n*) likely arose in Central Asia, and their closest sexual relatives appear to be *A. urmiana* and *A.* sp. Kazakhstan, an undescribed species from Kazakhstan (Muñoz *et al.*, 2010; Maccari *et al.*, 2013a).

Polyploid parthenogenetic *Artemia* reproduce through apomixis, while the mode of reproduction of *Ap*2*n* remains unresolved (Barigozzi, 1944). Authors have described at least four different types of automixis in *Ap*2*n* (Table 1). Some of these types should lead to the partial segregation of chromosomes. Since females are known to be heterogametic in the genus (ZW, Bowen, 1963, 1965; De Vos *et al.*, 2013), including *Ap2n* (Stefani, 1963), this should lead to the production of a high fraction of male offspring (Stefani, 1964). Parthenogenetic diploids do produce males that are functional and can transmit asexuality when mated with related sexual females (contagious asexuality Maccari *et al.*, 2014). However, these males have always been observed at low frequencies, typically below 1% (Bowen *et al.*, 1978;

MacDonald & Browne, 1987; Maccari *et al.*, 2013b). In diploid parthenogenetic lineages, offspring have been reported to be genetically identical to the mother, barring mutation or recombination (Abreu-Grobois, 1987), but only little genetic data is available to date to reach more precise conclusions. There are thus at least two lines of evidence (low frequencies of male production, production of genetically identical offspring) that suggest that maternal heterozygosity is largely maintained in offspring. Among the reproductive modes in Table 1, maternal heterozygosity is retained in modes (1)-(3) and in mode (5) if there is low or no recombination. Modes (1)-(3) appear unlikely given the cytological observations reported in the literature (see Table 1), but should not *a priori* be excluded. Mode (5) is automixis via central fusion, which retains maternal heterozygosity at the centromeres, but leads to a gradual loss of heterozygosity in centromere-distal regions due to recombination (Rizet & Engelmann, 1954; Pearcy *et al.*, 2006). Central fusion with low, but non-zero amounts of recombination could thus account for the high degree of heterozygosity retention, and at the same time for the low, but non-zero production of males.

To test the hypothesis that *Ap*2*n* reproduces by central fusion with low, but non-zero recombination (compared to other modes of reproduction listed in Table 1) we investigate the genetics of diploid parthenogenetic *Artemia* from two western Mediterranean populations. We verified their ploidy levels, followed the changes in heterozygosity in cultures maintained over two years (~30 generations) in the laboratory (to assess whether initial heterozygosity is retained even after many generations), and measured the population-wide level of heterozygosity at 12 microsatellite loci. The latter data were used to assess levels of inbreeding. Specifically, if maternal heterozygosity is fully retained (clonal-like reproduction), an excess of heterozygosity compared to Hardy-Weinberg proportions is expected. In contrast, if heterozygosity is lost (even if this loss is very rare, but still more frequent than new mutation), an excess of homozygosity is expected. The results strongly support automictic reproduction via central fusion, with low but non-zero rates of recombination. This settles a century-old controversy on the reproductive mode of these taxa, and opens new avenues for the investigation of the genomic consequences of asexuality and inbreeding along chromosomes.

Material and Methods

Artemia sampling

Live *A. parthenogenetica* were sampled in two populations. In the first population (Aigues-Mortes, Gard, France) samples were collected at 7 sites (*n* = 285 in total), between April 2010 and June 2013. In the second population (Odiel, Huelva, Spain), samples were collected at 5 sites (*n* = 70 in total) in September 2013 (Table 2). Sites within populations corresponds to different, but interconnected, ponds. *A. parthenogenetica* from Aigues-Mortes have been described as diploid, whereas *A. parthenogenetica* from Odiel have been described as a mix of diploid and tetraploid individuals (Amat *et al.*, 2005).

Table 2. List of the different *Artemia* samples use for flow cytometry, genotyping and *Fis* analyses. ARC: Artemia Reference Center (Ghent, Belgium). IATS: Instituto de Acuicultura de Torre de la Sal, CSIC, Castellón, Spain.

Species	Population, Country	Ploidy	Sample size flow cytometry (subsample size used for genotyping)	Sample size genotyping for *Fis* analyses	Reference Number
A. urmiana	Lake Urmia, Iran	2n	5 (-)	-	ARC1230
A. sp Kazakhstan	Unknown, Kazakhstan	2n?	9 (-)	-	ARC1039
A. parthenogenetica	Citros, Grece	4n	6 (10)	-	IATS cyst collection
A. parthenogenetica	Burajaloz, Spain	4n	10 (10)	-	IATS cyst collection
A. parthenogenetica	Odiel, Spain	2n, 4n	14 (14)	70	Wild-collected and laboratory samples
A. parthenogenetica	Aigues-Mortes, France	2n	13 (10)	285	Wild-collected and laboratory samples

Long-term maintenance of strains in the laboratory

We isolated three *A. parthenogenetica* females from Aigues-Mortes. These females reproduced asexually and were used to initiate asexual lines (PAM6, PAM7, PAM10) in the laboratory. PAM6 and PAM7 were isolated in March 2012, and PAM10 was isolated in August 2013. Individuals from these isofemale strains were genotyped at

different dates as indicated in Table 3 (until April 2015, i.e. 36 generations and 20 generations for PAM6/PAM7 and PAM10, respectively, conservatively assuming one generation per month under laboratory conditions).

Table 3. List of genotyped individuals (at different dates) for three different asexual strains founded by a single female and maintained in the laboratory.

Isofemale strains	Sample date	#individuals genotyped
PAM6	May 2012	1
	Sept. 2014	2
	April 2015	5
PAM7	May 2012	1
	Sept. 2013	7
	Sept. 2014	2
	April 2015	5
PAM10	Sept. 2013	7
	Sept 2014	2
	April 2015	5

Ploidy characterization

To verify ploidy levels, 2C DNA values were estimated through flow cytometry. Individuals from two related sexual species, *A. urmiana* and *A.* sp Kazakstan, (Muñoz *et al.*, 2010), and from cytogically characterized tetraploid populations, were used as references (Abatzopoulos *et al.*, 1986; Amat *et al.*, 1994). These reference samples were obtained from collections of diapause cysts (Table 2). These individuals were hatched from cysts and raised in the laboratory using standard conditions. In total, 57 *Artemia* individuals were analyzed by flow cytometry (Table 2). Individuals were rinsed in distilled water, blotted with paper towel and cut in half using a razor blade. The first half was stored in 96% ethanol for subsequent genotyping. The second half was cut in small fragments in a small plastic petri dish (5-cm diameter), together with a 0.5mm^2 leaf fragment of *Oryza sativa* ssp *japonica* cv Nipponbare (2C = 2X = 0.91pg, (Uozu *et al.*, 1997) in 1mL of modified LB01 nuclei extraction buffer (Dpoolež el *et al.*, 1989), with mercaptoethanol substituted by 40 mM Na2SO3). The extract was homogenized with a pipette and filtered through a 30-µm Partec filter placed above a 5-mL test tube. 5µL of RNAse (final concentration: 50µg/mL) and 40

µL of a 0.2 mg/mL propidium iodide solution (Invitrogen) were added, followed by vortexing for 5 seconds at low speed. Samples were incubated on ice for 10 minutes for staining. The nuclear DNA content of each individual was estimated using a Partec PAII laser Flow Cytometer (Partec GmbH, Münster, Germany) with a Blue Solid State laser (488 nm). DNA-PI fluorescence emission was measured at 600–640 nm. Instrument alignment and stability were monitored using 5 mL of a solution of Partec calibration beads. Histograms were analyzed using the Partec FloMax software, which determines peak position and coefficients of variation. Endopolyploidy (variation in ploidy levels among somatic cell within an individual) was observed in all samples. Only the first peaks and second peaks with at least 2,000 events per peak were used to estimate genome size. All analyzed peaks were at least two times higher than the "endopolyploid peaks". Nuclear DNA content was calculated from mean florescence intensity (FL, arbitrary units) on the gated data of the first and second peak as:

Artemia 2C value = (Second peak FL / First Peak FL) x 0.91 pg.

Protocol validity was verified using female chicken red blood cells. We found an average 2C value of 2.70 pg (SD=0.06, n=10), which was within the range of 2.15-3.01 pg reported for chicken in the literature (Mendonça *et al.*, 2010). To test for differences in average C values among samples, we used Welch two samples t-test in R (version 3.1.1.).

To verify that putative diploid and tetraploid individuals could be discriminated by genotyping, based on their allelic profiles, we used the second half of the body of 24 individuals from Aigues-Mortes and Odiel and 16 tetraploid individuals from Citros and Bujaraloz for genotyping analyses (Table 2). To increase our sample size of tetraploid individuals, we also genotyped four new individuals (i.e., individuals for which flow cytometry was not carried out) from Citros (Table 2).

Genotype characterization

DNA extraction

Genomic DNA of the 285 individuals from Aigues-Mortes and 70 individuals from Odiel was extracted using Sigma Extraction Solution (Sigma-Aldrich, Germany; extraction in 30µL at 95°C for 10 min, 20°C for 10min, product diluted with 50µL of

sterile deionized water). For small individuals (such as nauplii), we used half quantities of the buffer and dilution water.

Microsatellite amplification

We used a panel of nine (Nougué *et al.*, 2015) and three microsatellites, Apdq01TAIL, Apdq02TAIL and Apdq03TAIL (Muñoz *et al.*, 2008). The latter were multiplexed respectively using VIC, NED and FAM fluorescent dyes, Applied Biosystems, respectively, and the former as indicated in (Nougué *et al.*, 2015). For each multiplex, we used the following amplification protocol: 9µL of a PCR mix containing 5µL of Multiplex buffer, 1µL from a solution at 1/50 of the forward and reverse primers for each microsatellite fragments [100 µM] and 3µL of sterile water was added to 1µL of DNA extract. PCR amplification was conducted under the following thermocycler conditions: An initial denaturation step at 95°C for 15 min, 30 cycles consisting of 30 s at 95°C, 90 s at 60°C, and 60 s at 72°C finally, a supplementary extension step of 30 min at 60°C.

3µL of diluted of PCR product (between 10 and 15ng) was added to 15 ml of HI-DI TM formamide (Applied Biosystems) and 0.2 ml of GeneScan-500 LIZ size standard, and analyzed on an ABI PRISM 3130xL DNA Analyzer (Applied Biosystems) at the LabEx CeMEB sequencing platform (Montpellier, France). Fragment analysis and scoring were carried out using GeneMapper v. 3.7 (Soft Genetics). The DNA fragment sizes measured from the peaks were converted into discrete alleles by comparison with reference lists of allele sizes.

