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Evolution expérimentale de la gynodioécie chez un animal et conflits nucléo-cytoplasmiques

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« Ouvre Excel, tu vas voir. »
Patrice David – Mémoires d'une thèse

INTRODUCTION GENERALE 12

1. DIVERSITE ET EVOLUTION DES SYSTEMES SEXUELS 12
2. COEXISTENCE DE DEUX PHENOTYPES SEXUELS : LE CAS DE STERILITE MALE CYTOPLASMIQUE (SMC) 14
2.1 Trajectoires évolutives d'un conflit nucléo-cytoplasmique : SMC vs restaurateurs 16
2.2 Quelles estimations dans les études empiriques ? 21
3. LE PREMIER CAS DE SMC DECRIT CHEZ UN ANIMAL : *PHYSA ACUTA* 24
3.1 A PROPOS DE *PHYSA ACUTA* 24
3.2 DECOUVERTE D'UN VARIANT MITOCHONDRIAL TRES DIVERGENT ASSOCIE A DE LA STERILITE MALE 26
3.3 CONSEQUENCES DE CETTE DECOUVERTE 29
4. CONFLITS GENOMIQUES CHEZ LES ANIMAUX 29
4.1 DISTORTEURS DE SEXE-RATIO : LES BIAIS MEIOTIQUES 30
4.2 DISTORTEURS DE SEXE-RATIO POST SEGREGATION : LES SYMBIOTES 33
5. ETUDE DU CONFLIT NUCLEO-CYTOPLASMIQUE CHEZ *PHYSA ACUTA* 35

DISCUSSION GENERALE 17

1. INTERPRETATION GLOBALE DES RESULTATS 17
1.1 LA SMC CHEZ LA PHYSE A-T-ELLE DES CARACTERISTIQUES DIFFERENTES DE LA SMC CHEZ LES PLANTES ? 17
1.2 LA PERSISTANCE DU MITOTYPE D : UN PARADOXE 19
2. RETOUR SUR LA MESURE DU PHENOTYPE ET SA BINARISATION 20
3. CONSEQUENCES SUR LE MAINTIEN DU POLYMORPHISME DANS UN CONFLIT GENOMIQUE 22
3.1 DES DYNAMIQUES SIMILAIRES DANS D'AUTRES CONFLITS 22
3.2 LA COURSE AUX ARMEMENTS 22
4. PERSPECTIVES 23
4.1 BOUCLER LA BOUCLE DE L'EVOLUTION EXPERIMENTALE 23
4.2 TROUVER DES GENES DE RESTAURATIONS 24
4.3 PRECISER LES PHENOTYPES ET LES EFFETS SUR LA VALEUR SELECTIVE SELON L'ENVIRONNEMENT 24

Introduction générale

1. Diversité et évolution des systèmes sexuels

Les systèmes sexuels se définissent par la répartition des fonctions femelle et mâle ; chez les animaux comme chez les plantes à fleurs on en distingue deux grands types, les monomorphismes sexuels, composés d'un seul phénotype sexuel et les polymorphismes sexuels, composés d'au moins deux phénotypes sexuels. Grâce à la grande variabilité morphologique des fleurs, les angiospermes présentent une importante diversité de systèmes sexuels (Figure 1).

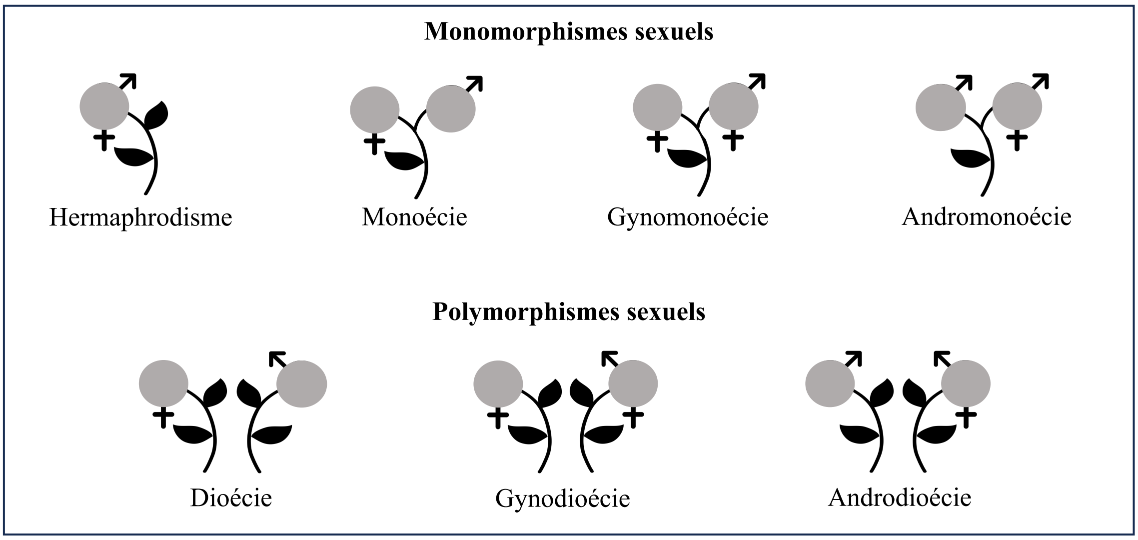


Figure 1 : Résumé non exhaustif des systèmes sexuels observés chez les angiospermes. Parmi ces systèmes, l'hermaphrodisme y est majoritaire et se retrouve dans plus de 75 % des espèces (Yampolsky & Yampolsky, 1922) alors que chez les animaux, la dioécie prédomine.

L'évolution des systèmes sexuels chez les angiospermes est étudiée depuis Darwin (1877) qui, dans son livre « The different forms of flowers », a notamment consacré une grande place à la gynodioécie – polymorphisme sexuel dans lequel les femelles coexistent avec les hermaphrodites. On peut noter que Darwin a porté une attention beaucoup moins importante au cas analogue de l'androdioécie (désignant la coexistence d'individus unisexués mâle avec des hermaphrodites) et ce lié au manque d'observations. Au cours des siècles qui ont suivi les travaux de Darwin, les biologistes de l'évolution ont cherché à comprendre l'origine et le maintien de ces systèmes sexuels (Lewis, 1941; B. Charlesworth & Charlesworth, 1978; Gouyon et al., 1991a; D. Charlesworth, 2006; Weeks, 2006; Renner, 2014). Une partie de ces études ont montré que la gynodioécie et l'androdioécie étaient des voies évolutives conduisant à la dioécie (Bawa, 1980; Barrett, 2010, Figure 2).

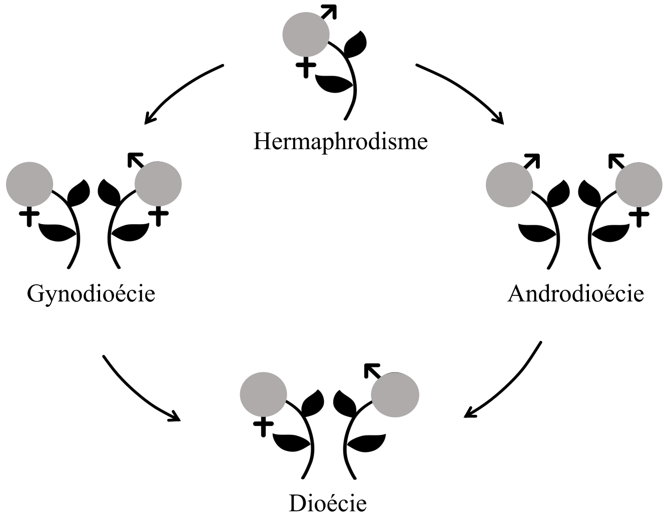


Figure 2 : Diagramme illustrant les transitions évolutives de l'hermaphrodisme vers la dioécie les plus communes (d'après Weeks, 2006). L'évolution de la dioécie *via* l'apparition d'individus unisexués femelles (resp. mâles) nécessite l'invasion de mutants stériles dans des populations monomorphes suivie par une sélection contre la fonction femelle (resp. mâle) chez les individus hermaphrodites (Charlesworth & Charlesworth, 1987; Maurice et al., 1994). L'émergence de la dioécie depuis la gynodioécie a été montré chez différentes espèces de Lamiaceae (Rivkin et al., 2016) mais aussi dans le genre *Silene* (Desfeux et al., 1997). Dans le genre *Acer* (les érables), la dioécie aurait évolué plusieurs fois à partir de l'androdioécie (Gleiser & Verdú, 2005). Une autre voie possible (non représentée ici) vers la dioécie est le passage par la monoécie (e.g. Dorken et al., 2002; Renner et al., 2007; Renner & Won, 2001).

Chez les angiospermes, l'évolution des sexes séparés à partir de l'hermaphrodisme est biaisée avec une forte prévalence d'intermédiaire gynodioïque plutôt qu'androdioïque (Charlesworth, 2006), alors que chez les animaux les patrons de transitions pourraient être différents. Tout d'abord, les transitions s'effectuent dans le sens opposé, c'est-à-dire depuis la dioécie vers l'hermaphrodisme (Eppley & Jesson, 2008). Ensuite, Weeks (2012) montre que les animaux ne présentent qu'une seule transition de l'hermaphrodisme vers la gynodioécie et 16 transitions de l'hermaphrodisme vers l'androdioécie. Notons que l'androdioécie chez les animaux diffère de l'androdioécie chez les plantes car les hermaphrodites ne peuvent pas transmettre de sperme à d'autres individus, seulement s'autoféconder (Weeks, 2012).

De manière générale, la gynodioécie est relativement commune chez les plantes puisqu'elle est retrouvée dans 2 % des genres et un tiers des familles (Dufay et al., 2014), alors qu'elle est extrêmement rare chez les animaux avec une seule espèce identifiée, jusqu'à récemment (2022), comme gynodioïque (*Epiactis prolifera*, une anémone, Dunn, 1975). Cependant, les données de cette étude suggèrent qu'il ne s'agit pas d'un polymorphisme sexuel, mais plutôt d'une modification de l'ontogénèse avec une maturité décalée de la fonction mâle, c'est-à-dire que les jeunes individus sont femelles et deviennent hermaphrodites en vieillissant. L'androdioécie est, elle, très rare chez les plantes (50 espèces d'après Weeks, 2012) comme chez les animaux (115 espèces dont une majorité de Crustacés, Weeks, 2012). La gynodioécie ou l'androdioécie envahiront chez un hermaphrodite si un mutant cause la stérilité d'un des deux sexes et augmente en fréquence. Un tel mutant, s'il est dans un gène nucléaire, sacrifie la moitié de sa valeur sélective et n'envahira donc que s'il compense cette perte en ayant deux fois plus de valeur sélective via la fonction qui lui reste. Or dans ce cas on s'attendrait à plus d'androdioécie que de gynodioécie, car les ovules consomment potentiellement plus d'énergie à produire et entretenir (même après pollinisation) que le pollen : donc économiser les ovules pourrait permettre de doubler la quantité de pollen plus souvent que l'inverse. Dans la partie suivante, nous allons voir que la forte prévalence de la gynodioécie résulte vraisemblablement d'un conflit intragénomique.

2. Coexistence de deux phénotypes sexuels : le cas de Stérilité Mâle Cytoplasmique (SMC)

Dans un système dioïque, les fonctions mâle et femelle sont nécessaires à la reproduction, ce qui crée une fréquence dépendance qui maintient obligatoirement le polymorphisme avec un sexe-ratio maintenu autour 50% en panmixie. Dans un système gynodioïque ou androdioïque, une fonction commune existe entre les deux types d'individus. La coexistence des phénotypes sexuels est donc moins évidente, et il existe des conditions sur la fertilité des phénotypes pour la fonction commune pour que le polymorphisme soit maintenu. Ces conditions ont été formalisées par les travaux de Charnov (1982) et Charlesworth & Charlesworth (1978), mais elles ne sont valables que lorsque le déterminisme du sexe est nucléaire.

Chez les espèces gynodioïques, le phénotype femelle est souvent induit par des gènes cytoplasmiques mitochondriaux (Saumitou-Laprade et al., 1994), appelés gènes de Stérilité

Mâle Cytoplasmique (SMC), car ceux-ci bloquent la production de gamètes mâles (Encadré 1). Ces gènes transmis uniquement par les ovules sont sélectionnés dès que les femelles (nommées aussi mâle-stériles, car résultant évolutivement de la stérilisation des parties mâles d'un hermaphrodite) sont meilleures que les hermaphrodites dans l'accomplissement de la fonction femelle, on parle d'avantage femelle (Lewis, 1941). Cet avantage peut être lié à une réallocation de ressources depuis la fonction mâle vers la fonction femelle ou à l'évitement de la dépression de consanguinité chez les femelles. Théoriquement, l'avantage femelle doit être supérieur à 2 – les femelles doivent produire deux fois de descendant que les hermaphrodites – si le déterminisme est purement nucléaire. En revanche, les modèles théoriques prédisent qu'un avantage femelle entre 1 et 2 est suffisant au maintien du polymorphisme sexuel si le déterminisme de la gynodioécie est nucléo-cytoplasmique (Lewis, 1941), ce qui est le cas chez la plupart des espèces gynodioïques étudiées (Charlesworth & Laporte, 1998).

Contrairement aux gènes mitochondriaux, les gènes nucléaires se transmettent à la fois par la fonction mâle et par la fonction femelle. En présence de SMC ils se retrouvent donc désavantagés car ils ne sont transmis que par la voie maternelle. Ainsi, la SMC induit la sélection de gènes nucléaires restaurant la fertilité mâle, appelés restaurateurs (Encadré 1, e.g. Belhassen et al., 1991; Delph et al., 2007). Dans ce cas, la gynodioécie est le résultat d'un conflit nucléo-cytoplasmique opposant les gènes SMC et les gènes restaurateurs. La SMC a été décrite chez beaucoup d'espèces de plantes – en 1972, 140 espèces issues de 47 genres et 20 familles étaient déjà identifiées (Laser & Lersten, 1972) – mais jusqu'en 2022 elle n'avait jamais été observée chez un animal (David et al., 2022). De multiples gènes SMC coexistent souvent dans les populations naturelles de plantes gynodioïques, comme par exemple chez *Beta vulgaris* (Cuguen et al. 1994), *Thymus vulgaris* (Belhassen et al., 1991), *Silene vulgaris* (Charlesworth & Laporte, 1998) ou chez les plantes cultivées (Kim & Zhang, 2018). Chez certaines plantes, aucun cytotype fertile n'est trouvé (comme chez *Plantago lanceolota*, Haan et al., 1997). Des études ont montré que dans la plupart des cas, un gène restaurateur (*Rf*) est spécifique d'un gène SMC, alors qu'un gène SMC peut être affecté par plusieurs restaurateurs (voir Tableau 1 Chen & Liu, 2014). Par exemple, chez le maïs les gènes *Rf1* et *Rf2* restaurent les individus porteurs du gène *cms-T*, *Rf3* le *cms-S* et *Rf4* le *cms-C* (Snyder & Duvick, 1969; Sisco, 1991; Kamps & Chase, 1997). Les cas où un gène restaurateur restaure plus d'un mitotype SMC sont rares. On trouve un tel exemple chez le riz commun, où *Rf5* et *Rf6* sont également capables de restaurer le mitotype CMS-BT (Huang et al., 2012, 2015; Kim & Zhang, 2018).

Encadré 1 : SMC et restaurateurs nucléaires (adapté de Delph 2007)

SMC

Chez la plupart des plantes étudiées la SMC est causée par des gènes chimériques, formés en associant des segments d'autres gènes mitochondriaux ainsi que de segments d'origine inconnue ; ensemble, ces segments associés forment des cadres de lecture ouverts (ORF) qui sont transcrits et traduits pour produire une nouvelle protéine (Hanson, 1991). De plus, ils sont souvent co-transcrits avec d'autres gènes mitochondriaux (Schnable & Wise, 1998). Dans certains cas, la SMC a été attribuée à des mutations de type "perte de fonction" ; cependant, la plupart des preuves sont cohérentes avec un rôle actif pour le nouvel allèle et ses produits, y compris plusieurs cas dans lesquels la nouvelle protéine semble agir comme une toxine qui interfère avec la respiration mitochondriale (Schnable & Wise, 1998; Wang et al., 2006). On pense que les gènes CMS apparaissent souvent à la suite d'une recombinaison intragénomique (Hanson 1991).

Restaurateurs

On pense que les restaurateurs proviennent des nombreux gènes nucléaires qui contrôlent l'expression des gènes mitochondriaux. Des données chez le pétunia, le radis et le riz suggèrent que les gènes restaurateurs ont été recrutés dans la famille des gènes répétés pentatricopeptides (35 acides aminés) (PPR), qui est impliquée dans le contrôle de l’expression des gènes des organelles (revue par Touzet & Budar, 2004). Les restaurateurs déstabilisent le transcrit produit par le gène CMS en entrant dans la mitochondrie et en modifiant l’ARNm.

2.1 Trajectoires évolutives d’un conflit nucléo-cytoplasmique : SMC vs restaurateurs

2.1.1 Les paramètres

Une abondante littérature s’est intéressée au maintien du polymorphisme nucléo-cytoplasmique chez les plantes (D. Charlesworth, 1981; D. Charlesworth & Charlesworth, 1987; Delannay et al., 1981; Ross & Gregorius, 1985; Frank, 1989; Gouyon et al., 1991; Maurice et al., 1994; Bailey et al., 2003; Dufaÿ et al., 2007; Dornier & Dufay, 2013). Les études théoriques de la gynodioécie nucléo-cytoplasmique considèrent généralement soit un déterminisme asymétrique du sexe avec un cytotype fertile et un cytotype stérile, soit un déterminisme symétrique avec deux cytotypes stériles (Figure 3). Quelques modèles, plus rares (Ehlers et al., 2005; Bailey & Delph, 2007), ont considéré un déterminisme quantitatif du sexe (Encadré 2). Lorsque le déterminisme est symétrique ou asymétrique, trois paramètres sont modélisés : 1) l’avantage femelle (défini dans le précédent paragraphe), c'est à dire l'augmentation de la valeur sélective femelle liée au fait de n'investir que dans la fonction femelle ; 2) l'effet du cytotype, car pour un même phénotype sexuel, la valeur sélective des phénotypes hermaphrodites à cytotype stérile mais restauré peut être plus faible que celle des hermaphrodites portant un cytotype fertile ; 3) le coût pléiotropique de la restauration. Concernant le coût de la restauration, trois expressions sont possibles : 1) un coût *constitutif* qui s'exprime indépendamment du cytoplasme auquel le restaurateur est associé (Bailey et al. 2003), 2) un coût *silencieux* affectant seulement les individus portant des cytotypes fertiles (Gouyon et al. 1991), 3) un coût *exprimé* affectant uniquement les individus porteurs de cytotypes stériles (Bailey et al. 2003). Ces différents types de coût peuvent se retrouver soit uniquement sur la fonction mâle, car les restaurateurs interviennent au niveau de la production de gamètes mâles, soit uniquement sur la fonction femelle, soit sur les deux.

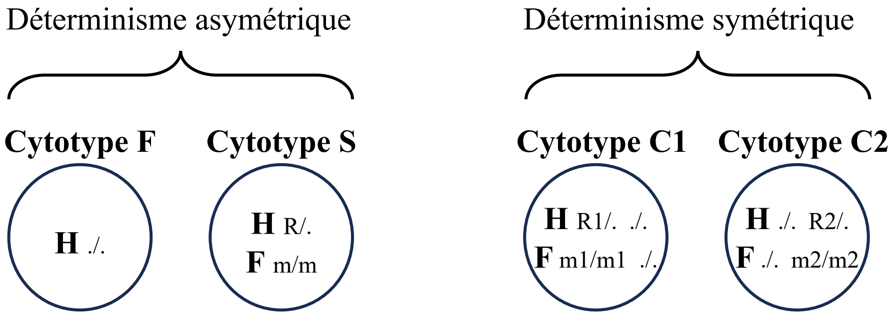
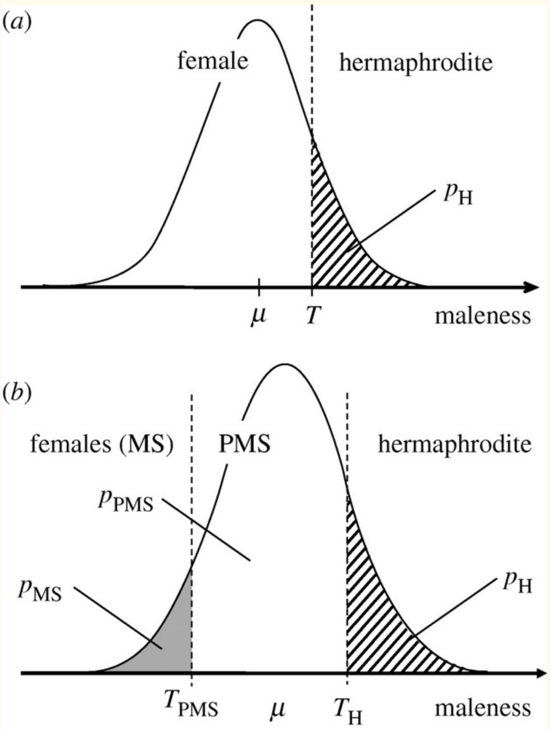


Figure 3 : Les différents types de déterminismes nucléo-cytoplasmique dans le cas d'un restaurateur dominant (d'après Maurice 2012). Déterminisme asymétrique (proposé chez *Beta maritima*) : tous les individus portant le cytotype F sont mâles-fertiles (H, hermaphrodites). Les individus portant le gène cytoplasmique de stérilité S sont mâles-fertiles s'ils portent le gène de restauration (R), on parle alors d'hermaphrodites restaurés, sinon ils sont femelles (F). Déterminisme symétrique (proposé chez *Plantago lanceolata*) : les individus sont mâles-fertiles s'ils portent le gène de restauration spécifique de leur gène cytoplasmique de stérilité. Des hermaphrodites et des femelles sont donc présents dans les deux compartiments cytoplasmiques.

Encadré 2 : Déterminisme quantitatif du sexe : le cas des modèles à seuil.

Dans les modèles à seuil, plusieurs locus ayant des effets additifs sont impliqués dans la restauration d'un seul SMC. L'émergence de ces modèles provient des données sur le thym (*Thymus vulgaris*) ayant montré que chez les femelles, les fleurs varient dans le développement des anthères (Thompson et al., 2002) et que les femelles plus proches morphologiquement des hermaphrodites donnaient un pourcentage plus élevé d'hermaphrodites dans leur descendance. Pour formaliser cette idée, un indice de « *masculinité* » (*maleness*) ou de restauration des individus est défini et dépend de nombreux facteurs additifs ; un seuil dans la valeur de cet indice détermine si l'individu est femelle ou hermaphrodite. Ces modèles permettent de considérer deux cas : un cas où le phénotype est binaire (femelle et hermaphrodite (a)), et un cas impliquant plus de deux phénotypes (femelle, hermaphrodite et individu partiellement mâle-stérile (b)).



Représentation graphique du modèle à seuil (d'après Ehlers et al. 2005). (a) Un modèle à seuil à deux morphes avec des femelles et des hermaphrodites. Un seul seuil *T* (ligne pointillée) est utilisé. (b) Un modèle à seuil à trois morphes avec des femelles, des hermaphrodites partiellement mâle-stériles (PMS) et des hermaphrodites. Deux seuils, T_{PMS} et T_H, sont utilisés.

2.1.2 Les prédictions

Il existe deux grandes classes de modèles s'intéressant au maintien du polymorphisme. Dans la première, seule la sélection est considérée pour maintenir le polymorphisme dans une population panmictique de taille infinie (e.g. Gouyon et al. 1991, Dufay et al. 2007) ; dans la seconde, la dérive et/ou la migration s'ajoute(nt) pour maintenir le polymorphisme dans une population structurée (e.g Couvet et al., 1998; Charlesworth, 2002).

Dans le cas où seule la sélection opère, la SMC est sélectionnée grâce à l'avantage femelle. Lorsque la SMC augmente en fréquence dans la population, les restaurateurs sont alors sélectionnés car ils ont un avantage sélectif qui est la production de grain de pollen. Une fois que les restaurateurs deviennent fréquents, la fréquence de femelle diminue et les cytotypes SMC sont principalement portés par des hermaphrodites restaurés. Si les hermaphrodites non restaurés ont une valeur sélective femelle plus importante que celle des individus porteurs de SMC, alors la fréquence de la SMC diminue (à cause du coût du cytotype). Enfin, les individus porteurs de restaurateurs subissent un coût silencieux de la restauration, ce qui entraine la contre-sélection des restaurateurs et les fait diminuer en fréquence. Les SMC qui sont devenues rares seront de nouveaux sélectionnées et vont augmenter en fréquence. Ce type de dynamique entraine des oscillations des fréquences de la SMC et des restaurateurs, que l'on appelle cycles limites (Gouyon et al. 1991, Bailey et al. 2003, Dufay et al. 2007). Ces cycles sont permis par une sélection fréquence-dépendante négative (Figure 4). Les modèles prédisent que les dynamiques cycliques peuvent être permanentes (Gouyon et al. 1991) ou lentement amorties vers un équilibre polymorphe (e.g. Charlesworth 1981, Delannay et al. 1981, Dufay et al. 2007) et que l'amplitude des cycles dépend à la fois du coût de la restauration et de l'avantage femelle. Dufay et al. (2007) montrent que si l'avantage femelle est relativement élevé par rapport au coût de la CMS, alors la CMS envahit la population et le polymorphisme au locus du restaurateur est maintenu, conduisant à une gynodioécie nucléaire.

Dans les modèles, différentes hypothèses ont été explorées pour la dominance de l'allèle restaurateur, la dominance du coût lui-même, le mode de coût (silencieux, exprimé ou constitutif) et l'incidence du coût sur la production de graines et/ou de pollen. Le fait que le coût de restauration soit dominant ou récessif a un impact relativement important sur la fréquence des femelles, ainsi que sur le maintien de la gynodioécie nucléo-cytoplasmique (Bailey et al. 2003). Les coûts dominants permettent d'obtenir les fréquences les plus élevées de femelles, mais les coûts récessifs permettent de maintenir les femelles lorsque la compensation et les coûts sont extrêmement faibles (Bailey et al. 2003). La gynodioécie nucléaire-cytoplasmique est stable sur un plus grand espace de paramètres lorsque les coûts des restaurateurs sont constitutifs (c'est-à-dire qu'ils affectent à la fois les individus CMS et non-CMS) par rapport à ceux qui n'ont que des coûts exprimés ou silencieux. Dufay et al. (2007) prédisent même que la gynodioécie nucléo-cytoplasmique n'évolue pas lorsque le coût est exprimé.

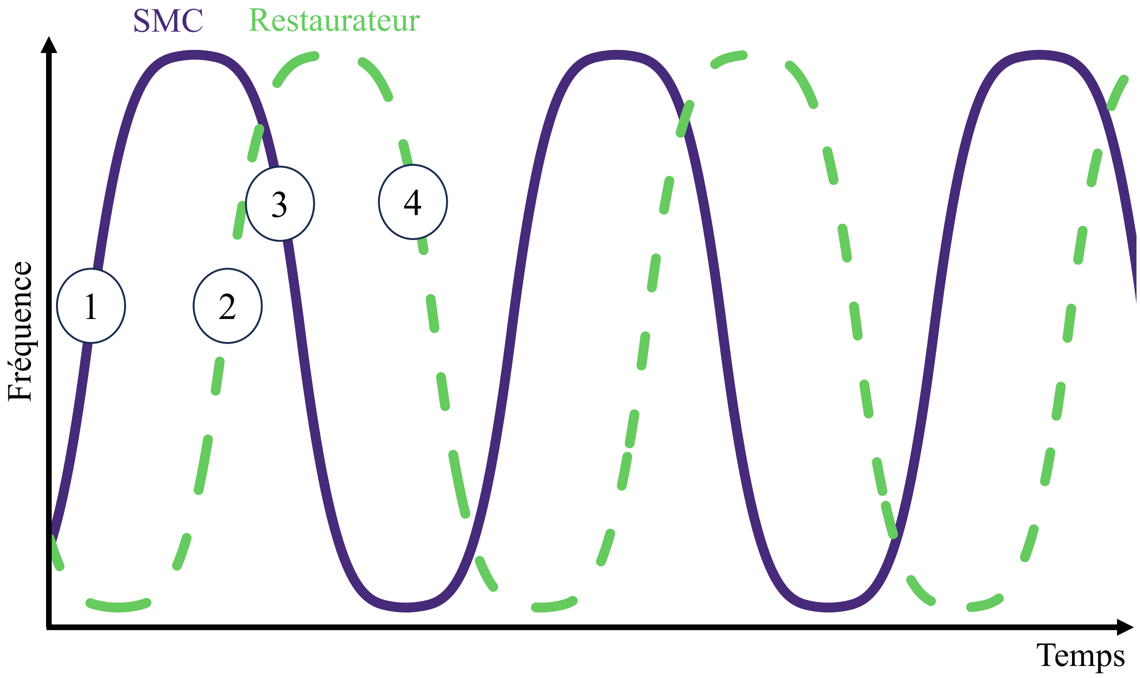


Figure 4 : Illustration de la dynamique de la SMC et des restaurateurs dans les cycles limite. 1) la SMC est sélectionnée grâce à l'avantage femelle, 2) le restaurateur, rare dans la population, augmente alors en fréquence – les individus porteurs de restauration produisent du pollen et ont donc un avantage sur la valeur sélective mâle des individus SMC, 3) l'effet de la SMC fait diminuer sa fréquence et 4) le coût silencieux de la restauration entraine une diminution de la fréquence des restaurateurs. Dans l'ensemble, l'avantage de la femelle, le coût de la restauration et le coût (ou effet) des gènes SMC permettent théoriquement à la sélection fréquence-dépendante de maintenir le polymorphisme nucléo-cytoplasmique, souvent par le biais d'importantes oscillations du sexe-ratio dans le temps, les gènes SMC étant sélectionnés lorsque les allèles des restaurateurs sont rares et les restaurateurs étant sélectionnés lorsque les gènes SMC sont fréquents.

Dans la deuxième classe de modèles, on retrouve une dynamique similaire aux premières phases décrites dans le paragraphe précédent (phases 1 et 2 de la Figure 4). Cependant, avec deux facteurs CMS et des allèles restaurateurs dominants spécifiques, Couvet et al. (1998) prédisent l'évolution d'un polymorphisme nucléo-cytoplasmique en métapopulation (population interconnectées) lorsque le flux de gènes entre populations est limité et que les dèmes s'éteignent localement – le polymorphisme est maintenu en l'absence de coût de la restauration. Dufay & Pannell (2010) montrent que la combinaison de la dérive et de la sélection entraîne la perte du polymorphisme sexuel dans des scénarios qui le maintiendraient sous l'influence de la seule sélection. De plus, bien que le flux de pollen n'empêche pas la perte du polymorphisme cytoplasmique au sein des dèmes, il permet le maintien du polymorphisme nucléo-cytoplasmique au niveau de la métapopulation. Au sein d'un dème, la dérive peut entrainer la perte de la gynodioécie nucléo-cytoplasmique en fixant certains gènes (Frank 1989). En revanche, au sein de la métapopulation, la migration entre les dèmes restaure le polymorphisme nucléo-cytoplasmique perdu localement (Frank 1989).

Les patrons prédits par les deux classes de modèles ont des conséquences différentes sur la diversité cytoplasmique. Le polymorphisme nucléo-cytoplasmique est stable dans le cas d'une sélection fréquence-dépendante négative et entraine une importante diversité des génomes cytoplasmiques (Touzet & Delph, 2009). Par la suite, on se focalisera sur les prédictions faites dans les modèles de fréquence-dépendance négative.

2.2 Quelles estimations dans les études empiriques ?

L'avantage femelle, le coût silencieux de la restauration, le coût des gènes SMC sont des paramètres importants influençant le succès reproducteur des individus dans les populations gynodioïques. Connaître les valeurs sélectives relatives de chacun des génotypes est une question centrale pour comprendre le maintien du polymorphisme sexuel associé à la gynodioécie nucléo-cytoplasmique. De nombreuses études empiriques ont donc cherché à estimer les paramètres définis précédemment mais également les fréquences de femelles et le déterminisme génétique des restaurateurs dans les populations naturelles. Ces estimations ne constituent cependant pas un test direct des dynamiques prédites par les modèles (Figure 4).

2.2.1 Fréquence de femelles dans les populations

Le sexe-ratio chez les espèces gynodioïques présente généralement de grandes variations d'une population à l'autre comme par exemple chez *Plantago coronopus* (Koelewijn & Van Damme, 1995), *Beta maritima* (Cuguen et al.,1994), *Plantago lanceolata* (Haan et al., 1997), *Raphanus sativus* (Murayama et al., 2004), *Daphne laureola* (Medrano et al., 2005), *Nemophila menziesii* (Barr, 2008). Ces variations sont parfois considérées comme une conséquence de la sélection fréquence dépendante. Les populations se trouveraient à différentes phases du cycle décrit précédemment (Figure 4), d'où ces variations (Gouyon et al. 1991). Cependant, il s'agit d'une vision très hypothétique de la dynamique de la gynodioécie nucléo-cytoplasmique qu'il est difficile de tester expérimentalement.

2.2.2 Déterminisme génétique de la restauration

Les modèles théoriques supposent généralement que la restauration est sous le contrôle d'un unique allèle (mais voir Bailey & Delph, 2007). Bien que cela semble être le cas chez les espèces cultivées (voir Table 2 de Delph et al. 2007 pour une revue), les études menées chez les d'autres espèces suggèrent un déterminisme génétique plus complexe. En effet, de nombreuses espèces gynodioïques présentent des phénotypes sexuels intermédiaires (comme définis dans les modèles seuils, voir Encadré 2), soit (i) en produisant des fleurs avec des anthères non déhiscentes, moins nombreuses ou en produisant une quantité ou une qualité moindre de pollen (par exemple, Koelewijn & Van Damme, 1996; Poot, 1997; Dufay et al., 2008), soit (ii) en portant un mélange de fleurs femelles et hermaphrodites (individus gynomonoïques, par exemple López-Villavicencio et al., 2005; Dufay et al., 2010). Cette variation intra-individuelle a été proposée comme étant le résultat d'une restauration polygénique (Koelewijn & van Damme, 1996 ; Ehlers et al., 2005). Dans le cas d'une détermination polygénique de la restauration, les individus porteurs de SMC peuvent rarement être entièrement restaurés et les valeurs sélectives mâles varient parmi les hermaphrodites restaurés (par exemple Dufay et al., 2008).

2.2.3 Avantage femelle

L'Avantage Femelle (AF) peut résulter de deux mécanismes non exclusifs : (i) la réallocation de ressource depuis la fonction mâle vers la fonction femelle permet aux individus mâle-stérile de produire plus de graines que les hermaphrodites (par exemple Avila-Sakar & Domínguez, 2000), (ii) les femelles ne pouvant pas s'autoféconder, elles évitent la dépression de consanguinité et produisent, en moyenne, des graines de meilleure qualité que les hermaphrodites qui s'autofécondent (par exemple Chang 2007). La méta-analyse de Shykoff et al. (2003) a mis en évidence que l'AF se retrouvait sur plusieurs traits : une production accrue de fleurs, de fruits et de graines chez les femelles mais aussi la production de graines ayant de meilleurs taux de germination que celles des hermaphrodites. Par la suite, Dufay & Billard (2012) ont montré une grande variation dans la valeur de l'AF allant de <1 à >30, avec cependant une grande majorité d'espèces ayant un AF compris entre 1 et 2, ce qui correspond aux attendus théoriques dans le cadre d'une gynodioécie nucléo-cytoplasmique (voir Section 2).

2.2.4 Effet du cytotype

Dans l'avant dernière phase du cycle (Figure 4, phase 3), une diminution de la fréquence des SMC est prédite si la valeur sélective femelle du cytotype non-CMS était plus élevée que celle du cytotype SMC. Des études moléculaires de la CMS chez les espèces cultivées montrent que la plupart des gènes CMS apparaissent *de novo* (Encadré 1) et que leur expression est souvent tissu-spécifique (Budar et al., 2003; Chase, 2007). On peut donc s'attendre à un coût lié à l'expression constitutive du gène CMS. Cet effet cytotype a été suggéré chez *Plantago lanceolata* (van Damme, 1984) et chez *Silene vulgaris* (Mccauley & Olson, 2003) où la valeur sélective femelle était mesurée en nombre de graines et/ou en poids par graine. Cependant, ces études ne s'appuyaient pas sur des marqueurs spécifiques de la CMS permettant de clairement distinguer les hermaphrodites restaurés des hermaphrodites non-restaurés.

2.2.5 Coût de la restauration

Dans la dernière phase du cycle limite (Figure 4, phase 4), les individus ayant à la fois un mitotype non-CMS et un allèle restaurateur expriment un coût silencieux de la restauration, et les restaurateurs devraient donc être contre-sélectionnés. Ce coût silencieux est nécessaire pour maintenir la gynodioécie nucléaire-cytoplasmique (Dufay et al. 2007) et il est largement trouvé par les espèces cultivées (voir Tableau 2 Delph 2007 pour une revue). Chez les espèces non cultivées, seules quelques études ont empiriquement observé des effets négatifs de la restauration (e.g. Koelewijn, 1996). Ces études suggèrent que le coût de la restauration peut prendre une multitude de formes en impactant une grande diversité de traits associés à la survie des individus et aux capacités reproductives mâles et/ou femelles, et ce, à différents stades de vie de la plante. Par exemple, Del Castillo et al. (2009) montrent que les gènes restaurateurs affectent la survie et la viabilité des graines chez *Phacelia dubia* ; de Haan et al. (1997) trouvent également un effet négatif de la restauration sur la fonction femelle chez *Plantago lanceolata*. Un coût sur la fonction mâle des individus non-CMS est observé chez *Lobelia siphilitica* (Bailey 2002) et *Beta vulgaris spp. maritima* (Dufay et al. 2008, De Cauwer et al. 2011). Cet ensemble d'exemples et développements théoriques laissent penser que l'évolution de SMC devrait être commune chez l'ensemble des organismes disposant d'organelle cytoplasmique (les Eucaryotes) et d'anisogamie (avec une hérédité asymétrique des organelles entre les deux types de gamètes). Pourtant, bien que les animaux semblent correspondre à ces deux critères, tous les cas connus historiquement sont exclusivement chez les plantes. Dans la partie suivante, nous nous intéresserons au premier cas découvert chez les animaux, le cas de l'escargot d'eau douce *Physa acuta* qui fait l'objet de ma thèse.

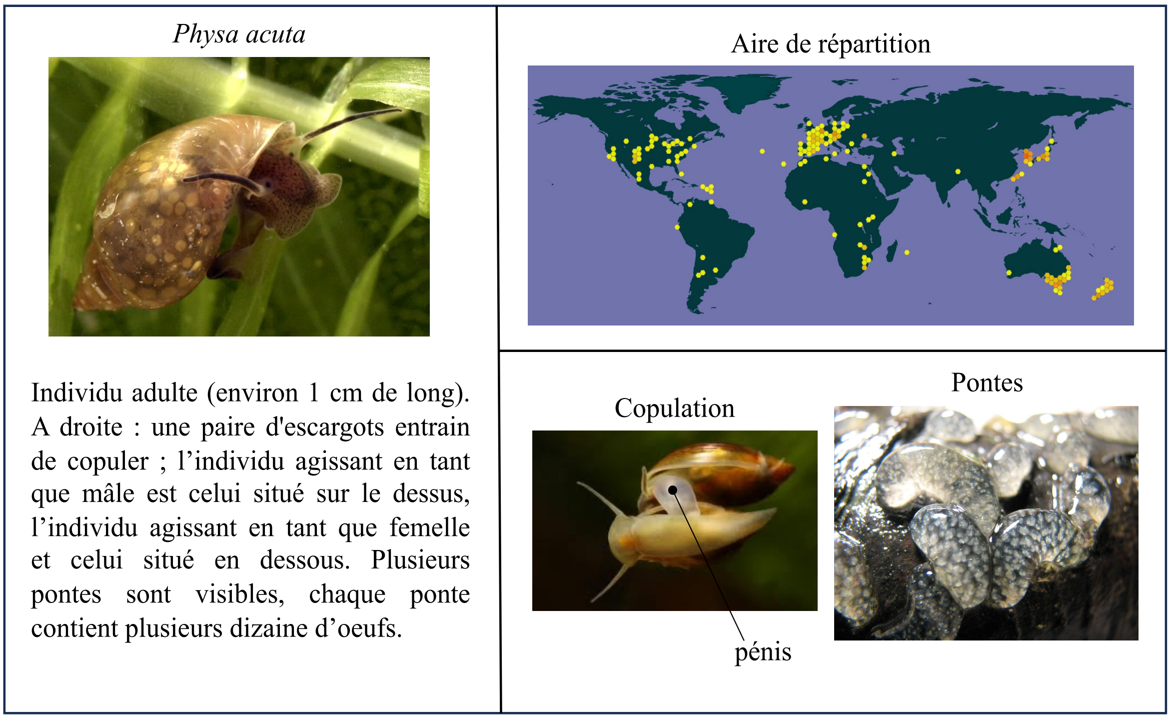
3. Le premier cas de SMC décrit chez un animal : *Physa acuta*

Comme illustré par les paragraphes précédents, la gynodioécie nucléo-cytoplasmique est bien connue chez les plantes. Étant donné les mécanismes communs de l'hérédité mitochondriale et nucléaire, on peut se demander si ce conflit nucléo-cytoplasmique se retrouve chez les animaux. En excluant les insectes, un tiers des espèces animales sont hermaphrodites (Jarne & Auld, 2006). Malgré cela, aucun cas de SMC n'a été trouvé jusqu'à récemment (David et al. 2022), et la gynodioécie reste extrêmement rare (Weeks 2012). Une explication possible à cette rareté serait que les génomes mitochondriaux chez les animaux sont plus compacts et structurellement stables, limitant ainsi l'émergence de variants mâle-stériles (Boore, 1999; Galtier, 2011). Une autre explication est le manque de détectabilité ; les gonades des animaux étant internes, il est difficile d'identifier des individus mâle-stériles. À ce jour, un seul animal présentant une stérilité mâle cytoplasmique a été identifié : *Physa acuta*, un escargot d'eau douce (David et al. 2022).

3.1 A propos de *Physa acuta*

P. acuta (Figure 5) est un escargot d'eau douce hermaphrodite de la famille des Physidae (Hygrophila, Gastropoda) (Wethington & Lydeard, 2007; Jarne et al., 2010). Cette espèce, connue sous différents noms (voir Dillon et al., 2002), est l'espèce d'Hygrophila qui présente la plus grande aire de répartition en raison d'une expansion récente (Figure 5). Elle est originaire de l'est des Montagnes Rocheuses en Amérique du Nord, et a envahi tous les continents à l'exception de l'Antarctique depuis le XVIII^{e} siècle (voir Bousset et al., 2014; Vinarski, 2017; Ebbs et al., 2018). Elle occupe une large gamme d'habitats et supporte bien les habitats anthropisés. Un aspect important est qu'elle peut être maintenue et élevée très facilement dans des conditions de laboratoire assez simples. Le délai entre la ponte et l'éclosion des œufs est d'environ une semaine à 25°C, et la maturité sexuelle est atteinte 5 à 6 semaines après l'éclosion des œufs.

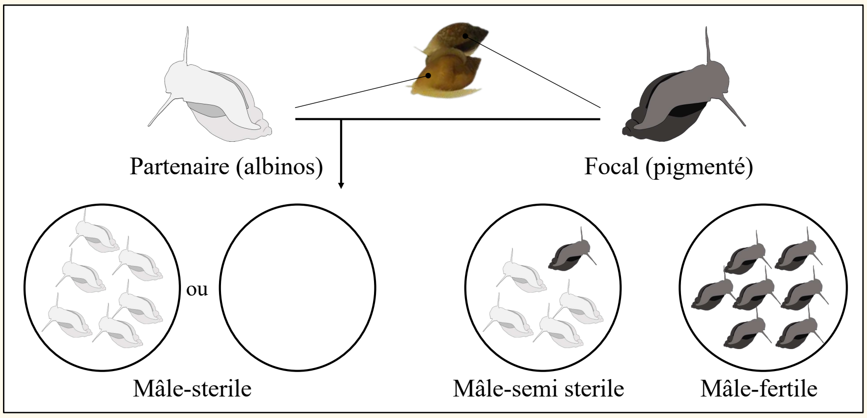
La physe recourt à l'autofécondation lorsqu'elle ne trouve pas de partenaire disponible, aussi bien dans les populations naturelles qu'en conditions de laboratoire (Jarne et al., 2000). Des expériences avec des marqueurs génétiques ont montré que l'appariement d'individus sexuellement matures aboutit en fait à 100% d'allofécondation (Pélissié et al., 2012; Wethington & Dillon, 1996; Janicke et al., 2014). L'allosperme peut être stocké et utilisé par un individu pour fertiliser ses œufs jusqu'à plusieurs semaines. La copulation est unilatérale chez *P. acuta*, un individu joue le rôle de mâle tandis que l'autre joue le rôle de femelle (le gonopore et le phallus sont situés à gauche chez les espèces à coquille senestre comme *P. acuta*, Figure 5). La physe pond des capsules gélatineuses pouvant contenir jusqu'à quelques dizaines d'œufs (Figure 5). Grâce à une souche albinos isolée et élevée au laboratoire (Noël et al., 2016), nous testons facilement la fertilité mâle chez cette espèce (Encadré 3).



Individu adulte (environ 1 cm de long). A droite : une paire d'escargots entrain de copuler ; l'individu agissant en tant que mâle est celui situé sur le dessus, l'individu agissant en tant que femelle et celui situé en dessous. Plusieurs pontes sont visibles, chaque ponte contient plusieurs dizaine d'oeufs.

Figure 5 : Illustration de la biologie de *P. acuta*. Sources images : GBIF (Global Biodiversity Information Facility); AquaPortail.

Encadré 3 : Comment tester la fertilité mâle chez *P. acuta*



Un individu focal pigmenté est croisé avec un partenaire albinos. La fertilité mâle du focal est évaluée à partir de la descendance de l'albinos : (i) pas d'œufs ou uniquement des descendants albinos, alors le focal est mâle-stérile, (ii) une majorité d'albinos et quelques pigmentés, alors le focal est mâle-semi stérile, (iii) de nombreux individus pigmentés, alors le focal est mâle-fertile. Ce sont les observations faites sur premiers croisements qui nous ont conduit à classer les phénotypes sexuels de cette manière. Par la suite il nous est apparu que la distribution du phénotype sexuel mâle n'était pas réellement binaire comme suggéré ici (voir Chapitre 2).

3.2 Découverte d'un variant mitochondrial très divergent associé à de la stérilité mâle

Très récemment, lors d'une étude du COI – un gène mitochondrial couramment utilisé en phylogéographie – David et al. (2022) ont découvert un ADN mitochondrial très divergent des séquences de physe déjà connues (appelées N pour « normal »). Cet ADN mitochondrial ou mitotype, nommé D pour « divergent », a été trouvé dans une population près de Lyon (France). L'étude menée par David et al. (2022) montre que la divergence n'affecte pas uniquement le COI mais tous les gènes mitochondriaux (Figure 6). L'analyse du comportement sexuel révèle que les individus porteurs du mitotype D ont un comportement mâle réduit (Figure 7) et que les valeurs sélectives mâles des individus D sont quasiment nulles (Figure 8). Les individus D ne parviennent pas à féconder leur partenaire car ils ne produisent pas de spermatozoïdes (Figure 8), le mitotype D est donc associé à de la stérilité mâle. Aucune différence de comportement ou de valeur sélective femelle n'a été observée entre les N et les D.

Le mitotype D chez *P. acuta* est le premier exemple de SMC chez les animaux hermaphrodites. Ce mitotype est associé à une divergence intraspécifique extrême due à un taux de mutation élevé et à des réarrangements structurels (David et al. 2022). Chez les plantes, des taux de substitution élevés peuvent être la trace d'une course à l'armement dans laquelle les mitotypes mâles-stériles doivent suivre l'évolution constante des gènes nucléaires rétablissant la fertilité mâle (Touzet & Budar, 2004; Touzet & H. Meyer, 2014). La divergence du mitotype D pourrait donc être liée à la présence de restaurateurs nucléaires ; cependant ils n'ont pas été mis en évidence par cette étude.

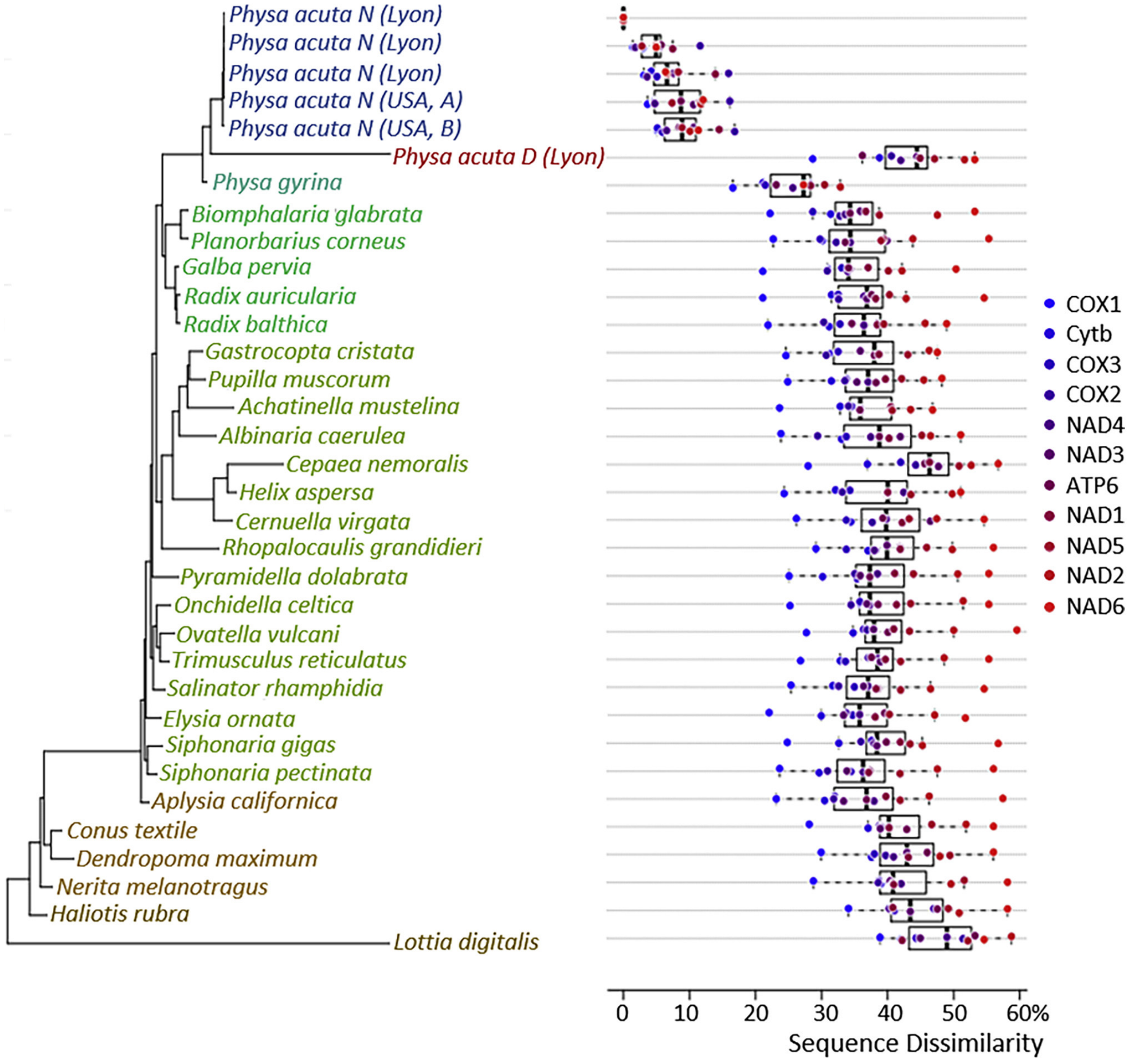


Figure 6 : Phylogénie basée sur les séquences mitochondriales complètes codant pour les protéines (issu de David et al. 2022). La distance nucléotidique avec le premier individu de *P. acuta* (mitotype N), pris comme référence, est indiquée pour 11 gènes codant pour des protéines mitochondriales (avec la médiane et les quartiles). *Lottia digitalis* est utilisé comme groupe externe.

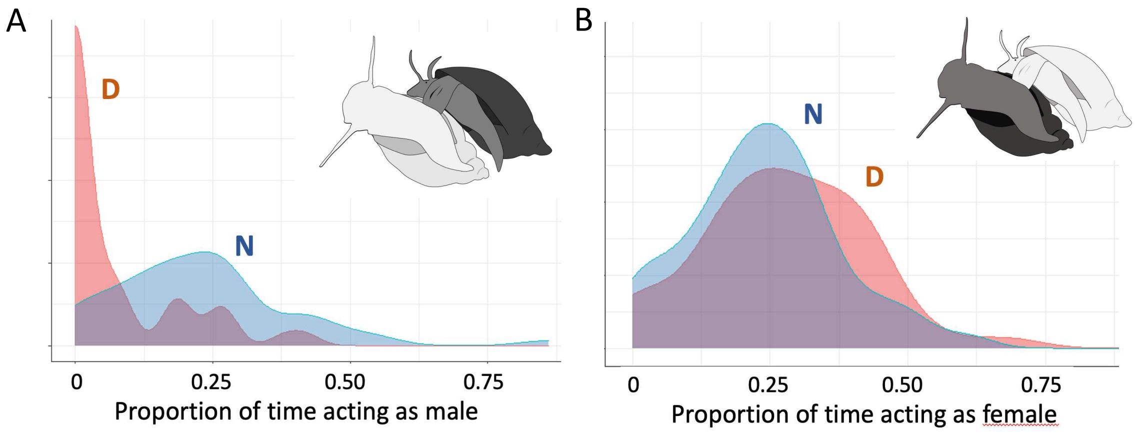


Figure 7 : Distribution du temps passé en comportement mâle (A) et femelle (B) par les individus D et N (issu de David et al. 2022). Les interactions sexuelles ont été enregistrées chronométrées pendant des sessions de 45 minutes. Chaque individu focal est associé à un partenaire vierge ; les dessins illustrent les positions de l'individu focal (en foncé) et de son partenaire (en clair), vus de dessous.

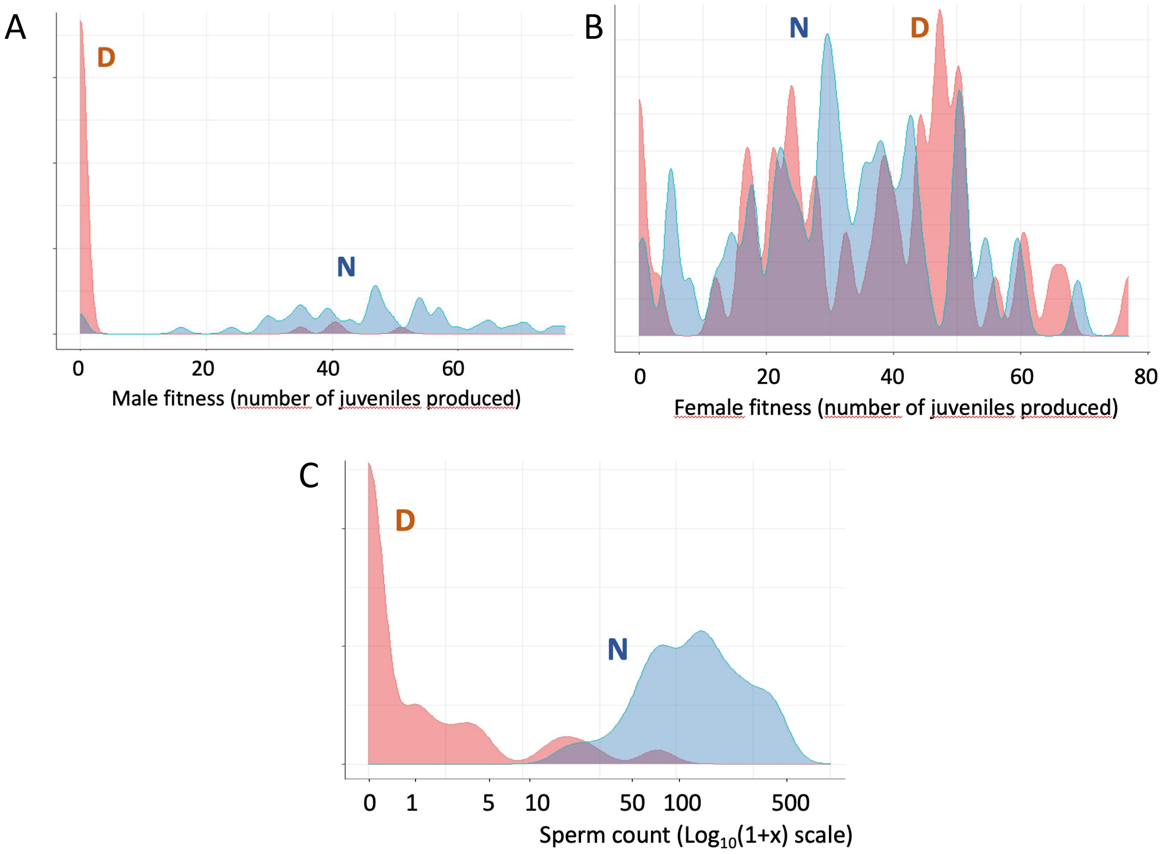


Figure 8 : Estimation des valeurs sélectives mâle (A) et femelle (B) ainsi que du nombre de spermatozoïdes produits (C) par les individus N et D (issu de David et al. 2022). La

valeur sélective mâle est comptée dans la descendance pondue par le partenaire et correspond au nombre de juvéniles engendrés par l'individu focal. La valeur sélective femelle est le nombre d'œufs pondus par le focal. Les spermatozoïdes sont comptés après dissection de la vésicule séminale.

3.3 Conséquences de cette découverte

Suite à la découverte de ce nouveau mitotype, la diversité mitotypique est explorée hors de la population lyonnaise d'origine. Cette exploration se fait au moyen de PCR réalisées sur le COI. Avec ce test, un profil atypique ne correspondant ni à N ni à D, est mis en évidence et le séquençage révèle qu'il s'agit d'un nouveau mitogénome que nous appelons « K ». Des études préliminaires semblent alors montrer que ce mitotype K est différent de D et de N, renforçant l'hypothèse discutée précédemment d'un conflit nucléo-cytoplasmique entre SMC et restaurateurs nucléaires.

4. Conflits génomiques chez les animaux

Comme nous l'avons vu avec l'exemple de la SMC, les modes de transmission entre les gènes mitochondriaux et nucléaires diffèrent, ce qui impacte la valeur sélective d'un organisme. En effet, une conséquence majeure de la transmission maternelle des mitochondries est que les mutations qui sont délétères pour la fonction mâle peuvent atteindre des fréquences élevées si elles sont neutres (ou avantageuses, ou même légèrement délétères) chez les femelles (Burt & Trivers, 2006, voir Encadré 4 sur la malédiction de la mère). Plus généralement, les conflits génomiques sont liés à l'apparition d'éléments génétiques égoïstes employant diverses stratégies pour maximiser leur transmission à la génération suivante. Il existe de nombreuses formes d'éléments génétiques égoïstes, souvent classées en fonction de leur mode d'action (Tableau 1). Seuls certains d'entre eux seront développés ici.

Tableau 1 : Listes non-exhaustives des différentes classes d'éléments génétiques égoïstes (d'après Price & Wedell, 2008).

Classes	Groupes	Exemple
Réplicateurs autonomes	Eléments transposables	DNA transposons
Elément de conversion	Conversion génique biaisée	Homing endonucléase
Distorteurs de ségrégation	Biais méiotiques	Biais de Sexe Ratio, chromosomes B, complexe *t*
Distorteurs post ségrégation	Endosymbiontes	*Wolbachia*

Encadré 4 : Malédiction de la mère

Physa acuta est le premier exemple de stérilité mâle cytoplasmique complète chez les animaux, cependant des conflits nucléo-cytoplasmiques sur la reproduction et médiés par les mitochondries sont déjà connus. En effet, le même raisonnement, basé sur les différents modes d'hérédité des gènes mitochondriaux et nucléaires, prédit que des variations mitochondriales n'entraînant pas de coûts sur la fonction femelle ou augmentant sa valeur sélective peuvent se propager dans une population, et ce même si ces variations sont délétères pour la fonction mâle. On parle de malédiction de la mère (« *mother's curse* », Camus et al., 2022). Des mutations mitochondriales individuelles entraînant des altérations de la fonction mâle ont été montrées chez diverses espèces comme l'humain (Wallace, 1989), les mouches des fruits (Clancy et al., 2011), ou encore les souris (Nakada et al., 2006). Des études menées chez *Drosophila melanogaster* ont mis en évidence des mutations ayant des effets antagonistes sur le sexe – elles indiquent que les mutations mitochondriales ayant des effets négatifs sur la fonction mâle peuvent s'être répandues en raison d'effets bénéfiques sur la fonction femelle (Camus et al., 2015; Camus & Dowling, 2018). L'évolution de gènes compensatoires dans le noyau des espèces gonochores, atténue les effets des mutations mitochondriales nuisibles aux mâles ; ce processus se manifeste sous la forme d'une réduction de valeur sélective des mâles lorsque l'ADN mitochondrial rencontre un fond nucléaire incompatible (Innocenti et al., 2011; Patel et al., 2016; Perlman et al., 2015; Camus & Dowling, 2018).

4.1 Distorteurs de sexe-ratio : les biais méiotiques

Les biais méiotiques (« *meiotic drive* ») sont des éléments génétiques égoïstes qui manipulent la méiose ou ses produits pour se propager dans les populations (Jaenike, 2001).

Ils peuvent être des tueurs de gamètes (« *gamete killers* »), des tueurs d'autosome (« *autosomal killers* ») éliminant les gamètes porteurs de l'allèle alternatif, ou encore des biais méiotiques femelle (« *female meiotic drives* ») utilisant la division cellulaire asymétrique de la méiose chez les femelles pour influencer quelle paire de chromosomes homologues est distribuée dans le noyau plutôt que dans les corps polaires (Price et al., 2020). Il existe moins d'exemples de biais méiotiques tueurs d'autosomes que de tueurs de gamètes. L'une des raisons expliquant cette différence est que les biais X (ou Y) entraînent une distorsion du sexe-ratio dans la descendance, les rendant facilement détectables contrairement aux biais autosomiques. Les biais tueurs d'autosomes les plus connus se retrouvent chez différentes espèces de souris avec l'haplotype *t* et chez *Drosophila melanogaster* avec le distorteur de ségrégation (Burt & Trivers 2006). De même que pour la CMS et les restaurateurs, Charlesworth & Hartl (1978) prédisent que chez *D. melanogaster*, le distorteur de ségrégation et la résistance associée suivent une dynamique cyclique.

Je choisis de m'attarder ici sur les tueurs de gamètes liés aux chromosomes sexuels en raison de leur effet sur les sexe-ratios et de l'asymétrie de transmission entre sexes (le X transmis principalement par les femelles, le Y uniquement par les mâles, les autosomes également par les deux sexes), deux caractéristiques qui engendrent des dynamiques évolutives similaires à celles des systèmes CMS-restaurateurs. Ces biais méiotiques provoquent une distorsion dans la transmission du sexe hétérogamétique (L. D. Hurst & Pomiankowski, 1991) en détruisant ou dégradant les gamètes qui ne portent pas l'élément génétique égoïste distorteur de sexe-ratio (appelé SR pour Sexe-Ratio). L'évolution de ces biais est favorisée par la différenciation entre les chromosomes X et Y (et Z/W)

4.1.1 Biais liés au chromosome X (« *X-drive* »)

Les biais méiotiques liés au chromosome X favorisent leur transmission au détriment du chromosome Y chez les mâles hétérogamétiques produisant ainsi un excès de femelles dans leur descendance. L'existence de tels biais est bien connue chez différentes espèces de *Drosophila* (James & Jaenike, 1990; Dyer, 2012; Helleu et al., 2016). Chez ces espèces les mâles porteurs d'un chromosome X^{SR} n'engendrent que des femelles, les spermatozoïdes porteurs de Y étant éliminés lors de la spermatogenèse (Policansky & Ellison, 1970). Cet avantage en termes de transmission entraîne la propagation accrue du chromosome X^{SR} par rapport aux chromosomes Y et X non SR, provoquant ainsi un conflit génomique (Burt et Trivers 2006).

Si les chromosomes X^{SR} se répandent dans les populations, on s'attend à ce que des suppresseurs de leur expression soient sélectionnés sur les chromosomes Y et sur les autosomes (Hurst & Pomiankowski 1991). Lorsqu'une population a un sexe-ratio fortement biaisé en faveur des femelles, tout variant produisant plus de mâles sera favorisé par sélection fréquence dépendante (Bull & Charnov, 1998). Des suppresseurs de biais portés par le chromosome Y et autosomiques ont été détectés chez un certain nombre d'espèces de drosophiles présentant un biais SR (voir par exemple Carvalho et al., 1997; Jaenike, 1999; Stalker, 1961). Chez *Drosophila simulans*, le chromosome Y présente des variations dans sa résistance au X^{SR}, les individus exprimant un large continuum de phénotypes allant de 95 % de femelles dans la descendance (forte sensibilité) à 50 % (résistance totale, Montchamp-Moreau et al., 2001). L'élément SR semble également supprimé par des locus autosomiques (Courret et al., 2019).

