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Evolutionary pathways driven by female mate choice: the curious case of the *Squalius alburnoides* allopolyploid fish complex

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Especialidade de Etologia

Miguel Filipe Morgado dos Santos

Tese orientada por:
Luís António de Matos Vicente
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Maria João Ivens Collares Pereira

Documento especialmente elaborado para a obtenção do grau de doutor

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NOTA PRÉVIA

A presente dissertação inclui resultados de trabalhos já publicados ou submetidos para publicação (**Capítulos 2 a 5**), de acordo com o previsto no nº 2 do artigo 25º do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, em Diário da República, 2.ª série, N.º 155, de 11 de Agosto de 2017. Tendo os trabalhos sido realizados em colaboração, o candidato esclarece que participou integralmente na sua concepção, na obtenção, análise e discussão de todos os resultados, bem como na redacção dos manuscritos.

Lisboa, 25 de Janeiro de 2018,
Miguel Filipe Morgado dos Santos

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RESUMO

A **hibridação**, *i.e.* o cruzamento entre duas espécies ou populações diferentes, é frequentemente inviável devido a incompatibilidades genéticas e, quando não o é, conduz normalmente à produção de descendência pouco viável ou, no limite, infértil. Estes constrangimentos levaram a que a hibridação fosse vista durante décadas como um mero erro esporádico, irrelevante para o processo evolutivo. No entanto, a descoberta de um número crescente de organismos híbridos viáveis, férteis e com populações estáveis foi alterando progressivamente a visão da comunidade científica em relação à hibridação, que é hoje considerada um importante mecanismo evolutivo que pode levar à formação de novas espécies (especiação híbrida). Os constrangimentos genéticos responsáveis pela infertilidade de uma parte considerável dos organismos híbridos bem-sucedidos foram ultrapassados através do desenvolvimento de modos de **reprodução assexuada**. Estes organismos passam, então, à sua descendência, um ou mais conjuntos de cromossomas inalterados geneticamente, produzindo assim linhagens clonais. Vários são os tipos de reprodução assexuada conhecidos, nomeadamente, partenogénese, ginogénese, androgénese e hibridogénese, variando nos mecanismos de hereditariedade. De todos estes, destaca-se a **hibridogénese**, em que os gâmetas sofrem redução de ploidia, sendo uma das linhagens, normalmente a paterna, excluída, e passando à descendência apenas os cromossomas maternos. Neste modo de reprodução, contrariamente aos restantes, há incorporação dos genomas de ambos os progenitores na descendência a cada geração, representando uma forma de reprodução hemiclonal, por oposição à clonalidade estrita. O carácter clonal dos organismos assexuados leva a rácios sexuais assimétricos, dado que as linhagens de clones herdam o mesmo sexo de geração para geração. Assim, nos casos em que são necessários gâmetas masculinos e femininos para se iniciar a embriogénese, os indivíduos assexuados parasitam sexualmente o sexo oposto de espécies simpátricas compatíveis. Em teoria, os organismos assexuados correm sérios riscos de extinção. Por um lado, o carácter clonal da sua reprodução leva à acumulação de mutações deletérias ao longo do tempo e à uniformidade genética da descendência, o que aumenta o risco de problemas de adaptação a ambientes instáveis. Por outro lado, o parasitismo sexual está putativamente associado a desequilíbrios populacionais, uma vez que os organismos assexuados têm, em teoria, o dobro do potencial de crescimento populacional dos organismos sexuados por não terem necessidade de produzir dois sexos. Esta discrepância leva teoricamente a um crescimento desproporcional do número de indivíduos assexuados na população, que rapidamente poderá levar à extinção das espécies sexuais simpátricas das quais dependem. A sobreexploração dos gâmetas destas espécies acaba, assim e consequentemente, por levar à extinção dos próprios organismos assexuados, que deixam de poder reproduzir-se devido à ausência dos seus hospedeiros sexuais. Independentemente destes constrangimentos, vários são os híbridos assexuados e bem-sucedidos que se encontram descritos, com populações abundantes, tendo frequentemente não só uma origem antiga como uma elevada variabilidade genética. Assim sendo, que ferramentas evolutivas levarão ao sucesso destes organismos, ultrapassando as desvantagens associadas à clonalidade e ao parasitismo sexual? Vários são esses mecanismos, como, por exemplo, a poliploidia, a introgressão de genes das espécies sexuadas e as diferenças ecológicas e reprodutoras entre organismos sexuados e assexuados (*e.g.* segregação de habitat, taxa de infecção patogénica distinta, fertilidade e sobrevivência diferenciais). No entanto, de todos esses mecanismos, destaca-se a **escolha de parceiros sexuais** por machos e fêmeas, que desempenha um papel relevante nos complexos híbridos e assexuados. Por um lado, nos sistemas ginogenéticos, a escolha das fêmeas assexuadas com base na genética dos machos não é tão relevante, uma vez que a sua descendência não herda o genoma dos machos escolhidos, sendo o esperma apenas necessário para despoletar a embriogénese. Nestes sistemas, é, então, a preferência dos machos das espécies simpátricas que desempenha um papel mais importante, sendo que estes machos têm vantagem em evitar reproduzir-se com as fêmeas assexuadas. Deste jogo, resulta a coexistência em equilíbrio entre linhagens sexuadas e assexuadas. Por outro lado, nos complexos hibridogenéticos, a escolha das fêmeas baseada na genética dos machos já tem uma importância superior, uma vez que o genoma destes é incorporado na descendência, sendo os seus genes funcionais, e importando, portanto, a qualidade genética do progenitor. A escolha de parceiros sexuais pelas fêmeas hibridogenéticas assume ainda uma relevância superior tendo em consideração que estes sistemas, contrariamente aos ginogenéticos, são normalmente caracterizados pela existência de dois sexos híbridos, pelo que o leque de potenciais parceiros sexuais para as fêmeas é mais alargado.

O objectivo da presente dissertação foi estudar a escolha de parceiros sexuais pelas fêmeas de um dos mais complexos sistemas hibridogenéticos conhecidos, o **complexo *Squalius alburnoides***, nomeadamente a sua relevância na dinâmica reprodutora, na composição genomotípica das populações e no futuro evolutivo deste complexo. Este ciprinídeo representa uma entidade biológica constituída por machos e fêmeas férteis de diferentes ploidias (2n, 3n e 4n) e combinações híbridas de genomas (*i.e.* genomotipos) de várias espécies parentais. A sua reprodução engloba modos sexuados e assexuados, misturando parasitismo sexual e autonomia reprodutiva. Deste modo, este complexo pode ser considerado um modelo ideal para estudar a selecção sexual com base genética e a sua influência nas possíveis rotas evolutivas dos organismos. Especificamente, foi estudado: a) o papel da escolha de parceiros pelas fêmeas hibridogenéticas/assexuadas na manutenção das populações no seu estado híbrido (*i.e.* parasitismo sexual) através de modelação teórica e dados empíricos; b) a influência da alocação de oócitos (uma típica medida de preferência em peixes com fertilização externa), da taxa de fertilização e da sobrevivência da descendência na dinâmica reprodutora das populações, através da junção de dados obtidos em cruzamentos direccionais e livres com análises de paternidade; e c) a influência da escolha de parceiros na genética das populações, nomeadamente o seu papel na introgressão dos genomas das espécies parentais, e também a influência dessa mesma introgressão nos padrões de preferência observados, juntando, uma vez mais, testes experimentais e ferramentas genéticas modernas.

Os resultados obtidos demonstraram que a preferência das fêmeas híbridas pelos diferentes tipos de macho desempenha um papel relevante na manutenção não só da dinâmica reprodutora das populações como também da variabilidade genética das mesmas. Os padrões de preferência destas fêmeas evidenciaram-se variados, sugerindo que escolhem diferentes tipos de machos de acordo com probabilidades particulares que levam à manutenção das populações com a composição de genomotipos que as caracterizam, ou seja, uma ordem de preferência que permite a estabilidade da dinâmica reprodutora. Estes dados foram corroborados por modelação teórica baseada na preferência das fêmeas e na frequência dos diferentes genomotipos e das espécies sexuadas simpátricas, demonstrando que a variação nestas variáveis é coerente com as composições genomotípicas e respectivas dinâmicas encontradas na natureza. Para além disso, demonstrou-se que o padrão de preferência das fêmeas influencia o sucesso reprodutor dos machos, estando essas mesmas preferências directamente relacionadas com o sucesso da descendência produzida (*e.g.* taxa de sobrevivência). Por último, a escolha de parceiros sexuais pelas fêmeas híbridas parece também assegurar a manutenção da variabilidade genética das populações. As fêmeas híbridas demonstraram uma preferência superior por machos que conferem maior variabilidade genética à descendência, favorecendo aqueles que: a) possuem modos de reprodução sexuada; b) lhes são menos familiares; e c) possuem genomas mais heterozigóticos. Estas estratégias parecem permitir a persistência das populações durante tempos evolutivos, criando condições para que possam eventualmente evolucionar para novas espécies através de especiação híbrida. A especiação híbrida ocorre, nestes casos, quando os híbridos recuperam a homologia cromossómica e retomam, assim, a possibilidade de haploidização por redução meiótica normal, *i.e.* sexuadamente. Estes híbridos são, então, capazes de desenvolver uma dinâmica própria, independente tanto de outros híbridos como das espécies parentais, abandonando, assim, a condição de parasitismo sexual. A escolha de parceiros sexuais parece, uma vez mais, desempenhar um papel fundamental nesta rota evolutiva, uma vez que o estabelecimento de preferências entre híbridos semelhantes acelera significativamente o processo. Em *S. alburnoides*, a tetraploidização é o caminho mais directo para a especiação híbrida, sendo que os tetraplóides balanceados preenchem todos os requisitos necessários, nomeadamente: a) conjuntos de cromossomas homólogos; b) meiose com recombinação como mecanismo de reprodução; c) machos e fêmeas com um rácio sexual equilibrado; e d) dinâmica reprodutora independente. De facto, três populações constituídas exclusivamente por tetraplóides já foram reportadas. Os resultados obtidos no âmbito desta tese indicam que a escolha de parceiros desempenha, de facto, um papel fundamental neste processo. A passagem de uma população triplóide (*i.e.* no seu estado tipicamente híbrido) para uma população tetraplóide (*i.e.* à beira da especiação) parece requerer que as fêmeas triplóides sigam um padrão específico de preferência, favorecendo os machos das espécies simpátricas aos restantes. Embora este padrão seja coerente com o carácter parasítico de *S. alburnoides*, o caminho para a tetraploidização pode não ser assim tão simples, uma vez que é expectável que as espécies simpátricas evitem reproduzir-se com os híbridos, tal como observado, tendência que poderá, pelo menos parcialmente, explicar a raridade das populações tetraplóides.

Por último, na presente dissertação descreveu-se também o primeiro caso conhecido de androgénese em vertebrados, observado em indivíduos e gâmetas não manipulados experimentalmente. Para além de esta descoberta ser uma novidade para o conhecimento acerca da reprodução como um todo, constitui ainda uma importante peça para a compreensão da dinâmica reprodutora de *S. alburnoides*, uma vez que este modo de reprodução assexuada poderá levar à independência reprodutora das populações híbridas, que poderão, assim, deixar de necessitar das espécies simpátricas para persistir.

Os vários resultados obtidos no âmbito da presente dissertação apontam para um papel fundamental da escolha de parceiros sexuais pelas fêmeas híbridas não só na dinâmica reprodutora e na composição genomotípica de *S. alburnoides*, mas também nos seus putativos caminhos evolutivos. Se, por um lado, as preferências das fêmeas parecem suportar a manutenção das populações no seu estado híbrido, levando à sua estabilidade reprodutora e mantendo a sua variabilidade genética, por outro lado, a escolha das fêmeas poderá também encaminhar as populações para a tetraploidização e, consequentemente, para um processo de especiação híbrida.

PALAVRAS-CHAVE | Reprodução assexuada; Hibridogénese; Dinâmica reprodutora; Introgressão; Androgénese.

SUMMARY

Many asexual animals are sexual parasites that reproduce with sympatric species, leading to genetic, ecological and behavioural challenges. Several mechanisms allow the coexistence of sexuals and asexuals, and, over time, the latter may regain sexual reproduction and develop an independent reproductive dynamics, paving the way to the arising of new species through hybrid speciation. Mate choice may play a relevant role in all evolutionary stages of asexual organisms, either contributing to their persistence in hybrid state or routing them towards hybrid speciation. Here, we studied mate choice in the allopolyploid cyprinid *Squalius alburnoides*, namely its role in driving the evolutionary pathways of this hybrid complex. Bridging together genetic, theoretical and experimental approaches, we aimed at assessing: a) the role of mate choice in driving population stability or routing populations towards hybrid speciation; b) the influence of egg allocation, fertilization rate and offspring survival in shaping the reproductive dynamics and genomotype composition of populations; and c) the gameplay between mate choice by hybrid females and genetic introgression and variability. In the one hand, mate choice by hybrid females seems able to uphold the persistence of populations in their hybrid state by maintaining the reproductive dynamics between the interdependent genomotypes and sympatric congeneric species, and also by guaranteeing the maintenance of high genetic variability. On the other hand, when particular conditions are met, mate choice by hybrid females may also route populations towards hybrid speciation, namely via tetraploidization. The findings reported herein also include the first ever described case of naturally occurring androgenesis in vertebrates. In a general perspective, *Squalius alburnoides*' hard-to-combine features challenge even the most conservative views about the role of hybridization in species' diversification. Studying this allopolyploid complex is like looking at a snapshot of evolution and, undoubtedly, a privilege to every passionate evolutionary biologist.

KEYWORDS | Asexual reproduction; Hybridogenesis; Reproductive dynamics; Introgression; Androgenesis.

CHAPTER 1

GENERAL BACKGROUND

Chapter 1 | GENERAL BACKGROUND

1.1 | *From hybridization to asexuality and sexual parasitism*

The biological concept of species as reproductively isolated populations [1-3] led to the generalized and naive belief among zoologists that hybridization was rare and irrelevant to evolution [4,5]. This view of hybridization as a sporadic and insignificant evolutionary mistake was uphold by evidence that the vast majority of interspecific crosses are likely unviable, and that most from the limited range of viable cases lead to the production of sterile hybrid offspring, due to the lack of homology between chromosome pairs needed for correct segregation during meiosis (postmating reproductive isolation; *e.g.* "Dobzhansky-Muller incompatibilities") [3,5,6-13]. Furthermore, from the even more limited range of viable cases in which fertile hybrid offspring is formed, hybrids find few to none mates of their own type and backcrosses with the parental species are likely unfit [14,15]. Consequently, hybrid animals were for long considered evolutionary dead-ends, posing only as mere errors of the evolutionary process. Over the decades, this view has been recurrently countered, with a large bulk of studies showing that hybridization is actually a fairly common phenomenon among animals, occurring on, at least, 10% of the known species [14], and poses as a strong evolutionary force, promoting genetic variation by means of introgression and routing populations towards speciation, either by reinforcing prezygotic isolation to avoid unfit hybrids [1,5,16-20] or by generating new species from the hybrids themselves (*i.e.* hybrid speciation) [15,21-41].

If the genomes of the parental species are compatible, allowing the occurrence of meiotic recombination during gametogenesis, hybrids may retain sexual reproduction. These organisms maintain the chromosome number of their parental species, being, thus, called homoploid hybrids, and, granted the required reproductive isolation, may be able to evolve into new species through the so-called recombinational or homoploid speciation [15,25-27,31,35]. However, many hybrids deal with significant chromosome incompatibilities precluding the occurrence of a standard meiosis, which lead to the development of unusual reproductive modes to dodge sterility, namely asexual clonal reproduction (see **Box 1**) [22,24,42-50]. Asexual reproduction has an incidence of 0.1% among vertebrates [51-55], and, within this group, asexual organisms are normally sexual parasites, whose reproduction is dependent on parental species [42,46,49,56-61], meaning that they must coexist and compete for mates and resources with their sexual hosts in mixed populations [49,62-64]. Being simultaneously hybrid, asexual and sexual parasite poses serious challenges for the persistence of these organisms [52,54], but there are several known cases of extant thriving hybrid asexual organisms among platyhelminthes, molluscs, insects, crustaceans, fishes, amphibians and reptiles [24,52,58,61,65], with abundant populations and vast distribution ranges [49,66,67], sometimes inhabiting beyond the distribution edges of their parental species [48,68-70]. So, how did these organisms surpass the theoretical disadvantages of being hybrids, asexuals and sexual parasites? The answers rely not only on genetics, but also on ecology and behaviour.

Box 1

Asexual reproduction is an altered reproductive system characterized by the lack of regular sexual mechanisms (*i.e.* normal amphimixis), namely by the **omission of meiotic reduction with recombination** during gametogenesis. Asexually reproducing organisms produce gametes **clonally**, with or without ploidy reduction, meaning their genome (one or more complete sets of chromosomes) is passed to the descendants unaltered by any means. In some cases, fertilization is completely abandoned (**parthenogenesis**), but, in others, it is still required (**gynogenesis**, **androgenesis** and **hybridogenesis**) (see section 1.2 for details on each known type of asexual reproduction mechanism).

Asexual organisms vary greatly in reproductive dynamics, ranging from **all-female** or, more rarely, **all-male** to populations with **both males and females**. Moreover, some asexual systems may also include standard sexual reproduction, with some individuals reproducing asexually, but others reproducing sexually within the same population. This marked variability in asexual systems leads to difficulties in generalizing terminologies and patterns, with a multitude of terms being used in the literature in an attempt to be consistent with the particular reproductive traits of each asexual complex. In addition to the term "asexual", several others have been suggested, such as "**unisexual**", "**nonsexual**" and "**quasi-sexual**", trying to be inclusive to all such organisms, from the ones with only one sex and dismissing fertilization to those with both males and females and requiring fertilization. However, none is comprehensive enough to include all the known cases, given the marked differences distinguishing them. Henceforward, the term "asexual" will be used throughout this dissertation whenever in wider and general context, although acknowledging it is a highly reductive term.

1.2 | *Dodging genetic challenges*

The lack of meiotic recombination [49,57,71] leads to the accumulation of deleterious mutations through generations and to mutational meltdown and fast extinction [72-80], an evolutionary constraint known as the Muller's ratchet [81,82]. Furthermore, the lack of recombination in most asexual organisms leads to offspring genetic uniformity and decreased variability [83], which comes exclusively from sporadic beneficial mutations occurring in the lineages of clones [84,85], posing as a serious risk for asexuals inhabiting changing environments, in which sex is useful by means of selection, facilitating the spread of advantageous mutations [79,85,86].