Statistical analysis

Expected heterozygosity (hereafter *He*), observed heterozygosity (hereafter *Ho*) and departure from Hardy-Weinberg expectation ($F_{IS} = 1 - Ho/He$) were estimated for each locus in both Aigues-Mortes and Odiel populations. *He*, *Ho* were estimated using Arlequin 3.5.1.2 software (Excoffier *et al.*, 2005). F_{IS} for each locus was calculated for each sites within populations, and averaged using sample-size-weighted means for *He* and *Ho* in each population. In the Aigues-Mortes samples, all samples were from the same site were pooled in the analysis, even if they were taken at different dates as there was virtually no population structure among sites within populations.

Results

Diploid status of the samples from Aigues-Mortes and Odiel

The results from the flow cytometry measurements are summarized in Fig. 1. *A. urmiana* and *A.* sp Kazakhstan had average 2C values of 4.23 pg (SD = 0.20) and 4.92 pg (SD = 0.14), respectively, whereas tetraploid individuals from Burajaloz and Citros had average 4C values of 12.53 pg (SD = 0.17) and 12.20 pg (SD = 0.06) respectively. This suggests that *A. urmiana* and *A.* sp Kazakhstan are diploid and that the genome size of tetraploid species is more than twice as large as the genome size of related diploid sexual species.

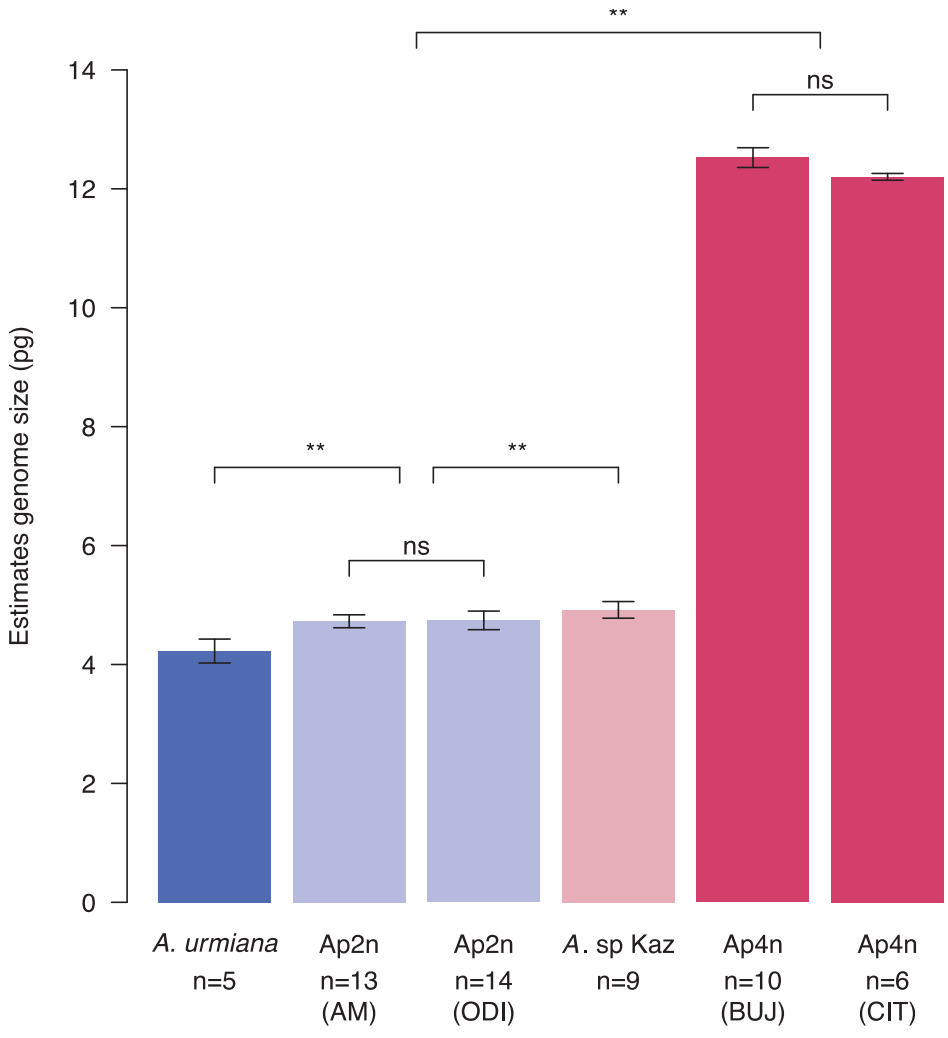

Figure 1: Estimated genome size (pg) of diploid sexual (*A. urmiana*, *A.* sp Kaz), diploid asexual (*Ap2n*) and tetraploid (*Ap4n*) asexual *Artemia* spp. Mean ± SD C-values are shown. Significant differences in *C-value* ($P < 0.004$) are indicated by '**'. n : sample size used for the flow cytometry analyses.

Individuals from Aigues-Mortes and Odiel had average 2C values of 4.72pg (SD = 0.11) and 4.74 pg (SD = 0.16) respectively. These values were not significantly different from one from another (t = -0.29, df = 23.32, *P*-value = 0.79). The average 2C value of individuals from Aigues-Mortes and Odiel was intermediate between *A. urmiana* and *A.* sp Kazakhstan. Interestingly, their genome size was significantly larger than that of *A. urmiana* (*t* = 5.43, df = 4.67, *P*-value = 0.004, individuals from Aigues-Mortes and Odiel pooled together), but significantly smaller than that of *A.* sp. Kazakhstan (*t* = -3.48, df = 13.27, *P*-value = 0.004). Their genome size was also significantly (2.6 times) smaller than that of tetraploid lineages (*t* = -131.51, df = 22.34, *P*-value << 10^{-6}). Considering that samples from Aigues-Mortes and Odiel have a genome size similar to the known diploid *A. urmiana* but about twice smaller than the reference tetraploid samples, we conclude that these parthenogenetic individuals are diploid.

Difference in allelic profiles between tetraploid and diploid individuals

All 20 tetraploid individuals, originating from two geographically distant populations had very similar allelic profiles. In particular, they all had identical alleles at Aupm7 and Aupm16 loci (four and three alleles, respectively). This feature clearly distinguishes them from *Ap2n*. Minor variation was observed between the two populations at one locus (Appm20: three alleles in all individuals from Bujaraloz, two alleles in all individuals from Citros), and three loci showed minor variation within populations (Apdq01TAIL, Apdq02TAIL, and Apdq03TAIL loci, but their interpretation is known to be problematic in polyploid samples (Maniatsi et al 2011). Hence, the allelic profiles of tetraploid individuals appear very homogeneous across populations and loci.

In contrast, only one out of the 12 microsatellite loci had identical allelic profiles across the 24 Aigues-Mortes and Odiel individuals genotyped. No individual had a haplotypic profile matching the characteristic allelic combination found at the eight loci in all tetraploid individuals. Only one locus (Apcpm1) had three alleles in 9 individuals from Odiel. This locus had the same two alleles in all the tetraploid samples, so this pattern might result from a duplication of the locus in some

individuals from Odiel. Based on these data, tetraploid and diploid samples can be clearly discriminated based on their combinations of alleles.

Long-term heterozygosity within lineages

Five out of 12 microsatellite loci tested were heterozygous in 2012 in the three long-term lines (PAM6, PAM7 and PAM10). These five loci were still heterozygous with the same allele identities in all samples through 2015. In addition, allele identity at the seven homozygous loci remained unchanged. Hence, over the ~36 (PAM6 and PAM7) or ~20 (PAM10) generations in the laboratory, the genotypes of the parthenogenetic lineages were transmitted intact, indicating that recombination is very rare or absent in diploid *A. parthenogenetica*. Nonetheless, some recombination, particularly in centromere-distal regions cannot be excluded as the chromosomal positions of these markers are unknown and because selection against recombinants in the cultures cannot be excluded.

Populations wide patterns of heterozygosity

There was very little genetic structure among different sampling sites within populations, but substantial differentiation between populations (F_{st} among sites was 0.00 and 0.02 in Odiel and Aigues-Mortes, respectively, but was 0.13 among the two populations). This pattern was to be expected, as the sampling sites within populations are regularly connected throughout the year. Fig. 2 shows the F_{is} per site and locus in each population, as well as the average computed across sites within a given population.

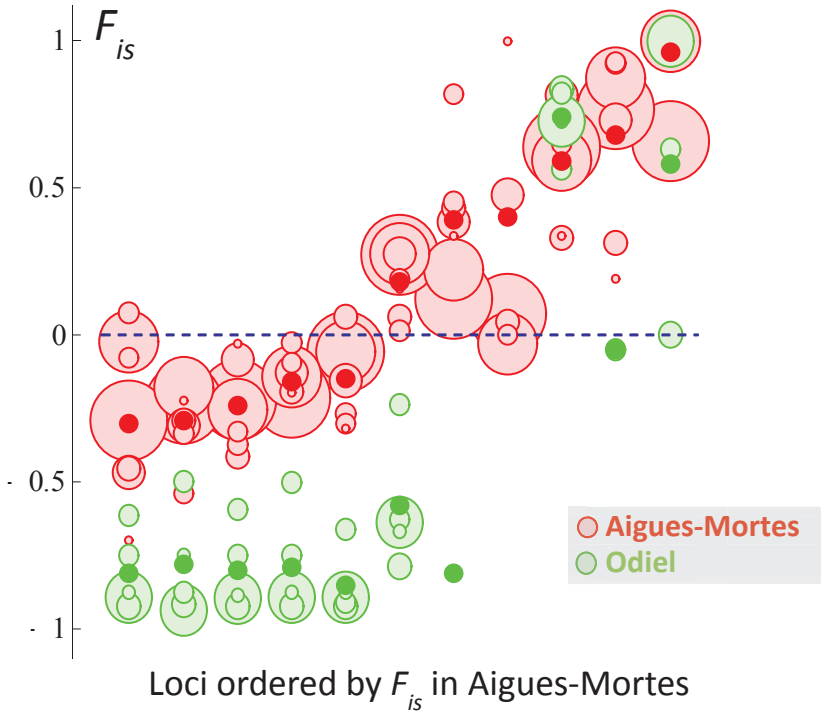

Figure 2. Estimated F_{is} for microsatellites genotyped in diploid parthenogens from Aigues-Mortes (Red) and Odiel (Green). Circles corresponds to values obtained for the different sampling sites within each population (7 sites in Aigues-Mortes, 5 sites in Odiel), with sample size reflected by the size of the circles. Plain dots correspond to population averagea. Loci are ordered by their mean F_{is} in Aigues-Mortes. F_{is} values for one locus could not be computed in Odiel as it was non-polymorphic in this population.