Des études théoriques se sont intéressées aux dynamiques évolutives des biais et des résistances. Ces études prédisent que la résistance liée au chromosome Y se propage rapidement dans les populations jusqu'à fixation (Thomson & Feldman, 1975; Clark, 1987) mais qu'elle peut aussi être maintenue à un équilibre stable en fonction des interactions fréquence dépendante entre le chromosome X^{SR} et le Y résistant (Carvalho et al., 1997). Les dynamiques évolutives des biais SR et des suppresseurs dépendent de plusieurs paramètres, tels que les coûts qui leur sont associés (Hall, 2004; Vaz & Carvalho, 2004). En cas de suppression totale, on s'attend à ce que la fréquence du biais diminue s'il est coûteux, ou évolue de manière stochastique s'il est neutre. Ce processus peut notamment générer des dynamiques cycliques (Hall 2004). Des études empiriques ont illustré cette dynamique cyclique en observant des variations géographiques des fréquences de SR et de suppresseurs (Atlan et al., 1997; Kingan et al., 2010), mais aussi en montrant l'existence d'une sélection positive des biais SR (Kingan et al. 2010). De plus, une étude d'évolution expérimentale récente (Bastide et al. 2022) a montré le déclin du chromosome X^{SR} sur 100 générations en présence d'un fond génétique où les suppresseurs étaient fixés.

4.1.2 Biais liés au chromosome Y (« *Y drive* »)

Les biais méiotiques liés au chromosome Y biaisent le sexe-ratio en faveur des mâles. Ces biais ont été documentés chez deux espèces de moustiques, *Aedes aegypti* et *Culex pipiens* (Wood & Newton 1991). Chez *Aedes*, certains chromosomes Y portent un allèle D (pour Distorteur) et les mâles produisent alors plus de 90% de fils. La présence de l'allèle D est variable selon les populations et partout où il a été observé il était associé à une résistance du chromosome X. Plusieurs études ont même révélé que la résistance du chromosome X produisait un biais en faveur des femelles (Suguna et al., 1977; Wood, 1976) où les suppresseurs étaient fixés.

Burt & Trivers (2006) suggèrent que les biais liés au chromosome X sont plus fréquents chez les drosophiles car les chromosomes X sont porteurs de plus de gènes que les Y et donc que la probabilité d'apparition d'une mutation tueuse de gamètes est plus importante sur les X que sur les Y. A l'inverse, chez les moustiques, les chromosomes X et Y sont peu divergents et ont à peu près le même nombre de gènes. A cause de la recombinaison ayant lieu chez les femelles, les complexes multilocus tueurs de gamètes peuvent être détruits sur le chromosome X mais pas sur le chromosome Y.

4.2 Distorteurs de sexe-ratio post ségrégation : les symbiotes

Un système de manipulation du sexe encore plus proche du système CMS-restaurateurs est rencontré dans le cas de symbiotes bactériens à transmission maternelle (la mitochondrie elle-même étant, en quelque sorte, une bactérie endosymbiotique avec une histoire de domestication plus longue que les autres). La distorsion du sexe-ratio peut être une des conséquences de la transmission maternelle car les lignées augmentant la fréquence des femelles seront favorisées par la sélection (Perlman et al., 2015). Les symbiotes microbiens peuvent distordre le sexe-ratio de plusieurs manières (Tableau 2), notamment en transformant les mâles infectés en femelles (féminisation), ou en tuant les fils des femelles infectées au début de leur développement (élimination des mâles).

Tableau 2 : Effets phénotypiques de la distorsion du sexe-ratio par des symbiotes (d'après Price & Wedell 2008).

Effets phénotypiques	Action	Exemple
Incompatibilité cytoplasmique	Croisements entre des femelles non-infectées et des mâles infectés non viables	Insectes
Elimination des mâles	Elimination des embryons mâles	Mouches, lépidoptères, coléoptères
Féminisation	Féminisation des mâles génétiques	Insectes, isopodes
Induction de parthénogenèse	Doublement des chromosomes haploïdes chez les mâles les transformant ainsi en femelles	Hyménoptères

4.2.1 Elimination des mâles et résistance

L'élimination des mâles (« *male-killing* ») est un processus utilisé par les endosymbiotes bactériens hérités de la mère pour tuer les embryons mâles. Plusieurs endosymbiotes pratiquent cette élimination, comme par exemple *Rickettsia*, *Spiroplasma,* et le plus connu *Wolbachia* ; on les retrouve principalement chez les insectes mais aussi chez les acariens (voir Table 1 Cordaux et al., 2011). La mort des mâles favorise la transmission de l'endosymbiote à la génération suivante notamment si les femelles qui les portent, disposant de plus de ressources, ont une plus grande probabilité de survie (Burt & Trivers 2006). Chez les coccinelles, la mort des mâles entraîne une réduction des interactions antagonistes entre frères et sœurs, une diminution de la consanguinité (Hurst et al., 2003), ainsi que la consommation des œufs non développés par les femelles (Hurst & Majerus, 1993).

Des suppresseurs de l'élimination des mâles ont été identifiés chez une espèce de coccinelle (*Cheilomenes sexmaculata*, Majerus & Majerus, 2010) et chez une espèce de papillon *Hypolimnas bolina* (Hornett et al., 2006). Chez cette dernière, l'effet du « *male-killing* » peut être complètement supprimé par un gène autosomal dominant de l'hôte. La propagation du suppresseur a été suivie dans les îles Samoa, où le sexe-ratio de *H. bolina* est passé de 99 % de femelles à la parité en dix générations.

4.2.2 Féminisation et résistance

Chez certaines espèces de crustacés mais aussi chez des insectes et des acariens, des bactéries interfèrent avec la différenciation sexuelle et transforment les mâles génétiques en femelles (voir par exemple Hiroki et al., 2002; Negri et al., 2006; Giorgini et al., 2010). Les femelles infectées produisent deux fois plus de filles que les femelles non infectées, ce qui permet une transmission deux fois plus importante de l'endosymbionte.

Chez l'isopode terrestre *Armadillidium vulgare*, la détermination chromosomique du sexe suit l'hétérogamie femelle, c'est-à-dire que les mâles sont ZZ et les femelles ZW (Legrand & Juchault, 1972). Cependant, de nombreuses mères produisent des descendances dont le sexe-ratio est biaisé en faveur des femelles, et ce en raison de la présence de *Wolbachia*. Les symbiotes *Wolbachia* transforment des mâles génétiques ZZ en femelles phénotypiques (Cordaux et al., 2004; Rigaud et al., 1997). Un système polygénique de résistance empêchant la féminisation a été identifié chez *A.vulgare* – la résistance limite la transmission du *Wolbachia* à la descendance (Rigaud & Juchault, 1992). Il existe chez *A.vulgare* un autre agent féminisant déclenchant un second conflit génomique, l'élément *f* qui est une partie du génome de *Wolbachia* ayant été transférée dans le génome nucléaire de l'hôte (Legrand & Juchault, 1984). La fonction mâle de l'hôte porteur de cet élément peut être restaurée par un gène autosomique et dominant appelé M. Ce gène M est en interaction épistatique avec le chromosome W mais pas avec *Wolbachia*, c'est-à-dire que le gène M ne rétablit pas un sexe-ratio équilibré chez les individus infectés par *Wolbachia*.

Nous avons vu que de nombreux cas de manipulation de la reproduction par des symbiotes à transmission maternelle sont documentés chez les animaux. En revanche, aucun cas clair de manipulation de la reproduction par des mitochondries n'avait été décrit avant la découverte de la CMS chez *P. acuta* (mais voir *Liposcelis bostrychophila*, Perlman et al. (2015) pour un cas où un génome mitochondrial très altéré est associé à un mode de reproduction avec clonalité femelle).

La physe est le premier exemple de CMS chez les animaux, nous permettant ainsi de voir si les observations faites chez les plantes sont généralisables. Avec un animal à générations courtes comme la physe, il devient possible de suivre les dynamiques évolutives en temps réel, ce qui est difficilement réalisable avec des modèles plantes. Des observations de ce type ont déjà été réalisées avec d'autres systèmes, comme par exemple chez la drosophile avec les biais méiotique X^{SR} et les suppresseurs associés (Bastide et al., 2022). De plus, la physe s'élève facilement en laboratoire et les croisements entre individus ne présentent pas de difficultés. Il est donc facile de réaliser des introgressions afin de placer des génomes mitochondriaux dans différents fonds nucléaires. En somme, *P. acuta* se prête à un travail qui est beaucoup plus difficile à envisager chez les plantes et nous permet ainsi d'aller plus loin dans le test des modèles théoriques élaborés dans ce domaine.

5. Etude du conflit nucléo-cytoplasmique chez *Physa acuta*

Au cours de ma thèse, j'ai étudié le conflit nucléo-cytoplasmique chez *Physa acuta*. Pour étudier ce conflit, il m'a fallu tout d'abord le mettre en évidence ; je me suis donc penchée sur l'étude du mitotype K (Chapitre 1). Dans ce premier chapitre, je me suis demandée si K était, comme D, associé à de la SMC et s'il existait du polymorphisme nucléaire contribuant à la restauration de la fonction mâle. J'ai ensuite exploré les trajectoires évolutives prédites par les modèles s'intéressant au maintien du polymorphisme chez les plantes (Figure 4) en réalisant une évolution expérimentale sur 11 générations (Chapitre 2). Dans cette évolution expérimentale, je me suis plus particulièrement intéressée à deux phases : (i) celle prédisant une diminution de la fréquence de SMC en raison de son coût (Figure 4, phase 3), (ii) celle prédisant que la restauration est soumise à un régime de sélection qui dépend de la fréquence de SMC et qu'un coût silencieux de la restauration entraine une diminution de la fréquence des restaurateurs (Figure 4, phase 4). Je me suis également intéressée à la spécificité et au déterminisme de la restauration chez *P. acuta* (Chapitre 3). J'ai regardé si la restauration mis en évidence dans le chapitre 1 était spécifique du mitotype K ou si elle pouvait également restaurer le mitotype D et j'ai testé l'hypothèse d'un restaurateur monolocus dominant. Enfin, à l'aide d'une approche de scan génomique j'ai souhaité explorer les conséquences génomiques de la restauration et de la SMC en population naturelle (Chapitre 4).

CHAPITRE 1

ETUDE DU MITOTYPE K

CHAPITRE 1 – CONTEXTE

Dans ce chapitre nous décrivons la découverte du mitotype K et la mise en évidence d'un conflit nucléo-cytoplasmique. Nous reconstituons la phylogénie du groupe *Physa* et étudions la divergence entre les trois mitotypes (N, D et K). Nous testons l'existence d'un fond nucléaire affectant le phénotype, comme attendu en présence de restaurateurs nucléaires de la fertilité. Pour cela, nous réalisons un protocole d'introgression sur 17 générations ainsi que des mesures de traits liéde reproduction ont été réalisées pour comparer les individus porteurs des types K et N.

Both nuclear and cytoplasmic polymorphisms are involved in genetic conflicts over male fertility in the gynodioecious snail *Physa acuta*

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Abstract: Gynodioecy, the coexistence of hermaphrodites with females, often reflects conflicts between cytoplasmic male sterility (CMS) genes and nuclear genes restoring male fertility. CMS is frequent in plants, and has been recently discovered in one animal: the freshwater snail *Physa acuta*. In this system, CMS was linked to a single divergent mitochondrial genome (D), devoid of apparent nuclear restoration. Our study uncovers a second, novel CMS-associated mitogenome (K) in *Physa acuta*, demonstrating an extraordinary acceleration of molecular evolution throughout the entire K mitochondrial genome, akin to the previously observed pattern in D. This suggests a pervasive occurrence of accelerated evolution in both CMS-associated lineages. Through a 17-generation introgression experiment, we further show that nuclear polymorphisms in K-mitogenome individuals contribute to the restoration of male function in natural populations. Our results underscore shared characteristics in gynodioecy between plants and animals, emphasizing the presence of multiple CMS mitotypes and cyto-nuclear conflicts. This reaffirms the pivotal role of mitochondria in influencing male function and in generating genomic conflicts that impact reproductive processes in animals.

Key words: gynodioecy, cytoplasmic male sterility, restoration, arms-race, mitochondrial genomes, *Physa acuta*

Introduction

Gynodioecy is a sexual polymorphism in which hermaphrodites coexist with male-sterile individuals, that are therefore functionally female (Saumitou-Laprade et al. 1994, Touzet 2012). This coexistence led Darwin, in 1877, to question the Linnean categories of sexual systems in plants and to become interested in the evolution of modes of reproduction. Botanists extensively described gynodioecy (reviewed in Barrett, 2010), recognizing its prevalence in angiosperms (approximately 20% of families, Dufay et al. 2014, Caruso et al. 2016). Regarded as a potential transition from hermaphroditism to dioecy (Barrett 2002), the evolutionary dynamics of gynodioecy have been thoroughly explored in plants, combining theoretical and empirical perspectives (Shykoff et al. 2003, Delph et al. 2007, McCauley & Bailey 2009). Gynodioecy in plants usually reflects the presence of mitochondrial Cytoplasmic Male Sterility (CMS) genes that suppress the male function in individuals that would otherwise be hermaphroditic (Chase 2007). Because of maternal inheritance, such genes can increase in frequency as soon as they slightly enhance female fitness, irrespective of their effects on male fitness (Lewis 1941, Lloyd 1974). However this suppression adversely affects nuclear genes, as half of a hermaphrodite's fitness relies on the male function. Consequently, some nuclear genes, known as restorers of male fertility, evolve to counteract CMS by converting individuals with male-sterilizing cytoplasms back into hermaphrodites (Cosmides and Tooby 1981).

Given the prevalence of the mito-nuclear conflict in plants, and the shared mechanisms of mitochondrial and nuclear inheritance, the question arises whether animals follow a similar evolutionary path. Although about one-third of non-insect animal species are hermaphrodite (Jarne & Auld 2006), gynodioecy is very rare (Weeks 2012). This may be explained by the compact and structurally stable nature of animal mitochondrial genomes, that may limit the emergence of male-sterile variants (Boore 1999, Galtier 2011). In addition some gynodioecious animals may remain undetected, as many hermaphroditic animals have concealed sperm-producing organs, making male-sterile individuals challenging to identify.

To date, only one animal with confirmed cytoplasmic male sterility (CMS) has been identified: *Physa acuta*, a freshwater snail (David et al. 2022). This snail provides a unique opportunity to assess how universal concepts developed in plants are. The initial study however revealed two major differences from known gynodioecious plants. First, only one CMS-associated mitochondrial type (D) was found, with extreme molecular divergence from normal cytotypes (N) at all mitochondrial genes. Second, there was no evidence for nuclear restoration, as nearly all captured D individuals exhibited zero male fitness (David et al. 2022).

In contrast, multiple CMS genes often coexist in natural populations of gynodioecious plants (e.g. in *Beta vulgaris* Cuguen et al. 1994, *Thymus vulgaris* Belhassen et al. 1991, *Silene vulgaris* Charlesworth and Laporte 1998) as well as in cultivated crops (28 CMS genes identified in 13 species, Kim & Zhang 2018). In some cases, no fertile (i.e. non-CMS or maintainer) cytoplasms can be found (e.g. *Plantago lanceolata,* de Haan et al. 1997). The different CMS types within a species have specific nuclear restorers, usually genes encoding for pentatricopeptide repeat (PPR)-containing proteins that act by suppressing the expression of mitochondrial genes that cause sterility (Dahan & Mireau 2013). For example, maize requires the genes Rf1 and Rf2 for cms-T, Rf3 for cms-S and Rf4 for cms-C (Snyder and Duvick 1969, Kamps and Chase 1997, Sisco 1991). The diversity of CMS types is explained by (i) the high rate of mitochondrial DNA rearrangements facilitating the production of new CMS mutants (Adhikari et al. 2019), (ii) frequency-dependent selection that maintains intermediate frequencies of CMS types and corresponding Rf alleles due to the pleiotropic costs of restoration on fitness (Gouyon et al. 1991, Bailey 2002, Del Castillo & Trujillo 2009, Caruso & Case 2013).

The presence of a single male sterility mitotype in *P. acuta* is unexpected, given that the high mutation rate thought to be responsible for the divergence of the D mitotype could lead to multiple CMS types. It remains unknown whether CMS can occur in this system without high molecular divergence, or whether sterility and divergence are interdependent. No evidence for restorers was found in *P. acuta* – although some D-mitotype individuals were male-fertile, their frequency was low (8%) and it is uncertain whether they were caused by nuclear alleles, *versus* an incomplete penetrance of the male sterility phenotype. If this species does indeed have a single male-sterile mitotype without restoration, the limits to CMS expansion might be very different from those usually acting in plants. To investigate this question, we explored mitochondrial diversity in populations around Lyon (France), where D individuals were discovered by David et al. (2022), looking for potential new mitotypes and evidence of restoration.

Here, we report the discovery of a new mitochondrial type, named K, in two populations near Lyon. We investigated its phylogenetic relationship with existing types, particularly its proximity to D and the non-CMS mitotype N. We then tested whether K mitotypes were also associated with a male-sterile phenotype and whether this phenotype was affected by the nuclear background, as expected in the presence of nuclear restorers of fertility.

Material and methods

Discovery of the K mitotype

We sampled *P. acuta* at four stations around the initial discovery site of the male-sterile D type in Lyon, France (October 2019, Table S1). Approximately one hundred G0 individuals from each site were mitotyped after some were allowed to lay separately to preserve progeny. DNA was extracted using a Chelex protocol (Gautam 2022), and we used the David et al. (2022) PCR test designed to identify D and N mitotypes. This involved two PCRs, each with specific primers of a 604-bp fragment (N-specific in the first PCR, D-specific in the second) as well as external universal primers of a 705-bp fragment in the mitochondrial COI gene (Folmer et al. 1994). The 705-bp fragment is amplified only when the specific primers fail to anneal. The resulting PCR products, run side by side on agarose gels, allow mitotype identification based on fragment size patterns (long-short for D, short-long for N, David et al. 2022).

While investigating, some individuals exhibited a long-long COI pattern (Figure S2), suggesting a mismatch with both N- and D-specific primers (Figure S3). Seven such individuals (two from Irigny and five from Erevan) were Sanger sequenced for the COI fragment amplified with "Folmer" universal primers, revealing a distinct sequence, that we termed “mitotype K”. Subsequently, a new PCR test was developed for one-step mitotype assessment (N, K, or D) based on COI sequences. The test used a mixture of five primers, including two universal external COI primers and three internal primers specific for N, D, and K. On an agarose gel, the resulting fragment size directly indicated the corresponding mitotype, with a long fragment indicating no match to N, D, or K. The primers and protocol for this "triplex" PCR test are detailed in Figure S4.

Introgression experiments.

Nuclear restoration is present when the propensity of a mitotype to produce male-fertile or male-sterile individuals, depends on the nuclear background to which it is associated. We tested this idea through an introgression experiment over 17 generations, inserting the mitochondrial lineages derived from wild-caught ancestors from Lyon, into the “naive” nuclear background of a laboratory population (see introgression protocol detailed Supplementary Text 1 and Figure S1). Introgression occurred within “matrilines”, defined as sets of focal individuals sharing the same ancestor by maternal descent, and hence the same mitochondrial DNA; there were 25 matrilines (12 K and 13 N). At each generation, the sperm donors were taken from a laboratory population of *P. acuta*, derived from a mixture of ten natural populations from Montpellier (300km south of Lyon, Noël et al. 2016), with an albino phenotype. The albino population is “naive” because it consists of 100% N-mitotype individuals and has been maintained in captivity for more than 80 generations: selection should not have maintained high frequencies of restorer genes, if they exist and have a cost. Within the matrilines, the nuclear genes (initially from Lyon) were generation after generation replaced by those of the albino population. We however selected only pigmented offspring to propagate each matriline, allowing us to distinguish them from sperm donors. We performed PCR tests at different generations to check that the mitotype remained the same within a matriline over generations (Supplementary Text 1).

At each of the 17 generations, we assessed the male-fertility status of the focal individual on the basis of the offspring laid by albino mates (Figure S1). The presence of pigmented juveniles indicates that the (pigmented) partner from the matriline is male-fertile. Albinism is driven by a single recessive allele: if an albino mother produces a pigmented offspring, the father must be a pigmented individual.

Behavioral and reproductive traits of K and N individuals.

In the introgression protocol, we investigated the sexual phenotype of the newly discovered mitotype K. Both behavioral and fitness traits were assessed for the matrilines K and N at G1 and G5, with two to three individuals from each matriline isolated until sexual maturity. We conducted observations of mating pairs during 45mn, each focal individual from a matriline (pigmented) paired with a N-mitotype albino mate, both previously virgin, with behaviors recorded and timed. After observations, snails were left together for three days, re-isolated for egg-laying in separate boxes for three more days, and then we collected their clutches. Female fitness was assessed by counting the number of eggs laid, male fitness by the number of juveniles sired by the focal in the progeny of its partner. Male fertility status was defined by the ability to stimulate oviposition when inseminating their virgin partner and to sire a majority of offspring (Supplementary Text 1).

Statistical analyses.

We used linear mixed models (LMM) and generalised linear mixed models (GLMM) with the lme4 R package (Bates et al. 2015) to analyse juvenile counts (fitness) and time spent in male or female behaviours. Poisson distributions were used for count variables and a Gaussian model for quantitative variables. To account for relatedness between individuals, a random factor 'matriline' was included, along with an individual random factor to deal with potential overdispersion in GLMMs.

Based on the 17-generation time course of inheritance of the male sterility phenotype within matrilines, we used Maximum likelihood estimation to determine the frequencies of polymorphic restorer alleles in the Lyon population (p0) and the albino population (p), under the assumption of mono-locus dominant restoration (i.e. recessive variant is sterile in the context of K), the most common restoration determinism observed in plant populations in which restoration is highly prevalent (Delph 2007). The likelihood was computed for each (p0, p) value, representing the probability of the observed male fertility status of founder K individuals and all their descendants within each matriline, conditional on their pedigree - in principle, the frequency of fertile individuals should start from p0 and progressively evolve towards p with successive generations of introgression (details of likelihood computation in Table S5). Multiple (p, p0) values (0 to 1 in steps of 0.01) were explored to determine the maximum likelihood, with 95% confidence intervals obtained using the profile likelihood method. All statistical analyses were performed in RStudio v. 4.3.1 (R Core Team 2023).

Mitochondrial genome sequence. To reconstruct the complete mtDNA of type K, short-read whole genome sequencing was performed on one individual (Supplementary Text 1, ENA project PRJEB50799). The genome of this individual was assembled using MEGAHIT (Li et al. 2015, 2016). We then extracted the contig corresponding to the mtDNA by blasting a COI sequence from a K mitotype against the assembly. The contig was then annotated using the MITOS webserver (Bernt et al., 2013). The absence of contamination and chimeras in the assembly was verified by looking at the coverage along the genome (Supplementary Text 1, Figure S4). The assembled and annotated mitochondrial genome were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB50799. Once the mtDNA was assembled, we sequenced ten individuals carrying the K mitotype. Ten K-mitotype individuals were bred (three from Irigny and six from Erevan; with the long-long band pattern), and sent for Illumina transcriptome sequencing (Supplementary Text 1).

Phylogenetic analyses.

The sequence alignments made in this section are available in Zenodo (10.5281/zenodo.10521893).

Physidae phylogeny. To investigate the phylogenetic origin of *P. acuta* mitotypes, we used mitochondrial protein-coding genes from K, D and N mitotypes, including newly acquired K sequences and D and N sequences from David et al. (2022). In addition, seven gastropod species from GenBank, consisting of three *Physidae* species and five other outgroup species (consistent with David et al. 2022, plus *Physa fontinalis*, Table S2 section 1), were included. The 11 protein-coding genes (excluding ATP8 and NAD4L) were first aligned without *P. acuta* K and D using MAFFT (Katoh et al. 2002). K and D mitotypes were then mapped onto this alignment using MACSE (Ranwez et al. 2011). G-blocks (50% gap allowed, Castresana 2000) were used to remove ambiguously aligned sites and coding sequences were concatenated. A phylogenetic tree was constructed using PhyloBayes (Lartillot et al. 2009) under a CAT-GTR+G+I model, focusing on the first and second codon positions to avoid saturation. Two independent chains were run and stopped after 455,785 steps (40,000 generations excluded as burn-in), after checking for convergence with the tracecomp program (effective sample size > 50 and discrepancy between chains < 0.3 for all statistics). The majority consensus tree, obtained from 41,578 trees sampled every 10 steps, was derived using a 10% threshold to resolve polytomies. This tree was used to estimate non-synonymous (dN) and synonymous (dS) substitution rates under the YN98 codon model. YN98 model parameters were estimated using bppml (Guéguen et al. 2013), and dN/dS, together with Kr/Kc, were calculated using substitution mapping with mapNH (Romiguier et al. 2012). Tree processing was performed using the R package ggtree (Yu et al. 2020).

Physa phylogeny. The COI sequence of the K mitotype was blasted against the NT database of GenBank to identify similar sequences. A COI dataset was then assembled using COI sequences retrieved from GenBank with more than 93% of identity to our K sequence (Table S4), seven K sequences from the Irigny and Erevan populations obtained by Sanger sequencing using the same primers as in the PCR tests, the D sequence and the five N sequences used in David et al. (2022), together with the *P. fontinalis* sequence used as an outgroup (Table S2 section 2). A phylogenetic tree was built using the same protocol described above.

Comparison of divergence among gastropods. To the COI dataset, we added COI sequences of other snails retrieved from GenBank and used in David et al. 2022 (Table S2 section 3). The sequences were aligned using MAFFT. Mean divergences between sequences of the K mitotype and between sequences from the three mitotypes (D, N and K) were then obtained for the COI using the APE package in R.

Results

K mitochondrial genome sequence

We developed a fast PCR protocol (see “triplex” PCR protocol, Figure S3) to distinguish a newly described male-sterile mitotype (K) from the previously found N and D mitotypes (respectively normal and male-sterile, David et al. 2022). The K mitotype was found in 12% of the individuals from the Irigny population and 32% from the Erevan population (Table S1). No K individuals were found in the Crêt-2 and Saint-Priest populations.

The seven K mitotype snails sequenced (two from Irigny and five from Erevan) shared an identical COI sequence (0% divergence). By blasting the K COI sequence in GenBank, six sequences between 100% and 93% of identity were found. These were from North America (Idaho), Singapore and South Africa and referenced as *Physa sp.* (Table S4). The average divergence between all 11 K sequences (seven from our populations and the six from Genbank) was 1.58% (±2.48% SD, uncorrected DNA distance). The K COI sequences from Singapore and South Africa were almost identical to our K sequences from Lyon (all had 100% identity, except one of the two South African samples at 97.4%). The COI sequences from North America were more divergent (nearly 7% divergence from the Lyon K sequences; and 5.9% (±1.3% SD) from all other K COI sequences combined - the seven from our populations plus the four from both Singapore and South Africa). A COI phylogenetic reconstruction grouped the K sequences into a single clade (pp= 1, Figure 1B) composed of two subclades: North American sequences, and all other localities including the French, South African and Singapore populations.

Comparison of divergence among gastropods. Based on COI gene divergences, the newly discovered K mitotype was 22.8 % (±0.79%SD, 11 individuals) divergent from the normal mitochondrial genome of *P. acuta* (5 individuals of N mitotype, among which the average divergence was 2.2% ±1.6%, Table S6). Interestingly, this divergence was even stronger when comparing K with the male-sterile D mitotype (28.1%, ±0.43% SD, average of five D individuals). The same observation, though at a higher divergence scale, was made for 11 additional mitochondrial genes: the K-N DNA divergence ranged from 35% to 57%, while the K-D divergence ranged from 41% to 61% (Table S6). The divergence between K and N (or D) was equivalent to that expected between snail clades that diverged hundreds of millions of years ago (Figure 1C).

Physidae phylogeny. In the phylogeny based on all mitochondrial genes, the K mitotype was not clearly grouped with either *N* or *D* (Figure 1A). Although K was clearly within the *Physidae* and more precisely the *Physella* clade (*Physa acuta* + *Physa gyrina*), deep nodes within this group were not well supported (posterior probabilities below 0.9) because the divergences between *P. gyrina* and the three mitotypes of *P. acuta are* probably too high, resulting in long-branch attraction artifacts (Figure 1A). For the terminal branches leading to each of the three mitotypes (K, D and N), the dN/dS ratios were low (0.035, 0.036 and 0.032), as were the Kr/Kc ratios (0.784, 0.798 and 0.725). The three mitogenomes N, K, D are of similar length (14.4 Mb) and they contain the same set of genes; however the K mitogenome differs from the N and D mitogenomes by multiple rearrangements (Figure S6).

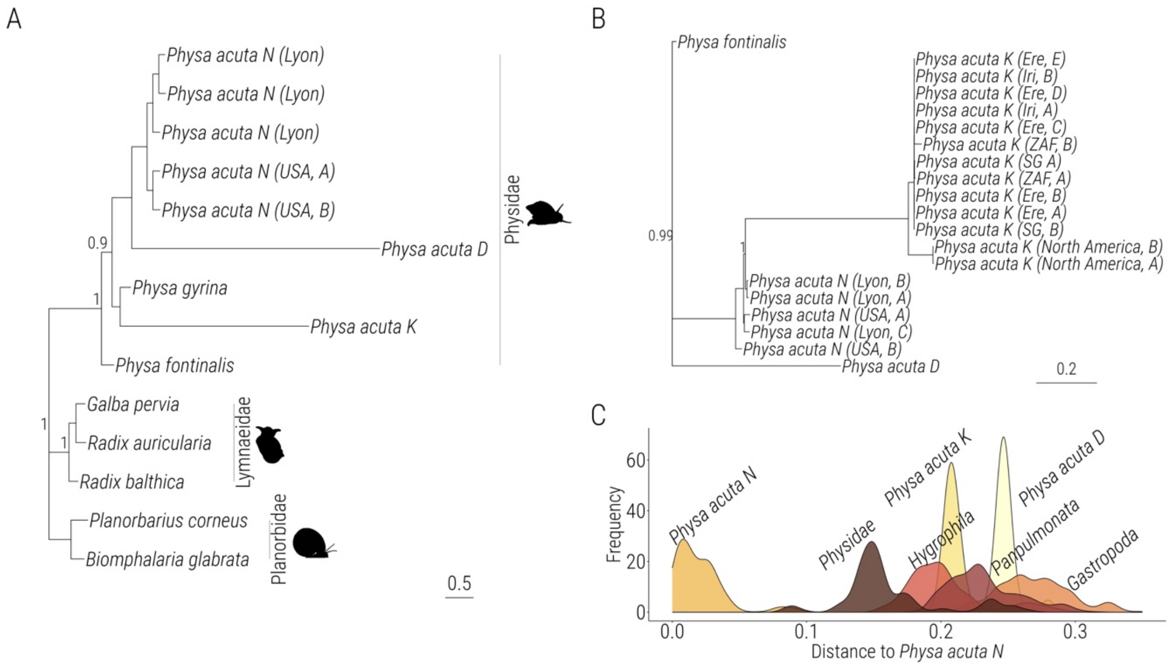

Figure 1: K mitotype is divergent from conspecific individuals and different from the D mitotype. Data used are given Table S2. **A:** Physidae phylogeny based on 11 mitochondrial protein coding genes and built with PhyloBayes (Lartillot et al. 2009) under a CAT-GTR+G+I model selecting only first and second codon positions because saturation. Data include 7 sequences from Genbank as well as 7 sequences of *P. acuta* (5N + 1D + 1K) from Lyon. Species from Lymnaeidae and Planorbidae families were used as outgroups. **B:** COI phylogeny of *Physa acuta*. *Physa fontinalis* was used as an outgroup. Ere: Erevan, Iri:

Irigny, ZAF: South Africa, SG: Singapore. Numbers at the nodes represent posterior probabilities; probabilities below 0.9 are not shown. **C:** Distributions of pairwise DNA distances at the COI mitochondrial gene in snails. All pairs include one individual of the N mitotype of *P. acuta*. Data include 304 sequences from Genbank as well as 41 new sequences of *P. acuta* (5D, 29N and 7K).

Introgression experiments

The control PCRs (N = 175, Supplementary Text 1) carried out during the introgression experiment showed that the mitotype was conserved within a matriline, i.e., the individuals within a matriline identified as K mitotype at G0 maintained the K mitotype in subsequent generations (G1, G2, G7 and up to G25).

Phenotyping

Behavioral and reproductive traits of K and N individuals

In G1, no significant difference in behavior (male or female) was found between K and N individuals (Figure 2A). Similar results were obtained for female and male fitness (Figure 2B and Table S3). However, at G5, K individuals exhibited a significantly reduced male activity compared to N mitotype individuals (Figure 2A). Out of 26 albinos paired with K snails, 18 did not lay any eggs or produced only albino hatchlings (*i.e.* self-fertilized brood). However, the female behavior and fertility of K snails did not differ from that of N snails, and they produced many offspring sired by albino mates (Figure 2B and Table S3).

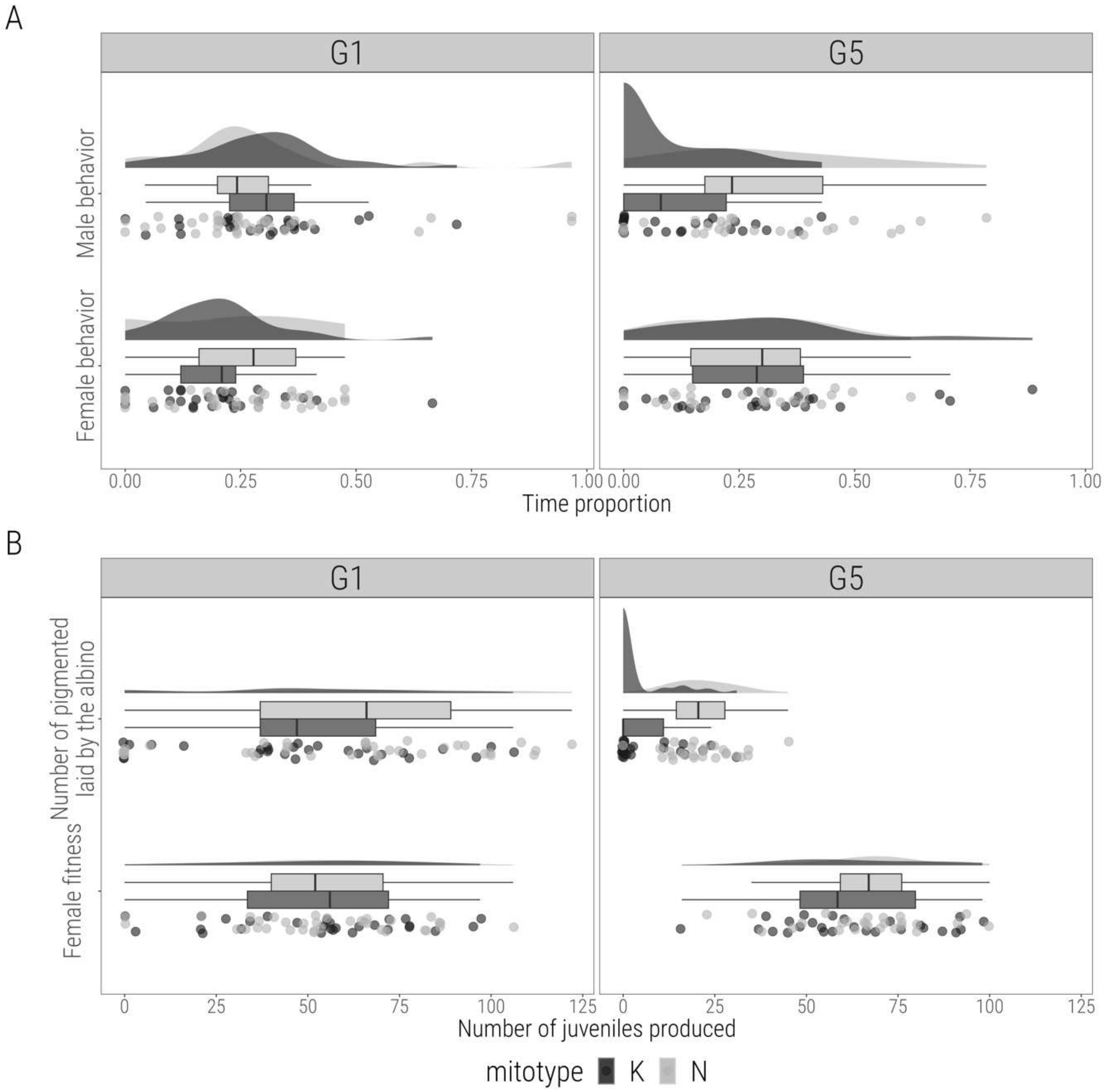

Figure 2: The K mitotype has a male-fertile phenotype at G1 then a male-sterile phenotype at G5. **A:** Distributions of time spent in male and female behavior by K and N individuals paired with virgin partners at both G1 and G5. **B:** Number of pigmented offspring laid by the albino partner. At the first generation individuals were homozygous pigmented so all their offspring were pigmented. After the first generation focal individuals were heterozygous at the albinism locus and produced a mix of albino and pigmented juveniles in their progeny. Thus, the offspring sired by the N individuals resulted in a 50-50% mix of albino and pigmented (i.e. the number of pigmented offspring laid by the albino in G5 was lower than in G1) – the number of pigmented offspring represents only one half of male fitness. Female fitness, i.e., number of eggs layed by focal snails.

Proportion of male sterility in K progressively increased with generation of introgression

The nuclear background of the mitochondrial lineages originating from wild-caught ancestors from Lyon was diluted into the “naive” nuclear background of the Montpellier albino population over 17 generations. This introgression protocol led to an increase in the proportion of male-sterile K individuals; after G11, male sterility stabilized at around 60% (Figure 3A). The proportion of male-sterile individuals in the N matrilines was consistently low and unaffected by introgression. From G1 to G17 male sterility was heritable, as male-sterile K mothers produced a majority of male-sterile offspring, significantly more than male-fertile mothers (Chi-squared test, X = 216.62, P < 0.001). However, male-sterile K mothers still had 27% of male-fertile offspring (Figure 3B).

The MLE of the restorer allele frequency (assuming a single-dominant gene) was 0.26±0.08 in the albino population and 0.56±0.08 in the K individuals from Lyon (Figure S5).

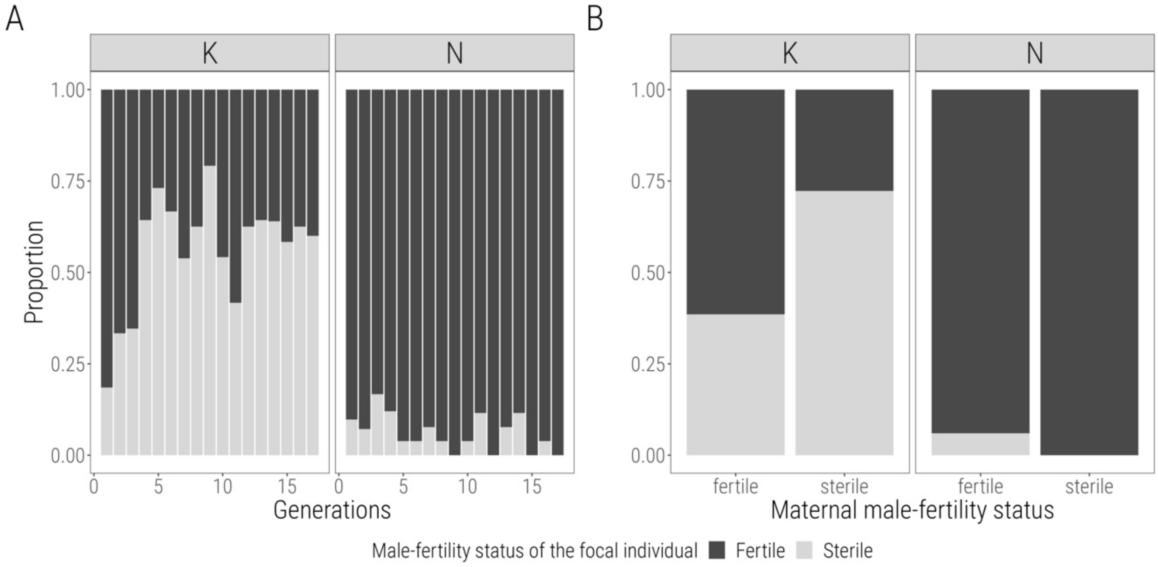

Figure 3: Male-sterility increases in K matrilines when introgressed into a naive genomic background, and is heritable. A: Proportion of male-sterile and male-fertile individuals in both K and N mitotype, from first-generation individuals taken from Lyon (nuclear background entirely from Lyon) to 17-th generation individuals whose genome has been near-completely replaced by that of the albino Montpellier laboratory stock. **B:** Male-fertility of daughters of male-sterile and male-fertile mothers (over all 17 generations). In both A and B, semi-sterile and fully male-sterile individuals were pooled into a single “sterile” category

Discussion

Highly divergent K mitotype is associated with CMS and restoration

David et al (2022) reported the first example of cytoplasmic male sterility in animals, linked to a highly divergent mitochondrial genome (the D mitotype). Here, we discovered another such mitotype, K. The mitochondrial nature of K sequences is confirmed by their high coverage, the absence of stop codons or frameshifts in coding sequences, and the maternal transmission of PCR patterns that persisted over more than 20 generations while the nuclear genome was progressively replaced by introgression. We did not obtain evidence for mitochondrial heteroplasmy either (Supplementary Text 1). The K mitotype shares its essential characteristics with D: a male sterility phenotype and high divergence from the normal mitochondrial type (N) without evidence of accelerated non-synonymous substitutions proportions (dN/dS is stable). However, the expression of male sterility is different from that of D. Indeed, first-generation K individuals, whose genome comes entirely from their natural populations of origin, mostly looked normal: when pairing N snails from an albino laboratory strain with K snails, both individuals mated in both sex roles and could sire offspring. It was only by introgressing the K mitotype into the genomic background of the "naive" albino population from Montpellier (which has been kept for more than 80 generations in the laboratory, without any contact with the K mitotype) that K snails started to show a reduction or suppression of male behavior and fertility. During introgression, K male-sterile mothers tended to produce more male-sterile offspring than male-fertile ones, showing that the male-sterile phenotype associated with the K mitotype is heritable. The results of the introgression experiment indicate the existence of nuclear polymorphisms that modulate the expression of the male sterility phenotype in K individuals, i.e., restorer genes. This stands in contrast to the D mitotype, in which almost all individuals from the natural population in Lyon were male-sterile (a small fraction was fertile), a situation that remained unchanged by introgression into the genomic background of the Montpellier albino stock (David et al. 2022). The K mitotype in *P. acuta* is therefore the first animal example of CMS with clear evidence of nuclear restoration.

High frequency of restoration in P. acuta natural populations

In plants, previous studies suggest that restorer alleles are more often dominant than recessive (reviewed in Delph et al. 2007). We were able to estimate the frequency of a putative restorer allele conferring male fertility to the K mitotype under a mono-dominant scenario. Interestingly the restorer frequency was estimated to be 26% in the Montpellier albino population, reflecting the fact that even after 17 generations of introgression (i.e., when the quasi-totality of the nuclear genome originates from the Montpellier albino stock), a significant fraction of male fertility still exists in K snails and male-sterile mothers continue to produce a fraction of male-fertile offspring. Under the same scenario, the frequency of the restorer allele in the original K population was then estimated to be around 56%. This latter result is consistent with the high frequencies of restorer alleles observed in some gynodioecious plant populations. For example, in natural populations of *Raphanus sativus Rf* frequencies range from 41% to 100% (Murayama et al. 2004); in wild rice species it is either 60% or 41.9%, depending on the CMS type (Li et al. 2005).

The presence of restorers in our albino population might appear paradoxical; after more than 80 generations maintained as a pure N mitotype population, one could expect restorer alleles to have been eliminated from this population. Two possibilities could explain the maintenance of the restorer gene: (i) the K mitotype was historically present in the original Montpellier source populations (which served as founders of our laboratory stock 80 generations ago) and the cost of restoration in the absence of CMS is low, so the restorer frequency is decreasing only slowly over generations, (ii) our scenario on the genetic architecture of restoration is too simple; it is possible, for example, that K male-sterility would not be total, even in a naive population without specific restorers (David et al 2022). In this case, there would be no nuclear background producing 100% of male sterile individuals, whatever the mitotype. The phenotype of K would be a partial reduction in male fertility (e.g., a decrease in sperm production, not a complete elimination) leading to a non-zero proportion of male fertile individuals under the conditions of our tests (pairing with virgin individuals). This situation would not be exceptional as threshold models have been used to understand complicated restoration patterns of inheritance of CMS in plants (Ehlers et al. 2005, Bailey & Delph 2007) – in such models, the genetic variance for a multigenic quantitative trait (liability) determines the fraction of male-sterile individuals in a given cytoplasm/mitotype, through a threshold function : individuals with a trait value above a given threshold will have a hermaphrodite phenotype, while the remaining individuals will have a female phenotype. As in plants, a combination of single locus and complex genetic basis of restoration may apply to *P. acuta*, with different relative prevalence in populations depending on their costs and on the abundance of CMS. Further studies are needed to clarify the genetic determinism of the restorer and its frequency, particularly by selecting nuclear backgrounds with contrasting frequencies of the restorer in the Montpellier populations.

The origin of highly divergent mitotypes and intragenomic conflicts

Our results showed that (i) The K mitotype is not an offshoot of the D mitotype, (ii) K and D are both very distant from the N mitotype and from each other, (iii) the order of genes differs among the three mitogenomes without any clear resemblance shared by the two CMS-associated ones. Unfortunately, the K and D mitotypes have diverged so much that it has become impossible to reconstruct their detailed origin. The K and D mitotypes branch at the root of the *Physa* group (including *acuta* and *gyrina* species), which is likely due to long-branch attraction artifacts and saturation that prevent to fully reconstruct their phylogenetic history. As suggested by David et al. (2022) elevated mutation rates may be responsible for the high divergence of the CMS mitotypes. As similar results are found in the K mitotype, the patterns observed may be driven by the same mechanism. However, an increase in the mutation rate alone would increase the substitution rate, but it should also increase the genetic diversity.

As for the D mitotype, the K mitotype has a high substitution rate but low diversity. The seven mitochondrial sequences from both the Erevan and Irigny populations were almost identical; the COI sequences originating from Singapore and South Africa were also sub-identical to the COI sequences from the Erevan and Irigny populations (0.34% of divergence). The combined observation of a high substitution rate, very low diversity and worldwide distribution suggests that this particular K haplotype has been recently selected and has invaded the *P. acuta* populations. The slightly more divergent (7%) variants of the K mitotype found in North America (Idaho) suggest that more diversity may be found in the native area of *P. acuta*. Indeed, *P. acuta*, although initially described in the Garonne River (France) (Draparnaud 1805), is thought to have originated in North America (where its diversity is maximal, and where sister species are found), and spread to the old world (Europe, Asia, Africa) only recently with human exchanges (it is now probably the most widespread freshwater mollusc, Ebbs et al. 2018). While this invasion has not been accompanied by spectacular losses in molecular diversity (Bousset et al. 2014), the native area of *P. acuta* probably harbours a diversity of CMS mitotypes. The high divergence of these mitotypes has probably hindered their inclusion in phylogeographic studies: people may discard them from analyses thinking their sample is contaminated by another species (Ng et al. 2015), or consider them as a cryptic taxon, basal in the Physid phylogeny (Young et al. 2021).

The logical link between genomic evolution of the mitochondrial DNA and male-sterility is still elusive. First, genomic instability of the mitochondrial DNA (whatever its cellular cause) may fuel the production of male-sterile variants (as in plants). Second, certain mutations could have an impact on the energy metabolism of sperm or sperm-producing cells without affecting females (see Havird et al. 2019). In addition, the fast molecular evolution of the mitochondrial DNA may favour the persistence of such variants over time, in the context of an arms race with nuclear genes. This hypothesis is in agreement with the apparent lack of accumulated polymorphism within male-sterile mitotypes (K and D) despite a mitogenome-wide increase in mutation rates, which suggest recurrent selection drafts. Finally, one cannot exclude that the instability of the mitogenome and the male-sterility phenotype are two consequences of a common cause (such as a maternally-transmitted cytoplasmic symbiont) – but we have no evidence for it so far.

Accelerated mitogenome evolution has also been observed in some plant clades where CMS is frequent (e.g., *Plantaginaceae* and *Caryophyllaceae*, Cho et al. 2004, Mower et al. 2007). It has been proposed that the driver of this accelerated evolution is antagonistic coevolution, known to drive rapid evolution in other contexts (Brockhurst et al. 2014, Ebert & Fields 2020). Indices of arms race evolution have indeed been observed in the main family of plant restorer genes (Fujii et al. 2011). The finding of male-fertility restorers in *P. acuta* opens up the possibility of a similar interpretation in our system. The arms race could be linked to the penetrance of the male sterile phenotype: a mutation conferring higher penetrance to a CMS mitotype would increase in frequency, while restorers would be selected to reduce the penetrance. In this context, the D mitotype (for which no restorers are yet known) should invade the populations, but -with the little available data- its frequencies seem to be relatively low at both local and global scale compared to K. We still have a very incomplete picture of a potentially highly dynamic system in which new divergent male-sterilizing mitotypes may emerge and spread quickly before being counteracted by restorers. Tracking the dynamics of divergent mitotypes in natural populations over time would be an essential source of information on this issue.

Theoretical studies of gynodioecy (reviewed in Delph et al. 2007) show that CMS polymorphism can be maintained under a form of balanced selection (Charlesworth 2002) when CMS mitotypes have a female advantage and restorers have sufficient costs. In our study, female advantage would result in higher female fitness of K when not restored (K>N in G5); while we would expect K<N when K is restored (in G1), resulting from costs of restoration. We did not find such differences. However this does not mean that differences would not appear in other conditions. Female advantage is frequent, but very variable and not systematically significant in plants, and probably depends on environmental conditions (Dufaÿ & Billard 2012). Restoration costs have been detected directly through their effects

on seed (de Haan et al. 1997, Del Castillo et al. 2009) or pollen production (Bailey 2002, Caruso & Case 2013), or inferred through equilibrium models from the variation in sex ratio among natural populations (Case & Caruso, 2010). In *P. acuta*, restoration costs bearing on sperm production or the number of eggs laid should similarly be investigated, contributing to accurately model the evolutionary dynamics of the first animal example male sterility of CMS.

Conclusion

While *Physa acuta* is the first example of complete cytoplasmic male sterility, it is by no means the only example of cyto-nuclear conflicts mediated by mitochondria on animal reproduction. Indeed, the same reasoning, based on the different modes of inheritance of mitochondrial and nuclear genes, predicts that in gonochoric animals, mitochondrial mutations harmful to males can persist or spread if they are not harmful to females, a phenomenon known as the mother's curse (Camus et al. 2022). The presence of nuclear restorers of CMS in *Physa* is paralleled by the evolution of compensatory gene or gene expression in the nucleus of gonochoristic species, mitigating the effects of male-harming mitochondrial mutations; this process manifests itself in the form of reduced male fitness when the mitochondrial DNA meets an incompatible nuclear background (Innocenti et al. 2011, Patel et al. 2016, Camus & Dowling 2018, Perlman et al. 2015). Maternally-transmitted bacterial symbionts sometimes have extreme adverse effects on male fitness (e.g. male killing) or feminize males, but to our knowledge, this was not known for mitochondria (Camus et al. 2022). Our findings reaffirm the pivotal role of mitochondria in generating genomic conflicts that impact reproductive processes in both gonochoric and hermaphroditic animals. CMS has long been considered impossible in animals because of the highly compacted and structurally stable mitochondrial genome, in contrast to plants where mitochondrial genomes are larger and prone to structural rearrangements that occasionally give rise to chimeric genes responsible for CMS. Here we show that not only does CMS exist in animals, but its dynamics are not so different from those observed in plants, suggesting that mechanisms of genomic conflicts between the sexes, if they are arguably under different degrees of constraint, are fundamentally common to most sexual organisms.

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Author contributions

PD conceived the project. TL, NS performed the mitochondrial assembly. NS, JR, TL, FL participated in the phylogenetic analyses. FL analysed the data. FL and PD wrote the manuscript. JR, NS, TL, EL and SP revised the manuscript. KB, PD conducted the experiments. KB designed the PCR test. SP, EL performed the fieldwork sampling. AB, LK performed the molecular works (DNA/RNA extractions and libraries preparation).

Data availability statement

The genomic and transcriptomic reads, genome assembly, annotated mitochondrial genome are available in ENA under project number PRJEB50799. The alignments used to produce the phylogenetic trees and to compute the divergence between gastropods have been deposited on Zenodo (10.5281/zenodo.10521893). The accession numbers are in the materials and methods section of the manuscript.

Conflict of interest statement

The authors declare they have no competing interests.

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Supplementary materials

Supplementary Text 1 – Material and Methods

The species

P. acuta (Physidae, Hygrophila, Gastropoda) is a freshwater snail native to North America with a cosmopolitan distribution that lives in various freshwater habitats (Dillon et al. 2002). Individuals lay egg capsules that hatch in 7-10 days and reach sexual maturity in six to eight weeks at 25°C. *P. acuta* is a simultaneous hermaphrodite and preferential outcrosser; sperm and oocytes are produced within the same gland (ovotestis) and, unlike plants, external morphology is identical between male-sterile and male-fertile individuals – the difference can only be made on the basis of the ability to sire offspring in controlled pairings, or on the basis of dissection and sperm counts (David et al. 2022). This species is a preferential outcrosser and two male-fertile individuals, especially if both are virgin, usually mate rapidly when paired (for example, 70% of them had copulated as males (and 88.6% as females) in the first 45mn after encountering a virgin mate in David et al. 2022). Matings are one-sided: at a given time, one individual is acting as a male and the other as a female; but they often switch roles for the next mating, that can take place immediately after the first one. Self-fertilization can occur as a reproductive insurance strategy when sexually mature individuals are deprived of mates; in that case, the onset of reproduction is delayed by about two weeks ("waiting time") compared to individuals with available mates (Tsitrone et al 2003).

Samples studied

The *P. acuta* snails studied here were sampled in October 2019 at 4 sites near Lyon (Saint-Priest, Erevan, Crêt-2, and Irigny, Table S1), around the population where the male-sterile D mitotype was initially found (Lyon, France). Some of the snails were frozen to extract DNA while others were kept alive. At the same time, we carried out a whole-genome sequencing analysis on several French populations, and found individuals with mitotype K in one of them: "Canet des Maures" (CDM, Table S1). One individual from the CDM population was used to reconstruct the complete mitochondrial genome of type K (see below). For the introgression experiment (see below), we also used a laboratory albino population of *P. acuta*, maintained as a large outbred population for >80 generations, and initially derived from a mix of ten natural populations from Montpellier (300km south of Lyon, Noël et al. 2016). This population is composed only of male-fertile, N-mitotype individuals with an albino phenotype. Albinism is due to a single recessive allele, body colour can thus be used to ascertain paternity: when an albino mother produces a pigmented juvenile, the father must be a pigmented individual.

During all experiments, the snails were maintained in 75-ml plastic boxes at 25°C in groundwater under a 12:12 photoperiod and fed ad libitum with boiled organic lettuce. Water was changed once or twice a week.

Introgression experiments

The aim of the introgression protocol was to progressively replace the nuclear DNA originating from wild-caught ancestors from Lyon (focal), by the "naive" nuclear background of the albino N population from Montpellier (partner), while keeping the ancestral (N or K) mitochondrial lineage from Lyon. The introgressed lines ("matrilines") were propagated by maternal descent, keeping only pigmented individuals, while the sperm donors came at each generation from the albino population. From G1 to G17 the same protocol was used; focal (individual from the matriline) were pigmented and their partners albino, the male-fertility status of each focal was assessed at each generation on the basis of the offspring of albino partners.

Generating the focal mothers. To generate the first generation of focal individuals, we placed all adults from Erevan and Irigny (i.e., G0 individuals, Table S1) in one large aquarium per population for one week, so that each individual had many opportunities to be inseminated. We then let individuals lay separately in individual boxes and raised the offspring used as focal in the first generation of introgression (G1).

Introgression. Introgression occurred within "matrilines", defined as sets of focal individuals sharing the same ancestor by maternal descent, and hence the same mitochondrial DNA; there were 25 matrilines (12 K and 13 N). At each generation, individuals from each matriline (focals) were kept virgin and, once mature, paired with a virgin N-mitotype albino partner. Eggs laid by the focal were then used to generate the next generation. After the first generation focal individuals were heterozygous at the albinism locus and produced a mix of albino and pigmented juveniles in their progeny. We propagated the matrilines by keeping only pigmented individuals, to distinguish them from their mates. We usually kept three pigmented individuals in each matriline at each generation to avoid accidental loss of matrilines due to mortality or failure to reproduce. This process was continued for 17 successive generations.

Assessing male-fertility status. The male fertility status of focal individuals was assessed at each generation on the basis of the offspring laid by albino mates: the presence of pigmented juveniles indicated male-fertility. Starting from the G2, the focal was heterozygous at the albinism locus, thus from the third generation on, a 100% siring success resulted in a mix of 50% albino and 50% pigmented juveniles.

Monitoring mitotypes. All the G0 individuals were sacrificed and their mitotype was determined using PCR tests (Figure S2). Then, throughout the experiment we tested again individuals at G1 (N = 75, three offspring per G0 mother), G2 (N = 50, all the focals and their albino partner) and G7 (N = 50, two offspring per focal mother). In parallel we also constituted, using excess individuals in G1, two large populations by pooling K offspring in one aquarium, and N offpring in another. The aquaria were conserved to this day as separate populations and 24 individuals sampled in each aquarium were mitotyped in G25.

Behavioral and reproductive traits of K and N individuals

In the G1 and G5 generations of the introgression protocol, which respectively represent matrilines before and after introgression, we assessed the sexual phenotype of matriline individuals. Following a methodology akin to that employed for mitotype D, we evaluated both behavioral and fitness traits. Two to three individuals from each N or K matriline were isolated until reaching sexual maturity at 50 days. The assessment of behavioral traits took place during 45-minute observations of mating pairs, starting immediately after pairing. Each pair comprised one pigmented focal individual from the matriline and one N-mitotype albino mate, both partners being virgin prior to mating. The behaviors of both male and female individuals were recorded and timed, focusing on the climbing and sitting actions of the male-acting individual. Subsequent to the observations, the snails were left together for three days, re-isolated for egg-laying in separate boxes for a further three days, and then removed.

The male-fertility status was established through the snails ability to (i) induce oviposition in their virgin partners (the normal consequence of insemination of a virgin partner) and (ii) sire a majority of the offspring laid by the partner. Male-sterile individuals either failed to inseminate, so that the partner produced no or purely self-fertilized (100% albino) offspring, or inseminated their partner but with very little sperm, so that very few outcrossed offspring were produced. This second case, which we defined as less than 10 outcrossed offspring, was termed 'semi-sterility' and could occur either because the outcrossed offspring represented a very low proportion of all offspring or because the number of eggs laid was abnormally low. Complete male sterility was declared when absolutely no pigmented offspring were produced. In the remaining individuals, more than half of the partner's offspring were outcrossed, with a total exceeding 10; these individuals were called male-fertile. In G1, because focal individuals were homozygous pigmented, this number of outcrossed offspring was obtained directly by counting pigmented juveniles. In G5, because focal individuals were heterozygous, the criteria were adjusted to more than 25% and 5 pigmented juveniles (representing an expected 50% or 10 outcrossed juveniles respectively). In the main text, we report results obtained by contrasting male-fertile with pooled male-sterile and male-semisterile phenotypes. However the semisterile individuals are not numerous and the results remain qualitatively the same when they are excluded or pooled with the fertile category.

Mitochondrial genome sequence

To reconstruct the complete mitochondrial genome of type K, a whole genome sequencing was performed on one individual (CDM029) sample in a population sampled at Canet des Maures (Var, France). The DNA libraries were prepared at the GenSeq technical platform (University of Montpellier, France) using an Illumina kit (*Nextera DNA Flex*). The libraries were sequenced at Montpellier GenomiX (Montpellier, France) with an NovaSeq6000 lane and validated using DNA quantification (concentration and fragment size) on Fragment Analyzer (Standard Sensitivity NGS kit) and qPCR (ROCHE Light Cycler 480). The genome was assembled using MEGAHIT (Li et al. 2015, 2016).

To ensure the absence of contamination and chimera in the assembly of the K mitochondrial genome, coverage was checked along the assembled contig. To do this, short pair-end reads were mapped onto the assembled genome using BWA (Li and Durbin, 2009). The resulting SAM file was converted to BAM using Samtools (Li et al. 2009), then coverage was measured on the mitochondrial contig using the function depth of Samtools.

While the average genome coverage is 25X (sd=57), the average coverage of the mitochondrial genome is 555X (sd=63) and is constant throughout the mitochondrial genome (Figure S3).

In order to assess the variability of the K, transcriptomes were sequenced for ten cultivated individuals carrying the K mitotype. We bred ten K-mitotype individuals (three from Irigny and six from Erevan; from different G0 mothers with the long-long band pattern), and sent them for Illumina transcriptome sequencing (extraction and sequencing protocol as in David et al. (2022)). Using Trimmomatic (v 0.39, Bolger et al. 2014), adapters were clipped from the sequences, reads were scanned with a 4-base sliding window and trimmed when the average quality per base was below 15. Resulting reads longer than 36 bases were kept. Paired-end transcriptomes were assembled de novo using Trinity v2.3.2 (Grabherr et al., 2011). Mitochondrial genes of *P. acuta* N were blasted against the assembled transcriptomes in order to find mitochondrial contigs. The longest and most expressed contigs were kept and annotated using the MITOS web server (Bernt et al., 2013) to obtain sequences of protein-coding genes. The genes were aligned using the MAFFT software (Katoh et al. 2002) and their divergence calculated in R using the APE package (Parais *et al.* 2004). As the sequences of the ten K individuals were nearly identical (max 0.2% divergence), we assumed that they belonged to the same mitotype and used only one of them to determine their position in the phylogeny. For the phylogenetic analysis (*Physidae* phylogeny), we used the sequences from the individual E3A1 because the sequences were of better quality (few indeterminate bases). Transcriptome reads and assembly have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB50799.We carried out blasts on the 10 transcriptomes to recover the sequences of mitochondrial protein-coding genes. These blasts did not reveal any cases of heteroplasmy. In fact, in each case where two different contigs were blasted, the second contig had extremely low coverage compared with the first, compatible with cross-contamination. No cases with two normally covered contigs were found.

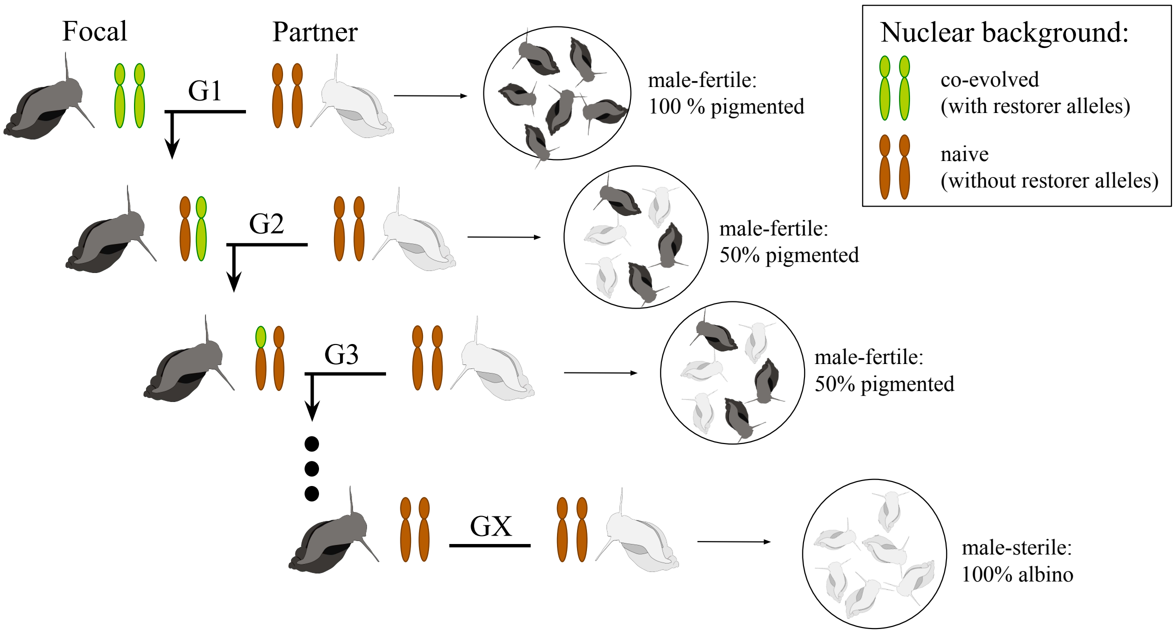

Figure S1: Introgression protocol. Nuclear DNA of the mitochondrial K originating from wild-caught ancestors from Lyon (called "co-evolved"), is progressively diluted into the "naïve" nuclear background of the N albino population kept in Montpellier, that provides sperm donors at each generation. Fathers are of the albino phenotype, mothers are pigmented. The G1 is 100% of Lyon origin (first-generation offspring of wild-caught mothers and fathers), introgressed generations are from G2 to G17. From the G2 on, mothers carry the albino allele in heterozygous form, inherited from the father. From the G3 on, offspring are a mix of albino and pigmented (heterozygous) juveniles at each generation, among which one pigmented individual is selected to become the mother of the next generation. The protocol was continued to G17.

Table S1: *Physa acuta* populations sampled in October 2019 near Lyon (France). Proportions of individuals carrying each of three mitotypes (K, N and D) are assessed from PCR tests made on wild-caught individuals (see Figure S2).