Studies have shown that, contrary to theoretical predictions, most asexual animals have old origins [87-94] and unexpectedly high levels of genetic and clonal variability [95-101], which may be due to mutation, but also to the simultaneous existence of several distinct clonal lineages [91,99,102-108]. How asexual organisms dodge the accumulation of deleterious mutations may remain on genetic tricks, via introgression, hybridogenesis and polyploidy. From the four asexual reproductive modes described among animals, namely parthenogenesis, gynogenesis, androgenesis and hybridogenesis, parthenogenesis is the only that totally dismisses the need of two sexes, with females producing clones of themselves from unfertilized unreduced oocytes, which start embryogenesis without the need of any male stimulus [109,110]. However, it seems to have a limited incidence among vertebrates [52,111,112]. All the remaining asexual reproductive modes require male and female gametes simultaneously. In gynogenesis (sperm-dependent parthenogenesis) and androgenesis [113], both oocyte and sperm are needed to trigger embryogenesis, but the genetic material of one gamete is discarded postfertilization, with offspring lacking genes from father or mother, respectively [8,42,46,98,114-122]. Although the need of male and female gametes may look like a disadvantage in comparison to full strict asexuality (parthenogenesis), it may be genetically advantageous. It may allow the introgression of genes from the sexual host into the asexual parasite, thus buffering the accumulation of deleterious mutations and increasing offspring genetic variability, a benefit that can be magnified in asexuals that parasitize multiple host species [48,49,58,93,98,123-128]. After fertilization of gynogenetic oocytes, for example, genome portions of the male host may remain by failure of the extrusion process in the form of functional microchromosomes, inheritable throughout generations [129-134].

Although this type of input of new genetic material into the asexual lineages only occurs by chance, introgression of genes from the sexual hosts is the rule among hybridogenetic systems and not the exception. In hybridogenesis, the genetic material of both mother and father are present in the offspring, with descendants benefitting from the genes of both progenitors, although, during gametogenesis of hybrids, no recombination between paternal and maternal chromosomes occurs, with the genetic lineage of one parent being discarded and only the genetic baggage of the other passing throughout the generations [43,47,49,50,71,98,135-139]. This means that there is an input of an entire fresh genome into the offspring of these asexuals on a generational basis, with hybridogenesis being considered a type of hemiclonal reproduction [140,141]. Although the lack of standard meiosis characterizes most asexual reproductive modes, there is one exception, meiotic hybridogenesis [116,139,142-144], in which recombination does, indeed, occur among homospecific chromosomes, putting hybridogenesis on the top of the list of the asexual reproduction modes allowing higher offspring genetic variability.

Meiotic hybridogenesis normally occurs in triploid forms, in which the heterospecific genome is discarded and the two remaining homologous genomes undergo regular reductional meiosis with recombination, leading to the production of haploid gametes.

A common direct consequence of the input of entire sets of chromosomes into the offspring is the increase of ploidy level, *i.e.* polyploidy, which offers additional genetic material and variability on which mutation and selection may act and also counters the accumulation of deleterious mutations. Coping with polyploidy may involve mechanisms of dosage compensation by gene-copy silencing, through which polyploids functionally work as diploids concerning gene expression **[145-148]**. Nevertheless, polyploidy is a fairly common phenomenon among "lower vertebrates" **[40,49,57,65,80,105,118,134,149-152]**.

1.3 | *Dodging ecological and behavioural challenges*

In a mixed population of sexual and asexual organisms connected through sexual parasitism, asexual parasites and sexual hosts may dive into an arms race **[153]**, constantly evolving new strategies to outsmart their counterparts via ecology and behaviour, a game that may theoretically result in the coexistence of both lineages over evolutionary timeframes. However, theory predicts that sexuality is in disadvantage due to the cost of producing two sexes, which are unnecessary for asexuals **[154-157]**. This leverage theoretically leads to the displacement and extinction of the sexual hosts and to the absolute dominance of the asexual parasites **[61]**, with asexual females often dominating wild populations of asexual systems **[49,158]**. The over-exploitation of the sexual hosts backfires at the asexual parasites, which can no longer reproduce due to the lack of oocytes and sperm required for fertilization in hybridogenetic individuals and for triggering fertilization in gynogenetic and androgenetic individuals, thus leading to extinction of both hosts and parasites **[117,159,160]**. Therefore, how did evolution trick theory to allow the persistence of thriving asexual animals in coexistence with their sexual hosts? Researchers have for long tried to answer this question through both empirical evidence and theoretical modelling, assessing the roles of mate choice, frequency-dependent mating success, fecundity, survival, sex ratio, ecological niches and competition **[63,161-170]**.

Although parthenogenetic females are less prone to contract contagious diseases given there is no physical contact between partners during mating, the same does not apply to gynogenetic and hybridogenetic females, which require partners to produce offspring. Some studies have shown that pathogens may favour the coexistence of sexual and asexual lineages by disproportionally reducing the fitness of asexuals and buffering their leverage **[171,172]**, with clonality being potentially linked to reduced MHC variability

[173]. If pathogens are narrowly specialized and adapt to preferentially infect hosts with more common genotypes, asexuals may be more prone to infection, given the clonal character of their lineages. Although asexual females may dodge this problem by avoiding to mate with infected males with a stronger effort than sexual females [174], sexuals may quickly evolve disease resistance by recombining defences and originating new allele combinations to outcompete pathogens, which is a lost arms race for asexuals due to the lack of meiotic recombination (*i.e.* "Red Queen hypothesis") [175,176]. However, empirical evidence on this subject is controversial, with some but not all studies reporting higher pathogen susceptibility in asexuals [177-184].

Besides pathogen susceptibility, other differential ecological traits between sexual and asexual lineages may contribute to their coexistence. In the one hand, asexuals may show lower fecundity or offspring survival than their sexual counterparts [185-189], although this is not a rule [190-195]. On the other hand, to reduce competition, asexual parasites and sexual hosts may displace ecological niches through microhabitat or diet partitioning [62,184,196]. Due to their clonal uniform genetics, asexual parasites are expected to have narrower ecological niches (specialists) than the more flexible sexual hosts (generalists) [57], which may lead to ecological segregation while facilitating coexistence, a pattern already uphold by some empirical evidence [185,197-199]. Moreover, the narrower range of optimal conditions of asexuals may represent a handicap that potentially reduces population growth leverage in changing environments and in nonaverage conditions (*e.g.* temperature) [200].

Given most asexual animals need the gametes of the host non-hybrid sexual species to reproduce, access to mates is directly linked to reproductive success, and, thus, mate choice is also relevant among asexual (non-parthenogenetic) organisms, despite the clonal character of their reproduction [58,59,201]. Generally, selection favours mate choice by females (intersexual selection) and competition among males (intrasexual selection), given the costs associated with mating failure are higher for females than for males. Indeed, while males produce virtually infinite sperm cells with low energetic cost, females invest much higher levels of energy to produce a small number of oocytes (*i.e.* "Bateman's principle") [202]. Thus, it is more advantageous for females to find quality males offering optimal genetics and reproductive resources (*e.g.* better spawning territories) that contribute to the success of the offspring, but it is more advantageous for males to maximize their reproductive success quantitatively by mating with multiple females. However, under particular conditions (*e.g.* females varying greatly in quality), mate choice may be advantageous also for males [203,204]. Mate choice studies on asexual systems often focus on male mate choice, not only empirically, but also via theoretical modelling [169,170,196,205,206], since males of the sympatric sexual species gain advantage on avoiding to mate with the asexual parasitic females, thus lowering their population growth potential and consequently allowing the coexistence of sexual and asexual lineages. The evolution of such male mate choice against asexual females is prone to occur because male genes are discarded immediately after fertilization of gynogenetic oocytes or after one or two generations in hybridogenetic lineages. Thus, the reproductive success of males mating with gynogenetic or hybridogenetic females is virtually zero. Since males favouring mating with sexual females will father more conspecific offspring, selection may shortly act to favour assortative male mate choice in host species parasitized by asexuals, with empirical evidence showing that host males do, indeed, discriminate between sexual and asexual females, avoiding to mate with the latter or priming less sperm when they do **[46,63,67,161,168,207-213]**, with only a few exceptions **[195,214]**. However, since several thriving asexual complexes exist worldwide, such male mate choice against asexuals is obviously not strong enough to completely prevent crosses between asexual females and sexual males, thus ensuring the persistence of the asexual lineages, which may occur through a variety of ways:

a. The occurrence of crosses between parasitic females and male hosts may be due to the low cost associated with male reproductive failure **[202]**, with errors being energetically less expensive than developing a strong mate choice against asexual females **[215]**;

b. The arms race between sexual and asexual lineages may lead the asexual parasites to become more attractive to male hosts, being more vigorous in attempts to mate. Furthermore, asexual females may be more aggressive and competitive for mates towards sexual females than towards other asexual females, or they may mimic the sexual females to attain, at least, similar reproductive success **[63,216-220]**;

c. Males courting asexual females may gain a compensation to their lost reproductive effort by being consequently more attractive to sexual females and being selected via mate choice copying **[221,222]**;

d. Males of the host species may deceive other males to reproduce with asexual females via male mate choice copying **[223]** by misleadingly pretending they are interested in them, to outcompete the fitness of other conspecific males **[224]**.

Notwithstanding, another scenario also posits a key role for male mate choice in driving population stability among sexual and asexual lineages, even if males do not evolve mate choice against asexual females, *i.e.* via negative frequency-dependent male mate choice **[161,225,226]**. This hypothesis states that males of the host species may not be choosy when the frequencies of sexual and asexual females in the population are similar, but strongly select sexual females as they become rarer due to the faster growth of the asexuals, thus maintaining oscillating but harmonious proportions of sexuals and asexuals.

Although male mate choice may be one of the mechanisms allowing the coexistence of the asexual parasites and their reproductive hosts in mixed populations, less is known about female mate choice in hybrid asexual complexes. Mate choice by asexual females is often neglected because gynogenetic females only need to guarantee access to mates of the sympatric species for the asexual lineage to persist, given the sperm of the host is only needed to trigger embryogenesis and all male genes are discarded after fertilization. Thus, since offspring success is independent of the genetic quality of the father, there is little evolutionary pressure for gynogenetic females to be choosy on a genetic basis [227,228], with embryogenesis being triggered even by unfertile sperm in some cases [229]. However, hybridogenetic females may gain advantage on stronger mate choice, given the genes of the male sexual host are incorporated within the offspring and, thus, directly influence the viability and success of the descendants. Moreover, contrary to gynogenetic systems, hybridogenetic complexes normally include fertile male hybrids [45,49,51,230] that may contribute to their reproductive dynamics. Thereby, hybridogenetic females have a wider range of mating options, including hybrid and non-hybrid males, than some other asexual systems in which, generally, only sexual males of the host species exist. This peculiarity further strengthens the evolutionary potential of female mate choice in hybridogenetic systems. Indeed, similarly to what is observed in gynogenetic systems regarding mate choice by male hosts, mate choice by hybridogenetic females may promote coexistence between sexual and asexual lineages, with empirical and theoretical evidence supporting this view [165-167,191,231-235]. The pattern seems to be the same: asexual females select the males with which they perpetuate the hybrid lineage, often favouring sexual hosts over their own “conspecific” hybrid males, while male and female sexual hosts avoid to mate with asexual females and males, respectively. This gameplay includes intersexual (female and male mate choices) and intrasexual (male-male competition) selections, upholding coexistence between asexual parasites and sexual hosts.

1.4 | *From asexuality and sexual parasitism to speciation*

As referred above, hybrid asexual parasites have evolved mechanisms to persist over time through a variety of genetic, ecological and behavioural adaptations that contour the putative obstacles to their establishment. Thus, the rarity of successful hybrid animal complexes, especially among vertebrates, does not seem to be related to the inherent theoretical long-term disadvantages of asexuality and sexual parasitism, but rather to difficulties in the formation of viable and fertile clonal organisms in the wild, since the genomic and ecological conditions for successful hybridization to occur between two species are often not met (*i.e.* "rare formation hypothesis") **[236]**. Anyway, it seems now clear that hybrid asexual complexes are not evolutionary dead-ends as believed for many decades **[4,5]**. On the contrary, these complexes have a high evolutionary impact on parental species, not only shaping the evolution of their behaviour as stated above, but also being able to:

a. Recreate the parental lineages from the hybrids **[195,237]**;
b. Contribute with functional genes through introgression from hybrids to the bisexual species **[238]**;
c. Amplify the incidence of fit genotypes generated by sexual reproduction **[239]**;
d. Shape the geographical distribution and expansion of the parental species **[240]**;
e. Accelerate evolution, leading to the arising of new species through hybrid speciation, sometimes almost instantaneously via polyploidy **[15]**.

Indeed, a large bulk of studies over the last decades have shown the strength and relevance of hybrid speciation in evolutionary processes, both of recombinational homoploid hybrid speciation and of allopolyploid hybrid speciation **[15,21-41]**. Hybrid speciation begins when the hybrid organisms become reproductively independent and isolated from their parental species and develop their own autonomous reproductive dynamics. Asexual hybrids are some steps behind hybrid speciation than homoploid hybrids, since the latter, maintaining sexual reproduction, have generally a shorter route towards reproductive autonomy. Thus, the major step towards hybrid speciation in asexual organisms is the recovery of sexual reproduction and, consequently, the abandonment of sexual parasitism, which may easily occur in genotypes with an even number of balanced genomes (*e.g.* diploids or tetraploids), potentially allowing chromosome homology needed for meiotic segregation **[241-243]**. The complete emancipation of the asexuals may occur in two different ways. In the one hand, hybrids may be able to recreate individuals of the parental species through crosses involving hybrids only, creating an enough proportion of nuclear non-hybrid partners to sustain the entire complex without the need of the actual parental species **[195,237]**. On the other hand, asexual complexes with fertile hybrid males and females may dismiss the need of the parental species at all **[70,230,241,244]**. When sexual reproduction is recovered in these autonomous hybrids, the way is paved to the arising of new standard bisexual species.

Extant animal species with putative hybrid origin exist among insects, molluscs, crustaceans, fishes, amphibians, reptiles, birds and mammals **[24,32,41,245,246]**.

Mate choice may play a key role in the reproductive isolation between the autonomous, now sexual, hybrids and their parental species via assortative mating. In the one hand, hybrids may show a distinct phenotype than parental species, and sexual selection may favour mating among similar phenotypes, thus directly upholding prezygotic isolation between hybrids and parental species **[247-249]**. On the other hand, assortative mating based simply on ploidy level may also occur, with individuals favouring for mating other individuals with the same ploidy, a pattern that arises from distinct mate choice patterns or mating strategies among ploidies (*e.g.* mating calls) **[250-255]**.

In short, here is the most likely route from the original hybridization event to hybrid speciation in asexual systems:

i. Two species hybridize. Their genomes are compatible enough to enable the formation of viable hybrid offspring, but not compatible enough to maintain standard meiosis with recombination, thus leading to the development of asexual reproduction to dodge sterility;

ii. With asexuality comes sexual parasitism, with asexuals depending on the sympatric parental species to attain reproduction. Sometimes the genome of the parasitized sexual species is incorporated into the offspring (hybridogenesis), sometimes it is not (gynogenesis);

iii. Coexistence between sexuals and asexuals is uphold by variable genetic, ecological and behavioural mechanisms until asexuals eventually evolve their own autonomous reproductive dynamics and become independent from the parental species;

iv. Some male and female hybrid forms regain chromosome homology and, consequently, sexual reproduction, bringing back meiosis and recombination and being able to perpetuate their own genomotype without the need of neither the parental species nor other hybrid forms;

v. These autonomous genomotypes develop assortative mating, with males and females of the same genomotype mating mainly with each other, establishing an independent reproductive dynamics within the whole hybrid complex;

vi. Behavioural prezygotic isolation settles among hybrid forms, and a new species emerges.

The intricate population dynamics of hybrid asexual complexes make these organisms extraordinarily interesting from an evolutionary perspective, from the moment they arise until they eventually stride towards hybrid speciation. The incidence of hybrid speciation among animals is surely underestimated, given the hybridization/asexual phase prior to hybrid speciation is transient and rapidly vanishes in evolutionary timeframes, before the arising of the new species. Thus, hybrid asexual complexes are snapshots of evolution in real time, sharing hard-to-combine genetic, ecological and behavioural peculiarities, and being, therefore, ideal study models to bridge together several fields in Biology **[15,24,49,51,54,55,58,60,61]**.

1.5 | *The* Squalius alburnoides *allopolyploid complex*

1.5.1 | *Hybrid origin, genetics and reproductive mechanisms*

Squalius alburnoides (Steindachner 1866) is a cyprinid fish endemic to the Iberian Peninsula **[49]**. This biological entity, firstly described as a *Leuciscus*, has been placed in distinct genera (*Tropidophoxinellus*, *Rutilus*, *Pararutilus*, *Leuciscus* again and *Iberocypris*), but is currently included in the *Squalius* genus **[256,257]**. *Squalius alburnoides* had a hybrid origin **[257-259]** among *Squalius pyrenaicus* (Günther 1868) (PP genome; 2n=50) and an extinct species belonging to the lineage of the extant *Anaecypris hispanica* (Steindachner 1866) (AA genome; 2n=50) **[260-264]**. As usual among hybrid organisms **[265]**, the original hybridization event was unidirectional, involving *S. pyrenaicus* females and males of the extinct ancestor, given only *S. pyrenaicus* mitochondrial DNA is found in *S. alburnoides* individuals **[266]** (**Fig. 1**). The diploid hybrids formed (PA genome; 2n=50) surpassed sterility by developing asexual reproduction, namely clonal gametogenesis, producing allodiploid unreduced sperm and oocytes (*pa* genome; 2n=50). Crosses among the newly formed hybrids (PA×PA) and backcrosses with the parental species (PA×PP and PA×AA) led to the production of a hybrid complex of diploid (PA genome), triploid (PAA and PPA genomes) and tetraploid (PPAA genome) males and females (2n=50, 3n=75, 4n=100 **[267,268]**) with distinct combinations and proportions of the parental genomes (*i.e.* genomotypes) (**Fig. 1**). Past crosses between hybrids (P mitochondrial DNA) and the extinct paternal AA species (A mitochondrial DNA) allowed the reconstitution of the AA genomotype. This lineage of cytoplasmic hybrids (nuclear non-hybrid AA genome with P mitochondrial DNA) outlived the paternal ancestor of the complex and is still part of the reproductive dynamics of some *S. alburnoides* populations [269]. Reconstituted AA females are exceedingly rare, with only two individuals being found so far [259,270], and, thus, this lineage is considered all-male [269].