The different loci exhibited widely different F_{is} estimates (averaged across sites within populations), with values across loci well spread between-0.30 and +0.96 in Aigues Mortes, and between -0.85 and +0.58 in Odiel. Importantly, most F_{is}-values at individual loci were consistent when estimated across different sites. Mean F_{is} estimates in Aigues-Mortes and Odiel were positively correlated (Pearson Correlation r = 0.84, df = 10, P-value = 0.0025). However, loci tended to exhibit lower F_{is} in Odiel (10 loci out of 11, which were polymorphic in both locations), especially for loci presenting an excess of heterozygotes (negative F_{is}). The proportion of null alleles at a given locus was estimated though the proportion of individuals with no amplification for this locus. This proportion did not covary with mean F_{is} neither in Aigues-Mortes (r = 0.17, df = 11, P-value = 0.62) nor in Odiel (r = -0.18, df = 10, P-value = 0.60). Possible positive biases in estimates due to cryptic population subdivision (Wahlund effect) are also likely to be negligible, given the range of value observed, the

negligible differentiation within populations and the repeatability of estimates across sites.

Discussion

Automixis in *Artemia*

In this study, we first showed that the parthenogenetic populations studied were indeed diploid. We then showed that three independent asexual lines grown in the laboratory for approximately 36, 36 and 20 generations, respectively, maintained their initial genotype at five heterozygous and seven homozygous loci. This observation alone allows ruling out several types of reproduction (cases 4 and 6-9 in Table 1) because for each of these at least some segregation (i.e., loss of heterozygosity compared to the initial genotype) would have been expected. In contrast, this transmission pattern across generations is consistent with apomixis (mode 1) and several types of automixis (2, 3, and 5 with low recombination). Next, we investigated the population-wide level of heterozygosity. We found very heterogeneous departures from Hardy-Weinberg expectation among loci, from excess of heterozygotes to strong excess of homozygotes. Specifically, F_{is} ranged from -0.30 to +0.96 in Aigues Mortes, with values across loci being well spread in this interval, and from -0.85 to 0.58 in Odiel. F_{is} estimates were consistent when measured in different sites within each population, and, most importantly mean F_{is} estimates for individual loci were highly positively correlated between these two populations, which are more than a thousand kilometers apart. It is very unlikely that natural selection could maintain a large number of different asexual lineages at similar frequencies in these two distant and ecologically different populations, so as to create a strong correlation in F_{is}-values across populations. A much more likely explanation is that *Ap*2*n* reproduce by a form of automixis that preserves heterozygosity at a large number, but not at all loci (central fusion, case 5 in Table 1). Even though the genetic map positions and the positions relative to the centromeres of their respective chromosome are unknown for the microsatellite loci used in this study are unknow, it can be expected that their position relative to the centromere varies. Hence, loci with negative F_{is}-values would be loci at proximal positions relative to centromeres and thus recombine rarely enough (or never) so that they accumulate heterozygosity via mutation. In contrast, loci with positive F_{is}-values would be loci at more distal

positions and thus recombine more frequently, leading to a loss of heterozygosity and to an overall deficit in heterozygotes. As loci are very likely to share their chromosomal position across populations, this scenario would explain the positive correlation in F_{is} between the two populations. The overall higher excess of heterozygotes in Odiel would then simply reflect a lower overall effective recombination rate in this population.

Our results are consistent with automixis by central fusion (case 5 in Table 1) only if recombination rates are low but non-zero in both populations. With too high recombination rates, given the time-scale of mutation, all loci should present a strong deficit in heterozygotes, except for a few loci being located almost at the centromere, and no gradual change in F_{is}-values would be expected. The hypothesis of low rates of recombination is corroborated by two observations: First, we did not observe any loss of heterozygosity after ~36 generations in the laboratory, and second the sexual species most closely related to asexual diploids (*A. sp. Kazakhstan*) exhibits unusually low recombination rates (unpublished data). Non-zero rates of recombination, which are inconsistent with modes 1-4 in Table 1, are also supported by several line of evidence. First, the genetic diversity of *Ap2n* is clearly higher than would be expected in clonal organisms and is also higher than in apomictic, tetraploid *A. parthenogenetica*. Second, the strong variation in F_{is}-values and the positive correlation of these values across populations is inconsistent with total absence of recombination.

All these lines of evidence suggest that *Ap2n Artemia* are very likely to reproduce via a form of automixis that is genetically equivalent to central fusion with low, but non-zero rates of recombination. The careful observations of Stefani (1960) were thus probably the most accurate among the cytological studies. According to his description, meiosis I starts normally but, at anaphase I, the first division spindle breaks in the middle and the two half-spindles reunite in a single metaphase plate (i.e., the first division is aborted mid-way, which is equivalent to central fusion, Asher, 1970), then meiosis II occurs normally. Similar types of automixis have been described in Lepidoptera (*Apterona*, *Solenobia*, *Luffia*, Narbel-Hofstetter, 1964) and in Crustaceans (*Daphnia pulex*, Hiruta *et al.*, 2010). This mechanism, associated with low (and across populations slightly variable) rates of recombination would fully account for all our observations.

Rare males

Central fusion in combination with low and variable recombination also provides a simple explanation for the putative origin of rare males. If the sex-determination locus is close to the centromere, it remains heterozygous most of the time, leading to female offspring. However, rare recombination events lead to segregation at the sex locus (and the production of two third of ZW females, and one third of ZZ males, assuming WW lethality). Hence, three times the proportion of rare males in the progeny of a lineage should be a rough estimate of the genetic distance to the centromere of the sex-determining locus. This proportion has been shown to vary in the range 0% - 1.7% across lineages in diploid parthenogenetic lineages of *Artemia* (Maccari *et al.*, 2013b), which is consistent with the idea that there is some differences in recombination rates among parthenogenetic lineages. Similarly in our data, recombination needs to be slightly lower in Odiel than in Aigues-Mortes to explain stronger the excess of heterozygosity observed in the former population. Interestingly, recombination might be directly selected against in this system, to prevent segregation at the sex-determination locus (the latter contributing largely to the segregation load, see Antonovics & Abrams, 2004 for similar ideas).

A new method to uncover the mode of automixis

The interpretation of population genetic data from asexual populations has always been a difficult problem, because the well-known patterns expected for sexual populations are no longer valid. Frequencies at neutral marker are largely influenced by indirect selection at linked selected loci. Departure from Hardy-Weinberg can be very large, and in any direction, depending on subtle details of the reproductive modes (see Table 1). Linkage disequilibria are likely to be so high that it is often clearer to “count” clones than trying to interpret the data at the locus level. Finally few comprehensive tools are available to analyze multilocus data from asexual populations (Meirmans & Van Tienderen, 2004; Arnaud-Haond *et al.*, 2007). There is however a positive side of all these difficulties: different reproductive modes can leave subtle but long-lasting genomic footprints. In particular, the mean and variance of deficit/excess of heterozygotes across loci can be very informative on the reproductive system. It is well known that a strong signature of apomixis is the large heterozygote excess at all loci, so large that it can even lead to the so-called Meselson effect, whereby mean divergence among homologue chromosomes is

larger than diversity within the sub-population of carriers of each homologue (Birky, 1996). The expected distribution of *Fis* has even been theoretically investigated under partial apomixis (Stoeckel & Masson, 2014). The reverse pattern of a uniform deficit of heterozygotes has also been widely used to infer rates of self-fertilization. Near complete loss of heterozygosity is also indicative of specific forms of automixis (mode n° 4, 7, 8 in Table 1). However, as we show here, a strong variation across loci of excess/deficit of heterozygote is strongly indicative of a mode of automixis where recombination rate determines the level of heterozygosity (mode n°5, 6, 7). An overall bias towards an excess in heterozygotes being indicative of central fusion (n°5, as in our case), while an overall bias toward a deficit in heterozygotes being indicative of terminal fusion (n°6). Confirmation of these patterns could be obtained by analyzing genetic variation between mother and offspring, but only if recombination rates are high enough to detect loss-of-heterozygosity at least for some loci. If in contrast, recombination is low, as they appear to be in Artemia, documenting large F_{is} heterogeneity among loci can be used as an alternative, population based approach. This is reinforced by showing that the variation across loci is consistent in different and distant populations, as marker position on chromosomes is not expected to vary across populations. Indeed strong variation in F_{is} has been found in species known to reproduce by central fusion or in which mother-offspring data was conclusive (Pearcy *et al.*, 2006; Kellner & Heinze, 2011; Rey *et al.*, 2011; Kronauer *et al.*, 2012). Further confirmation could be obtained if the map/physical position relative to the centromere is available for these markers. However, for obvious reasons, chromosome mapping in asexuals is almost impossible by conventional methods (but is becoming feasible using new sequencing technologies). If, on the contrary, the species investigated is already known to reproduce via central fusion, patterns of F_{is} could be used to map loci with respect to centromeres. Hence, the method proposed here complements traditional cytological approaches by providing data on the amount of recombination in automictic species, where crossing-overs could not be observed directly (e.g. Hiruta et al 2010).

Within genome heterogeneity of heterozygosity

Asexuality is characterized by a diversity of reproductive modes, with different population genetics consequences. Understanding and documenting this diversity is important also important to understand the evolutionary transitions between sexual

and asexual reproduction (Schwander *et al.*, 2010; Neiman *et al.*, 2014). In addition, the forms of automixis generating within-genome heterogeneity (cases 5 and 6 in table 1) may be particularly insightful to study long-term genomic consequences of breeding systems, as they generate a gradient of heterozygosity of loci across each chromosome, driven by their genetic distance to the centromere. For instance, in the case of central fusion with low recombination (as we describe here for *Artemia*), pericentric regions should present large excess of heterozygotes and accumulate a large genetic load (e.g. recessive lethals), while telomeric regions should present almost no heterozygosity and be much less loaded with deleterious mutations. This contrasted situation would allow comparing, *everything else being equal*, the relative genomic impacts of 'selfing' and 'asexuality' (e.g. dN/dS, $\pi N/\pi S$, distribution of transposable elements, genetic load etc.), (Glémin & Galtier, 2012).