Site	Latitude	Longitude	K	N	D	number of individuals tested
Irigny	N 45°68’20.1”	E4°83’38.7”	12 %	85 %	3%	100
Erevan	N 45°74’42.1”	E 4°81’58.4”	32 %	68 %	0%	124
Crêt-2	N45°80’47.6”	E4°99’69.8”	0 %	100 %	0%	125
Saint-Priest	N45°71’72.3”	E4°93’01.8”	0 %	100 %	0%	114

Table S2: Accessions of mitochondrial sequences used in this study. Section 1: Accessions of complete mtDNA sequences that were used in Fig. 1A, in combination with our own sequences of *P. acuta* from Lyon. For each sample, elements of taxonomy are provided (from lower to higher: family, clade 2, clade 1). For *Physa gyrina*, we reassembled the mtDNA from the available transcriptome reads; the accession provided is that of the reads in the SRA database. **Section 2:** Accessions of COI sequences used in Fig. 1B. **Section 3**: Accession of COI sequences used in Fig. 1C. Shaded accessions correspond to sequences from this study.

Section 1: complete mt DNA sequences used in Figure 1A

Accession	Genus	species	Family	Clade 2	Clade 1
KP098541	Radix	balthica	Lymnaeidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
KP098540	Radix	auricularia	Lymnaeidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
JN564796	Galba	pervia	Lymnaeidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
MT862415	Planorbarius	corneus	Planorbidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
AY380567	Biomphalaria	glabrata	Planorbidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
SRX565294	Physa	gyrina	Physidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
JQ390525	Physa	acuta (isolate A)	Physidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
JQ390526	Physa	acuta (isolate B)	Physidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
MT483691	Physa	fontinalis	Physidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
PRJEB50799	Physa	acuta (Lyon)	Physidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
PRJEB50799	Physa	acuta (Lyon)	Physidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
PRJEB50799	Physa	acuta	Physidae	Hygrophila	Heterobranchia,

		(Lyon)			Euthyneura, Panpulmonata
PRJEB50799	Physa	acuta D	Physidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata
PRJEB50799	Physa	acuta K	Physidae	Hygrophila	Heterobranchia, Euthyneura, Panpulmonata

Section 2: COI sequences used in Figure 1B

Accession	Genus	species	type	Family
PRJEB50799	Physa	acuta (Lyon)	N	Physidae
PRJEB50799	Physa	acuta (Lyon)	N	Physidae
PRJEB50799	Physa	acuta (Lyon)	N	Physidae
JQ390525	Physa	acuta (USA, A)	N	Physidae
JQ390526	Physa	acuta (USA, B)	N	Physidae
PRJEB50799	Physa	acuta	D	Physidae
MT483691	Physa	fontinalis		Physidae
KP182981.1	Physa	acuta (Singapore)	K	Physidae
KP182982.1	Physa	acuta (Singapore)	K	Physidae
ON953193.1	Physa	acuta (South Africa)	K	Physidae
ON953200.1	Physa	acuta (South Africa)	K	Physidae
OK510774.1	Physa	acuta (North America)	K	Physidae
OK510777.1	Physa	acuta (North America)	K	Physidae
PRJEB50799	Physa	acuta (Erevan)	K	Physidae
PRJEB50799	Physa	acuta (Erevan)	K	Physidae
PRJEB50799	Physa	acuta (Erevan)	K	Physidae
PRJEB50799	Physa	acuta (Erevan)	K	Physidae
PRJEB50799	Physa	acuta (Erevan)	K	Physidae
PRJEB50799	Physa	acuta (Irigny)	K	Physidae
PRJEB50799	Physa	acuta (Irigny)	K	Physidae

Section 3: COI sequences of gastropods used in Figure 1C

num_accession	species	species_short	genus	family	cat_dist_Pacuta	clade2	clade1	subclass	type
NC_023253	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
JQ390526	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
GU247996	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
GU247995	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
GU247993	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651203	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651193	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651192	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651188	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651186	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651185	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651181	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651174	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651203	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY282589	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038389	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038372	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038371	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038368	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038367	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038366	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038365	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038361	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038360	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038357	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038356	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
EU038355	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KJ769128	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KJ7691	Physa_acuta	acuta	Physa	physid	0	Hygro	Panpul	Heterobr	N

27				ae		phila	monata	anchia	
KJ769126	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KJ769125	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KJ769124	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KJ769123	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
JQ390526	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
JQ390525	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280445	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280444	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280443	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280442	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280441	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280440	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280439	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280438	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280437	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280436	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
KT280435	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651184	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651183	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651177	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651176	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651171	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
AY651170	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
E2_Folmer	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	K
E11_Folmer	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	K
E17_Folmer	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	K
E19_Folmer	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	K
E26_Folmer	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	K
I4_Folmer	Physa_acuta	acuta	Physa	physidae	0	Hygrophila	Panpulmonata	Heterobranchia	K
I5_Folm	Physa_acuta	acuta	Physa	physid	0	Hygro	Panpul	Heterobr	K

er				ae		phila	monata	anchia	
AY651198	Physa_zionis	zionis	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038396	Physa_sp_A	sp_A	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038395	Physa_sp_A	sp_A	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038392	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038391	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038387	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038386	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038385	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038384	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038383	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038382	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038380	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038379	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038378	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038359	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038358	Physa_ancillaria	ancillaria	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038377	Physa(Aplexa)_elongata	elongata	Physa(Aplexa)	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038375	Physa(Aplexa)_elongata	elongata	Physa(Aplexa)	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038363	Physa_pomilia	pomilia	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038354	Physa_pomilia	pomilia	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038353	Physa_pomilia	pomilia	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651196	Physa_hendersoni	hendersoni	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651195	Physa_hendersoni	hendersoni	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651194	Physa_hendersoni	hendersoni	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038398	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038374	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038373	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651200	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651199	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY6511	Physa_gyrina	gyrina	Physa	physid	1	Hygro	Panpul	Heterobr	N

97				ae		phila	monata	anchia	
AY651191	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651187	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651182	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651178	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651173	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651172	Physa_gyrina	gyrina	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038369	Physa(Stenophysa)_marmorata	marmorata	Physa(Stenophysa)	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
EU038370	Physa(Stenophysa)_marmorata	marmorata	Physa(Stenophysa)	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651190	Physa_fontinalis	fontinalis	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651189	Physa_fontinalis	fontinalis	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651205	Physa_spelunca	spelunca	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
AY651204	Physa_spelunca	spelunca	Physa	physidae	1	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_01	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_02	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_03	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_04	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_05	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_06	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_07	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_08	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	D
P_ac_09	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_10	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_11	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_12	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_13	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	D
P_ac_14	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_15	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_16	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_17	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_18	Physa_acuta	acuta	Physa	Physid	0	Hygro	Panpul	Heterobr	N

				ae		phila	monata	anchia	
P_ac_19	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_21	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_22	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_23	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_24	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	D
P_ac_25	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_26	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_27	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_28	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_29	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	D
P_ac_30	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_31	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_32	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_33	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	D
P_ac_34	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
P_ac_35	Physa_acuta	acuta	Physa	Physidae	0	Hygrophila	Panpulmonata	Heterobranchia	N
MH087513	Pseudosuccinea_columella	columella	Pseudosuccinea	Lymnaeidae	2	Hygrophila	Panpulmonata	Heterobranchia	N
KM612193	Lymnaea_stagnalis	stagnalis	Lymnaea	Lymnaeidae	2	Hygrophila	Panpulmonata	Heterobranchia	N
MN601426	Galba_mweruensis	mweruensis	Galba	Lymnaeidae	2	Hygrophila	Panpulmonata	Heterobranchia	N
AY651207	Biomphalaria_obstructa	obstructa	Biomphalaria	Planorbidae	2	Hygrophila	Panpulmonata	Heterobranchia	N
MF458795	Planorbarius_corneus	corneus	Planorbarius	Planorbidae	2	Hygrophila	Panpulmonata	Heterobranchia	N
KM612167	Planorbella(Helisoma)_trivolvis	trivolvis	Planorbella(Helisoma)	Planorbidae	2	Hygrophila	Panpulmonata	Heterobranchia	N
KF958031	Planorbella(Helisoma)_campanulata	campanulata	Planorbella(Helisoma)	Planorbidae	2	Hygrophila	Panpulmonata	Heterobranchia	N
EU038397	Gyraulus_parvus	parvus	Gyraulus	Planorbidae	2	Hygrophila	Panpulmonata	Heterobranchia	N
EU038390	Glyptophysa_sp	sp	Glyptophysa	Planorbidae	2	Hygrophila	Panpulmonata	Heterobranchia	N

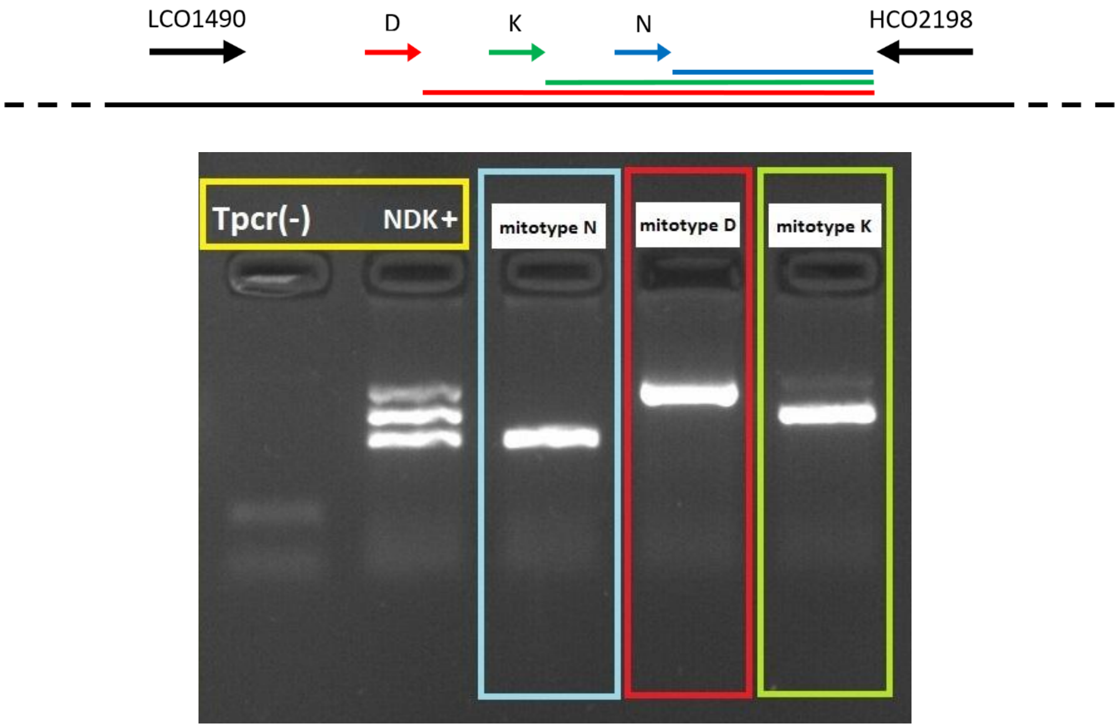

Figure S3: Mitotype identification by PCR. One PCR was performed per individual using five primers per PCR, the two external, universal COI “Folmer” primers and three internal specific primers (ATTACACTTAGCTGGGTTATCA for N, GGGACAACTAGGGTCTTAACTAC for D and TTGCTCCCTCCTTCTTTCACA for K mitotypes, blue, red and green respectively). PCR products were run on an agarose gel (2%) giving a typical pattern depending on the mitotype (an example of gel with individuals from three maternal lines of each mitotype is given). Short fragments span 288 bp for N, 607 bp for D and 426 bp for K respectively; if none of the N, D, or K primers matches a 705 bp fragment is amplified (represented by the black line). Amplifications were carried out in 10-µL final reaction volume comprising 5-µL of 2X Sigma-Aldrich REDExtract-N-Amp PCR ReadyMix, 0.25-µL of each primer (10 µM), 1.75-µL of PCR-grade Water. The PCR conditions were as follows: 5 min at 95°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 50°C and extension for 40 sec at 72°C, completed with a final extension step for 10 min at 72°C.

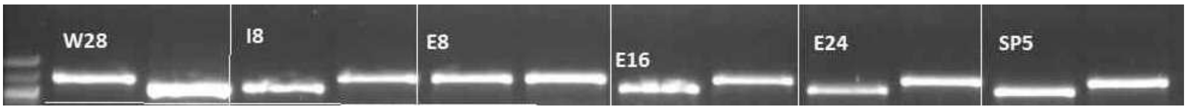

Figure S2: One example of pattern found using the “N versus D” PCR test from David et al. 2022. This test uses two separate amplifications to produce either a long band- short band pattern typical of the D mitotype (example: individual W28, an individual from the Lyon Autoroute population), or a short-long pattern typical of the N mitotype (I8, an individual from Irigny; E16 and E24, two individuals from Erevan; SP5, an individual from Saint-Priest). The E8 individual (from the Erevan population) shows a long-long pattern indicating that none of the internal primers specific to D or N has been able to block the amplification of the long fragment by the external, universal primers. Individuals such as E8 turned out, after sequencing, to belong to a third very divergent mitotype named K.

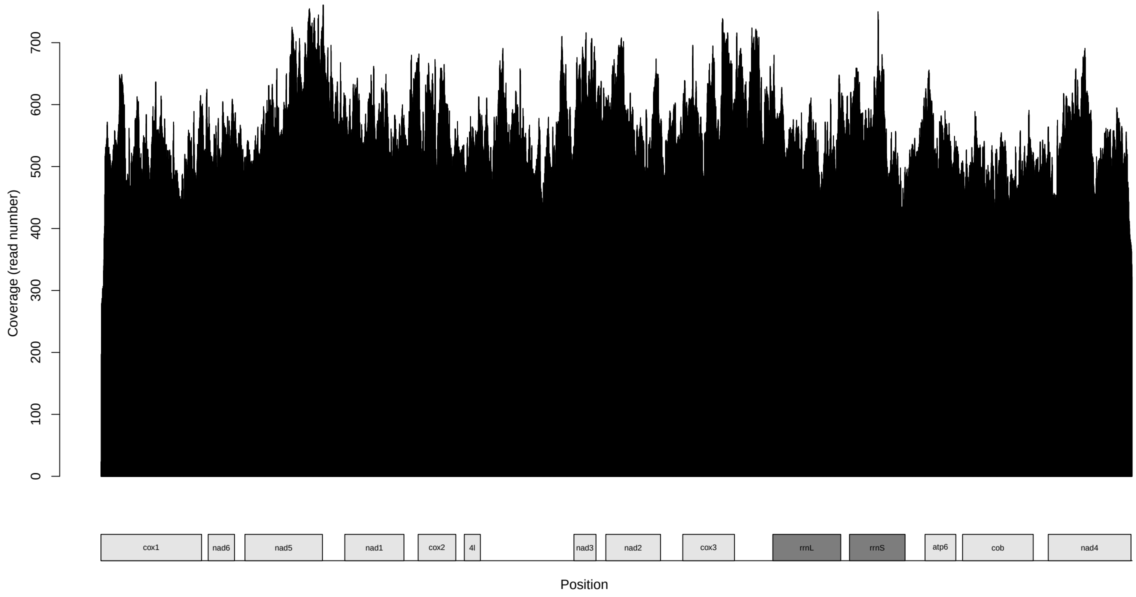

Figure S4: Coverage along the K mitochondrial genome.

Table S3: Results of linear models on behavioral, copulation and reproductive traits of *P. acuta* K and N at G1 and G5. Performances of N and K mitotypes are reported either as trait means and SE (for quantitative traits) or as successes / failures (success = at least one copulation observed, or at least one egg or juvenile produced, failure = no copulation, egg or juvenile). After the first generation focal individuals were heterozygous at the albinism locus and produced a mix of albino and pigmented juveniles in their progeny. Thus the number of pigmented offspring was divided by two from G1 to G5 for the N individuals.

The last columns indicate the random effects included in the model and the distribution of the variable. An “individual” (ind) random effect was added when appropriate in order to account for overdispersion (GLMMs). The “matriline” random effect accounts for correlations between related individuals that have inherited copies of the same mitogenome by maternal descent from a common ancestor collected in natura. (a)The difference in female fertility between K and N individuals in G5 remained non-significant (means ± SE : N : 65.88±3.63 ; K : 60.22±4.89 ; LRT test $\chi^2 = 0.69$, $P = 0.41$) when only male-sterile K individuals and male-fertile N individuals were included in the comparison.

Traits	Generations	Number of pairs	Mean ± se success / failures		Test of type effect df = 1	Random effects included	Model type
			Type N	Type K			
Behavioral and copulation data							
Male time	G1	63	0.29±0.04	0.30±0.03	$\chi^2 = 0.001$, $P = 0.98$	Matriline	Gaussian
	G5	52	**0.30±0.03**	**0.12±0.03**	**$\chi^2 = 13.85$, $P = 1.9\ 10^{-5}$**		
Male copulation (yes/no)	G1	63	28/7	24/4	$\chi^2 = 0.10$, $P = 0.75$	Matriline, ind	Binomial
	G5	52	**22/4**	**10/16**	**$\chi^2 = 19.56$, $P = 9.8\ 10^{-6}$**		
Female time	G1	63	0.25±0.03	0.21±0.03	$\chi^2 = 0.78$, $P = 0.38$	Matriline	Gaussian
	G5	52	0.26±0.03	0.30±0.04	$\chi^2 = 0.88$, $P = 0.35$		
Female copulation (yes/no)	G1	63	25/10	19/9	$\chi^2 = 0.13$, $P = 0.71$	Matriline, ind	Binomial
	G5	52	21/5	22/4	$\chi^2 = 0.44$, $P = 0.51$		
Fitness traits							
Number of pigmented offspring laid by the albino partner	G1	63	60.74±6.11 (37/4)	49.07±5.81 (22/5)	$\chi^2 = 1.85$, $P = 0.17$	Matriline	Gaussian
	G5	52	**21.50±1.86 (25/1)**	**6.00±1.82 (8/18)**	**$\chi^2 = 35.58$, $P = 2.44\ 10^{-9}$**		
Female fitness	G1	63	55.00±4.02	53.00±4.85	$\chi^2 = 0.06$, $P = 0.81$	Matriline	Gaussian
	G5	52	65.92±3.49	62.50±4.04	$\chi^2 = 0.16$, $P = 0.69$		

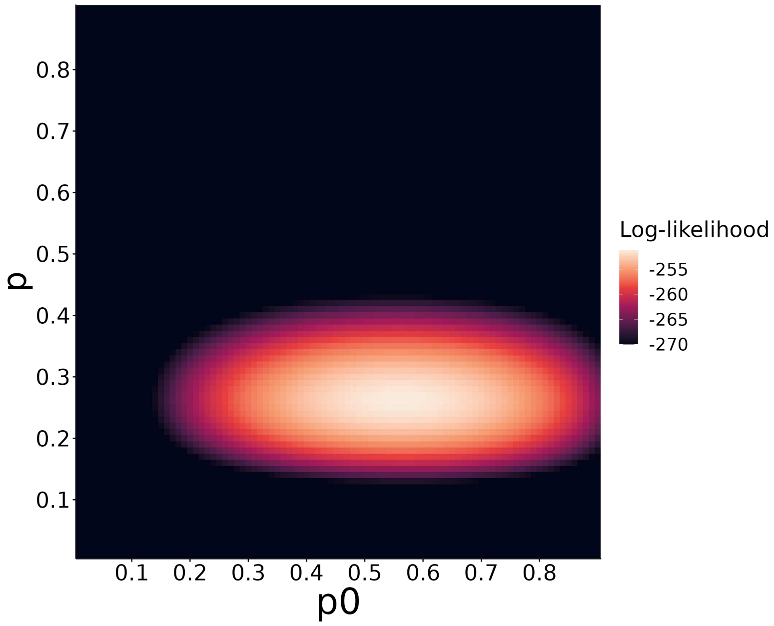

Figure S5: Maximum likelihood estimation for the restorer frequency in the focal population K (p0) and in the albino population (p). Log-likelihood surface in the (p0, p) plane.

Table S4: Similar sequences found by blasting the COI sequence of the K mitotype in Genbank.

Species	Accession	% identity	Publication
Physa sp.	KP182981.1	100	Ng, T. H., Tan, S. K., & Yeo, D. C. (2015). Clarifying the identity of the long-established, globally-invasive Physa acuta Draparnaud, 1805 (Gastropoda: Physidae) in Singapore. *BioInvasions Record*, *4*(3).
	KP182982.1	100	
Physa sp.	ON953193.1	100	Molaba, G. G., Molefe-Nyembe, N. I., Taioe, O. M., Mofokeng, L. S., Thekisoe, O. M. M., & Mtshali, K (2022). Molecular Characterization of Larval Trematodes from Naturally Infected Freshwater Snails in South Africa. *Available at SSRN 4254241*.
	ON953200.1	97.37	
Physinae sp.	OK510774.1	93.25	Young, M. K., Smith, R., Pilgrim, K. L., & Schwartz, M. K. (2021). Molecular species delimitation refines the taxonomy of native and nonnative physinine snails in North America. *Scientific reports*, *11*(1), 1-13.
	OK510777.1	93.09	

Table S5: Conditional probabilities used to assess the maximum likelihood estimation. Maximum likelihood estimation was used to determine the frequency of restorer alleles, if any, in the Lyon population (p0) and in the albino population (p). The data used are the male-fertility phenotypes of 69 individuals over 17 generations and 12 K matrilines – at each generation several daughters of the same mother are raised and mated in parallel (among which only one propagates the line); this increases the sample size, and provides backup individuals in case one of them fails to produce live offspring. We hypothesized that the restored phenotype is controlled by a single dominant allele (*f*). Male-fertile individuals can be heterozygous (*fs*) or homozygous (*ff*) while sterile ones are all *ss*. The conditional likelihood of each individual (L) is computed iteratively based on the male-fertility status of (i) the mother, (ii) its own phenotype, (iii) all her daughters. From G1 to G17, the probabilities were calculated iteratively as indicated in the table. Let us take the example of a male-fertile offspring of a fertile mother, that only has fertile daughters (top line of the table). The mother is either *ff* or *fs* with probabilities $\frac{p(ff)_{mom}}{p(F)_{mom}}$ and $\frac{p(fs)_{mom}}{p(F)_{mom}}$ given by the previous iteration of the computation (where $p(F)_{mom} = p(ff)_{mom} + p(fs)_{mom}$. The focal individual can be *ff* or *fs* given that it has produced no sterile daughter ; its probability to be *ff*, conditional on maternal and daughter phenotypes, and knowing that the paternal allele is *f* with probability p, is $p(ff) = \frac{p(ff)_{mom}}{p(F)_{mom}} p + \frac{p(fs)_{mom}}{p(F)_{mom}} \frac{1}{2} p$. The probabilities of the *fs* genotype can be found the same way, and the likelihood of the observed fertile daughter (conditional on the phenotype of her mother and daughters) is p(*ff*)+p(*fs*). The case of a fertile individual with fertile mother and at least one sterile daughter is treated separately because in that case the individual must be *fs* so p(*ff*) is 0. For a sterile individual with a fertile mother (irrespective of daugthers' phenotypes) the mother must be heterozygous and the likelihood of the individual is $\frac{1}{2}(1-p)$.

At G1, because the male-fertility status of the mother was unknown, probabilities were defined as follows. If the focal individual was fertile :

$$p(fs) = 2p_0(1-p_0)$$

$$L = p(ff) + p(fs)$$

with $p(ff) = 0$ if the individual had at least one sterile daughter, or $p(ff) = p_0^2$ if all the daughters were fertile.

If the focal individual was sterile:

$$p(fs) = 0$$

$$p(ff) = 0$$
$$L = (1 - p_0)^2$$

The (conditional) likelihood of each individual was then log() transformed. All the conditional log-likelihoods were summed up over individuals and matrilines to find the sample likelihood, and different sets of values for p and p0 were explored.

Mother	Daughters	Focal individual	Probabilities and likelihood values
Fertile	all fertile	Fertile	$p(ff) = \frac{p(ff)_{mom}}{p(F)_{mom}} p + \frac{p(fs)_{mom}}{p(F)_{mom}} \frac{1}{2} p$ $p(fs) = \frac{p(ff)_{mom}}{p(F)_{mom}} (1 - p) + \frac{p(fs)_{mom}}{p(F)_{mom}} \frac{1}{2}$ $L = p(ff) + p(fs)$
	≥ 1 sterile	Fertile	$p(ff) = 0$ $p(fs) = \frac{p(ff)_{mom}}{p(F)_{mom}} (1 - p) + \frac{p(fs)_{mom}}{p(F)_{mom}} \frac{1}{2}$ $L = p(ff) + p(fs)$
		Sterile	$p(ff) = 0$ $p(fs) = 0$ $L = \frac{1}{2}(1 - p)$
Sterile	all fertile	Fertile	$p(ff) = 0$ $p(fs) = p$ $L = p(ff) + p(fs)$
	≥ 1 sterile	Fertile	$p(ff) = 0$ $p(fs) = p$ $L = p(ff) + p(fs)$
		Sterile	$p(ff) = 0$ $p(fs) = 0$ $L = (1 - p)$

Table S6: Divergence observed between K mitotype and N and D mitotype at 11 mitochondrial genes. The percentage of divergence on the COX1 gene was estimated on 13 K mitotype (our sequences plus available sequences from Genbank, Table S2), 1 D mitotype and 5 N mitotype sequences (there are many published N sequences but we kept only those for which other mitochondrial genes are available, see M&M). For the other ten genes, we took only one K mitotype as all the available sequences were only from this study and were all identical.

Gene	Divergence K/N	Divergence K/D	Divergence N/N
ATP6	41.2% (±0.5% SD)	44.1%	7.3% (±4.1% SD)
COX1	22.8% (±0.8% SD)	28.1% (±0.4% SD)	2.2% (±1.6% SD)
COX2	40.3% (±1% SD)	42.9%	13.8% (±7.2% SD)
COX3	43.3% (±0.3% SD)	42.4%	4.16% (±2.4% SD)
CYTB	35.8% (±0.4% SD)	40.9%	4.27% (±2.3% SD)
NAD1	45.2% (±0.8% SD)	50.4%	12.50 (±6.8% SD)
NAD2	57.4% (±0.6% SD)	57.4%	8.12% (±4.9% SD)
NAD3	47.8% (±0.9% SD)	52.3%	10.42% (±6.2% SD)
NAD4	48.8% (±0.7% SD)	52.1%	3.99% (±1.6% SD)
NAD5	42.9% (±0.2% SD)	47.5%	5.82% (±3.5% SD)
NAD6	52.9% (±1% SD)	61.0%	8.00% (±4.8% SD)

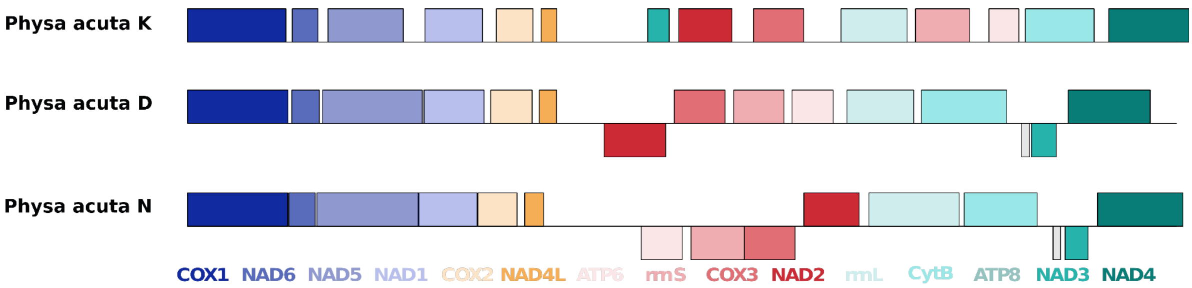

Figure S6: Structural variants in the assembled mitogenomes of D and N individuals of *P. acuta*. In *Physa acuta* K, rearrangements have occurred at the level of ATP8 and even if certain pieces of mitochondrial DNA look like the ATP8 sequence, we have several hypotheses as to its position. We therefore believe that ATP8 is present in the genome but that it is too divergent (like the rest of the genome) to be detected with certainty. In *Physa acuta* D we were lucky that ATP8 remains in synteny with NAD3, allowing us to be confident that the gene is located there.

CHAPITRE 2

EVOLUTION EXPERIMENTALE

CHAPITRE 2 – CONTEXTE

Les modèles s'intéressant au maintien du polymorphisme SMC/restaurateurs, prédisent des oscillations de fréquences produisant des dynamiques cycliques (Gouyon et al. 1991, Bailey et al. 2003, Dufay et al. 2007). Ces cycles sont permis par une sélection fréquence-dépendante négative et leur amplitude dépend à la fois du coût de la restauration et de l'avantage femelle.

Dans ce chapitre, nous décrivons la constitution de populations expérimentales à partir d'individus fondateurs N et K ayant le même fond nucléaire. Les individus étant des descendants de la population d'origine des K, leur fond nucléaire est supposé avoir un fort potentiel de restauration. Nous cherchons à constituer deux types de populations, des populations pour lesquelles la restauration est fixée et la fréquence SMC évolue et des populations pour lesquelles la SMC est fixée la fréquence des restaurateurs évolue. Avec ces populations expérimentales, nous étudions deux prédictions des modèles SMC/restaurateur : i) celle prédisant une diminution de la fréquence de SMC en raison de son coût (manuscrit 1), (ii) celle prédisant que la restauration est soumise à un régime de sélection qui dépend de la fréquence de SMC et qu'un coût silencieux de la restauration entraine une diminution de la fréquence des restaurateurs (manuscrit 2).

- MANUSCRIPT 1 -

Experimental evolution reveals counter-selection on Cytoplasmic Male Sterility in resistant nuclear backgrounds

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In preparation

Abstract:

Genomic conflicts arise when different compartments of the genome are subject to selection pressures favoring opposite effects on a phenotype. Thus, resistances or suppressors may evolve, that counter-select a selfish variant and prevent its fixation. The interplay of selfish alleles and resistance is likely to generate strong selection and fast evolutionary trajectories; however such dynamics are usually inferred from measures of fitness-related traits rather than directly observed. This applies to the textbook example of genetic conflict: cytoplasmic male sterility (CMS) in plants, where maternally-transmitted genes suppress the male function of hermaphrodites, and nuclear genes called restorers of male-fertility, frequently evolve as resistance mechanisms. Here we used a new biological model, a freshwater snail with rapid generations, to test whether this resistance indeed imposes counter-selection on CMS. During 11 generations of experimental evolution, we observed rapid decreases in frequency of CMS-associated mitogenomes in a nuclear background restoring male fertility, consistent with a fitness disadvantage of ~20% compared to non-CMS mitogenomes. Costs of CMS are also expressed in the form of reduced hatching rate of isolated clutches, but this reduction seems insufficient to explain the large fitness differential within populations, suggesting that counter-selection may be amplified by strong competition.

Key words: cytoplasmic male sterility, restoration, experimental evolution, cost, *Physa acuta*

Introduction

Gynodioecy is a common sexual system in plants (Dufay et al., 2014) characterized by the coexistence of hermaphrodites and male-sterile individuals - functionally females (Delph et al., 2007; Touzet, 2012). Gynodioecy is thought to be a possible intermediate stage in the transition from hermaphroditism to dioecy (Bawa, 1980; Barrett, 2002). In plants, this mating system is generally determined by the interplay of nuclear and cytoplasmic genes: cytoplasmic (usually mitochondrial) genes causing male sterility are called CMS genes (for Cytoplasmic Male Sterility) and nuclear genes opposing the effects of CMS are called restorers of male fertility (de Haan et al., 1997). Hence, individuals carrying a CMS gene express a female phenotype only if restorer alleles are absent from the nuclear background. In several gynodioecious species multiple cytotypes and nuclear restorer alleles coexist in populations (e.g., maize Gabay-Laughnan & Laughnan, 1994, *Plantago lanceolata* de Haan et al. 1997, *Thymus vulgaris* Manicacci et al., 1997, *Silene nutans* Garraud et al., 2011, *Beta vulgaris ssp. maritima* Laporte et al., 2001, *Plantago coronopus* van Damme et al., 2004). The CMS-restorer system is a textbook example of genetic conflict, as selection favours cytoplasmic genes which maximise female allocation on the one hand, and nuclear genes that restore the balance between female and male functions on the other hand. For this reason, a large body of theoretical studies have elaborated models to predict changes in frequency of CMS and restorer alleles, and to understand how and when cytoplasmic and nuclear polymorphisms can persist in populations (Charlesworth, 1981; Gouyon et al., 1991; Couvet et al., 1998; Bailey et al., 2003).

The evolutionary dynamics of CMS mainly depends on the positive effects of CMS alleles on female fitness in the absence of restorers (called female advantage, Lewis, 1941; Lloyd, 1975) and on the negative pleiotropic effects of restorer alleles on either male or female fitness (i.e., cost of restoration, Charlesworth 1981, Gouyon et al. 1991, Maurice et al., 1994). Female advantage can result from reallocation of resources saved from pollen production or from avoidance of self-fertilization by male-sterile individuals (e.g. Avila-Sakar & Domínguez, 2000). For example, Shykoff et al. (2003) found that females produced more flowers, fruits and seeds than hermaphrodites, and that their seeds were larger and germinated better. Similar observations of female advantage are common in gynodioecious plants, although quantitatively very variable (Dufay & Billard, 2012). In contrast only a few studies have empirically observed costs of restoration on the female function (*Plantago lanceolata* de Haan et al. 1997, *Phacelia dubia* Del Castillo & Trujillo, 2009) or on the male function of non-CMS individuals (*Lobelia siphilitica* Bailey, 2002). Even if more such data were available, measures of individual fitness traits are not easily combined to obtain an estimate of realized fitness of all genotypes in an evolving population. Thus, measures of reproductive traits are not precise and/or complete enough to obtain fitness values that one could directly inject into evolutionary models, and to test whether they correctly explain the maintenance of cytonuclear polymorphism (e.g. Barr, 2008; Cuguen et al., 1994; Koelewijn & Van Damme, 1995). As an alternative to detailed estimates of fitness traits, experimental evolution in artificial populations can be used to observe selection in action, though it requires several generations, and therefore lots of time. In essence the maintenance of polymorphism requires that selection on CMS changes sign as the restorer increases in frequency (being positive when the restorer is rare, and negative when it is frequent), and vice-versa, in such a way that fixation is prevented at both loci (Charlesworth 1981, Gouyon et al. 1991, Dufaÿ et al., 2007). Here we use experimental evolution to test the idea that when restoration is frequent in the population, CMS is indeed counter-selected, as is theoretically required to prevent its fixation. To this end, we use a recently discovered biological model of CMS that has the advantage of fast generations and easy molecular determination of CMS.

CMS has been looked for in animals for a long time without success (Vellnow et al., 2017). However, it has been recently reported in *Physa acuta*, a freshwater snail (David et al., 2022). In this species, two mitochondrial types conferring male sterility, D and K, have been discovered so far. The D type was discovered first, but evidence for corresponding nuclear restoration is still lacking (David et al. 2022). Recently however, the second CMS mitotype K revealed a different situation, with evidence that K-specific restorer genes are abundant in natural populations (Laugier et al., 2024). Owing to its short generation time (around 2 months), this snail system is a more tractable model than most gynodioecious plants to launch an experimental evolution protocol.

Our approach is inspired by theoretical studies that predict, under wide parameter ranges, limit cycles in CMS (oscillations of female frequencies) through a form of frequency-dependent selection (D. Charlesworth, 1981; Delannay et al., 1981; Frank, 1989; Gouyon et al., 1991; Bailey et al., 2003; Dufaÿ et al., 2007; Dornier & Dufay, 2013). Dufay et al. (2007) modelled the competition between CMS and fertile cytotypes within a gynodioecious population, and described a typical cycle in five steps (Figure 1). Polymorphism may be lost at any of these stages if the parameters are not adequate, for example, if female advantage is very high and cost of restoration low, CMS may go to fixation followed by restorers (reversion to 100% hermaphroditic state), or if restorers have a low cost they may go to fixation and CMS may be lost. High variation in frequencies of male-sterile individuals among populations of gynodioecious species, for example in *Plantago coronopus* (Koelewijn & Van Damme, 1995), *Beta maritima* (Cuguen et al.,1994), *Plantago lanceolata* (de Haan et al. 1997), *Raphanus sativus* (Murayama et al., 2004), *Daphne laureola* (Medrano et al., 2005), *Nemophila menziesii* (Barr 2008), is consistent with these populations being at different phases of the limit cycle, thereby indirectly supporting the conceptual model. However, there usually remains ambiguity as to whether a certain female frequency signifies a high frequency of CMS and of restorers or simply a low prevalence of CMS. None of the phases of the theoretical cycle has been actually observed by monitoring the dynamics over several generations. Our information on the evolution of these systems therefore remains mostly indirect.

In this study, we used *Physa acuta* to start exploring empirically whether actual evolutionary trajectories resemble theoretical scenarios, focusing, for a start, on one phase of the theoretical cycle. The cyto-nuclear model of gynodioecy applies to the recently discovered mitotype K. The K individuals sampled in natural populations so far carried restoration at a high frequency, an initial situation corresponding to phase 4 of the cycle (Figure 1) where CMS is expected to decrease. We followed the evolution of the K mitotype, starting from different frequencies, during 11 generations in experimental populations. We predicted (according to the model above) that the frequency of K should decrease in these populations. In addition, we measured components of male and female fecundity of both CMS (K) and non-CMS (N) individuals from our experimental populations to (i) try to identify which traits could be involved in the observed selection on CMS, if any; and (ii) check that restoration remained at high frequency during experimental evolution, so that conditions of selection on CMS remained approximately constant.

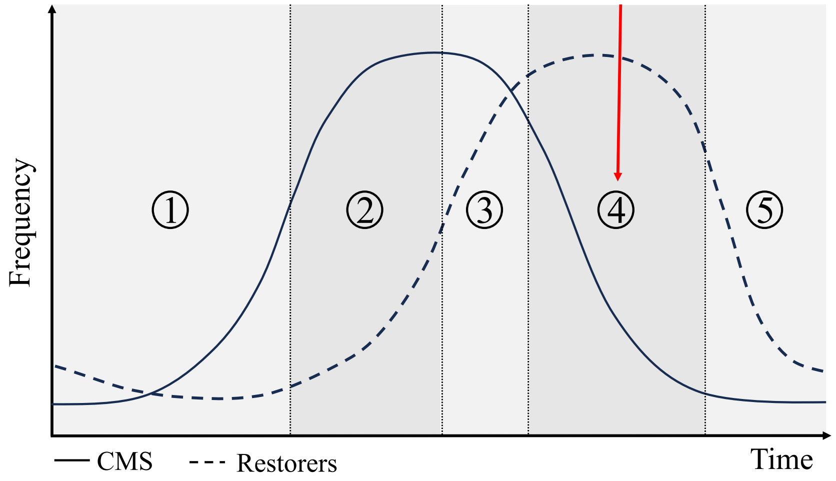

Figure 1: Conceptual figure of the dynamics between the CMS and the restorer genes. From Dufay et al. (2007), CMS and restorers can follow evolutionary cycles that can be

described in five steps, starting from a population with initially low frequency of CMS and restorer alleles: 1) CMS benefits from a female advantage and increases in frequency, 2) once CMS is frequent, the restorers have a selective advantage and increase in frequency, 3) as a consequence, the number of females decreases, as CMS is increasingly carried by restored hermaphrodites, 4) CMS decreases in frequency in favour of the male-fertile cytotype, 5) among the -now majoritarian- individuals with a male-fertile cytotype, those that carry restorer alleles pay a cost of restoration, ; therefore restorer alleles are counter-selected and the population reverts back to is initial state (rare CMS and rare restorer alleles). The red arrow underlines the phase of the cycle we expect to be relevant to the experimentally evolving populations in this paper.

Material and methods

The species

P. acuta (Physidae, Hygrophila, Gastropoda) is a freswhater snail native with cosmopolitan distribution (Dillon et al., 2002). Individuals lay egg capsules that hatch in 7-10 days; sexual maturity is reached between six and eight weeks at 25°C. *Physa acuta* is a simultaneous hermaphrodite and preferential outcrosser; sperm and oocytes are produced within the same gland (ovotestis) and, unlike plants, external morphology is identical between male-sterile and male-fertile individuals – the difference can only be made based on the ability to sire offspring in controlled pairings, or based on dissection and sperm counts (David et al. 2022).

In this species, Laugier et al. (2024) discovered a novel cytoplasmic male sterility (CMS) associated with a distinct mitochondrial genome, referred to as the K mitotype. Notably, the K mitotype shares key characteristics with the first CMS-associated mitotype found (the D mitotype, David et al. 2022), including male sterility and significant divergence from the normal mitochondrial type (called N). However, the expression of male sterility in K (unlike in D) depends on the nuclear background. Initially, K individuals collected in natural populations near Lyon (France) appeared male-fertile, displaying mating behaviors and a male fitness similar to those of the N mitotype. After 17 generations of introgression into the background of a laboratory population, however, most K individuals (67%) became male-sterile, showing that the male fertility in the natural population was due to restorer alleles (at a yet unknown number of loci) present at high frequency in this population, and rare in the laboratory population.

Source populations

To form our experimental populations, we used two laboratory populations of *P. acuta*, one 100% N and the other 100% K, each of which was maintained separately as a large outbred populations for seven generations. Both were initially derived from a mix of the two natural populations from Lyon where the K mitotype was first found, sampled in October 2019 (Erevan and Irigny, Laugier et al. 2024). In these natural populations, the restoration was estimated to have a high frequency (22 of 27 K individuals were recorded as male-fertile).

To assess male fecundity, we also used a laboratory albino population of *P. acuta*, maintained as a large outbred population for >80 generations, and initially derived from a mix of ten natural populations from Montpellier (300km south of Lyon, Noël et al., 2016). This population is composed only of male-fertile, N-mitotype individuals with an albino phenotype. Because albinism is due to a single recessive allele, body colour can thus be used to ascertain paternity of descendants from crosses with individuals from experimental populations: when an albino mother produces a pigmented juvenile, the father must be a pigmented individual.

Experimental evolution design

We followed 15 experimental populations over 11 generations. Each experimental population was initiated as a mix of K and N individuals with one of four possible initial frequencies: 5%K, 25%K, 50%K, or 75%K. The treatment 5%K was replicated three times (replicates 5%K^A, 5%K^B and 5%K^C), the treatments 25%K, 50%K were replicated five times (replicates: 25%K^A to 25%K^E and 50%K^A to 50%K^E respectively) and the treatment 75%K was replicated six times (replicates 75%K^A to 75%K^F). The populations were propagated by 150 adult individuals each and replicates were not all synchronous. The different numbers of replicates per treatment reflect the fact that we lost some replicates and had to restart new ones to replace them in the course of the generations. A first wave of populations (all but 75%K^F, 5%K^B, 5%K^C) were founded between February and May 2021; of which we lost two (75%K^B, 75%K^C) at respectively the second and first generation due to a technical problem (bacterial contamination). Population 75%K^F was started in November 2021, and 5%K^B, 5%K^C were started in January 2022.

Creating the experimental populations

We aimed to constitute the populations so that the founding N and K individuals had the same nuclear background, thereby avoiding a linkage disequilibrium between their mitochondrial and nuclear background. For that purpose, the founding populations were established with descendants of pair-crosses between the 100% N and 100% K laboratory populations (Figure 2). From these populations, 88 N and 88 K individuals (hereafter G_0 individuals) were raised in isolation to sexual maturity (age 50 days) and then paired (one K with one N) for three days. All the G_0 individuals were re-isolated in separate boxes for three days to lay eggs, then sacrificed to confirm their mitotype using PCR tests. These crosses ensure that among the offspring of a given pair, individuals with mother K and father N have their maternal K mitotype, but otherwise the same nuclear background as individuals with mother N and father K.

Three weeks after the eggs hatched, the (still immature) offspring of all pairs were grouped in two tanks: one with the N mitotype offspring (mother N father K) and one with the K mitotype offspring (mother K father N). After ten days, we used these offspring to create the first generation of experimental populations (G_1) based on the initial frequencies chosen and for a total of 200 individuals per population. One week after, we randomly removed 50 individuals per population and therefore kept 150 in each population. We replicated this protocol four times in total: (i) February 2021 to generate all the populations of the A and B replicates except 5%K^B, (ii) April 2021 to generate all the populations of the C, D and E replicates except 5%K^C (iii) September 2021 to generate the F replicate (iv) October 2021 to generate the B and C replicates of the 5%K treatments (Table S1).

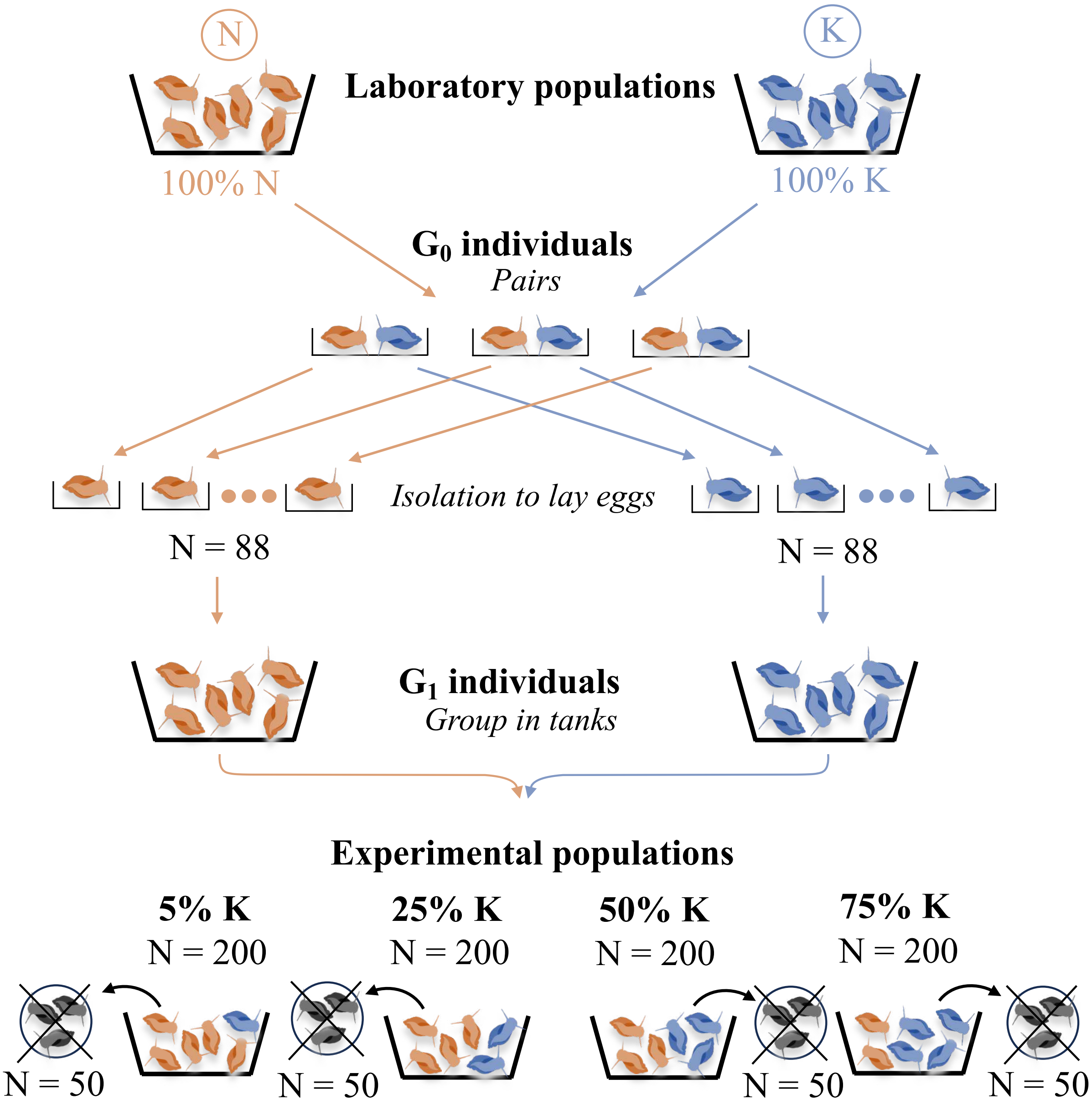

Figure 2: Constitution of the first generation of experimental populations. Experimental populations originated from laboratory populations which derived from the same two natural populations near Lyon (France). In each laboratory population, we randomly sampled 88 individuals (G0 individuals). At sexual maturity, we made N-K pairs and subsequently isolated them for egg laying. The hatchlings produced by both parents are full-sibs to each other but differ in mitotype (transmitted by the maternal individual). Upon hatching, the offspring (G1 individuals) were grouped and raised in two tanks, with each tank representing one mitotype. These (still immature) offspring were then used to form the four type of experimental populations. Each experimental population consisted of 200 individuals, distributed as follows: 10 K and 190 N in the 5%K, 50 K and 150 N in the 25%K, 100 K and

100 N in the 50%K, 150 K and 50 N in the 75%K. Then, we randomly removed 50 individuals from each populations to obtain N=150.

Rearing conditions and passage of generations

Each snail population was maintained in a 1.5L tank filled with groundwater and kept at 25°C. The snails were under 12:12 photoperiod and fed with boiled organic lettuce, grinded more or less finely depending on the age and size of snails. We fed the snails and renewed the water in each tank twice a week. The position of each tank on the shelves was changed regularly to avoid micro-environmental effects.

Each generation started with eggs laid by previous-generation adults during 10 days in a fresh aquarium. Parental adults were removed and the eggs on the walls of the aquarium kept to hatch. Hatching took place between 1-2 weeks after egg collection, yielding large numbers of juveniles that we raised together for 2 more weeks. At this date, juveniles were still immature, and only 150 of them were transferred to a new aquarium (density regulation). After this age mortality is normally very low so that the number stays nearly constant in the aquarium until reproduction. Once the 150 individuals reached maturity– we considered sexual maturity reached when at least twenty clutches were visible in the tank – we moved them into a fresh tank to let them lay. After ten days, we collected the clutches to start the new generation and transferred the adults into a second tank for another ten days to obtain back-up clutches. We sacrificed the 150 adults after collecting the backup clutches and preserved them in alcohol at -20°C.

Accidental loss of populations

Episodes of bacterial contamination (of unknown source) took place in August 2021 and November 2021. They affected only some populations and we were able to renew most of them using the back-up tanks, sometimes having to break the population into small groups to prevent spread of disease, with some losses. The contamination occurred: (i) at G_3 for 75%K^B and 50%K^B, (ii) G_2 for populations 25%, 50% and 75% of D, C and E replicates, (ii) at G_5 for 25%K^A, 50%K^C and G_6 for 25%K^B. Ultimately, only the adult population 25%K^A did not reach the prescribed N = 150 and was limited to N = 46 at G_5. We lost entirely three populations (75%K^B, 25%K^C, 75%K^C Table S1). In addition, populations at 25%K^D and 50%K^D were lost at G_9 due to accidental overfeeding of the juveniles.

Changes in frequencies over generations

We followed the evolution of the N and K mitotype frequencies by doing a PCR test on 50 out of 150 individuals randomly picked from stored samples. The test has been fully described in Laugier et al. (2024) (see Figure S1 for a summary). Briefly it consists in one PCR with a mix of primers designed to be specific to each of the mitotypes, amplifying different fragments of the COI mitochondrial gene, with fragment size typical of each mitotype; the mitotypes are divergent enough that nonspecific priming does not occur. Universal external primers from Folmer et al. 1994 are also present in the mix and amplify a long fragment in case none of the specific primers matches -thus failure in amplification (no fragment) can be distinguished from a potential new mitotype (amplification of the long fragment). In most cases, we ran a PCR test for each generation until the G_6 and then one in two generations until G_{11}. Populations lost in the early generations (before G_4) were excluded from the monitoring (75%K^B and 75%K^C).

Reproductive traits at the sixth generation

We assessed adult body weight, female fecundity and male-fertility status of several individuals randomly picked in each 25%K, 50%K and 75%K population at generation G_6. The mitotype of each individual was not known until after the end of measurements, when the individual could be sacrificed and was mitotyped using the PCR protocol described above. Therefore, to ensure that enough K individuals were tested, the total number of tested individuals was increased when the initial frequency of K was low (n = 81, 51, 42 for resp. 25%K, 50%K, 75%K treatments). The 5%K populations were not tested because the frequency of K individuals at G_6 was too low to get any meaningful comparison between N and K. The replicate 75%K^F could not be tested due to a bacterial contamination of their partners after crossing.

The focal individuals were collected from the tanks at an early stage (three weeks after the eggs hatched) and each snail was raised in isolation to sexual maturity (age 50 days). Then, each focal individual was paired with a virgin partner from the laboratory albino population. Pairing took place for three days, then individuals were isolated and laid eggs for another three days. Eggs of the focal individuals were counted and eggs of the albinos were kept for 14 days, then live juveniles produced by the albinos were counted and we recorded their morphs (pigmented or albino). Female fecundity of focal snails was estimated as the number of eggs laid by the focal snail. Our estimate of male fecundity for focal snails was the number of pigmented juveniles obtained from clutches laid by the albino partner (juveniles sired by the focal individual). From this, we determined the male-fertility status of each focal individual. Male-fertile individuals normally stimulate egg-laying when inseminating their virgin partner and sire the vast majority (usually 100%) of the offspring produced (which are therefore all pigmented). When individuals are male-sterile, they do not inseminate their partner, which can result in either the partner not laying any egg, or its laying self-fertilized eggs that develop into 100% albino juveniles. In some cases, pigmented juveniles were obtained, but their total number was abnormally low (<10), suggesting reduced (though not completely suppressed) male fertility. We recorded these individuals as "semi-sterile". An individual was considered fertile if two conditions were fulfilled: it sired more than half of the offspring of its partner and the total number of sired juveniles was >10. If we did not find any albino among the offspring sired by the focal, we stopped counting the pigmented offspring at 20 and recorded the individual as male-fertile and its male fitness as 20+; if one or more albino were present, we counted all the offspring.

Egg survival and development of N and K offspring

Most of the mortality in laboratory populations occurs at an early stage (egg, embryo, hatchlings). We therefore assessed whether juvenile survival differences existed between K and N individuals. This cannot be done using clutches laid in the aquaria where clutches of the two mitotypes are mixed in unknown frequency, and do not survive well if detached from the walls to put them into separate boxes. Therefore, we created pair crosses (N = 81) between virgin K and N individuals from the ancestral 100%N and 100%K populations and checked the survival of eggs laid separately by both members of each pair (eggs with the same genetic makeup at nuclear genes but differing only in mitotype). To that end, after pairing, K and N parents were left to lay eggs separately for two successive periods of three days. After each period we obtained K and N eggs that we counted and then we let them develop either at 25°C (first period) or at 20°C (second period). We added the 20°C treatment because we realized that the actual temperature in the rooms where the experimental populations were kept could sometimes fall a few degrees below the intended 25°C, i.e. between 20 and 25°C; and we also wanted to increase our chances to see any difference in egg survival by challenging energetic metabolism a little. Three days after being collected, eggs were provided with food, and left to hatch for another 10 days (25°C) or 13 days (20°C). After this period we counted live juveniles, unhatched juveniles and undeveloped eggs remaining in the box. We called "undeveloped" any egg where the embryo has not reached the stage at which the shell and the eyes are visible, and "unhatched juveniles" those that passed this stage but did not hatch (though some of them were still alive and mobile within the egg membrane). Using these counts we estimated "survival rate" as the proportion of eggs that became live juveniles, "developmental success" the proportion that passed the undeveloped stage, "hatching rate" the proportion of the latter that hatched and "hatchlings survival rate" the proportion of hatchlings that were still alive at the time of count.

Statistical analyses

All statistical analyses were carried out in R 4.3.1 (R Core Team 2023).

Estimation of the population parameters

From the monitoring of the K frequencies, we aimed to estimate a selection coefficient (s) assuming that the fitnesses of the mitotypes were as follows: $W_N = 1, W_K = 1 + s$ and remained constant over the 11 generations of the experiment and across all populations. We had to take genetic drift into account given the finite size of our experimental populations (150 adults, with a lower number for 25%K^A at G_5, 46 adults); however effective population size (N_e) is rarely equal to census size so we authorized a reduction in effective size compared to census size (i.e. an increase in genetic drift compared to expectation under equal contribution of all adults to the next generation) in our populations. To infer the selection coefficient and the reduction in effective size from the selection-drift trajectories of our populations, we estimated their likelihoods by simulation, using the `Infusion` package (Rousset et al., 2017). In this method, selection-drift trajectories are simulated for many parameter values, and machine-learning techniques (here Random Forest methods for non-parametric regression) are used to learn the relationship between simulated trajectories and the parameter values driving them. The data and the simulated trajectories are then reduced to values of the parameters predicted by the trained learner, and the likelihood surface for the parameters, given their predicted values for the data, is inferred. This learning process is repeated iteratively to delineate the region of parameter space with high enough likelihood, appropriate for the evaluation of confidence intervals.

Within the inference workflow, we simulated for each population (i) a selection and drift step modelling changes in gene frequencies, (ii) a sampling and genotyping step to account for the sampling design (i.e., the number of individuals typed by PCR at selected generations, reproducing the structure of our data). In the selection step, we first computed the expected frequency Ep_{n+1} of K in one population at generation n+1 as a function of the frequency p_n at generation n as:

$$Ep_{n+1} = \frac{p_n(1+s)}{1+p_n s}$$

Then we modelled drift by picking a fixed number of adults set at N = 150 with the exception of 25%K^A at G_5 which experienced a bottleneck (N = 46). Under the Wright-Fisher model, the number of adults of K mitotype should be a binomial draw with mean $N\,Ep_{n+1}$. However, as explained above, the variance in the contribution of each adult to the next generation is probably higher, by a factor to be estimated, than binomial variance. To account for this, we introduced an overdispersion parameter (*d*), and drew the number of adults of type K in a beta-binomial with mean $N\,Ep_{n+1}$ with a variance of change in frequency increased by $1 + d$ (relative to binomial). This procedure amounts to $d = \frac{\sigma_d^2}{2} - 1$ with σ_d^2 the variance of number of offspring among adult individuals (e.g., Wright 1938). The corresponding reduction in effective size compared to the Wright-Fisher model is:

$$N_e = \frac{N}{1+d}$$

. The genotyping step was modelled as a random sampling without replacement in the total number of individuals at a given generation (G_n). The number of individuals sampled depended on the number of successfully genotyped individuals (i.e., individuals for which we could successfully assess if they were N or K mitotype).

We inferred the likelihood surface as described above, first using the default simulation design implemented in the `Infusion` package, which in this 2-parameter model leads to a total of 5000 simulations, before doubling the number of simulations. Confidence intervals were obtained in a standard way from the inferred likelihood profiles for each parameter. In the end, we obtained a likelihood surface as a function of *s* and *d*, and determined the position of its maximum (our estimates of the two parameters) as well as profile-likelihood 95% confidence intervals (i.e. upper and lower limits for each parameter, at which log profile likelihood was the global maximum minus 1.92). We checked the coverage of such confidence intervals in a simulation study of inferences on 200 datasets simulated under our estimates of *s* and overdispersion, obtaining coverages of 94% and 96% respectively for these parameters.

Reproductive traits at G_6

We used linear mixed models (LMM) and generalised linear mixed models (GLMM) with the `lme4` R package (Bates et al., 2015) to analyse egg counts (female fecundity), body weight and male-fertility status. Binomial distributions were used for male-fertility and a

Gaussian model for body weight. Similar results were obtained using both a Gaussian model and a Poisson model (with overdispersion) for the egg counts. Furthermore, the egg counts followed a quasi-normal distribution, consequently we chose to exclusively present the results obtained with the Gaussian model. Mitotype, treatment (i.e. initial frequency of K) and their interaction were added as fixed effects. To account for common history of individuals within each population, a random factor 'replicate (within treatment)' was included, along with its interaction with mitotype. As it did not affect the results, individuals recorded as semi-sterile were grouped with the sterile category.

Egg survival and development

The survival rate was analysed by binomial GLMMs (number of juveniles versus number of eggs that did not became juveniles) including, as fixed effects, temperature (25 or 20°C), mitotype (K versus N) and their interaction, and as random effects the identity of the N-K pair (in interaction with temperature), the identity of the mother (nested within pairs), and a unique observation identifier for each box of juveniles (there are two boxes for each mother one at 20°C and one at 25°C, the box effect therefore includes both the mother-temperature interaction and any other box-specific source of variation). Together the random factors account for genetic and maternal effects, and overdispersion. To precise the stage at which differences among mitotypes could emerge, we repeated binomial models on different variables; numbers of developed versus non developed eggs; among developed eggs, number that hatched versus that did not; among hatched eggs, number of hatchlings that were found alive versus not. In all LMs and GLMMs, tests of fixed effects were performed using likelihood-ratio tests, comparing models with and without the effect of interest, keeping the complete random effect structure and other fixed effects in the two models.

Results

Evolution of the K frequency across 11 generations

The K mitotype frequency was monitored over 11 generations by PCRs. The mitotype was successfully assessed for an average of 48.9 ± 11.9 SD individuals per analysed generation and population (total 6208 individuals). Over the 11 generations of experimental evolution, the K frequency decreased in most of the populations and reached zero for: 5%K^A, 5%K^B, 5%K^C, 25%K^A, 25%K^E, 50%K^E, 75%K^E (Figure 3A, Table S2). In three populations (out of 16), however, the proportion of K either appeared stable (25%K^B, 50%K^D- the latter was prematurely interrupted at G_9 by accident), or increased markedly between the 6th and 7th generation (50%K^{A}; the K mitotype frequency increased from 50% to 98% between G_6 and G_7 and was still 98% at G_{11}).

Estimation of the population parameters

From the time-series of K frequency, we estimated the selection coefficient (*s*) on the K mitotype to be negative *s* = - 0.18 (95% confidence interval [-0.09, -0.27]). The effective population size (N_e) of our experimental populations was lower than census size, as the *overdispersion* was estimated at 5.81 and significantly >0 (interval [2.67, 10.86]) (Figure 3B, Figure S2). The corresponding estimated N_e was, for N = 150, N_e = 22.03 (interval [12.65, 40.87]).

We re-ran the inference workflow excluding an apparent outlier: the 50%K^{A} population for which the K frequency increased until G_{11} while it decreased in the others (see Figure 3B). The estimated parameter values showed no significant difference compared to when the population was included: *s* = - 0.21 (interval [-0.12, -0.29]), overdispersion = 4.89 (interval [1.94, 9.24]).

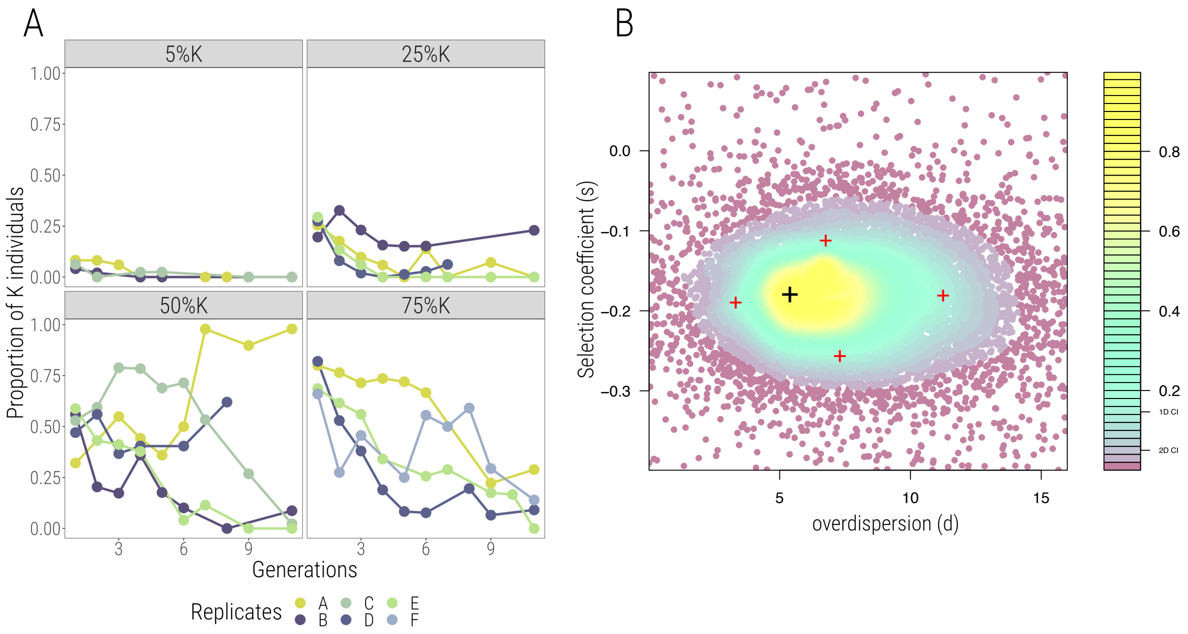

Figure 3 Evolution of the K frequency and populations parameters. A: Evolution of K frequency over the 11 generations of experimental evolution. Populations lost before the G_3 were not represented (75%K^{B} and 75%K^{C}). As they were lost before G_{11}, curves of both 25%K^{D} and 50%K^{D} populations stopped at respectively G_7 and G_8. B: Summary likelihood ratio surface of both the overdispersion parameter (*overdisp*) and the selection coefficient (*s*). Each point corresponds to a sample-generating parameter vector in the reference table.

The color scale represents the ratio of likelihood of such parameters relative to the inferred maximum. The black cross locates the summary-MLE and the red crosses locate the the bounds of the profile-likelihood based confidence intervals.

Reproductive traits at G6

Reproductive phenotype and mitotype could be assessed for (i) 70 ± 8.3 (mean ±standard deviation) individuals in the four replicates of the 25%K treatments, (ii) 40 ± 8.0 individuals in the five replicates of the 50%K treatments, (iii) 30 ± 11.5 individuals in the three replicates of the 75%K treatments.