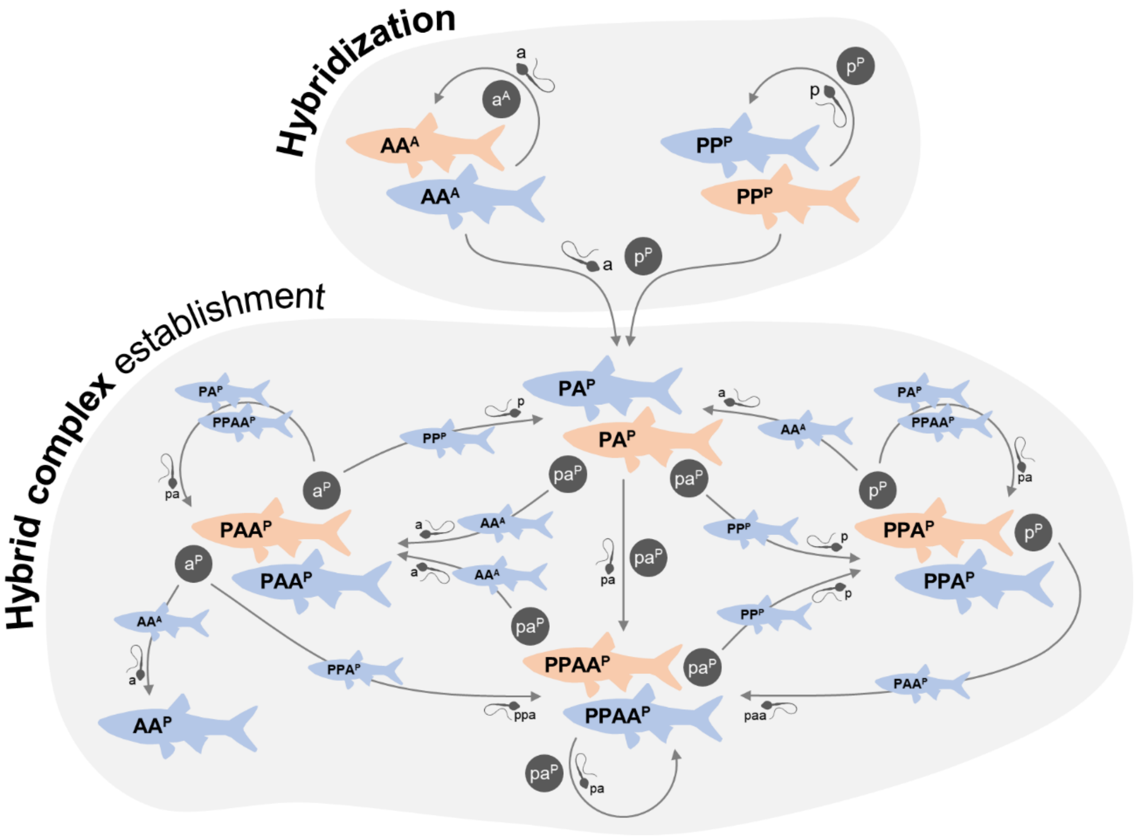

Figure 1. Original hybridization event and reproductive framework among hybrid and parental species leading to the establishment of the *S. alburnoides* allopolyploid complex. For details on reproductive modes and mechanisms, see text. Pink: females; blue: males. A/a, P/p: genomes of the extinct paternal and *S. pyrenaicus* maternal ancestors, respectively, in individuals (uppercase) and gametes (lowercase). Superscript letters: mitochondrial genomes.

All known *S. alburnoides* genomotypes are fertile and produce gametes trough a vast range of sexual and asexual reproductive mechanisms [143,261,267,268,271,272], namely:

a. Reductional meiosis with recombination (sexual reproduction) in AA males and in PPAA males and females, producing haploid *a* sperm and diploid *pa* sperm and oocytes, respectively;

b. Clonal gametogenesis in PA males and females and in PAA and PPA males, producing diploid *pa* sperm and oocytes and triploid *paa* and *ppa* sperm, respectively;

c. Meiotic hybridogenesis in PAA and PPA females, producing haploid *a* and *p* oocytes, respectively.

Ploidy levels higher than 4n are likely unviable, and other genome combinations of tetraploids (PAAA and PPPA) are extremely rare with unknown reproductive modes if any. Gynogenesis was only observed in a laboratorial cross, in a very low frequency of eggs, and meiotic hybridogenesis and clonal oogenesis occurring simultaneously in a single batch was once reported in a PAA female producing reduced (*a*) and unreduced (*paa*) oocytes, respectively **[273]**. Similarly, standard hybridogenesis was only inferred to occur once in a few diploid and triploid females **[259]**, but it was never observed again nor demonstrated using modern genetic tools. Thus, the apparent rarity of these occurrences suggests they were exceptional cases.

As in most hybrid complexes, the majority of *S. alburnoides* populations are highly biased towards females, a pattern that reflects the sex ratio of particular genomotypes, especially of triploids, which dominate most populations **[49,260]**. The reason behind these biased sex ratio is probably related to the underlying sex determination system, which is still unknown, but probably has a strong genetic basis, given perceived links between genotype and sex in *S. alburnoides* (*e.g.* the presence of an all-male lineage, AA). Studies have shown that the expression of certain sex-related genes differ between males and females, but results do not completely uphold the observed biases in *S. alburnoides* sex ratios **[274-276]**. Although hermaphroditism has been also reported in *S. alburnoides* **[277]**, the actual incidence of hermaphroditism at the population level is still unknown, but the rarity of such occurrence suggests it was likely exceptional and has virtually little to no impact on the dynamics of the complex.

Squalius alburnoides populations vary greatly in dynamics and genomotype composition, both in space and time. This variation is caused, among other factors, by changes in the species of the *Squalius* genus that coexist with *S. alburnoides* throughout the distribution range of the complex. There are three bisexual *Squalius* species that are sympatric and serve as sexual hosts to *S. alburnoides*, namely *S. carolitertii* (Doadrio 1988) (CC genome; 2n=50) in northern drainages (Douro, Vouga and Mondego), *S. pyrenaicus* (PP genome; 2n=50) in central-southern drainages (Tagus, Sado, Guadiana, Almargem, Guadalquivir and Odiel) and *S. aradensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira 1998) (QQ genome; 2n=50) in a southwestern drainage (Quarteira) **[49]**. Generally allopatric among themselves, all these bisexual species serve as sexual hosts to *S. alburnoides* hybrids, which parasitize their gametes to achieve reproduction (**Fig. 2**). Being hybridogenetic, *S. alburnoides* complex is constantly incorporating the genomes of the different sexual hosts (C, P or Q) into the offspring, leading to the substitution of the P ancestral genome by the ones of the respective sympatric *Squalius* species, and this, consequently, promotes a high divergence in genomotype composition among populations in distinct geographical areas (**Fig. 2**). Specifically, *S. alburnoides* populations most commonly include:

a. CA, CAA, CCA and CCAA genomotypes when sympatric with *S. carolitertii* (CC);
b. PA, PAA, PPA and PPAA genomotypes when sympatric with *S. pyrenaicus* (PP);
c. QA, QAA, QQA and QQAA genomotypes when sympatric with *S. aradensis* (QQ).

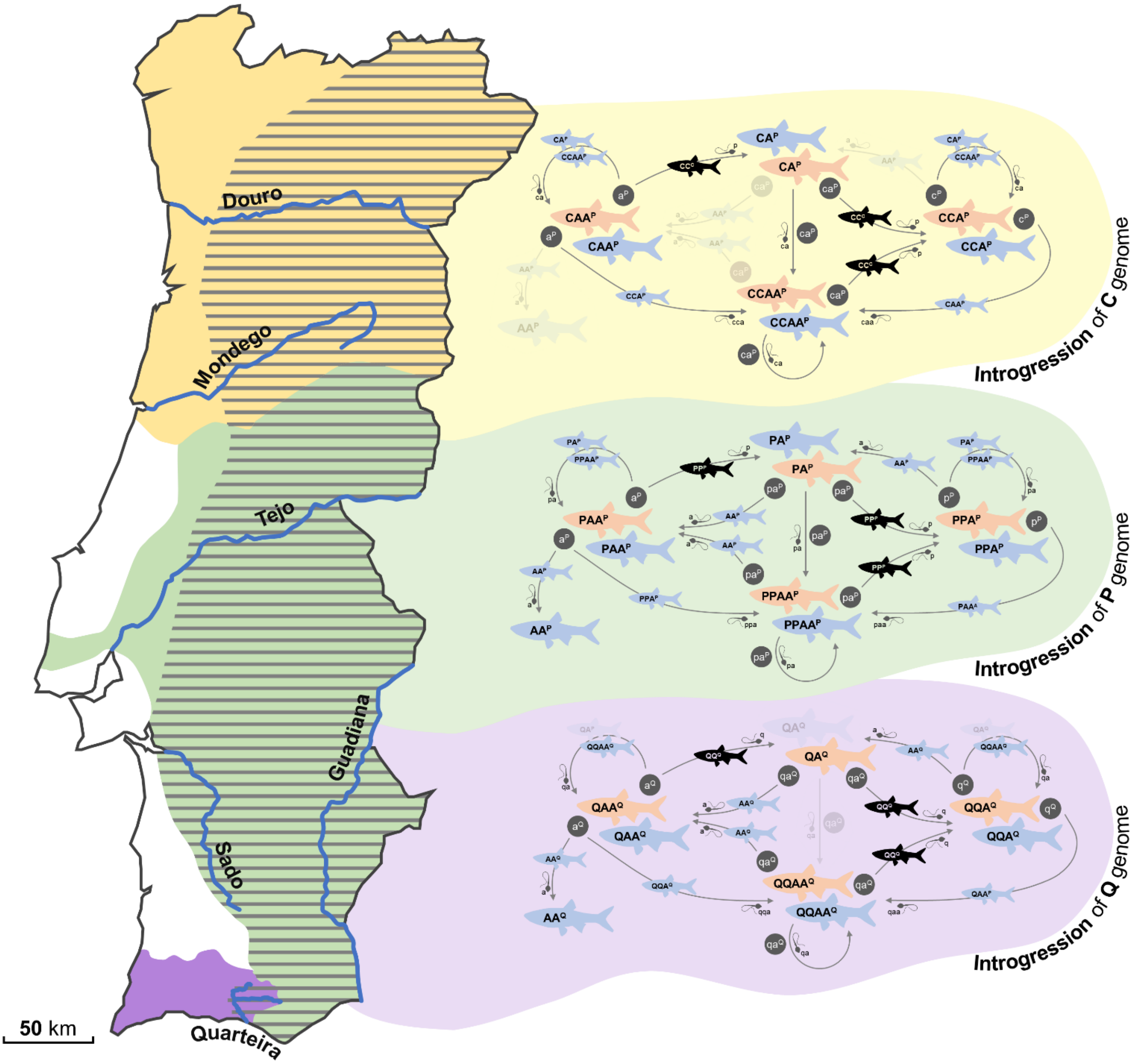

Figure 2. Distribution ranges of *S. alburnoides* (dashed), *S. carolitertii* (yellow), *S. pyrenaicus* (green) and *S. aradensis* (purple) across Portuguese drainages and the general reproductive dynamics of the respective populations. For Spanish distribution ranges, please see [49]. For details on reproductive modes and mechanisms, see text. Pink: *S. alburnoides* females; blue: *S. alburnoides* males; black: males of the sympatric

sexual species; watermarked: genomotypes never found on the respective drainage. A/a, C/c, P/p, Q/q genomes: genomes of the extinct paternal ancestor, *S. carolitertii*, *S. pyrenaicus* and *S. aradensis*, respectively, in individuals (uppercase) and gametes (lowercase). Superscript letters: mitochondrial genomes.

The introgression of the genomes of the sympatric *Squalius* species into the hybrid complex surely buffers the long-term genetic disadvantages associated with asexuality (*e.g.* accumulation of deleterious mutations and reduced genetic variability) **[260]**. Recently, *S. alburnoides* was found to hybridize also with *Alburnus alburnus* (Linnaeus 1758) **[278]**, though still modestly since this species was only recently introduced in Iberian freshwaters, but raising the question on whether the hybrid complex is putatively capable of also using exotic species as sexual hosts.

Although the distribution of *S. alburnoides* genomotypes as well as the distribution of the sympatric *Squalius* species may suggest that the hybrid complex had multiple ancestral parental species or original hybridization events (*i.e.* multiple origins), this is still a controversial topic. In the one hand, one study confirmed that hypothesis, suggesting five independent origins for the *S. alburnoides* complex starting in the Upper Pliocene and lasting a long period of geological time, namely in Douro, Tagus, Guadiana-Guadalquivir, Sado and Quarteira **[91]**. On the other hand, another study contradicted this hypothesis, suggesting a single origin for *S. alburnoides* in the bulk of Iberia (Tagus-Guadiana) in the Middle Pleistocene (~700.000 years ago) **[93]**.

1.5.2 | *Ecology and reproductive traits*

Squalius alburnoides is generalist in microhabitat and food usage **[279-283]**. Studies discriminating genomotypes suggest they occupy distinct ecological niches, showing spatial segregation and favouring different microhabitats, and adjust their diet according to food availability **[284,285]**. This possibly diminishes competition and facilitates coexistence among the various *S. alburnoides* genomotypes. Niche differences among genomotypes are accompanied by differences in morphology, evident by morphometry but not to the naked eye, with genomotypes varying in the shape and size of the body and particular structures, also when compared with the sympatric *Squalius* species **[286-288]**. Morphometric studies have shown that the congeneric bisexual species are generally larger (*e.g.* ~111 mm **[288]**) than all *S. alburnoides* genomotypes (*e.g.* ~45-88 mm **[287,288]**), and nuclear hybrids are also larger (*e.g.* ~68-88 mm **[287,288]**) than nuclear non-hybrid AA males (*e.g.* ~45 mm **[287]**). Although these differences suggest a genome-dosage effect and an additive inheritance pattern on individual size, phenotype may also be influenced by paternal genomotypes, as, for instance, triploid offspring with the same genomotype but fathered by different male genomotypes showed differences in growth rate [289].

Generally, females grow larger and live longer than males (6 years *vs.* 4 years of maximum age), although individuals lack evident sexual dimorphism [290-293]. Both males and females mature in the second year of life, and reproduction occurs between March and July [290,291,293]. *Squalius alburnoides* is a multiple spawner with external fertilization, with females laying batches of oocytes several times throughout the reproductive season [290,293]. Diploid and triploid females are very similar in reproductive traits, but diploid females seem to lay slightly more and larger oocytes than triploid females, possibly due their ploidy level (haploid *vs.* diploid, respectively) [293]. Diploid females laying more and larger eggs may guarantee them a reproductive advantage, which may uphold, at least partially, the higher proportion of the triploid genomotype in natural populations in which triploids are fathered by allodiploid females (see below).

Very little is known about *S. alburnoides* reproductive behaviour. The only study performed so far reported a mate preference by hybrid females towards AA over nuclear hybrid males [294], though variation in female genomotype was not accounted and males of the sympatric *Squalius* species were not included in experiments. As previously mentioned, mate choice is one of the main mechanisms upholding the persistence of hybrid complexes over time or routing them towards hybrid speciation. Thus, the lack of in-depth studies focusing on mate choice in *S. alburnoides* was normally considered a major gap in the understanding of this hybrid complex, its dynamics and evolutionary pathways.

1.5.3 | *Reproductive dynamics, tetraploidization and hybrid speciation*

Although the replacement of the ancestral maternal genome by the genomes of the sympatric *Squalius* species leads to obvious differences in genomotype composition among *S. alburnoides* populations [93], it does not influence the reproductive dynamics of the complex, since the reproductive mechanisms of each genomotype are unaltered regardless of the genomes they comprise (*e.g.* CAA, PAA and QAA females all reproduce through meiotic hybridogenesis, producing haploid *a* oocytes) [49]. The differences in reproductive dynamics among natural populations are rather caused by other factors, also varying among drainages, such as the frequency of each genomotype, the presence/absence of AA males and the sex ratio. The reconstituted AA non-hybrid males are only present in central-southern populations, being absent from northern drainages [93,101,295]. The reason for this differential distribution may be related to temperature, with AA males being possibly not adapted to the lower temperatures of the northern drainages [93]. Moreover, the sex ratio of diploid hybrids also differs among drainages, with males and females being found in northern drainages (though mainly males), but only females being found in southern drainages [49]. The reason behind this differential sex ratio is still unknown, but it obviously leads to marked differences in the reproductive dynamics of populations. Considering the variation on these variables, three types of populations may be distinguished, with marked differences in reproductive dynamics:

a. **Triploid-dominated populations via allodiploid females**: This reproductive dynamics is typical of southern and some central populations, characterized by the dominance of triploid females, the presence of AA males and the absence of allodiploid males. Triploid females (PAA/QAA genomotypes; *a* oocytes) are produced in crosses between allodiploid females (PA/QA genomotypes; *pa*/*qa* oocytes, respectively) and AA males (*a* sperm). In turn, allodiploid females result from crosses between the triploid females and males of the sympatric bisexual *Squalius* species (PP/QQ genomotypes; *p*/*q* sperm, respectively) or between females of the sympatric *Squalius* species (PP/QQ genomotypes; *p*/*q* oocytes, respectively) and AA males. Lastly, AA males are produced from crosses between triploid females and AA males. The influence of the females of the sympatric *Squalius* species in the reproductive dynamics of the hybrid complex seems limited to this type of population, since the mitochondrial genomes of the hybrids and of the sympatric *Squalius* species are similar only where AA males are present, suggesting that these males are the only reproducing with non-hybrid females, possibly via sneaking behaviour, parasitizing crosses of the sympatric species [93,287,296];

b. **Triploid-dominated populations via allodiploid males**: This reproductive dynamics is typical of northern and some central populations, characterized by the dominance of triploid females, the absence of AA males and the presence of allodiploid males. Triploid females (CAA/PAA genomotypes; *a* oocytes) are produced in crosses between triploid females and diploid males (CA/PA genomotypes; *ca*/*pa* sperm, respectively). In turn, diploid males and females (CA/PA genomotypes; *ca*/*pa* sperm and oocytes, respectively) result from crosses between the triploid females and males of the sympatric *Squalius* species (CC/PP genomotypes; *c*/*p* sperm, respectively). The presence of allodiploid males and females simultaneously in the same populations offers the hybrid complex a direct path for tetraploidization (CA/PA males, *ca*/*pa* sperm × CA/PA females, *ca*/*pa* oocytes = CCAA/PPAA offspring) [271], an unavailable route in populations without allodiploid males;

c. **Tetraploid-dominated populations**: This reproductive dynamics was only found in three populations (Lodeiro and Paiva Rivers, Douro drainage, and in Peal del Becerro Spanish cave, Guadalquivir drainage) [241,297], characterized by the dominance of the symmetrical tetraploid genomotype with a balanced sex ratio (CCAA/PPAA genomotypes; *ca*/*pa* sperm and oocytes, respectively) (**Fig. 3**).

Tetraploidization is the obvious route towards hybrid speciation in *S. alburnoides* (**Fig. 3**), since tetraploids are the only autonomous genomotype of this hybrid complex, dispensing the involvement of any other hybrids or sympatric species in their independent reproductive dynamics, given crosses between male and female tetraploids father tetraploid offspring via sexual reproduction (reductional meiosis with recombination). If assortative mating settles among tetraploids, the establishment of a new species is on the verge, although with twice the number of chromosomes of the parental species, following genome diploidization (*i.e.* 4n=100 becoming 2n=100) (**Fig. 3**).