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Annexe 3

Temporal characterization of sediment core from Aigues-Mortes saltern, A. franciscana invasion and development of new SNP's markers.

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Cette annexe présente les méthodes et résultats préliminaires obtenus sur l'extraction, l'analyse des sédiments et la création d'une banque de SNP sur différentes espèces d'artémies. L'ensemble est rédigé en anglais en prévision de la rédaction future d'un papier sur ce sujet.

1. Extraction et caractérisation temporelle d'une carotte de sédiments

Methods

Artemia samples

Artemia are anostracan that can both have ovoviviparous, when environmental conditions are optimal, and oviparous reproduction (Criel & MacRae 2002). These resistance eggs are called cysts. They may endure extreme environmental conditions (e.g. anoxia, heat, dessication or radiation; Clegg and Conte 1980; Sorgeloos et al. 1987) and still be able to hatch. One female Artemia can produce more than a hundred cysts per brood (Browne *et al.* 1988) and, at the end of the reproductive season tones of cysts can be produced. Theses dormant cysts are dehydrated; they float on salted waters (Clegg 2005) and are moving along water current.

In saltern, water circulates from the sea to the different ponds by channels. The ponds are connected to the channels through "martelières" (sluice). The l'Abbé site (43°31'24.72"N; 4°14'12.67"E) in the Aigues-Mortes saltern is one of such martelière that connected a pond and a channel where cysts tend to accumulate. It was shut down in the early 1960's (pers. com.) and silted up ever since. Cysts accumulated in the sediments, creating a long time series. As the l'Abbé site is connected to the rest of the saltern, the evolution of its population reflects the entire saltern *Artemia* population (Nougué et al. in prep).

Core extraction and time analyses

A total of 8 cores were retrieved from the l'Abbé site (Figure S1). The first one was manually retrieved on the 1st of February 2012 (see below for details) and was used to estimate the introduction level of *A. franciscana* in the sediments. The three next were retrieved on the 26th of June 2013 using a manual corer (see below for details) and were used to date the sediments.

Extraction and analysis on the first core.

A hole of 1 m deep was dug with one vertical side (Figure S1). Cuboids of 16 x 16 x 3.5 cm were retrieved from the vertical side of the hole, divided in two equal sub cuboids and placed in individual hermetic bags. A total of 19 horizons were retrieved.

Cysts were separated from sediments using saturated brine. Cysts in the core were dehydrated and then floated on brine while sediments sank. Surface water were pumped and filtered to retrieve cysts.

For each core horizon, the DNA from 20 individual cysts was extracted using 15µL of E buffer (HotSHOT, Sigma-Aldrich), then placed in a thermocycler (95°C for 10 min plus 20°C for 10 min) and finally, extract was diluted with 25µL of sterile water. To discriminate between *A. parthenogenetica* and *A. franciscana,* Ap02 and Af03 microsatellite markers (Muñoz *et al.* 2008b) were respectively used with the following protocol. PCR mix, containing 5 µL of Multiplex buffer, 1 µL of both forward and reverse primers [2 µM] and 2 µL of sterile water, was added to 1 µL of DNA sample. Thermocycler program was as follow: initial denaturation ran for 15 min at 95°C, 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1min30, and extension at 72°C for 1 min and, a final extension ran for 30 sec at 60°C. Ap02 and Af03 PCRs were conducted in parallel for each cyst individual. Cyst species identification was read on the amplification result after migration on 2% agarose gel for 30 min.

Extraction and analysis on the other cores.

Manual corer consisted in 1m PVC tubes that were pushed directly through the sediments. A pipe connected to an air compressor was attached at the bottom of the tube. Indeed, as the sediments are full of water, they are quite viscous and therefore

difficult to wind up only using vacuum force. The pipe helps creating an air bubble beneath the corer and eases the core extraction.

Cores were opened and photographed, than the chemical composition of the core was characterized using the Avaatech X-ray Fluorescence (XRF) CoreScanner from the Edytem lab (Université de Savoie, France). Avaatech XRF Core Scanner systems perform non-destructive analysis of elements from Mg right through to U along the core axis. Theses analysis allowed us to correlate the cores, to look for sedimentation cycling (year markers) and for special sedimentation event. We performed a Principal Component Analysis (PCA) to explicit correlations between diverse elements that are known to be associated with different types of sediments. Sulfur (S), brome (Br) and copper (Cu) are usually associated with organic matter of high oxygenation, iron (Fe) and manganese (Mn) are associated with organic matter of low oxygenation, while potassium (K) and silicon (Si) are associated with sand. We chose to illustrate the results of the XRF core scanner for the Br element as this element helped us mark sandy events easily distinguishable in all cores to be correlated. The correlation of cores is essential when analysis involves destructive protocols, such as molecular biology and gamma spectrometry, which might require back up samples.

To distinguish between recent and older core horizons both continuous time detection and the detection of event-marker are useful. The gamma spectrometry of both ^{137}Cs (Caesium-137) and ^{210}Pb (Lead-210) on several core horizons was realized in the Géosciences lab (Université Montpellier 2, France). While ^{210}Pb is a sedimentation indicator, ^{137}Cs mark nuclear bombing events with global implications (see Ayrault et al. 2009 for details).

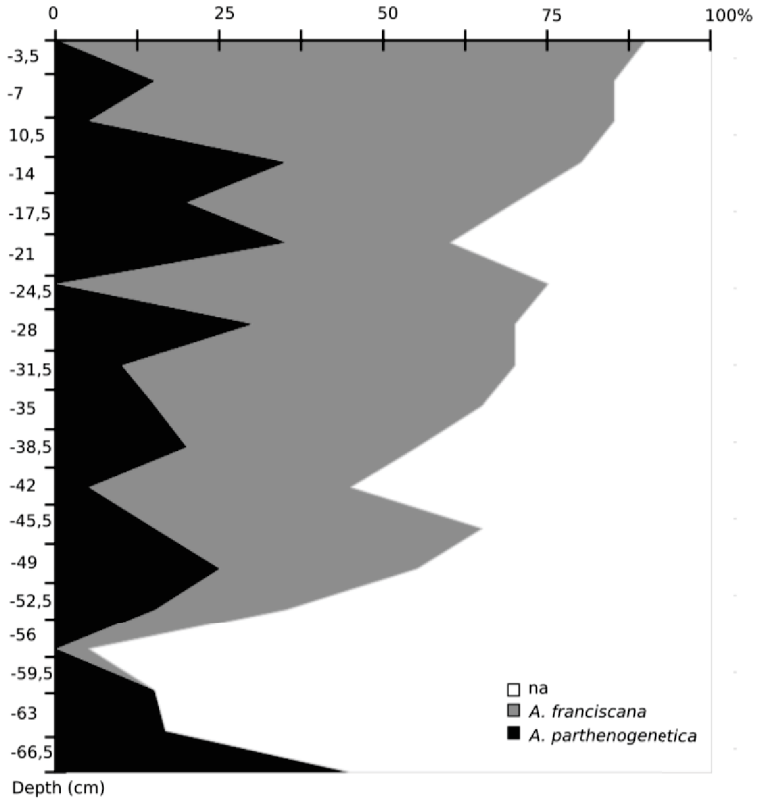
Results

DNA quality along the first sediment core

The proportion of *A. franciscana* decreases along the core horizons (Figure 1), from 90% on the top horizon to 0% in the last three horizons. On the other hand, we did not detect a decrease in the proportion of *A. parthenogenetica*. The proportion of non-readable results increased from 10% to a maximum of 95% along the core horizons. This indicates a degradation of the DNA quality in the samples. DNA

fragment were mostly smaller than 200 pb which is too small for microsatellite amplification (Figure S2).

Figure 1: Proportion of *A. parthenogenetica* and *A. franciscana* along the core horizons. na: non identifiable cyst; *A. franciscana* and *A. parthenogenetica*: cyst identified as sexual and non-sexual species.

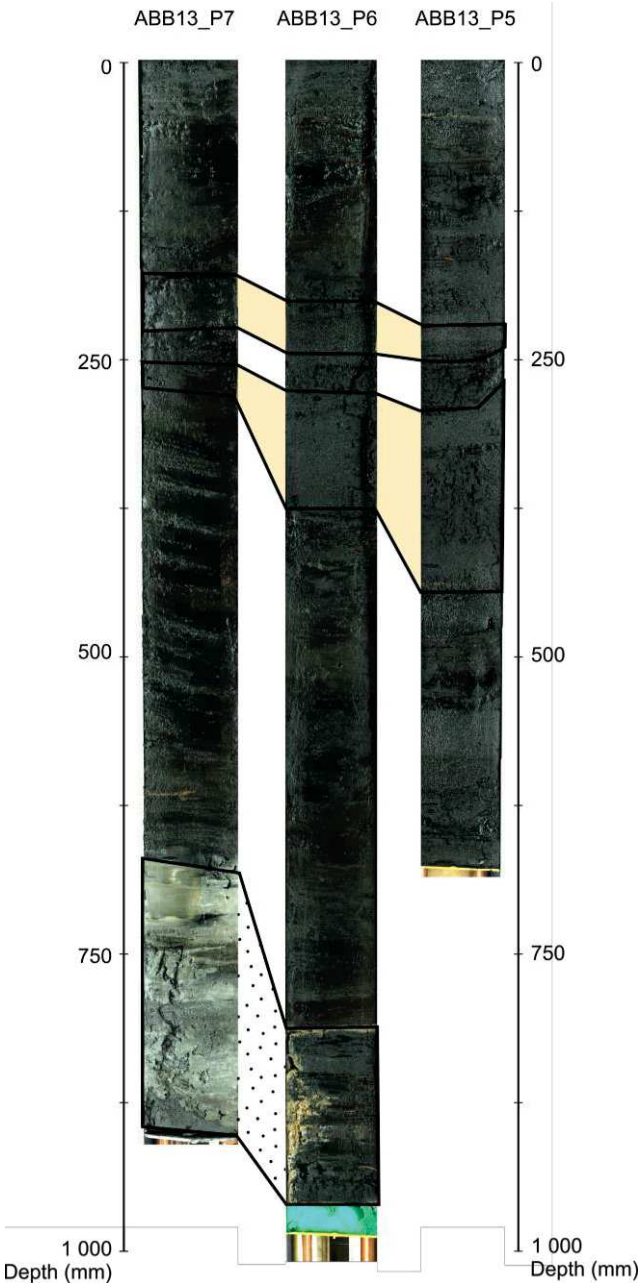


Time analysis

Core correlation

Visual observation of the cores (Figure 3) revealed that only ABB13_P6 and ABB13_P7 contained the complete sediment sequence that followed the martelière closure. Indeed, in both these cores stirred sediments (with no horizons; dote-shaded zone in Figure 3) are visible from 810 to 960 mm and 670 to 900 mm, respectively in ABB13_P6 and ABB13_P7; they correspond to the bottom of the channel that used to be maintained by digging out the sand that might have silted up throughout the year before the martelière shut down. No cycling was clearly observable on the three cores. However, two sandy horizons were visible on the three cores (yellow shaded zones on Figure 3).