Out of 280 (respectively 200, 90) albinos paired with snails from the 25%K (resp. 50%K, 75%K) treatment, 23 (respectively 27, 21) laid less than ten pigmented offspring and were therefore considered as male-sterile or semi-sterile. The proportion of individuals that belong to one of these categories was always low (<0.15, Figure 4A) and did not depend on the mitotype ($p = 0.81$), nor on the treatment ($p = 0.06$) or their interaction ($p = 0.58$, Figure 4B). No significant effect of mitotype, treatment or their interaction was found on either the female fecundity of the focal individuals (resp. $p = 0.79$, $p = 0.12$, $p = 0.50$, Table S3, Figure 4C) or their body weight (resp. $p = 0.16$, $p = 0.54$, $p = 0.14$, Table 1, Figure 4D).

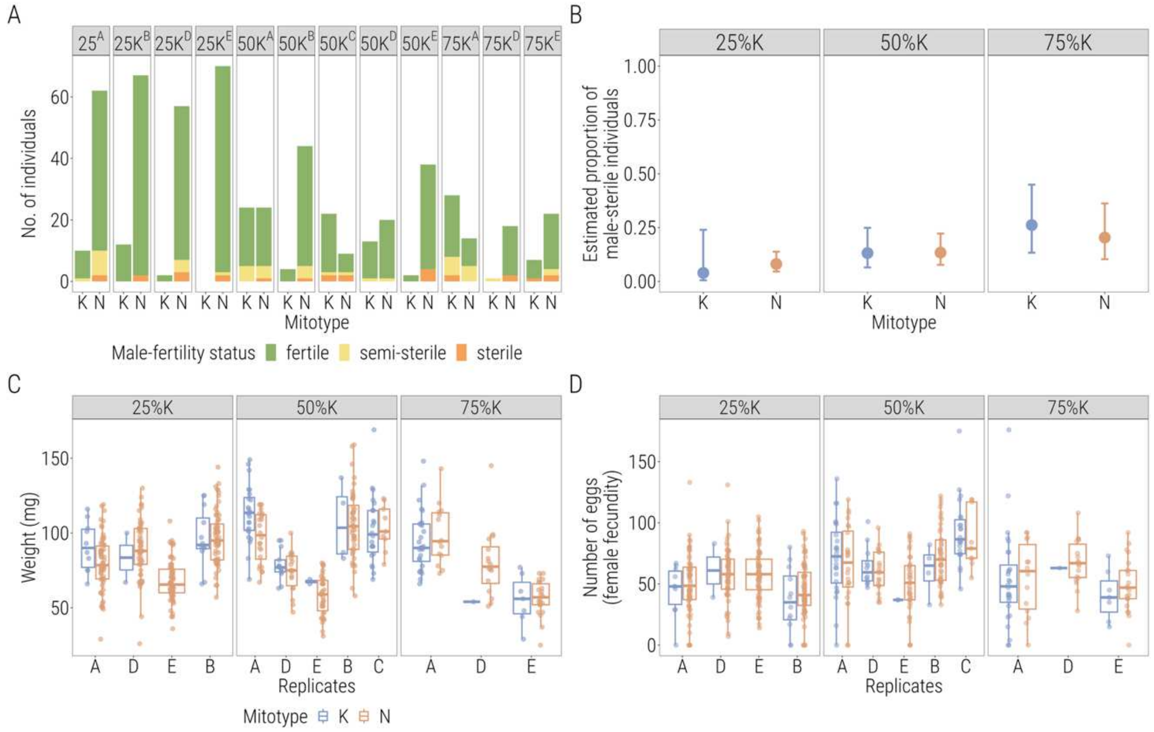

Figure 4: Reproductive traits at the sixth generation. Male-fertility status was assessed by the proportion of juveniles sired by the focal individual. A: Number of male-fertile, male

semi-sterile and male-sterile individuals of K and N mitotype in each population. The number of male-sterile individuals did not significantly vary between ‘K and N irrespective of treatment. On average, 88% of the individuals were fertile. B: Estimates and 95% confidence intervals of the male-sterile proportion for each treatment in both mitotypes (predictions of the GLMM with mitotype, treatment and interaction, delogited). C and D: Female fecundity and weight of the focal individual paired at G_6 with an albino partner. For each replicate, we represented one boxplot (median and quartiles) per mitotype; as the number of K individuals was already low at G_6, we sometimes detected very few or none of them. Female fecundity of focal snails was estimated as the number of eggs laid in three days. No significant differences were found between mitotypes or between treatments; their interaction was also non-significant.

Egg survival and development

The probability that an egg survives to the hatchling stage was not significantly affected by temperature, but we found a marginally significant effect of the mitotype: although both mitotypes had a high survival overall, it was slightly higher for N (87.6%±1.1%SE, versus 83.2% ± 1.6% in K, $p = 0.04$, Table 1, Figure 5). When breaking down survival into successive steps, we found that mitotype effects were significant at two steps: N eggs had a lower probability to remain undeveloped ($p < 0.001$, Table 1; Figure 5); and once developed, they hatched with a higher probability than K eggs ($p = 0.028$, Table 1; Figure 5). These effects were more pronounced at 20°C although the mitotype-temperature interaction was only significant for the hatching stage (Table 1). Once hatched, the survival rate of hatchlings was the same for both mitotypes ($p = 0.96$, Table 1).

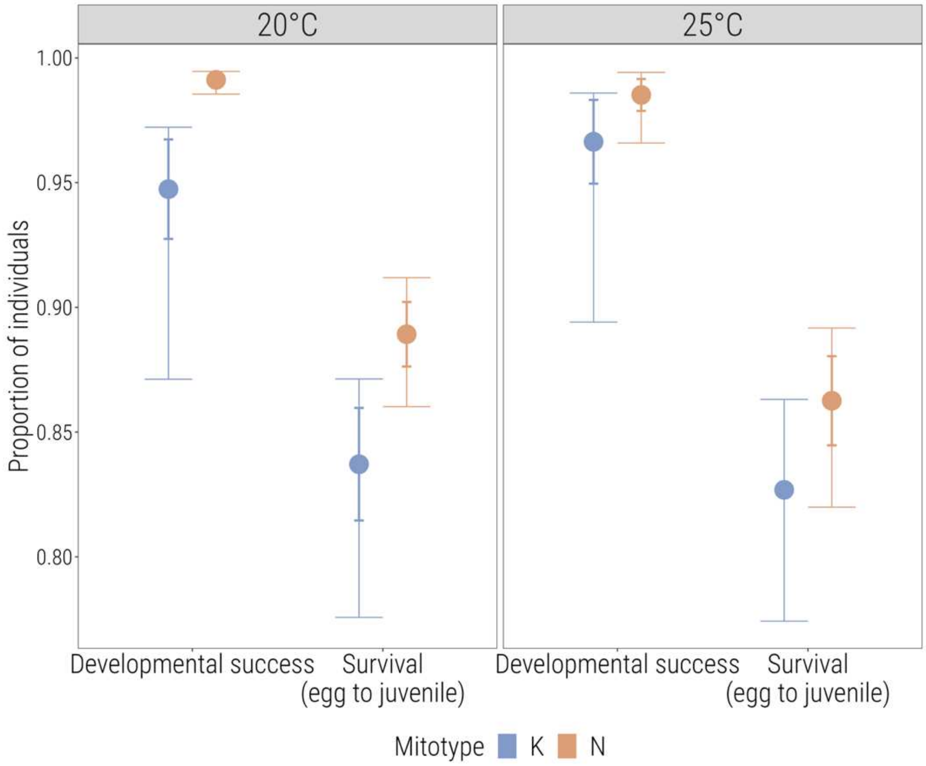

Figure 5: Development and survival of eggs of both mitotypes at 20°C and 25°C. The developmental success is the proportion of eggs that passed the undeveloped stage and the survival rate is the proportion of eggs that became live juveniles. Both rates were monitored at 20°C and 25°C. The dots are the average rates (weighted average); the error bars represent standard errors (thick lines) and the 95% confidence intervals fine lines) baqsed on 10,000 bootstraps.

Table 1: Results of GLMMs on egg survival and development. Fixed effects are tested by LRT on binomial models (logit scale, see M&M for the random effect structure common to all models), all chi-squares with 1 df. *p*-values lower than 0.05 are in bold, and the direction of effect is then indicated. When the mitotype-temperature interaction was significant (Hatching rate), we tested the mitotype effect separately at each temperature. Means are given for K and N separately on the original scale (weighted arithmetic means over all K or all N progeny) with SE and 95% confidence intervals obtained by 10,000 bootstraps.

Trait	Temperature	Mitotype	Interaction	mean K ± SE [95% CI]	mean N ± SE [95% CI]
Survival (egg to juvenile)	$\chi^2 = 0.173$, p = 0.678	$\chi^2 = 4.082$, p = **0.043** (N>K)	$\chi^2 = 0.325$, p = 0.55	0.832 ± 0.016 [0.796 - 0.859]	0.876 ± 0.011 [0.851- 0.895]

Developmental success	$\chi^2 = 8.359$, p = **0.0038** (t25>t20)	$\chi^2 = 12.227$, p = **0.0005** (N>K)	$\chi^2 = 0.088$, p = 0.767	0.956 ± 0.013 [0.918 - 0.974]	0.988 ± 0.003 [0.979 - 0.993]
Hatching rate of developed eggs	$\chi^2 = 3.161$, p = 0.075	$\chi^2 = 4.810$, p = **0.028** (N>K)	$\chi^2 = 5.193$, p = **0.023**	0.955 ± 0.006 [0.942 - 0.966]	0.962 ± 0.008 [0.939 - 0.974]
at 20°C		$\chi^2 = 8.885$, p = **0.0029** (N>K)		0.960 ± 0.007 [0.943 - 0.972]	0.984 ± 0.003 [0.976 - 0.989]
at 25°C		$\chi^2 = 0.062$, p = 0.803		0.950 ± 0.010 [0.924 - 0.966]	0.939 ± 0.016 [0.895 - 0.962]
Survival after hatching	$\chi^2 = 3.8503$, p = 0.050 (t25>t20)	$\chi^2 = 0.025$, p = 0.960	$\chi^2 = 1.019$, p = 0.296	0.911 ± 0.011 [0.887 - 0.930]	0.921 ± 0.009 [0.902 - 0.938]

Discussion

CMS frequency decreases in populations with high restoration potential

Here we tested a key assumption of models for the maintenance of cytonuclear polymorphism in male fertility: the CMS mitotype should have lower fitness when restorers of male fertility are in high frequency. The experimental populations were designed to set a context of high restoration and we indeed found a relative fitness reduction of about 18% for a CMS mitotype. In congruence with this strong reduction of fitness, we observed a decrease in the K mitotype frequency over the 11 generations of experimental evolution; it was even completely lost in seven populations. However, individual population trajectories were highly variable; especially in one population (50%K^A) the K mitotype increased, and nearly reached fixation in a very short time, near generation six. The variability of results probably reflects drift (as attested by the large overdispersion estimated, suggesting an effective size closer to a few tens of individuals than to the number of adults, usually maintained at N=150). The peculiar behaviour of population 50%K^A did not strongly influence our estimates of selection and drift, and we this particular population did not stand out in terms of relative female fertility of the K snails compared to the N so that we have no strong evidence that anything special (beyond chance) happened to it.

The male-fertility tests performed at the sixth generation confirmed that most K-mitotype individuals remained restored (i.e. male-fertile) as long as they persisted in the experimental populations (there were not enough K individuals remaining to test this after the 6th generation). Indeed, the proportion of male-fertile individuals was on average of 88%, with no significant difference among treatments or mitotypes. The fact that individuals failing to sire offspring were found, within each type of population, in the same proportion regardless of the mitotype (N or K) suggests that most of these failures do not represent cytoplasmic male sterility. Indeed, one difficulty with snails (compared to plants) is that male-sterility is not visible externally, so we have to test it by pair-mating focal individuals with virgin mates; even normal individuals sometimes fail to sire offspring in this test, usually with a frequency of around 10-15% (Laugier et al. 2024, David et al. 2022), that one may expect to vary depending on the age and physical condition of both partners and environment at the time of the pairing. We nonetheless observed a tendency towards slightly higher failure rates for both mitotypes in populations with higher initial frequency of K (75%K > 50%K > 25%K), which we could not relate to any obvious difference in condition (as attested by female fertility or body weight of focal individuals, or fertilities of their partners). One possibility is that populations with higher initial frequency of K had a lower effective size during their history because of selective elimination of many individuals (given the 18% female fitness disadvantage of K versus N). Also, the male fitness of restored K individuals in competitive condition within our populations may not be as high as that of N individuals, (e.g. Dufay et al., 2008); both factors could decrease the effective size in populations with higher initial proportion of K-mitotypes, that would then be more subject to accumulation of inbreeding and deleterious mutations. However, we have no way to properly distinguish this from simple chance, given the low number of populations per category. Whatever the cause, the fraction of individuals failing to inseminate their partner was low and not related to the mitotype, confirming that the K remained mostly restored during the experiment.

Context-dependent cost of CMS and restoration

Monitoring the survival rate from eggs to juveniles in the ancestral populations revealed that N individuals developed better than the K at 20°C and that, once developed, they hatched more than K individuals. Although not significant, a similar effect was found at 25°C. This differential survival rate between N and K indicates a disadvantage of the K mitotype consistent with what we observed in the experimental populations. The difference between CMS and non-CMS individuals reported here is related to what Dufay et al. 2007 called a cytotype effect or cost (Dufay et al. 2007, see Table 2). Our experimental evolution started with restorer alleles in high frequency and CMS mostly carried by restored hermaphrodites. In this situation, Dufay et al. 2007, predicted a decrease of the CMS frequency if the female fitness of the non-CMS mitotype was higher than that of the CMS ("cytotype effect"). This effect has been documented in *Plantago lanceolata* (Van Damme, 1984) and in *Silene vulgaris* (Mccauley & Olson, 2003). Studies of the molecular mechanism of CMS in crops have revealed that most male-sterilising genes arise *de novo* and that they are constitutive in plant tissues (Budar et al., 2003; Chase, 2007). We can therefore expect an additional cost associated with the constitutive expression of sterilising genes specific to the CMS cytoplasm.

In congruence with the theoretical prediction and empirical observations made in plants, our results showed a greater developmental success in the non-CMS individuals. However, this difference is of the order a few %, which does not seem strong enough to explain the considerably lower fitness of the K (18% female fitness disadvantage) in the experimental populations. Moreover, neither body weight nor the female fertility measured at the sixth generation suggested any advantage of the N type. This suggests that the impact of the restored CMS on female fitness was underestimated in the experimental conditions in which we measured traits. Indeed, the individuals were raised without competition, in isolation for 50 days – after 50 days we considered that all snails had reached sexual maturity – then each snail was paired, nearly all of them laid and eggs hatched and survived with high probability. In the experimental evolution protocol, a population was considered sexually mature when a sufficient number of clutches were visible, but it is probable (given our low effective size estimates) that among the 150 individuals present, many of them never managed to contribute to the next generation, leaving the opportunity to express higher contrasts in female fitness between the two mitotypes than they do in isolation. The environment-dependent expression of the CMS cost suggested here could be related to observations made on the female advantage and the cost of restoration. Indeed, the magnitude of the female advantage varies greatly among species but also among populations of the same species (Dufay & Billard, 2012). Several studies have suggested that these variations were environment-dependent (e.g Asikainen & Mutikainen, 2003). Caruso et al., (2012) have argued that ecological conditions can alter the cost of restoration, as environmental factors often shape the relationship between an individual's genotype and its phenotype. These genotype-by-environment interactions may cause variations in the expression of the cost of restoration, depending on the environment. Overall, we still need to carry more investigations to identify the conditions in which the selective advantage of N over K mitotypes in the presence of restoration is expressed.

In the last phase of the theoretical cycle, individuals with both a non-CMS mitotype and a restorer allele express a cost of restoration (called silent cost), therefore restorers are expected to be counter-selected. This 'silent' cost is necessary to avoid restorer fixation and maintain nuclear-cytoplasmic gynodioecy (Dufay et al. 2007) and it is exhibited by crop species (see Table 2 in Delph 2007). Three experimental studies on pollen viability in *Lobelia siphilitica* (Bailey, 2002), seed quality in *Plantago lanceolata* (De Haan et al., 1997) and pollen quality and viability in *Beta vulgaris* spp. *maritima* (Dufay et al. 2008, De Cauwer et al., 2011)) also suggested a silent cost of the restorers. However, the elimination or decrease in frequency of the K mitotype in our populations was rapid and by the sixth generation, remaining K individuals were still mostly male-fertile, suggesting that restorer alleles remained common, and that the silent cost, if any, is not strong enough for it to decrease before CMS individuals become very rare (and stay so for a sufficient number of generations). The controlled environment in which our study was done (25°C and competition) may also influence the expression of this cost. Theoretical models suggest that a necessary condition for gynodioecy to persist is that restorers should not be maintained in the absence of their corresponding CMS cytoplasm (unless there is a population structure effect). However, the cost of restoration has been estimated in only one or two populations per species (Bailey, 2002; Dufay et al., 2008; Del Castillo & Trujillo, 2009) as its estimation requires crossing designs and/or molecular markers of the CMS and the restorers.

In this study, we found a high cost of CMS in the presence of restoration and that a high frequency of restoration was therefore associated with evolution towards a low frequency of CMS. A different pattern is observed in many plants as medium-high frequencies of restorers seem often associated with a broad range of CMS frequencies. In natural populations of *Beta vulgaris* spp. *maritima* CMS frequency varies widely (0 - 88.9%) and the restoration rate is estimated between 42.5 and 100% in CMS individuals (De Cauwer et al., 2012). The same trend is found in *Raphanus sativus*, the CMS frequency varies from 0 to 100%, while the restorer frequency ranged from 41 to 100% (Murayama et al. 2004). However, in *Plantago lanceolata* the frequencies of both CMS and restorers seem highly variable (de Haan et al., 1997a; de Haan et al., 1997b). The variations observed in *B. vulgaris* and *R. sativus* suggest that the CMS cost in presence of high frequency of restorers might be lower in plants than what we observed in *P. acuta* laboratory populations. If our observations (fast counter-selection of CMS in the presence of restoration, relative to the cost of restoration in the absence of CMS) can apply to natural populations, we would expect many *P. acuta* populations to be with high frequency of restoration but low frequency of the K-mitotype. This is the case of the original populations where K was discovered near Lyon (K frequencies 12% and 32%, 81% of them restored) but more populations should be investigated.

Similar dynamics are found in other genetic conflicts

Outside plants, there is no other example of confirmed CMS than *Physa acuta*. However conflicts of similar nature between mitochondrial and nuclear genes can impact male fitness in separate-sex animals, a phenomenon known as the mother's curse (Camus et al., 2022). Studies on *Drosophila* in particular suggest that different nuclear backgrounds affect the impact of mitochondrial mutations harmful to males (e.g. in *Drosophila* Mossman et al., 2016), and that environment plays a role in mediating sex-specific mito-nuclear effects (G × E effects). Besides cytonuclear interactions (e.g. Rand et al., 2001), the maintenance of polymorphism within genomic conflicts may therefore depend on environmental factors. It would be interesting to know if male-harmful mitochondria are indeed counter-selected when corresponding compensatory mutations in the nuclear genome compensate their effects, as in the case for CMS in *Physa*, but to our knowledge such data are still absent.

Mitochondria are not the only one subverting the rules of Mendelian inheritance as other selfish genetic elements are also found in animals (see Price & Wedell, 2008; Price et al., 2020 for reviews). Sex chromosome meiotic drive elements reduce male fertility as they induce failure of spermatogenesis and have been observed with both Y and X chromosome (Price & Wedell 2008). The X-linked meiotic drivers favor the transmission of their carriers at the expense of the Y chromosome in heterogamic males, thus producing female-biased sex ratios by killing sperm after meiosis (Jaenike, 2001). Similarly to restorers counter-acting the effect of CMS, a rapid evolution of resistance against sex-linked drivers is also expected (Hurst & Pomiankowski, 1991). Studies performed on *Drosophila* have found resistant Y and autosomal suppressors associated with X-linked meiotic drivers (i.e. sex-ratio drive, e.g, Stalker, 1961; Carvalho et al., 1997; Montchamp-Moreau et al., 2001; Courret et al., 2019). In this system, theoretical studies predicted frequency-dependent interactions between the *sex-ratio* drive and the resistant Y (Carvalho et al. 1997) and as predicted for CMS systems the evolutionary dynamics depend on several parameters, such as costs (Vaz & Carvalho, 2004). Predicted outcomes are also cyclical frequencies of the sex chromosome and associated changes in the sex-ratio (Hall, 2004). The occurrence of this cyclical dynamic has been suggested by geographical variations in sex-ratio drive and suppressor frequencies (Atlan et al., 1997; Kingan et al., 2010). In *Drosophila simulans*, a recent experimental evolution ran over 100 generations (Bastide et al., 2022) have observed a slow decline of the X-linked meiotic driver in the presence of a genetic background consisting entirely of suppressors, as situation reminiscent of our observations on the decrease of CMS in the presence of restoration in *Physa acuta*, although at a slower speed. Bastide et al. (2022) concluded that both the drive and its suppressor were under reciprocal frequency-dependent selection. Models have long suggested similarities in the dynamics in different types of genomic conflicts, such as cyclical changes and frequency-dependence (e.g. Charlesworth & Hartl, 1978; Gouyon et al., 1991). Our data now provide the first evidence that outcomes of experimental evolution may also be similar between the CMS-restorer system and the other selfish gene-suppressor system studied by experimental evolution so far, the *sex-ratio* meiotic drive (e.g. Carvalho et al., 1997; Capillon & Atlan, 1999; Bastide et al., 2022).

Conclusion

We observed over 11 generations of experimental evolution, the counter-selection of cytoplasmic male sterility in a hermaphrodite. This evolutionary dynamics in *Physa acuta* occurred rapidly and was driven by strong fitness differentials between CMS and non-CMS mitotypes, which remain difficult to detect under optimal conditions. According to theoretical predictions (Gouyon et al. 1991, Dufay et al. 2007), this counter-selection of the CMS contributes to the maintenance of the polymorphism. Genomic conflict over sex-determination can also occur between nuclear genomes and bacterial endosymbionts (see Cordaux et al., 2011 for a review). In *Armadillidium vulgare* maternally-inherited sex ratio distorters (*Wolbachia* endosymbionts) are found along with a polygenic system of resistance genes reducing the rate of *Wolbachia* transmission. In this species, costs associated with the selfish element is also found as females infected by *Wolbachia* exhibit lower reproductive success (Rigaud & Moreau, 2004), lower immunocompetence (Sicard et al., 2010) and lower cognitive capabilities (Templé & Richard, 2015) relative to uninfected genetic females. Such costs may be sufficient to cause *Wolbachia* loss at individual or family levels, although they may not be high enough to cause *Wolbachia* loss at population level, especially as beneficial effects of *Wolbachia* infection have also been reported in *A. vulgare*. In *A. vulgare* populations, the polymorphism is stable (with 80 – 90% of females, Cordaux & Gilbert, 2017) but in *Hypolimnas bolina*, a species where *Wolbachia* induces male-killing, frequencies are contrasted among populations as shown by Hornett et al. (2006). Indeed, a suppressor host gene spread in one natural population of *H. bolina* and shifted the sex-ratio from 99% of females to parity within barely 10 generations. This example, in a single natural population, illustrates the speed at which evolution can proceed in the context of such conflicts, similar to what we observed in our experimental evolution replicates. Experimental evolution could, in these conditions, be a valuable tool to explore different aspects of these conflicts, especially how their outcome may vary with environmental conditions such as temperature, competition, or stress, and contribute to explain some of the (often considerable) spatial and temporal variability in sexual morph frequencies observed among populations within plant and animal species.

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Supplementary materials

Table S1: Experimental population origin. At G_1, the experimental populations were constituted with K and N offspring of the G_0 crosses this based on the initial frequencies chosen and for a total of 150 individuals per population. Because of bacterial contamination or overfeeding of the juveniles, some replicates were lost before the end of the experiment (G_{11}). Therefore, we here indicated the generation at which we encountered technical issues and the last viable generation of each population. Populations lost in G_1 or G_2 were not analysed.

Treatment	Replicates	G1 date	Technical issues	Last viable generation
5%K	A	03/11/21	none	G_{11}
5%K	B	01/07/22	none	G_{11}
5%K	C	01/07/22	none	G_{11}
25%K	A	03/11/21	G_5	G_{11}
25%K	B	03/23/21	G_6	G_{11}
25%K	C	05/05/21	G_2	G_1
25%K	D	05/12/21	G_2/G_9	G_9
25%K	E	05/19/21	G_2	G_{11}
50%K	A	03/11/21	none	G_{11}
50%K	B	03/23/21	G_3	G_{11}
50%K	C	05/05/21	G_2/G_5	G_{11}
50%K	D	05/12/21	G_2/G_9	G_9
50%K	E	05/19/21	G_2	G_{11}
75%K	A	03/11/21	none	G_{11}
75%K	B	03/23/21	G_3	G_2
75%K	C	05/05/21	G_2	G_1
75%K	D	05/12/21	G_2	G_{11}
75%K	E	05/19/21	G_2	G_{11}
75%K	F	11/16/21	none	G_{11}

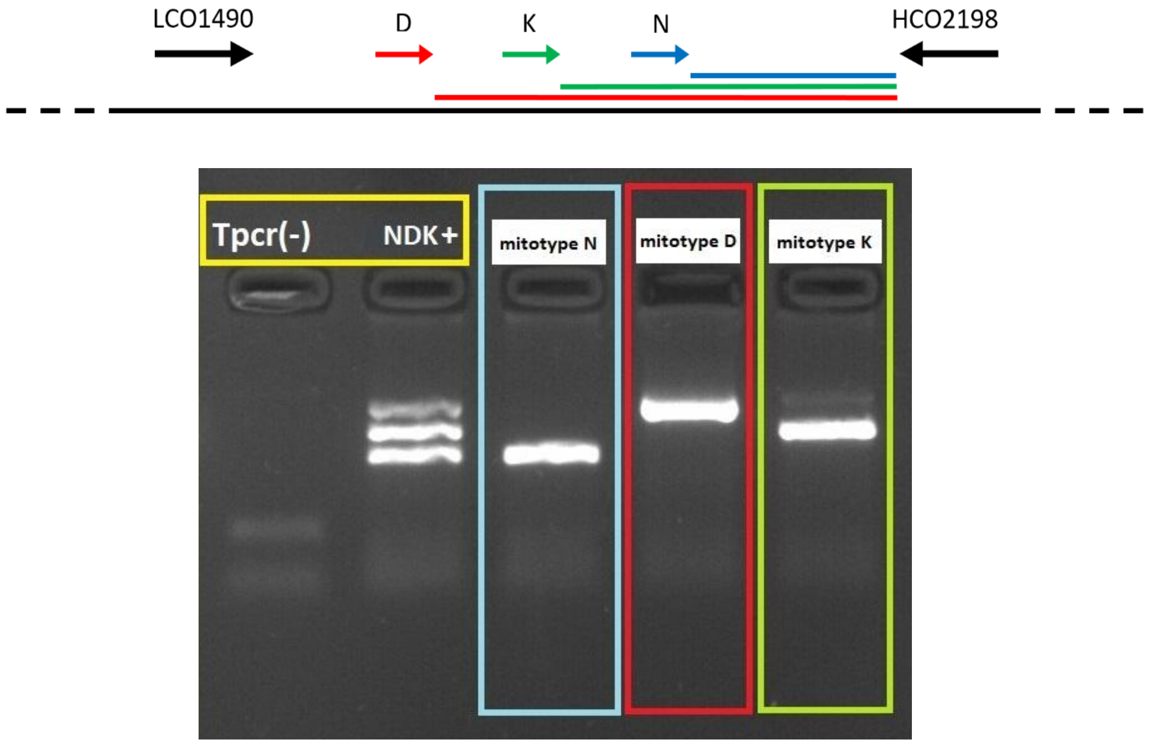

Figure S1: Mitotype identification by PCR (from Laugier et al. *in press*, repeated here for convenience). One PCR was performed per individual using five primers per PCR, the two external, universal COI “Folmer” primers and three internal specific primers (ATTACACTTAGCTGGGTTATCA for N, GGGACAACTAGGGTCTTAACTAC for D and TTGCTCCCTCCTTCTTTCACA for K mitotypes, blue, red and green respectively). PCR products were run on an agarose gel (2%) giving a typical pattern depending on the mitotype (an example of gel with individuals from three maternal lines of each mitotype is given, with a positive control NDK+ made with a mix of DNA from three individuals one of each mitotype). Short fragments span at 288 bp for N, 607 bp for D and 426 bp for K respectively. Amplifications were carried out in 10-µL final reaction volume comprising 5-µL of 2X Sigma-Aldrich REDExtract-N-Amp PCR ReadyMix, 0.25-µL of each primer (10 µM), 1.75-µL of PCR-grade Water. The PCR conditions were as follows: 5 min at 95°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 50°C and extension for 40 sec at 72°C, completed with a final extension step for 10 min at 72°C.

Table S2: Results of the PCR tests done to follow the evolution of the K mitotype frequency. PCR test were usually ran on 50 individuals randomly picked-up out from the storage tube. For some populations, PCRs did not work and we had a lot of NA (number of NA below). In these cases, we used other individuals from the storage tube and ran extra PCRs when possible.

Replicates	Treatments	Generations	K frequency	Number of K	Number of N
A	25	1	0.255	13	38
A	25	2	0.176	9	42
A	25	3	0.098	5	46
A	25	4	0.059	3	48
A	25	5	0	0	36
A	25	6	0.139	10	62
A	25	7	0	0	45
A	25	9	0.071	3	39
A	25	11	0	0	48
A	50	1	0.321	26	55
A	50	2	0.431	22	29
A	50	3	0.549	28	23
A	50	4	0.442	23	29
A	50	5	0.36	18	32
A	50	6	0.5	24	24
A	50	7	0.978	45	1
A	50	9	0.898	44	5
A	50	11	0.979	47	1
A	75	1	0.8	40	10
A	75	2	0.765	39	12
A	75	3	0.714	35	14
A	75	4	0.736	39	14
A	75	5	0.72	36	14
A	75	6	0.667	28	14
A	75	9	0.222	10	35
A	75	11	0.289	13	32
B	25	1	0.196	10	41
B	25	2	0.327	17	35
B	25	3	0.231	12	40
B	25	4	0.157	8	43
B	25	5	0.151	8	45
B	25	6	0.152	12	67
B	25	11	0.229	11	37
B	50	1	0.56	28	22
B	50	2	0.204	10	39
B	50	3	0.173	9	43

B	50	4	0.36	18	32
B	50	5	0.176	9	42
B	50	6	0.101	10	89
B	50	8	0	0	48
B	50	11	0.087	4	42
C	50	1	0.529	27	24
C	50	2	0.594	22	15
C	50	3	0.788	41	11
C	50	4	0.784	40	11
C	50	5	0.689	31	14
C	50	6	0.714	35	14
C	50	7	0.533	24	21
C	50	9	0.268	11	30
C	50	11	0.023	1	43
D	25	1	0.274	14	37
D	25	2	0.08	4	46
D	25	3	0.019	1	50
D	25	4	0	0	31
D	25	5	0.013	1	75
D	25	6	0.027	2	71
D	25	7	0.062	6	91
D	50	1	0.470	24	27
D	50	2	0.56	28	22
D	50	3	0.367	18	31
D	50	4	0.404	19	28
D	50	6	0.404	19	28
D	50	8	0.62	31	19
D	75	1	0.82	41	9
D	75	2	0.529	27	24
D	75	3	0.38	19	31
D	75	4	0.189	7	30
D	75	5	0.083	1	11
D	75	6	0.077	2	24
D	75	8	0.196	9	37
D	75	9	0.065	3	43
D	75	11	0.091	4	40
E	25	1	0.294	15	36
E	25	2	0.135	7	45
E	25	3	0.06	3	47
E	25	4	0	0	46
E	25	6	0	0	74
E	25	7	0	0	49
E	25	9	0	0	20

E	25	11	0	0	43
E	50	1	0.588	30	21
E	50	2	0.431	22	29
E	50	3	0.412	21	30
E	50	4	0.378	17	28
E	50	6	0.04	2	48
E	50	7	0.114	4	31
E	50	9	0	0	18
E	50	11	0	0	50
E	75	1	0.686	35	16
E	75	2	0.615	32	20
E	75	3	0.56	28	22
E	75	4	0.341	15	29
E	75	6	0.256	10	29
E	75	7	0.289	13	32
E	75	9	0.175	7	33
E	75	10	0.167	6	30
E	75	11	0	0	44
F	75	2	0.66	33	17
F	75	3	0.275	11	29
F	75	5	0.456	21	25
F	75	6	0.25	11	33
F	75	7	0.556	25	20
F	75	8	0.5	19	19
F	75	9	0.591	26	18
F	75	11	0.294	15	36
A	5	1	0.082	8	89
A	5	2	0.082	4	45
A	5	3	0.06	3	47
A	5	4	0	0	46
A	5	5	0	0	50
A	5	7	0	0	50
A	5	8	0	0	44
A	5	9	0	0	49
A	5	11	0	0	44
B	5	1	0.139	6	37
B	5	2	0.041	2	47
B	5	4	0.020	1	48
B	5	5	0	0	44
B	5	9	0	0	40
B	5	11	0	0	46
C	5	1	0	0	46
C	5	2	0.062	3	45

C	5	4	0	0	48
C	5	5	0.024	1	41
C	5	9	0.024	1	40
C	5	11	0	0	46

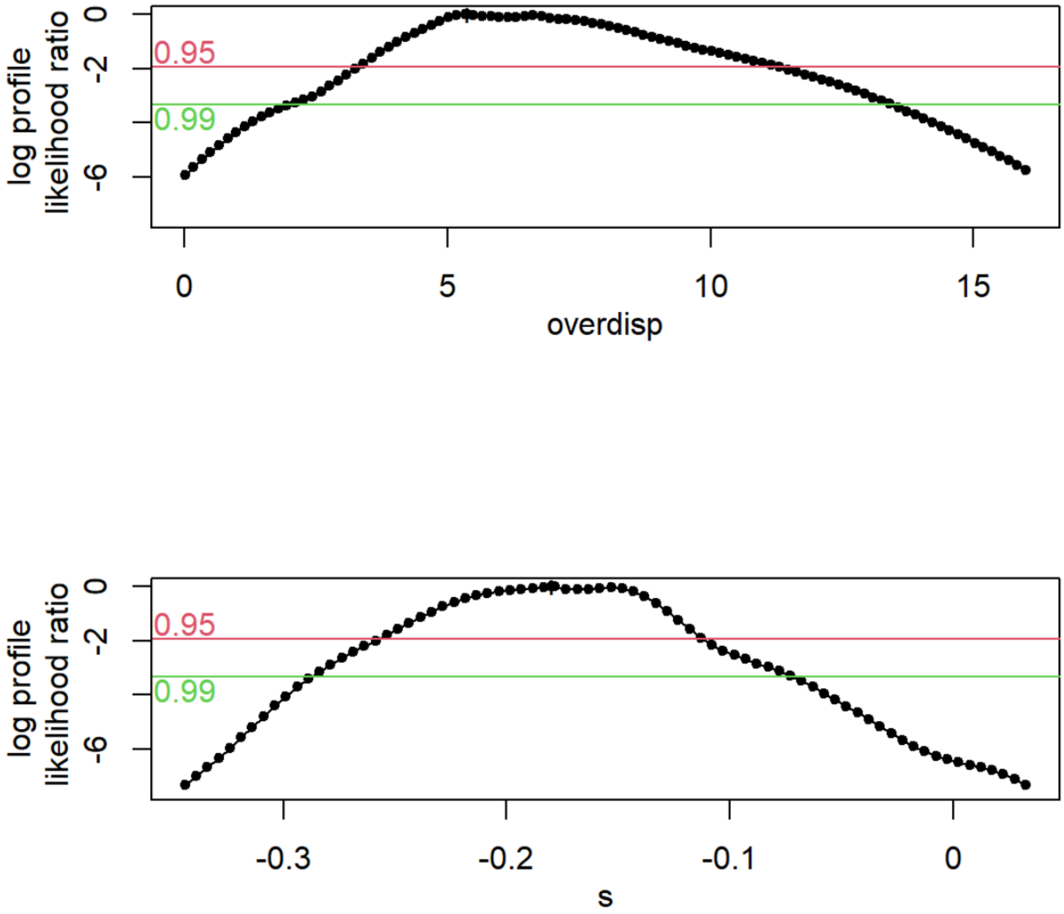

Figure S2: Log-likelihood ratio profile of the overdispersion (*d*) and the selection coefficient (*s*). The red and green lines represent the bounds of confidence intervals at 95% and 99%. The zero corresponds to the maximum likelihood estimate (likelihood ratio = 1).

Table S3: Results of linear models on male-fertility status, female fitness and body weight of *P. acuta* K and N at G_6. Performances of N and K mitotypes are reported either as trait means and SE (for quantitative traits) or as successes / failures (success = male-fertile, failure = male-sterile). To account for common history of individuals within each population, a random factor 'replicate (within treatment)' was included, along with its interaction with mitotype.

Traits	Number of pairs	Estimates ± se success / failures		Test of type effect df = 1	Model type
		Type N	Type K		
Male-fertility status	570	394/51	105/20	$\chi^2 = 0.06$, $p = 0.81$	Binomial
Female fitness	566	53.445± 4.802	52.382± 6.283	$\chi^2 = 0.068$, $p = 0.79$	Gaussian
Body Weight	562	83.653± 9.041	87.37± 9.141	$\chi^2 = 1.89$, $p = 0.17$	Gaussian

CHAPITRE 2

EVOLUTION EXPERIMENTALE

- MANUSCRIPT 2 -

Experimental evolution reveals frequency-dependent selection of resistance to cytoplasmic male sterility

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In preparation

Abstract

Genetic conflicts originate from the spread of selfish variants that enhance their own transmission while decreasing that of other genes. Suppressors often evolve to oppose these effects, but as for other forms of resistance the selective pressures are shaped by the environment. Cytoplasmic male sterility (CMS) is the textbook example of genetic conflict. It is caused by mitochondrial genes that abolish the male function of hermaphrodites. Nuclear suppressors, called restorers of male-fertility frequently evolve to counteract CMS. Such restorers would be selected in high CMS were not costly, male-sterile individuals would disappear from populations; however. Here, we used experimental evolution to obtain an integrated view of the fitness costs of restoration in the snail *Physa acuta*. Starting from the same, highly restored, base population, we observed over 11 generations a significant decrease in the restoration potential of populations evolved without CMS while it was maintained in populations with CMS, implying the existence of significant fitness costs of restoration.

Key words: genetic conflicts, cytoplasmic male sterility, restoration, resistance cost, experimental evolution, *Physa acuta*

Introduction

The cost of resistance has been studied in various contexts, notably in host-pathogen systems or insecticide resistance (e.g. Antonovics & Thrall, 1994; Shi et al., 2004). Classically it is considered that *de novo* mutations conferring resistance carry a fitness cost in the absence of their selective agent (Vanderplank, 1989). Indeed, based on Fisher's representation of adaptation (1930) mutations that provide a large fitness advantage in a new environment are likely to move the multidimensional phenotype away from its optimum in the previous environment, where the population has had a long time to adapt. Costs of resistance are necessary to maintain polymorphisms in heterogeneous environments (e.g. May et al., 1983; Antonovics & Thrall, 1994; Vale et al., 2008 ; Karasov et al., 2014). For example the frequency of insecticide-resistant acetylcholinesterase reflects the interplay of positive selection in treated areas during the warm season and costs expressed in nontreated places and during yearly interruptions of treatment in winter (Lenormand et al., 1999). Costs are often context-dependent and can be expressed on a variety of traits. For example in *Arabidopsis thaliana*, plants carrying the resistance gene to the bacterial pathogen *Pseudomonas syringae* exhibit a lower shoot biomass and show an average decrease in seed production (Tian et al., 2003). In mosquitoes the cost of insecticide resistance is exacerbated during overwintering in caves (Gazave et al., 2001).

Resistance evolution takes place not only against external pathogens or environmental stressors, but also, in the context of genetic conflicts, to limit the deleterious impacts of selfish variants within the individual. Selfish genetic element are genes that employ various strategies that ensure they get transmitted to a greater extent to the next generation than the rest of the genome (Burt & Trivers, 2006). Selfish genetic elements are remarkably abundant and diverse, ranging from meiotic driver chromosomes to heritable symbiotic micro-organisms (Price & Wedell, 2008); their effects are usually counteracted by resistance factors (e.g. Dermitzakis et al., 2000). Meiotic drive chromosomes alter the meiosis so that the driving chromosome is present in more than 50% of the gametes, i.e. if linked to a sex chromosome they promote sex ratio distortion. A selfish sex ratio-distorting gene is found in several *Drosophila* species (e.g. James & Jaenike, 1990; Dyer, 2012; Helleu et al., 2016) along with Y-linked and autosomal suppressors of the drive (e.g. Stalker, 1961; Carvalho et al., 1997; Jaenike, 1999). Among the *Drosophila* species, four of them were found to be polymorphic for the Y-linked suppressors, supporting the hypothesis that drive suppression could have a cost *(D. paramelanica,* Stalker, 1961; *D. mediopunctata*, Carvalho et al., 1997; *D. quinarian* Jaenike, 1999; *D. simulans,* Montchamp-Moreau et al., 2007).

Theoretical studies predicted frequency-dependent interactions between the sex-ratio drive and the suppressor depending on different parameters including the cost (Carvalho et al., 1997; Vaz & Carvalho, 2004). Unfortunately we know of no experiment directly observing how suppressors evolve over several generations in the absence of their corresponding distorter. Suppressors are not the only resistance mechanism expressed against the drive. Indeed, theory predicts that selfish genes could promote polyandry in females (i.e., females mating with more than one male) because it reduced the risk of mating with males carrying the sex ratio-distorting gene (Haig & Bergstrom, 1995). After ten generations of experimental evolution, an increased proportion of polyandry have been observed in *Drosophila pseudoobscura* (Price et al., 2008). The fact that the degree of polyandry was initially lower in the absence of the distorter, suggests that increased polyandry is costly and that it would come back to its initial value if the distorter is removed (although this has not been verified).

Symbiotic micro-organisms also subvert the sex-ratio in their host progeny using a variety of strategies (see Cordaux et al., 2011 for a review). Because they are located in the cytoplasm, endosymbionts have a maternal inheritance and therefore favour nuclear factors that promote male production. One of the well-known example is the feminization of *Armadillidium vulgare* by *Wolbachia*. In this isopod crustacean, females are the heretogametic sex – males are ZZ and females are ZW – and *Wolbachia* converts genetic males ZZ into phenotypic female (Rigaud et al., 1997; Cordaux et al., 2004). Thus, in the infected populations the W female chromosome is eliminated and the sex-determination is under the control of *Wolbachia*. However, a polygenetic system of resistance genes can prevent feminization by resisting the transmission of *Wolbachia* to offspring (Rigaud & Juchault, 1992). *Wolbachia* strategies to favour females over males host also includes male-killing (i.e. male hosts are eliminated early in embryogenesis, Majerus & Hurst, 1997). In the ladybirds *Cheilomenes sexmaculata* a single dominant allele rescues male progeny of infected females from the effect of the male-killer (Majerus & Majerus, 2010). In these systems, no costs of the resistance genes were reported.

The cost of resistance to a maternally transmitted selfish genetic element has been extensively studied in the context of gynodioecy, a sexual polymorphism in which hermaphrodites coexist with male-sterile individuals (e.g. Darwin, 1877; Gouyon et al., 1991; de Haan et al., 1997; Bailey, 2002; Dufay et al., 2008; McCauley & Bailey, 2009; Case & Caruso, 2010; Dornier & Dufay, 2013). Gynodioecy is generally determined by a nucleo-cytoplasmic conflict between the mitochondrial genes causing male-sterility, called

CMS (for Cytoplasmic Male Sterility) and the nuclear genes restoring male-fertility, called restorers (Chase, 2007). Restoration is often determined by one or a few alleles in cultivated species (see Table 2 Delph et al., 2007 for a review), but studies in several other species suggest a more complex genetic determinism, as many gynodioecious species also exhibit intermediate sexual phenotypes (Ehlers et al., 2005). In order for a species to exhibit nuclear-cytoplasmic gynodioecy, there must be polymorphism at the cytoplasmic loci causing male-sterility but also among the nuclear loci involved in restoration of male-fertility. The stability of such polymorphism depends on a variety of factors, including the positive effects of CMS alleles (i.e., seed fitness advantage of females) and negative pleiotropic effects of restorer alleles (i.e., cost of restoration, (Charlesworth, 1981; Delannay et al., 1981; Frank, 1989; Gouyon et al., 1991), otherwise restorers go to fixation and the population comes back to the hermaphrodite state, whether CMS itself persists or not. Theoretical studies have defined three types of cost of restoration (Dufaÿ et al., 2007): (i) a *constitutive* cost which is cytoplasm independent (Bailey et al. 2003), (ii) a *silent* cost expressed when the restorer does not change the sexual morph, i.e. when it is not paired with the cytoplasm it can restore (Gouyon et al., 1991), (iii) an *expressed* cost concerning only restorers associated with a male-sterile cytoplasm they are able to restore (Bailey et al., 2003). The silent cost is necessary to avoid restorer fixation and maintain nuclear-cytoplasmic gynodioecy (Gouyon et al., 1991; Dufaÿ et al., 2007). This cost has been documented in crop species (see Table 2 in Delph et al., 2007) and three experimental studies on pollen viability in *Lobelia siphilitica* (Bailey, 2002), seed quality in *Plantago lanceolata* (de Haan et al., 1997) and pollen quality and viability in *Beta vulgaris* spp. *maritima* (Dufay et al., 2008; De Cauwer et al., 2011) also suggested a silent cost of the restorers. However, these costs are rarely reported in plants as different crosses have to be performed to test their existence and because ecological conditions can alter the cost of restoration (Caruso et al., 2012).

Because of long generation times, evolutionary dynamics of the CMS-restorer systems in plants are usually inferred from measures of fitness-related traits rather than directly observed (réf). However, the recent discovery of CMS in animal with rapid generations (David et al., 2022) provides an opportunity to monitor these dynamics in time. In the freshwater snail *Physa acuta*, a mitochondrial type called mitotype K has been identified in natural populations near Lyon (France), where it co-exists with the normal type N. While all N snails are male-fertile, mitotype K causes cytoplasmic male-sterility (Laugier et al., 2024). The same study provided evidence that K-specific restorer genes are abundant in these natural populations (Laugier et al. 2024). Here we investigated the evolution of restoration potential when the corresponding CMS cytoplasm (mitotype K) is removed. If restorers indeed incur a silent cost, we expect that their frequencies will diverge between populations maintained with and without CMS, and more specifically, starting from a high level in the source population, they should remain high in CMS-rich populations and decrease in CMS-poor ones.

We formed eight experimental populations divided into two treatments, 100%N and 100%K. We let these populations evolve for 11 generations and at the last generation we carried out a test of the restoration potential. The restoration potential was tested by crossing the experimental lines with K-mitotype individuals with two different nuclear backgrounds: (i) one background where restoration frequency was high, so that restorer alleles transmitted by the tested experimental line would be expected to be mostly in the homozygous form (ii) one background where restoration frequency was low, so that restorers would be mostly in the heterozygous form. Based on theoretical models, we predicted that after 11 generations of evolution the restoration potential of 100%N populations (i.e. where CMS is absent) will be lower than the restoration potential of 100%K populations. We also expected the restoration potential to be higher when the partner originated from a population with a high frequency of restoration.

Materials and Methods

The species

P. acuta (Physidae, Hygrophila, Gastropoda) is a freshwhater snail native with cosmopolitan distribution that lives in various freshwater habitats (Dillon et al., 2002). Individuals lay egg capsules that hatch in 7-10 days; sexual maturity is reached between six and eight weeks at 25°C. *Physa acuta* is a simultaneous hermaphrodite and preferential out-crosser, though it can self-fertilize if no mate is available for some time (Tsitrone et al., 2003); sperm and oocytes are produced within the same gland (ovotestis) and, unlike plants, external morphology is identical between male-sterile and male-fertile individuals – the difference can only be made based on the ability to sire offspring in controlled pairings, or based on dissection and sperm counts (David et al., 2022).

In this species, a first CMS-associated mitochondrial DNA called mitotype D was identified by David et al. (2022), and a second one, called mitotype K, by Laugier et al. (2024). Both D and K mitochondrial genomes are very divergent from the normal, male-fertile type (N). Although the K mitotype induces male-sterility, in natural populations found so far, this phenotype is mainly suppressed by (still unidentified) nuclear restorers. Male-sterile phenotypes re-appear in association with the K mitotype when the latter is introgressed into a laboratory population kept away from CMS for many generations, in which the restoration potential is very low (74% male-sterile phenotypes).

Populations

In the experiment we used four different laboratory populations called KF, KST, alb-2, NF distinguished by their origin (Table 1, Table S2) – either Lyon or Montpellier. KF and NF originate from natural populations near Lyon (France) where the K mitotype has been discovered and is mostly restored: they have been founded by pooling together on one side first-generation laboratory offspring with the K mitotype (population KF) and on the other side those with the N mitotype, and have been maintained as separate large outbred populations since then. The frequency of restoration was initially high in Lyon (the male-fertility status of 27 K-mitotype individuals was assessed and 22 of them were recorded as male-fertile, thus restored, Laugier et al. 2024), and we assume it has stayed so at least in the KF population (where all individuals have the K mitotype, and therefore selection favors the maintenance of restoration). Our populations from Montpellier (alb-2, NF) have been established and maintained for a much longer time in the laboratory (now >100 generations). They all derive from a common stock initiated in 2008 by pooling together 10 natural populations from the outskirts of Montpellier (300km south of Lyon); while we do not know the frequencies of mitotypes at that time in the natural populations, 100% of the individuals from our Montpellier laboratory populations are of the normal N type. Among them, one population alb-1 has an albino phenotype (which allows to distinguish snails easily in crosses) and was used in a long-term introgression protocol: the K mitotype from lyon was thus inserted into the Montpellier background (Laugier et al. 2024). At each generation, the sperm donors were albino individuals taken from the A_1 population, while the introgressed individual (playing the role of the mother) was pigmented. We renewed the lines using maternal offspring of the pigmented individual, and among them, keeping only the pigmented ones to propagate the lines (a mix of albino and pigmented is produced at each generation, as the mother is heterozygous). The individuals used in this study come from this introgressed K population at generations 30 (and later), and we consider that the original nuclear background from Lyon has been completely replaced by that of Montpellier (except for a small chromosomal region around the pigmentation locus). Introgressed individuals are mostly male-sterile (only 67% of them are able to obtain paternities from a virgin partner, Laugier et al. 2024) and will be referred to as the “KST population”. We do not know whether the 33% residual male-fertility is due to a low frequency of restorer alleles or to a complete absence of restorers with an incomplete penetrance of the sterility phenotype; in any case restorers, if present, are in much lower frequency in KST than in KF.

To assess the restoration status in G11 (see below), we also used another population derived from Montpellier: the alb-2 (100% N) population. This population has an albino phenotype, but determined by another locus than in the albino individuals used for introgression of KST. Albinism in *P. acuta* is controlled by two epistatic recessive unlinked loci (*ALB1* described in Noël et al. 2016 and A*LB2* recently established Supplementary Text 1). Both loci are required to possess a dominant wild-type allele for the snail to be pigmented, in any other case, the snail is hypochromatic. A different morph is associated to each locus: (i) the morph A1 is homozygous for both recessive allele *alb-1* at the first locus and for the dominant wild-type at the second locus (*a1/a1 P2/P2*) – the populations used to introgress KST belonged to this morph, (ii) the morph alb-2 is homozygous for the wild type at the first locus and for the recessive allele *alb-2* at the second locus (*P1/P1 a2/a2*). The two morphs differ in both their shell and eye colour, alb-1 individuals have beige bodies and eyes less dark than normal, while alb-2 individuals have yellow-orange bodies and completely colourless eyes. The two morphs complement, i.e. any outcrossed offspring produced by a KST and a A2 parent will be pigmented, as it has received a wild-type allele at each locus.

Experimental evolution design

We followed four 100% N (100%N^A, 100%N^B, 100%N^C, 100%N^D) and four 100% K (100%K^A, 100%K^B, 100%K^C, 100%K^D) experimental populations over 11 generations. The populations were propagated by 150 adult individuals each and replicates were not all synchronous. A first wave of populations (replicates A and B of each treatment) was founded in March 2021. Populations 100%N^C, 100%$N^{D,}$ 100%K^C were started in November 2021 and 100%K^D in January 2022.

Creating the experimental populations evolution design

We aimed to constitute the founding populations with the same initial nuclear background. To that end we used individuals from the NF and the KF laboratory populations which were at that time at their 7th generation since foundation (Table 1) and followed the same protocol as Laugier et al. (2024) (Figure S1). From these populations, 88 N and 88 K individuals (hereafter G_0 individuals) were raised in isolation to sexual maturity (age 50 days) and then put in pairs (one K individual with one N) for three days. All the G_0 individuals were re-isolated three days to lay eggs in separate boxes, then sacrificed to confirmed their mitotype using PCR tests. These crosses ensure homogeneity of the nuclear background, as among the offspring of a given pair, individuals used to populate the 100%K replicates, i.e. with mother K and father N have their maternal K mitotype, but otherwise the same nuclear background as individuals with mother N and father K, used to populate the 100%N replicates. Thus, the initial frequencies of restorers were equal among all populations, and represented the average between frequencies in the KF and NL populations at the time of foundation. Three weeks after the eggs hatched, the (still immature) offspring of all pairs were grouped in two tanks: one with the N mitotype offspring (mother N father K) and one with the K mitotype offspring (mother K father N). After ten days, we used these offspring to create the first generation of experimental populations (G_1) for a total of 200 individuals per population. One week after, we randomly removed 50 individuals per population, leaving 150 in each. We replicated this protocol for each wave of experimental populations.

Rearing conditions and passage of generations

Each snail population was maintained in a 1.5L tank filled with water pumped from the groundwater on the CEFE-CNRS campus and kept at 25°C as well as the room temperature. The snails were under 12:12 photoperiod (neon tubes) and fed with boiled organic lettuce, grinded more or less finely depending on the age and size of snails. We fed the snails and renewed the water in each tank twice a week. The position of each tank on the shelves was changed regularly to avoid micro-environmental effects.

Each generation started with eggs laid by previous-generation adults during 10 days in a fresh aquarium. Parental adults were removed and the eggs on the walls of the aquarium kept to hatch. Hatching took place between 1-2 weeks after egg collection, yielding large numbers of juveniles that we raised together for 2 more weeks. At this date, juveniles were still immature, and only 150 of them were transferred to a new aquarium (density regulation). After this age mortality is normally very low so that the number stays nearly constant in the aquarium until reproduction. Once the 150 individuals reached maturity (G_n) – we considered sexual maturity reached when at least twenty clutches were visible in the tank – we moved them into a fresh tank to let them lay. After ten days, we collected the clutches to start the new generation and transferred the adults into a second tank for another ten days to obtain back-up clutches. We sacrificed the 150 adults after collecting the backup clutches and preserved them in alcohol at -20°C cold room.

Restoration test at the eleventh generation

At the eleventh generation, we evaluated the restoration potential of the populations. For each population, we tested 50 focal individuals. The test consisted in two sets of crosses (Figure 2.1 crosses with KST, Figure 2.2 crosses with KF) to determine whether a focal individual transmitted restorers, i.e. whether the focal could sire a male-fertile offspring. These two sets of crosses differed in the expected frequency of restorer alleles inherited from the mothers (i.e., KST and KF individuals). Thus, the restoration potential is tested (i) in the homozygous state using a population with a high restoration frequency KF, (ii) or in the heterozygous state using a population with low restoration (KST).

The focal individuals were collected from the tanks at an early stage (three weeks after the eggs hatched) and each snail was raised in isolation to sexual maturity (age 50 days). Each focal was first paired with a virgin KST albino partner then with a virgin KF partner (Figure 1 1.A). All pairings took place for three days, then partners were isolated and laid eggs for another three days. The live juveniles produced by the albinos KST were counted and we recorded the morphs (pigmented or albino) to assess the male-fertility status of the focal (Box 1). When a focal was male-fertile, we randomly picked a pigmented offspring among the juveniles produced by the albino KST partner. Regarding the cross with the KF, the male-fertility status of the focal was assessed only by the presence or absence of juveniles – KF individuals being pigmented, they only laid pigmented offspring if any. Thus, the focal was considered male-fertile when the KF partner produced live juveniles and male-sterile when the KF partner did not lay eggs (Figure 2.A). Again, when a focal was male-fertile, we randomly picked a pigmented offspring among the juveniles produced by the KF partner. Due to a data storing error, data recording the male-fertility status of the focal were lost in three populations (100%N^C x KST, 100%K^B x KST, 100%K^D x KF).

The juveniles taken from the progeny of the KST and the KF (sired by male-fertile focal individual and called "$\text{Focal}_{\text{off}}$") were raised in isolation to sexual maturity, then paired with a virgin alb-2 partner (Figure 1 2.A, 2.B). From these last crosses we determined the male-fertility status of the $\text{Focal}_{\text{off}}$ (Box 1, Figure 1 2.A, 2.B).

Assessing male-fertility status

The male-fertility status of a focal snails is assessed from the number of pigmented juveniles (sired by the focal individual). Male-fertile individuals normally stimulate egg-laying when inseminating their virgin partner and sire the vast majority (usually 100%) of the offspring produced (which are therefore all pigmented). When individuals are male-sterile, they do not inseminate their partner, which can result in either the partner not laying any egg, or its laying self-fertilized eggs that develop into 100% albino juveniles. In some cases, pigmented juveniles are obtained, but their total number was abnormally low (<10), suggesting reduced (though not completely suppressed) male fertility. These individuals are recorded as "semi-sterile". An individual is considered fertile if two conditions are fulfilled: it sires more than half of the offspring of its partner and the total number of sired juveniles is >10. If we do not find any albino among the offspring sired by the focal, we stop counting the pigmented offspring at 20 and record the individual as male-fertile and its male fitness as 20+; if one or more albino are present, we count all the offspring.

Data analyses

We used Generalised Linear Mixed Models (GLMM) with binomial distributions using the `lme4` R package (Bates et al., 2015) to analyse the male-fertility status (a binary variable, fertile or not fertile). The crossing steps (A and B, Figure 2) were modelled independently and the treatment (100%N or 100%K), the maternal type (KF or KST) and their interaction were added as fixed effects. To account for common history of individuals within each population, a random factor 'replicate (within treatment)' was included, along with its interaction with the maternal type. The individuals recorded as semi-sterile were grouped with the male-sterile category.

Results

Male-fertility status could be assessed for (i) 42.63 ± 17.27 (mean ± standard deviation, crossing step A) and 39.63 ± 5.32 (crossing step B) individuals in the four replicates of the 100%N treatment, (ii) 36.75 ± 22.71 (crossing step A) and 41.38 ± 5.40 (crossing step B) individuals in the four replicates of the 100%K treatment.

At the first crossing step, the proportion of individuals that were successful as males was high in both treatments (on average 82%, and always >75%, Figure 2A). No significant effect of the maternal type (KF or KST), the treatment or their interaction were found on the male-fertility status (Table 2, Figure 2C). At the second crossing step, the proportion of male-sterile individuals increased in the 100%N populations (Figure 2B). A significant effect of the treatment on male-fertility status was detected (Table 2), the individuals from the 100%N populations being more male-sterile than the individuals from the 100%K populations. The estimated proportion of male-sterile individuals did not differ between maternal types (KST and KF); for both of them, it was around 0.28 in the 100%N populations and 0.08 in the 100%K populations (Figure 2D). No effect of the interaction between the maternal type and the treatment was detected (Table 2).

Discussion

The restoration potential decreases in non-CMS populations suggesting a cost of restoration

Here we looked for cost of restoring male-fertility in CMS: the restorers should be counter-selected in absence of CMS. In this experimental evolution, the 100%N and the 100%K populations were initially in a context of high restoration. Because restorers were in equal initial frequencies in both treatment (100%N and 100%K), our results shown a decline of the restoration potential over the eleven generation of experimental evolution. However, one population (100%N^{A}) exhibited a different pattern as the restoration potential was still high (>90% of male-fertile in the cross with the KST), probably reflecting drift. This population may have fixed restorer alleles early in the generations, when this was likely because they were still in high frequency as in the parental population.

Through the conditions for siring success, we could have selected K individuals carrying more restorers than the average of their population whereas this selection could not have occurred in the N individuals as they are all male-fertile. However, results of the first crosses showed that the K individuals were as male-fertile as the N regardless of the individuals they were paired with. This ensured that we had not selected the restoration in the 100%K populations, which could have explained the differences in the restoration potential observed between the 100%N and 100%N populations at the second cross. Les K étaient, autant qu’on peut en juger, tous restaurés dans les pops 100K (est-ce que c’est les mêmes K qui sont infertiles lors des deux croisements A???)

Unexpectedly, the restoration potential is higher in the crosses with the KST

As the restoration potential was higher in the KF population than in the KST population, we expected crosses with KF mothers to produce more male-fertile offspring than crosses with the KST ones. In the case of a dominant mode of action, this effect would be more visible when the father comes from a 100%N population (NF, where restorers are at lower frequency) than when it comes from a 100%K population (KF, where restorers are in very high frequency, so that the father nearly always transmits them to the offspring). In the case of restoration determined by recessive alleles, the reverse would be expected. Surprisingly, we did not find a significant effect of the interaction between the treatment and the maternal type.

As in other gynodioecious species (e.g. Charlesworth & Laporte, 1998; van Damme & van Delden, 1982; Koelewijn & Van Damme, 1995), we found individuals with a male semi-sterile phenotype in the second crossing step. We choose to binarise the phenotype by pooling the semi-sterile category with the sterile category, even though this intermediate phenotype was clearly observed – semi-sterile individuals produced from one to ten offspring whereas fertile individuals usually sired more than twenty. This binarisation might not be appropriate to analyse the male-fertility status and could explain why the interaction effect was not significant. Indeed, in the not fully restored populations ($100N^B$, $100N^C$, $100N^D$), we found more semi-sterile individuals than in the restored populations. On average, the proportion of male semi-sterile individuals was higher when individuals were crossed with a KF mother (7% vs 5 %). One explication could be that the restorers which decreased in frequency often have a partial effect on the phenotype: the restoration decreases progressively, creating first partial male-sterile and then fully male-sterile individuals. Partial male-sterile are found either because restorer alleles are less efficient when heterozygous or because there several loci control the restoration as suggested by Ehlers et al. (2005). The occurrence of semi-sterile in the cross with the KF mother suggests that restoration is not dominant, i.e. even if restorers are inherited from the mother, the alleles of the father decrease the male-fertility rate suggesting codominant or quantitative multilocus determination.

In this experiment, we considered that the cost of restoration could be express as a binary phenotype. However, the effect of the restorer on the male-sterile phenotype might be a binary trait (i.e. restored individual will express a male-fertile phenotype), this might not be the case for the cost of restoration. We expect individuals carrying restorers to have a reduced male and/or female fitness compared to individuals deprived of restorers (e.g. de Haan et al., 1997; Bailey, 2002). Under this assumption, individuals heterozygous at the restorer allele could have intermediate fitness values. For example in insecticide resistance, compensatory mutations arise after the evolution of resistance allele to reduce its cost; in absence of the insecticide the resistance allele is lost, then the compensatory mutations (e.g Lenormand et al., 1999). Selection for resistance or restoration in the absence of the target element could be more complicated than just the counter-selection of an unfavourable allele at a locus.

Implication of the restoration cost in maintenance of the polymorphism

By comparing the decline of CMS monitored in Laugier et al. *in press* in high restoration context with the decline of the restorers in absence CMS, we observed a stronger selection acting against CMS. Indeed, after 11 generations of experimental evolution, the CMS frequency was low and reached zero in some populations ranging from 5 to 75% of K individuals. Here XXX à voir en function des estimations faites plus haut On considère qu'on part à 100% de restauration ?

Discuter éventuellement sur la vitesse de cette sélection comparée à ce qui se passe sur la CMS : à quel endroit du cycle ca-t-on se trouverle plus souvent ? que nous manque-t-il pour le savoir ? cohérence avec le fait que les seules populations analysées pour l'instant aient des fréquences modérées de CMS et pourtant élevées de restauration… qu'attend-on dans les autres?

Conclusion

Here showed the counter-selection of the restoration male-function in absence of the male sterilizing cytoplasm after 11 generations of experimental evolution. Our study confirmed a prediction of the limit cycles (Gouyon et al., 1991; Dufay et al. 2007): when the frequency of CMS is low restorers decrease in frequency because they express a silent cost of restoration. However, the expression of this cost to be determined; it could be express on male and/or female function as found in plants (de Haan et al., 1997; Bailey, 2002; Dufay et al., 2008; De Cauwer et al., 2011). A large literature has investigated the cost of resistance, notably in the context of meiotic drive where suppressors could be rapidly eliminated in absence of the drive (Curtsinger & Feldman, 1980).

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Table 1: Populations used in the experiment.