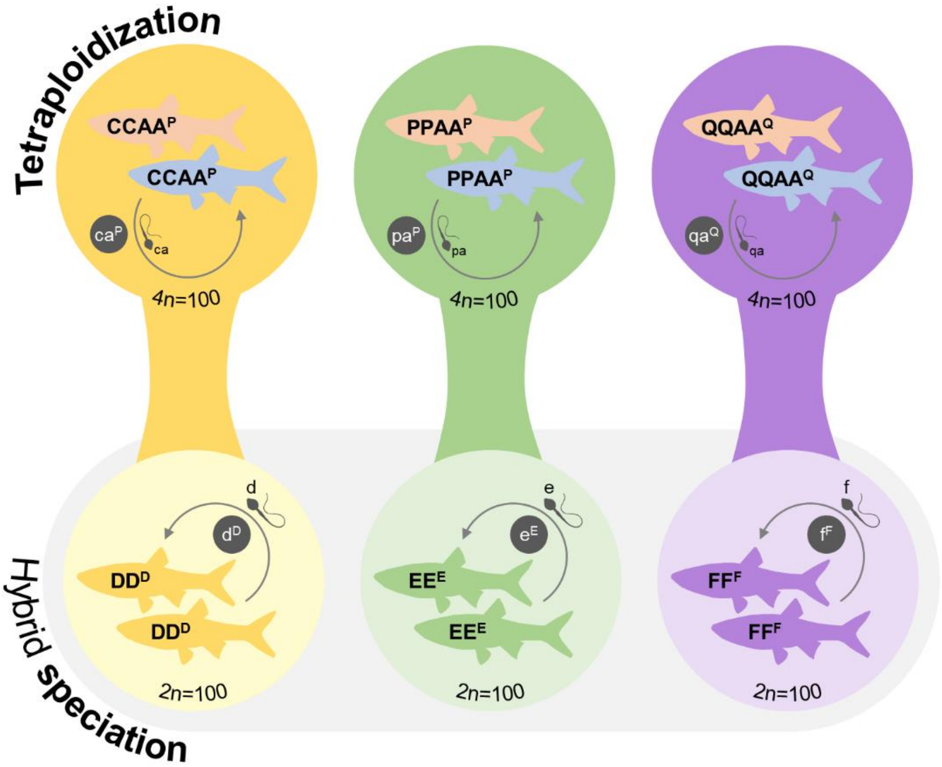

Figure 3. Tetraploidization and consequent arising of new independent sexual species through hybrid speciation, following genome diploidization. Pink: *S. alburnoides* females; blue: *S. alburnoides* males. A/a, C/c, P/p, Q/q: genomes of the extinct paternal ancestor, *S. carolitertii*, *S. pyrenaicus* and *S. aradensis*, respectively,

in individuals (uppercase) and gametes (lowercase). D/d, E/e, F/f: genomes of the newly formed species, in individuals (uppercase) and gametes (lowercase). Superscript letters: mitochondrial genomes.

The diversity of *S. alburnoides* populations and reproductive dynamics is a window of opportunity to study the interesting and peculiar behavioural, ecological and evolutionary topics offered by such unique hybrid asexual organisms. Ranging from hybrid populations maintained by intricate reproductive dynamics among several genomotypes to populations on the verge of hybrid speciation (see **Appendix 1** for a complete diagram on *S. alburnoides* evolutionary pathways), *S. alburnoides* is undoubtedly a valuable animal model to answer key questions in Evolutionary Biology.

1.6 | *Objectives and structure of the present thesis*

In this PhD thesis, we studied the influence of mate choice and reproductive traits in the dynamics of *S. alburnoides*, assessing their role in maintaining natural populations in their hybrid state or routing them towards hybrid speciation. To achieve this main goal, we conducted a series of experiments involving theoretical modelling and empirical testing, bridging together behavioural and genetic tools, aimed at accomplishing the following specific objectives:

i. Assess the mate choice patterns of *S. alburnoides* females in a population with high diversity of male genomotypes. We designed a theoretical equation to predict the future of this model population based on female preferences and genomotype frequency, in order to ascertain which mate choice patterns allow the persistence of the complex in its hybrid state and which route the population towards hybrid speciation. For the purpose, we merged together empirical and theoretical outcomes, and the resulting article is already published in an international journal (**Chapter 2**);

ii. Assess the role of pre- and postzygotic reproductive traits in the dynamics and genomotype composition of the same model *S. alburnoides* population. Specifically, we translocated a previously genetically screened random sample of *S. alburnoides* and *S. pyrenaicus* to an exterior pond, in which fish could freely mate during an entire reproductive season, without any human interference. Offspring was then collected and genetically analysed, using microsatellite parentage analysis, to determine the reproductive success of each parental fish translocated to the pond. We complemented this experiment by testing egg allocation, fertilization rate and offspring survival in an extra set of directional crosses between hybrid females and males of the several genomotypes in the population, to unveil the processes behind the differences in reproductive success observed among the fish reproducing in the pond. Altogether, we assessed the role of reproductive traits in the genomotype composition and maintenance of the model population, and the resulting article was already published in an international journal (**Chapter 3**);

iii. Investigate the role of mate choice by hybrid females in driving introgression of the genomes of distinct *Squalius* sympatric species into the hybrid complex, using as study model a population in which *S. pyrenaicus* and *S. aradensis* were found in sympatry with *S. alburnoides*. We performed a series of mate choice affiliation trials to assess the preference of *S. alburnoides* females towards all the available male genomotypes to evaluate whether and which of the three genomes (A, P and Q) were preferentially introgressed into the hybrid complex via female mate preferences. Introgression levels of each genome were assessed through next-generation genotyping. We bridged together the results of affiliation trials with the in-depth genotyping data to evaluate the role of mate choice in shaping the future of the hybrid and non-hybrid populations, and the respective manuscript was already submitted to an international journal (**Chapter 4**).

During the accomplishment of these specific objectives, we found by serendipity a whole new reproductive mode for *S. alburnoides* – androgenesis – that was the first reported case of such asexual reproductive mode among vertebrates in a natural context and without any experimental manipulation. Although the extension of its incidence in natural populations is still to be assessed, androgenesis represents an easy alternative for the reproductive independence of *S. alburnoides* hybrid populations, through which hybrids may become autonomous from the *Squalius* hosts and abandon sexual parasitism, the first step towards hybrid speciation. This unexpected result in the context of this thesis was already published in an international journal (**Chapter 5**).

The above experiments were accomplished using samples from distinct populations of *S. alburnoides* and from sympatric congeneric species, namely from Ribeira do Cobrão (Ocreza River, Tagus drainage), Ribeira dos Moinhos and Ribeira da Fonte Menalva (Quarteira drainage), Ribeira do Almargem (Almargem drainage) and Ribeira do Arade (Arade drainage). Several genetic markers were used throughout the studies, namely nuclear markers (*β-actin*), SNP's obtained from next-generation genotyping (3.376 loci analysed, chosen from a total of 427.624 loci identified), microsatellites (LCO1, LCO3, LCO4, LC27, LC288, LC290, Lsou05, Lsou08, Lsou34 and SpyrAMT) and mitochondrial markers (D-Loop/Control Region). Experiments on mate choice and reproductive traits took place on several experimental setups, as specifically stated in each chapter, including: a) a branched tank with divisions aimed for affiliation trials; b) tanks especially designed for the collection of eggs of fishes with external fertilization; and c) an exterior pond. Field and laboratorial procedures followed the recommended ethical guidelines **[298-300]** and the Portuguese legislation regarding animal capture, manipulation and experimentation for scientific purposes (Instituto da Conservação da Natureza e das Florestas – ICNF, permit numbers 140/2012/CAPT and 239/2013/CAPT). Because studied taxa are listed as threatened **[301-303]**, sample sizes were generally small, to avoid natural stock depletion. All efforts were made to minimize accidental deaths and stress on fish throughout the studies. Captured fish were returned alive to the sites of origin after the experiments.

The findings uncovered in the present thesis were integrated altogether in a General Discussion to picture the transversal influence of reproductive behaviour in all stages of *S. alburnoides* evolution, from the maintenance of the hybrid populations and mating dynamics with the sympatric host species to tetraploidization and hybrid speciation. The general outcome of such joint findings is discussed in-depth from ecological, behavioural and evolutionary perspectives, opening a path for future research (**Chapter 6**).

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CHAPTER 2

Mate choice drives evolutionary stability in a hybrid complex

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RESEARCH ARTICLE

Mate Choice Drives Evolutionary Stability in a Hybrid Complex

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Abstract

Previous studies have shown that assortative mating acts as a driver of speciation by countering hybridization between two populations of the same species (pre-zygotic isolation) or through mate choice among the hybrids (hybrid speciation). In both speciation types, assortative mating promotes speciation over a transient hybridization stage. We studied mate choice in a hybrid vertebrate complex, the allopolyploid fish *Squalius alburnoides*. This complex is composed by several genomotypes connected by an intricate reproductive dynamics. We developed a model that predicts the hybrid complex can persist when females exhibit particular mate choice patterns. Our model is able to reproduce the diversity of population dynamic outcomes found in nature, namely the dominance of the triploids and the dominance of the tetraploids, depending on female mate choice patterns and frequency of the parental species. Experimental mate choice trials showed that females exhibit the preferences predicted by the model. Thus, despite the known role of assortative mating in driving speciation, our findings suggest that certain mate choice patterns can instead hinder speciation and support the persistence of hybrids over time without speciation or extinction.

Introduction

Many studies have shown that assortative mating acts as a driver of speciation [1–3], especially through the reinforcement of pre-zygotic isolation [4–8]. However, the relationship between assortative mating and hybrid speciation is still not well understood. Hybrid speciation occurs when two species reproduce to form hybrid organisms which over time evolve into a new species, with or without genome multiplication (allopolyploid and homoploid hybrid speciation, respectively) [9, 10]. By recreating the original hybridization events through experimental crosses between the parental species, some empirical studies suggested a key role for mate choice in driving homoploid hybrid speciation [11–13]. However, the role of mate choice in

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allopolyploid speciation remains unknown. Here, we studied mate choice in a well-established allopolyploid organism which may be on the verge of hybrid speciation [14].

Squalius alburnoides is an Iberian freshwater fish originated by the hybridization of females of the still sympatric *Squalius pyrenaicus* (PP genome, P oocytes) with males from an extinct species related to the extant *Anaecypris hispanica* (AA genome, A sperm) [15]. These intergeneric crosses produced fertile hybrids (PA genome) with clonal gametogenesis (PA gametes). In turn, crosses between these allodiploids and backcrosses with the parental species originated a successful hybrid complex that includes fertile males and females with distinct ploidies ($2n = 50$, $3n = 75$ and $4n = 100$) and different combinations of the parental genomes (genomotypes) (reviewed in [16]). This diversity of fertile genomotypes enables a multiplicity of crosses, with females being able to mate with several distinct male genomotypes (Fig 1A).

Squalius alburnoides natural populations vary in their composition of genomotypes. Two distinct population types with utterly distinct reproductive dynamics may be defined, namely triploid-dominated and tetraploid-dominated populations (Fig 1B). The overall sex-ratio of triploid-dominated populations is highly female-biased, with males only representing around 15% of the allotriploid genomotype (PAA) that dominate in such populations. PAA females may breed with any of the male genomotypes available in the population, namely allodiploid (PA), allotriploid (PAA) and balanced tetraploid (PPAA) males, but also males from the sympatric *S. pyrenaicus* non-hybrid species (PP) (Fig 1A). PAA females reproduce by meiotic hybridogenesis, a reproductive mode in which the heterospecific genome (P) is discarded and the remaining homologous genomes (AA) undergo meiosis, producing haploid oocytes (A) [16]. Consequently, these females may generate three types of offspring: a) PAA offspring from crosses with PA or PPAA males (which produce PA sperm through clonal gametogenesis and meiosis, respectively), restocking the triploid genomotype in the population; b) PA offspring from crosses with the sympatric *S. pyrenaicus* non-hybrid species (which produces, bisexually, P sperm through meiosis); and c) PAAA offspring from crosses with PAA males (which produce PAA sperm through clonal spermatogenesis) (reviewed in [15, 16]). In triploid-dominated populations, most genomotypes are interdependent, meaning their production depends exclusively on crosses involving other genomotypes (Fig 1A). Thus, triploid-dominated populations rely on the maintenance of a high variability of genomotypes in order to persist over time.

The same requirement does not apply to the tetraploid-dominated populations because they are mainly composed by the only *S. alburnoides* self-sustainable genomotype (PPAA). The PPAA genomotype has a balanced sex ratio, with males and females producing allodiploid (PA) sperm and oocytes through meiosis [16]. Thus, the offspring produced by crosses between PPAA males and females is also PPAA, not requiring the involvement of any other genomotype and also of the sympatric *Squalius* non-hybrid species. This independency not only allows for a much simpler reproductive dynamics in tetraploid-dominated populations (Fig 1A), but also potentiates hybrid speciation through assortative mating [14]. That is, if PPAA females show a stronger preference for PPAA males over other male genomotypes (assortative mating), this would favor the evolution of a new independent species, an evolutionary pathway not available in triploid-dominated populations due to their obligatory genomotype interdependency. In fact, classic assortative mating is not possible to occur among the PAA genomotype because crosses between PAA males and females do not father PAA offspring, meaning PAA females have to mate disassortatively in order to produce offspring of their own type. Moreover, the offspring produced when PAA females mate assortatively (PAAA genomotype) is extremely rare in natural populations, which suggests that assortative mating is unlikely to be occurring among the PAA genomotype. Thus, triploid-dominated populations only succeed if PAA females have a less strict mate choice pattern, allowing them to mate with distinct male

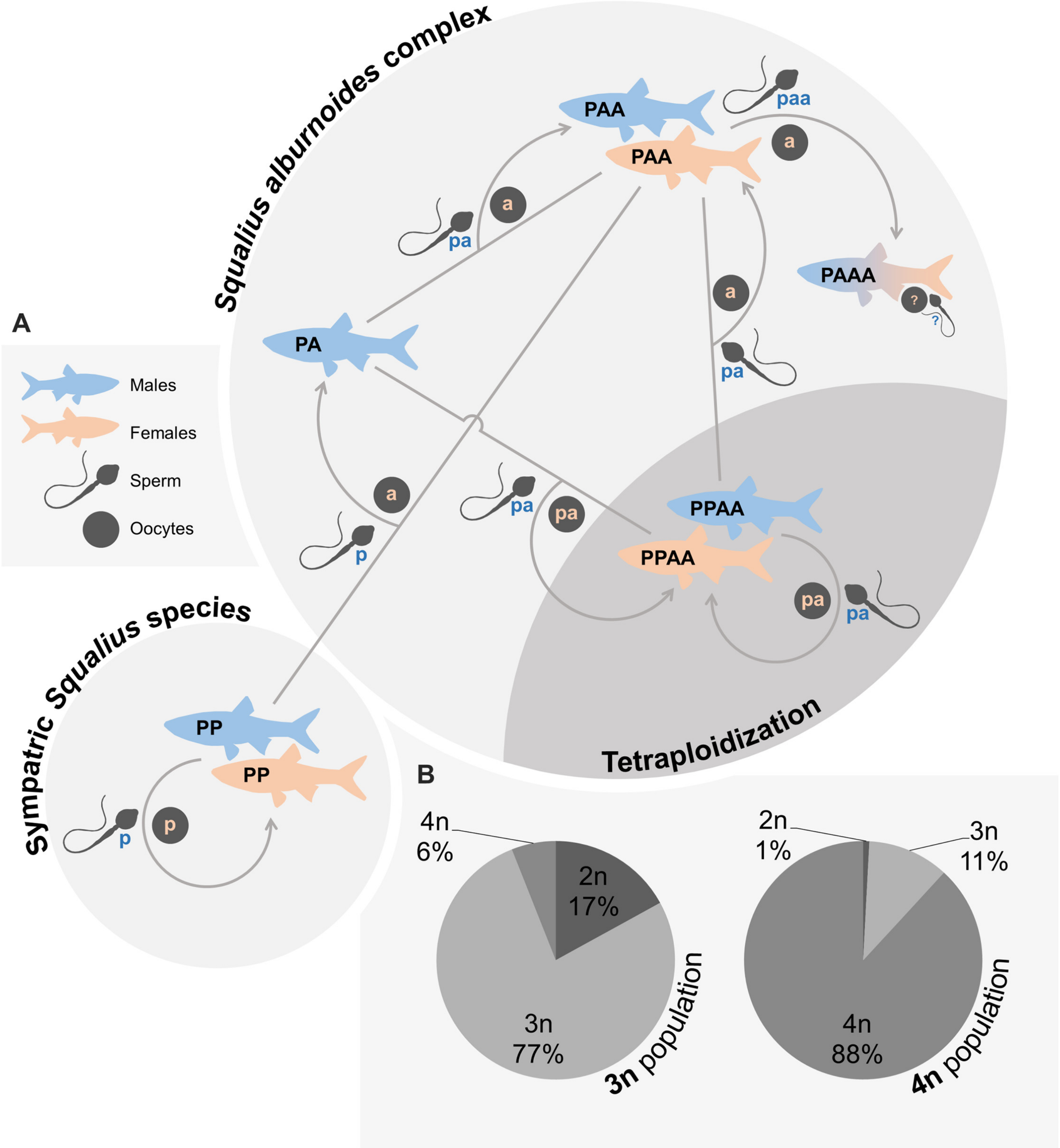

Fig 1. Reproductive dynamics and example genomotype compositions of the *S. alburnoides* complex. A. Simplified diagram of *S. alburnoides* reproductive dynamics in a triploid-dominated (light grey area) and in a tetraploid-dominated (dark grey area) populations. See Introduction for more details about the reproductive modes of each genomotype. The genome nomenclature used was based on central and southern populations where the bisexual *Squalius* non-hybrid species is *S. pyrenaicus* (PP). Other non-hybrid *Squalius* species are also sympatric with *S. alburnoides* in other geographic regions (*S.*

carolitertii, CC genome, in northern populations and *S*. *aradensis*, QQ genome, in an isolated southwestern population), but their involvement in the reproductive dynamics of the complex is identical to the one shown. Very little is known about the extremely rare PAAA genomotype and, thus, their sex ratio and reproductive modes remain unknown. For other reproductive dynamics found in natural populations see the review by [16]. **B.** Examples of triploid-dominated and tetraploid-dominated populations. The shown ploidy levels only refer to the *S*. *alburnoides* genomotypes, being the proportions of the diploid *Squalius* non-hybrid species not represented on the charts.

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forms and, therefore, maintain genomotype variability. Indeed, other studies have suggested that mate choice plasticity allows the maintenance of polymorphisms in natural populations of several species [17–21].

The two *S. alburnoides* population types are not evenly found in the wild. Whereas triploid-dominated populations abound across *S. alburnoides* distribution range, only two tetraploid-dominated populations have been found so far [14]. This pattern suggests that the reproductive strategies ruling each population type may not be equally successful. The flexible mate choice patterns occurring among the triploid genomotype seem to overrule the effect of the assortative mating occurring among the tetraploid one, thus preventing tetraploidization and maintaining most populations triploid-dominated (i.e. in their hybrid state). If so, mate choice may be hindering hybrid speciation in *S. alburnoides* complex.