Figure 3: Visual correlation of sediment cores. The dote-shaded zone corresponds to the bottom of cores ABB13_P6 and ABB13_P7 where sediments were stirred. The yellow shaded zones correspond to clear sandy areas.



X-ray Fluorescence observations showed alternating between organic matter containing elements such as sulfur, copper and bromine and sands containing elements like potassium or silicon. Indeed, the PCA results exhibit a first dimension, explaining 41.42% of the variation (Figure 4), which segregate elements corresponding to organic vs. non-organic matter (clay and sandstone). The second axis, explaining 27.66% of the variation, segregate low from highly oxygenated

organic matter elements. In Figure 5, the three cores present alternative horizons with high and low quantity of bromine (element present in organic matter). Mirror graphs (results not shown) can be produced using potassium (element present in sandstone), which again point out the alternating of organic and sand horizons in the cores. The two sandy horizons that were visually observable are also observable on the three bromine graphs (yellow zones in Figure 5), as well as the stirred sediments in the bottom of ABB13_P7 (dote-shaded zone in Figure 5).

Figure 4: PCA on XRF CoreScanner results for ABB13_P5. Chemical element associated with clay (**triangle**; Rb: rubidium), sandstone (**square**, K: Potassium; Si:Silicon) and organic matter of high (**empty circle**; Fe: iron; Ca: calcium; Ti: titanium; Mn: manganese) or low oxygenation (**full circle**; S: sulfur; Br: bromine; Cu: copper; Sr: strontium).

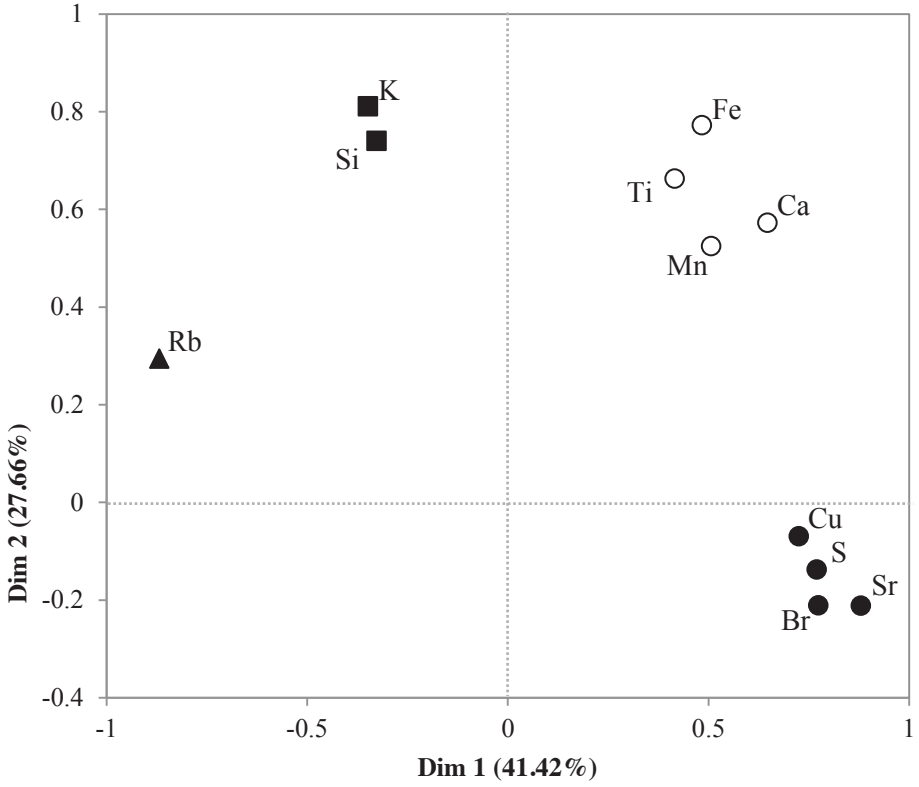
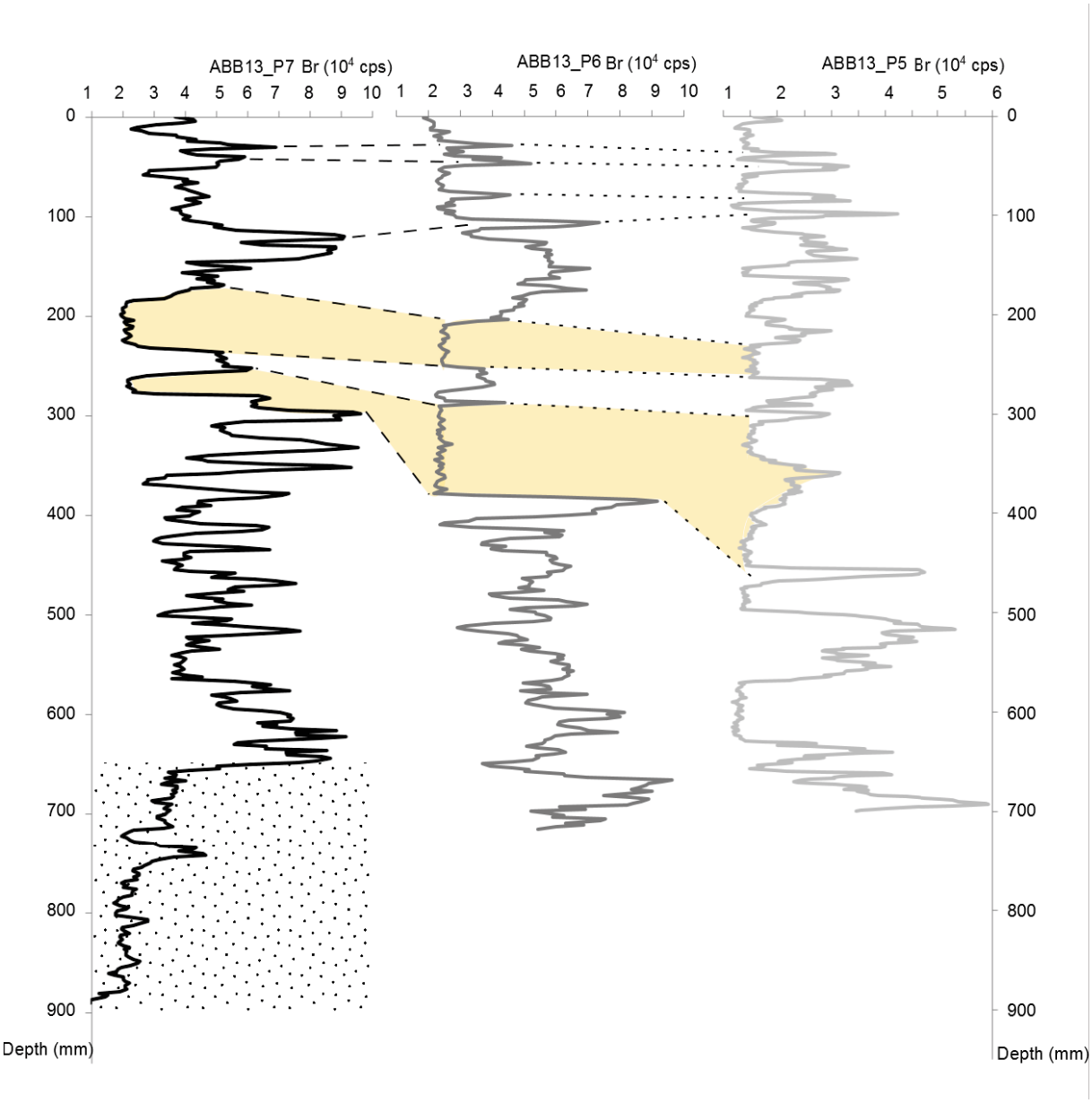


Figure 5: Core correlation using bromine concentration along the cores horizons. Horizontal axis corresponds to the counts per second (cps) recorded at 30kV for bromine, while vertical axis corresponds to the depth in millimeter for each core. On ABB13_P7 core, the dote-shaded zone corresponds to the bottom of the core where sediments were stirred. Yellow shaded areas mark the sand area spotted on the cores. Dashed lines are correlations between ABB13_P7 and ABB13_P6 while dotted lines are correlations between ABB13_P6 and ABB13_P5.



Core timing

To distinguish between recent and older core horizons we used both (Figure 6) continuous time detection (particle diameter and ^{210}Pb) and the detection of event-marker (^{137}Cs). The ^{210}Pb signal should follow a logarithmic decay. However, as the particle diameter and previous results displayed the predominance of sand in the sediments. Lead tends to be washed away from such sediments; the interpretation of the ^{210}Pb graph is, therefore, quite difficult.

The particle diameter negatively correlates with the bromine graph (Figure 5). The signal for the two sandstone horizons is visible with high diameter particle from 200 to 350 mm deep (labeled Sand 1 and Sand 2 in Figure 6). Another high diameter particle horizon was detected between 50 and 100 mm deep (Figure 6). It corresponds to low bromine density in ABB13_P6 (Figure 4). This corresponds to another sandy horizon (labeled Sand 3 hereafter).

Finally, ^{137}Cs was detected all along the core depth expose the fact that the entire time series was posterior to 1954. A maximum of 14.35mBq/g was detected at 620mm deep, signaling the fallout maximum of 1963. However, we were not able to detect the Tchernobyl impact of 1986. Two reasons might be suggested: (i) the scale was too large for such a singular event; (ii) the time series stops prior to 1986. In the first case, a finer sampling might be realized especially between 100 and 200mm depth for the quantity of ^{137}Cs seems to increase in this area of the core.

The three sediment cores retrieved from the Aigues-Mortes saltern are composed of successive layers of sand and organic matter more or less oxygenated. Three major sand layers (Sand 1 to 3 in Figure 6) helped us correlate the cores. Theses sandy horizons might corresponds to extreme meteorological events (wind storms, rain). The presence of a weather station next to the saltern (Quillé 2000) that analysed the weather archives of the area since 1951 helped us determine possible events that might have produced those sandy horizons. Indeed, we found eight extremely windy events from November 1982 to Mars 2008 (Table 2) that might correspond to the formation of these sand layers.