Populations	Nuclear DNA origin	Mitochondrial DNA origin	Mitotype	Phenotype
KF	Lyon	Lyon	K	pigmented
KST albino	Montpellier	Lyon	K	alb-1
Alb-1	Montpellier	Montpellier	N	alb-1
Alb-2	Montpellier	Montpellier	N	alb-2
NF	Lyon	Lyon	N	pigmented

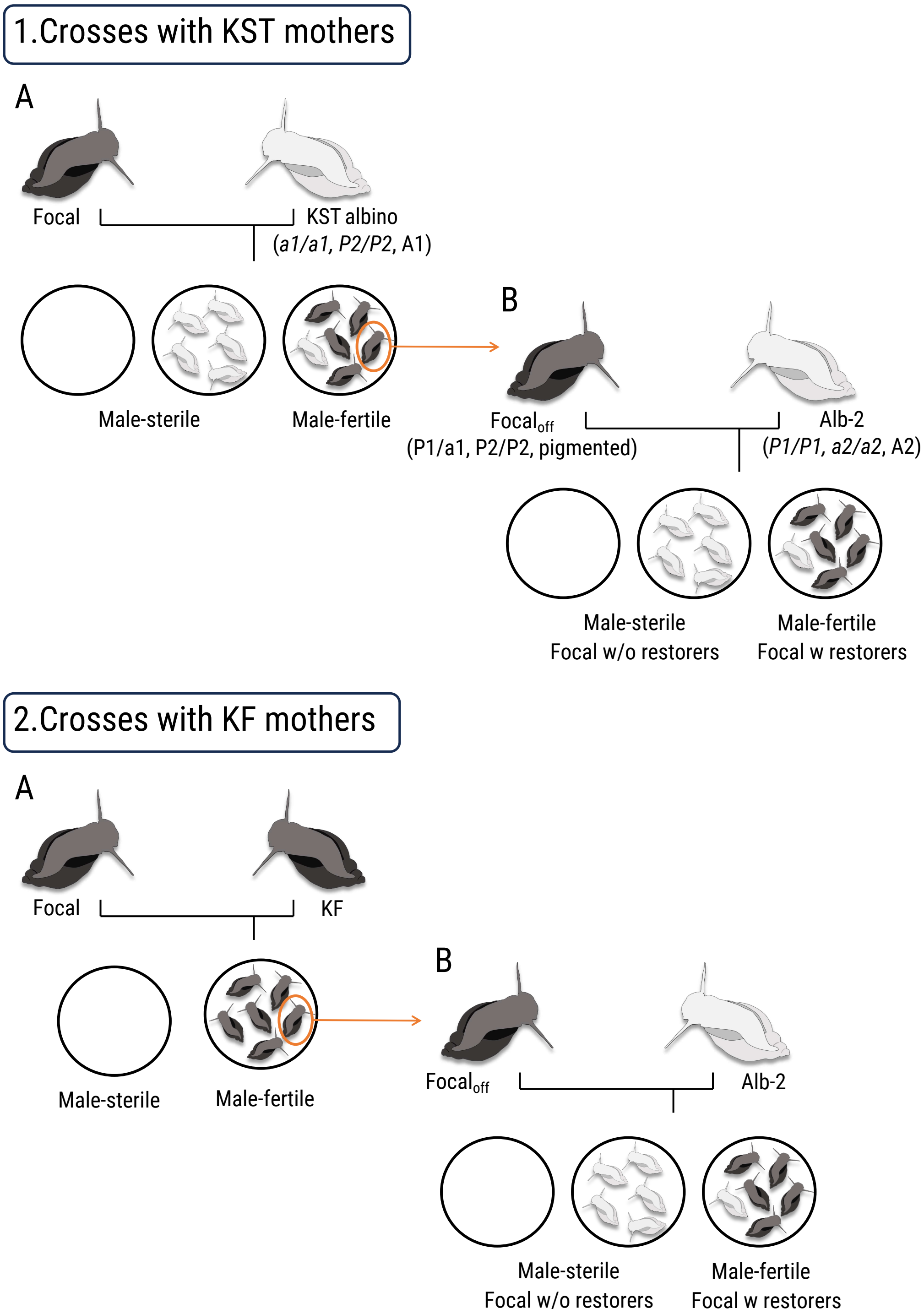

Figure 1: Protocol of the restoration test at the eleventh generation. We distinguished two sets of crosses 1) with KST individuals, 2) with KF individuals. Focal individuals were from the 100%N and 100%K experimental population and for each of the eight population we tested 50 individuals. A focal individual was first paired with a virgin KST albino partner (1.A) then with a virgin KF partner (2.A). From these crosses we determined the male-fertility status of the focal individual (Box 1) and isolated one offspring for each cross in

which the focal individual was identified as male-fertile (1.B and 2.B). In the 1.B and 2.B crosses we used alb-2 partners to be able to assess the male-fertility status: any cross-fertilized offspring laid by the alb-2 has a pigmented phenotype due to complementation (the alb-2 allele of the alb-2 individual is masked by the dominant wild-type allele given by the focal, and the alb-2 individual also transmits a dominant wild-type allele for the first pigmentation locus). In contrast, all self-fertilized offspring (if any) are phenotypically albino like their mother (see *Populations*). As did not affect the results, individuals recorded as semi-sterile were grouped with the sterile category for data analyses. Thus, from this restoration test, we differentiated three main categories of focal individuals: (i) male-sterile at first cross (A), (ii) male-fertile at the first cross (A) and siring male-sterile offspring (B), (iii) male-fertile at the first cross (A) siring male-fertile offspring (B).

Table 2: Results of the linear models on male-fertility status at the eleventh generation. We used Generalised Linear Mixed Models (GLMM) with binomial distributions. We added treatment (0%K or 100%K), maternal type (KF or KST) and their interaction were added as fixed. To account for common history of individuals within each population, a random factor ‘replicate (within treatment)’ was included, along with its interaction with the maternal type.

	Effects	Test of type effects, df = 1
Crossing step A	Treatment:maternal type	$\chi^2 = 0.27$, $p = 0.60$
	Treatment	$\chi^2 = 0.00$, $p = 0.95$
	Maternal type	$\chi^2 = 0.01$, $p = 0.92$
Crossing step B	Treatment:maternal type	$\chi^2 = 0.07$, $p = 0.80$
	Treatment	**$\chi^2 = 5.30$, $p = 0.02$**
	Maternal type	$\chi^2 = 0.01$, $p = 0.93$

Significant effects are shown in bold.

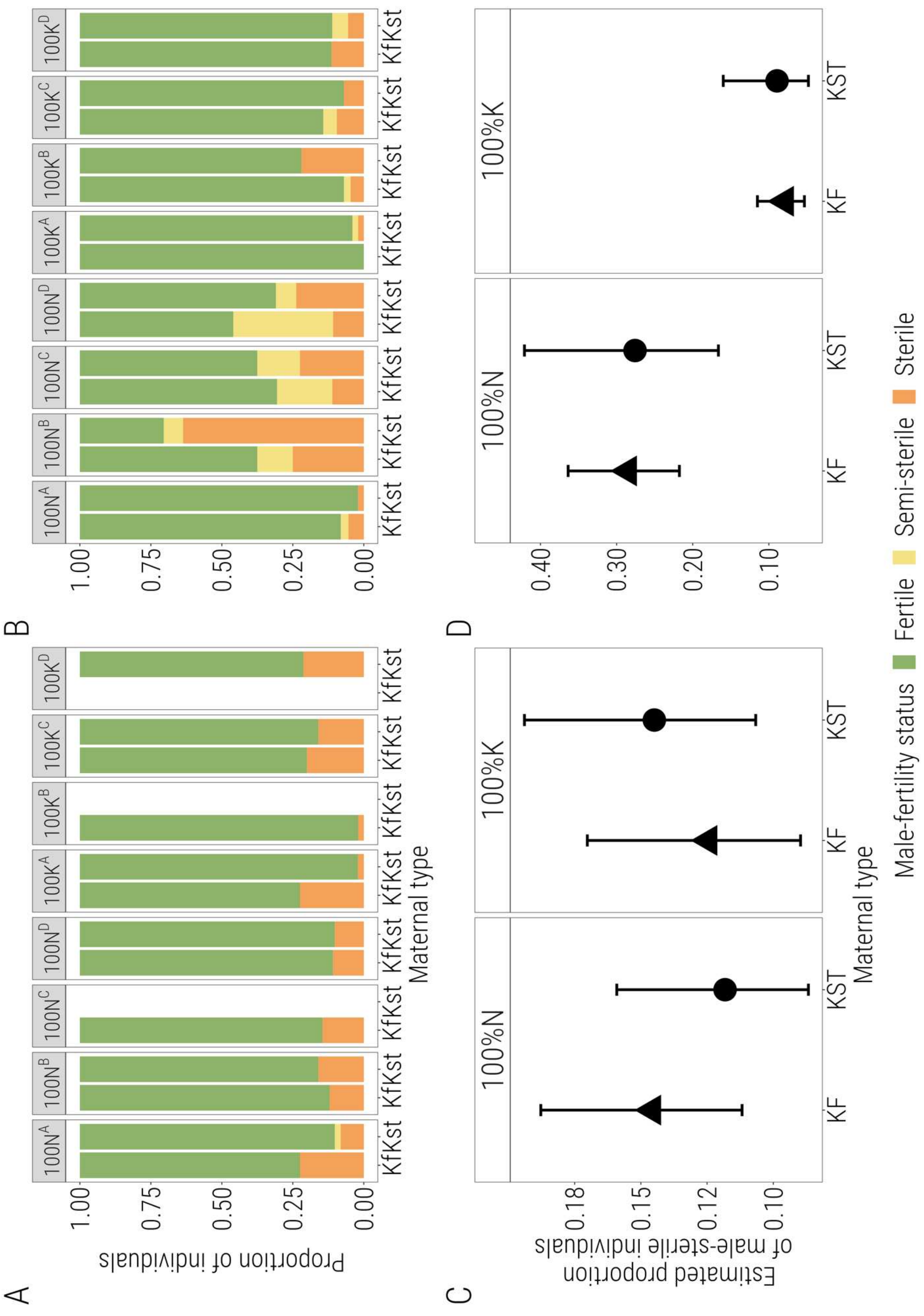
A
B
C
D
Proportion of individuals
1.00
0.75
0.50
0.25
0.00
100N^A
100N^B
100N^C
100N^D
100K^A
100K^B
100K^C
100K^D
Kf Kst
Maternal type
Estimated proportion of male-sterile individuals
0.18
0.15
0.12
0.10
0.40
0.30
0.20
0.10
100%N
100%K
KF
KST
Male-fertility status
Fertile
Semi-sterile
Sterile

Figure 2: Male-fertility test at the eleventh generation shown that the restoration potential decreased in the 100%N experimental populations. Male-fertility status was assessed by the proportion of juveniles sired by the focal individual and the offspring of the focal individual (Figure 1). For the male semi-sterile, we considered a threshold of less than 10 juveniles sired. The left panels (A and C) are the results obtain at the first crossing step when a focal individual is crossed with a KF and a KST. The absence of bar for 100%N^C x KST, 100%K^B x KST, 100%K^D x KF is due to a loss of data. The right panels (B and D) are the results obtain at the second crossing step when the offspring sired by the focal individual is crossed with an alb-2 individual. A and B: Proportion of individuals in the three categories of male-fertility status. For the eight populations, we distinguished the two maternal types (KF and KST). C and D: Estimates of the effect size and standard error of the male-sterile proportion for each treatment. We grouped the male semi-sterile, the male-sterile and considered them as male-sterile. At the second crossing step, the proportion of male-sterile individuals was significantly higher in the 100%N treatment.

Supplementary – Text 1: Origin and extraction of the alb-2 stock (from Skarlou & Laugier *in prep*, repeated here for convenience)

Phenotypes of three adults from the pigmented, alb-1 and alb-2 populations maintained in Montpellier.

The alb-2 stock was initiated from a single individual with depigmented phenotype that appeared within a self-fertilized brood of a parent from the Montpellier pigmented stock, generation 74 in December 2020 (see Figure 1). We suspected it could be a different mutant from the alb-1 albino stock already available, because (Dillon et al., 2002) mentioned the existence of two complementary albino strains in *Physa acuta*. In addition the adult phenotype of the new albino was slightly different from the alb-1: the dark pigments seemed completely absent, so that the body appeared slightly more orange (versus beige to yellowish for the alb-1), and the eyes were barely visible. These traits are however very difficult to ascertain on hatchlings or juveniles because the eyes of alb-1 hatchlings are barely visible too. We first let the newly discovered albino individual self-fertilize and obtained four G_1 offspring, each of which was crossed with a different individual belonging to the alb-1 population (complementation test). All the G_2 obtained were pigmented, suggesting that the new type, now named alb-2, and the alb-1 were mutated on different genes and that both mutations were recessive to their respective wild-type pigmented allele. From the supposedly double-heterozygous G_2 we created a G_3 (avoiding crosses between full sibs) among which we selected only the non-pigmented phenotypes (which therefore were, according to our hypothesis, homozygous for albinism at least at one of the two loci; however we could not at that stage recognise different albino phenotypes from one another with certainty). Within the G_3 we wanted to select those that were homozygous for the albinism allele at the second locus, but homozygous for the wild-type allele at the first; i.e. individuals that, upon crossing with the alb-1 stock, would give 100% pigmented phenotypes. To that end we paired each of 155 G_3 with a different virgin DS individual of albino phenotype. The DS are male-sterile so they act as females in this cross, and their genetic background is that of the alb-1 population. We let the DS partners lay and in case the offspring were 100% pigmented, we selected the corresponding G_3 individual. We found 4 such individuals and left them together to mate; their albino offspring constituted the G_4. This $G_4$was, given the low numbers of ancestors involved, rather inbred; to constitute a population with a larger genetic basis, we mated 22 of these G_4each to a different pigmented individual from the Montpellier pigmented population. The G_5 obtained was 100% pigmented (as expected) and we retained 150 individuals that were left to mate in two large aquaria. We retrieved a large number of G_6 juveniles among which we selected 100 from the minority that had the alb-2 phenotype; these were used to found the alb-2 stock which has since then been kept as a large autonomous population (5 aquaria of ~100 individuals each with regular exchange of individuals among them). At the seventh generation we also crossed eight alb-2 each to a distinct alb-1 individual and checked that all offspring were pigmented as expected.

60

31

Table S2: Experimental populations origin and populations used to perform the restoration test at G_{11}. At G_1, the experimental populations were constituted with K and N offspring of the G_0 crosses this based on the initial frequencies chosen and for a total of 150 individuals per population. As the replicates not synchronised, the partners used for the G_{11} restoration test were of different generations.

			Generation at the G_{11} crosses		
Treatment	Replicates	G_1 date	K_f	K_{st}	A_2
100%N	A	03/11/21	G_{15}		G_{14}
100%N	B	03/23/21	G_{15}		G_{15}
100%N	C	11/16/21	G_{19}		G_{15}
100%N	D	11/16/21	G_{19}		G_{16}
100%K	A	03/11/21	G_{20}		G_{14}
100%K	B	03/23/21	G_{20}		G_{16}
100%K	C	11/16/21	G_{23}		G_{16}
100%K	D	01/07/22	G_{24}		G_{17}

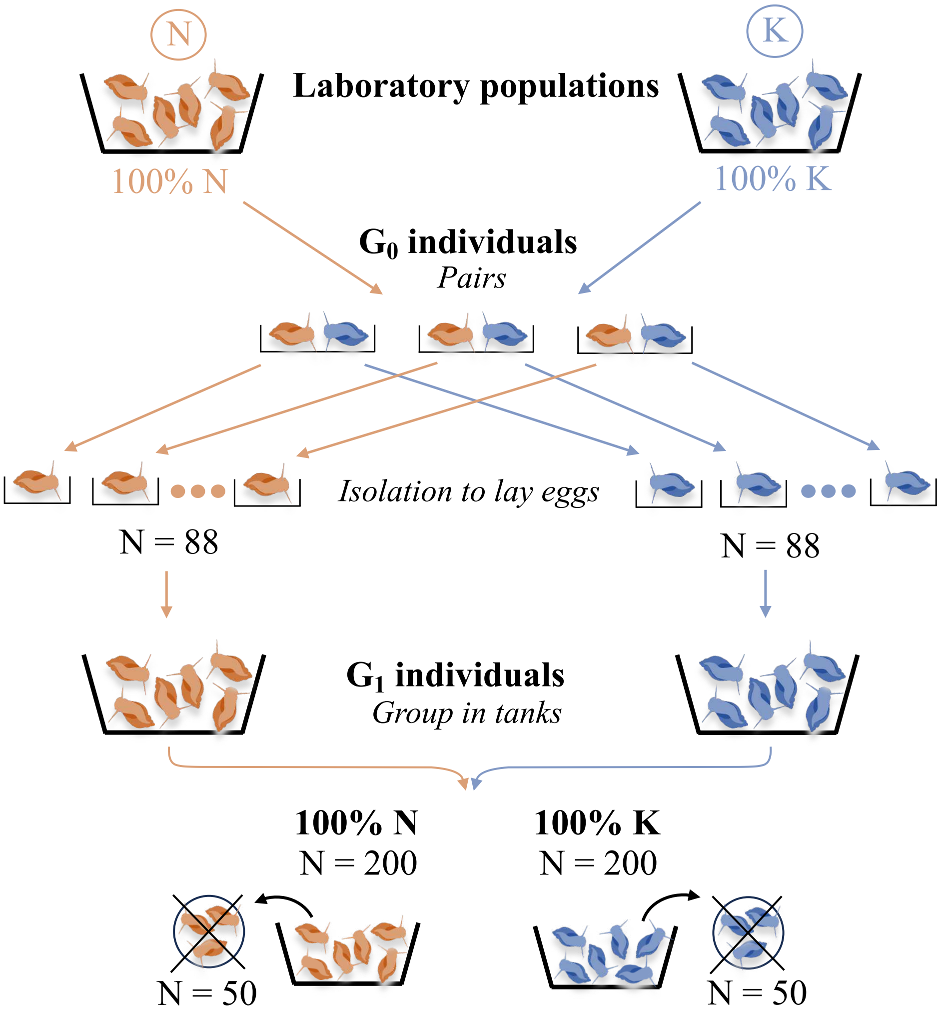

Figure S1: Constitution of the first generation of experimental populations (from Laugier et al. *in prep*, repeated here for convenience). Experimental populations originated from laboratory populations which derived from the same two natural populations near yon (France). In each laboratory populations, we randomly sampled 88 individuals (G_0 individuals). At sexual maturity, we paired them and subsequently isolated them for egg laying. Upon hatching, the offspring (G_1 individuals) were grouped and raised in two tanks, with each tank representing one mitotype. These (still immature) offspring were then used to form the four type of experimental populations. Each experimental population consisted of 200 individuals, then, we randomly removed 50 individuals from each populations to obtain N=150.

Supplementary – Text 2: Estimation of the restorer frequency. Here, we aimed to observe the evolution of the restorer frequency in both the 100%K and the 100%N experimental populations. Because we did not measure the restoration potential at the first generation of experimental evolution, we estimated the restorer frequency in the population of origin of both K and N individuals (Lyon, Laugier et al. 2024). From this data and the results obtained here, we calculated the frequencies using two hypotheses, (i) a dominant restorer associated with a fully-penetrant CMS, (ii) a dominant restorer associated with a partially penetrant CMS.

We defined x as the average failure rate of a cross between a N individual and a KST mother and p as the frequency of the restorer. We used the proportion of male-sterile K individuals estimated by Laugier et al. (2024) – 5 out of 27 K individuals were male-sterile – and x was estimated at 0.12 from the GLMM model (Figure 2C).

Dominant restorer with fully penetrant CMS

$$P_S = (1-(1-p)^2)\,x + (1-p)^2$$

CHAPITRE 3

SPECIFICITE & DETERMINISME DE LA RESTAURATION

CHAPITRE 3 – CONTEXTE

La plupart des modèles SMC/restaurateurs considèrent la présence d'allèles restaurateurs dominants (par exemple Charlesworth, 1981 ; Delannay et al., 1981 ; Dufay et al., 2007) comme c'est notamment le cas chez les espèces cultivées (Delph 2007). Cependant, dans les populations naturelles, la restauration semble avoir une détermination génétique plus complexe (Charlesworth & Laporte, 1998 ; Koelewijn, 2003).

Dans ce chapitre, nous explorons le déterminisme génétique et la spécificité de la restauration chez *Physa acuta*. Nous testons l'existence d'allèles restaurateurs dominants dans les populations naturelles où le mitotype K est présent. Nous testons également si ces restaurateurs sont spécifiques du mitotype K ou s'ils ont également un effet sur la fertilité mâle du mitotype D.

Complex genetic determination of male-fertility restoration in the gynodioecious snail *Physa acuta*

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In preparation

Abstract

Male fertility in plants is often controlled by the interaction between mitochondrial and nuclear genes. Some mitotypes confer cytoplasmic male sterility (CMS), making the individual male-sterile, unless the nuclear background contains alleles called restorers, that suppress the effects of CMS and restore the hermaphroditic condition. Restorers in cultivated crops are often alleles with strong and dominant effect, but in wild plants, data often suggest more complex systems. Here, we characterized the inheritance and specificity of restoration in a new CMS model, the freshwater snail *Physa acuta*. We explored two different populations (i) a naive population i.e., without contact CMS in the past 80 generations, (ii) a non-naive population, where CMS is present and largely restored. We found that restoration potential was indeed heritable in both contexts and specific to a CMS type. However our results suggest that restoration of male fertility could be of a different nature depending on population history: in naïve populations not coevolved with CMS the background variation may include alleles that happen to act as weak quantitative modifiers of the penetrance of CMS, while in populations coevolved with CMS, selection may have favored, when such variants were available, the emergence of strong alleles with a dominant effect.

Key words: cytoplasmic male sterility, restoration, genetic determination, *Physa acuta*

Introduction

Gynodioecy is a sexual polymorphism, common in angiosperms (Dufay et al., 2014; Caruso et al., 2016), whereby hermaphrodites coexist with male-sterile individuals (i.e., functionally females, Saumitou-Laprade et al., 1994). Gynodioecy represents a well-known example of genetic conflict (Burt & Trivers, 2006) because sex determination in gynodioecious systems is often cyto-nuclear. In that case, cytoplasmic male sterility (CMS) genes, usually associated with the mitochondrial genome, suppress pollen production and render an individual functionally female unless CMS is counteracted by one or more nuclear genes that restore the male function (Cosmides & Tooby, 1981; Saumitou-Laprade et al., 1994; Werren & Beukeboom, 1998). An individual is phenotypically female if it carries CMS without the proper nuclear gene known as a restorer. It is a hermaphrodite if it carries either (i) a male-fertile cytoplasm, or (ii) a CMS cytoplasm paired with the proper nuclear restorer. CMS has been documented in 140 species of angiosperms (Laser & Lersten, 1972), and twenty-eight CMS genes from 13 crop species have been identified (Chen & Liu, 2014).

It appears that cyto-nuclear gynodieocious systems often contain multiple forms of CMS (e.g. *Oryza sativa, Zea mays*, see Chen and Liu 2014 for a review), each requiring its own mode of restoration (A. de Haan et al., 1997; Charlesworth & Laporte, 1998; van Damme et al., 2004). Fourteen restorer genes (*Rf*) from nine crops have been isolated (see Table 1 Kim & Zhang, 2018), with the majority of them encoding PPR (Pentatricopeptide Proteins Repeat). However, *Rf* genes encoding for other proteins have been discovered, including peptidase in sugar beet (Yamamoto et al., 2008; Matsuhira et al., 2012) and aldehyde dehydrogenase in maize (Cui et al., 1996). In most cases, a *Rf* gene is specific to one CMS, whereas a CMS gene can be affected by multiple *Rf* genes (see Table 1 Chen and Liu 2014). For example, in *Z. mays* the CMS-T is restored by both *Rf1* and *Rf2* (Cui et al., 1996; Dewey et al., 1987; Dill et al., 1997) and in *Plantago coronopus* at least five restorer alleles seem to be involved in the restoration of CMS (Koelewijn & Van Damme, 1995). Cases in which a gene restores more than one CMS mitotype are rare. Such an example is found in the common rice, where *Rf5* and *Rf6* (initially identified in CMS-HL individuals) are also able to restore CMS-BT mitotype (Huang et al., 2012, 2015; Kim & Zhang, 2018).

The maintenance of cyto-nuclear polymorphism has been extensively modelled (e.g. (Charlesworth & Ganders, 1979; Couvet et al., 1998; Gouyon et al., 1991 ; Bailey et al., 2003 ; Dufaÿ et al., 2007) and theoretical studies have shown that the stability of this polymorphism depends on a variety of factors, including the positive pleiotropic effects of CMS alleles (i.e., seed fitness advantage of females, or "female advantage", Lewis, 1941) and negative pleiotropic effects of restorer alleles ("cost of restoration", Charlesworth & Ganders, 1979; Delannay et al., 1981; Frank, 1989; Gouyon et al., 1991). Models often assume that restoration of male fertility is achieved through one allele (but see Frank 1989) and a variety of assumptions regarding its dominance of restoration have been modeled (Delph et al., 2007). The majority of those models considered the presence of dominant restorer alleles with only a couple a cases using a recessive inheritance (Ross & Gregorius, 1985). Regarding empirical studies, most of the crop species exhibited dominant restorer alleles (see Table 2 Delph 2007 for a review but see also *Plantago lanceolata*, van Damme, 1984 ; de Haan et al., 1997). However, in natural populations the restoration of male fertility suggests a more complex genetic determination (Charlesworth & Laporte, 1998; Koelewijn, 2003; Touzet et al., 2004). Indeed, several nuclear loci may act in an additive manner (Charlesworth & Laporte, 1998; Dufay et al., 2008) and/or through a threshold effect (Ehlers et al., 2005). For example, Koelewijn & van Damme (1995) suggested that at least five restorer loci were involved in restoring male sterile cytoplasms of *Plantago coronopus*, and Dudle et al., (2001)found that sex determination in *Lobelia siphilitica* seemed to vary among cytoplasms with one CMS gene being restored by a single dominant allele, while restoration of other CMS could only be explained by the action of several nuclear loci and/or epistatic effects. Inspired by those examples, Elhers et. al (2005) developed a threshold model for sex-determination in gynodioecious species and showed that treating restoration of CMS as a quantitative trait can be an alternative way of explaining complicated patterns of restoration in CMS species. Following on those steps, Bailey & Delph (2007) described a model of cyto-nuclear controlled gynodioecy, in which sex was determined by two CMS types each one interacting with multiple restorer alleles. The used assumptions reflected several well-studied cases of gynodioecy, such as, *P. coronopus, P. lanceolata, S. vulgaris*, and *T. vulgaris* and showed that females can be maintained in the population (Belhassen et al., 1991; de Haan et al., 1997; Koelewijn, 2003; Mccauley & Olson, 2003).

A mix of several determinisms is an interesting situation to consider. Dominant and specific restorers acting on one or a few CMS mitotypes, found in present-day plant populations, are strong-effect alleles that may result from a history of coevolutionary arms-race with their specific CMS gene (e.g. Sloan et al., 2012), or at least from the selective sweep of a favorable mutant (Bergero et al., 2019). When a new CMS type first appears, it is unlikely that such strong-effect specific restorer alleles are already ready and waiting. However, the standing variation may contain genotypes that happen to reduce the penetrance of CMS, not necessarily in a way that fully restores male-fertility, and not necessarily in a specific way. Such a background variation may contribute to the cases of complex inheritance in plants, but is expected to be more visible when there are not yet specific large-effect restorers segregating in the population, a relatively rare condition (refs).

As illustrated above, CMS is well understood theoretically and prevails in plants but is exceedingly rare among animals (Vellnow et al., 2017). However, CMS has been recently reported in one animal: *Physa acuta*, a freshwater snail (David et al., 2022). In this species, two mitochondrial types conferring male sterility, D and K, have been discovered so far, both of them showing an extreme molecular divergence from one another, as well as from normal, fertile cytotypes (collectively called N), at all mitochondrial genes. The D mitochondrial type was discovered first, but evidence for corresponding nuclear restoration is still lacking (David et al. 2022). Recently however, the second CMS mitotype K revealed a different situation, with evidence that most K individuals captured in natural populations have a male-fertile phenotype, i.e., there exist genes that restore male fertility in K-mitotype snails, while male-sterility reappears when the K mitotype is introgressed into a “naïve” laboratory genetic background maintained without contact with CMS (Laugier et al., 2024).

In this study, we explored the inheritance and specificity of restoration in *Physa acuta*. We aimed to characterize the variation in restoration for the K mitotype, specifically to test if the data are consistent with the existence of dominant-restorer alleles in natural populations where K is present. We also tested whether these restorers are specific to K or also have an effect on male-fertility of the D mitotype. Finally, we were interested in the determination of male-fertility in our laboratory population, where the K-mitotype, although mostly male-sterile, still maintains a fraction of male-fertile individuals. This population may represent a truly “naïve” state, i.e. the state of a population in which CMS would be newly introduced and confronted to standing variation; or it may have inherited, from many generations ago, the same restorer alleles as in natural populations where K is present, but these alleles would now be at a low frequency.

To achieve these objectives, we phenotyped CMS K- and D-mitotype individuals within two different nuclear backgrounds (Lyon and Montpellier). The Lyon population has been in contact with both K and D, while the Montpellier has not. We used two different approaches to reveal genetic variation in restoration potential within Lyon and Montpellier population: we produced populations selected against and for restoration, and we analysed inbred lines. This approach enables us to explore restoration in various contexts and characterize it accordingly. Answers to these questions will provide insight into the CMS evolutionary dynamics and, for the first time, explore genetic variation for CMS restoration in animals.

Material and methods

Study system

Physa acuta is a cosmopolitan, hermaphroditic, predominantly outcrossing, freshwater snail, very common in natural freshwaters worldwide, and easy to raise in the laboratory. The generation time is about 6-8 weeks in the laboratory at 25°C ; we maintain this snail in standard conditions (25°C, 12/12 photoperiod) using ground water changed at least once a week, and grinded boiled lettuce ad libitum as food. Cytoplasmic male sterility has been first described by David et al. (2022) in this species, later completed by Laugier et al. (2024). Briefly two very divergent mitogenomes, named D and K, have been found to coexist with the normal (N) type in wild populations near Lyon (France). While N individuals are regular hermaphrodites, D and K are associated with male-sterile phenotypes, unable to play the male role in sexual interactions and to sire offspring. Nearly all the D (92%, David et al. 2022) individuals are male-sterile. In contrast, nuclear backgrounds restoring male-fertility of K snails are found in natural populations where K is present in Lyon (France). However the same K mitotype, when introgressed into the nuclear background of a population from Montpellier (hereafter « naive », maintained for >80 generations in the laboratory without contact with CMS), produces around 70% male-sterile phenotypes (Laugier et al., 2024). The nature of the ~30% residual fertility is still hypothetical, it could be either that restorer genes are segregating at low frequency in the naive population (despite the absence of K mitotypes) ; or that the penetrance of CMS is incomplete, always leaving a fraction of individuals with nonzero male fitness.

General structure of the experiments

Our objective was to characterize, within both the naive (Montpellier) and the non-naive (Lyon) backgrounds, genetic variation for the potential to restore male fertility in CMS mitotypes. We did this in two ways (i) first, we tested whether we could select for high versus low restoration potential within the naive population (ii) second, we produced inbred lines from both populations and characterized genetic variance in restoration potential among lines (trying, in particular, to find lines fixed for either restorer or maintainer alleles). In both cases, lines were of the N mitotype, and their restoration potential was evaluated by crossing them with either a K or a D snail, and evaluating the male fertility of the offspring. In these crosses, the paternal strains were N while the maternal ones were K or D. As mentioned above, all (or nearly all) our D individuals are un-restored, mostly male-sterile individuals. However, two K strains are available, one with the original background from Lyon (with high restoration potential and mostly male-fertile, noted KF) and one with the naive background from Montpellier (low restoration potential, mostly male-sterile, noted KS). We used both types to be in position to observe the effects of potential restorer alleles provided by the paternal strain either in a predominantly homozygous (cross with KF) or heterozygous (cross with KS) form.

Source populations and their history in the laboratory

The Montpellier population has been founded in 2007 by a mix of ten natural populations sampled around Montpellier, and maintained in the laboratory, in high numbers, since then (Figure 1). The albino stock was separated from the main population in the first few laboratory generations (see Noël et al., 2016). It has been maintained as an autonomous large outbred population in aquaria to this day (estimated age in 2023: 96 generations) and is entirely of the N mitotype and male-fertile. Albinism is coded by a recessive allele, and albino mothers produce pigmented babies when inseminated by a wild type snail. More recently, a new type of albinotic mutants appeared within the Montpellier stock, with a slightly different phenotype (see Figure S1 and S1). After some crosses (see S1) we were able to establish it as an independent outbred stock (called alb-2) that complements with the first albino stock (now called alb-1): alb-1 and alb-2 are mutated at different loci, and crosses between them produce 100% pigmented individuals, as well as crosses between any of the two and pigmented homozygous snails.

The Lyon populations have been founded from two populations sampled in 2019 near Lyon (Irigny and Erevan, see Laugier et al. 2024) mixed together. They initially contained a mix of K and N mitotypes, the former with mostly male-fertile phenotypes (see above) and all pigmented (wild-type). After mass-mating, we collected progenies from individual mothers that were typed by PCR as either K or N; and the K and N progenies were pooled into two separate tanks, to initiate pure-K and pure-N stocks (Figure 1). Each stock was kept as a separate population (hereafter KF and NF populations) and maintained as a large outbred population to this day (in 2023, KF and NF were at generation 20 after foundation). Each of the outbred populations (alb-1, alb-2, NF, KF) was maintained throughout as a metapopulation of 5 aquaria, each with 100-150 snails at reproductive stage, freely mating (« mass-mating »), with regular exchange of ~10% individuals among aquaria; the eggs are separated from the parents to hatch as soon as there are enough on the aquarium walls, and a new cohort is started. Aquaria are fed once to twice a week depending on life stage.

A last source of snails for our experiments is a set of lines in which the D or the K mitotypes have been introgressed into the naive Montpellier alb-1 background (David et al 2022, Laugier et al 2024). Briefly this introgression takes place in sets of « matrilines » each founded by a D or K ancestor from Lyon (wild population) and propagated by maternal descent. At each generation each snail from a matriline is inseminated by an individual taken at random from the alb-1 stock so that over generations the genome of the founder is progressively replaced by the alb-1 background. At the time of the experiment the D matrilines were at the 41th generation and the K matrilines at the 20th generation, their genome can be considered as totally replaced. Matrilines are maintained pigmented and heterozygous to distinguish them from their albino mates: at each generation we obtain a mix of albino and pigmented progeny, of which we keep only the latter to propagate the matriline. For the experiment however it was practical to use the albino progeny. Both K and D matrilines are mostly male-sterile (as mentioned above); for this reason we will refer to them as KS and DS respectively (by contrast, KF snails are restored and mostly male-fertile).

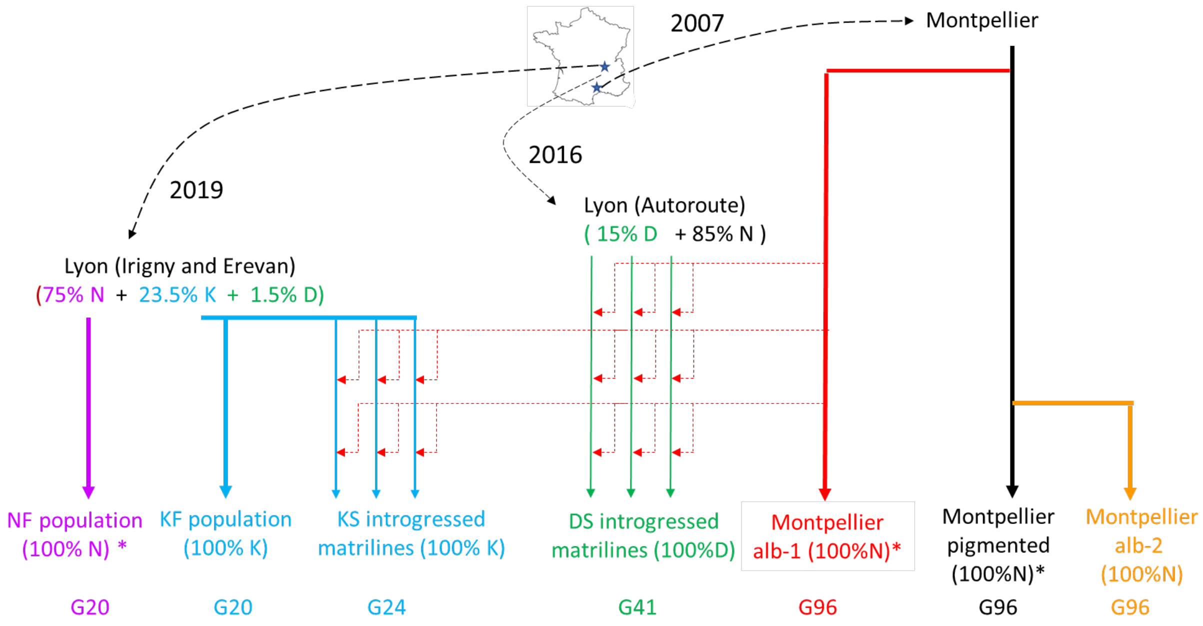

Figure 1: **Source populations and their history in the laboratory since extraction from natural populations (indicated by dashed arrows, with year of sampling).** Thick lines indicate propagation in large outbred populations in aquaria. Fine lines indicate propagation in matrilines, with insemination at each generation by sperm donors from another population (dotted arrows). The percentages of each mitotype (K, D or N) are indicated for the natural populations at sampling (from David et al.2022, Laugier et al. 2024) and for populations kept in the laboratory. The number of generations elapsed since extraction from natural populations in late 2022 – early 2023 (when most of the experiment took place) is indicated as G_{XX} below each stock. The numbers of independent matrilines are 12 for KS and 11 for DS. Populations from which inbred lines were extracted are indicated by a star (*). The coordinates of the populations of origin can be found in Noël et al. 2016 (Montpellier), David et al. 2022 (Lyon, Autoroute), and Laugier et al. 2024 (Lyon, Irigny and Erevan)

Selection for high and low frequency of restoration of the K mitotype

The aim of this selection was to mimick the arrival of a CMS mutant in a naive population and test whether there was variation from which restoration potential could be rapidly selected in such a situation. This was envisaged for the K mitotype because the penetrance of the male-sterile phenotype in KS individuals (mitotype K introgressed into the naive alb-1 background) is around 60-70% ; if the residual fertility is genetic it can increase or decrease by selection. On the other hand, the residual fertility of DS snails is too low to start a similar selection.

The selection protocols are detailed in supplementary S2. In short, to select against restoration, we kept the (N-mitotype) alb-1 sperm donors that, when crossed with a KS snail, produced only male-sterile K daughters. The selected sperm donors where then grouped and constituted the first generation of selection for low-frequency-of-restoration (LFR). We re-used offspring of these individuals as sperm donors for a second round of selection. The LFR individuals used here are therefore considered to be at their second generation of selection against restoration. The selection for high frequency of restoration (HFR) is simpler because selecting for fertility is easier than for sterility: we simply kept the offspring sired by male-fertile KS individuals at each generation. The HFR population was selected for two successive generations, as the LFR. Expected results of such a selection in the simplest scenario (fully penetrant, monodominant restorers) are given in S2.

Constitution of inbred lines

Inbred lines allow in principle to fix restoring and non-restoring genotypes in the homozygous form. We created inbred lines from N-mitotype ancestors originating from either the Lyon or the Montpellier laboratory populations (more lines were made from Montpellier where restoration is supposed to be rare). Each ancestor produced a line by at least two generations of selfing (enforced by letting a virgin snail reproduce in isolation) and single-individual descent. Lines are not fully homozygous at the second generation (there remains ¼ of the initial heterozygosity) but we had to find a compromise with the time required and with the potential loss of lines by inbreeding depression over generations. In this experiment we used 9 lines from Lyon and 40 from Montpellier.

Crosses

In total we tested the restoration potential of focal individuals from two selection lines (HFR and LFR) and from 49 inbred lines of two origins: Montpellier (naive population= and Lyon (experienced population). To that end these focal individuals were used to inseminate three types of individuals: DS, KS and KF. The resulting progeny has the focal individual as father and DS, KS or KF mother. In each progeny we grew one to three G1 offspring until maturity and tested them for male fertility. If the father transmits restorer alleles, these alleles are present in the tested offspring either in a mostly heterozygous form (mothers DS or KS, with absent or rare restoration), or in a mostly homozygous form (mothers KF, with a high rate of restoration). The total number of progenies tested was 1508, with the repartition indicated in Table 1.

Table 1: Sample sizes by category. For each combination of paternal and maternal types, we provide N_1/N_2, where N_1 is the number of G_1 individuals successfully assessed for male fertility, and N_2 is the number of G_0 mothers (each mother produced 1 or 2 G_1 individual).

		Maternal			
		KS	KF	DS	Total
Paternal	Lyon inbred lines	164/82	36/21	65/47	265/150
	Montpellier inbred lines	350/231	220/130	211/168	781/529
	Total inbred lines	524/313	256/140	276/215	1056/668
	HFR selected population	52/31	49/27	32/22	133/80
	LFR selected population	248/105	39/20	32/25	319/150
	Total selected populations	300/136	88/47	64/47	452/230

Assessment of male sterility

The assessment of male sterility of an individual is made by pairing it to a virgin partner for three days, letting the latter lay for three days in isolation, and observing the progeny. The studied individual is said to be male-sterile if either the partner does not lay any egg (failure to stimulate egg-laying, which is a normal outcome of insemination) or it lays eggs but the juveniles are 100% self-fertilized. Partners of male-fertile individuals in this situation normally produce several tens of eggs and juveniles are nearly all outcrossed. A small number of individuals had an intermediate status: outcrossed individuals were obtained but in abnormally low numbers (<10) ; in this case we recorded them as semi-sterile. In the analyses we grouped these together with fully male-sterile individuals (without appreciable consequence on the results). It is, in any case, important to realize that the male-fertility test is not perfect: even male-fertile individuals (with fertile mitotype) occasionally fail to inseminate their partner during pairing, with a probability <10% in our lab conditions.

We used body pigmentation to recognise outcrossed offspring from self-fertilized ones. Depending on the paternal pigmentation genotype, we used either albino alb-1 or alb-2 partners in such a way that any pigmented offspring they produced must have been sired by the tested individuals. We therefore simply counted the number of pigmented offspring in the progeny after incubating clutches for 13 days (we stopped counting at 20 and noted 20+ if all offspring were pigmented, and counted exhaustively all pigmented and albino offspring otherwise, including those that were well-developed though not yet hatched, if any). In some cases (heterozygous individuals tested with an alb-1 partner) outcrossed offspring were expected to be 50% pigmented, 50% albino instead of 100% pigmented; in this case we set the threshold for semi-sterility to 5 pigmented (i.e. the expectation for 10 outcrossed offspring) instead of 10 (see above).

We tested male fertility in G1 individuals and in their DS, KS, KF mothers too. Many of the latter were pigmented and mated with albino N-mitotype fathers; thus the father could act as a partner for testing the male-sterility of the mother, and we kept the father's progeny to that end. In case the father was a pigmented individual and/or the mother an albino, this was not possible so we provided a second partner to the mother, after she had laid eggs, and this second partner was alb-2 so that outcrossed offspring would be recognised (alb-1 and alb-2 are complementary lines and produce pigmented babies upon cross-fertilization).

Because of the size of the experiment, it was spread over nearly two years from 2022 to early 2024, with four phases treated as temporal blocks.

Statistical analyses

We analysed separately the differences between the selected populations (HFR and LFR) on one hand, and the variation among inbred lines on the other hand. The male-fertility status of the G_1 individuals from selected lines was treated by GLMMs as a binary 0/1 variable. The main model included the maternal type (KF, KS or DS), the selected line (HFR or LFR) and their interaction as fixed effects, and the identity of the mother and the block as random effects. We also made more detailed models including the maternal status (fertile or sterile) in the fixed effects. The male-fertility status of the G_1 individuals from inbred lines was treated in a similar way, except that the fixed effects included: the geographic origin of the inbred line (Montpellier versus Lyon), the maternal type (KF, KS or DS) and their interaction; and the random effects included the identity of the inbred line, in interaction with maternal type, in addition to block and mother identity. All tests were performed by likelihood-ratio chi-squares, comparing models with and without the effect of interest with all other effects kept. The models were run using the `lme4` package in R (Bates et al., 2015).

Predictions

The selected lines inform on the nature of the variation in male-fertility observed when CMS is introduced into a naive background (the Montpellier laboratory population), i.e. why is there a residual male fertility in K individuals introgressed into this background ? If there exist restorer alleles with large effects, we expect the selection protocol to be efficient, and the frequency of male-fertility of K individuals sired by HFR fathers should exceed that of their congeners with LFR fathers. Depending on dominance, this effect should be more visible in different circumstances. A dominant allele transmitted by fathers makes a difference when the mother does not herself bring the same allele (in our case, with male-sterile mothers, and/or with mothers from the KS strain). A recessive allele, on the contrary, would have more effects when the mother is male-fertile and/or from the KF strain. Finally, the specificity of restoration with respect to the mitotype can be tested by checking whether the LFR/HFR difference, if any, affects male-fertility when the mother is of the D mitotype.

The inbred lines allow to document the nature and specificity of restoration in both the Montpellier and Lyon populations. Based on previous studies (Laugier et al. 2014) we expect that K-mitotype individuals sired by Lyon lines (a largely restored population) will have more male-fertile offspring than those sired by Montpellier. We expect, if restoration has a simple genetic basis, that some lines will be homozygous for restorers and others for maintainer alleles, generating high variation in male-fertility among lines, either when crossed with KS / male-sterile mothers (if restorers are dominant) or when crossed with KF / male-fertile mothers (if recessive). Similar effects could be observed upon crossing with DS if there exist restorers efficient against CMS brought by the D mitotype; restoration may be specific (in that case, the same lines should restore fertility in both D and K) or not.

Results

Male-sterility in the maternal generation

The phenotypes of the mothers used to test the restoration potential in inbred lines and selected (HFR/LFR) lines were distributed in the same way and conformed to expectations based on previous studies (see Material and methods): mothers with CMS introgressed into the alb-1 Montpellier background were mostly male-sterile with (in total) 5.7% of male-fertile individuals among D-mitotype mothers (DS) and 27.2% in K-mitotype ones (KS). In contrast nearly all (92.3%) mothers of the KF type were male-fertile. In both datasets the proportion of male-fertile individuals differed very significantly among types (Binomial GLMMs, χ^2_{1df} =119.9, and 36.6 respectively for the inbred lines and selected lines datasets, both $p < 0.001$).

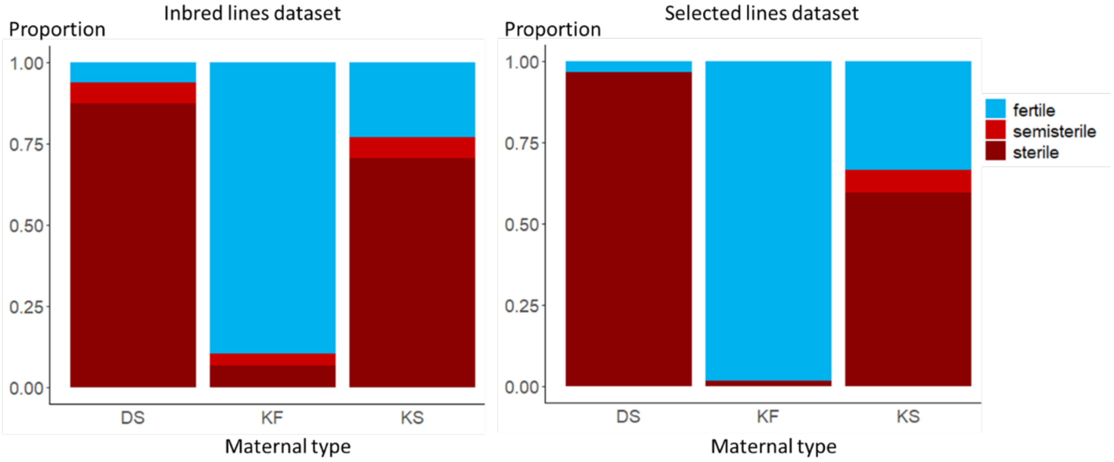

Figure 2: Proportions of individuals with male-sterile, male-fertile and male-semi-sterile phenotype in the maternal generation. Proportion are represented for both datasets as a function of maternal type DS: D-mitotype introgressed into the alb-1 background from Montpellier; KF : K-mitotype within its native background (Lyon) ; KS : K-mitotype

introgressed into the alb-1 background from Montpellier. The two datasets are presented separately because they were made at different times.

Male sterility in the offspring generation: LFR versus HFR

The proportions of male-fertile offspring closely mirror those observed in their mothers (Figure 3, compare with Figure 2), and the maternal type (DS, KS, or KF) accordingly has a very significant effect on the probability of being male-fertile ($p < 0.001$, Table 2). The paternal line (HFR or HFR) has a weakly significant effect ($p = 0.039$, Table 2), and this effect is perceptible only when the mother has a K mitotype introgressed into the alb-1 Montpellier background (KS mothers, Table 2). In this category the proportion of male-fertile offspring is slightly higher when the father comes from the LFR line than when the father comes from the HFR line.

The effect of the maternal phenotype (i.e. whether the mother was a male-sterile or a male-fertile snail) cannot be studied in the DS and KF categories because of insufficient numbers (only one DS individual with a male-fertile mother; only two KF individuals with a male-sterile mother). In the KS category however, numbers are more balanced (98 individuals with male-fertile mothers, versus 202 with male-sterile or -semisterile mothers), and the maternal phenotype partially predicts the offspring's one. Indeed, male-fertile mothers tend to have more male-fertile offspring, whatever the paternal line; the influence of the paternal line remains significant, and in the same direction as previously, when the maternal phenotype is taken into account (GLMM: effect of paternal line $\chi^2_{1df} = 4.65$, $p = 0.031$; effect of maternal phenotype $\chi^2_{1df} = 4.48$, P = 0.034 ; interaction $\chi^2_{1df} = 0.15$, $p = 0.70$; Figure 3).

Note that full-sibs (offspring sharing the same mother and father) tend to have similar phenotypes as attested by significant effects of mother identity (Table 2) on top of what is already explained by the fixed effect of maternal type. This mother effect is not completely predicted by maternal phenotype (among KS mothers) as it remains approximately the same in the model with the maternal phenotype included as a fixed effect ($\chi^2_{1df} = 4.80$, variance = 0.72, $p = 0.014$). Note that the mother effect is also, surprisingly, significant within the KF category (Table 2) ; this is because among the few (four) individuals with KF mother and HFR father observed to be male-sterile, two have the same mother which is unlikely by chance.

Table 2: Generalised linear mixed models on the binomial male-fertility variable (fertile versus sterile or semi-sterile). LRT tests are given for fixed and random factors (χ^2_{df} and corresponding *p*-values). For random factors we also provide estimates of variance (v, on logit scale) and *p*-values account for the one-sided alternative (variances cannot be negative). Note that the very high « maternal ID » variance in the KF category is an effect of the logit scale when proportions are close to 0 or 100% (here 92% individuals are fertile): in these conditions a very large variance in the logit scale makes a small variance in actual proportions. The block random factor was omitted in this table because in all models the estimated variance was 0.00. *p*-values are bolded when <0.05.

Data	Fixed effects			random effects
	maternal type (DS, KF, KS)	paternal type (HFR, LFR)	Interaction	maternal ID
All	$\chi^2_{df2} = 64.36$ p = **1.06 10^{-14}**	$\chi^2_{df1} = 4.25$ p = **0.039**	$\chi^2_{df2} = 0.91$ $p = 0.634$	$\chi^2_{df1} = 6.79$, v = 1.01 p = **0.0045**
only DS		$\chi^2_{df1} = 0.356$ $p = 0.551$		$\chi^2_{df1} = 0.00$, v = 0.00 $p = 0.500$
only KF		$\chi^2_{df1} = 0.001$ $p = 0.970$		$\chi^2_{df1} = 10.07$, v =91.72 p = **0.008**
only KS		$\chi^2_{df1} = 4.23$, p = **0.039**		$\chi^2_{df1} = 6.79$, v = 0.76 p = **0.010**

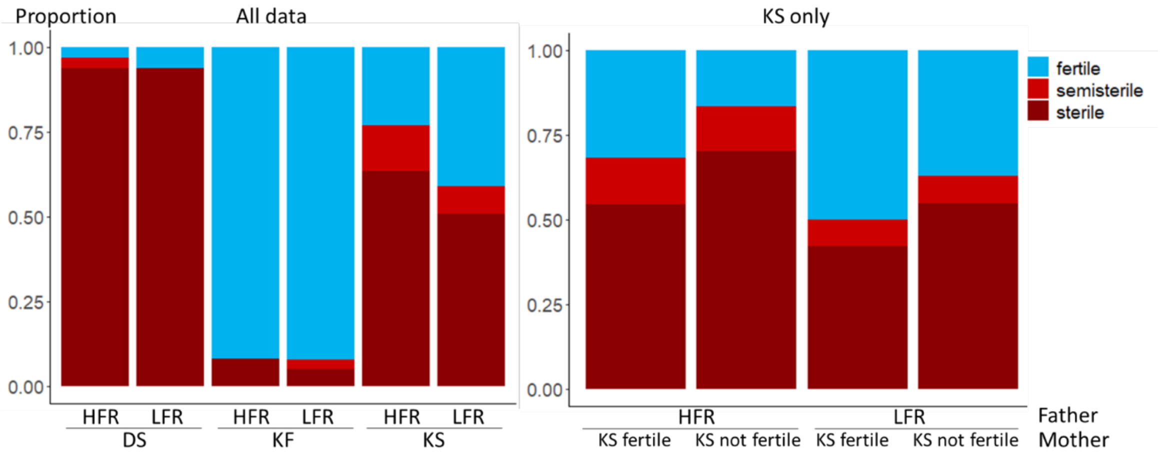

Figure 3: Proportions of individuals with male-sterile, male-fertile and male-semi-sterile phenotype. Left: proportions of male-fertile and male-sterile phenotypes as a function of paternal selection line (HFR versus LFR), maternal type (DS, KF, KS). Right: detailed view of proportions in offspring of KS mothers, as a function of paternal line and maternal phenotype (male-fertile versus not male-fertile). Similar divisions were not considered in the DS and KF category because of the low numbers of male-fertile and non male-fertile mothers respectively.

Male-sterility in the offspring generation: inbred lines

As previously, maternal types (DS, KS or KF) induced large contrasts in the proportion of male-sterility, with DS being predominantly male-sterile, KF predominantly male-fertile, and KS in an intermediate situation (Table 3, Figure 4). Only in the KS category, the origin of the paternal inbred line made a difference: paternal lines from Montpellier (laboratory population entirely of the N mitotype) had largely male-sterile offspring, while paternal lines from Lyon (where K and D were initially found) had more than 50% male-fertile offspring. In these KS offspring, the variance explained by paternal lines was significant (Table 3), on top of what was already explained by the geographic origin, and the among-lines variance, when estimated separately, was higher in the Lyon population compared to Montpellier (Table 3). This can be visualised by representing BLUPs (Best Linear Unbiased Predictors) associated with paternal lines in the linear models: the Montpellier inbred lines span a range of male-fertility between 0 and 50% while the Lyon lines cover essentially the whole range (0-100%) (Figure 4). The apparent continuity of the distribution does not seem to be an artefact of the GLMM (which assumes an underlying normal distribution on the logit scale for random effects) as it is also observed when plotting the raw percentages of male-fertile individuals (supplementary Figure S4). In contrast with KS, the variance among paternal inbred lines is estimated to be zero in the KF category. However it is significant in the DS category, in which the distribution of BLUPs spans the 0-50% interval with an apparently random distribution of lines from Montpellier and Lyon. The predicted male-fertility of offspring of an inbred line in a DS context is not significantly correlated to the same line in a KS context (Spearman ρ = 0.22, NS, Figure 4).

Models including the maternal phenotype (male-fertile or not) in the predictors, in addition to paternal population of origin, showed no significant effect in KS ($\chi^2_{1\mathrm{df}} = 0.018$, $p = 0.895$), and a weakly significant effect in the DS context ($\chi^2_{1\mathrm{df}} = 4.37$, $p = 0.037$) where male-fertile mothers had more male-fertile daughters than male-sterile mothers (see plots in

S3). The number of male-sterile mothers was insufficient to test a difference in the KF category.

Table 3: Generalised linear mixed models on the binomial male-fertility variable (fertile versus sterile or semi-sterile), inbred lines dataset. Legend as in Table 2. The block random factor was omitted because the estimated variance was 0.00. (1) the test gives the significance of the variance explained by the paternal line, in interaction with maternal type. Because of the significance of the interaction, the variances (ni logit scale) explained by paternal line are examined within each maternal type separately. In each case we also provide separate estimates of variance for the inbred lines derived from Montpellier and Lyon populations.

Data	Fixed effects			Random effects	
	maternal type (DS, KF, KS)	paternal origin (Lyon, Montpellier)	interaction	maternal ID	paternal line
all	$\chi^2_{df2} = 80.61$ $p <$ $\mathbf{10^{-15}}$	$\chi^2_{df1} = 9.73$ $p =$ **0.002**	$\chi^2_{df2} =$ 5.37 $p = 0.068$	$\chi^2_{df1} = 7.16$ v = 0.92 $p =$ **0.0037**	$\chi^2_{df5} =16.75$ $p =$ **0.005** (1)
only DS		$\chi^2_{df1} =1.439$ $p = 0.203$		$\chi^2_{df1} =0.18$ v = 0.29 $p = 0.387$	$\chi^2_{df1} = 6.58$, v = 0.80 $p =$ **0.005** (in Montpellier: v= 0.77, in Lyon : v=0.79)
only KF		$\chi^2_{df1} = 0.28$, $p = 0.597$		$\chi^2_{df1} = 0.89$ v = 1.32 $p =$ 0.174	$\chi^2_{df1} = 0.00$, v = 0.00, $p = 0.5$ (in Montpellier: v = 0.00, in Lyon : v = 0.00)
only KS		$\chi^2_{df1} = 14.84$, $p =$ **0.0001**		$\chi^2_{df1} =6.54$, v = 1.00 , $p =$ **0.005**	$\chi^2_{df1} =32.4$ v = 1.58 , $p =$ $\mathbf{6.2\ 10^{-9}}$ (in Montpellier: v = 1.45,

in Lyon : v= 1.97)

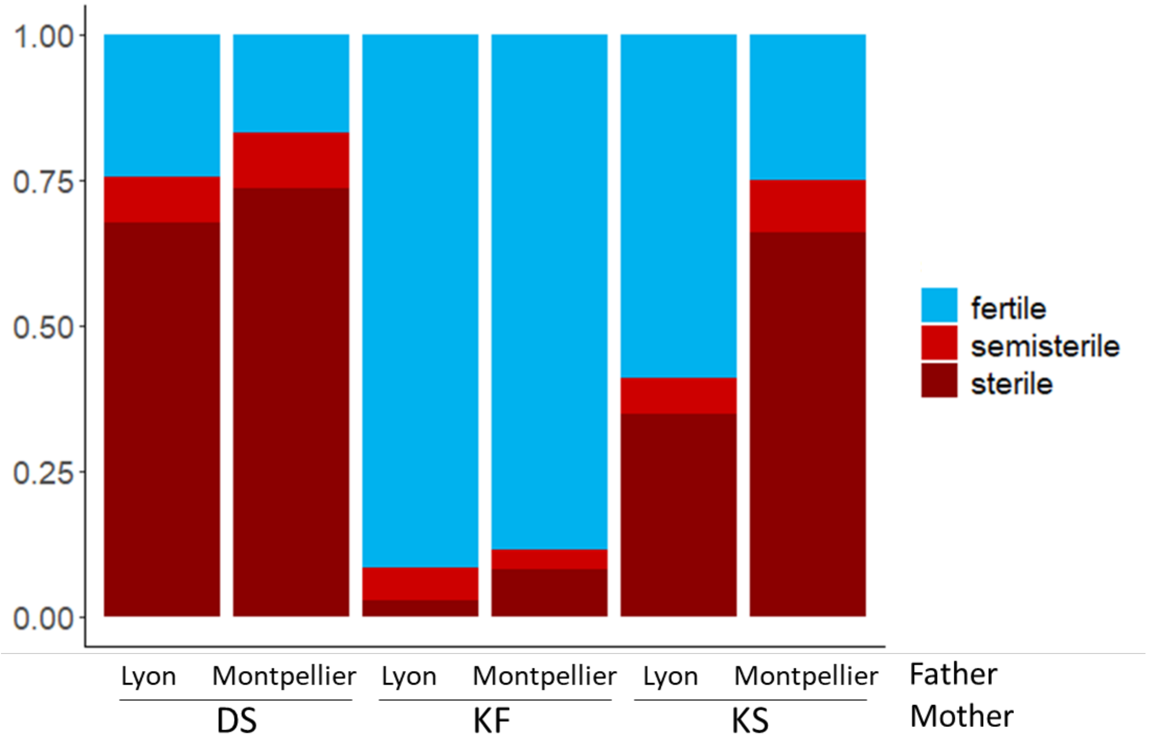

Figure 4: Proportions of male-fertile and male-sterile phenotypes as a function of the origin of the paternal inbred line (Lyon or Montpellier) and the maternal type (DS, KF, KS).

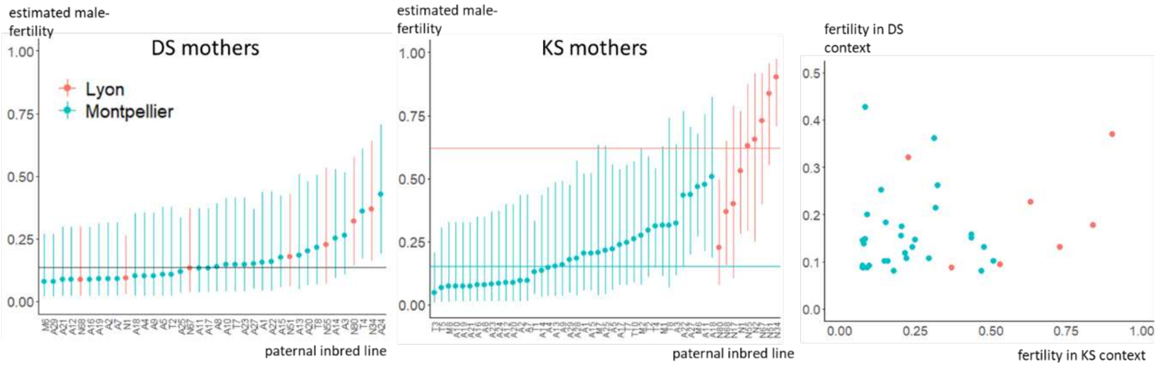

Figure 5: Proportions of male-fertile individuals in offspring sired by various inbred lines from Montpellier (red) and Lyon (blue), in a DS maternal context (left) or KS maternal context (middle). The estimates are extracted from binomial GLMMs (BLUPs), de-logited to put them back into natural scale, and presented with 95% confidence intervals. The lines are arranged by increasing male-fertility, and separated by origin in the KS context, as the population of origin has a significant effect in this context (in DS context, this is not

the case). Horizontal lines provide mean proportions of male-fertile individuals estimated by the model, over all paternal lines (DS context) or separately for each population of origin (KS context). Note that there are more paternal lines tested in KS than in DS context. The performance of lines tested in the two contexts are plotted together in the right panel (each dot is a paternal inbred line). The correlation is not significant (Spearman's $\rho = 0.22$, $p = 0.184$).

Discussion

This experiment was designed to characterize the restoration in *P. acuta* using two different nuclear backgrounds (i) a naive background, originated from Montpellier, which has never been in contact with N individuals at least during the previous 90 generations, (ii) a non-naive background, originated from Lyon, which has been in contact with the K and the D mitotypes in its recent history. Our results show that the two nuclear backgrounds had different genetic determination and that restoration, when present, was mitotype-specific, as in most plant examples.

Male fertility is restored by two different systems in Physa acuta

In the Montpellier background the K mitotype induced male sterility with an average penetrance close to 70%. Results from the selection experiment point to a significant but limited heritability of this penetrance, as (i) within the KS stock (Montpellier background), male-fertile mothers produced slightly more male-fertile daughters than male-sterile mothers, regardless of the father, and (i) fathers from the two selected populations, both derived from the same background after two generations, also differed in their restoration potential. However the latter difference was counter prediction as the lines selected to have a low restoration frequency (LFR) sired more male-fertile offspring than the lines assumed to have a high restoration frequency (HFR). This means that selection on the LFR and the HFR lines was ineffective, and has probably been overwhelmed by drift. This outcome is unexpected if restoration depends on fully-penetrant, dominant alleles, in which case large differences among LFR and HFR, in the direction of selection, would be expected (see supplement S2). However, if genetic variation is not large, and is expressed as a quantitative degree of penetrance (rather than binary 100% sterile or 100% fertile), the male-fertility phenotype of a few offspring becomes an imprecise indicator of the breeding value of each individual, lowering the efficiency of our selection process relative to drift, especially with the low numbers of individuals that passed the selection criteria. The distribution of male fertility in offspring of inbred lines derived from the Montpellier population strengthens this view, as overall, we have no convincing indication that any of the lines produced more than 50% male-fertile daughters, and the distribution seemed continuous between 0 and 50%, though mostly concentrated near 0.

From these results, we concluded that male-fertility of the K mitotype in the Montpellier nuclear background was expressed as a quantitative trait with a small amount of genetic variance that can be transmitted both by fathers and mothers, and some nongenetic variance (i. e. the same genotype can be male-fertile or male-sterile, with a genetically determined proportion, as in the threshold trait model Ehlers et al., 2005). In addition, we did observe cases of partial male-sterility ("semi-steriles") although their low number did not allow a separate analysis, suggesting that binary classification is a simplification of true phenotypic distributions. Indeed, in case of polygenic determination of restoration, individuals carrying CMS may rarely be fully restored and male fitness may vary quantitatively among restored CMS hermaphrodites (e.g. Dufay et al., 2008). This result is similar to previous studies on non-crop species that rejected Mendelian models of restoration (e.g. *Thymus vulgaris,* Belhassen et al., 1991; Charlesworth & Laporte, 1998; *Lobelia siphilitica,* Dudle et al., 2001). Ehlers et al. (2005) suggested that scenarios considering restoration of male fertility as a threshold trait may fit these data better than multilocus Mendelian models while producing overall similar expectations as single-allele evolutionary models regarding sex-ratio dynamics.