In order to test this hypothesis, we simulated the theoretical effect of a wide range of mate preferences and genomotype frequencies in shaping the composition of natural populations over time. This theoretical approach was complemented by experimental trials, aimed at studying mate choice in *S. alburnoides* PAA females, allowing them to choose among the available male genomotypes. The results obtained were assessed in order to evaluate how the observed mate choice patterns of the most common and abundant female genomotype influence the genomotype composition of the offspring produced and whether such mate tendencies route or counter tetraploidization and, consequently, hybrid speciation.

Materials and Methods

Ethics Statement

Fish captures were carried out with the permission of Instituto da Conservação da Natureza e das Florestas (permit numbers 140/2012/CAPT and 239/2013/CAPT). Although the taxa studied are threatened, the population chosen for sampling (Ocreza River, Tagus drainage) was not imperiled and the sample size was chosen to avoid depletion of the natural stock. Fishes were handled following recommended ethical guidelines [22]. Electrofishing was performed in low duration pulses to avoid killing juveniles (300 V, 2–4 A) and the transport to the laboratory was made in appropriate aerated containers. The portion of fin used for genomotype assessment was minimum and the removal was performed in a peripheral area of the fin in order to guarantee a fast regrowth of the tissue and minimize fish discomfort after awakening from the anesthesia (0.1 g/L MS-222, 0.2 g/L $NaHCO_3$). The study was not carried out on private land and all specimens were returned alive to the collecting site after the experiments.

Theoretical Modeling

We formulated a theoretical model for the dynamics of the genomotype frequencies in a *S. alburnoides* population, using female mating preference and *S. pyrenaicus* frequency as model parameters.

The frequency of each male and female genomotype in a population determines the encounter probability of two particular genomotypes. This encounter probability would be directly proportional to mating success if there were no other factors, such as differential mate choice, affecting or biasing cross occurrence. In the former case, the probability of a female mating with a male of a given type would be a linear function of the frequency of those males. Adding the effect of female mate preference leads to a non-linear response and an increase of the mating probability with the favored males (Fig 2). Note that, because *S. alburnoides* is a multiple spawner, females can breed with distinct male genomotypes and produce distinct offspring in a single reproductive season. Considering that, at time t, a particular male genomotype (M_j) occurs with a frequency f_{Mj} in the population, and a particular female genomotype (F_i), occurring with f_{Fi} frequency, has $\varphi_{Fi\rightarrow Mj}$ preference for that male genomotype, then the probability ($p_{Fi\times Mj}$) of a cross between females i and males j is:

$$p_{F_i\times M_j}(t) = \frac{f_{F_i}(t)f_{M_j}(t)\varphi_{F_i\rightarrow M_j}}{\sum_{k=1}^{n_F} f_{F_k}(t)\sum_{l=1}^{n_M}(f_{M_l}(t)\varphi_{F_i\rightarrow M_l})}$$

This probability also represents the proportion of the particular offspring genomotype arising from crosses between females i and males j, assuming that: a) the encounter probability of a pair of genomotypes is only a function of their frequencies in the population, not being affected by other factors such as differential spatial segregation, search rates and conspicuity, among others; b) males are not choosy, meaning they breed indifferently with any female genomotype; c) individual females have similar reproductive successes per reproductive season, regardless of genomotype; d) male genomotypes have similar reproductive capabilities, being equally able to fertilize oocytes (e.g. same sperm quality); and e) the viability and survival of the offspring produced is similar for all cross types. Note that our model aims at assessing how mate choice shapes population dynamics and does not address whether the simulated preferences are adaptive.

We inferred mate preferences ($\varphi_{Fi\rightarrow Mj}$) for each female genomotype from the reproductive dynamics of each population type, that is, triploid- and tetraploid-dominated populations. Thus, we assumed assortative mating to occur only among the self-sustainable PPAA genomotype and allowed PAA females to have a more flexible mate choice pattern, due to their obligatory reproductive interdependency. Although there might be up to four male genomotypes in triploid-dominated populations (Fig 1A), we grouped males according to their functional role in their reproductive dynamics because some males, namely PA and PPAA, produce the same sperm type and, consequently, father the same offspring. We considered three male groups: a) type I males, comprising PA and PPAA males, which produce PA sperm and father PAA offspring with PAA females; b) type II males, the ones from the sympatric *S. pyrenaicus* bisexual species (PP genome, P sperm), which father PA offspring with PAA females; and c) type III males, the PAA ones, which produce PAA sperm and father PAAA offspring with PAA females. In triploid-dominated populations, PAA is the most frequent genomotype, followed by the PA and, lastly, by the PAAA one (absent in most populations). These relative frequencies suggest that the male genomotypes which father PAA offspring (PA and PPAA males) may have a higher reproductive success than the ones fathering PA offspring (PP males) and a much higher than the ones fathering PAAA offspring (PAA males), being thus denominated here as type I, type II and type III males, respectively. These differential male reproductive successes may be due to a higher preference of PAA females towards type I males than towards the type II ones ($0<\varphi_{FPAA\rightarrow MII}< \varphi_{FPAA\rightarrow MI}<1$). Nonetheless, we simulated the entire range of preferences towards these two male types (0.002 steps), allowing either type I or type II males to be favored by PAA females (Table 1). We assumed that females reject type III males ($\varphi_{FPAA\rightarrow MIII} = 0$; Table 1) because the offspring produced from crosses between PAA males and females (PAAA genomotype) is absent in the vast majority of natural populations and, when present, occurs at extremely low frequencies (~1:500).

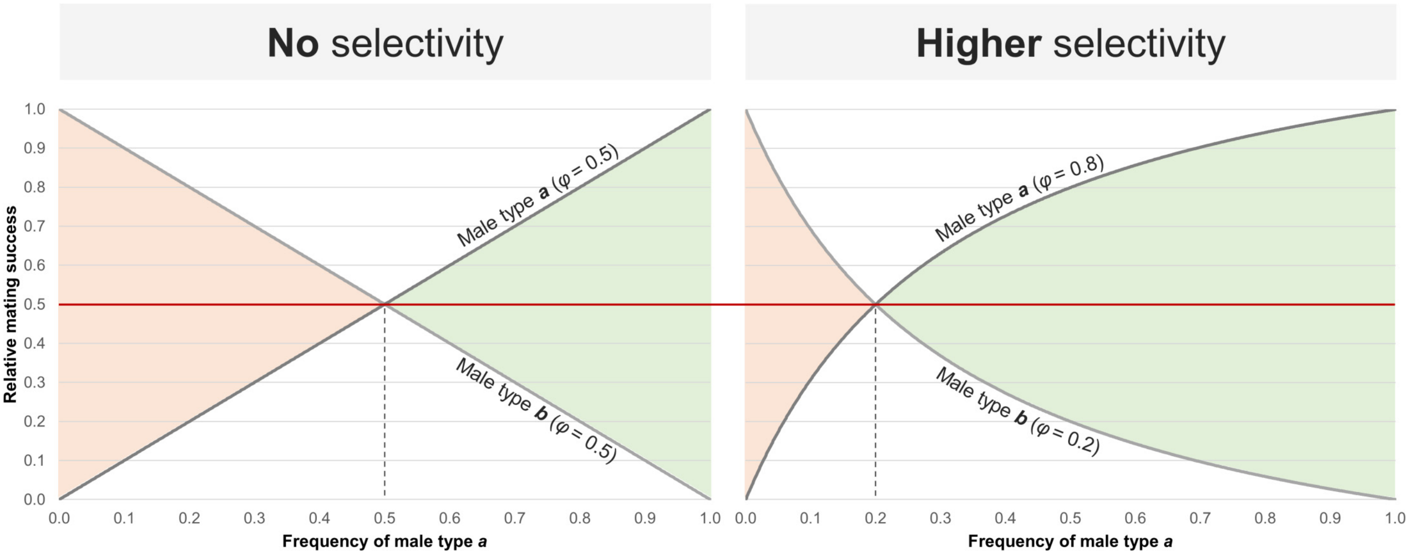

Fig 2. Relative mating success of two theoretical male types as a function of their frequency and female preference (φ). The intersection between both lines bounds two areas: the red area, in which male type *b* has a higher relative mating success, and the green area, in which male type *a* has a higher relative mating success. If female preference is similar towards both male types ($\varphi = 0.5$; i.e. females choosing male type *a* or male type *b* for mating is equally probable), the male relative mating success depends exclusively on their frequency in the population (assuming that the effect of all other synergistic factors affecting male mating success are similar for both male types). However, if females show a higher preference for a particular male type (e.g. $\varphi = 0.8$ for male type *a*), the frequency-based functions of the male relative mating success deviate, increasing the green area and decreasing the red area, that is, increasing the relative mating success for male type *a* and decreasing it for male type *b*.

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We assessed multiple initial population compositions by varying the relative proportions of each genomotype, namely by increasing or decreasing the frequency of PAA and PPAA genomotypes (Table 2) and, thus, simulating triploid- and tetraploid-dominated populations. For each initial population composition, we ran our model until genomotype frequencies reached stability. Offspring composition at each generation *t* was calculated using the genomotype frequencies at *t*-1 and used as the new parental composition for the next generation (*t*+1). Offspring sex ratios (R_g) applied in the model for each genomotype were based on joint data from well-studied triploid- and tetraploid-dominated populations ($R_{FPA} = 0.00$, $R_{MPA} = 1.00$; $R_{FPAA} = 0.85$, $R_{MPAA} = 0.15$; $R_{FPPAA} = 0.50$, $R_{FPPAA} = 0.50$). Note that the sympatric *S. pyrenaicus* (PP), whose males also participate in *S. alburnoides* reproductive dynamics (Fig 1A), does not belong to the hybrid complex itself. It is an autonomous non-hybrid species with even sex ratios ($R_{FPP} = 0.50$, $R_{MPP} = 0.50$) and independent population dynamics. For this reason, its frequency among the overall fish population (*S. alburnoides* plus *S. pyrenaicus*) was kept fixed over all generations of each simulation. The entire range of possible PP frequencies ($0<f_{PP}<1$, 0.002 steps) was tested in the model.

Table 1. Preferences of PAA and PPAA females towards type I, type II and type III males simulated in the model. The preferences of PAA females consist in a flexible mate choice pattern because it includes a certain degree of preference towards both type I and type II males, whereas PPAA females only favor type I males for mating. Note that PPAA females also produce offspring of their own genomotype in crosses with PA males (see text for further details).

	Type I males	**Type II males**	**Type III males**	
Females	PA and PPAA	PP	PAA	**Mate choice pattern**
PAA	$0<\varphi_{FPAA\to MI}<1$	$\varphi_{FPAA\to MII} = 1\text{-}\varphi_{FPAA\to MI}$	$\varphi_{FPAA\to MIII} = 0$	Flexible
PPAA	$\varphi_{FPPAA\to MI} = 1$	$\varphi_{FPPAA\to MII} = 0$	$\varphi_{FPPAA\to MIII} = 0$	Assortative mating

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Table 2. Ranges of genomotype frequencies used to generate the initial population compositions for the model. All initial genomotype compositions were aimed at recreating triploid- and tetraploid-dominated populations and used as starting points for all sets of simulations.

Population initial composition	Genomotype frequencies ($f_{PA}+f_{PAA}+f_{PPAA}$ = 1.0)		
	PA	PAA	PPAA
Triploid-dominated	$0.0<f_{PA}<0.5$	$0.5<f_{PAA}<1.0$	$0.0<f_{PPAA}<0.5$
Tetraploid-dominated	$0.0<f_{PA}<0.5$	$0.0<f_{PAA}<0.5$	$0.5<f_{PPAA}<1.0$

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Thus, the overall dynamics of our theoretical model is

$$f_g(t+1) = R_g \sum_i \sum_j (p_{F_i \times M_j}(t) b_g(F_i \times M_j))$$

where b_g is a binary variable assuming a value of 1 when the cross between females *i* and males *j* originates offspring of genomotype *g* and a value of 0 otherwise. All simulations were performed in R software v2.15.2 [23].

Mate Choice Trials

We assessed mate preferences of PAA females through mate choice experiments. Trial females were allowed to choose among the male genomotypes present in a triploid-dominated population (Tagus drainage). This population was also used as one of the main references for the model, namely regarding sex-ratios. A random sample (N = 41) of *S. alburnoides* (N = 25: f_{MPA} = 0.28, f_{FPAA} = 0.56, f_{MPAA} = 0.12, f_{MPPAA} = 0.04) and *S. pyrenaicus* (N = 16: f_{FPP} = 0.50, f_{MPP} = 0.50) was collected from Ocreza River during the reproductive season. The capture was performed randomly, trying to cover all available habitats in order to guarantee that the genomotype composition of the sample would be representative of the one found in the studied population. Note that *S. alburnoides* and *S. pyrenaicus* are threatened fishes, classified as Vulnerable and Endangered [24], respectively, which raises ethical challenges to the capture of larger samples. The individuals were sexed by applying a mild and brief pressure on the abdomen, forcing the extrusion of a few gametes. The fish were transported to the laboratory and small fin clips were used to assess the genomotype of each individual by flow cytometry [25] and sequencing of the *β-actin* gene [26]. Individuals were kept together in a maintenance tank (250 L) with a 14h/10h light/dark cycle, mimicking the natural conditions of the reproductive season, and were fed twice a day with an adequate amount of frozen bloodworms and brine shrimp. The water quality was assessed on a weekly basis.

The experimental trials started after a two-week habituation period to captivity, also ensuring that the small portion of tissue collected from the terminal edge of the fins was fully regrown. Individual recognition was performed using scale patterns [27]. Each experimental trial was conducted in a mate choice tank, specially designed for the purpose (Fig 3). In each trial (N = 11), a single individual of each male genomotype (PA, PAA, PPAA and PP) was inserted randomly in each of the male compartments and a single PAA female in the central neutral area of the experimental tank. Due to the rarity of some male genomotypes, some stimulus males were used more than once in the affiliation trials (contrary to females, which were never repeated). Note that adult genomotypes have distinct characteristic lengths [28, 29], making it impossible to isolate the effect of fish size (mean standard lengths: ♂PA, 5.38 cm; ♀PAA, 5.84 cm; ♂PAA, 7.20 cm; ♂PPAA, 5.40 cm; ♂PP, 7.09 cm). Trial females (N = 11) were allowed to swim freely across the tank and visit each of the males during a period of 1h 30min. The trials were recorded using a digital camera for ulterior video analysis in which the proportion of time spent by females near each male was measured. The first half hour of each trial was

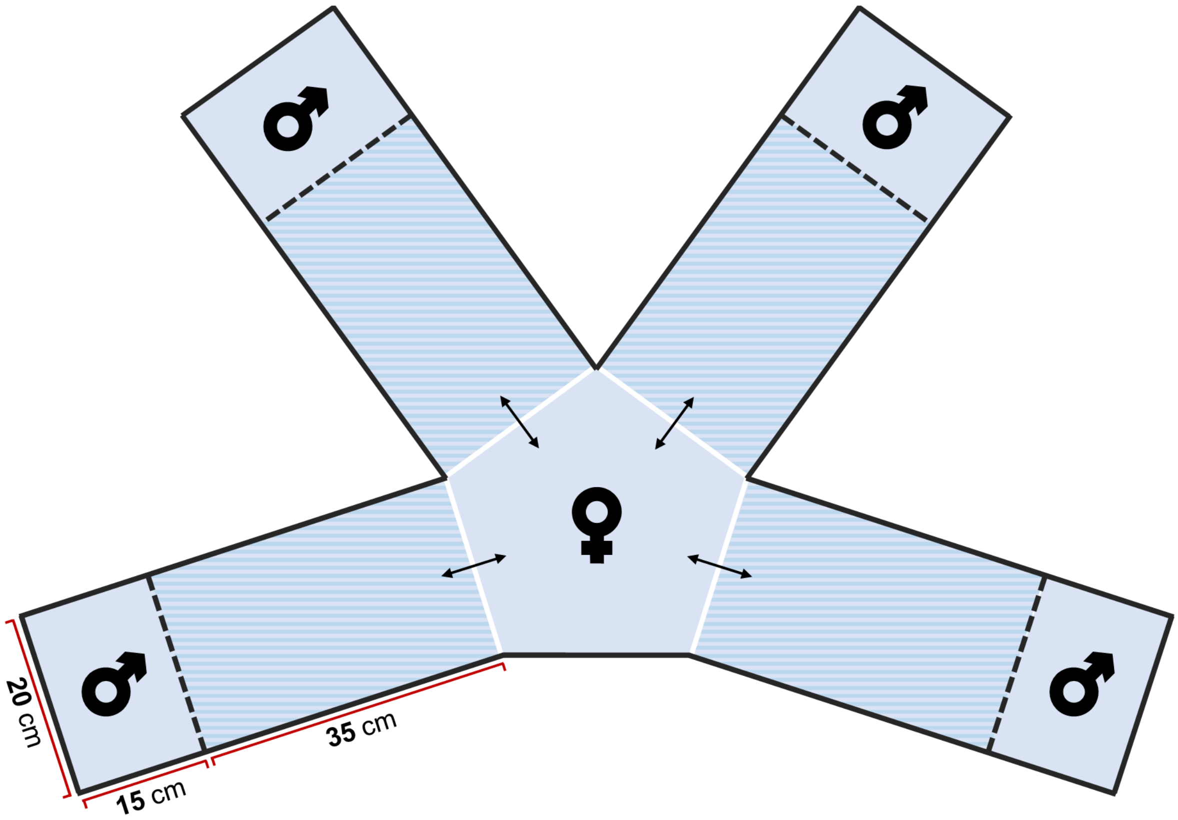

Fig 3. Experimental tank specially designed for the study of *S. alburnoides* mate choice. The choice areas for each male genomotype are dashed. The proportion of time spent by females in each of these areas was used as a measure of preference, being the central area considered neutral. Male compartments were delimited by transparent perforated acrylic plates, allowing the passage of all types of stimuli between male and female.

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considered habituation period to the experimental tank and, thus, discarded from the analyses. The proportion of time spent by females near each male was used as a measure of preference [30] and compared among male genomotypes (PA, PAA, PPAA and PP) and groups (type I, type II and type III) using repeated measures ANOVA. Normality and sphericity assumptions were tested with Shapiro-Wilk's and Mauchly's tests, respectively. When needed, all frequencies were transformed using the arcsine of the square root in order to achieve normality. Repeated measures ANOVA is quite robust dealing with normality violations, thus, slight deviations were considered acceptable. When our data violated the sphericity assumption, a Greenhouse-Geisser correction was used. Post-hoc tests were carried out using a Bonferroni correction for multiple comparisons. Lastly, the genomotype composition of the sample collected and the female preferences obtained from the mate choice trials, particularly the frequency of the sympatric *S. pyrenaicus* (f_{PP}) and the preference of PAA females towards type I males ($\varphi_{FPAA \rightarrow MI}$), were used to run the model. All statistical procedures were performed in StatSoft Statistica v12 [31].