Figure 6: ABB13_P6 core datation. To distinguish between recent and older core horizons we used both continuous time detection (particle diameter and ^{210}Pb) and the detection of event-marker (^{137}Cs).

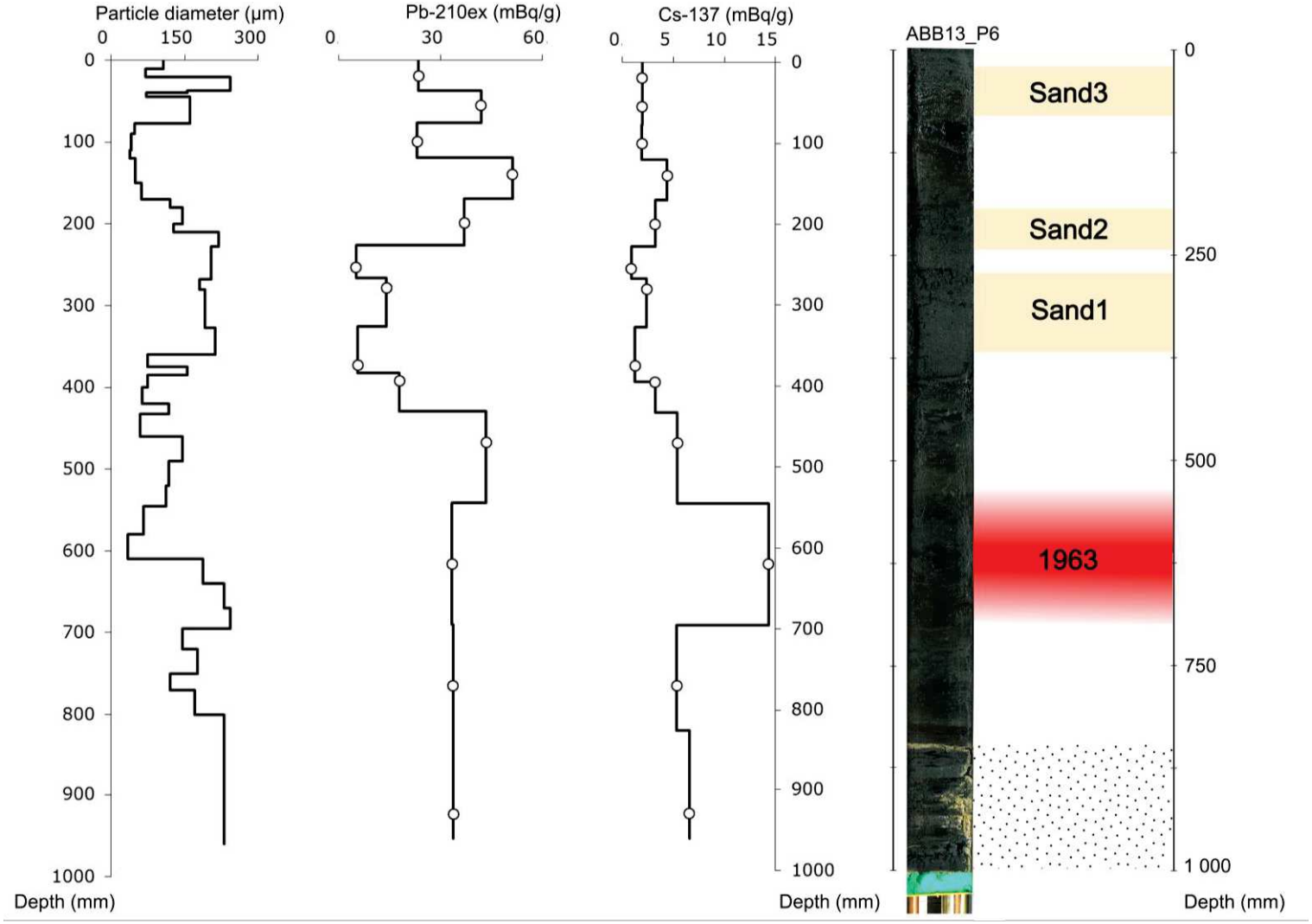


Table 2: Windy events between 1982 and 2009 in Aigues-Mortes.

	Wind speed (km/h)	Duration (days)
November 1982	120 (max 130)	2
February 1984	120	1
October 1987	120	1
September 1992	120	1
December 1997	120 (max 140)	3
August 1999	140	1
December 1999	140	1
Mars 2002	120	1

The use of ^{137}Cs confirmed that the martelière's closure was prior to 1963 (maximum of the radiations fallout). We were not able to detect the Tchenobyl radiation pic of 1986 so we could not confirm that the surface sediments were posterior to the 1980's or not. A finer analysis between 100 and 200 mm depth might help us answering this question. Finally, the use of microsatellite markers helped us make a first estimation on the beginning of the *A. franciscana* invasion of the saltern.

This study was a first analysis that will help us better sample the core in order to study the impact of *A. franciscana* invasion on the local *A. parthenogenetica* population in Aigues-Mortes.

2. Caractérisation de nouveaux marqueurs SNP et détails de la phylogénie du genre *Artemia*

Methods

Artemia samples used for SNPs characterization

Artemia samples for SNP characterization originated from laboratory collections. In this experiment only adult *Artemia* were used. A total of 39 individuals were used: 25 *A. parthenogenetica* and 7 couples of sexual *Artemia* from various origins (see Table 1).

Table 1: STACKS analysis results for asexual (A) and sexual (B) artemia samples. RAD-Tags is the number of reads available after NGS; **Reads** is the number of utilized reads in the STACKS analysis; **Stacks** is the number of polymorphic loci found in the analysis; **Coverage** is the mean number of read linked to a stack.

Table 1A.

Sample	Location		RAD-Tags	Reads	Stacks	Coverage
Caitive_1	France	Aigues-Mortes (Gard)	1 575 734	1 518 777	143 113	10.31
Caitive_2			8 588 515	8 469 112	308 001	26.80
Caitive_3			334 305	316 290	40 866	7.49
Caitive_4			232 643	220 023	29 947	7.12
Caitive_5			6 630 376	6 533 302	281 530	22.62
Caitive_6			7 000 620	6 903 204	294 401	22.90
Caitive_7			9 763 802	9 640 306	313 874	30.00
Caitive_8			8 213 946	8 105 935	299 929	26.39
Caitive_9			480 528	456 161	56 151	7.87
Caitive_10			11 017 240	10 882 596	322 676	32.94
Caitive_11			10 532 368	10 408 083	316 146	32.19
Caitive_12			7 981 494	7 878 692	297 836	25.86
S9_3			6 537 483	6 435 949	304 196	20.64
PAM10			7 175 773	7 067 543	284 212	24.20
PAM6			5 507 586	5 424 682	274 436	19.33
PAM7			7 188 950	7 088 852	289 712	23.87
LAPIX		La Palme (Aude)	11 116 527	10 969 716	344 174	31.19
ANK	Madagascar	Ankiembe	11 718 672	11 532 910	368 894	30.51
LM5	Spain	La mata	11 484 253	11 339 001	328 306	33.81
LM7			7 119 842	7 031 857	293 792	23.45
ODIEL		Odiel	8 785 824	8 667 374	310 767	27.19
PACO50**	Iran	Lake Urmia	10 775 934	10 648 474	323 223	32.19

Table 1B.

Species	Location		Sexe	RAD-Tags	Reads	Stacks	Coverage
A. tibetiana* (ARC1347)	China	Lagkor Co	f	6 371 120	6 262 772	286 059	21.34
			m	6 426 098	6 315 071	279 549	22.01
A. sinica* (ARC1188)		Yimeng area	f	9 127 458	9 001 490	312 931	28.04
			m	10 902 907	10 754 062	323 889	32.35
A. salina**	Spain	La Malaha	f	6 788 836	6 732 300	257 858	25.42
			m	9 625 215	9 554 271	282 536	32.93
A. franciscana* (ARC1574)	USA	San Francisco Bay	f	7 232 331	7 166 026	267 947	26.15
			m	9 658 125	9 559 093	275 928	33.67
A. franciscana* (ARC1710)		Great Salt Lake	f	7 620 422	7 546 209	269 875	27.22
			m	7 623 174	7 551 221	284 427	25.96
A. franciscana	France	Aigues-Mortes	S9_1	7 187 435	7 126 368	252 030	27.66
			S9_2	8 133 905	8 058 378	259 981	30.21
A. kazakhstan* (ARC1039)	Kazakhstan		f	11 395 205	11 251 871	326 246	33.70
			m	9 951 613	9 846 933	319 715	30.22
A. urmiana* (ARC1542)	Iran	Lake Urmia	f	13 779 176	13 572 580	344 795	38.58
			m	7 810 547	7 700 846	315 919	23.77
A. urminana (ARC1230)*			?	9 880 904	9 769 242	321 139	29.82

Footnote: All samples were collected by the authors except for * curtesy of C. Mahieu from the Artemia Research Center (Gent, Belgium) and ** curtesy of F. Amat from the Instituto de Acuicultura de Torre de la Sal (Castellon, Spain).

These crustaceans are non-selective filter-feeder and there gut is full of algae or bacteria (Allender *et al.* 2010). However, to do RAD sequencing, DNA requires being as pure as possible. Therefore, two different protocols were tested to discard those gut pollutions: one using marble, the other using bleach and dissection. In the marble protocol, Artemia were left alive in a salted water solution (salt concentration varying among species) with marbles (glass marbles, 20 to 80µm; SIGMA Sephadex G25 fine) for 3 to 4 days. Then, they spent half an hour in salted water solution with 2% bleach. They were rinsed three times for 10 min in salted water solution than stocked in 96° ethanol at -20°C. In the bleach and dissection protocol, living artemia spent 10 min in salted water solution with 2% bleach. They were then transferred to a salted solution for 10 min. Then to a sterile salted solution for 10 min and were directly dissected (gut track removal). The gutless artemia was rinsed with 96° ethanol than stocked in 96° ethanol at -20°C. This second protocol was used on all *A.*

parthenogenetica samples from Aigues-Mortes saltern and the *A. sinica* male and female samples.

DNA extraction and RAD Library Preparation

The DNAEasy Blood and Tissue kit (Qiagen) was used for DNA extraction, following manufacturers' instruction with some modifications prior to DNA washing and elution. Elution used nuclease free water instead of buffer in order to allow sample concentration if needed.