Results obtained from the crosses with KF mothers (i.e. with a background from Lyon) suggested that male-fertility of the K-mitotype has a very different genetic basis in the original Lyon population (a population where K naturally occurs) compared to the laboratory Montpellier population. The results are consistent with one or a few dominant restorer alleles acting on the K mitotype fixed or segregating at very high frequency in the Lyon nuclear background. Indeed, mothers from the KF population were nearly all (>90%) male-fertile and their daughters too, even when their father came from the Montpellier population (HFR, LFR or Montpellier inbred lines) where there is no or rare restoration (as discussed above), as expected from transmission of dominant restorers by mothers. Reciprocally, mothers from Montpellier, although predominantly male-sterile themselves, produced high frequencies of male-fertile daughters when fertilized by fathers from inbred lines derived from the Lyon population. It is interesting to note however, that if dominant restorers were fixed in Lyon, we would expect all these inbred lines to restore fertility with high efficiency ; this is the case of only three of the 9 lines (75 to 100% fertility) , the rest being distributed in the 25%-75% range, with an overall estimated mean of 62%. The contrast in male-fertility frequencies in Montpellier-Lyon crosses, depending on whether the father or the mother comes from the Lyon population, is likely to reflect the recent history of the Lyon stock in the laboratory. The KF mothers come from a 100% K population maintained autonomously for 20 generations, during which all sperm donors must have been of the K-mitotype. Therefore the frequency of any restorer of male-fertility was positively selected, and maintained high, or perhaps increased, compared to the natural population. On the other hand, fathers from inbred lines from Lyon were derived from a population that, although founded from the same natural population, was maintained in parallel with 100% N mitotype. In these conditions, restorers were silent (in the sense that all individuals were male-fertile anyway) and if they were costly, may have decreased in frequency. This hypothesis of a divergent selection on restorers depending on mitotype frequencies will however have to be checked by looking at more replicates than just one population of each type. Interestingly, the inbred lines from Lyon do not show a strikingly bimodal distribution, suggesting that several loci with cumulative effects may be involved in restoration.

Our results highlighted two different restoration systems in *P. acuta*. In plants, restorer alleles with different effects have been reported. For example, in *Plantago* genus, multiple independent restoration systems were identified with both dominant and recessive effects sometimes involving more than one locus (Koelewijn & Van Damme, 1995; E. van Damme, 1984). In *Lobelia siphilitica* one CMS gene is restored by a single dominant allele, while restoration of other CMS is controlled by several loci and/or epistatic effects (Dudle et al. 2001). In these examples, one restoration system corresponds to one CMS type, however in *P. acuta* we found that two restoration systems acting together on the same CMS type K.

The two restoration systems are specific of the K mitotype

The D-mitotype individuals were on average 90 – 100% male-sterile irrespective of the nuclear background. The low proportion of male-fertile individuals was consistent with the previous study of David et al. (2022) which found 8% of male-fertile D individuals in the original population. Here, we found no evidence for a dominant restorer with large penetrance; however we observed the existence of genetic variation for penetrance among inbred lines from both Montpellier and Lyon, without evidence for a difference between the two origins (we would expect a higher rate of male-fertility in Lyon if this variation has been specifically maintained by selection for restoring male fertility, as the Montpellier population had no recent contact with the D mitotype). This lack of difference between Montpellier and Lyon, and the fact that the lines with the highest male-fertility rate in the context of the D mitotype were not necessarily the ones that efficiently restore fertility in the K-mitotype, suggest that the two types of variation act in a mitotype-specific manner. The specificity of the restorers has been also observed in plants where different CMS genes can coexist within a population each with its own specific nuclear restorer alleles (e.g. van Damme & van Delden, 1982; Manicacci et al., 1997).

In the wild the D mitotype seems rare as it was found only in one population and at low frequency (14%, David et al. 2022). Thus, suppression of the male sterility may not be frequent enough for a restorer to be selected. The high evolutionary rate detected in the D mitotype (David et al. 2022) could result from an arms race with a restorer gene which is now extinct or rare, or has lost its ability to restore fertility following a recent evolution of the D mitochondrial genome itself. According to the frequency-dependent model, as soon as a CMS gene is present in a population, there is a strong selection for any nuclear mutation that counteracts the phenotypic effects of CMS. Restorers should spread rapidly and if they have no or little fitness cost, the polymorphism is transient. However, nuclear restoration is expected to be rare under the hypothesis of a high cost of restoration (Gouyon et al., 1991; Dufay et al., 2007). Founder effects causing a reduction of the nuclear diversity could also lead to the local absence of certain nuclear restorers in a recent founded population (e.g. Manicacci et al. 1996; Laporte et al., 2001). Therefore, we can hypothesize that the evolution of restorers specific to the D mitotype might be constrained by a high cost or founder effects.

Two stages in the maintenance of cyto-nuclear polymorphism

Most models have studied the maintenance of the cytoplasmic and nuclear genetic variation in gynodioecious populations assuming strong and dominant restorer alleles at a few loci. Such alleles are indeed the most likely to be under strong selection and undergo selective sweeps in populations invaded by new CMS genes. Depending on their associated costs, they may either reach fixation (“hard-sweep”) or stabilize at intermediate or cyclically variable frequencies (“soft-sweep”) together with their corresponding CMS cytotypes (Gouyon et al., 1991; Dufaÿ et al., 2007). Gynodioecious populations observed today are mostly in the polymorphic state and the restorers found indeed include large-effect alleles with dominant action (e.g. de Haan et al., 1997; Dufay et al., 2008, 2009). In addition this action is usually specific to each cytotype, revealing either a arms-race coevolutionary history specific to each cytotype-restorer pair, and/or the fact that each new CMS cytotype has a mode of action that differs so strongly from previous ones that existing restorers are unlikely to neutralize it. In *Physa acuta,* the K mitotype, and the restorers present in the natural population where it was found, are likely to be in this situation, as we found restoration to be strong (though possibly distributed into several loci), dominant (same effect in the heterozygous state in daughters, than in homozygous state in mothers) and specific (no cross-mitotype effect).

However, while such situations are expected to be frequent in stable gynodioecious populations, they are probably not representative of initial stages when a new CMS mutant appears in a "naïve" population or species, where no selection has ever taken place on nuclear genes to suppress its effect. According to Lewis (1941), the spread of CMS in the population will in this case become limited only by pollen limitation when females become so abundant that many of them lose their ovules by lack of pollen to fertilize them. Our results suggest however a way by which such populations may lower the impacts of pollen (or, in the case of *Physa*, sperm) limitation: CMS may not be fully penetrant, leaving a residual production of male gametes that is at least sufficient to fertilize the eggs of mates that have not had previous contacts with male-fertile congeners. It is likely that even in a naïve population, pre-existing quantitative genetic variation includes variants that act on this penetrance. This probably corresponds to the situation of the K-mitotype when introgressed into the "naïve" Montpellier population, and of the D mitotype in both the Montpellier and Lyon populations. Here, we mean by "naïve" that it has either never had any history of selection in presence of the K and D mitotypes, or that the contact is too recent for large-effects restorers to have emerged, or too ancient for them to have persisted if they ever existed. The background variation acting on incomplete penetrance of CMS could produce a reservoir of variation within the population, by which male fertility can start to increase, until the appearance of complete penetrant restorers.

The emergence of restoration could therefore often take place in two stages: a first stage with predominantly quantitative, weak modifiers of CMS penetrance, and a second stage when dominant, strong restorer alleles appear and are selected at one or several loci, ending up with either fixation or a mixed determinism of male-fertility. Although theoretical models generally consider a simple genetic determination for restoration (but see Frank, 1989; Bailey & Delph, 2007), empirical studies on wild plants have often rejected this assumption as mentioned above (e.g. Charlesworth & Laporte, 1998; Koelewijn, 2003). A complex determination of restoration implies that within a population, CMS individuals may be partially restored (i. e. male fertility may be more of a continuous than binary 0/1 distribution) and this could affect the degree of selection for restorer alleles. Then, the positive selection of restorers could slow down and ultimately modify the conditions of maintenance of cytonuclear polymorphism, thereby favouring the maintenance of females in gynodioecious populations (Bailey & Delph, 2007).

Conclusion

This study shows that as in plants, the genetic determination of male-sterility in *Physa acuta* is complex and suggests that it encompasses two different layers: one of weak, quantitative modifiers of the penetrance, not necessarily coevolved with CMS, the other of strong-effect restorer alleles with dominant action, that were selected in the presence of CMS. Moreover, the way the phenotype is measured could influence our perception of the restoration potential. Indeed, the male fertility status was evaluated in the absence of competition as individuals were always paired with virgin mates; the restoration of the male fertility might be considered less efficient when measured in a population with competition for the male function. Thus, it will be interesting to evaluate male fitness in competition, e.g. when partners are not virgin, and have been first paired with a male-fertile partner before being paired with focal individuals. The genetic determinism of the restoration may influence the maintenance of the cyto-nuclear polymorphism by acting on the strength of the restoration cost. This could be evaluated by looking at the correlations between the penetrance of the nuclear backgrounds and the cost of restoration on male and/or female fitness. However, studies performed in plants suggest that costs are difficult to detect (but see de Haan et al., 1997; Bailey, 2002; Dufay et al., 2008). Finally, because our results suggest that the restoration of the male function is a quantitative trait, identification of QTLs for male-fertility restoration would be necessary.

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Supplementary materials

S1: Origin and extraction of the alb-2 stock

Figure S1: Phenotypes of three adults from the pigmented, alb-1 and alb-2 populations maintained in Montpellier.

The alb-2 stock was initiated from a single individual with depigmented phenotype that appeared within a self-fertilized brood of a parent from the Montpellier pigmented stock, generation 74 in December 2020 (see Figure 1). We suspected it could be a different mutant from the alb-1 albino stock already available, because Dillon (ref) mentioned the existence of two complementary albino strains in *Physa acuta*. In addition the adult phenotype of the new albino was slightly different from the alb-1: the dark pigments seemed completely absent, so that the body appeared slightly more orange (versus beige to yellowish for the alb-1), and the eyes were barely visible. These traits are however very difficult to ascertain on hatchlings or juveniles because the eyes of alb-1 hatchlings are barely visible too. We first let the newly discovered albino individual self-fertilize and obtained four G_1 offspring, each of which was crossed with a different individual belonging to the alb-1 population (complementation test). All the G_2 obtained were pigmented, suggesting that the new type, now named alb-2, and the alb-1 were mutated on different genes and that both mutations were recessive to their respective wild-type pigmented allele. From the supposedly double-heterozygous G_2 we created a G_3 (avoiding crosses between full sibs) among which we selected only the non-pigmented phenotypes (which therefore were, according to our hypothesis, homozygous for albinism at least at one of the two loci; however we could not at that stage recognise different albino phenotypes from one another with certainty). Within the G_3 we wanted to select those that were homozygous for the albinism allele at the second locus, but homozygous for the wild-type allele at the first; i.e. individuals that, upon crossing with the alb-1 stock, would give 100% pigmented phenotypes. To that end we paired each of 155 G_3 with a different virgin DS individual of albino phenotype. The DS are male-sterile so they act as females in this cross, and their genetic background is that of the alb-1 population. We let the DS partners lay and in case the offspring were 100% pigmented, we selected the corresponding G_3 individual. We found 4 such individuals and left them together to mate; their albino offspring constituted the G_4. This G_4 was, given the low numbers of ancestors involved, rather inbred; to constitute a population with a larger genetic basis, we mated 22 of these G_4 each to a different pigmented individual from the Montpellier pigmented population. The G_5 obtained was 100% pigmented (as expected) and we retained 150 individuals that were left to mate in two large aquaria. We retrieved a large number of G_6 juveniles among which we selected 100 from the minority that had the alb-2 phenotype; these were used to found the alb-2 stock which has since then been kept as a large autonomous population (5 aquaria of ~100 individuals each with regular exchange of individuals among them). At the seventh generation we also crossed eight alb-2 each to a distinct alb-1 individual and checked that all offspring were pigmented as expected.

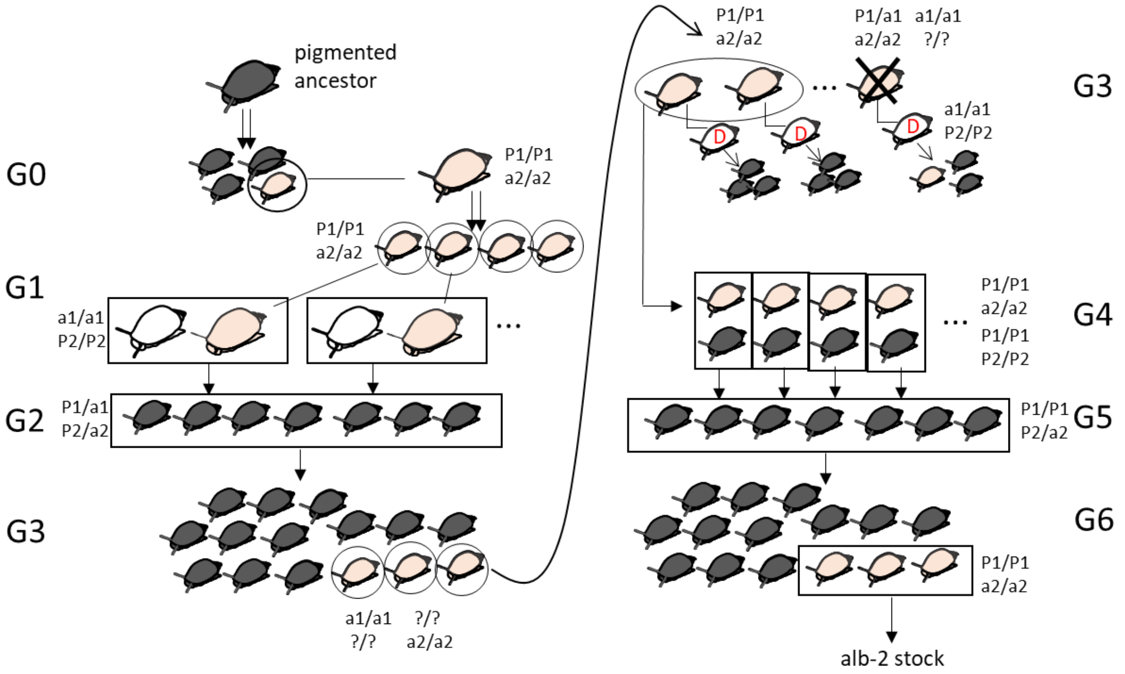


Figure S2: Derivation of the alb-2 stock. Dark snails are of pigmented phenotype, light ones of albino phenotypes (the white and pink serve to distinguish snails but the two forms of albino phenotype were not recognised visually). The inferred genotypes at two loci (each with a pigmented dominant P; and an albino recessive a) are indicated but were not known, they were inferred a posteriori from the results of the crosses. G_0: an albino individual is identified in the self-fertilized brood of a pigmented ancestor from the Montpellier stock. G_1: the albino is replicated by selfing, each of four offspring is mated with an alb-1 individual. G_2: all offspring are pigmented and mated to one another. G_3: the albino phenotypes are selected and test-crosses made with D individuals with alb-1 background; G_3 that produce 100% pigmented offspring are selected (N = 4) and mass-mated together the others are eliminated. G_4: each of 22 offspring is mated with an individual of the Montpellier pigmented stock, to enlarge the genetic basis of the alb-2 population. G_5: all individuals are pigmented and left to mass-mate together (N=150). G_6: A minority of albino phenotypes are found; they are selected (N>150) to found the alb-2 population.

S2: selection of the HFR and LFR lines

Selection of the HFR (« high frequency of restoration ») line.

The base population is the alb-1 Montpellier stock (with N mitotype), and the KS matrilines which have the same nuclear background as the alb-1 stock, except that the mitotype is K. Most KS individuals are male-sterile, however between 1/3 and ¼ of them are « male-fertile » (in the sense that they can fertilize a virgin N individual, and obtain >10 paternities this way).

The HFR selected line resulted from two successive crosses between K and N individuals as follows. In the first cross, multiple pair-crosses between KS pigmented and alb-1 individuals were made (e.g K_1xN_1, K_2xN_2, K_3xN_3 … K_nxN_n). Note that all the KS individuals used were daughters of male-sterile mothers. In each pair, the two partners were then isolated to lay separately. By phenotyping the progeny mothered by N individuals, we could assess male-fertility in its K mate (the K individual was said to be male-fertile if >5 pigmented babies were obtained from the N partner). The selection step to increase restoration frequency was as follows: If, according to the fertility test, the K individual of a pair was male-fertile, the offspring of both members of the pair were considered part of the first selected generation, otherwise they were discarded. In addition we kept only pigmented offspring of the K progeny, and albino offspring of the N progeny; the conserved offspring were raised to adulthood and used for a second cross. In this second cross, pairs with a K individual (pigmented) and a N individual (albino) were made again, taken from different families to avoid inbreeding. We again conserved only pairs for which the K individual turned out to be male-fertile, thus imposing a second selection step. In these pairs we conserved the N offspring only, and pooled them together to constitute de HFR stock.

Below we give an expectation for the change of frequency of a restorer allele R during selection (noting m the corresponding maintainer allele), under the simplest scenario of monodominant restoration with full penetrance (i.e. RR and Rm individuals are male-fertile, and mm individuals male-sterile, in a K cytoplasm), noting S0, S1, S2 successive generations of selection. To simplify matters we assume that the starting frequency of R is 1/3 in the alb-1 source population (corresponding approximately to the observed frequency of male-fertile phenotypes in K offspring of male-sterile K mothers). In these conditions the frequency of R in the HFR population is expected to be 50/96 and the daughter of a male-sterile KS mother and HFR father should be male-sterile with probability 46/96 instead of 2/3.

Table S1: Expected frequencies during selection of the HFR line, assuming monodominant restoration.

	K individuals			N individuals		
Genotype	RR	Rm	mm	RR	Rm	mm
Gametic frequencies in alb-1				f(R)=1/3		f(m)=2/3
Parents (S_0) before selection	0	1/3	2/3	1/9	4/9	4/9
S_0 after selection	0	1	0	1/9	4/9	4/9
S_1 before selection	1/6	3/6	2/6	1/6	3/6	2/6
S_1 after selection	1/4	3/4	0	1/6	3/6	2/6
S_2	25/96	50/96	21/96	25/96	50/96	21/96
Gametic frequencies in HFR				f(R)= 50/96		f(m)=46/96

Selection of the LFR (« low frequency of restoration ») line.

The LFR population derives from the same sources as the HFR and started with similar pair-crosses in the S0. This time, we conserved the N partner when its K mate was male-sterile i.e. the progeny of the N individual was either missing, or devoid of pigmented offspring ; in all cases we waited until the N individual self-fertilized **(Supp Fig.XX (3))**. In parallel two pigmented babies of the male-sterile K mother were grown to adulthood and tested for their male sterility **(Supp Fig.XX (4))**. If both were male-sterile (like their mother), suggesting that their father did not transmit them restoration genes, the self-fertilised progeny of their N Albino father was kept. On the other hand if either of them was male-fertile, the self-fertilised progeny was discarded. This whole protocol was repeated several times, adding the self-fertilized progenies each time to the same aquarium (approximately 15 progenies total) resulting in a self-sustained population called LFR 1 (Low Frequency of Restoration, 1st generation of selection). The population produced G1 individuals that we used as N partners for new KS individuals, to repeat the whole cycle of selection, yielding the LFR 2 population used in this study. The complexity of the selection step, requiring to eliminate most of the progenies, explains the low number of families that founded the LFR population so this population was exposed to important genetic drift compared to the ancestral alb-1 stock ; however the selection applied is very strong so it should be visible, if it applies to alleles with strong effects. For example, in the monodominant restoration scenario (see above) a *RR* parent is systematically eliminated by the selection process (because all his offspring are male-fertile) ; a *mm* parent is systematically kept (because all his offspring are male-sterile) and a heterozygous *Rm* parent is retained with probability ¼ (the probability that it gives the *m* allele to two independent offspring). To give a rough idea, if the frequency of R was initially 1/3, it should be 1/10 after the first generation of selection, and 0.037 after two generations.

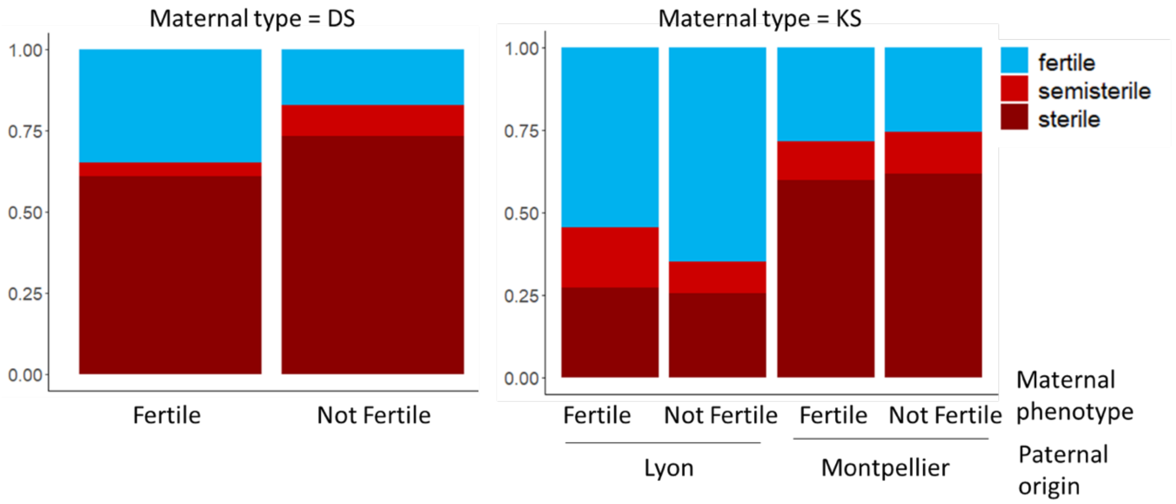


Figure S3: Additional plots, effect of maternal phenotype in the inbred lines dataset. Only the DS and KS categories are represented because there are not enough male-sterile mothers in the KF category. The number of male-sterile mothers is also not high in the DS category (N=23), so percentages should be taken with caution. The KS dataset has been split by paternal origin because this effect is significant which is not the case in the DS dataset (see main text).

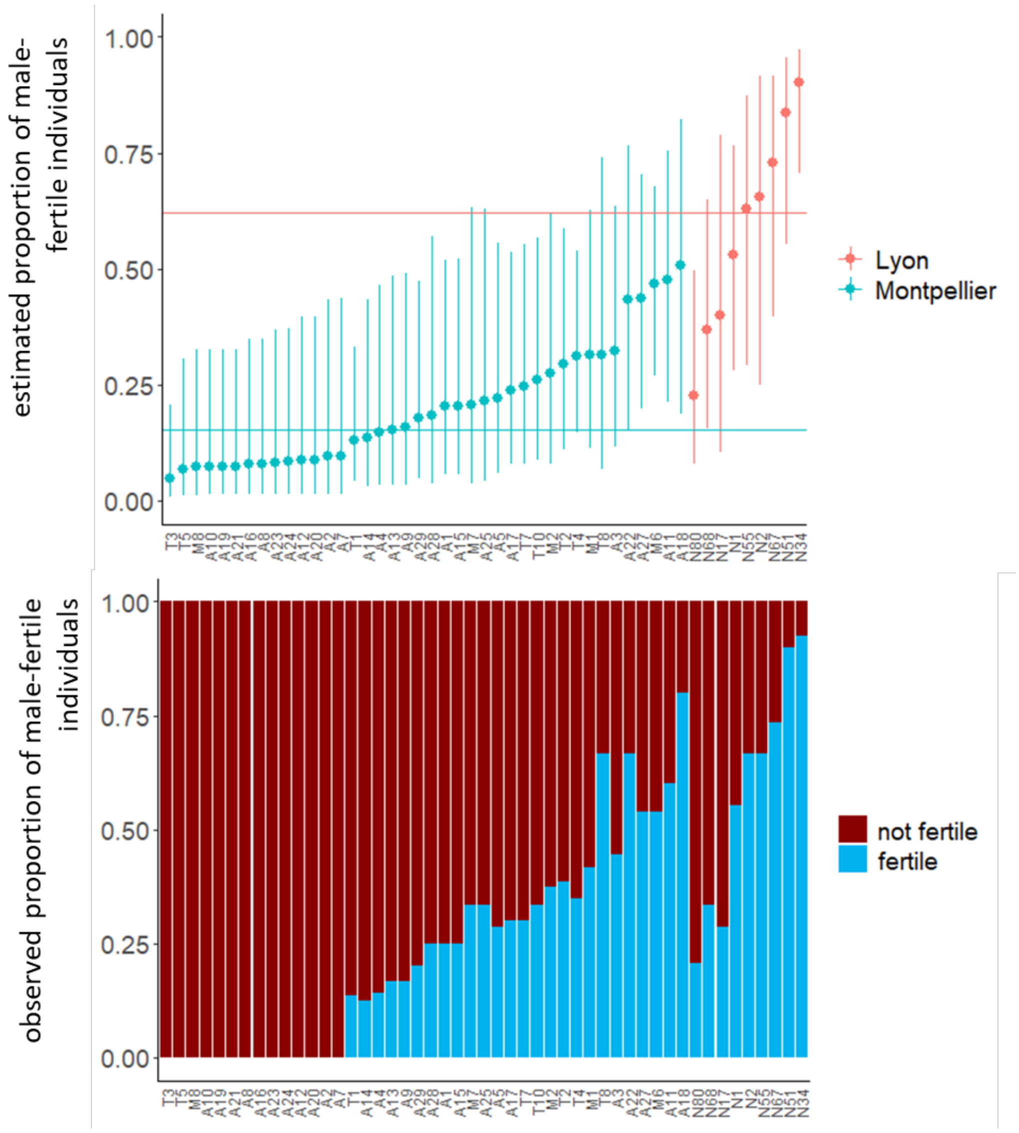


Figure S4: Additional plots, model estimates (top) versus raw proportions (down) of male-sterile individuals for each inbred line crossed with KS mothers. Inbred lines are arranged in the same order in the two graphs.

CHAPITRE 4

SCAN GENOMIQUE

CHAPITRE 4 – CONTEXTE

Dans ce dernier chapitre, j’avais pour objectif d’explorer les conséquences génomiques de la restauration et de la SMC en populations naturelles. J’ai obtenu les données pour ce chapitre au début de ma dernière année et j’en ai commencé l’analyse. Malheureusement, une erreur de la personne en charge des serveurs a entraîné la suppression de nombreuses données au sein de l’équipe de mon laboratoire d’accueil, incluant celles de ce chapitre. Nous avons cependant pu obtenir une copie des données brutes. L’analyse de ces données est un long processus du fait du grand nombre d’individus et de la couverture génomique importante du séquençage (séquençage de 300 génomes de 1,2 Gb en couverture 30X). Dans le temps qui m’était imparti, il m’était impossible de recommencer les analyses depuis le début et d’obtenir des résultats avant le rendu de ce manuscrit. Je compte tout de même poursuivre ce travail et je souhaite en inscrire une trace dans ma thèse. Je présente donc ici une brève introduction visant à expliquer les questionnements liés à ce chapitre ainsi que début de matériel et méthodes. Dans ce dernier figure ce que j’ai déjà effectué et en partie perdu (paragraphe “Material and methods”) ainsi que ce que je prévois de faire dans la suite des analyses (paragraphe “Plan for the rest of the analyses”).

Introduction

Gynodioecy is a sexual polymorphism, common in angiosperms (Dufay et al., 2014; Caruso et al., 2016), whereby hermaphrodites coexist with male-sterile individuals (i.e., functionally females, Saumitou-Laprade et al., 1994). Gynodioecy represents a well-known example of genetic conflict (Burt & Trivers, 2006) because sex determination in gynodioecious systems is often cyto-nuclear. In that case, cytoplasmic male sterility (CMS) genes, usually associated with the mitochondrial genome, suppress pollen production and render an individual functionally female unless CMS is counteracted by one or more nuclear genes that restore the male function (Cosmides & Tooby, 1981; Saumitou-Laprade et al., 1994; Werren & Beukeboom, 1998).

The exact molecular mechanism for cytoplasmic male sterility has been only explored in crop species so far (reviewed in Delph et al., 2007; Chen & Liu, 2014). In crops, CMS is caused by chimeric genes formed from segments of other mitochondrial genes as well as from segments of unknown origin; together, these segments form open reading frames (ORFs) that are transcribed and translated to produce a novel protein (Chen & Lui 2014). In addition, they are often co-transcribed with other mitochondrial genes. In some cases, CMS has been attributed to loss-of-function mutations; however, most evidence is consistent with an active role for the novel allele and its products, including several cases in which the novel protein appears to act as a toxin that interferes with mitochondrial respiration (Wang et al., 2006). CMS genes are thought to be the result of intragenomic recombination (Hanson, 1991). At least ten mitochondrial genes have been found to be involved in the formation of CMS genes; *cox1*, *atp8* and *atp6* being the most frequent. In the majority of cases, restorer genes produce proteins with the ability of acting directly on the transcription of the CMS gene (reviewed in Chen and Liu 2014).

More than half of the identified restorer genes encode *PPR* (Pentatricopeptide Repeat) proteins (Dahan & Mireau, 2013) – a group of RNA-binding proteins – which tend to exist as tandem gene clusters (Delannoy et al., 2007). For example, a genome wide distribution analysis of *PPR* genes indicates that although the vast majority of *PPR* genes are dispersed throughout the *A. thaliana* genome (Lurin et al., 2004), a loose cluster of *PPR* genes is present on the long arm of chromosome 1 with 19 genes in close vicinity of each other (Desloire et al., 2003). In some *PPR* gene clusters, more copies of the *PPR* genes are present in restorer lines than in non-restorer lines, suggesting that some restorer genes originated recently through duplication events (Hernandez Mora et al., 2010). Most eukaryotic genomes harbor only a few *PPR* encoding genes, but in plants this gene family has greatly expanded (Lurin et al. 2004). However, restoration of fertility includes several other well-documented mechanisms, which do not involve *PPR* proteins, including include glycine rich proteins, alcohol dehydrogenase, acyl-carrier proteins and a peptidase (Cui et al., 1996; Yamamoto et al., 2008; Matsuhira et al., 2012).

The maintenance of the nucleo-cytoplasmic polymorphism has been extensively studied from both theoretical and empirical perspectives (Lewis, 1941; Charlesworth, 1981; Gouyon et al., 1991; Koelewijn, 2003; Touzet et al., 2004; Delph et al., 2007; Dufay et al., 2008). Theory suggests that the stability of this polymorphism depends on a variety of factors, including the positive pleiotropic effects of CMS alleles (i.e., seed fitness advantage of females, or “female advantage”, Lewis 1941) and negative pleiotropic effects of restorer alleles (“cost of restoration”,(Charlesworth & Ganders, 1979; Delannay et al., 1981; Frank, 1989). As pointed out by Delph et al. (2007), field studies often attempt to quantify compensation and/or cost whereas studies using agricultural species focus on identifying the molecular action of the genetic components in crops and the genes associated with CMS and restorers.

CMS has been looked for in animals for a long time without success (Vellnow et al., 2017). However, CMS has been recently reported in one animal: *Physa acuta*, a freshwater snail (David et al., 2022). In this species, two mitochondrial types conferring male sterility, D and K, have been discovered so far, both of them showing an extreme molecular divergence from one another, as well as from normal, fertile cytotypes (collectively called N), at all mitochondrial genes. The D mitochondrial type was discovered first, but evidence for corresponding nuclear restoration is still lacking (David et al. 2022). Recently however, the second CMS mitotype K revealed a different situation, with evidence that nuclear polymorphisms can modulate the expression of the male sterility phenotype in K individuals, i.e., there exist K-specific restorer genes (Laugier et al., 2024). The *P. acuta* system has two new features, it is the only case of CMS in animals, and there is an accelerated evolution of the mitochondrial genomes. This could suggest a new restoration mechanism, or, on the contrary, a convergence between the patterns observed in plants and in animals, via different evolutionary pathways.

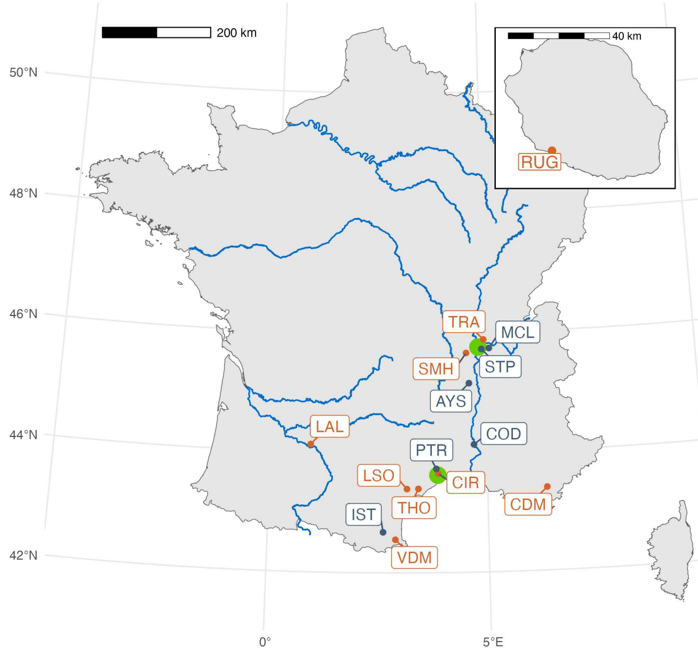
P. acuta is a widespread species found in a diverse range of habitats. Populations have already been identified (Montpellier and Lyon, Noël et al., 2016, David et al. 2022, Laugier et al. 2024) with contrasted frequencies of CMS-associated mitotype and restoration – CMS mitotypes were absent in the Montpellier population and estimated at 15% and 32% in the Lyon populations. Here, we aimed to explore the genomic consequences of the presence of male-sterility and restorers in natural populations. The objective was to observe genome variation in populations with a context for selection of restoration and populations without a context for selection of restoration. As we have confirmed the existence genes restoring male-fertility in the K-mitotype individuals (Laugier et al; 2024), we selected populations with highly contrasted frequencies of K and hope to identify restorer genes by looking for changes in allele frequencies. We sequenced a total of 300 individuals equally distributed among 15 populations. An assembly of *P. acuta* reference genome was also required in order to perform the analyses.

Material and methods

Populations and sample studied

To assemble the reference genome of *P. acuta*, we used an individual inbred for 28 generations. This individual originated from ancestral wild-caught snails that were part of a larger sample taken from a wild "Pont-Romain" population in the Lirou river (PTR population Figure 1, Table S1) on October 2011.

The distribution of the mitotypes in *P. acuta* populations was monitored during the year 2019 (P. David unpublished data). Sample sites were first chosen along the Rhône River southwards in steps of 25 km, as rivers are dispersal corridors for freshwater snails, then the sampling was extended to random sites in metropolitan France and overseas territories. In each site, 100 individuals were sampled and stored in 96% ethanol. We assessed the mitotype of the individuals by using a PCR multiplex test (fully described in Laugier et al. 2024). Briefly, it consists in one PCR with a mix of primers designed to be specific to each of the mitotypes, amplifying different fragments of the COI mitochondrial gene, with fragment size typical of each mitotype; the mitotype are divergent enough that nonspecific priming does not occur. Universal primers from Folmer et al. (1994) are also present in the mix and amplify a long fragment in case none of the specific primers matches – thus failure in amplification can be distinguished from a potential new mitotype. The PCR tests were performed on all the sampled individuals and from these results we assessed the proportion of the three mitotype in each population. Among these populations, we selected isolated sites (i.e, sites no belonging to the same drainage basin) and populations with contrasted frequencies of N and K mitotype to perform a genome scan approach (Figure 1 and Table S1). Two types of populations were selected: (i) seven populations with high frequencies of K individuals (>20%), i.e. populations in a context with selection for restoration, (ii) eight populations with a few K individuals or none (<5%), i.e. populations without context for the selection of restoration. We randomly took 20 individuals per population (N = 300).



Populations	%N	%K	%D
AYS	98	2	-
IST	98	2	-
MCL	100	-	-
PTR	100	-	-
STP	98	2	-
COD	100	-	-
THO	95	5	-
CDM	77	23	-
LAL	50	50	-
RUG	45	55	-
TRA	52	48	-
VDM	29	71	-
LSO	29	64	7
SMH	77	22	1
CIR	50	44	5

Figure 1: Distribution of the 15 populations used and their associated mitotype frequencies. The green points represent Lyon (North) and Montpellier (South). Populations with low frequencies of K are in blue and populations with high frequencies of K are in orange. Sampling sites were located in metropolitan France, expected for one population sampled in the Réunion island. Most of the sites were lakes or ponds.

Reference genome assembly

We extracted DNA from the foot of the reference individual using a Blood & Cell culture kit (Quiagen) and sent it for PacBio Hifi sequencing. Long reads were assembled de novo using Hifiasm (Cheng et al. 2021). BUSCO (Benchmarking Universal Single-Copy Orthologs) v.5.1.3 (Manni et al. 2021) analysis of the assembly using the mollusca *odb10* dataset demonstrated a low level of duplication (92.4% of single-copy orthologs). Genome annotation was performed by the National Center for Biotechnology and Information NCBI. The assembled and annotated reference genome is available in NCBI under accession number GCF_028476545.12.

Whole-genome sequencing

DNA was extracted using NucleoSpin Tissue, Mini kit for DNA from cells and tissue kit (Macherey-Nagel) from the remaining tissue of each individual which were stored after the mitotype assessment by PCR (see above). DNA concentration was quantified with a Qubit 2.0 fluorometer using QuantiFluor dsDNA System kit (Promega). The DNA libraries were prepared at the GenSeq technical platform (University of Montpellier, France) using Illumina kit (Nextera DNA Flex). The libraries were sequenced at Montpellier GenomiX (Montpellier, France) with an NovaSeq6000 lane and validated using DNA quantification (concentration and fragment size) on Fragment Analyzer (Standard Sensitivity NGS kit) and qPCR (ROCHE Light Cycler 480).

To ensure the mitotype of each individual (Table S2), genomes were assembled using MEGAHIT (Li et al., 2015, 2016). Short pair-end reads were mapped onto the assembled genome using BWA (Li & Durbin, 2009). The resulting SAM file was converted to BAM using Samtools (Li et al., 2009).

Mapping samples

FASTQ files from the two types of populations were aligned to the reference genome using BWA-MEM (Li 2013). Coverages were measured on the mitochondrial contig using the function depth of Samtools.

Plan for the rest of the analyses

Variant calling

The next step will be to compare the two types of populations and identify different selection regimes. Analyses will be performed in ANGSD (Korneliussen et al., 2014), GATK (McKenna et al., 2010) and Freebayes (Garrison & Marth, 2012) using genotype likelihoods. We are planning on calling variants using several variants caller in order to compare the result obtain with each of them and the most appropriate for our data.

Genomic comparisons

For each population location we calculated pairwise nucleotide diversity (θ_π) for HR and LR populations separately, and F_{ST} between HR and LR populations at each location. Statistics will be calculated in non-overlapping 10kb windows, and windowed θ_π values will be normalized by dividing by the number of sites in each window. We aim to identify shifts in allele frequency as well as local reductions or augmentations in genetic diversity for detecting selective sweeps thanks to new deep learning-based methods (Lauterbur et al., 2023)

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Supplementary materials

Table S1: Populations used to perform the genome scan approach. The name of the site along with the coordinates are provided. The type of environment in which the snails were sampled is also indicated.

Name of the sites	Latitude	Longitude	Environment
AYS: Ay Saitillieu	45.15094	4.61841	River
IST: Ille-sur-Têt	42.67596	2.61222	Marsh
MCL: Montcul	45.72939	5.10841	Isolated pond
PTR: Pont Romain	43.72957	3.83009	Stream
STP: Saint-Priest	45.71723	4.93018	Isolated pond
COD: Codolet	44.1282	4.70702	Lake (Gravière)
CDM: Cannet des Maures	43.38728	6.36067	Marsh
LAL: Lalande	44.1283	0.92833	Pond
THO: Thongue	43.39869	3.41643	River
RUG: Réunion Gol	21.28534	55.3976	River
TRA: Tramoyes	45.87486	4.98095	Connected pond
VDM: Villelongue-del-Monts	42.55247	2.89104	Lake (Gravière)
LSO: Lignan-su-Orb	43.39642	3.15442	Marsh
SMH: Saint-Martin-en-Haut	45.65579	4.56455	Isolated pond
CIR: Cirad	43.65106	3.87128	River (Lez)

Table S2: Accessions and mitotypes of the individuals used in the genome scan approach.

Accession	Population	Mitotype	Accession	Population	Mitotype
AYS001_S1	AYS	N	MCL193_S255	MCL	N
AYS005_S2	AYS	N	MCL194_S242	MCL	N
AYS008_S226	AYS	N	MCL195_S243	MCL	N
AYS014_S227	AYS	K	MCL196_S128	MCL	N
AYS015_S3	AYS	N	MCL197_S129	MCL	N
AYS016_S4	AYS	N	MCL198_S130	MCL	N
AYS021_S228	AYS	N	MCL199_S131	MCL	N
AYS032_S5	AYS	N	MCL201_S132	MCL	N
AYS038_S6	AYS	N	MCL202_S133	MCL	N
AYS044_S7	AYS	N	MCL203_S134	MCL	N
AYS053_S8	AYS	N	PTR100_S135	PTR	N
AYS056_S9	AYS	N	PTR101_S136	PTR	N
AYS057_S10	AYS	N	PTR102_S137	PTR	N
AYS065_S11	AYS	N	PTR103_S138	PTR	N
AYS068_S12	AYS	N	PTR104_S139	PTR	Nx
AYS071_S13	AYS	N	PTR106_S140	PTR	N
AYS076_S14	AYS	N	PTR108_S141	PTR	N
AYS078_S15	AYS	N	PTR109_S142	PTR	N
AYS084_S16	AYS	N	PTR110_S143	PTR	N
AYS086_S276	AYS	N	PTR113_S144	PTR	N
CDM006_S229	CDM	N	PTR114_S145	PTR	N
CDM013_S230	CDM	K	PTR115_S146	PTR	N
CDM017_S231	CDM	K	PTR117_S147	PTR	N
CDM024_S232	CDM	N	PTR118_S148	PTR	N
CDM028_S17	CDM	N	PTR119_S149	PTR	N
CDM029_S224	CDM	K	PTR120_S150	PTR	N
CDM031_S18	CDM	N	PTR122_S151	PTR	N
CDM034_S225	CDM	K	PTR123_S152	PTR	N
CDM045_S19	CDM	N	PTR124_S153	PTR	N
CDM050_S277	CDM	K	PTR129_S154	PTR	N
CDM051_S278	CDM	K	RUG001_S155	RUG	N
CDM053_S20	CDM	N	RUG003_S244	RUG	K
CDM054_S21	CDM	N	RUG004_S156	RUG	K
CDM060_S22	CDM	K	RUG008_S157	RUG	K
CDM061_S23	CDM	N	RUG013_S158	RUG	K
CDM064_S24	CDM	N	RUG016_S159	RUG	N
CDM072_S25	CDM	N	RUG019_S160	RUG	K
CDM080_S26	CDM	N	RUG021_S161	RUG	N
CDM085_S27	CDM	N	RUG024_S245	RUG	K
CIR001_S28	CIR	K	RUG028_S246	RUG	K

CIR002_S29	CIR	K
CIR003_S30	CIR	N
CIR005_S31	CIR	K
CIR006_S32	CIR	K
CIR008_S33	CIR	K
CIR009_S34	CIR	K
CIR010_S35	CIR	N
CIR011_S36	CIR	K
CIR012_S37	CIR	K
CIR013_S38	CIR	K
CIR014_S39	CIR	K
CIR015_S40	CIR	K
CIR016_S41	CIR	K
CIR017_S42	CIR	K
CIR018_S43	CIR	D
CIR019_S44	CIR	K
CIR020_S45	CIR	N
CIR021_S46	CIR	K
CIR022_S47	CIR	N
CIR023_S48	CIR	N
CIR024_S49	CIR	N
COD101_S50	COD	N
COD102_S51	COD	N
COD103_S52	COD	N
COD104_S53	COD	N
COD106_S54	COD	N
COD107_S55	COD	N
COD108_S56	COD	N
COD109_S57	COD	N
COD110_S58	COD	N
COD111_S59	COD	N
COD113_S60	COD	N
COD114_S61	COD	N
COD115_S62	COD	N
COD116_S63	COD	N
COD117_S64	COD	N
COD118_S65	COD	N
COD119_S66	COD	N
COD120_S67	COD	N
IST082_S68	IST	N
IST084_S69	IST	N
IST085_S70	IST	N
IST086_S71	IST	N
RUG032_S162	RUG	N
RUG033_S163	RUG	N
RUG037_S164	RUG	K
RUG042_S165	RUG	K
RUG067_S166	RUG	K
RUG068_S247	RUG	N
RUG071_S167	RUG	K
RUG074_S168	RUG	K
RUG076_S248	RUG	N
RUG085_S249	RUG	N
SMH102_S250	SMH	N
SMH103_S251	SMH	K
SMH104_S169	SMH	N
SMH105_S170	SMH	N
SMH106_S171	SMH	N
SMH107_S172	SMH	N
SMH108_S173	SMH	N
SMH109_S174	SMH	N
SMH110_S175	SMH	N
SMH111_S176	SMH	D
SMH112_S177	SMH	K
SMH113_S178	SMH	N
SMH114_S179	SMH	N
SMH115_S180	SMH	N
SMH117_S181	SMH	N
SMH118_S182	SMH	N
SMH119_S183	SMH	N
SMH121_S280	SMH	N
SMH122_S256	SMH	K
SMH128_S281	SMH	K
STP101_S252	STP	N
STP102_S253	STP	N
STP103_S254	STP	N
STP104_S184	STP	N
STP105_S282	STP	N
STP106_S185	STP	N
STP108_S186	STP	N
STP109_S187	STP	N
STP110_S283	STP	N
STP111_S188	STP	K
STP112_S189	STP	N
STP113_S190	STP	N
STP114_S191	STP	N

IST091_S72	IST	N
IST094_S73	IST	N
IST096_S74	IST	N
IST100_S75	IST	N
IST101_S76	IST	N
IST102_S77	IST	N
IST103_S78	IST	N
IST104_S79	IST	N
IST105_S80	IST	N
IST106_S81	IST	N
IST107_S82	IST	N
IST108_S83	IST	N
IST109_S84	IST	N
IST111_S85	IST	N
IST112_S86	IST	N
IST113_S87	IST	N
LAL002_S233	LAL	K
LAL006_S88	LAL	K
LAL007_S89	LAL	K
LAL010_S90	LAL	N
LAL017_S91	LAL	N
LAL021_S92	LAL	N
LAL024_S93	LAL	K
LAL028_S94	LAL	N
LAL034_S95	LAL	K
LAL040_S96	LAL	N
LAL044_S234	LAL	K
LAL046_S97	LAL	N
LAL048_S98	LAL	N
LAL050_S99	LAL	N
LAL052_S100	LAL	N
LAL056_S235	LAL	K
LAL062_S101	LAL	K
LAL063_S102	LAL	N
LAL066_S103	LAL	K
LAL077_S236	LAL	N
LSO005_S104	LSO	K
LSO010_S237	LSO	D
LSO013_S105	LSO	N
LSO014_S106	LSO	K
LSO016_S107	LSO	K
LSO018_S238	LSO	N
LSO021_S108	LSO	K
STP115_S192	STP	N
STP116_S284	STP	N
STP117_S285	STP	N
STP118_S193	STP	N
STP121_S286	STP	N
STP122_S287	STP	N
THO104_S194	THO	N
THO105_S195	THO	N
THO106_S196	THO	N
THO107_S197	THO	N
THO108_S198	THO	N
THO109_S199	THO	K
THO110_S200	THO	N
THO111_S201	THO	K
THO112_S202	THO	N
THO113_S288	THO	N
THO114_S289	THO	N
THO115_S203	THO	N
THO116_S204	THO	N
THO117_S205	THO	K
THO118_S290	THO	N
THO119_S206	THO	N
THO120_S207	THO	N
THO122_S208	THO	N
THO124_S291	THO	N
THO125_S292	THO	N
TRA332_S293	TRA	N
TRA333_S209	TRA	K
TRA335_S210	TRA	K
TRA338_S211	TRA	K
TRA339_S212	TRA	N
TRA340_S213	TRA	K
TRA341_S294	TRA	K
TRA343_S295	TRA	N
TRA344_S214	TRA	K
TRA345_S257	TRA	N
TRA346_S296	TRA	N
TRA348_S297	TRA	K
TRA350_S215	TRA	N
TRA351_S298	TRA	N
TRA352_S258	TRA	K
TRA355_S259	TRA	K
TRA357_S260	TRA	N

LSO022_S239	LSO	N
LSO025_S240	LSO	D
LSO026_S109	LSO	N
LSO027_S110	LSO	K
LSO028_S111	LSO	K
LSO030_S112	LSO	N
LSO032_S113	LSO	K
LSO034_S241	LSO	K
LSO038_S114	LSO	K
LSO039_S115	LSO	N
LSO040_S116	LSO	K
LSO041_S279	LSO	D
LSO043_S117	LSO	K
MCL106_S118	MCL	N
MCL107_S119	MCL	N
MCL109_S120	MCL	N
MCL110_S121	MCL	N
MCL111_S122	MCL	N
MCL113_S123	MCL	N
MCL116_S124	MCL	N
MCL117_S125	MCL	N
MCL120_S126	MCL	N
MCL121_S127	MCL	N
TRA358_S216	TRA	K
TRA359_S217	TRA	K
TRA360_S218	TRA	N
VDM101_S219	VDM	K
VDM102_S220	VDM	K
VDM103_S261	VDM	K
VDM104_S221	VDM	K
VDM105_S262	VDM	K
VDM106_S263	VDM	N
VDM107_S264	VDM	K
VDM108_S265	VDM	K
VDM109_S266	VDM	K
VDM110_S267	VDM	K
VDM111_S222	VDM	N
VDM112_S268	VDM	K
VDM113_S269	VDM	K
VDM114_S270	VDM	K
VDM115_S271	VDM	K
VDM116_S272	VDM	N
VDM117_S273	VDM	K
VDM118_S274	VDM	K
VDM119_S275	VDM	K
VDM120_S223	VDM	K

Discussion générale

L'objectif de cette thèse était d'étudier le conflit nucléo-cytoplasmique chez *Physa acuta*. Dans cette discussion, je confronterai nos résultats aux prédictions théoriques ainsi qu'aux observations empiriques faites chez les plantes. Je reviendrai sur notre mesure du phénotype et son codage « binaire » à deux états : fertile et stérile. Cette étude s'inscrivant dans le contexte des conflits génétiques, je comparerai plus largement mes résultats à ceux obtenus dans d'autres conflits et leurs conséquences sur le maintien du polymorphisme. Enfin, je donnerai des perspectives d'études futures de la SMC chez la physe.

1. Interprétation globale des résultats

1.1 La SMC chez la physe a-t-elle des caractéristiques différentes de la SMC chez les plantes ?

1.1.1 Retour sur les prédictions théoriques

Les résultats principaux ce cette thèse sont issus de l'évolution expérimentale dans laquelle il s'agissait de tester deux des hypothèses clef des modèles de maintien de la SMC (Gouyon et al., 1991; Dufaÿ et al., 2007). Conformément aux prédictions, nous avons observé que 1) le mitotype associée à la SMC a une valeur sélective plus faible lorsque les restaurateurs de la fertilité mâle sont très fréquents et donc diminue en fréquence (Chapitre 2, manuscrit 1), 2) la restauration est soumise à un régime de sélection qui dépend de la fréquence de SMC : elle se maintient à des valeurs plus élevées quand la SMC est très abondante, que quand elle est absente (Chapitre 2, manuscrit 2). Une autre des prédictions de ces modèles est la sélection d'un variant SMC grâce à l'avantage femelle. Pour tester l'existence d'un tel avantage, il faut comparer les valeurs sélectives femelles d'individus K mâle-stérile avec celles d'individus hermaphrodites N ; or l'obtention d'individus K mâle-stérile a nécessité un long protocole d'introgression, nous disposons donc de données limitées pour cette mesure (Chapitre 1). Toutefois, les valeurs sélectives mâle et femelle obtenues à la cinquième génération d'introgression ont montré que les K mâle-stérile pondaient autant d'œufs que les N, suggérant que nous n'avons pas détecté d'avantage femelle sur ce trait (ce point sera discuté plus loin).

1.1.2 Retour sur les observations empiriques

Les dynamiques évolutives de SMC n'ont jamais été observé en temps réel chez les plantes qui sont des modèles biologiques ayant des temps de génération longs (en général 1 an est nécessaire pour les plantes gynodioïques annuelles, beaucoup plus pour des plantes pérennes comme le Thym). Toutefois, des mesures indirectes de traits existent et nous permettent de comparer nos résultats. Comme nous l'observons chez la physe, certaines études ont documenté l'existence d'un coût de la SMC comme chez *Plantago lanceolata* (E. van Damme, 1984) et *Silene vulgaris* (Mccauley & Olson, 2003) mais aussi l'existence de coûts de la restauration détectés par leurs effets sur la production de graines (de Haan et al., 1997; Del Castillo & Trujillo, 2009) ou de pollen (Bailey, 2002; Caruso & Case, 2013), ou déduits par des modèles d'équilibre à partir de la variation du sex-ratio parmi les populations naturelles (Case & Caruso, 2010). Chez *P. acuta*, l'existence du coût de la restauration est suggérée par les résultats de l'évolution expérimentale mais il ne semble pas très élevé et nous ne savons pas encore sur quel trait il s'exprime. De la même manière que chez les plantes, le coût de la restauration pourrait porter sur la fonction mâle, i.e. la production de sperme et/ou la fonction femelle, i.e. le nombre d'œufs pondus.

1.1.3 Aucun avantage femelle associé au mitotype K ?

Dans les populations naturelles, les fréquences de K sont très variables allant de 0% à 71% (voir Chapitre 4, Figure 1). Comme nous n'avons pas détecté d'avantage femelle (voir Section 1.1.1), cette observation peut sembler paradoxale. Cependant, nous ne pouvons pas conclure qu'il n'existe pas d'avantage femelle associé au mitotype K sur la base de ce seul résultat. Dans ces expériences, les valeurs sélectives ont été mesurées en l'absence de compétition – les partenaires sont toujours vierges et appariés avec un seul individu focal – ce qui ne reflète pas la réalité écologique. De plus, chez plusieurs espèces de plantes gynodioïques, aucun avantage femelle n'est trouvé bien que de nombreux traits soient comparés entre les femelles et les hermaphrodites (Dufay & Billard, 2012). Par exemple, chez *Beta vulgaris* (Dufay et al., 2009), *Daphne laureola* (Alonso & Herrera, 2001) et *Raphanus sativus* (Murayama et al., 2004) de fortes fréquences de femelles (allant jusqu'à 56%) sont maintenues dans différentes populations sans qu'on ait détecté d'avantage sélectif. Dufay & Billard (2012) font l'hypothèse que l'avantage femelle se produit sur certains traits non étudiés comme la probabilité de floraison et l'âge à la première floraison ou la survie des adultes. Chez la physe, nous pouvons non seulement faire une hypothèse similaire mais également supposer que les conditions de compétition et les conditions environnementales ont un impact sur la valeur de ces traits comme le suggère la mesure des taux de survie fait à différentes températures (20°C et 25°C) dans le Chapitre 1, manuscrit 2.

Les données de l'évolution expérimentale nous permettent d'expliquer la variation des fréquences de K observées en population naturelle. Nous avons constaté que le coût de la restauration n'est pas aussi important que le coût de la SMC ; la fréquence des restaurateurs diminue donc moins vite quand la SMC est en forte fréquence que la fréquence de la SMC le fait quand les restaurateurs sont en forte fréquence. La présence de restaurateurs peu coûteux se maintenant relativement longtemps dans les populations limite la fréquence de la SMC (valeur extrême : 71%, Chapitre 4, Figure 1) et le fort coût de la SMC en présence de restaurateurs peu expliquer les faibles fréquences de mitotype K : en imaginant que les fréquences de SMC et restaurateurs varient dynamiquement dans les populations, celles-ci passeront plus de temps dans la situation forte fréquence de restaurateurs-faible fréquence de SMC que dans la situation inverse. Un schéma différent est observé chez de nombreuses plantes, car une fréquence moyenne à élevée de restaurateurs semble souvent associée à une large gamme de fréquences de SMC. Dans les populations naturelles de *Beta vulgaris spp. maritima*, la fréquence de la SMC varie considérablement (0 - 88,9 %) et le taux de restauration est estimé entre 42,5 et 100 % chez les individus SMC (De Cauwer et al., 2012). La même tendance est observée chez *Raphanus sativus*, la fréquence des SMC varie de 0 à 100%, tandis que la fréquence des restaurateurs varie de 41 à 100% (Murayama et al., 2004). Les variations observées chez *B. vulgaris* et *R. sativus* suggèrent que le coût de la SMC en présence d'une fréquence élevée de restaurateurs pourrait être plus faible chez ces plantes que chez P. acuta.

1.2 La persistance du mitotype D : un paradoxe

Les fréquences de D trouvées en populations naturelles sont sûrement l'élément le plus paradoxal du système physe. Nous n'avons pas trouvé de restaurateurs associés à ce mitotype (Chapitre 3) et une étude récente (Monnet et al. *in prep*, voir quelques résultats dans l'Annexe 1) montre que les individus D bénéficient d'un avantage femelle important, ils sont en moyenne 20 % plus gros que les N et pondent 41 % d'œufs en plus. Avec ces deux résultats, on s'attendrait à trouver des fréquences élevées de D, or ce n'est pas le cas (Annexe 2), les fréquences n'excèdent jamais 7 %. Une fois encore, on peut supposer que les conditions environnementales jouent un rôle dans la propagation limitée des D, notamment la température. Ou qu'il existe des restaurateurs propres au mitotype D dans certaines populations, que nous n'avons pas encore trouvés.

Un autre scénario est de considérer que l'invasion par des femelles peut conduire à une baisse de densité de la population, si celles-ci ne trouvent plus de donneur de sperme pour féconder leurs œufs. Dans un tel scénario, on peut imaginer que nos observations soient biaisées : nous ne pouvons observer que le début de l'invasion SMC associée au D car dès que celui-ci se répand, la population se renouvelle moins bien, et disparaît ou la densité devient trop faible pour qu'elle soit échantillonnée. Sur plus de 80 sites échantillonnés, une seule population est polymorphe de type N/D (avec 2% de D) ; la plupart sont soit purement N, soit N/K et certaines contiennent les mitotypes N/K/D (Annexe 2). Si l'invasion du mitotype D conduit au suicide cela pourrait expliquer la rareté des populations de type N/D. Un avantage du mitotype K sur le mitotype D limiterait l'invasion de ce dernier. Cependant, cette hypothèse semble peu probable au vu de nos résultats (un important avantage femelle du D et aucun avantage femelle du K). Une structure en métapopulation pourrait également permettre la persistance du mitotype D grâce à une transmission de dème en dème. En effet, même si l'invasion du D entraîne le « suicide » d'un dème, il est possible qu'il se transmette entre-temps à d'autres dèmes si la migration est suffisante, persistant ainsi en faible fréquence dans la métapopulation générale, i.e. grâce à une dynamique épidémique.

2. Retour sur la mesure du phénotype et sa binarisation

Comme évoqué précédemment, les mesures de valeur sélective ont toujours été effectuées en l'absence de compétition, les individus étant maintenus vierges jusqu'au moment des croisements. Or d'après les différents résultats obtenus, il semble que la valeur de traits tel que la distribution du potentiel de restauration ou l'avantage femelle dépende beaucoup de la manière dont nous mesurons le phénotype. En effet, restaurer la fertilité mâle sur un seul partenaire vierge semble plus facile que restaurer une fertilité mâle comparable à celle d'un mitotype mâle-fertile dans une population où les individus sont en compétition pour fertiliser les ovules disponibles. Il est donc probable que nous détections des variations de traits bien plus importantes en présence de compétition. Cette hypothèse est confirmée par des données préliminaires (P. David, données non publiées) montrant que la valeur sélective mâle d'un individu K introgressé (Chapitre 1) est nulle en cas de compétition avec un individu N, ce qui contraste fortement avec la fertilité de 30 % en l'absence de compétition. Ces données préliminaires sont issus de croisements entre des individus N et K introgressés mis en compétition dans un même aquarium. L'un des deux types d'individus est homozygote albinos, l'autre hétérozygote pigmenté-albinos. Les fréquences des morphes observées dans les descendances semblent être compatibles avec l'idée que les individus K sont tous fécondés par des individus N et les N aussi.

Une des réflexions récurrentes ayant accompagnée cette thèse est : comment prendre compte les individus semi-stériles et quel seuil fixé pour cette catégorie ? Dans tous les manuscrits, nous avons choisi un seuil de 10 – un individu était considéré semi-stérile s'il engendrait entre 1 et <10 descendants – et nous avons groupé les individus mâle semi-stérile avec les individus mâle-stérile, binarisant ainsi le phénotype. Dans les premières expériences, une distribution bimodale se dessine clairement avec une partie des individus à 0 et une autre dessus de 10 et très peu d'individus entre ces deux catégories. Cependant, cette répartition a parfois été moins nette, notamment lors des mesures du potentiel de restauration à la onzième génération où beaucoup d'individus se répartissaient entre 5 et 10. Dans le cadre de cette expérience, une des explications possibles est la baisse de fréquence des restaurateurs crée d'abord des individus partiellement mâle-stérile puis des individus totalement mâle-stérile. Cela pourrait arriver par exemple s'il existe plusieurs allèles ou locus, ou des allèles à dominance partielle, impliqués dans la restauration. En outre, si la plupart des modèles, dans un objectif de simplification, considèrent qu'un allèle restaurateur a un certain coefficient de dominance (fixé), il est très possible que selon le phénotype considéré le mode d'action des allèles varie (cf plus haut) : un seul allèle restaurateur pourrait suffire à rétablir la valeur de référence pour le caractère à distribution quasi-binaire « capacité à obtenir des paternités avec un partenaire vierge » (aux semi-stériles près) alors même que le caractère « fitness mâle ou fitness femelle en conditions de compétition dans la population » ne retrouverait qu'une fraction de sa valeur normale. En d'autres termes ce qui est dominant et/ou épistatique pour un caractère « stérilité mâle » mesuré individuellement avec un certain protocole peut être codominant ou additif pour la mesure de fitness qui est importante pour l'évolution des fréquences.

De manière générale, la binarisation du phénotype pourrait ne pas être appropriée pour analyser la fertilité mâle. Des mâle-stériles partiels (PMS, voir Ehlers et al., 2005) sont signalés chez d'autres espèces gynodioïques (par exemple *Silene vulgaris* ; Charlesworth & Laporte, 1998 ; *Plantago* sp., van Damme & van Delden, 1982; Koelewijn & Van Damme, 1995) et, comme Ehlers et al. (2005) le souligne, l'importance de ces morphes intermédiaires pour l'évolution de la SMC n'a pas encore été étudiée en détail. Une approche par modèle de seuil (voir Introduction Encadré 2) pourrait permettre de prendre en compte la catégorie semi-stérile – dans ces modèles, la variance génétique d'un trait quantitatif multigénique détermine la fraction d'individus mâles-stériles dans un cytoplasme donné, par le biais d'une fonction de seuil. Ainsi, les individus dont la valeur du trait est supérieure à un seuil donné auront un phénotype hermaphrodite, tandis que les individus restants auront un phénotype femelle. L'emploi du telle approche est également justifiée par notre étude du déterminisme de la restauration. Nous avons en effet conclu que la stérilité mâle du mitotype K dans le fond nucléaire montpelliérain était exprimée comme un trait quantitatif agissant sur la pénétrance du phénotype mâle-stérile (Chapitre 3). Ainsi, comme suggéré par Ehlers et al. (2005) il serait plus adapté de considérer des scénarios où la fonction mâle est un caractère à seuil que d'employer des modèles mendéliens multilocus.

3. Conséquences sur le maintien du polymorphisme dans un conflit génomique

3.1 Des dynamiques similaires dans d'autres conflits

Les travaux présentés ici, nous ont permis d'étudier le maintien du polymorphisme dans un conflit génétique et nos résultats s'accordent avec les dynamiques retrouvées dans d'autres conflits, notamment celui opposant les distorteurs de sexe-ratio et leur suppresseurs chez différentes espèces de drosophiles (e.g. Jaenike, 1999). Les dynamiques évolutives de ce conflit sont similaires à celle que nous retrouvons chez la physe. Quand les suppresseurs du distorteur X sont fixés chez *Drosophila simulans*, on observe que le distorteur décroit de la même manière que la SMC quand la restauration est fixée (Chapitre 3). Cette décroissance est cependant plus lente dans le cas des distorteurs que dans le cas de la SMC : pour une fréquence initiale de 66%, le distorteur persiste encore à une fréquence substantielle dans la moitié des six populations expérimentales après près de cent générations (Bastide et al., 2022). Une autre étude montre l'augmentation en fréquence de chromosome Y et des autosomes résistants quand ils sont introduits dans des populations contenant des distorteurs (Capillon & Atlan, 1999).

Nous avons suggéré un effet de l'environnement et notamment de la température sur le maintien du polymorphisme chez la physe. Cette hypothèse est appuyée par des études montrant un effet de l'environnement sur les interactions mito-nucléaires ; chez la drosophile, le type d'alimentation modifie les effets de l'ADN mitochondrial et de l'ADN nucléaire (e.g. Mossman et al., 2016).