Results

For all initial genomotype compositions (see Table 2), we simulated 500 distinct PP frequencies ($0<fPP<1$, 0.002 steps) and 500 distinct mate preferences of PAA females towards type I males ($0<\varphi_{FPAA \rightarrow MI}<1$, 0.002 steps; Table 1), totalizing 250 000 distinct scenarios per initial genomotype composition. Equilibrium was reached for both triploid- and tetraploid-dominated populations under multiple scenarios. No relation was found between the initial genomotype composition and the evolutionary pathway followed by simulated populations because the equilibrium reached was similar for any initial composition. However, the two parameters we studied, namely, mate preferences and frequency of the sympatric *S. pyrenaicus*, influenced the final equilibrium (Fig 4). Most scenarios that favored tetraploidization led populations to be exclusively composed by the self-sustainable PPAA genomotype ($f_{PPAA} = 1.0$), whereas the ones favoring the dominance of the PAA genomotype also allowed the maintenance of the PA one (Fig 4), derived from the PAA females' variable preference degree towards type II males (PP genome, P sperm). In general, these theoretical genomotype compositions do not differ significantly from the ones observed in natural populations (Fig 1B).

The overall outcome of our theoretical model shows that the persistence of triploid- and tetraploid-dominated populations are favored by opposite forces, although a narrow range of scenarios allowed the co-existence of both triploid and tetraploid genomotypes in the same population (Fig 5). Whereas lower PP frequencies and stronger preferences of PAA females towards type I males (PA and PPAA) seem to favor the persistence of triploid-dominated populations, higher PP frequencies and stronger preferences of PAA females towards type II males (PP) seem to route populations towards tetraploidization (Fig 5). From all 250 000 simulated scenarios, 55.8% led to tetraploid-dominated populations, 38.8% stabilized in triploid-dominated ones, 2.9% allowed the equilibrated coexistence of the PAA and PPAA genomotypes, and 2.5% led populations to be exclusively composed by the PAA genomotype (Fig 5). Note that populations only constituted by the PAA genomotype are not viable because *S. alburnoides* triploid-dominated populations cannot persist without genomotype variability (see Introduction; Fig 1A). Thus, this last outcome was considered to represent extinction.

In order to experimentally evaluate the role of female preferences (one of the main parameters of our model) in *S. alburnoides* reproductive dynamics, we assessed the mate preferences of PAA females in affiliation trials (Fig 6A; S1 Table). Repeated measures ANOVA revealed that tested PAA females ($N = 11$) showed differential mate preferences towards the available male genomotypes (i.e. PA, PAA, PPAA and PP males) ($F_{3,30} = 3.834$, $p = 0.019$). Post-hoc tests using the Bonferroni correction revealed that PAA females had a significant higher preference for PPAA males than for PA ($p = 0.041$) and PAA males ($p = 0.035$). Males from the sympatric *S. pyrenaicus* non-hybrid species (PP) were in an intermediate position, not differing significantly from any of the *S. alburnoides* male genomotypes. Note that, as previously stated, adult genomotypes have distinct typical lengths [28, 29], which does not allow to exclude the effect of fish size from our results. However, fish size seems unlikely to play a major role because females showed distinct affiliation tendencies towards male genomotypes with similar average standard lengths (PA vs. PPAA; PP vs. PAA; Fig 6A).

In order to compare the experimental results with the outcome our model, the set of preference levels obtained from the mate choice trials was reorganized by male type, namely type I (PA and PPAA), type II (PP) and type III males (PAA). Thus, PAA females' preference levels towards PA and PPAA male genomotypes (type I males) were summed for each tested female, reorganizing the data according to the offspring genomotype females would produce with each male group (PAA, PA and PAAA with type I, type II and type III males, respectively). All statistical analyses were repeated for this new male structure (Fig 6B). Repeated measures ANOVA revealed that PAA females showed a differential affiliation tendency towards the three male groups ($F_{2,20} = 6.597$, $p = 0.006$). Post-hoc tests using the Bonferroni correction revealed that PAA females' preference towards type I males were significantly higher than towards type III males ($p = 0.007$) and nearly significantly higher than towards type II males ($p = 0.052$). However, their affiliation tendency was similar between type II and type III males.

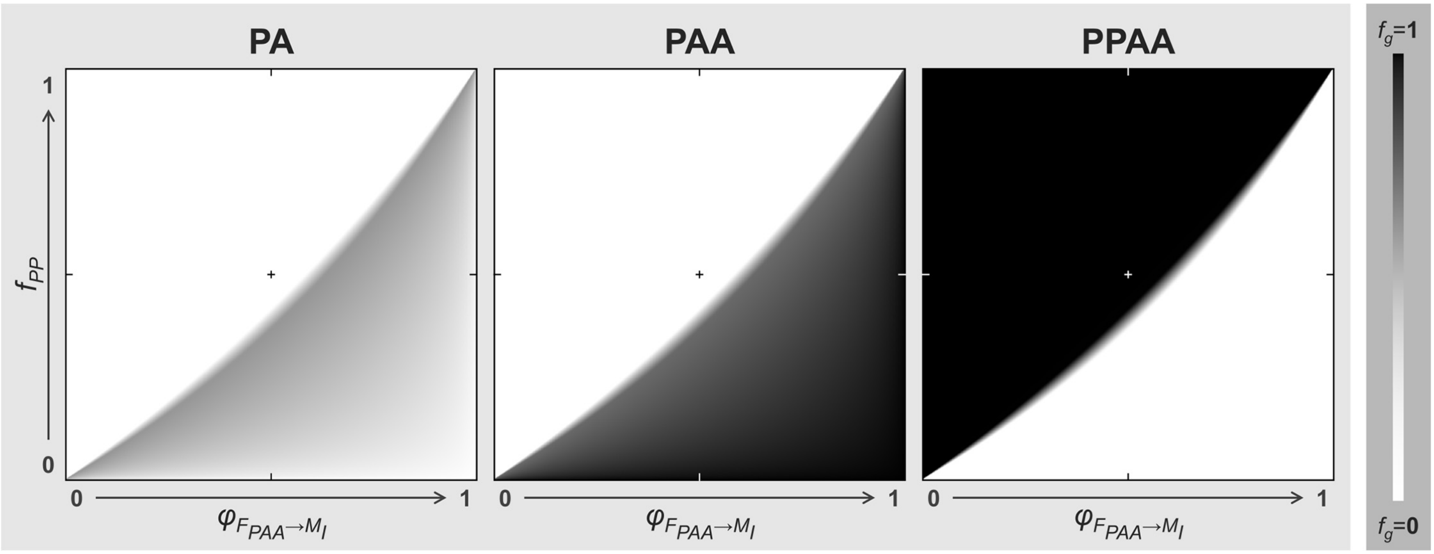

Fig 4. Genomotype frequencies at the equilibria predicted by the model. Relative frequencies of the three *S. alburnoides* genomotypes (PA, PAA and PPAA) range from $f_g = 0$ (white) to $f_g = 1$ (black) as a function of PAA females' preference towards type I males ($\varphi_{FPAA\rightarrow MI}$; Table 1) and of the frequency of the sympatric *S. pyrenaicus* non-hybrid species (f_{PP}). Note that, although a $0<f_{PP}<1$ range is shown on the *y*-axis, the model cannot operate on $f_{PP} = 1$ because it represents a population exclusively constituted by *S. pyrenaicus* specimens, in which the *S. alburnoides* ones are absent.

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Lastly, the frequency of the sympatric *S. pyrenaicus* species observed in the studied natural population ($f_{PP} = 0.39$) and the average joint preference of PAA females towards type I males (PA and PPAA) obtained from the mate choice trials ($\varphi_{FPAA\rightarrow MI} = 0.58$) were used to run the

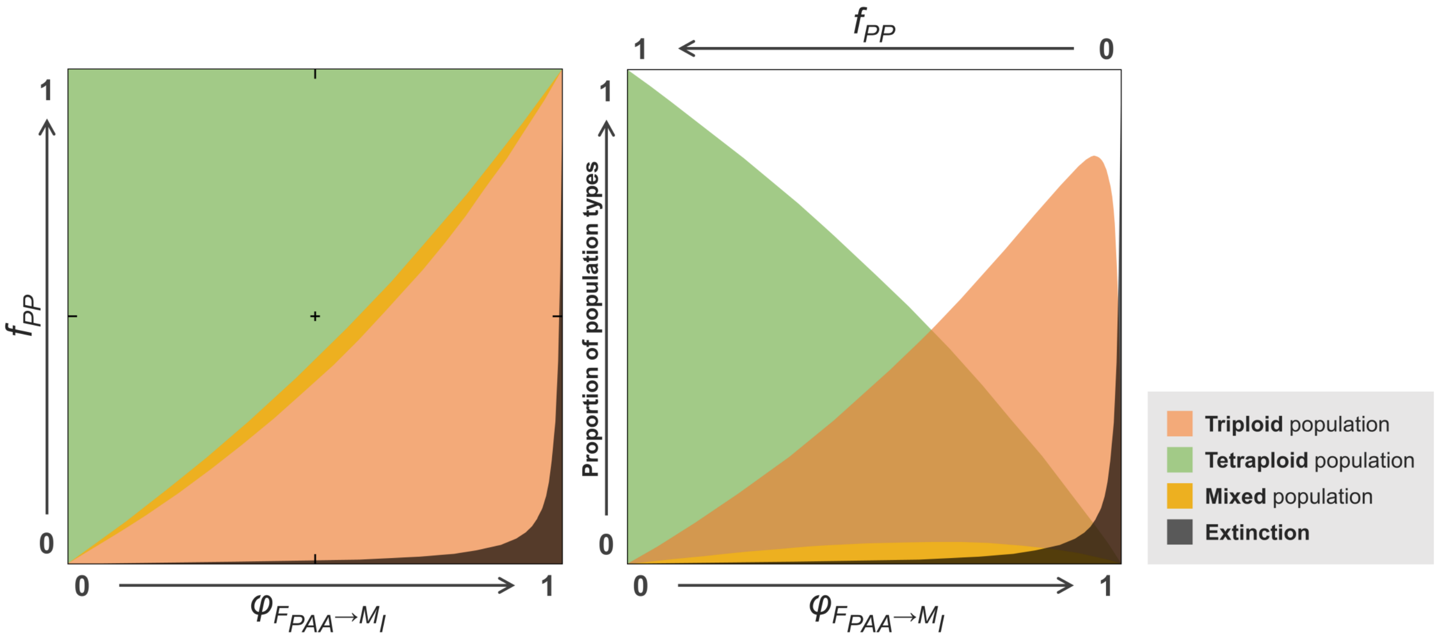

Fig 5. Range of scenarios leading to all population types predicted by the model. Tetraploid-dominated populations exclusively constituted by the PPAA genomotype are represented in green, whereas triploid-dominated populations composed by the PAA and the PA genomotypes are shown in red. Yellow represents the narrow area of scenarios leading to stable populations comprising the three genomotypes (PA, PAA and PPAA) in equilibrium. Lastly, the dark area represents populations exclusively constituted by the interdependent PAA genomotype and, therefore, the respective set of scenarios was considered to lead populations to extinction (see text for more details).

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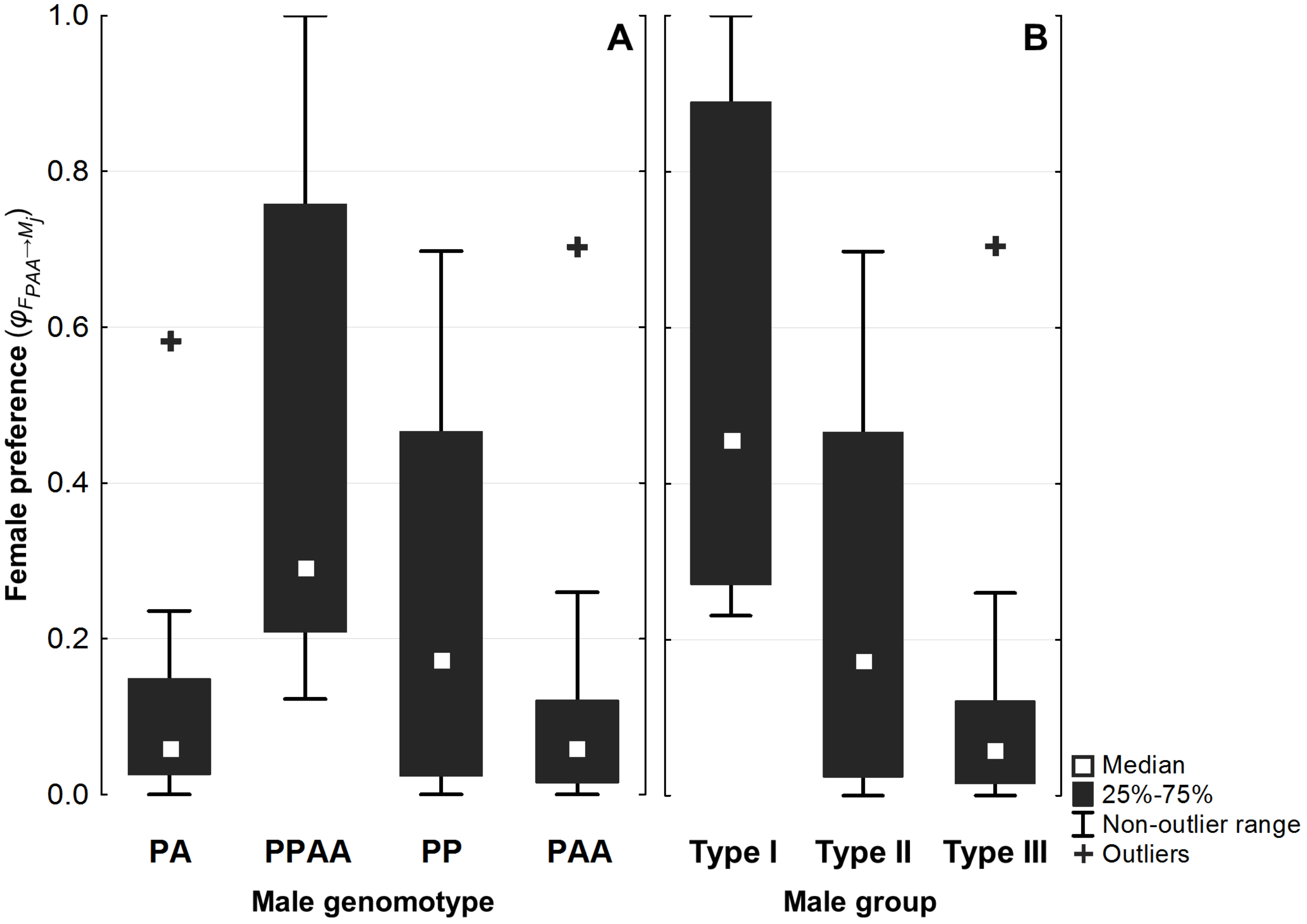

Fig 6. Mate choice results. Proportion of time spent by tested PAA females ($N = 11$) near each male genomotype (**A**: PA, PPAA, PP and PAA) and by male group (**B**: type I, type II and type III). These results obtained from the affiliation trials were used as measures of female preference (means: $\varphi_{FPAA\rightarrow MPA} = 0.13$, $\varphi_{FPAA\rightarrow MPPAA} = 0.45$, $\varphi_{FPAA\rightarrow MPP} = 0.26$, $\varphi_{FPAA\rightarrow MPAA} = 0.13$; $\varphi_{FPAA\rightarrow MI} = 0.58$, $\varphi_{FPAA\rightarrow MII} = 0.26$, $\varphi_{FPAA\rightarrow MIII} = 0.13$).

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theoretical model. Simulated *S. alburnoides* genomotype frequencies ($f_{PA} = 0.38, f_{PAA} = 0.62, f_{PPAA} = 0.00$) were close to the ones observed in the referred natural population ($f_{PA} = 0.28, f_{PAA} = 0.68, f_{PPAA} = 0.04$).

Discussion

The irrelevant role of the initial *S. alburnoides* genomotype composition over the final equilibrium reached in each simulation reveals that even a low frequency of the PPAA genomotype in a triploid-dominated population may tetraploidize it and even a low frequency of the PAA genomotype in a tetraploid-dominated population may triploidize it. Thus, the evolutionary route a given population will follow is independent of its current genomotype composition, but, according to our findings, seems highly influenced by female preferences and frequency of the sympatric *Squalius* species. A closer look at the role of both these forces in shaping *S. alburnoides* genomotype composition over time reveals that their relevance relies on the production of PA males. This intermediary genomotype seems to play a central role in tetraploidizing populations, but it is also indispensable for the persistence of triploid-dominated populations. This happens because both PAA and PPAA females restock their own genomotype by crossing with PA males. On the one hand, when the frequency of the sympatric *S. pyrenaicus* (PP) is high and/or when PAA females show a higher preference towards these males (conditions that favor tetraploidization; Fig 5), crosses between PAA females (A oocytes) and PP males (P sperm) become more frequent, increasing the frequency of the PA genomotype in the population. In turn, crosses between PPAA females (PA oocytes) and PA males (PA sperm) become more frequent, leading to an overall increase of the PPAA genomotype and, consequently, tetraploidizing the population. On the other hand, when the sympatric *S*. *pyrenaicus* non-hybrid species (PP) is less frequent and/or the PAA females' preference towards type I males (PA and PPAA) is higher than it is towards the type II ones (PP) (conditions that favor triploidization; Fig 5), crosses between PAA females (A oocytes) and PA males (PA sperm) become more frequent, leading to an overall increase of the PAA genomotype and, consequently, triploidizing the population. Although this last scenario seems to lead to a struggle between the dominance of PAA and PPAA genomotypes (triploidization vs. tetraploidization), it actually leads to the persistence of triploid-dominated populations. Although, at first sight, this outcome may look unexpected due to the fact that PAA females, contrary to the PPAA ones, need an intermediary step (PA production; Fig 1A) in order to restock their own genomotype, the explanation lies on the characteristic female-biased sex ratio of the PAA genomotype. Its higher proportion of females (~85%) represents an advantage that compensates the assortative mating occurring among the PPAA genomotype and hinders tetraploidization in some scenarios (Fig 5).

If the stability of triploid- and tetraploid-dominated populations depended exclusively on the two factors assessed by our theoretical model (frequency of the sympatric *Squalius* bisexual species and mate choice pattern of PAA females) and if the observed values of both these variables were random among natural populations, the overall outcome of our model indicates that 55.8% of *S*. *alburnoides* natural populations would be tetraploid-dominated (against 38.8% of triploid-dominated ones), because the set of conditions favoring tetraploidization is wider than the one leading to triploidization. However, triploid-dominated populations abound across *S*. *alburnoides* geographic range and only two tetraploid-dominated populations were found so far, suggesting that the values composing the range of the studied factors do not seem to be equally probable to occur among natural populations. Both known tetraploid-dominated populations [14] occur in northern Portugal, a geographic area where the sympatric *Squalius* species, *S*. *carolitertii* (CC genome), has a Least Concern status, being more common and abundant than the sympatric species of southern regions, *S*. *pyrenaicus* and *S*. *aradensis*, classified respectively as Endangered and Critically Endangered [24]. However, although this higher frequency of the bisexual *Squalius* species might have helped the tetraploidization of those tetraploid-dominated populations (composed almost exclusively by CCAA males and females), most populations of the northern region are also triploid-dominated (CAA genomotype). In these other populations, the preference of triploid females towards type I males is probably high enough to promote triploidization and counter the effect of the assortative mating occurring among the tetraploid genomotype (Fig 5). Note that, although the genomotypes of the northern region include C and not P genome, their reproductive modes are the same and, thus, the reproductive dynamics of those populations is similar to the one shown in Fig 1.