The final library should contain 2.1µg of DNA. Measurements of concentrations were done using NanoDrop to check for minimal concentration of 700ng of DNA used in the restriction enzyme (RE) digestion. Here, the pstI RE enzyme was used. DNA was mixed with water and RE specific buffer. The mix was placed at 37°C for 45 min. The enzyme was heat-inactivated by placing the mix at 80°C for 20 min. Cool down at room temperature for 10 min.

P1-ligation mix includes 1µL 10X NEB buffer 2, 3µL Bar-coded P1 adapter [100nM], 0.6µL rATP [100mM], 0.5µL concentrated T4 DNA-ligase, 2.9µL of water. Vortex for 2 sec than spin down for 5 sec at 5000 rpm. Incubate at room temperature for 45 min. Heat-inactivate T4 DNA ligase by placing samples at 65°C for 20 min at 350 rpm. Cool down at room temperature for 10 min.

Samples were multiplexed by combining 1 to 2µg of DNA in a maximum volume of 300µL than size selection (300 to 500bp) was done on a 1.25% of agarose gel using the MinElute Gel Extraction kit (Qiagen). P2-ligation mix includes 5µL of 10X NEB buffer 2, 1µL P2 adapter [10µM], 0.5µL rATP [100µM] and 0.5 T4 DNA-ligase. Vortex for 2 sec than spin down for 5 sec at 5000 rpm. Incubate at room temperature for 30 min. Purify with QIAQuick column kit (Qiagen) than, elute in 52µL of EB buffer.

In this step, high fidelity amplification on P1 and P2 adapter-ligated DNA fragments is performed. First, a test amplification is done to help determining the library quality. The final PCR mix includes 20.5µL of water, 25.5µL of 2X Phusion High-Fidelity Master Mix (Thermo Scientific), 1.2µL of Forward and Reverse Solexa primer [10µM] and 5µL of RAD-library template. Vortex for 2 sec than spin down for 5 sec at 5000 rpm and place 8.9µL into 6 PCR tubes. Cycling conditions are 98°C for 30 sec, 18 cycles of 98°C for 10 sec, 65°C for 30 sec and 72°C for 30 sec and one final

elongation of 72°C for 5 min. Combine the 6 PCR volumes into one and purify with the MinElute Purification kit. Elute in 25µL of EB buffer. Migrate on a 1.25% agarose gel and select for 350 to 600bp than elute using the MinElute Gel Extraction kit (Qiagen).

NanoDrop was used to check final concentration between 10 and 20ng.µL^{-1}. Single end sequencing was performed with Illumina sequencer at Genoscreen (Lille, France).

Quality filtering and SNP calling

Reads quality was checked using FASTQC tool (Andrew 2010). All sequences presented a Phred score above 30, so all the 100bp length sequences were conserved for further analysis. The libraries were demultiplexed using the process_radtags program from the STACKS pipeline (Catchen *et al.* 2011). Single errors within the barcode were automatically corrected by the software. We eliminated from further analysis three *A. parthenogenetica* (Caitive_3, Caitive_4 and Caitive_9) for extremely low number of RAD-tags compared to others (less than 500 000, see Table 1B for details). The final quality filtered and demultiplexed data set contained about 8.25 million reads in *A. parthenogenetica* and about 8.77 million reads in sexual species of *Artemia* (see Table 1 for details), each 95bp in length.

All reads were used for a *de novo* assembly in ustacks (STACKS pipeline, Catchen *et al.* 2011). A 'stack' is a set of identical sequences in the terminology of this pipeline; several of these stacks may then be merged to form putative loci. We set a minimum stack size of 2 reads (-m) and excluded all stacks with coverage lower than this threshold; lower coverage stacks may result from sequencing error and will generally provide low confidence in genotype calls. We set the maximum distance between stacks (-M) within a locus as 3, meaning that stacks that are merged to form a locus are allowed a maximum of three base pair differences with any other stack included in the locus. Note that because this parameter constrains the number of pairwise differences between stacks, the total number of base pair differences at a locus can be higher than three when more than two stacks are merged. We excluded putative loci with unusually high coverage (i.e. 'lumberjack stacks' of ustacks) because these loci probably derive from multiple copies of similar sequences present

in the genome or from highly repetitive regions. They are thus likely to contain non-orthologous sequences.

As we did not had a genetic map for *Artemia* to map the loci, we created a reference catalog with cstacks (STACKS pipeline, Catchen *et al.* 2011). We set a maximum of 2 mismatches (-n) between samples when generating the catalog. Finally, we reattributed standardized loci names to samples with sstacks (STACKS pipeline, Catchen *et al.* 2011). Under these parameters, the de novo assembly produced from 143 113 to 368 894 loci in A. parthenogenetica and from 257 858 to 344 795 loci in sexual *Artemia* (see Table 1 for details).

SNP matrix and tree construction

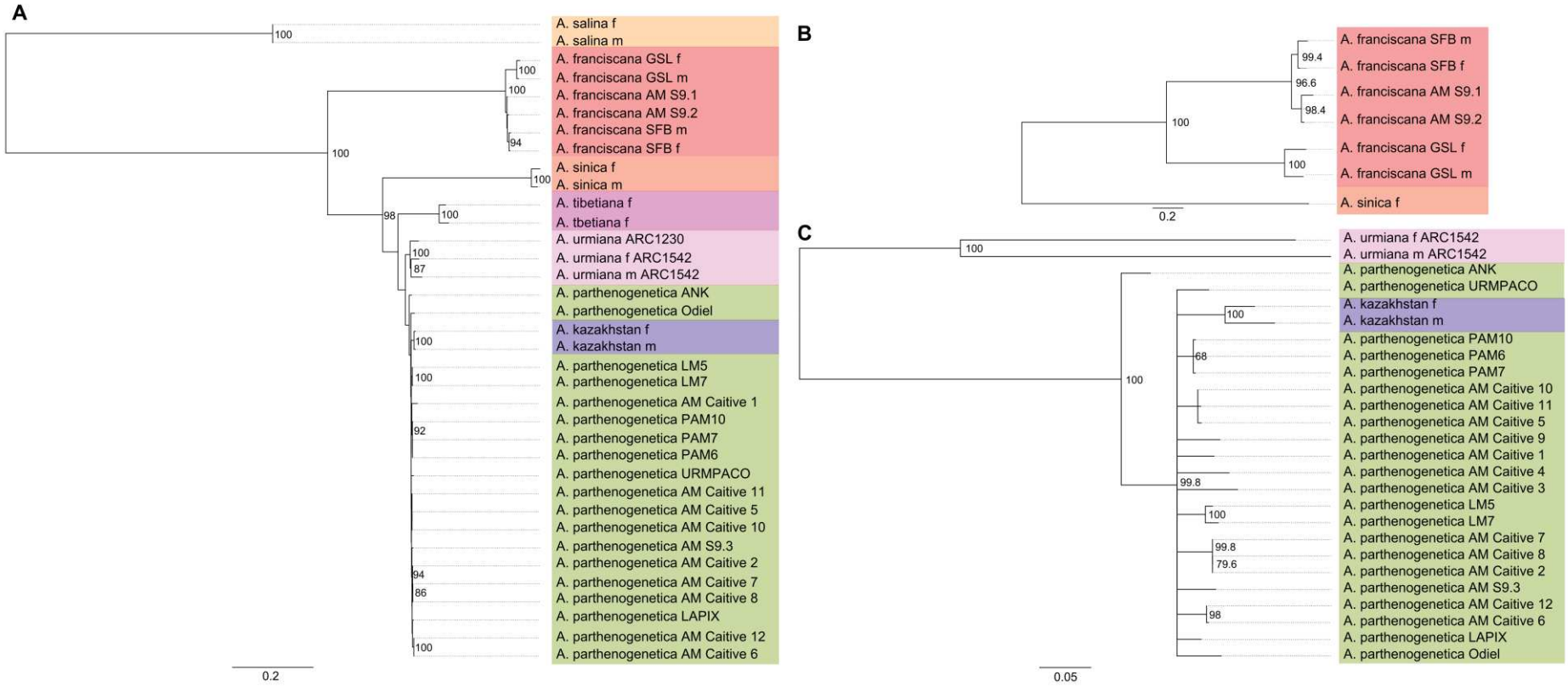
We build a SNP matrix using populations (STACKS pipeline, Catchen *et al.* 2011). We set one population per individual so as to get individual diagnostic SNPs. A locus had to be present in a minimum set of 25 individuals (-p) to be processed in the global phylogeny as well as the focus on *A. parthenogenetica* ans 6 individuals in the focus on the *A. franciscana* phylogeny. We obtained phylogenies by Maximum Likelihood (ML) method using the PHYML software (Guindon *et al.* 2005). We employed the GTR (General Time Reversible) substitution models determined by MODELTEST (Posada & Crandall 1998). We rooted phylogenies with the *A. franciscana* individuals originating from San Francisco Bay (SFB, California) or the Great Salt Lake (GSL, Utah). Bootstrap supports were calculated using 1000 replicates.

Results

We were able to generate a total of 3762 diagnostic SNP's loci that helped us built the phylogeny presented in Figure 2A. The present phylogeny matched previous ones found in the literature (Baxevanis *et al.* 2006; Muñoz *et al.* 2008a, 2010; Munoz & Pacios 2010; Munoz *et al.* 2013).

We generated 21 769 diagnostic SNPs to have a higher resolution on the phylogeny of *A. franciscana* individuals (Figure 2B). We were able to determine that the sexual *Artemia* sampled in the Aigues-Mortes saltern are closely related to *A. franciscana* from the SFB than GSL. The clustering of individuals from AM and SFB may have two meanings. First, the introduction of A. franciscana in the Mediterranean saltern is

Figure 2: Artemia phylogeny using SNP. A. General phylogeny using 3762 diagnostic SNPs; **B.** *A. franciscana* phylogeny using 21 769 diagnostic SNPs; **C.** *A. parthenogenetica* phylogeny using 895 diagnostic SNPs. Colors correspond to the different species: *A. salina* in yellow; *A. franciscana* from various origins in red; *A. sinica* in orange, *A. tibetiana* in pink, *A. urmiana* in light pink, *A. kazakhstan* in purple and *A. parthenogenetica* lineages from various origins in green. Boostrap values > 85% are presented on the tree nodes.



dated from the late 1960's (Rode *et al.* 2013). Thus, both populations may have diverged from one another since that moment. Second, A. franciscana from both SFB and GSL were introduced in the AM saltern (Rode *et al.* 2013). The GSL population was introduced later and the level of interbreeding capacity between the GSL and SFB population isn't well known. However, an admixture signal could correspond to this phylogeny. Signal for admixture should be more precisely looked for to distinguish between these two hypotheses.