3.2 La course aux armements

La gynodioécie étant vue comme une voie évolutive depuis l'hermaphrodisme vers la dioécie (Barrett, 2002), on peut se demander si la physe pourrait évoluer une séparation des sexes. Chez certains clades où la gynodioécie est fréquente, comme *Silene* (Spigler & Ashman, 2012), on trouve des taux d'évolution accéléré à la fois chez les espèces gynodioïques et chez les espèces hermaphrodites (Sloan et al., 2012, 2014). Ces observations suggèrent que le polymorphisme peut être parfois maintenu mais également que les restaurateurs et des mutants SMC peuvent parfois se fixer. Dans ce cas, le polymorphisme sexuel est perdu et les individus redeviennent hermaphrodites jusqu'à l'apparition d'une nouvelle mutation SMC. On observe ainsi une course à l'armement entraînant une évolution accélérée du génome nucléaire et mitochondrial (Fujii et al., 2011).

L'existence de mitotypes SMC extrêmement divergents et la découverte de restaurateurs (Chapitre 1) chez la physe nous conduit à une interprétation similaire. La course aux armements pourrait être liée à la pénétrance du phénotype mâle-stérile : une mutation conférant une pénétrance plus élevée à un mitotype SMC augmenterait en fréquence, tandis que les restaurateurs seraient sélectionnés pour réduire la pénétrance. Dans ce contexte, le mitotype D (pour lequel aucun restaurateur n'est encore connu) devrait envahir les populations, mais comme nous l'avons vu, ses fréquences semblent être relativement faibles par rapport au mitotype K. Nous avons encore une image très incomplète d'un système potentiellement très dynamique dans lequel de nouveaux mitotypes divergents liés à de la SMC peuvent émerger et se propager rapidement avant d'être contrés par des restaurateurs. Le suivi de la dynamique des mitotypes divergents dans les populations naturelles au fil du temps serait une source essentielle d'informations sur cette question.

4. Perspectives

4.1 Boucler la boucle de l'évolution expérimentale

Les modèles d'évolution du conflit entre SMC et restaurateurs, dont le comportement type est reflété par les cycles limites prédits notamment par Dufaÿ et al. (2007) sont fondés sur un système de fréquence-dépendance réciproque, le régime de sélection sur la SMC s'inverse selon que les restaurateurs sont rares ou fréquents, le régime de sélection sur les restaurateurs s'inverse selon que la SMC est rare ou fréquente (voir Introduction, Figure 4). Nous avons exploré deux aspects de cette dynamique en évolution expérimentale, (i) la diminution de la fréquence de SMC en présence de restaurateurs et (ii) l'existence d'un régime de sélection différent en phase de forte fréquence de SMC par rapport à une phase de faible fréquence de SMC.

Concernant ce deuxième aspect, il serait nécessaire de quantifier la sélection sur les restaurateurs en suivant leurs fréquences dans le temps. Ce suivi nous permettrait d'aller au-delà de l'observation d'une différence entre deux régimes de sélection car nous pourrions mesurer des coefficients de sélection. Les populations expérimentales 100%N et 100%K sont encore disponibles au laboratoire et ont maintenant atteint la 19e génération d'évolution expérimentale. Nous pourrions réaliser un protocole similaire à celui effectué à la 11e génération (Chapitre 2, manuscrit 2) afin d'obtenir un deuxième point temporel estimant la fréquence des restaurateurs. D'après nos observations (Chapitre 2, manuscrit 2), le coût de la restauration semble faible, cependant pour conclure sur ce point, une deuxième mesure du potentiel de restauration est nécessaire.

Plus généralement, pour explorer toutes les étapes des cycles limites, il nous faut nous intéresser à la sélection de la SMC en l'absence de restaurateur. Pour cela, nous pouvons utiliser le fond nucléaire introgressé car nous avons vu que celui-ci a une faible variation quantitative de fertilité-mâle résiduelle et est dépourvu de restaurateurs à effets forts (Chapitre 1, Chapitre 4). Une expérience similaire pourrait être réalisée avec le mitotype D afin de mieux comprendre son maintien dans les populations naturelles. Il s'agirait de créer plusieurs traitements associés à différentes fréquences initiales d'individus D placés avec des individus N en fréquence complémentaire. Si le mitotype D se maintient grâce à une dynamique épidémique, on s'attend à ce qu'il envahisse les populations et conduise éventuellement au suicide évolutif, c'est-à-dire à un renouvellement des générations de plus en plus difficile, la fécondité femelle étant de plus en plus limitée par la rareté des individus mâle-fertiles.

4.2 Trouver des gènes de restaurations

L'approche de scan génomique devrait nous permettrait d'identifier des gènes liés à la restauration. Nous pourrons notamment rechercher des différences de fréquences dans les membres de la famille de protéines Pentatricopeptide Repeat (PPR) ; ces protéines sont responsables de la restauration de la fonction mâle chez les plantes cultivées (Delph et al., 2007) et sont présentes chez animaux (Giegé, 2013, notamment les mammifères) où un motif semble très conservé (motif PROP ; Manna, 2015).

En parallèle du scan génomique, maintenant que nous disposons de fonds génétiques avec restauration quasi-fixée d'un côté, et quasi-absente de l'autre, une étude par QTL dans des descendances F2 où le caractère « restauration » ségrégerait est envisageable. Il s'agira alors de rechercher, dans les séquences des F2, quels marqueurs sont co-transmis avec la capacité de restauration tel que nous pouvons la mesurer en laboratoire.

4.3 Préciser les phénotypes et les effets sur la valeur sélective selon l'environnement

Un des thèmes récurrents qui émergent de nos expériences est la nature complexe du phénotype de fertilité mâle et l'importance du contexte environnemental et de compétition dans sa quantification. La mesure des effets et coûts de la SMC et des restaurateurs doit a minima, être complétée par des mesures en conditions de compétition. Pour cela, nous pourrions évaluer la valeur sélective mâle en compétition en croisant tout d'abord le partenaire avec un individu mâle-fertile puis avec l'individu focal (dont le sperme doit donc entrer en compétition avec le sperme de l'individu mâle-fertile pour obtenir des paternités) – cette méthode a été utilisée par Timothée Chenin dans un stage de M1 que j'ai co-encadré avec P. David, dont seule une partie des résultats a été analysés jusqu'ici – l'exploitation du reste est en cours. Une autre méthode possible est d'utiliser des groupes ou nous mettons en compétition des génotypes (restauré/non restauré ou SMC/non SMC) chacun associé à un morphe différent (albinos/pigmenté) de manière à ce que les fréquences des morphes dans les petits produits par chaque type indiquent le taux de paternité relatif des différents génotypes. De telles expérimentations sont également en cours au laboratoire (Patrice David, Romann Charbonnier, Céline Froissard).

Une étape bloquante pour caractériser, notamment les coûts de la restauration, est de pouvoir disposer de génotypes connus (avec et sans restaurateurs) dans le même fond nucléaire, ce qui n'était pas facile jusqu'ici (les génotypes restaurés étaient de fond lyonnais, les génotypes non restaurés de fond montpelliérain) ; avec les résultats de l'évolution expérimentale ces génotypes deviennent peu à peu disponibles car nous obtenons des populations à forte et faible fréquence de restauration issus du même fond génétique de départ (lyonnais). Si, par ailleurs, le scan génomique ou les QTLs nous fournissent des marqueurs de séquence associés aux restaurateurs, le travail de comparaison sera grandement facilité (les génotypes avec et sans restaurateurs pouvant être distingués moléculairement même quand le mitotype est non-SMC).

Enfin l'exploration d'aspects plus fonctionnels de l'action de la SMC, restaurée ou non, sur le phénotype permettra de mieux comprendre son mécanisme : la divergence extrêmement forte des séquences codantes des mitochondries SMC par rapport aux mitochondries normales doit avoir des conséquences sur le métabolisme, l'expression des gènes, les traits d'histoire de vie et leur réaction à la température (qui affecte le métabolisme, et pour laquelle nous avons observé des effets). De telles expériences sont déjà en cours via les travaux de Nathanaëlle Scalier mais aussi la thèse de Sophie Bérerd encadrée Emilien Luquet, Sandrine Plénet.

Physa acuta est un nouveau modèle d'étude fascinant qui se prête à un travail qui est beaucoup plus difficile à envisager chez les plantes, nous permettant ainsi d'aller plus loin dans le test des modèles théoriques élaborés dans ce domaine. Comme nous l'avons vu au travers de ces perspectives, de nombreuses questions restent encore à explorer chez la physe alors, c'est parti

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ANNEXE 1

AVANTAGE FEMELLE DU MITOTYPE D

Evaluation de l’avantage femelle des individus D chez *Physa acuta*

D’après Master 1 de François Monnet (2019), sous la direction de Patrice David et Mathilde Dufaÿ : La fonction mâle est-elle limitante dans une espèce gynodioïque ?

Préambule : Cette annexe est un extrait du stage de François Monnet et présente quelques résultats sur l’évaluation de l’avantage femelle associé au mitotype D. Seuls le matériel et méthode et les résultats sont retranscrits ici. Avant 2022, le mitotype D était appelé W (pour Weird), cette appellation a été conservée dans ce manuscrit.

Matériel et méthode

Matériel biologique utilisé

Les escargots utilisés lors de cette expérience descendent d'individus de la population lyonnaise (David et al., 2022). L'échantillon originel a subi une introgression de 11-12 générations (Figure S1) avec une population *albinos* issue de la région de Montpellier (Noël et al., 2016). Les individus possèdent donc un génome nucléaire quasi- exclusivement Montpelliérain (rendant plus improbable encore la présence de gènes nucléaires restaurateurs, la SMC étant absente à Montpellier). Grâce à cette méthode il a été obtenu 4 populations sources pour l'expérimentation : une population SMC albinos (Walb), une population SMC pigmenté (Wpig), une population hermaphrodite albinos (Nalb) et une population hermaphrodite pigmenté (Npig). Les individus pigmentés sont hétérozygotes au locus du gène codant l'albinisme (l'allèle d'albinisme est récessif). Les morphes permettent de diagnostiquer la paternité des descendants. Un descendant pigmenté d'une mère albinos doit provenir d'une allofécondation par un père pigmenté, l'albinisme étant récessif.

Populations expérimentales et traitements

360 individus vierges, de même âge, issus des 4 populations sources (Walb, Nalb, Wpig, Npig) ont été élevés jusqu’à maturité (6 semaines) en conditions standard (boites de 5, puis boîtes individuelles). Ils sont alors entrés dans le protocole expérimental, en constituant des populations de 10, avec 6 fréquences d'individus de cytotypes W et N: 0W/10N – 2W/8N – 5W/5N –8W/2N – 9W/1N – 10W/0N. Chaque fréquence était répliquée 6 fois pour un total de 36 populations. Face au nombre important de manipulations, les populations ont été divisées en deux blocs de 18 (chacun avec 3 populations de chacune des 6 fréquences) décalés intégralement d'une semaine.

L'albinisme a été réparti de manière (i) à ce que les deux cytotypes soient associés chacun à un morphe différent dans chaque aquarium ce qui permet ultérieurement d'estimer leur paternité relative (ii) à limiter un possible biais en inversant la correspondance morphe-cytotype d'un aquarium à l'autre (autant d'aquariums Walb+Npig que de Wpig+Nalb au sein de chaque traitement). Dans les populations homogènes (10W/0N ou 0W/10N) cinq individus étaient albinos et cinq pigmentés.

Avant de constituer les populations, chaque individu a été marqué individuellement avec une pastille collée sur la coquille portant une couleur et un numéro – ceci nous permet de reconnaître chaque individu même en mélange avec d'autres. Les populations ont chacune été mises dans des aquariums (23,2*15,3*16,6cm, remplie aux 2/3 d'eau de source à 25°C) avec de la nourriture en abondance (salade bio cuite et broyée). Après 3 jours les individus ont été sortis et pesés (Mettler PM100, précision 1 mg). Chaque individu a été isolé dans une boîte individuelle (5,6*4*4,2cm, eau de source 25°C), nourri et laissé pondre pendant 2 jours. Au terme de la période d'isolement chaque population a été réunie dans son aquarium pour une période de 5 jours. Au total 3 périodes de pontes ont été réalisées de cette manière avec comptage systématique du nombre de pontes et d'œufs pour chaque individu. Les pontes de la première et troisième période ont été conservées et entretenues (sans déshydratation et avec ajout de salade très fine après une semaine) pour estimer le taux d'éclosion après deux semaines.
Après la dernière ponte, les individus sortent du protocole expérimental.

Analyse statistique des résultats

La fitness a été étudiée au travers de 3 variables réponses :

1. 1- Le poids, mesuré lors de la constitution des populations ainsi qu'à chacune des 3 isolations pour pontes, exprimé en milligramme.
2. 2- Les pontes, mesurées à chacune des 3 isolations pour pontes, exprimées en quantité d'œufs pondus.
3. 3- La survie des juvéniles, mesurée pour la 1ère et 3ème ponte, en nombre de juvéniles ayant éclos et survécu par rapport au nombre d'œufs pondus.

Les données ont été analysées à travers des modèles linéaires mixtes (LMM) ou généralisés (GLMM) réalisés avec le logiciel R version 3.6.0 (package lme4). Dans ces modèles, les facteurs fixes sont le traitement (T: 6 types de population de 0W10N à 10W0N), le cytotype de l'individu (C: individu de type W ou N) et le morphe de l'individu (M: individu pigmenté ou albinos). Le facteur cytotype (C) permet l'étude de l'avantage femelle, le facteur traitement (T) celui de la limitation par la fonction mâle et la combinaison des deux (T*C) permet d'investiguer un possible effet d'interaction. S'y ajoute un effet bloc (bloc 1 et 2) et un facteur aléatoire « population » (chacun des 36 réplicats/aquariums).

Notons que les facteurs T et C sont corrélés par nature. Par exemple dans le traitement 10W0N il n'y a plus qu'un cytotype (W). La distribution des échantillons selon les facteurs est donc non-orthogonale.

Pour limiter l'impact de cette colinéarité nous avons divisé le jeu de données différemment en fonction du facteur étudié. Pour l'analyse de l'effet C, les traitements homogènes 10W0N et 0W10N (où un seul des cytotypes est présent) ont été retirés de l'analyse. Pour l'analyse de T, les cytotypes W et N ont été traités de manière séparée et tous les traitements possibles dans chaque cytotype (y compris 10W0N pour le cytotype W et 0W10N dans le cytotype N) ont été inclus.

Les tests des effets fixes et de leurs interactions sont des tests de likelihood-ratio (LRT) par simplification de modèle. On estime les déviances de deux modèles, avec et sans l'effet, et la différence est comparée à un chi-deux à *n* degrés de liberté (nombre de paramètres estimés pour l'effet). Notre procédure générale consiste à partir du modèle le plus complexe, et à retirer progressivement des effets non significatifs (interactions triples, puis doubles, puis effets principaux), jusqu'à un modèle minimal comprenant des effets tous significatifs (qui peut être le modèle nul). Lorsqu'une interaction est significative (par exemple T :C), cela signifie que l'effet d'un facteur (C) n'est pas le même selon les niveaux de l'autre (T). On doit alors séparer le jeu de données selon les niveaux du premier (T) pour traiter le second (C).

Les modèles de départ (les plus complexes) sont les suivants :

- Pour le jeu de données avec les deux cytotypes, mais sans les traitements 0W10N et 10W0N:

$$\boldsymbol{Y} \sim \mathbf{1} + \boldsymbol{Bloc} + \boldsymbol{T} * \boldsymbol{C} * \boldsymbol{M} + (\mathbf{1} | \boldsymbol{pop})$$

qui inclut les effets fixes T, C, M et leurs interactions doubles et triple, l'effet fixe Bloc, et l'effet aléatoire population (Y= variable réponse).

- Pour les jeux de données séparés par cytotype :

$$\boldsymbol{Y} \sim \mathbf{1} + \boldsymbol{Bloc} + \boldsymbol{T} * \boldsymbol{M} + (\mathbf{1} | \boldsymbol{pop})$$

Les distributions associées aux modèles sont : Gaussienne (Poids des individus), Poisson (pour les fécondités exprimées en nombre entiers d'œufs), Binomiale (pour les survies juvéniles exprimées en nombre d'œufs ayant et n'ayant pas donné un descendant vivant à 14j). Les fonctions de lien étaient respectivement identité (modèle gaussien), logarithme (modèle poisson) et logit (modèle binomial). Pour les deux derniers cas la présence de surdispersion nous a conduit à ajouter un facteur aléatoire « individu » dans le modèle, permettant d'ajuster un niveau de variance individuelle en plus du niveau population (Browne et al., 2005; Elston et al., 2001). Par ailleurs, pour le modèle sur les poids, les moyennes et les variances des poids semblaient différer entre les deux blocs ; nous avons donc centré-réduit les données au sein de chaque bloc plutôt que de simplement fitter l'effet fixe bloc – ceci contrôle à la fois les différences de moyenne et de variance, et rend inutile l'ajout de l'effet fixe bloc aux modèles ; cet effet a donc été supprimé.

Les attendus donnés en introduction devraient se retrouver dans l'analyse statistique par un effet cytotype traduisant un avantage femelle pour les W et par un effet traitement chez chaque cytotype traduisant une limitation par la fonction mâle. Il est en revanche attendus que la limitation ne soit pas parallèle chez les deux cytotypes. En effet les traitements 9W1N sont particuliers car les N ne peuvent pas être allofécondés contrairement au W, ils se retrouvent alors dans un cas de figure similaire aux W des traitements 10W0N.

Enfin, deux erreurs de manipulations ont dû être prises en compte. (i) Certaines données de poids en bloc 1, première mesure, ont été perdues et remplacées par une nouvelle pesée deux jours plus tard (ceci a été pris en compte en ajoutant deux jours de plus pour les mesures concernées). (ii) Une partie du bloc 2 a été mise en aquarium commun une semaine trop tôt. Il n'existe donc pas de données de 3ème ponte et de survie pour 1/3 des populations de chaque traitement du 2ème bloc (1/6e du total), le nombre de populations restant équilibré entre les traitements.

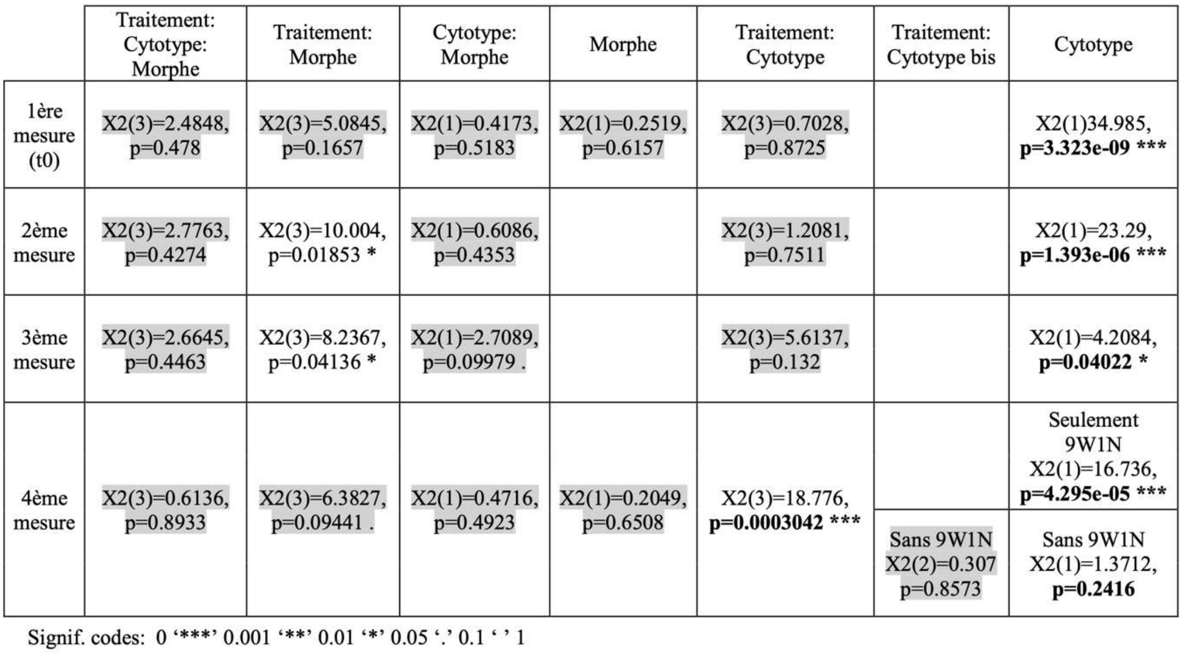
Résultats

Poids

L'analyse du poids sans les traitements 10W0N et 0W10N (Tab.1) montre un effet du facteur cytotype dès le début de l'expérience avec un avantage pour les W (Fig.1 A&B) qui sont en moyenne 20% plus gros que les N. Cet effet perd en intensité au fil du temps jusqu'à la troisième mesure. À la quatrième mesure, une interaction significative montre que la différence entre cytotypes dépend du traitement (Tab.1 et Fig.1 A&B). Wans le traitement 9W1N, les individus N sont beaucoup plus gros (31%) que les W, alors qu'il n'y a pas de différence significative entre cytotypes dans tous les autres traitements. Le comportement particulier du traitement 9W1N est visible dès la 2e mesure (Tab.1) mais l'interaction traitement/cytotype n'est significative qu'à la 4e.

Tab.1 Analyse du poids normalisé sans traitement 10W0N ni 0W10N.

Les deux blocs ont été mélangés après réduction-centrage des données au sein de chaque bloc (donc aucun effet bloc n'a été fitté). Les tests sont des test de Chi-2 (entre parenthèses, nombre de degrés de liberté) correspondant au likelihood-ratio (LRT) de deux modèles, l'un avec, l'autre sans l'effet considéré. Les interactions d'ordre supérieur sont enlevées, si non significatives, pour tester les interactions d'ordre inférieur ou les effets fixes. Tous les effets non-significatifs sont enlevés du modèle final (en gris). Lorsqu'une interaction est significative (cas de traitement-cytotype 4e mesure), le jeu de données est divisé pour tester les facteurs sous-jacents (par exemple, ici, l'interaction traitement-cytotype p=0.0003 vient d'un effet différent du cytotype dans le traitement 9W1N par rapport aux autres ; on examine d'un côté ce traitement (dans lequel on teste l'effet cytotype) et de l'autre côté tous les autres (on vérifie que l'interaction Traitement :cytotype n'est pas significative et on teste cytotype). L'effet traitement a été conservé mais non testé dans ce modèle : il est testé au sein de chaque cytotype en incluant tous les traitements, y compris 10W0N (cytotype W) et 0W10N (cytotype N) (Tab.2).



	Traitement: Cytotype: Morphe	Traitement: Morphe	Cytotype: Morphe	Morphe	Traitement: Cytotype	Traitement: Cytotype bis	Cytotype
1ère mesure (t0)	X2(3)=2.4848, p=0.478	X2(3)=5.0845, p=0.1657	X2(1)=0.4173, p=0.5183	X2(1)=0.2519, p=0.6157	X2(3)=0.7028, p=0.8725		X2(1)34.985, **p=3.323e-09** ***
2ème mesure	X2(3)=2.7763, p=0.4274	X2(3)=10.004, p=0.01853 *	X2(1)=0.6086, p=0.4353		X2(3)=1.2081, p=0.7511		X2(1)=23.29, **p=1.393e-06** ***
3ème mesure	X2(3)=2.6645, p=0.4463	X2(3)=8.2367, p=0.04136 *	X2(1)=2.7089, p=0.09979 .		X2(3)=5.6137, p=0.132		X2(1)=4.2084, **p=0.04022** *
4ème mesure	X2(3)=0.6136, p=0.8933	X2(3)=6.3827, p=0.09441 .	X2(1)=0.4716, p=0.4923	X2(1)=0.2049, p=0.6508	X2(3)=18.776, **p=0.0003042** ***		Seulement 9W1N X2(1)=16.736, **p=4.295e-05** ***
						Sans 9W1N X2(2)=0.307 p=0.8573	Sans 9W1N X2(1)=1.3712, **p=0.2416**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

En traitant séparément les deux cytotypes (Tab.2) on observe chez les N un effet traitement significatif seulement à partir de la 3ème mesure ; cet effet est dû aux individus du traitement 9W1N qui ont beaucoup plus grossi que les autres (Fig.1 A&B). Chez les W on observe un effet similaire mais lié au traitement 10W0N, les individus W de ce traitement prennent plus de poids que ceux des autres traitements (Fig.1 A&B).

Notons que dans certains de ces modèles on remarque des différences d'effet du traitement (non attendues) suivant le morphe (interactions T:M significatives, (Tab.2). Cependant (i) les effets cytotypes sont testés dans un modèle où l'interaction T:M est prise en compte (ii) les effets traitements vont dans la même direction et sont globalement significatifs au sein des deux morphes séparément.

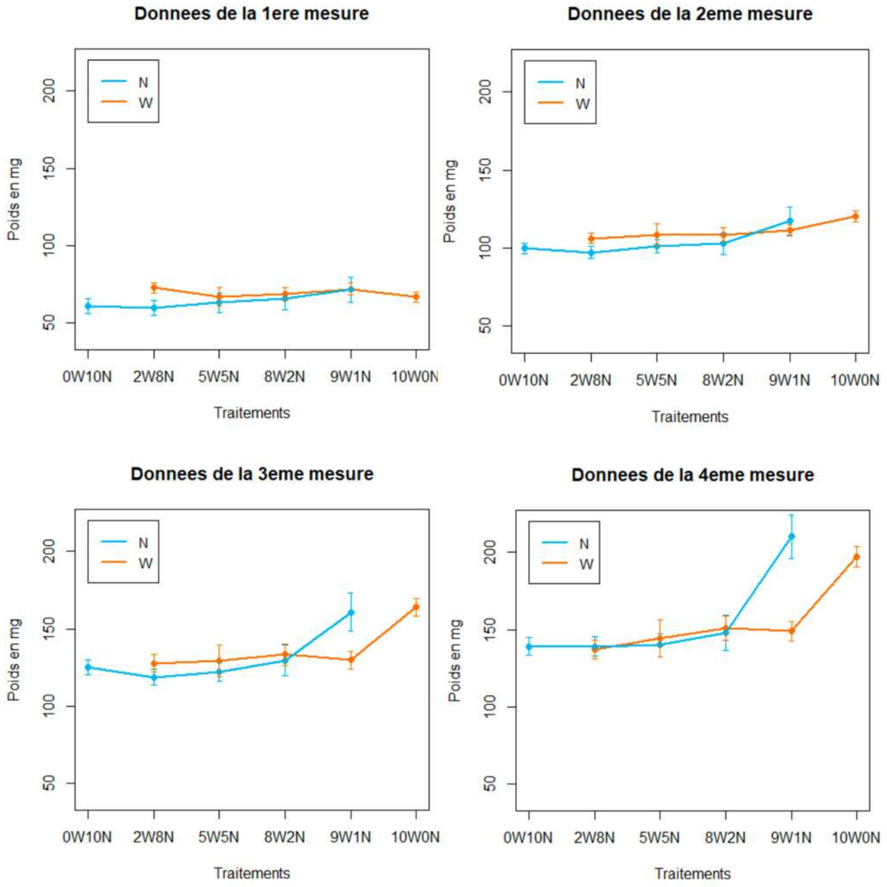


Fig.1A Evolution du poids des individus du bloc 1. Les erreurs standards sont représentées.

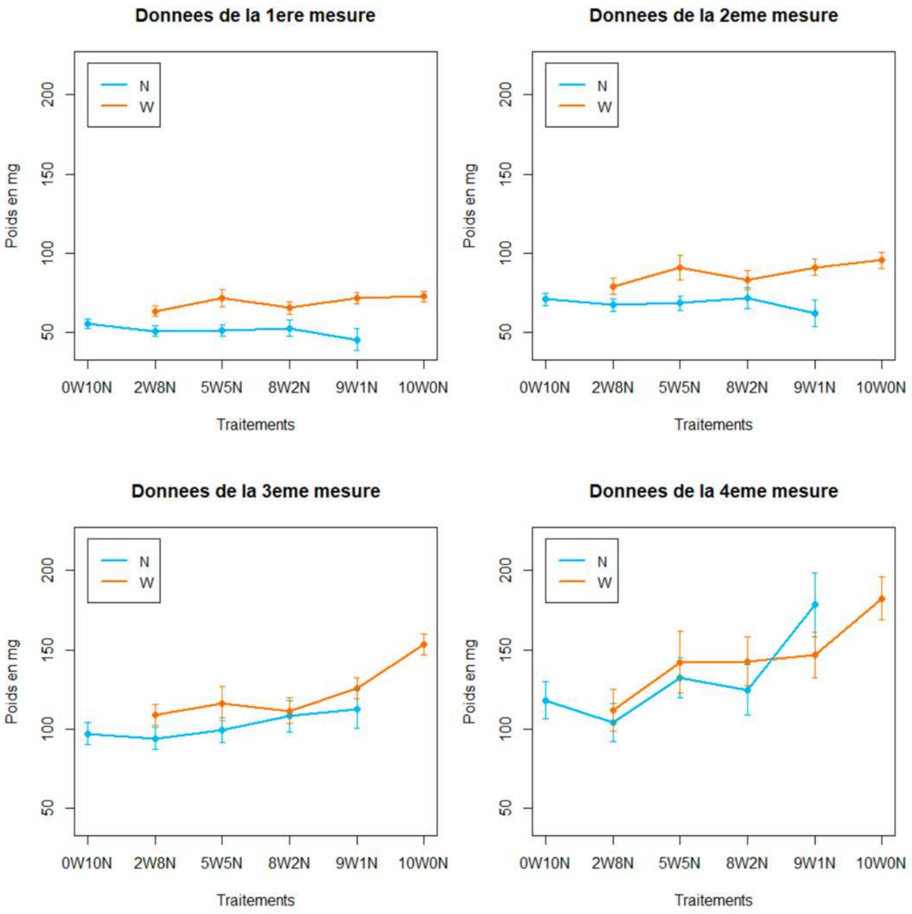


Fig.1B Evolution du poids des individus du bloc 2. Les erreurs standards sont représentées.

Tab.2 Analyse du poids normalisé avec cytotypes séparés. Même convention que table 1.

N	1ère mesure (t0)			
	T:M	M	T	
	X2(4)=2.8564, p=0.5821	X2(1)=2.3447, p=0.1257	X2(4)=1.0908, p=0.8957	
	2ème mesure			
	T:M	M	T	
	X2(4)=2.9322, p=0.5692	X2(1)=0.4589, p=0.4981	X2(4)=2.2284, p=0.6938	
	3ème mesure			
	T:M	M	T	**T sans 9W1N**
	X2(4)=4.4487, p=0.3487	X2(1)=0.3174, p=0.5732	X2(4)=11.696, **p=0.01976 ***	X2(3)=2.7769, **p=0.4273**
	4ème mesure			
	T:M	M	T	**T sans 9W1N**
	X2(4)=1.6628, p=0.7975	X2(1)=0.0332, p=0.855	X2(4)=30.718, **p=3.496e-06 *****	X2(3)=2.608, **p=0.456**
W	1ère mesure (t0)			
	T:M	M	T	
	X2(4)=10.34, p=0.03508 *		Pigmentés X2(4)=4.7935, p=0.3091	
			Albinos X2(4)=6.7111, p=0.152	
	2ème mesure			
	T:M	M	T	**T sans 10W0N**
	X2(4)=16.294, p=0.002649 **		Pigmentés X2(4)=7.5355, p=0.1102	
			Albinos X2(4)=18.103, **p=0.001178 ****	X2(3)=3.2274, **p=0.3579**
	3ème mesure			
	T:M	M	T	**T sans 10W0N**
	X2(4)=9.5875, p=0.04798 *		Pigmentés X2(4)=11.208, **p=0.02432 ***	X2(3)=3.2693, **p=0.3519**
			Albinos X2(4)=40.487, **p=3.432e-08 *****	X2(3)=3.8871, **p=0.2739**
	4ème mesure			
	T:M	M	T	**T sans 10W0N**
	X2(4)=10.313, p=0.03547 *		Pigmentés X2(4)=14.126, **p=0.006903 ****	X2(3)=2.5853, **p=0.4601**
			Albinos X2(4)=30.227, **p=1.718e-06 *****	X2(3)=9.6035, **p=0.02226 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Pontes

L'analyse des pontes en excluant les traitements 10W0N et 0W10N montre une interaction significative C:T dans les deux premières pontes (Tab.3). Dans tous les traitements les W sont plus féconds que les N (+41%) mais cette différence est beaucoup plus prononcée pour le traitement 9W1N où les N ne pondent pas du tout (1^{e} mesure) ou très peu (2^{e} mesure) (Fig.2).

À la $3^{ème}$ mesure cette particularité disparaît, l'interaction C :T devient non significative, mais le cytotype W reste plus fécond que N sur l'ensemble des traitements (effet C significatif, tab.3, tab.4). Sur l'ensemble, la figure 2 et le tableau 4 nous indiquent que les individus W (SMC) pondent presque systématiquement plus que les individus N.

L'amplitude de l'avantage femelle peut être estimée avec le traitement 2W8N comme référence. On choisit ce traitement car c'est le traitement avec des individus des 2 cytotypes qui présente le moins de limitation par la fonction mâle (donc le moins d'effet pouvant biaiser l'estimation de l'avantage femelle). L'amplitude de l'avantage femelle est calculée à l'aide de cette formule:

$$Avantage\ femelle = \frac{Pontes\ des\ D}{Pontes\ des\ N}$$

L'avantage femelle pour l'ensemble des pontes est estimé à 1,4. Les W ont donc pondu dans l'ensemble 1,4 fois plus d'œufs que les N dans le traitement 2W8N.

En analysant les cytotypes séparément (Tab.5) on constate chez les N un effet traitement significatif qui s'estompe au fil des mesures. Cet effet est principalement dû au traitement 9W1N comme vu précédemment. Chez les W il y a un effet traitement persistant dans le temps qui est principalement dû au traitement 10W0N, où les individus ne pondent que très peu sur toute la durée de l'expérience (Fig.2). Cependant, leur ponte n'est pas nulle. En effet, parmi les traitements 10W0N (sans hermaphrodite normal, donc théoriquement sans fonction mâle disponible), 26 des 60 individus W ont eu des pontes.

Survie juvénile

Aucun facteur n'a d'effet significatif, que ce soit dans l'analyse du traitement (Tab.6) ou dans celle du cytotype (Tab.7).

Parmi les traitements 10W0N, 12 des 30 individus albinos ont eu des descendants survivants. 9 de ces 12 individus albinos ont eu des descendants pigmentés, preuve d'une allofécondation (l'allèle d'albinisme étant récessif) (Tab.8).

Parmi les 6 individus N des traitements 9W1N, 3 ont eu des descendants survivants. Les partenaires potentiels (les 9 individus W) étant d'un morphe différent on peut en déduire le type de fécondation des N. Les différentes fréquences de pigmentation indiquent un cas d'autofécondation et 2 cas d'allofécondation (Tab.9).

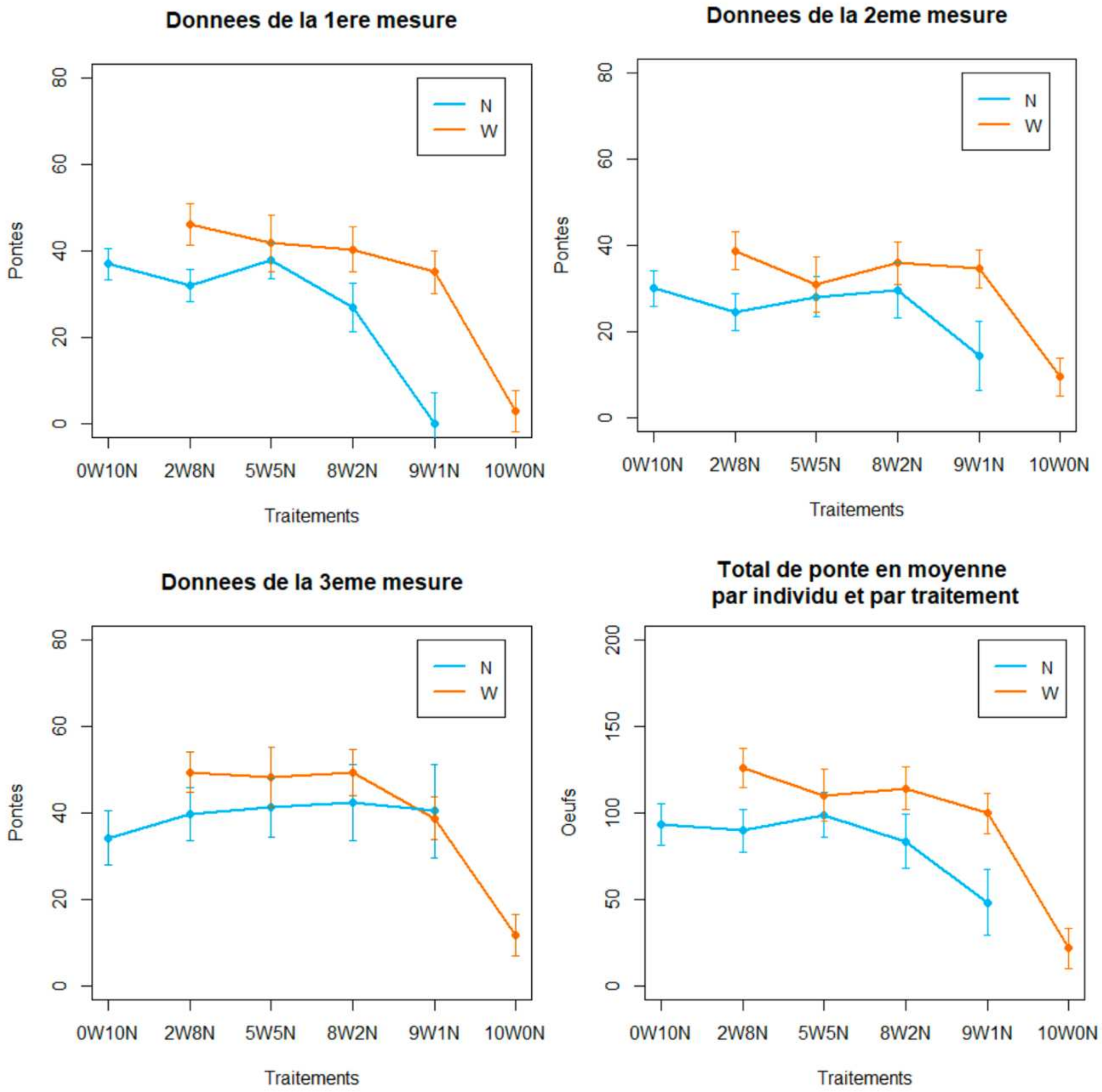
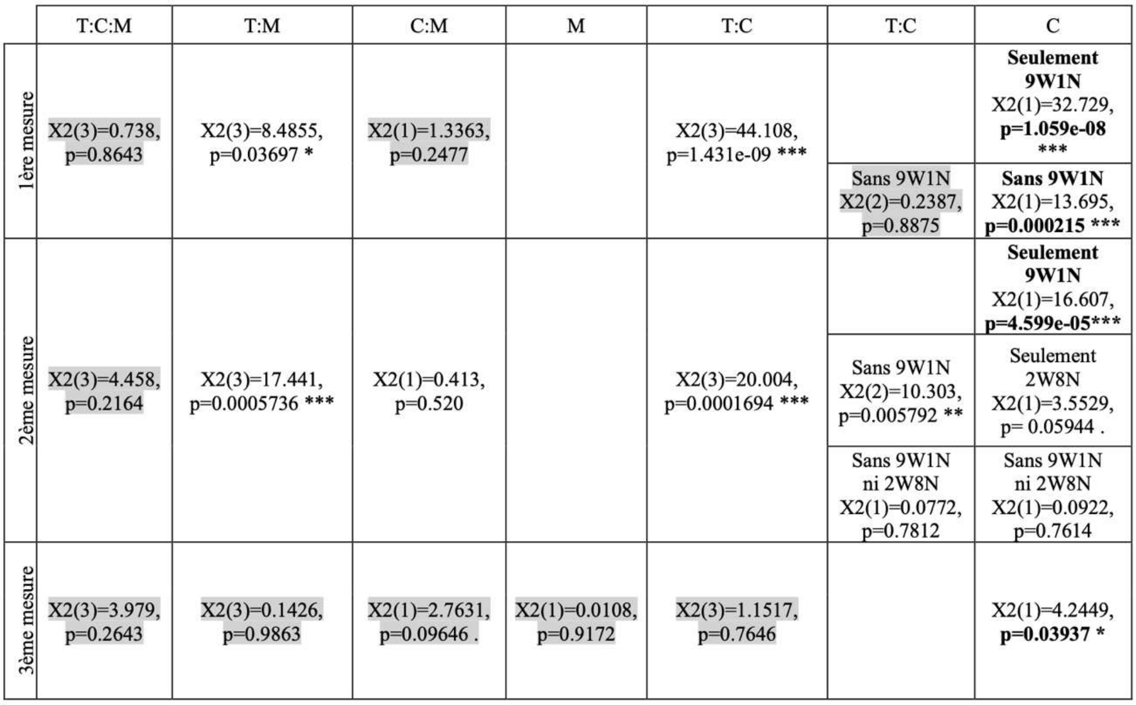


Fig.2 Pontes par session et total cumulé sur 3 sessions.

Tab.3 Analyse des pontes sans traitement 10W0N ni 0W10N. Mêmes conventions que table 1.



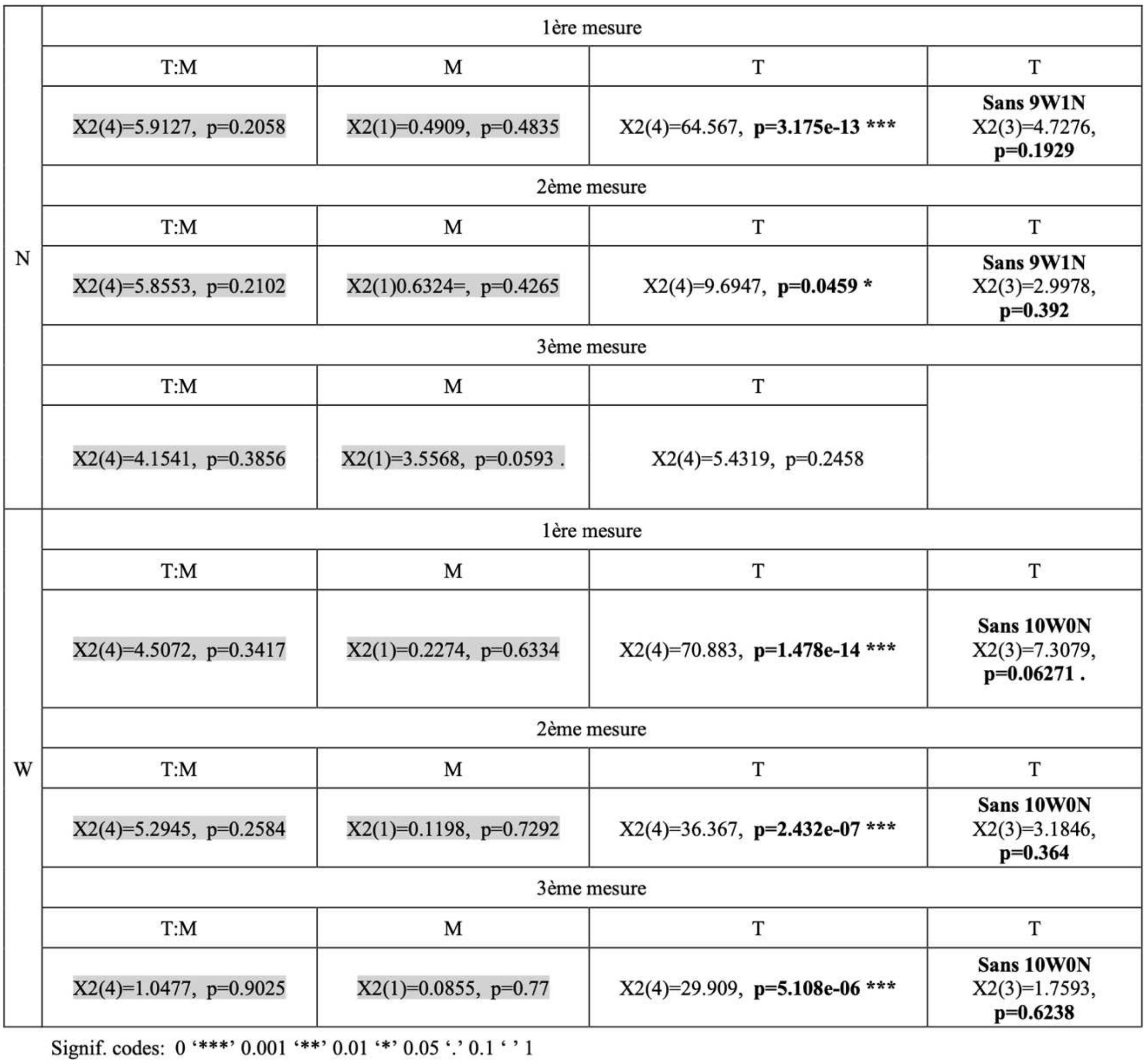
	T:C:M	T:M	C:M	M	T:C	T:C	C
1ère mesure	X2(3)=0.738, p=0.8643	X2(3)=8.4855, p=0.03697 *	X2(1)=1.3363, p=0.2477		X2(3)=44.108, p=1.431e-09 ***		**Seulement 9W1N** X2(1)=32.729, **p=1.059e-08 *****
						Sans 9W1N X2(2)=0.2387, p=0.8875	**Sans 9W1N** X2(1)=13.695, **p=0.000215 *****
2ème mesure	X2(3)=4.458, p=0.2164	X2(3)=17.441, p=0.0005736 ***	X2(1)=0.413, p=0.520		X2(3)=20.004, p=0.0001694 ***		**Seulement 9W1N** X2(1)=16.607, **p=4.599e-05*****
						Sans 9W1N X2(2)=10.303, p=0.005792 **	Seulement 2W8N X2(1)=3.5529, p= 0.05944 .
						Sans 9W1N ni 2W8N X2(1)=0.0772, p=0.7812	Sans 9W1N ni 2W8N X2(1)=0.0922, p=0.7614
3ème mesure	X2(3)=3.979, p=0.2643	X2(3)=0.1426, p=0.9863	X2(1)=2.7631, p=0.09646 .	X2(1)=0.0108, p=0.9172	X2(3)=1.1517, p=0.7646		X2(1)=4.2449, **p=0.03937 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Tab.4 Différence de ponte moyenne par traitement (ponte W – ponte N)

Traitement	1ere mesure	2eme mesure	3eme mesure	Total
2W8N	14.08	14.29	9.58	37.95
5W5N	4.00	2.83	7.04	13.87
8W2N	13.45	6.31	6.99	26.74
9W1N	35.09	20.18	-1.70	53.57
Moyenne	16.66	10.90	5.48	33.04

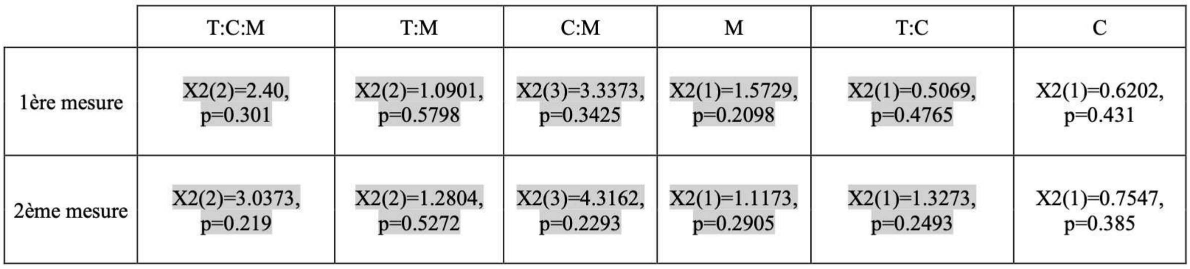
Tab.5 Analyse des pontes avec cytotypes séparés. Mêmes conventions que table 1.



		T:M	M	T	T
N	1ère mesure	X2(4)=5.9127, p=0.2058	X2(1)=0.4909, p=0.4835	X2(4)=64.567, **p=3.175e-13 *** **	**Sans 9W1N** X2(3)=4.7276, **p=0.1929**
	2ème mesure	X2(4)=5.8553, p=0.2102	X2(1)0.6324=, p=0.4265	X2(4)=9.6947, **p=0.0459 * **	**Sans 9W1N** X2(3)=2.9978, **p=0.392**
	3ème mesure	X2(4)=4.1541, p=0.3856	X2(1)=3.5568, p=0.0593 .	X2(4)=5.4319, p=0.2458	
W	1ère mesure	X2(4)=4.5072, p=0.3417	X2(1)=0.2274, p=0.6334	X2(4)=70.883, **p=1.478e-14 *** **	**Sans 10W0N** X2(3)=7.3079, **p=0.06271 .**
	2ème mesure	X2(4)=5.2945, p=0.2584	X2(1)=0.1198, p=0.7292	X2(4)=36.367, **p=2.432e-07 *** **	**Sans 10W0N** X2(3)=3.1846, **p=0.364**
	3ème mesure	X2(4)=1.0477, p=0.9025	X2(1)=0.0855, p=0.77	X2(4)=29.909, **p=5.108e-06 *** **	**Sans 10W0N** X2(3)=1.7593, **p=0.6238**

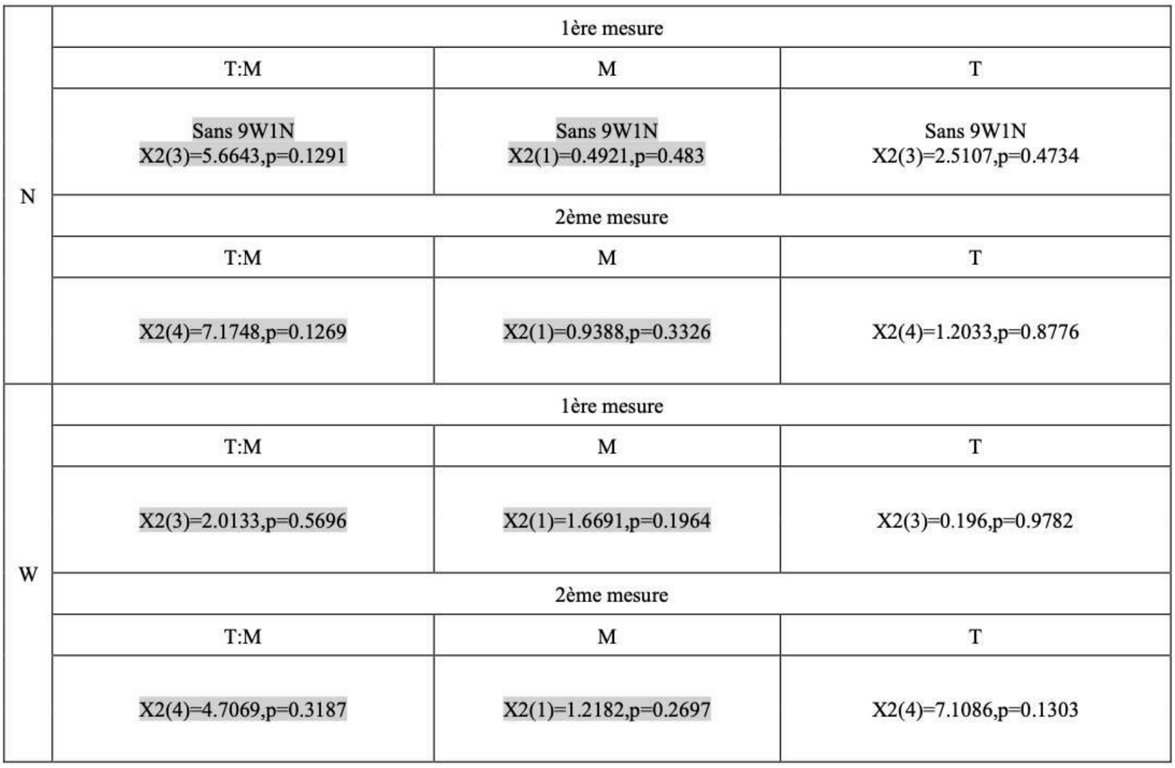
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Tab.6 Analyse de la survie juvénile sans 10W0N ni 10W0N. Mêmes conventions que table 1.



	T:C:M	T:M	C:M	M	T:C	C
1ère mesure	X2(2)=2.40, p=0.301	X2(2)=1.0901, p=0.5798	X2(3)=3.3373, p=0.3425	X2(1)=1.5729, p=0.2098	X2(1)=0.5069, p=0.4765	X2(1)=0.6202, p=0.431
2ème mesure	X2(2)=3.0373, p=0.219	X2(2)=1.2804, p=0.5272	X2(3)=4.3162, p=0.2293	X2(1)=1.1173, p=0.2905	X2(1)=1.3273, p=0.2493	X2(1)=0.7547, p=0.385

Tab.7 Analyse de la survie juvénile avec cytotype séparés. Mêmes conventions que table 1. Dans l'analyse des N, le traitement 9W1N a été retiré de l'analyse lors de la première mesure. Ce retrait était nécessaire car aucun individu n'avait pondu dans ce traitement, il était donc impossible de monter un modèle valide avec les N de ce traitement.

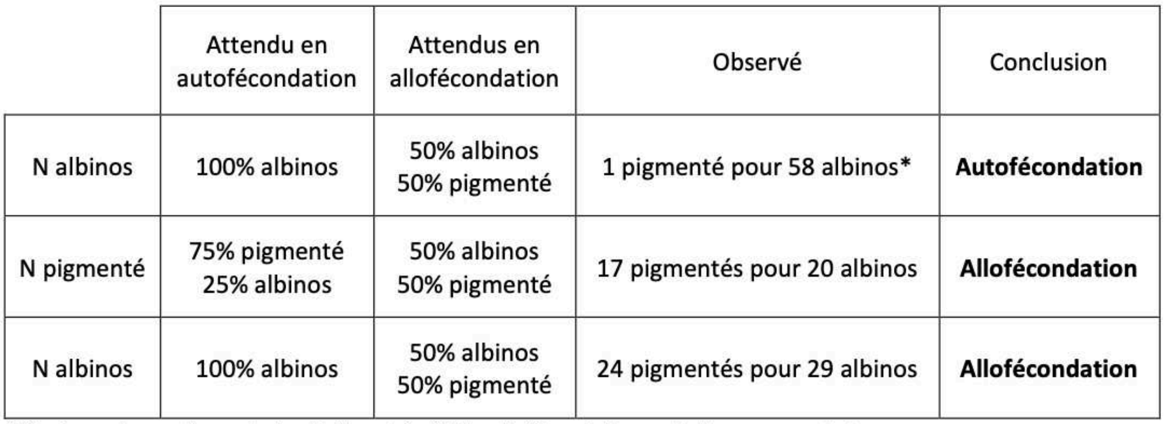


	1ère mesure		
	T:M	M	T
N	Sans 9W1N X2(3)=5.6643,p=0.1291	Sans 9W1N X2(1)=0.4921,p=0.483	Sans 9W1N X2(3)=2.5107,p=0.4734
	2ème mesure		
	T:M	M	T
	X2(4)=7.1748,p=0.1269	X2(1)=0.9388,p=0.3326	X2(4)=1.2033,p=0.8776
	1ère mesure		
	T:M	M	T
W	X2(3)=2.0133,p=0.5696	X2(1)=1.6691,p=0.1964	X2(3)=0.196,p=0.9782
	2ème mesure		
	T:M	M	T
	X2(4)=4.7069,p=0.3187	X2(1)=1.2182,p=0.2697	X2(4)=7.1086,p=0.1303

Tab.8 Individus W issus des traitements 10W0N ayant pondu. La dernière colonne renseigne sur le nombre d'individus albinos ayant obtenu des descendant albinos et pigmentés. 26 individus sur 60 ont pondu, toutes mesures confondues.

		nombre d'individus ayant pondu			Individus allofécondés
	Population	1ère mesure	2ème mesure	3ème mesure	
Bloc 1	VI	2	4	3	**2**
	VIII	3	6	7	**5**
	XVIII	1	0	0	0
Bloc 2	CVI	0	0	0	0
	CXII	0	1	2	0
	CXVIII	3	4	7	**2**

Tab.9 Individus N issus des traitements 9W1N ayant pondu.



	Attendu en autofécondation	Attendus en allofécondation	Observé	Conclusion
N albinos	100% albinos	50% albinos 50% pigmenté	1 pigmenté pour 58 albinos*	**Autofécondation**
N pigmenté	75% pigmenté 25% albinos	50% albinos 50% pigmenté	17 pigmentés pour 20 albinos	**Allofécondation**
N albinos	100% albinos	50% albinos 50% pigmenté	24 pigmentés pour 29 albinos	**Allofécondation**

L'unique pigmenté provient soit d'une très faible allofécondation, soit d'une erreur de typage.

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Figure supplémentaire

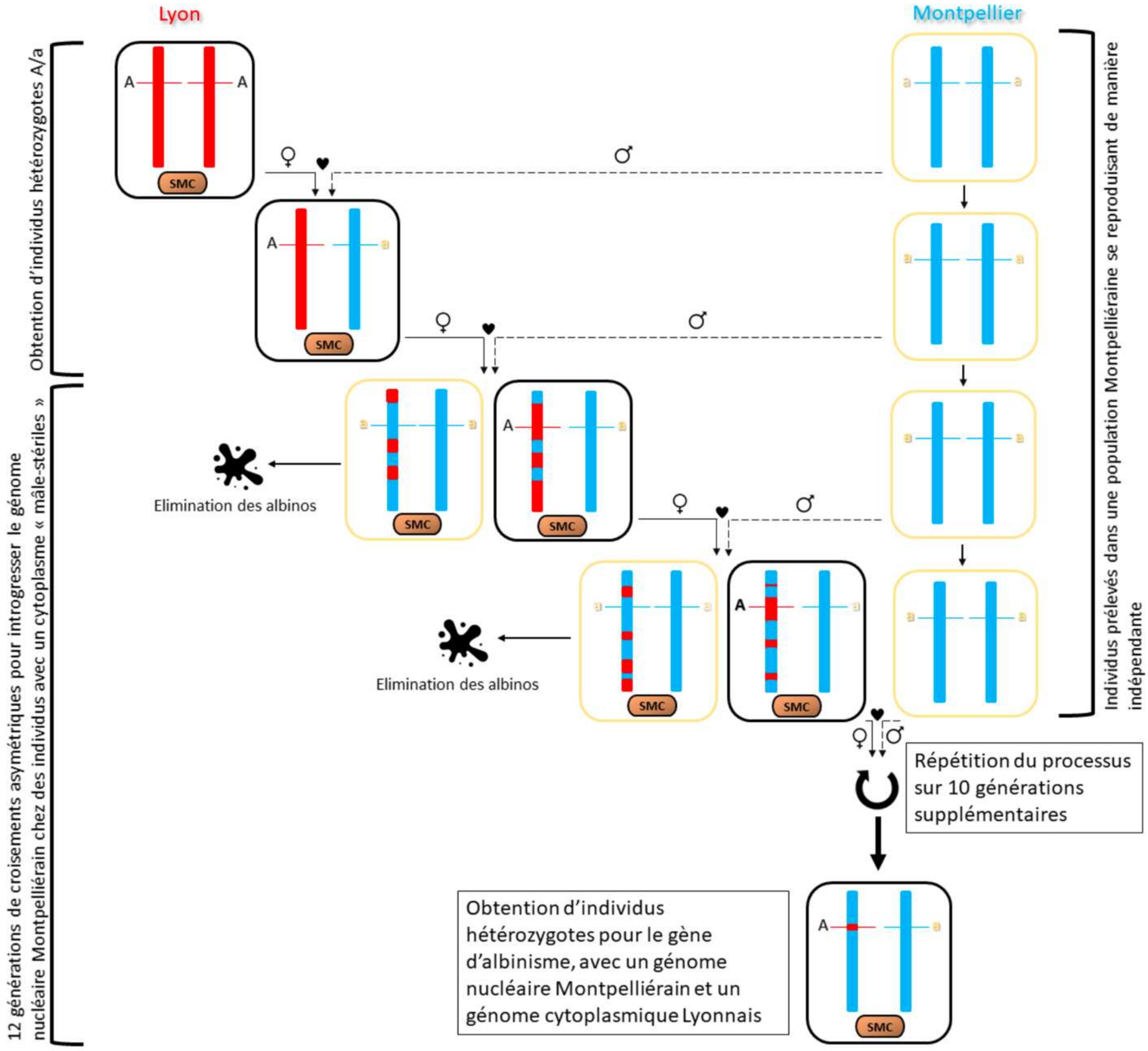

Figure S1 : Introgression.

Deux ensembles d'individus de Lyon ayant un cytoplasme déterminé (W ou N ; on représente ici le cas d'un individu W mâle-stérile, avec sa mitochondrie SMC) ont été inséminés par des escargots albinos de Montpellier. Ce premier croisement a permis d'obtenir des hétérozygotes avec un cytoplasme W (ou N) lyonnais. Seules les pontes des individus Lyonnais sont collectées, de cette manière il est assuré de ne récupérer que des individus ayant hérités des cytoplasmes lyonnais (W ou N). Ces hétérozygotes ont été à nouveau inséminés par des albinos Montpelliérains. Parmi les descendants il a été obtenu des albinos a/a et des pigmentés A/a, seuls les pigmentés ont été conservés. Cette étape est répétée pour un total de 12 générations, de manière à obtenir au fil du temps un génome nucléaire quasi-

Montpelliérain. Il subsistera notamment une portion de génome Lyonnais au niveau du locus du gène de pigmentation, du fait de la sélection et du balayage sélectif qui en résulte (il est considéré que cette portion est trop petite pour que la probabilité de présence d'un gène de restauration de la fonction mâle n'y soit pas négligeable).

ANNEXE 2

POPULATIONS ECHANTILLONNEES

Tableau des populations de *Physa acuta* échantillonnées en France entre 2019 et 2020 par E. Luquet, S. Plénet, P. David (données non plubliées).

Nom de la population	**Dates d'échantillonnage et fréquences des mitoytpes**
Agulla (AGU)	02/07/2020 **98%N 2%K**
Amblérieu (AMB)	02/07/20 **100%N**
Angoulême (ANG)	01/07/2020 **100%N**
Arche (ARC)	03/07/20 **100%N**
Arras-sur-Rhône (ASR)	10/07/20 **87%N, 11%W, 2%K**
Autoroute (AUT)	28/02/2020 **96%N, 4%K**
Ay Satillieu (AYS)	11/08/2020 **96%N, 3%W, 1%K**
Bénovie (BEN)	15/11/2019 **92%N, 8%K**
Braume (BRA)	15/11/2019 **45%N, 55%K**
Barrage de Vaugris (BRV)	30/05/2020 **90%N, 8%W, 2%K**
Bielefeld (BLF)	25/09/2020 **98%N, 2%K**
Botte Sainte-Croix (BSC)	28/06/20 **100%N**
Boistray (BTY)	25/06/2020 **100%N**
Chiova d'Asinu (CDA)	12/06/2020 **100%N**
Cannet des Maures (CDM)	16/08/2020 **77%N, 23%K**
Challans (CHL)	18/09/2020 **100%N**
Chasselay (CLY)	12/01/2020 **100%N**

Chaume (CME)	30/06/2020 **100%N**
Cirad (CIR)	Attention que 18 individus **50%N, 5%W, 44%K**
Codolet (COD)	28/07/2020 **100%N**
Collias (COL)	02/08/2020 **77%N, 23%K**
Crêt 1 (CR1)	16/10/2019 **100%N**
Crêt 2 (CR2)	16/10/2019 **100%N**
Cruas (CRU)	21/07/2020 **93%N, 7%K**
Duchère (DUC)	10/11/2019 **100%N**
Erevan (ERE)	18/10/2019 **68%N, 32%K**
Lovagny Fier (LOF)	25/07/2020 **93%N, 7%K**
Genas (GEN)	24/10/2019 **100%N**
Grange écrasée (GRE)	21/07/2020 **68%N, 28%W, 4%K**
Hermite (HER)	26/10/2019 **100%N**
Irigny (IRI)	28/06/2020 **68%N, 2%W, 30%K**
Irigny sud (IRS)	28/06/2020 **80%N, 20%K**
Ille-sur-Têt (IST)	02/08/2020 **100%N**
Lalande (LAL)	19/06/2020 **48%N, 52%K**
Lauzerte (LAU)	12/09/2020 **100%N**
Ligne à haute tension (LHT)	16/10/2019 **100%N**

Limonest (LIM)	02/11/2019 **97%N, 3%K**
Lignan sur Orb (LSO)	04/09/2020 **29%N, 7%W, 64%K**
Loire-sur-Rhône (LSR)	30/05/2020 **96%N; 4%K**
Lac d'Yzeron (LYZ)	18/05/2020 (7 individus) **100%N**
Mauves (MAU)	10/07/20 **96%N, 4%K**
Montcul (MCL)	21/06/2020 **100%N**
Morlet (MOR)	23/06/2020 (62 individus) **98%N, 2%W**
Orbieu (ORI)	04/09/2020 **72%N, 1%W, 27%K**
Ozon (OZO)	26/06/2020 **100%N**
Etang de la Passe (PAS)	29/06/20 **100%N**
Palau del Vidre (PDV)	13/07/2020 **53%N 46%K**
Pizay (PIZ)	30/10/2019 **100%N**
Pont-Saint-Esprit (PSE)	28/07/2020 **93%N, 5%W, 2%K**
Pont Romain (PTR)	15/11/2019 **100%N**
Quincieux (QCX)	25/06/2020 **75%N, 25%K**
Reart (REA)	11/06/2020 **100%N**
Remoulins (REM)	03/08/2020 **79%N, 1%W, 20%K**
Rizière Figares (RFI)	29/07/2020 **100% N**
Rizière Gageron (RGA)	29/07/2020 **100%N**
Rochemaure (ROC)	21/07/2020 **100%N**

Rizière Saint-Gilles (RSG)	29/07/2020 **99%N, 1%W**
Réunion Divon (RUD)	04/01/2021 **95%N, 5%K**
Réunion Gol (RUG)	12/2020 **45%N, 55%K**
Serrières (SER)	30/05/2020 **88%N, 10%W, 1%K**
Etang du Sepey (SEP)	29/06/20 **100%N**
Salses-le-Château (SLC)	09/07/2020 **100%N**
Saint-Martin-en-Haut (SMH)	18/05/2020 **77%N, 1%W, 22%K**
Salagou (SAL)	**87%N, 13%K**
Sorgues (SOR)	28/07/2020 **84%N, 16%K**
Saint-Priest (STP)	22/10/2019 **100%N**
Thongue (THO)	04/09/2020 **100%N**
Tramoyes (TRA)	26/10/2019 **46%N, 53%K**
Vallabregues (VAL)	28/07/2020 **98%N, 2%K**
Villelongue-del-Monts (VDM)	17/07/2020 **26%N 74%K**
Vernaison (VER)	16/02/2020 **100%N**
Vorla (VOR)	16/10/2019 **100%N**
Verneuil-sur-Seine (VSS)	05/10/2020 **96%N, 4%K**
Castagnon (CAS)	1/09/2020 **100%N**
VVF (VVF)	18/05/2020 **100%N**

ANNEXE 3

AVANTAGE MÂLE CHEZ ESPECE ANDRODIOÏQUE

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Male fertility advantage within and between seasons in the perennial androdioecious plant *Phillyrea angustifolia*

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- **Background and Aims** Androdioecy, the co-occurrence of males and hermaphrodites, is a rare reproductive system. Males can be maintained if they benefit from a higher male fitness than hermaphrodites, referred to as male advantage. Male advantage can emerge from increased fertility owing to resource reallocation. However, empirical studies usually compare sexual phenotypes over a single flowering season, thus ignoring potential cumulative effects over successive seasons in perennials. In this study, we quantify various components of male fertility advantage, both within and between seasons, in the long-lived perennial shrub *Phillyrea angustifolia* (Oleaceae). Although, owing to a peculiar diallelic self-incompatibility system and female sterility mutation strictly associated with a breakdown of incompatibility, males do not need fertility advantage to persist in this species, this advantage remains an important determinant of their equilibrium frequency.
- **Methods** A survey of >1000 full-sib plants allowed us to compare males and hermaphrodites for several components of male fertility. Individuals were characterized for proxies of pollen production and vegetative growth. By analysing maternal progeny, we compared the siring success of males and hermaphrodites. Finally, using a multistate capture–recapture model we assessed, for each sexual morph, how the intensity of flowering in one year impacts next-year growth and reproduction.
- **Key Results** Males benefitted from a greater vegetative growth and flowering intensity. Within one season, males sired twice as many seeds as equidistant, compatible hermaphroditic competitors. In addition, males more often maintained intense flowering over successive years. Finally, investment in male reproductive function appeared to differ between the two incompatibility groups of hermaphrodites.
- **Conclusion** Males, by sparing the cost of female reproduction, have a higher flowering frequency and vegetative growth, both of which contribute to male advantage over an individual lifetime. This suggests that studies analysing sexual phenotypes during only single reproductive periods are likely to provide inadequate estimates of male advantage in perennials.