The results of the experimental mate choice trials were in agreement with the dominance of triploid populations in nature because PAA females showed a higher affiliation tendency with type I males (PA and PPAA), crosses that produce PAA offspring, thus, contributing to triploidize populations. However, female preference differed significantly between the two type I male genomotypes, with PPAA being favored over PA. This difference may be related to the fact that PPAA males undergo meiosis as reproductive mode and, therefore, contribute to a higher genetic variability of the offspring, a factor already proposed as relevant when choosing a mate [32–34]. The same does not apply to the PA males because they produce their gametes through clonal gametogenesis and, thus, the genetic variability of their offspring only comes from the mother. Future additional mate choice trials in a triploid-dominated population in which the PPAA genomotype is absent may be useful to understand the observed difference, in order to assess if the preference of PAA females towards PA males is higher when no PPAA males are available.

Type III males (PAA) were the least preferred choice of triploid females, a predictable result considering the extreme rarity of the PAAA genomotype (the offspring produced from crosses between PAA males and females) in natural populations. However, this result is still particularly interesting because PAA females seem to avoid mating with males of their own genomotype (disassortative mate choice) in order to produce offspring of their own genomotype (assortative mate choice). To our knowledge, this assortative-disassortative mate choice pattern was never reported before.

Because hybrid organisms with nonsexual reproductive modes have altered gametogenesis and lack regular sexual mechanisms (i.e. normal amphimixis), they were for long considered evolutionary dead-ends [35]. However, several studies over the last decades have shown otherwise [16, 36]. Actually, the intricate reproductive dynamics of most hybrid complexes allow a multiplicity of alternative evolutionary pathways along which organisms may evolve. Our results suggest a key role for mate choice in driving such pathways. Although assortative mating may favor tetraploidization and route populations towards hybrid speciation, the other mate choice patterns occurring among hybrids seem able to counter its effect and maintain populations in its triploid-dominated state. Nonetheless, the role of the bisexual *Squalius* species in *S. alburnoides* reproductive dynamics seems to be equally relevant in routing populations towards tetraploidization. Hybrid speciation seems only possible if the sympatric parental species is frequent in the population, suggesting that sympatry is mandatory for speciation in this hybrid complex, contrary to what is commonly argued [37–40]. Indeed, parental bisexual species may play a persistent key role in hybrid systems, an effect that can ultimately influence mate choice [30, 41].

Our findings add an important and almost neglected piece to the puzzling persistence of some hybrid animal populations without speciation or extinction. Among vertebrates, namely in amphibians and fishes [16, 40, 42–46], several successful hybrid populations have been reported over the years, some with independent reproductive dynamics, but, to our knowledge, this is the first assessment on the influence of mate choice in routing the evolutionary pathways of such organisms, bridging theoretical and experimental approaches. The role of mate choice uncovered in our study may be applicable to other similar hybrid systems, that is, hybrid populations upheld by sexual and nonsexual reproductive modes.

Supporting Information

S1 Table. Data obtained from mate choice trials. Preference values refer to the proportion of time tested females (PAA, $N = 11$) spent interacting with each male genomotype.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MMS HMP LV MJCP. Performed the experiments: MMS. Analyzed the data: MMS HMP LV MJCP. Contributed reagents/materials/analysis tools: HMP LV MJCP. Wrote the paper: MMS.

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2.7 | *Supplementary material*

S1 Table | Data obtained from mate choice trials. Preference values refer to the proportion of time tested females (PAA, *N* = 11) spent interacting with each male genomotype.

Females (PAA)	**Preference**			
	PA ♂	**PAA ♂**	**PPAA ♂**	**PP ♂**
#1	0.060	0.705	0.171	0.036
#2	0.000	0.000	1.000	0.000
#3	0.583	0.038	0.292	0.078
#4	0.039	0.016	0.231	0.698
#5	0.148	0.260	0.123	0.360
#6	0.132	0.069	0.757	0.024
#7	0.125	0.083	0.533	0.173
#8	0.236	0.121	0.209	0.378
#9	0.035	0.052	0.291	0.593
#10	0.026	0.059	0.429	0.466
#11	0.024	0.009	0.949	0.009

CHAPTER 3

Reproductive dynamics shapes genomotype composition in an allopolyploid complex

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Reproductive dynamics shapes genomotype composition in an allopolyploid complex

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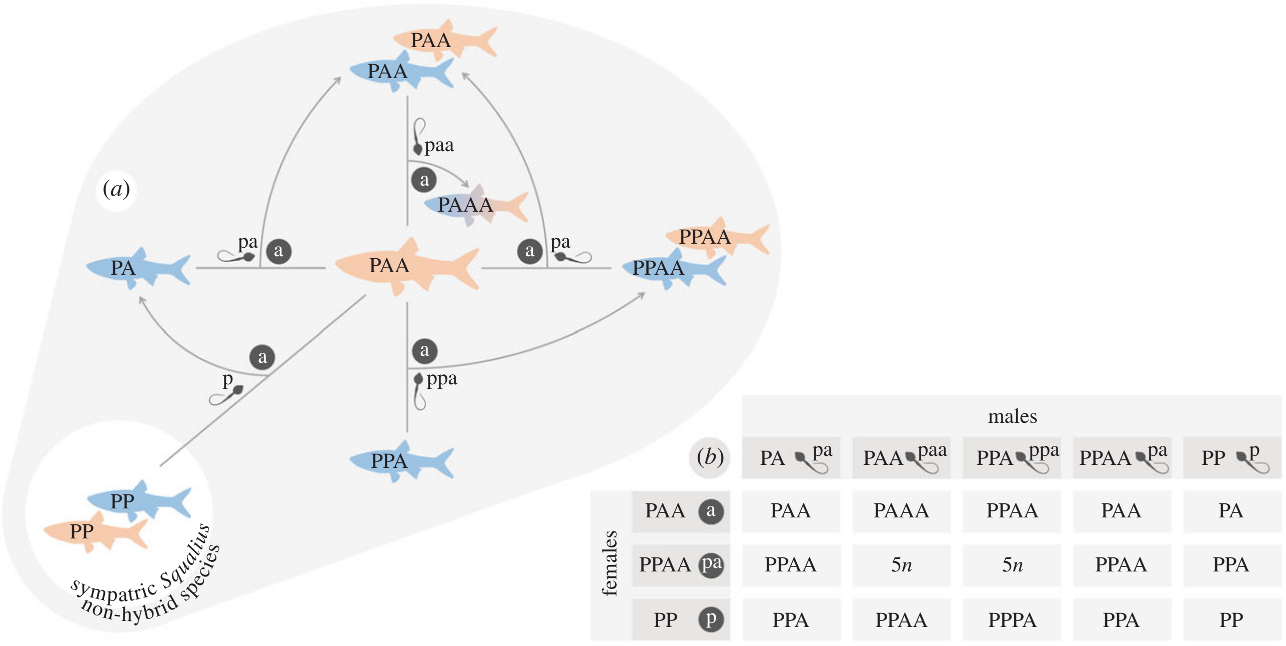
Hybrid complexes are composed of organisms with multiple combinations of parental genomes (genomotypes) that interconnect through nets of crosses. Although several such complexes are well established without speciation or extinction, mechanisms shaping their dynamics remain poorly understood. In this study, we quantified the reproductive success of the allopolyploid Iberian fish *Squalius alburnoides* in experimental free-access and directional crosses involving the most common genomotypes. Specifically, we analysed the paternity of the offspring produced when females had free access to male genomotypes and quantified variations in egg allocation, fertilization rate, and offspring survival among crosses involving each male genomotype. The composition of the offspring produced from free-access crosses varied significantly from that expected from random mating, suggesting that offspring production and viability are not independent of parental male genomotype. Moreover, directional crosses producing the genomotype most commonly found in wild populations appeared to be the most successful, with females laying more eggs, and fertilization rate and offspring survival being the highest. These results suggest that reproductive dynamics plays a relevant role in structuring the genomotype composition of populations and opens a path to future research on the ecology and evolutionary biology of allopolyploids and their multiplicity of possible evolutionary pathways.

1. Introduction

Successful homoploid hybrids and allopolyploid complexes have been reported in various taxonomic groups, showing stable population dynamics or even evolving into new species through hybrid speciation [1–4]. Multiple mechanisms, such as mate choice, egg and sperm allocation, and offspring survival at early ontogenetic stages, may shape the dynamics of such hybrid populations to variable extents [4–11]. Clarifying those mechanisms is crucial to advance our knowledge regarding hybrids' ecology and evolution, namely, in allopolyploid vertebrates.

Increasingly recognized as one of the most well-established hybrid vertebrates known to date [4,12], the *Squalius alburnoides* (Steind. 1866) fish complex is an ideal model to study mechanisms shaping the dynamics of allopolyploids. This Iberian complex arose from intergeneric hybridization, involving *S. pyrenaicus* (Günther 1868) females (PP genome) and males from an already extinct species of the *Anaecypris hispanica* (Steind. 1866) lineage (AA genome; reviewed in [4]). It includes hybrid males and females with several ploidies ($2n = 50$, $3n = 75$, and $4n = 100$) and various combinations of the parental genomes (i.e. genomotypes) [4]. All these genomotypes are fertile and able to cross to produce offspring and to breed with sympatric bisexual *Squalius* species. The vast majority of populations are dominated by allotriploids, namely, by the PAA genomotype in central and southern rivers. This highly female-biased genomotype is entirely maintained by crosses with other genomotypes (figure 1), because neither spontaneous



(b)	PA pa	PAA paa	PPA ppa	PPAA pa	PP p
PAA a	PAA	PAAA	PPAA	PAA	PA
PPAA pa	PPAA	5*n*	5*n*	PPAA	PPA
PP p	PPA	PPAA	PPPA	PPA	PP

Figure 1. (*a*) Simplified reproductive framework of the *S. alburnoides* allopolyploid complex in the Ocreza River (Tagus drainage, Central Portugal), showing the core of its reproductive dynamics and its relationship with the sympatric bisexual *S. pyrenaicus*. Males and females are represented in blue and pink, respectively. (*b*) Table of all crosses that could theoretically occur in the referred population. Reproductive modes include meiotic hybridogenesis in PAA females, clonal spermatogenesis in PA, PAA, and PPA males, and regular meiosis in PPAA and PP individuals; 5*n* offspring is unviable. Dark grey circles represent eggs. Capital letters refer to fish genomes, and small letters to gamete genomes: A, a from the *Anaecypris*-like paternal ancestor of the complex; P, p from the *S. pyrenaicus* maternal ancestor of the complex. (Online version in colour.)

parthenogenesis nor gynogenesis occurs in this complex and, thus, PAA females cannot restock their own genomotype without male genome incorporation (reviewed in [4]). This strict genomotype interdependency suggests that the structure of *S. alburnoides* populations may depend on an intricate reproductive dynamics, promoting the prevalence of the PAA genomotype, although empirical evidence to corroborate this is still mostly lacking. Previous studies have suggested that *S. alburnoides* females may display differential mate preferences among male genomotypes [13], favouring the ones with which they produce PAA offspring [14]. However, it is unclear whether mate preferences actually also influence reproductive success, offspring production, and genomotype composition.

In an attempt to clarify the mechanisms shaping the structure of *S. alburnoides* populations, we analysed the reproductive success of PAA females in free access and directional crosses involving *S. alburnoides* males with distinct genomotypes (PA, PAA, PPA, and PPAA) and *S. pyrenaicus* males (PP). Specifically, we conducted two sets of experiments in order to (i) assess the paternity of the offspring produced when PAA females have free access to all male genomotypes and (ii) quantify egg allocation, fertilization, and offspring production by individual mating pairs involving PAA females and males of each genomotype. Results obtained in both experiments were assessed to explore the interplay between genomotype composition and reproductive dynamics, and the way natural selection acting on early ontogenetic stages may shape population structure in *S. alburnoides*.

2. Material and methods

(a) Fish sampling and genomotype assessment

Mature individuals used in free-access and directional crosses were sampled in the Ocreza River (Portugal), where diverse genomotypes of *S. alburnoides* co-occur with *S. pyrenaicus* (figure 1). Sampling was conducted early in the reproductive season (April), using short pulses and moderate voltage electrofishing (300 V, 2–4 A). During this period, individuals could be easily sexed by applying a mild pressure on the abdomen and observing gamete discharge. Fish showing no physiological stress or injuries were transported to the laboratory in separate aerated vats, and the remaining returned to the river.

Because *S. alburnoides* genomotypes are morphologically similar, the ploidy and genome combination of each individual were unknown until assessment. In the laboratory, individuals were anaesthetized ($0.1\ \mathrm{g\ l^{-1}}$ MS-222, $0.2\ \mathrm{g\ l^{-1}}$ $NaHCO_3$), measured for standard length (SL, mm) and photographed on their left and right sides for further scale pattern interpretation and individual recognition [15]. Small clips of the caudal fin were collected for genomotype assessment through flow cytometry [16] and Sanger sequencing of the β-actin gene (PCR conditions: 35 cycles of 94°C, 30 s; 55°C, 40 s; 72°C, 90 s) [17]. DNA extraction followed an adapted phenol–chloroform protocol [18].

(b) Assessment of the offspring produced in free-access crosses

An experimental population of 33 *S. alburnoides* (5.6 cm mean SL, 4.0–7.2 cm) and 19 *S. pyrenaicus* (7.6 cm mean SL, 5.3–10.6 cm) was established in an artificial pond, under natural light and temperature conditions, in January 2011. The genomotype of *S. alburnoides* individuals was assessed as described above. The experimental population included 23 PAA females, representing the dominant genomotype in central and southern rivers [4,14], and a high diversity of male genomotypes, that approached the proportions found in the wild, namely, six PA, one PAA, two PPA, one PPAA, and also 10 PP individuals. In addition, nine PP females were translocated to the pond to assess whether the eventual absence of offspring from PP males was due to their lack of interest towards PAA relative to PP females or due to a general failure in reproduction. The experimental population is illustrated in the electronic supplementary material, figure S1.

The artificial pond had a volume of 4 200 l (300 cm length × 200 cm width × 50 cm mean depth (25–90 cm)). The bottom of the pond was covered with a layer of cobbles (2–15 cm), to provide adequate substrate for fish spawning [19], and around 25% of the surface was occupied with the macrophytes *Ranunculus* sp. and *Juncus* sp., to provide cover and shelter for the fish [20]. Two pumps and a UV lamp were used to prevent water stagnation and deterioration. Overall, habitat conditions in the pond were close to those found in Iberian rivers during seasonal drought, when fish concentrate in isolated pools [21]. Fish were fed twice a day with commercial flakes for one month to prevent eventual lows in prey availability and facilitate adaptation to the pond conditions. The pond was monitored weekly for water pH (7–10) and inspected for dead fish (never detected) and larvae (first spotted on April). In October, parental fish and offspring were captured using electrofishing and transported to the laboratory in aerated vats. The pond was then emptied to assure complete fish collection.

In the laboratory, YOYs (youngs-of-the-year) were identified using length frequency distributions and maintained in a 500 l tank. All individuals were assessed for ploidy level, and a sample of 100 YOYs was randomly selected for sex and paternity assessment. Individuals were sacrificed with an overdose of MS-222 anaesthetic and sexed as described in [22]. Paternity was assessed through microsatellite genotyping, using nine microsatellites with high variability among cyprinids [23–25]. Moreover, we used an extra microsatellite found by MM Coelho team (2013, unpublished data), after sequencing a genomic fragment containing the intron region of the *aminomethyltransferase* gene (AMT), from which the primers were designed. Excepting LCO1, LCO3, and LCO4, all microsatellites were genotyped using primers with a M13 tail, following [26]. Complete information on the 10 microsatellites is in the electronic supplementary material, table S1.

Paternity was primarily assessed by comparing microsatellite alleles between offspring and parental individuals. When microsatellites were unable to distinguish between pairs of female progenitors, a mitochondrial fragment including the d-loop/control region, the tRNA–Phe gene, and the beginning of the 12S gene (PCR conditions: 35 cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 90 s) [27] was amplified and sequenced for ambiguous individuals. Overall, male progenitors were identified for all YOYs and female progenitors for 91 YOYs.

(c) Assessment of the offspring produced in directional crosses

Directional crosses were conducted during 2012 and 2013, using additional fish samples collected in the Ocreza River. In total, 29 mating pairs involving PAA females and 12 PA males, five PAA males, four PPAA males, two PPA males, and six PP males were used in directional crosses conducted in three experimental outdoor tanks, under natural conditions of light and temperature. To control for size effects on fecundity, females were selected to show the least variation in length as possible, ranging between 5.7 and 7.2 cm. No similar size selection was possible for males, which generally differ in length among genomotypes [4], with PP males being the largest.

Because spontaneous reproduction is hard to accomplish in captivity for isolated pairs of both *S. alburnoides* and *S. pyrenaicus* [19], outdoor tanks were compartmentalized for holding six mating pairs each. Tanks were 130.0 cm × 70.0 cm × 50.0 cm (length × width × height) and compartments (43.3 cm × 25.0 cm) were divided by transparent perforated acrylic plates (8.0 mm diameter holes). This allowed all sorts of stimuli in the water to be shared among fishes, but breeding to occur only between mates in each compartment. Because *S. alburnoides* is a multiple bottom spawner [13,28], whose eggs stick to the substrate after fertilization, the holes were located on the top half of the acrylic plates, near the water surface, to prevent cross fertilization, and the bottom of each compartment was delimited by an acrylic net (1 cm^2 square holes) to avoid egg predation. Moreover, thin malleable acrylic sheets (0.5 mm) were put below the bottom nets, so that the stuck eggs of each batch could be removed through independent lateral compartments (10 cm width) without disturbing the mates.

Each year, experiments lasted three months (May–July). Water temperature in the tanks was similar between years for average (20.5°C versus 19.9°C, $t_{182} = 1.44$, $p = 0.152$) and maximum (25.4°C versus 24.9°C, $t_{182} = 0.76$, $p = 0.449$) values, and the pH ranged between 7.0 and 8.0. Water was filtered using pumps connected to UV lamps. In general, tanks held mating pairs with the same combination of genomotypes. In the only case in which two distinct male genomotypes were held in the same tank, the perforated acrylic plates were replaced by watertight opaque glass and separate filters and UV lamps were used to avoid water and stimuli mixture. In all cases, fish were fed twice a day with frozen bloodworms and brine shrimp.