Parthenogenetic lineages from Mediterranean French and Spanish origins are related to *A.* kazakhstan. Their again, this correlate with previous findings on the origin of asexuality in the *Artemia* genus (Maccari *et al.* 2014). We were only able to generate 895 diagnostic SNPs to have a higher resolution on the phylogeny of *A. parthenogenetica* individuals (Figure 2C). The SNP's loci used to build this phylogeny were not resolute enough to discern between the different parthenogenetic lines.

The development of new SNP markers should help us better follow allele variations throughout core sediments. The invasion of the saltern by *A. franciscana* may have different impact on the local *A. parthenogenetica* population. First, the invasive species may occupy a previously empty niche (Godoy *et al.* 2009). In such case, we do not expect to detect any other variation in *A. parthenogenetica* allele frequencies than mutation/migration and drift. We expect low drift as the Aigues-Mortes population is quite large and diverse (Nougué *et al.* in prep). Second, the invasive species may supersede individuals occupying several niches. Two possible scenarios can occur in such cases: either the superseded clones disappear, involving a reduction in the genetic variation among *A. parthenogenetica* lineages; or they adapt to other environments, involving a character displacement (Brown & Wilson 1956) that can be measured by the acceleration of the variation in allele frequencies of *A. parthenogenetica* with possible detection of selection.

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Supplementary Material

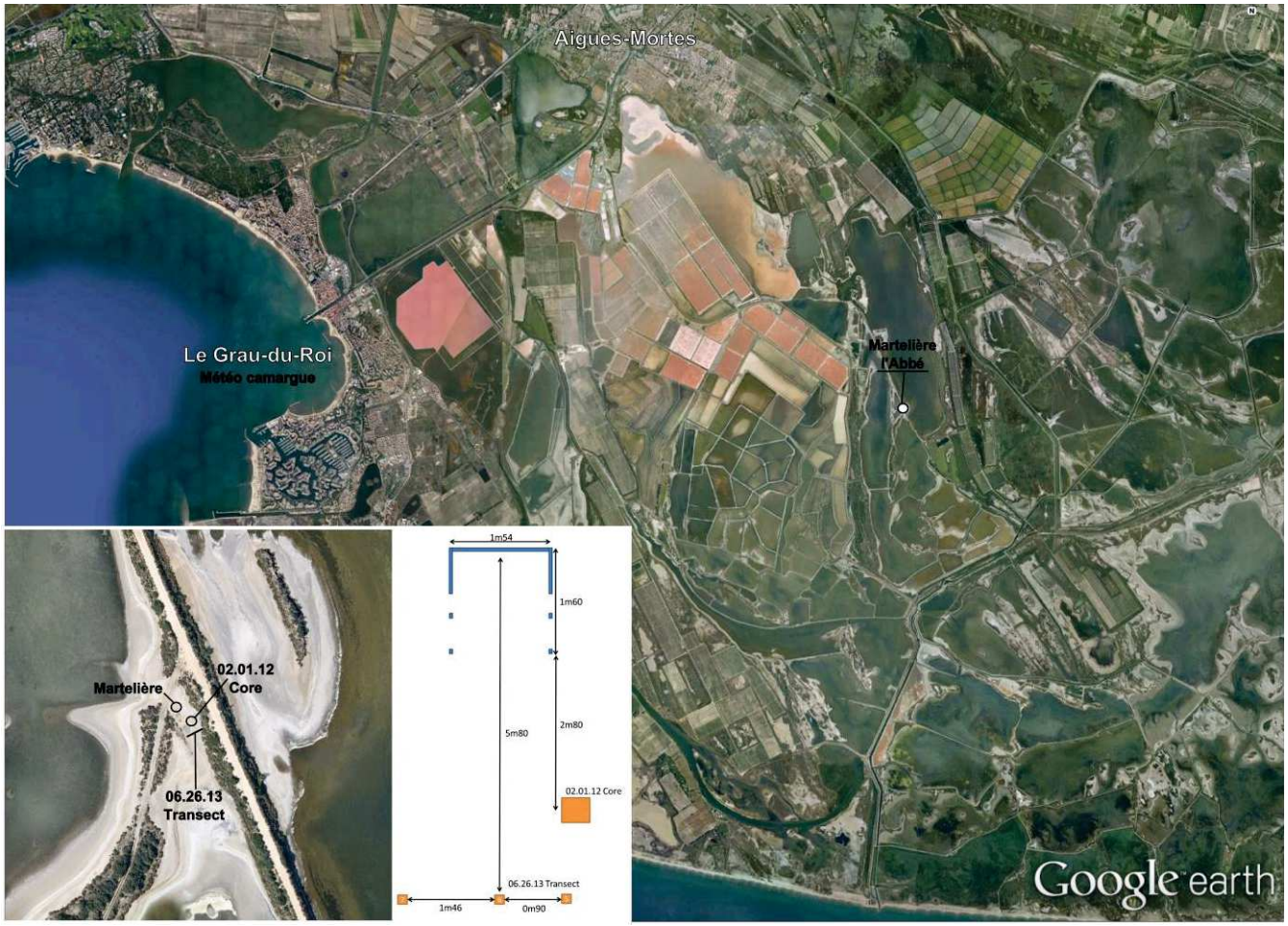

Figure S1: Martelière l'Abbé (43°31'24.72"N, 4°14'12.67"E) position in Aigues-Mortes saltern and relative sampling distance to the martelière.

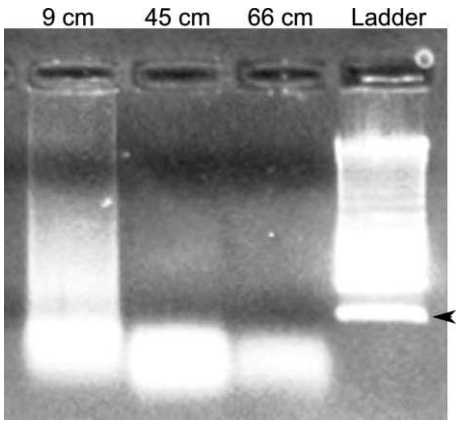

Figure S2 : Migration de l'ADN pour trois horizons sédimentaires. La profondeur pour chaque horizon est indiquée au-dessus du puit correspondant. La flèche sur le ladder indique 100 pb.

Ecologie évolutive des limites de niche : cas de l'adaptation à la salinité de l'artémie
Le concept de niche a été défini par Hutchinson comme un espace multidimensionnel de variables environnementales où l'espèce survie. Au cours de ce travail, je me suis intéressée à différentes limites conceptuelles et opérationnelles du concept de niche. En m'appuyant sur le cas de l'adaptation à la salinité chez le genre *Artemia* – branchiopode extrêmophile – nous nous sommes intéressés : (i) aux mécanismes à l'origine du maintien du polymorphisme génétique d'une large population clonale, qui m'a permis de m'interroger sur l'échelle utile à l'application du concept de niche ; (ii) à l'impact de la flore bactérienne sur l'adaptation de l'artémie aux faibles salinités, qui m'a permis d'évaluer plus globalement l'impact que les interactions biotiques peuvent avoir dans le contexte multidimensionnel de la niche ; (iii) aux effets de la plasticité et de la qualité d'habitat sur l'adaptation de l'artémie aux fortes salinités, qui pose des questions opérationnelles sur l'évaluation de l'influence de ces facteurs sur la niche. Le travail détaillé dans ce manuscrit s'appuie sur des méthodologies variées et a apporté des éléments de réponses aux problématiques posées. Tout d'abord, nous avons pu montrer que la diversité génétique d'une large population clonale était structurée par des déterminants environnementaux tels que la salinité ou la température. Ce travail a aussi montré que dans le cas d'une population asexuée, le concept de niche pouvait s'appliquer à un groupe d'individu génétiquement proche et pouvant (selon le mode de reproduction) appartenir à une lignée commune. Ensuite, nous avons montré que la niche de la flore intestinale de l'artémie facilite la digestion des algues, mais contraint leur tolérance aux faibles salinités. Il faut alors envisager que les interactions biotiques peuvent avoir différents effets (parfois même contradictoires) sur les différents axes de la niche de l'espèce focale. Enfin, nous avons apporté des solutions méthodologiques pour évaluer séparément l'impact de la plasticité et de la qualité d'habitat sur l'adaptation des artémies aux fortes salinités. Au final, nous avons apporté des solutions conceptuelles et/ou opérationnelles permettant de solidifier le concept de niche qui est une notion clé en écologie évolutive.

Mots clés : Génétique des populations clonales, Microbiota, Plasticité phénotypique, Structuration génétique spatio-temporelle, Mutualisme, Courbe de tolérance.

Evolutionary ecology of niche limits: the adaptation to salinity of *Artemia*
Hutchinson defined the niche concept as the multidimensional space of environmental variables where the specie survives. During this work, I focused on several conceptual and operational limits of this concept. Basing our work on the adaptation to salinity of the genus Artemia – an extremophile branchipod – we studied: (i) mechanisms involved in the polymorphism maintenance in a large clonal population, which asked the question of the scaling in the use of the niche concept; (ii) impact of the gut microbiota on the adaptation to low salinities, which asked the question on the impact of biotic interactions on the niche; (iii) the effects of habitat quality and phenotypic plasticity on the tolerance to high salinities, which asked operational questions on the evaluation of theses factors and there impact on the niche. The work detailed in this manuscript is based on a large variety of methodologies and helped providing elements of answers to solve the problematic. First, we showed that the important diversity found in the large clonal population was structured by environmental variables such as salinity and temperature. Therefore, in the case of a large asexual population, the niche concept can apply to a group of genetically close individual that might share (depending on the reproduction mode) common ancestry. Then, we showed that the niche of the gut microbiota, associated with *Artemia* for algae digestion, constrained their host tolerance to low salinities. Thus, biotic interactions may have different effects (even conflicting sometimes) on the different axes of their host niche. Finally, we provided some methodological solutions to evaluate separately the impact of plasticity and habitat quality on the adaptation of *Artemia* to high salinities. In the end, we provided conceptual and/or operational solutions that strengthen the evolutionary ecology key concept of the niche.

Key words: Clonal population genetics, Microbiota, Phenotypic plasticity, Spatio-temporal genetic structure, Mutualism, Tolerance curve.