Key words: Reproductive systems, androdioecy, *Phillyrea angustifolia*, male advantage, perennial plants, flowering rhythmicity, multistate capture–recapture model.

INTRODUCTION

Flowering plants exhibit a remarkable diversity of reproductive systems regarding the distribution of male and female reproductive functions among individuals/flowers within species (Barrett, 2002). Most species are hermaphroditic, but unisexual individuals, either male sterile or female sterile, have evolved within hermaphroditic species in a number of independent lineages (Barrett, 2002). The former reproductive system is known as gynodioecy, the latter as androdioecy. Gynodioecy is relatively common within Angiosperms (found in 2 % of genera and one-third of families; Dufay *et al.*, 2014), whereas functional androdioecy is remarkably rare (Charlesworth, 1984; Vassiliadis, 1999). The rarity of androdioecy has traditionally been explained by highly restrictive conditions for its maintenance. Early theoretical studies have shown that males have to sire more than twice as many offspring as hermaphrodites only to be maintained (Lewis, 1941; Lloyd, 1975). One central parameter in both theoretical and empirical studies of androdioecy is thus the ratio of the number of offspring produced by males over the number of offspring produced by hermaphrodites through their pollen. This ratio, relative to hermaphrodites, is called ‘male advantage’, and in the simplest models its magnitude must exceed two to explain the maintenance of this reproductive system. In these models, the frequency of males at equilibrium should also increase with the magnitude of male advantage (Lewis, 1941; Lloyd, 1975). Although such predictions suggest that a non-negligible frequency of males will be maintained in populations in a very restricted set of conditions, functional androdioecy (co-occurrence of functional hermaphrodites and males) has been described in several perennial species, such as *Tapiscia sinensis* (Zhou *et al.*, 2016), *Laguncularia racemosa* (Landry and Rathcke, 2007), *Cardamine amara* (Tedder *et al.*, 2015), *Ulmus minor* (López-Almansa *et al.*, 2003), *Fraxinus ornus* (Dommée *et al.*, 1999), *Fraxinus lanuginosa* (Ishida and Hiura, 1998), *Datisca glomerata* (Liston *et al.*, 1990) and *Phillyrea angustifolia* (Lepart and Dommée, 1992). This opens the question of how such strong constraints on male advantage predicted by the models can be satisfied in these species.

Among potential sources of male advantage, the most classically cited is reallocation of resources no longer used for female reproduction towards male reproduction (Charlesworth and Charlesworth, 1981). In the following, we take the convention to call this component of male advantage a 'male fertility advantage' as a way to underline that it is not frequency dependent (unlike advantages that might arise from differences in the frequency of compatible mates for example). The male fertility advantage can take several forms, including (1) a higher production (López-Almansa *et al.*, 2003; Tedder *et al.*, 2015; Zhou *et al.*, 2016; Duan *et al.*, 2019) or viability (Zhou *et al.*, 2016) of pollen; (2) a more efficient dispersal of pollen (owing to a higher investment in attractive floral traits and/or traits related to plant architecture, depending on the pollination mode); or (3) an increased competitive ability of pollen grains, i.e. better pollen tube growth and pollen germination on a compatible stigma (Ishida and Hiura, 1998; Zhou *et al.*, 2016; Duan *et al.*, 2019). In perennial plants, a higher flowering frequency and/or a higher individual survival of males relative to hermaphrodites could also contribute to a male advantage over the lifetime of an individual. Given that female function is usually considered as physiologically more costly than male function (Obeso, 2002), one expects a sharper trade-off between reproduction and vegetative growth and/or reproduction the next season in hermaphrodites compared with males, resulting in a higher number of flowering episodes in males. Although reproductive trade-offs have been studied in many perennial and iteroparous species (Renner and Ricklefs, 1995; Sakai, 2001; Ishida and Hiura, 2002; Vamosi *et al.*, 2003; Dufay and Billard, 2012; Zhou *et al.*, 2016; Duan *et al.*, 2019), most of the time fitness was measured over a single flowering episode (but see Penagos Zuluaga *et al.*, 2020). Here, we study how reallocation of resources might impact relative male fertility advantage, both within seasons and integrating over successive seasons in an androdioecious perennial plant: *Phillyrea angustifolia*.

Phillyrea angustifolia (Oleaceae) is a long-lived perennial shrub, in which males co-occur with functional hermaphrodites (producing functional pollen grains; Vassiliadis *et al.*, 2000) within populations, at high frequencies [(0.295–0.775), average male frequency = 0.468; Husse *et al.*, 2013]. We must acknowledge that the *Phillyrea* system deviates from the basic model of evolution of androdioecy cited above (Lewis, 1941; Lloyd, 1975). This species exhibits a diallelic self-incompatibility (DSI) system, which results in two compatibility groups of hermaphrodites, Ha and Hb. Fertilization is not possible within a group of hermaphrodites, but males can fertilize both groups (Saumitou-Laprade *et al.*, 2010). For this reason, and also because of a sex ratio distortion favouring males in crosses between males and Hb individuals (see Materials and methods for more details), a male fertility advantage owing to resource reallocation is no longer necessary to maintain androdioecy in *Phillyrea*: even with a relatively low fertility, males can reach high frequencies (Billiard *et al.*, 2015). However, the magnitude of reallocation-based male fertility advantage remains important to predict male frequencies at equilibrium. Moreover, given that *Phillyrea* is a long-lived perennial, taking several successive seasons into account is crucial to estimate all forms of reallocation during the life history of males and hermaphrodites correctly. As already mentioned, this issue is general to many other androdioecious systems, whether or not they share the peculiar mating system of *Phillyrea*.

In this study, we aimed at measuring possible components of male fertility advantage generated by resource reallocation (on top of the compatibility and the distortion advantages already documented in this species; Fig. 1). By surveying >1000 plants produced by a single controlled crossing experiment (Carré *et al.*, 2021), we were able to compare males (M) and hermaphrodites Ha and Hb, of the same age, in a uniformly dense stand over several flowering episodes.

Our first aim was to compare males and hermaphrodites with respect to several components of male fertility at the scale of one flowering season: pollen number per flower, estimates of flower number and pollen size as it can be related to pollen viability (Kelly *et al.*, 2002). We also compared several traits related to vegetative growth because larger plant size or height can translate into a higher efficiency of pollen dispersal in wind-pollinated plants (Burczyk *et al.*, 2004; Oddou-Muratorio *et al.*, 2005; Fénart *et al.*, 2007; De Cauwer *et al.*, 2010). We predict that males should out-compete hermaphrodites in one or several components of male fertility, owing to resource reallocation. We also expect males to show stronger vegetative growth and hermaphrodites to display a sharper trade-off between reproduction and growth.

Second, we quantified directly the male siring success of males versus hermaphrodites Ha during three consecutive flowering episodes by analysing maternal progenies of open pollinated hermaphrodites Hb. Although we expect males to be more efficient in siring seeds compared with hermaphrodites, the exact magnitude of this difference cannot be predicted because it does not strongly constrain the frequency of males in populations (Billiard *et al.*, 2015).

Third, in order to investigate additional components of male advantage at the scale of several flowering seasons, we compared the flowering rhythmicity of sexual phenotypes over 4 years. Although little used in mating system evolution studies, matrix models from evolutionary demography theory (Caswell, 2000) can be used to tackle this issue using long-term multiseason data in long-lived perennials. To do so, we estimated probabilities of transitions between flowering states using a multistate capture–recapture model, which is part of the family of matrix population models (Caswell, 2000). We expect males to flower more frequently than hermaphrodites because of the female cost of reproduction.

Finally, we compared the two categories of hermaphrodites for their components of male fertility and vegetative growth, because a previous model predicted that evolution towards dioecy might result in the loss of male function in the Ha hermaphrodites and the disappearance of the Hb group (Billiard *et al.*, 2015).

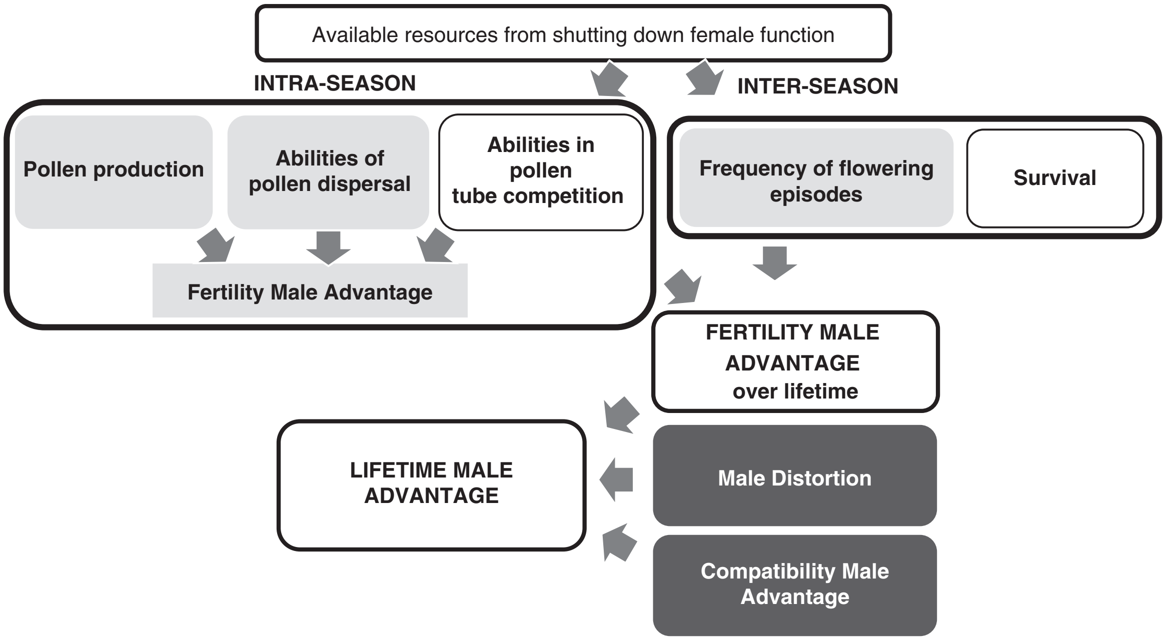

Fig. 1. Components influencing male advantage in perennial plants at the flowering season scale and at the lifetime scale. Light grey boxes represent traits evaluated in this study; dark grey boxes represent male advantage components specific to *Phillyrea angustifolia*. In addition, age at maturity, vegetative growth and individual size were surveyed in the present study because they have the potential to impact pollen production at the scale of one and/or several flowering episodes.

MATERIALS AND METHODS

Species and collection

Phillyrea angustifolia is an anemophilous evergreen shrub that can reach 4 m in height. It possesses a half-buried lignotuber, which contains numerous dormant buds that allow its regeneration after a disturbance (grazing or fire). This particularity confers to the shrub a form of bush whose age is impossible to evaluate from the growth rings of the branches. In males, flowers contain two stamens and an ovary containing four ovules, but the shutdown of stigma production impedes any seed production.

The flowering takes place in March–April and lasts 3–4 weeks within a population. The branches formed during year $n - 1$ carry, in the axil of each leaf, two tight inflorescences of four to nine flowers, which take part in the flowering of year n, in addition to a vegetative bud, which allows the growth of the branch that will ensure the flowering of the year $n + 1$. The growth of this branch begins after the flowering and occurs simultaneously with fruit maturation until the end of summer. A second phase of growth can be observed in autumn, after the fruits have fallen. Although *P. angustifolia* has been described as a seed masting species (Herrera *et al.*, 1998), this was not particularly noticeable in our collection.

The reproductive system is characterized by an original sporophytic self-incompatibility system (homomorphic DSI; Saumitou-Laprade *et al.*, 2010) and a female sterility mutation fully associated with a breakdown of incompatibility (Billiard *et al.*, 2015). Under equal proportions of Ha and Hb individuals, mating opportunities of hermaphrodites are halved, providing a 2-fold advantage of males over hermaphrodites (Saumitou-Laprade *et al.*, 2010). In addition, the segregation distortion of sex ratio occurring between males and Hb hermaphrodites results in 100 % males in progeny, which allows maintenance of >50 % males in populations.

A population of full sib progenies segregating for sex and DSI phenotypes was produced in March 2012 by crossing a hermaphrodite Ha with a male, having the putative genotypes allowing segregation of males, Ha and Hb in the progeny (Billiard *et al.*, 2015; Carré *et al.*, 2021). The sowing was done in September 2013, and the seedlings started to sprout in October 2013. A total of 1065 F1 individuals were transplanted before maturity in April 2014 into an experimental garden (15 rows of 71 plants) within the Plateforme des Terrains d'Expérience du LabEx CeMEB, (CEFE, CNRS) in Montpellier, France. Among these individuals, 613 hermaphrodites and 402 males have been scored (Carré *et al.*, 2021), whereas 50 individuals could not be phenotyped, either because they never flowered or because they died during the test period. A proportion of these individuals was measured for several possible components of fitness, as described below.

In 2018 and 2019, we removed a proportion of the individuals to release competition for space among plants and allow a more efficient individual survey of flowering. In December 2018, we removed 208 individuals [69 M, 73 Ha, 62 Hb and 4 not flowering (NF)] in every other row over the first 12 rows of the experimental garden (see Supplementary Data Table S1). The same was done in December 2019 over the 13 following rows ($n = 211$; 89 M, 66 Ha, 49 Hb and 7 NF).

Measurements and survey

Monitoring of sex, incompatibility group and flowering Every spring from 2015 to 2019, individuals were scored for their intensity of flowering and assigned to one of the following categories: no flowering (no flowers or only a very low number of flowers); medium flowering (occurrence of flowers only on a part of the branches or over the whole individual but at low density); and intense flowering (medium to high density of flowers over the whole individual). At their first episode of flowering, individuals were scored for sexual phenotype and for DSI group. Sexual phenotype was determined visually, based on the presence/absence of stigma. For assignment to DSI groups (Ha or Hb), we used the stigma test previously described by Saumitou-Laprade *et al.* (2010, 2017). A twig bearing inflorescences with at least one open flower was taken from each individual, the open flowers and leaves were removed, and the branch was soaked in a vase containing water and placed under a bell jar to avoid unwanted pollination. After 16 h of maturation at 20 °C, six newly opened flowers were collected, emasculated and placed on an agar medium in a Petri dish. Three flowers were pollinated with Ha pollen and three with Hb pollen previously collected from testers with known phenotype. After 16 h at 20 °C, the pollinated flowers were fixed in formaldehyde, acetic acid and alcohol, then stained with Aniline Blue in order to observe the growth of the pollen tubes by fluorescence microscopy. When several pollen tubes converge through the stigmatic tissue towards the style and, after attrition, one or two tubes cross the style until entrance of the ovary, compatibility is assessed: the sample and the tester belong to two different self-incompatibility (SI) groups. On the contrary, the absence of pollen tubes or the presence of only short pollen tubes growing within the stigma but never reaching the style indicate incompatibility: the sample and the tester belong to the same SI groups.

Pollen production and size In spring 2016, pollen production at the flower level was measured for 98 individuals (34 M, 32 Ha and 32 Hb). Individuals were chosen randomly over the whole parcel among flowering individuals. Within inflorescences with >10 % of open flowers, 15 mature floral buds were collected before stamen dehiscence, immediately fixed in 96 % ethanol and stored until use. For the counting experiment, two stamens per bud were collected, stored separately in two Eppendorf tubes and placed in an oven at 64 °C for 10 min to evaporate ethanol traces and force dehiscence. One millilitre of distilled water was then added, and dehisced stamens in water were sonicated for 5–10 min in an ultrasonic bath before analysis with a particle counter (CASY_model TT) following Dufaÿ *et al.* (2008). Particles of size ranging from 13.5 and 25.2 µm were retained and summed to obtain an estimate of the total number of pollen grains per stamen. For each plant surveyed, we obtained an average number of pollen grains per stamen, in addition to the average size of pollen grains. Variances in the number of pollen grains and pollen grain size were also calculated for each individual.

Plant size and vegetative growth In autumn 2016, 26 hermaphrodites (9 Ha and 17 Hb) and 19 males were surveyed for estimation of vegetative growth in 2016. Selection of surveyed plants was done among plants that either did not flower ($n = 17$) or flowered intensively ($n = 28$) during spring 2016, in order to assess the possible impact of flowering cost on vegetative growth. All hermaphrodites that had flowered during this year were bearing fruits. In November 2016, at the end of the vegetative growth phase, five stems per plant were collected. The biomass produced in 2016 was weighed, and for each subsample, the cumulated length of shoots and whorls and the stem diameter were measured.

In addition, in order to assess possible sex differences in cumulated vegetative growth after several years, we performed three measurements of vegetative biomass. In December 2018, on all phenotypes for sex and SI group that had been removed from the experimental garden ($n = 204$; 69 M, 73 Ha and 62 Hb), we harvested the lignotuber and the aerial part of the individuals by cutting the roots at the tuber level, and we measured the total mass, in addition to the mass of the tuber alone. In spring 2019, we measured the maximum height and the crown of all remaining living individuals ($n = 811$; 336 M, 240 Ha and 235 Hb). Finally, in December 2019, we measured total mass and mass of tuber alone on the second group of individuals removed from the collection ($n = 204$; 89 M, 66 Ha and 49 Hb), using the same protocol as in 2018.

Male fertility advantage at the scale of a flowering episode At the scale of one flowering season, we define male fertility advantage as the ratio of average siring success of males over the average siring success of compatible hermaphrodites. The segregation distortion that operates in crossings between males and Hb hermaphrodites offers the opportunity to quantify the male fertility advantage of males relative to Ha hermaphrodites. Only Ha and males can sire seeds on Hb individuals, and owing to segregation distortion, all offspring sired by males will be male, whereas crosses between Ha and Hb produce hermaphrodites exclusively. A proxy of male advantage at the scale of one flowering season can thus be calculated based on the frequency of males in the progeny of an Hb mother and the frequency of males among the potential mating partners of the same mother plant. Maternal progenies of Hb individuals were collected in summer 2017 ($n = 24$), 2018 ($n = 21$) and 2019 ($n = 25$). For each of these three years, the choice of Hb mothers was limited by the flowering intensity. In order to have enough fruits, only the individuals that had flowered intensively were chosen. Moreover, the choice of the individuals was constrained by their location on the experimental parcel, because we wanted plants to be distributed evenly. Individuals located at the edge of the parcel and therefore having fewer potential fathers around them were avoided. As a result of these constraints, some mothers were selected only one year, whereas others were used for two or three seasons (Supplementary Data Table S2). An average of 45 seeds were collected per mother plant, and two sex-specific single nucleotide polymorphisms were used on DNA extracted from embryos to assess the proportion of males in each maternal progeny (Saumitou-Laprade *et al.*, in prep.). Both single nucleotide polymorphisms co-localize with the recently mapped sex on chromosome 12 in *P. angustifolia* (Carré *et al.* 2021) and are fully linked to the male phenotype of individuals in the collections tested so far (from Cadiz in Spain to Camargue in France).

The frequency of males among potential mating partners [M/(M + Ha)] represents the expected sex ratio in the seeds of Hb hermaphrodites under the hypothesis of equality of male fertility of M and Ha, i.e. no fertility advantage; it was assessed based on the frequency of blooming males and hermaphrodites Ha around each considered mother plant, at different spatial scales (see description of analyses below). The realized male fertility advantage was then assessed as the ratio of observed over expected male frequencies in Hb seeds. Note that unfortunately this methodology does not allow us to compare male fertility between Hb and the other two morphs, because the absence of distortion in male × Ha crosses prevents us from using progeny sex ratio as a marker of paternity.

Data analyses

Given that environmental variation occurring within our common garden might lead to spatial variation in both vegetative and reproductive traits, we first ran statistical analyses with a random effect of spatial distribution by coding each individual with its spatial coordinates (rows and columns). All statistical analyses were conducted in RStudio v.1.1.453 (R Core Team, 2018). A random spatial effect was included in the *Matern* function of the *spaMM* package (Rousset and Ferdy, 2014; Supplementary Data Table S3). Given that no significant spatial effect was detected (Supplementary Data Table S4), we performed common linear or multinomial models using the *lm* function from the *stats* package (R Core Team, 2018) and the *multinom* function from the *nnet* package (Venables *et al.*, 2002). The validity of the linear regression model was checked with a function from the *lmtest* package (Zeileis and Hothorn, 2002). We ran three groups of analyses: (1) potential components of male fertility advantage (pollen and vegetative traits at the scale of the flowering episode); (2) estimation of the overall male fertility advantage at the scale of the flowering episode; and (3) comparison of frequency and intensity of flowering among sexual phenotypes, in order to improve our understanding of male fertility at the scale of an individual lifetime.

Flowering intensity and pollen production The age at maturity was investigated for the three sexual phenotypes with a multinomial model. Regarding flowering intensity, we compared the record of flowering (no/medium/intense) between the three sexual phenotypes for each year separately, by using a multinomial model. Given that only a few individuals bloomed in 2015 ($n = 10$), analyses of flowering intensity were conducted from 2016 to 2019 only, by doing pairwise tests.

Linear models were used to analyse pollen traits (average number of grains per stamen and average size of grains), by testing for an effect of sexual phenotype (hermaphrodite Ha, hermaphrodite Hb or male) and flowering intensity recorded the same year (medium vs. intense flowering). Linear models were also used to study the relationship between variance in pollen traits and the sexual phenotype.

Plant size and vegetative growth Regarding plant size, we first ran Spearman tests to analyse correlations between (1) plant weight and tuber weight and (2) plant height and plant diameter. Following this, we ran linear models to analyse plant height and plant weight, by testing for an effect of plant sexual phenotype. Given that plant weight was measured during two consecutive years, we also tested for an effect of plant age. Regarding annual vegetative growth, we initially analysed correlations among measured traits, applying a Spearman test and correcting for multiple comparisons (Holm–Bonferroni). Given that all measured traits were highly correlated, we focused on the average biomass produced per stem, by running linear models with plant group and flowering intensity the previous spring as explanatory factors (comparing plants that did not flower the previous year with the ones that flowered intensively). Significance of the main factors and their interaction was analysed by running analyses of variance on the results of the models. Normality of model residuals was checked with a Shapiro test, homoscedasticity with the Breush–Pagan test and autocorrelation with the Durbin–Watson test. Tukey's HSD tests were run for post-hoc analyses.

Frequency and intensity of flowering over several flowering episodes In order to detect whether the flowering intensity recorded in one year affected flowering patterns the following year, and whether such rhythmicity varied between sexual phenotypes, we applied multistate capture–recapture models. Such models provide estimates of the probability for an individual to go from a flowering intensity state at year 'y' to a flowering intensity state at year y + 1 (transition probability, φ). We performed transition probability estimations using the R package 'RMark' (Laake and Rexstad, 2008), which implements the program MARK (White and Burnham, 1999). Death rate was low (<3 %); therefore, we removed the dead individuals and used a fixed survival probability of $S = 1$. Given that flowering intensity was assessed for each individual, we used a fixed detection probability of $P = 1$. We then fitted the models using different combinations of the three factors: sexual phenotype, year and flowering intensity, and their interaction. Models were ranked using Akaike's information criterion (AIC; Akaike, 1973). We performed these models on individuals that had been kept alive on the parcel until 2019 ($n = 809$) and for three transitions among years (2016 to 2017, 2017 to 2018, and 2018 to 2019).

Male fertility advantage at the scale of a flowering episode In order to estimate and analyse a fertility male advantage (ratio of male fitness of males over male fitness of hermaphrodites) on each Hb maternal progeny i, we used the observed frequency of male offspring (mo_i) and the frequency of the two types of potential fathers (M_i, frequency of bloomed males; and Ha_i, frequency of bloomed hermaphrodites Ha around the mother i, with the sum of these two frequencies being equal to one). The average siring success of potential male mating partners around the mother i is thus mo_i/M_i, and the average siring success of potential Ha hermaphrodites mating partners around the same mother is $(1 - mo_i)/Ha_i$. This defines the fertility male advantage estimated on mother plant i as:

$$\propto_i = \frac{mo_i \times Ha_i}{M_i\,(1 - mo_i)}$$

Notably, the frequencies M_i and Ha_i that need to be used in this estimate depend on the spatial scale at which mating events occur. As a first step, we thus defined the spatial scale at which the frequency of blooming males around mother plants best fitted male offspring frequency in their progeny. We considered squares around mother plants of increasing sizes, from a radius of one individual around the mother to the whole parcel. For each scale, three general linear models of male offspring proportion were run, the first including only the frequency of blooming males (hereafter, Males model), the second testing for additional effects of the frequency of intensively and moderately flowered individuals among males and the frequency of intensively hermaphrodites Ha (All freq model), and the third being a null model (Null model) including all flowered individuals (Ha + M) regardless of their flowering intensity. The rationale for testing the All freq model was to test whether an overall larger pollen production in males contributed to their siring success. Models were then ranked by AIC. The normality of model residuals was checked with a Shapiro test, homoscedasticity with the Breush–Pagan test and autocorrelation with the Durbin–Watson test. As a second step, we calculated the male fertility advantage for each maternal lineage (α_i) at the scale that was defined as more suitable for our data. We averaged all values of α_i to obtain an estimate of the male fertility advantage over one flowering season ($\bar{\alpha}$).

RESULTS

Flowering intensity and pollen production

Individuals started to flower in 2015, but most of them reached maturity in 2016. None of the sexual phenotypes was more precocious than the others (Fig. 2). From 2017, nearly half of the males flowered intensely each year, whereas only one-quarter or less of the hermaphrodites were found in this category (Fig. 3). This difference was statistically significant between males and both SI phenotypes of hermaphrodites in 2017, 2018 and 2019. The intra-individual variances for both number and size of pollen grains were not impacted by the sexual phenotype ($P = 0.21$ and $P = 0.54$, respectively).

The number of pollen grains per stamen was significantly different between sexual phenotypes and varied positively with flowering intensity (Fig. 4A; Table 1). Hermaphrodites Ha produced significantly fewer pollen grains than Hb ($P < 0.05$) and marginally fewer pollen grains than males ($P = 0.07$). No significant difference was found between males and Hb ($P = 0.96$). Individuals with intense flowering produced more pollen grains per stamen than individuals with a medium flowering intensity ($P < 0.01$), with this effect being similar for all sexual phenotypes (no significant interaction between sexual phenotype and flowering intensity, $P > 0.05$).

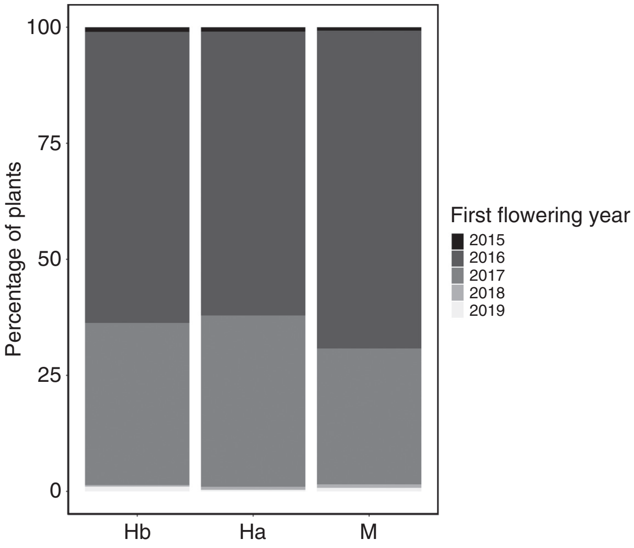

Fig. 2. Distribution of the first flowering year for each sexual phenotype (Ha, Hb and M). Differences among sexual phenotypes were not statistically significant.

Similar effects were found on the size of pollen grains, with their average size depending on both sexual phenotype and flowering intensity, with no significant effect of interaction (Fig. 4B; Table 1). Hermaphrodites Ha had smaller pollen grains than both males and hermaphrodites Hb ($P < 0.0001$). Plants with intense flowering had larger pollen grains than medium flowering plants ($P < 0.05$).

Plant size and vegetative growth

Shoot biomass was significantly different between sexual phenotypes (Table 1), with males producing significantly heavier shoots than hermaphrodites Ha and Hb. Shoot biomass also depended on the interaction between sexual phenotype and flowering intensity: among plants that flowered intensively the previous spring, only hermaphrodites showed a decrease in vegetative growth (Fig. 5).

Tuber weight was highly correlated with the total weight of plants in both 2018 ($R^2 = 0.86$, $t = 23.167$, d.f. = 198, $P < 0.001$) and 2019 ($R^2 = 0.79$, $t = 19.142$, d.f. = 209, $P < 0.001$) for the three sexual phenotypes. The total weight depended on plant age and on sexual phenotype (Table 1; Fig. 6), with higher values observed for both years in males compared with hermaphrodites Ha and Hb. Finally, maximum plant height was correlated with plant crown in 2019 ($R^2 = 0.50$, $t = 16.44$, d.f. = 808, $P < 0.001$). Again, plant height was significantly different between sexual phenotypes (Table 1), with males and hermaphrodites Ha being larger than hermaphrodites Hb ($P < 0.001$ and $P < 0.05$, respectively).

Frequency and intensity of flowering over several flowering episodes

Based on the AIC, the best-fitting model of flowering rhythmicity included interactions between sexual phenotype, year ‘y’ and flowering intensity state at year ‘y’ (Table 2). We thus obtained three matrices per sexual phenotype, one for each transition between two consecutive years (Table 3). Overall, males had a greater probability than hermaphrodites to remain in an intense flowering state (IF) for two consecutive years (Table 3). This probability was at least three times higher in males compared with hermaphrodites Ha and about two times higher compared with hermaphrodites Hb.

Male fertility advantage at the scale of a flowering episode

The average proportion of male offspring within Hb maternal progenies was 0.65, 0.77 and 0.70 in 2017, 2018 and 2019, respectively. For each year, the best model was the one that included an effect of male frequency around the

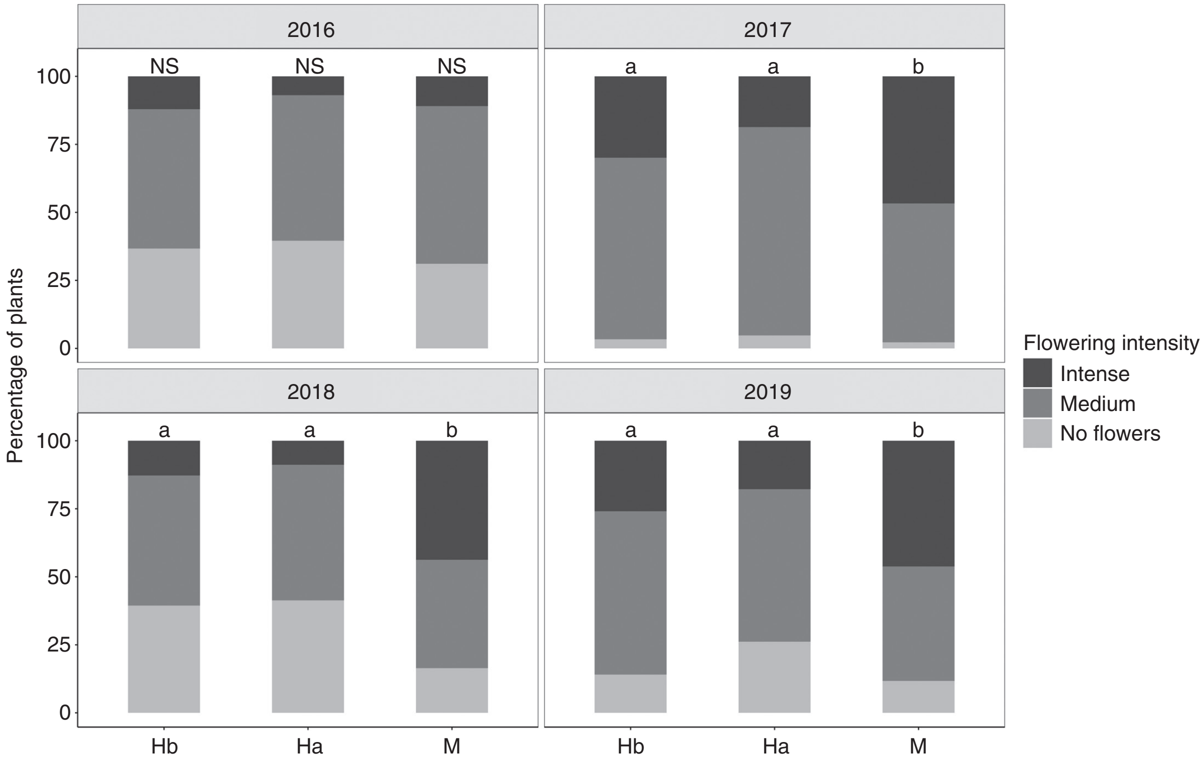

FIG. 3. Relative frequencies of the three categories of flowering intensity (intense flowering, medium flowering and no flowering) from 2016 to 2019 for each sexual phenotype (Ha, Hb and M). NS: non-significant differences among sexual phenotypes. Groups a and b show significant differences from pairwise comparisons run from multinomial models.

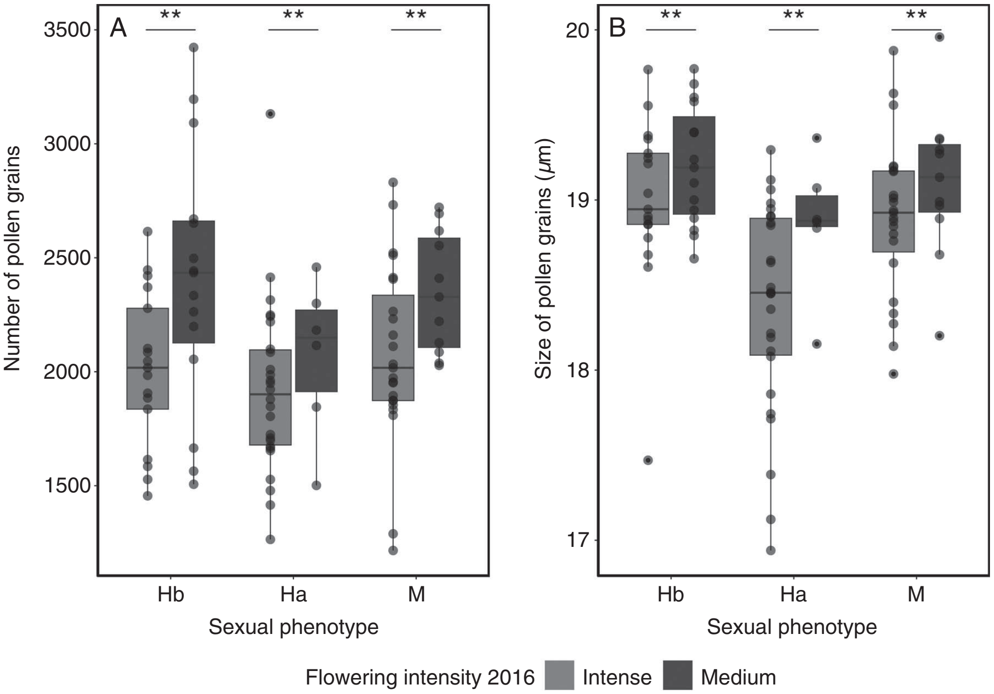

FIG. 4. Boxplots of both the average number of pollen grains per stamen (A) and the average size of pollen grains (B) for each sexual phenotype (Hb, Ha and M). Differences between sexual phenotypes in the average number and size of pollen grains were analysed for plants that either had a medium flowering intensity or that had flowered intensively. **Significant differences with $P < 0.01$.

Table 1. *Effect of sexual phenotype, flowering intensity and plant age on the different traits. For pollen traits and shoot biomass, linear models included the effects of sexual phenotype (Ha, Hb and males), flowering intensity (no/medium/intense) and their interaction. For the plant weight, we considered an effect of plant age, the sexual phenotype and their interaction. For the plant height, the linear model included only an effect of sexual phenotype. The number of degrees of freedom (d.f.), Fisher's statistic* (F) *and the* P*-value from the linear models are reported. Significant effects are in bold.*

Traits	Factors	Linear regression		
		d.f.	***F***	***P*-value**
Number of pollen grains per stamen in 2016	Sexual phenotype	**2/95**	**3.61**	**<0.05**
	Flowering intensity in 2016	**1/95**	**10.50**	**<0.01**
	Sexual phenotype × flowering intensity in 2016	2/95	0.56	0.57
Average size of pollen grains in 2016	Sexual phenotype	**2/95**	**13.86**	**<0.001**
	Flowering intensity in 2016	**1/95**	**6.19**	**<0.05**
	Sexual phenotype × flowering intensity in 2016	2/95	0.57	0.57
Shoot biomass in 2016	Sexual phenotype	**2/38**	**8.13**	**<0.01**
	Flowering intensity in 2016	1/38	3.30	0.07
	Sexual phenotype × flowering intensity in 2016	**2/38**	**5.32**	**<0.01**
Weight in 2018 and 2019	Sexual phenotype	**2/402**	**11.29**	**<0.001**
	Year	**1/402**	**10.45**	**<0.01**
	Sexual phenotype × year	2/402	1.20	0.30
Height in 2019	Sexual phenotype	2/805	12.06	**<0.001**

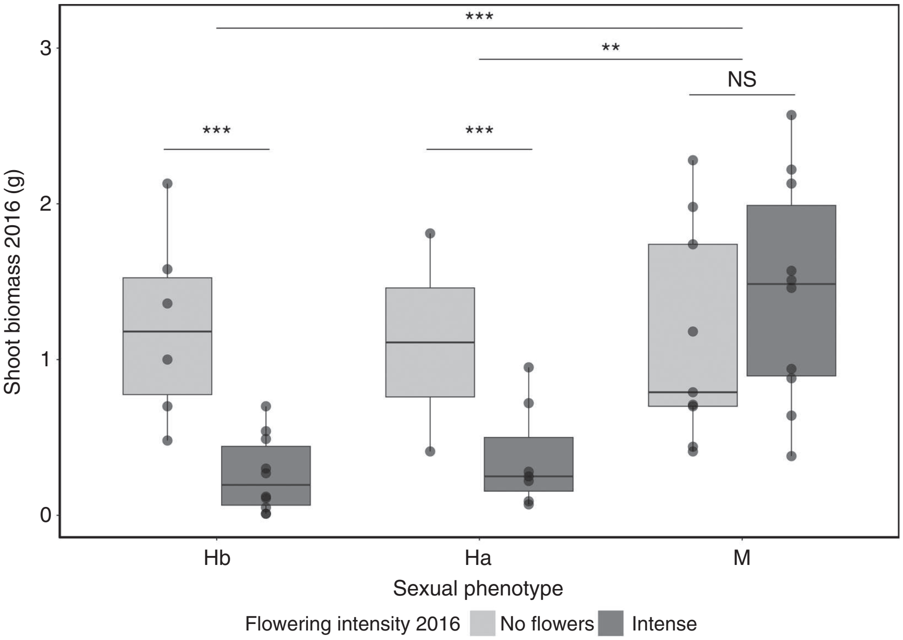

Fig. 5. Shoot biomass produced in 2016 on plants that either did not produce any flower during spring or plants that flowered intensively. Individuals with medium flowering were not surveyed for this analysis. NS: non-significant differences among the two categories of flowering intensity. ***Significant differences with $P < 0.001$. **Significant differences with $P < 0.01$.

mother at the lowest spatial scale (scale 1; Table 4). Note, however, that the proportion of males around the mother had a significant effect at scale 1 in 2018 (slope of the linear regression = 0.12, $P < 0.001$) and in 2019 (slope = 0.05, $P = 0.05$), but this was only marginal in 2017 (slope = 0.07, $P = 0.09$).

To compare with expectations if no fertility male advantage occurred, we calculated an expected ratio of male offspring based on the frequencies of compatible flowering individuals at the closest neighbourhood around each surveyed mother (scale 1). For all three years, we found a significant excess of male offspring in progenies compared with expectations ($P < 0.001$;

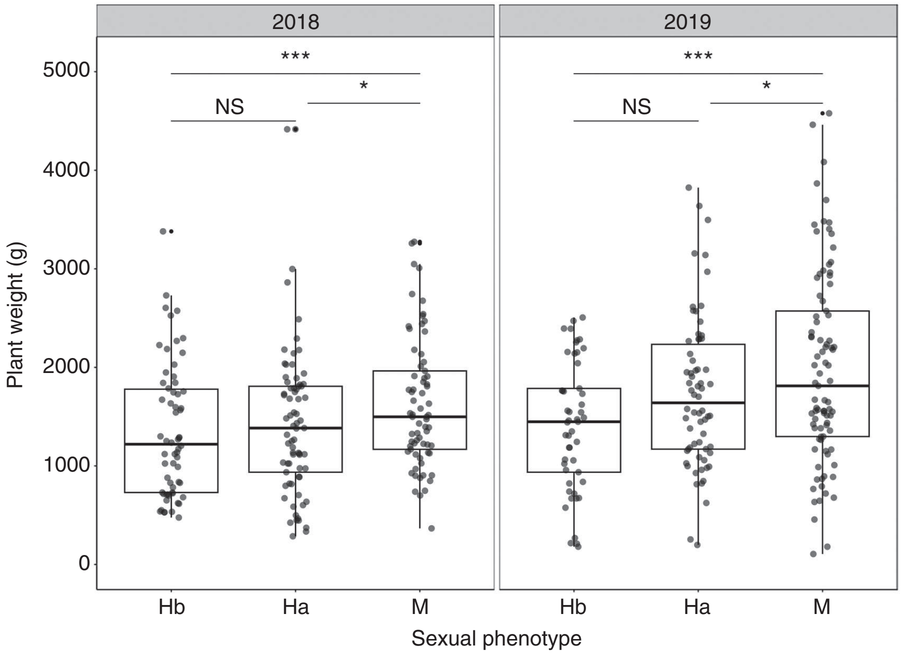

FIG. 6. Biomass weight of hermaphrodites (Ha and Hb) and males (M) in 2018 and 2019. Male biomass is higher than hermaphrodite biomass Ha and Hb. NS: non-significant differences among the two categories of flowering intensity. ***Significant differences with $P < 0.001$. *Significant differences with $P < 0.05$.

TABLE 2. *Models used to estimate transition probabilities between the three flowering intensity states across consecutive years (φ) ranked by Akaike information criterion (AIC) values. These models used combinations and interaction of the factors sexual phenotype (Ha, Hb and M), year 'y' (from 2016 to 2019) and flowering intensity state at year 'y' (no flowers, medium, intense).*

Models	AIC
φ ~ flowering intensity state at year 'y'	4546.21
φ ~ flowering intensity state at year 'y' + sexual phenotype + year 'y'	4509.25
φ ~ flowering intensity state at year 'y' × sexual phenotype	4407.15
φ ~ flowering intensity state at year 'y' × year 'y'	4216.36
φ ~ flowering intensity state at year 'y' × sexual phenotype × year 'y'	4107.49

Fig. 7; average expected male ratios: 0.56, 0.65 and 0.58 in 2017, 2018 and 2019, respectively). Consistently, average male fertility advantage (males relative to Ha) reached values around or even higher than two: 2017, $\bar{\alpha} = 2.46$ [95 % confidence interval (CI) = 0.998, 3.924]; 2018, $\bar{\alpha} = 1.98$ (95 % CI = 0.749, 3.209); and 2019, $\bar{\alpha} = 2.23$ (95 % CI = 1.041, 3.426).

DISCUSSION

Our study revealed several differences between sexual phenotypes in terms of flowering, vegetative growth patterns and realized male reproductive success. Below, we discuss these results, attempting to analyse the proximal causes of male fertility advantage (Fig. 1). Initially, we discuss the differences between males and hermaphrodites that might ultimately lead to differences in male fitness. Then we discuss implications and limits of our estimated difference in realized male fitness between males and hermaphrodites. Finally, because our study also revealed differences between the two categories of hermaphrodites, we discuss the possible implications for the evolution of reproductive systems.

Sex-specific variation in flowering intensity and pollen production: exploring across-season trade-offs

In several androdioecious species, males have been found to produce more pollen than hermaphrodites, either by increasing their production of pollen per flower (*Tapiscia sinensis*: Zhou *et al.*, 2016; *Osmanthus delavayi*: Duan *et al.*, 2019) or by increasing their total number of flowers (*Laguncularia racemosa*: Landry and Rathcke, 2007; *Cardamine amara*: Tedder *et al.*, 2015). In *P. angustifolia*, males showed equivalent pollen production per stamen and pollen size compared with one of the two DSI groups of hermaphrodites, suggesting that the shutdown of seed and fruit production does not necessarily translate into a larger investment in male function at the flower level. Because this species produces very high densities of small flowers, we could not estimate with precision the number of flowers produced. However, our semi-quantitative estimation of flowering intensity suggests that males produced more flowers than both categories of hermaphrodites, which should result in an increase of pollen production at the plant level. Such an effect was not detected for the first flowering episode, but seemed to be stable over the three following seasons.

Interestingly, our results showed that when hermaphrodites produced a large number of flowers (intense flowering), this had several negative impacts on following episodes of growth and flowering, whereas such effects were absent or, at least,

Table 3. *Transition matrices of flowering intensity states for each transition among years. Each matrix contained six transition probabilities obtained by RMark. Transition probabilities from a flowering intensity state at year 'y' to a flowering intensity state at year 'y + 1' read in column. For example, a hermaphrodite Hb in a non-flowering (NF) state in 2016 had a probability of 0.07 to remain in a an NF state in 2017. Transition probability to switch from an NF state in 2016 to a medium flowering (MF) state in 2017 is 0.74. Transition probability to switch from NF to an intense flowering (IF) state is 0.19.*

Sexual phenotype	Transition		
	2016–2017	2017–2018	2018–2019
Hb	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.07 & 0.18 & 0.05 \\ 0.74 & 0.52 & 0.50 \\ 0.19 & 0.30 & 0.45 \end{pmatrix} \end{array}$	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.60 & 0.38 & 0.32 \\ 0.20 & 0.53 & 0.51 \\ 0.20 & 0.09 & 0.17 \end{pmatrix} \end{array}$	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.29 & 0.07 & 0.05 \\ 0.58 & 0.64 & 0.46 \\ 0.13 & 0.29 & 0.49 \end{pmatrix} \end{array}$
Ha	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.06 & 0.01 & 0.04 \\ 0.89 & 0.69 & 0.68 \\ 0.05 & 0.30 & 0.28 \end{pmatrix} \end{array}$	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.67 & 0.37 & 0.27 \\ 0.33 & 0.54 & 0.54 \\ 0 & 0.09 & 0.19 \end{pmatrix} \end{array}$	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.67 & 0.37 & 0.27 \\ 0.33 & 0.54 & 0.54 \\ 0 & 0.09 & 0.19 \end{pmatrix} \end{array}$
M	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.07 & 0.05 & 0 \\ 0.72 & 0.495 & 0.16 \\ 0.21 & 0.50 & 0.84 \end{pmatrix} \end{array}$	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.63 & 0.20 & 0.06 \\ 0.37 & 0.48 & 0.31 \\ 0 & 0.32 & 0.63 \end{pmatrix} \end{array}$	$\begin{array}{cc} & \begin{array}{ccc} NF & MF & IF \end{array} \\ \begin{array}{c} NF \\ MF \\ IF \end{array} & \begin{pmatrix} 0.38 & 0.14 & 0.007 \\ 0.56 & 0.53 & 0.28 \\ 0.06 & 0.33 & 0.713 \end{pmatrix} \end{array}$

Table 4. *Akaike information criteria of the linear models used to analyse the proportion of male offspring at different scales. Levels of scale were defined by adding individuals in the group of potential fathers, with increased radius around the mother. Scale 1 is the lowest spatial scale, in which potential fathers belonged to the eight individuals surrounding the mother. In scale 2, potential fathers belonged to the group of eight from scale 1 plus 16 additional individuals. Scale 3 included individuals from scales 1 and 2 plus 24 additional individuals. Scale 4 included individuals from scales 1–3 plus 32 additional individuals. 'Males model' includes the frequency of only blooming males at a given scale. 'All freq model' includes additional effects of frequency of intensively flowering (IF) and moderately flowering (MF) individuals among males and the frequency of intensively flowering (IF) hermaphrodites Ha. The null model includes all flowering individuals regardless of their flowering intensity. The selected model is highlighted in bold.*

	Year 2017			Year 2018			Year 2019		
Scale	Males model	All freq model	Null model	Males model	All freq model	Null model	Males model	All freq model	Null model
1	**173.27**	175.39	174.19	**151.58**	153.46	163.23	**158.41**	161.67	163.23
2	173.44	174.88	174.19	165.09	167.37	163.23	165.00	165.44	163.23
3	175.93	179.07	174.19	165.21	168.65	163.23	177.11	178.72	163.23
4	176.15	179.84	174.19	165.10	163.83	163.23	178.38	177.84	163.23

not visible in males. Hermaphrodites that had flowered intensively during spring 2016 showed a reduced ability to produce new shoots until the following autumn season. Trade-offs between growth and reproduction have been shown in several long-lived perennial plant species (e.g. Humanes *et al.*, 2011; Hossain *et al.*, 2017), and our results confirm the general view that such a trade-off can be driven mainly by the cost of female reproductive function. In our species, seed maturation and stem growth occur in the same period of time and should be in direct competition, which explains why males seem largely to escape this cost of reproduction.

Although this sex-specific trade-off was investigated on only one year, the fact that males had reached a larger size and weight than hermaphrodites after 4 or 5 years suggests that this occurs repeatedly over time, as suggested by Pannell and Ojeda (2000). This trade-off also explains, at least in part, the observed differences in the rhythmicity of flowering between males and hermaphrodites. Indeed, the fact that flowers are carried mainly by shoots that had been produced the previous year is entirely consistent with the low probability for hermaphrodites to flower intensively in two consecutive years. Importantly, because the present study focused on components of male fertility, it did not include any measurement of seed production in hermaphrodites. Given that male flowers differ from hermaphrodite flowers exclusively by the absence of stigmata and contain fully developed ovules, the cost of ovule production cannot explain the results; in contrast, development and maturation of fruits which will occur from fertilization in March until the next autumn could explain the differences observed between males and hermaphrodites. Given that we could observe a relatively large variation in seed set both among individuals and among years (P. Saumitou-Laprade, P. Vernet, P.-O. Cheptou & M. Dufay,

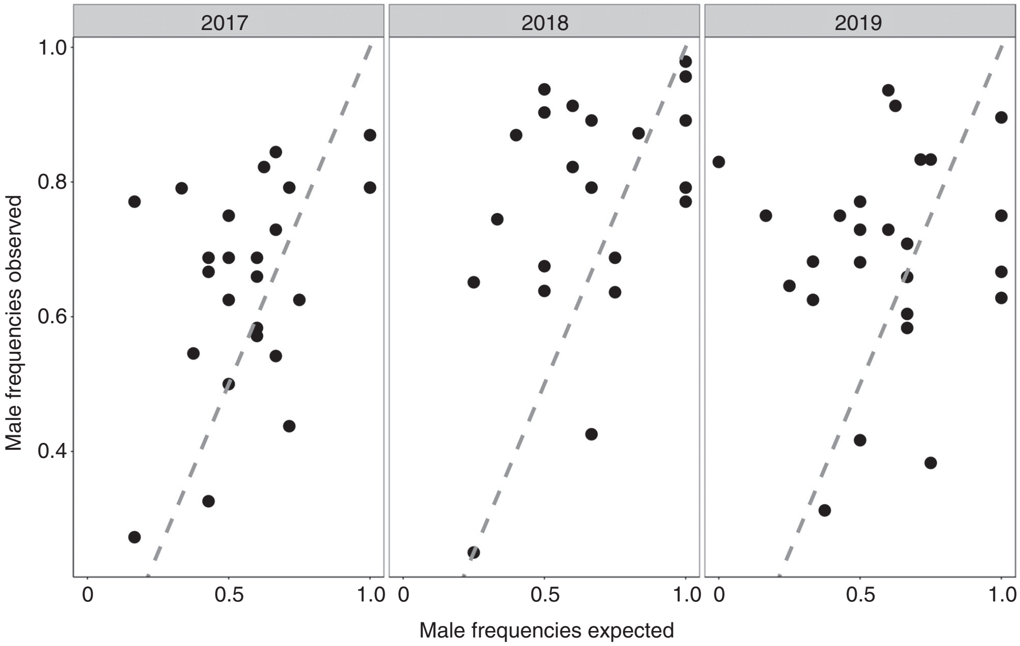

Fig. 7. Observed male offspring frequencies produced by Hb hermaphrodites, as a function of expected male offspring frequencies in the absence of male advantage, during flowering episodes in 2017, 2018 and 2019. Expected frequencies were calculated based on frequencies of flowering individuals in the closest neighbourhood of maternal plants (scale 1). Dots above the dashed lines are progenies in which observed male offspring were more frequent than expected.

pers. obs.), this means that our proxy of flowering intensity is an imperfect estimate of seed production in hermaphrodites. Although this does not impede the result that cost of female reproduction plays a significant role in the establishment of a male advantage, further studies should attempt to dissect the underlying mechanisms in more detail .

Published studies on andro- or gynodioecious species rarely include monitoring of plant reproduction over several years with individuals of the same age (but see Dommée *et al.*, 1999; Penagos Zuluaga *et al.*, 2020). In our case, it not only allowed us to investigate the proximal cause for a possible male advantage, but it also highlighted the fact that proxies of male fertility advantage at the level of one flowering season might strongly underestimate the difference in male fertility between males and hermaphrodites over their lifetime. The same process can accumulate over a high number of flowering episodes, and it is also likely that the difference between males and hermaphrodites increases with age. For instance, one possible important aspect of plant survival in natural populations of *P. angustifolia* is competition for space and light (J. Lepart, pers. com.), and the higher ability of males to grow that we detect in this study could ultimately translate into a higher survival rate.

Males benefit from a strong male fertility advantage at the level of the flowering season

The results discussed so far were based on sex-specific variation in several phenotypic traits that could ultimately translate into a male fertility advantage. To complement these findings, we also measured a direct proxy of male fertility advantage by analysing the observed prevalence of male offspring in several maternal progenies in comparison to the expected one calculated from the proportion of males among the potential fathers. Given that potential fathers included only compatible fathers (i.e. males and one category of hermaphrodites), this estimate of male fertility advantage is completely independent from their compatibility advantage. Moreover, because we considered only blooming potential fathers, this proxy does not include the fact that hermaphrodites seem to flower less often than males. Nevertheless, we found male advantage values around two for three consecutive years, a high magnitude of fertility male advantage. Noteworthy, when males cross with Hb hermaphrodites, the segregation distortion, already mentioned, results from an elimination through incompatibility of pollen grains encoding for the hermaphrodite phenotype, which represent half of their pollen grains, (P. Saumitou-Laprade, unpubl. results; and see discussion by Billiard *et al.*, 2015). This means that even with only half of their pollen grains being usable, males managed to sire twice as many offspring as their Ha competitors in our experiments.

Several limits of our estimate of male fertility advantage should be acknowledged. First, the transplanted individuals used in this study were from a full-sib family and were all the same age. These conditions allowed us to control for inter-individual variance related to age and genotype. However, the male fertility advantage and its components were estimated for only one family of individuals; therefore, the results could vary if other crosses were to be considered. Second, given that it compared males and hermaphrodites Ha (that might have a lower male reproductive success than the other category of hermaphrodites; see below) we might overestimate the average difference between males and hermaphrodites within the species. Also, we did not measure female fitness in hermaphrodites, which might affect lifetime fitness. Finally, our results possibly reflect sex differences in pollen dispersal, which was estimated in a very dense collection of plants, a situation that does not necessarily reflect what occurs in natural populations. That being said, our analyses of maternal progenies showed that males benefitted from a strong (~2-fold) advantage in terms of access to mates. Surprisingly, the fact that males flowered more intensively than hermaphrodites among the potential fathers did not explain our data better, suggesting that other phenotypic traits of males strongly contribute to the male fertility advantage. In wind-pollinated plant species, pollen dispersal seems to vary greatly according to plant height but also to population density (Burczyk *et al.*, 2004; Oddou-Muratorio *et al.*, 2005; Fénart *et al.*, 2007; De Cauwer *et al.*, 2010). The fact that males in our collection express larger size than hermaphrodites could thus explain our results, in part. Another possible advantage of males could rely on a higher germination rate of their pollen grains or better pollen tube growth, as found in some androdioecious species (Ishida and Hiura, 1998; Zhou *et al.*, 2016; Duan *et al.*, 2019), including *Fraxinus lanuginosa*, which is another species in Oleaceae (Ishida and Hiura, 1998). To date, variation in pollen germination has not been investigated in *P. angustifolia*, with the only indirect evidence being a larger size of pollen grains produced by males compared with Ha hermaphrodites.

The two categories of hermaphrodites differ in several proxies of male fertility

Besides the various lines of evidence that males invest more resources in flowering compared with hermaphrodites and benefit from a higher access to mates, our result shed light on several differences between the two categories of hermaphrodites, with Ha producing fewer and smaller pollen grains and flowering less often compared with Hb. Interestingly, Ha individuals did not show lower values for all investigated traits in this study, because we found them to reach larger plant sizes compared with the Hb group. Although this needs to be verified, such lower performance of Ha plants might be restricted mainly to traits linked with male reproductive function. The sex ratio distortion detected in *P. angustifolia* should lead mechanistically to a deficit of Hb individuals in populations (Billiard *et al.*, 2015), as observed in several natural populations of the species (Saumitou-Laprade *et al.*, 2010). Therefore, because pollen produced by Ha hermaphrodites should be compatible with a low proportion of individuals, one would expect that the selective pressures acting on Ha that would ultimately increase their investment in reproductive female function, at the expense of male reproduction. The first results obtained in the present study are consistent with these predictions; one interesting follow-up of these preliminary results will be to compare hermaphrodites for their female fitness. Another interesting follow-up will be to modify theoretical models in order to analyse how this differential investment in male function among hermaphrodites affects the ratios of Ha, Hb and males at equilibrium.

Conclusion

By comparing the siring success of males and hermaphrodites, this study demonstrates a strong male advantage as pollen donors. It also shows that males have a higher frequency of flowering and better vegetative growth, thus contributing to male advantage over the lifetime of individuals. Our results shed light on the fact that integrating lifetime reproductive success over several seasons using multistate capture–recapture models allows the unbiased estimation of sexual type fitness. In populations of the perennial species *P. angustifolia*, the combined effects of accelerated male growth, the disinvestment of the male pathway by Ha hermaphrodites and the disappearance of Hb hermaphrodites through segregation distortion might favour the transition towards dioecy.

SUPPLEMENTARY DATA

Supplementary data are available at *Annals of Botany* online and consist of the following.

Table S1: map of the experimental parcel. Table S2: all Hb mothers used to evaluate the male fertility advantage in 2017, 2018 and 2019. Table S3: testing for spatial effects, a general example. Table S4: results of spatial effects.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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Résumé

Les conflits génomiques apparaissent lorsque différents compartiments du génome sont soumis à des pressions de sélection favorisant des effets opposés sur un phénotype. L'origine de ces conflits provient d'éléments génétiques égoïstes employant diverses stratégies pour maximiser leur transmission à la génération suivante. Ainsi, des résistances ou des suppresseurs peuvent évoluer, contre-sélectionnant un variant égoïste et empêchant sa fixation. La stérilité mâle cytoplasmique (SMC) chez les plantes est un exemple bien connu de conflits dans lequel les gènes transmis par la mère suppriment la fonction mâle des hermaphrodites, et des gènes nucléaires appelés restaurateurs de la fertilité mâle, évoluent pour contrer les effets de la SMC.

La SMC a été longtemps recherchée chez les animaux, sans succès. Cependant, elle a été récemment reportée chez *Physa acuta*, un escargot d'eau douce. Chez cette espèce, le génome mitochondrial associé à de la SMC présente une divergence moléculaire extrême par rapport aux cytotypes normaux (N) et ce au niveau de tous les gènes mitochondriaux. Toutefois, aucune preuve de restauration nucléaire n'a été découverte jusqu'à présent. Cet escargot facile d'élevage a un temps de génération court et offre donc une occasion unique d'évaluer les concepts développés chez les plantes.

L'objectif de cette thèse était d'étudier un conflit nucléo-cytoplasmique au travers de la SMC chez *P. acuta*. Par ces travaux j'ai montré l'existence d'un nouveau génome mitochondrial divergent associé à de la SMC ainsi que l'existence d'un polymorphisme nucléaire contribuant à la restauration de la fonction mâle. La mise en évidence de restaurateurs m'a permis d'explorer les trajectoires évolutives prédites par les modèles de maintien du polymorphisme chez les plantes grâce à 11 générations d'évolution expérimentale. Ces résultats ont montré une sélection fréquence-dépendante des restaurateurs et une contre-sélection de la SMC dans un contexte où les restaurateurs sont abondants. J'ai également constaté que le potentiel de restauration est spécifique d'un type de SMC et que son déterminisme génétique est complexe. Enfin, à l'aide d'une approche de scan génomique j'ai souhaité explorer les conséquences génomiques de la restauration et de la SMC en population naturelle.

Mes travaux ont permis pour la première fois de suivre en temps réel l'interaction entre un variant mitochondrial égoïste et leur résistance.

Abstract

Genomic conflicts arise when different compartments of the genome are subjected to selection pressures favoring opposite effects on a phenotype. These conflicts originated from the emergence of selfish genetic elements employing various strategies to maximise their transmission to the next generation. In this context, resistances or suppressors can evolve, counter selecting a selfish variant and preventing its fixation. This applies to the textbook example of genetic conflict: cytoplasmic male sterility (CMS) in plants, in which maternally transmitted genes suppress the male function of hermaphrodites, and nuclear genes called restorers of the male fertility evolve to counteract the effects of CMS.

CMS has been looked for in animals, without success. However, it has recently been reported in *Physa acuta*, a freshwater snail. In this species, the mitochondrial genome associated with CMS showed extreme molecular divergence from normal cytotypes (N) at all mitochondrial genes. To date, no evidence of nuclear restoration has been found. However, this snail has a short generation time and therefore offers a unique opportunity to evaluate concepts developed in plants.

The aim of this thesis was to study a nucleo-cytoplasmic conflict through CMS in *P. acuta*. I highlighted the existence of a new divergent CMS-associated mitochondrial genome and found nuclear polymorphisms contributing to the restoration of male function in natural populations. I used this new CMS type and the identified restorers to explore the evolutionary trajectories predicted by plant models through 11 generations of experimental evolution. These results showed a frequency-dependent selection of restorers and a counter-selection of CMS in high restoration context. I found that the restoration potential was specific to CMS type and that its genetic determinism was complex. Finally, using a genome scan approach, I aimed to explore the genomic consequences of the restoration and the CMS in natural populations.

For the first time, we here observed the dynamics of the interaction between a selfish mitochondrial variant and their resistance.