Eggs produced by each mating pair were collected the day after spawning, rinsed with water, counted, and the bottom of each compartment was vacuumed to guarantee complete egg collection. Eggs laid in consecutive days were considered as a batch and were transferred to Petri dishes in groups of 100. Daily, groups of eggs were inspected under a stereo-microscope, and unfertilized and dead eggs and embryos were counted and removed. Larvae feeding exogenously and swimming properly were transferred to aerated containers (3 l) and fed daily with ground commercial flakes. A month after spawning, larvae were counted and the experiments were concluded.

(d) Data analyses

Analyses focused on variation in reproductive success of PAA females in relation to PA, PAA, PPA, PPAA, and PP males (henceforth designated as male genomotypes). Whenever relevant, regression analyses were conducted to account for effects of fish size on the response variables characterizing reproductive success (see below). Likewise, *t*-tests were used for assessing variation in response variables between two samples (e.g. sexes), after verification of conformity to assumptions (see below). Significance of statistical testing was assessed at $p < 0.05$, and analyses were performed using StatSoft Statistica software [29]. For clarity, variables are presented in original units in all figures.

To assess whether male genomotypes varied in reproductive success when PAA females had free access to mates, we compared the proportion of YOYs produced by each male against the proportions expected if females paired randomly with them. Likewise, we evaluated the extent of variation in reproductive success among PAA females through comparisons of YOYs produced against those that would be expected if the reproductive success of PAA females was similar. Using this approach, we assumed that offspring viability and survival were similar among crosses, and recognized that variation in reproductive success may reflect the influence of multiple factors, such as intersexual and intrasexual selection, egg allocation, fertilization rate, and larval survival. Nevertheless, measurable deviations from randomness in the proportion of YOYs produced would indicate variation in reproductive success irrespective of the exact mechanisms involved. The analyses were performed with observed versus expected χ^2-tests.

Reproductive success in directional crosses was assessed from the variation in egg allocation, fertilization rate, and larval survival among mating pairs involving different male genomotypes. Egg allocation was estimated from the total number of eggs laid by females throughout the reproductive season and from the number and average size of each batch. Fertilization rate was assessed as the proportion of fertilized eggs in batches with, at least, 100 eggs, which generally averaged 93% (±12%, ranging from minimum 50 to maximum 100%) of the total number of eggs laid by each female. Offspring survival was calculated from the proportion of larvae at the end of the experiments for samples with, at least, 50 fertilized eggs, which included, on average, 74% (±39%, 0–100%) of the total fertilized eggs of each female. Prior to analyses, variables were inspected for skewness and transformed to dampen the influence of exceptionally large numbers whenever necessary. Each variable was assessed for normality and homogeneity of variances using the Shapiro–Wilk and Levene tests, respectively, and tested for variation among male genomotypes using one-way ANOVA and Tukey honestly significant difference (HSD) post hoc tests. Tukey HSD multiple comparisons and grouping were performed following the procedure described in [30], i.e. first comparing the largest mean against the smallest, then against the next smallest and so on, until the largest has been compared with the second largest, and, thereafter, performing the same procedure for the second largest and so on.

3. Results

(a) Offspring produced in free-access crosses

No YOYs of *S. pyrenaicus* were captured in the artificial pond, but there were 261 YOYs of *S. alburnoides*. All but one of these individuals were triploid (99.6%), averaging 5.9 cm SL (±0.8, 4.2–7.7). The only diploid was an allodiploid PA male which was 5.1 cm, that was produced androgenetically by a PA male. It showed no female nuclear genomic contribution, but the sequence of the mitochondrial gene differed from that of the progenitor male, matching one of the PAA females, likely the mother [31].

Among the YOYs analysed for sex, genomotype, and paternity, there was a strong prevalence of females (6 : 1), which tended to be significantly larger than males (5.6 ± 0.7 cm, 4.3–6.9 versus 4.9 ± 0.5 cm, 4.2–6.3; $t_{97} = 3.88$, $p < 0.001$). With the exception of the allodiploid male (PA), all the remaining individuals were PAA.

The 13 males PAA, PPA, and PP fathered no offspring, with the six PA and the single PPAA males fathering 89.0% and 11.0% of the YOYs analysed, respectively (table 1). These proportions varied significantly from those expected if crosses were independent of male genomotype, i.e. including all five male genomotypes and all 20 male individuals in the analysis ($\chi^2_4 = 188.23$, $p < 0.001$). Considering PA and PPAA male genomotypes only, these proportions did not vary significantly from those expected from random mating ($\chi^2_1 = 0.88$, $p = 0.348$). However, there were significant variations in reproductive success among PA males ($\chi^2_5 = 313.67$, $p < 0.001$), with a single individual fathering 86.5% (77–89%) of the offspring produced by this genomotype (male coded as *e* in table 1). When this individual was excluded from analysis, no differences were found among the remaining PA males ($\chi^2_4 = 6.33$, $p = 0.176$), and the proportion of offspring fathered by the PPAA male became significantly higher than expected ($\chi^2_1 = 16.08$, $p < 0.001$). Most males reproduced with more than one female, but the PA male fathering the most YOYs crossed with more females than the remaining PA males (13 versus one to three females, respectively; table 1). The number of YOYs produced was independent of the length of PA and PPAA males ($R^2 = 0.27$, $F_{1,5} = 1.86$, $p = 0.231$).

The YOYs analysed for paternity were mothered by 15 out of the 23 PAA females in the pond. There was no variation in length between females with (6.5 ± 1.3 cm, 3.7–7.9) and

Table 1. Paternity and maternity of the sub-sample ($N = 100$) of the offspring produced in the artificial pond, where females had free access to males with distinct genomotypes, expressed in number of YOYs per cross. Total crosses' count exclude YOYs with undetermined maternity. Note that no descendants from PAA, PPA, and PP males were found. Similarly, PP females produced no offspring. See text for further details. ND = undetermined maternity.

			PAA females																total YOYs		total crosses	
			a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	ND				
males	PPAA	a		1	1	1	5							1		1		1	11	11	6	6
	PA	b	1			1								1				1	4		3	
		c							3	1								1	5		2	
		d										1							1	89	1	14
		e	4		10	3		4	3	3	10	23	2	3	1	5	1	5	77		13	
		f		1															1		1	
		g																1	1		0	
total YOYs			5	2	11	5	5	4	6	4	10	24	2	5	1	6	1	9	100			
total crosses			2	2	2	3	1	1	2	2	1	2	1	3	1	2	1	—				

without (6.0 ± 0.8 cm, 4.5–6.9) offspring ($t_{21} = 0.92$, $p = 0.367$). Moreover, the number of YOYs produced was independent of female length ($R^2 = 0.15$, $F_{1,21} = 3.60$, $p = 0.071$), and most females with offspring (nine out of 15) reproduced with more than one male. Individual females mothered a proportion of offspring significantly different than expected if all of them had the same reproductive success ($\chi^2_{14} = 75.65$, $p < 0.001$), and their individual reproductive success was not independent of male genomotype (PA versus PPAA) ($\chi^2_{14} = 51.72$, $p < 0.001$). A similar result was found if only the offspring fathered by PA males was considered ($\chi^2_{52} = 132.52$, $p < 0.001$), indicating that distinct PAA females had higher reproductive success with distinct PA males. However, this pattern was lost if the PA male that produced the most YOYs was excluded from analysis ($\chi^2_{18} = 27.00$, $p = 0.079$). Moreover, in this case, females showed similar tendencies for crossing with PA and PPAA male genomotypes ($\chi^2_{9} = 12.98$, $p = 0.163$).

(b) Offspring produced in directional crosses

More than 100 eggs were produced per mating pair, except in three crosses involving one PPAA and two PP males. Because we cannot be sure that females used in these crosses were fertile, they were discarded from analyses, reducing the sample size to 26 crosses. Overall, females laid an average of 1 026.0 eggs (±620.4, 139–2 324) and spawned 2.9 times (±1.6, 1–6), with batches including on average 329.3 eggs (±124.3, 102–595). There were no associations between female length and total number of eggs ($R^2 = 0.00$, $F_{1,13} = 0.01$, $p = 0.912$), number of egg batches ($R^2 = 0.05$, $F_{1,13} = 0.67$, $p = 0.429$), and average batch size ($R^2 = 0.00$, $F_{1,13} = 0.01$, $p = 0.929$). Similarly, no associations were found between male length and total number of eggs ($R^2 = 0.14$, $F_{1,13} = 2.04$, $p = 0.177$), number of egg batches ($R^2 = 0.15$, $F_{1,13} = 2.23$, $p = 0.159$), and average batch size ($R^2 = 0.19$, $F_{1,13} = 3.15$, $p = 0.099$).

Egg allocation showed considerable variation among crosses involving different male genomotypes. The number of egg batches laid by females remained virtually the same ($F_{4,21} = 2.39$, $p = 0.084$), but there were significant variations in the total number of eggs ($F_{4,21} = 4.10$, $p = 0.013$), with females laying fewer eggs with PP than with PA and PAA males (figure 2*a*), but showing no significant difference between PPA and PPAA males and the remaining male genomotypes. Similarly, there were significant differences in the average batch size ($F_{4,21} = 9.30$, $p < 0.001$), with females laying fewer eggs per batch with PP males than with any other male genomotype (figure 2*b*).

Fertilization rate was estimated for only 25 crosses, given eggs produced in the only batch laid by a mating pair involving a PA male were infected by fungi and lost. Overall, the average fertilization rate was 44.35% (±33.10%, 0.00–92.67%), with no eggs being fertilized by PP males. The fertilization rate was independent of the length of females ($R^2 = 0.15$, $F_{1,13} = 2.22$, $p = 0.160$) and males ($R^2 = 0.14$, $F_{1,13} = 2.14$, $p = 0.167$).

The proportion of fertilized eggs varied significantly among crosses involving different male genomotypes ($F_{4,20} = 6.88$, $p = 0.001$), with fertilization being lower for PP than for PA, PAA, and PPAA males, and PPA males fertilizing fewer eggs than PA males (figure 2*c*). When crosses involving PP males were excluded from the analysis, the average fertilization rate increased to 52.80% (±29.09%, 1.00–92.67%), and there were no significant differences among crosses ($F_{3,17} = 3.00$, $p = 0.060$), meaning all other male genomotypes were equally successful at fertilizing eggs. However, considering the much lower fertilization rate observed for PPA males (figure 2*c*), the lack of significance was likely related to the small sample sizes.

Considering all mating pairs, the fertilization rate tended to increase with the total number of eggs ($R^2 = 0.25$, $F_{1,23} = 7.81$, $p = 0.010$; figure 3*a*) and batches ($R^2 = 0.19$, $F_{1,23} = 5.45$, $p = 0.029$) laid by females, but showed only a nearly significant association with average batch size ($R^2 = 0.14$, $F_{1,23} = 3.80$, $p = 0.064$) (figure 3*b*).

The larval survival rate was only assessed for crosses involving PA, PAA, and PPAA males, given no eggs were fertilized in batches produced in crosses involving PP males and the average fertilization rate was only 4.3% in those involving PPA males (figure 2*c*). For the same reason, two mating pairs involving PA and PAA males, with average fertilization rates of 8.5% and 5.3%, respectively, were also excluded from the analysis. For the remaining 17 mating pairs, fertilization rates of the analysed batches ranged between 53.0 and 100.0%, and larval survival rate averaged 29.69% (±17.0%, 7.0–59.0%).

Larval survival varied significantly among crosses ($F_{2,14} = 4.43$, $p = 0.032$), being higher in those involving PPAA than PA and PAA males (figure 2*d*). Regardless of parental male genomotype, larval survival significantly decreased with average batch size ($R^2 = 0.39$, $F_{1,15} = 9.44$, $p = 0.008$; figure 3*c*), but showed no association with the total number of eggs ($R^2 = 0.06$, $F_{1,15} = 0.99$, $p = 0.336$) and batches ($R^2 = 0.05$, $F_{1,15} = 0.76$, $p = 0.397$) and fertilization rate ($R^2 = 0.20$, $F_{1,15} = 3.76$, $p = 0.072$). Moreover, larval survival was independent of the length of females ($R^2 = 0.32$, $F_{1,7} = 3.26$, $p = 0.114$) and males ($R^2 = 0.23$, $F_{1,7} = 2.11$, $p = 0.190$).

4. Discussion

Despite the high diversity of genomotypes and reproductive modes in the *S. alburnoides* complex, central and southern populations are generally dominated by PAA females. The results of our study suggest that this may reflect, at least partially, the influence of mate selection and reproductive success of male genomotypes. Indeed, the genomotype composition of the offspring produced by PAA females having free access to mates differed from the expected if mating preferences and reproductive success were similar among male genomotypes. Moreover, egg allocation and fertilization were superior when PAA females mated with PA and PPAA males, with which they produce PAA offspring, in comparison to PP males, with which they produce PA offspring. Furthermore, survival seemed to be higher in offspring fathered by PPAA males.

Although sample sizes used in our study may be regarded as small, this was a logistical limitation that reflected the scarcity of some genomotypes in the wild (e.g. PPA). Moreover, there are severe legal restrictions regarding the capture of *S. pyrenaicus* (PP), which lists as ‘Endangered’ [32]. Although small sample sizes together with the absence of balanced experimental design could have limited our results to some extent, we are confident that the patterns now perceived provide valid insights into the reproductive dynamics of the *S. alburnoides* complex, which should deserve further research (see below).

Results of free-access crosses revealed that mating choices and offspring viability are unlikely to be similar among male

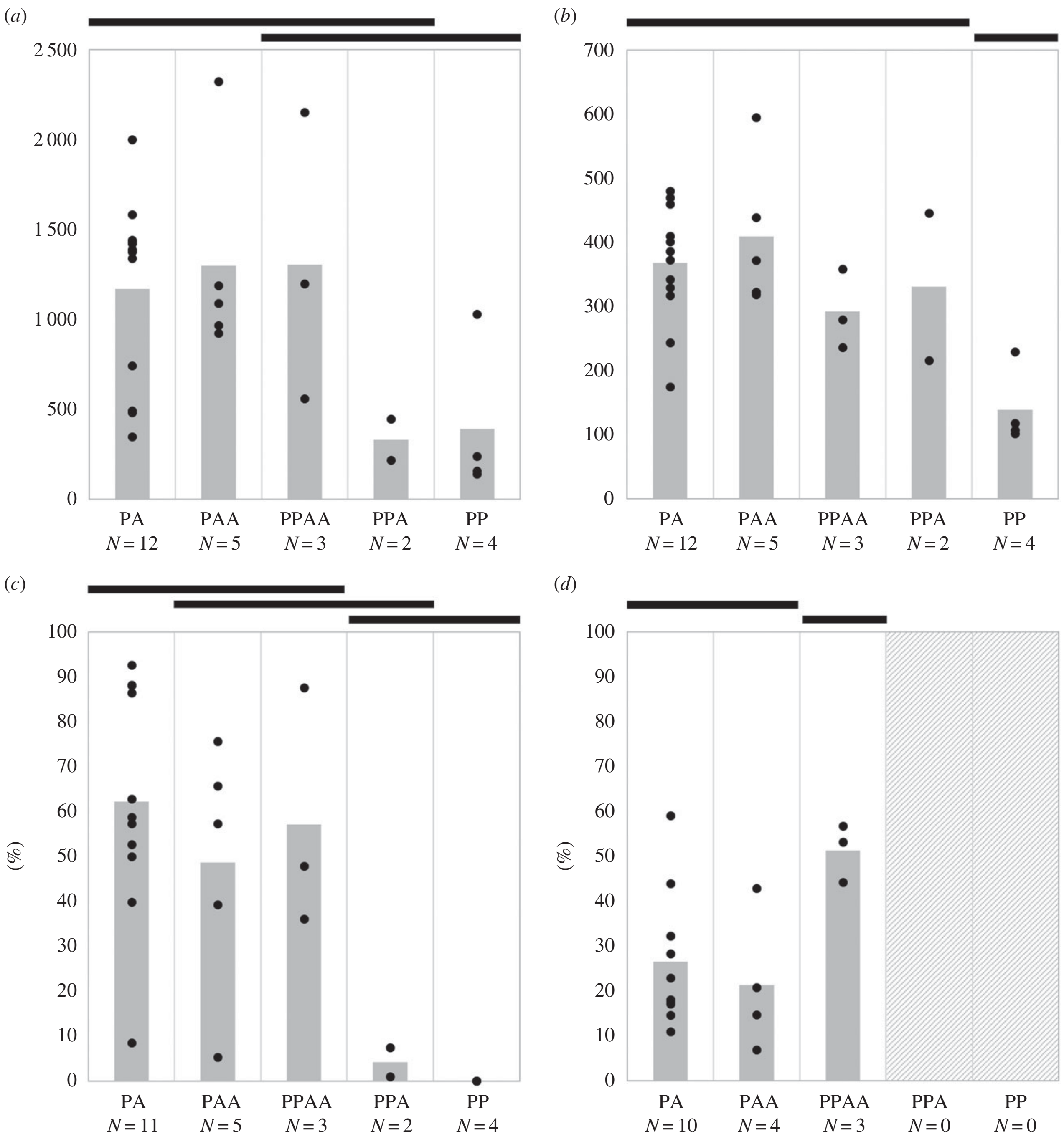


Figure 2. Variation in reproductive traits among directional crosses between PAA females and PA, PAA, PPAA, PPA, and PP males. Tukey HSD grouping (see text for more details) is shown above each graph, represented by black bars, and the total number of crosses analysed for each male genomotype is shown below the *x*-axis. (*a*) Total number of eggs, (*b*) batch size, (*c*) fertilization rate, and (*d*) larval survival. Dots represent mating pairs, and grey bars the average values obtained for each male genomotype.

genomotypes and individual mating pairs. Although the experimental population included males with five genomotypes (PA, PAA, PPA, PPAA, and also PP), only PA and PPAA males produced offspring with PAA females. Moreover, a single PA male fathered 77% of all the offspring, suggesting that the offspring produced by some mating pairs may be much more successful than that produced by other pairs of the same genomotypes. Ignoring this 'individual' effect may lead to misleading conclusions about population structuring in studies focusing on overall genomotype patterns and should, thus, be prevented in further studies.

Patterns of individual variation in offspring production perceived in free-access crosses were likely expressed pre- and post-zygotically. Indeed, PAA females differed not only in egg production in relation to male genomotype, but also in fertilization rate and offspring survival, suggesting that natural selection is probably in action in both phases. This is consistent with previous studies showing that *S. alburnoides* females are choosy and favour certain male genomotypes, independently of their frequency or density [13,14].

Overall, PP males appeared to be the least favourite of PAA females. The differential allocation hypothesis predicts that choosy females invest more reproductive resources towards high-quality than low-quality males, drawing a positive relationship between energetic investment and reproductive success [33,34], and meaning that differential allocation is directly influenced by mate choice. In fishes with external fertilization and displaying no parental care, differential allocation is often expressed in the number of eggs laid by choosy females [35–38]. In directional crosses,

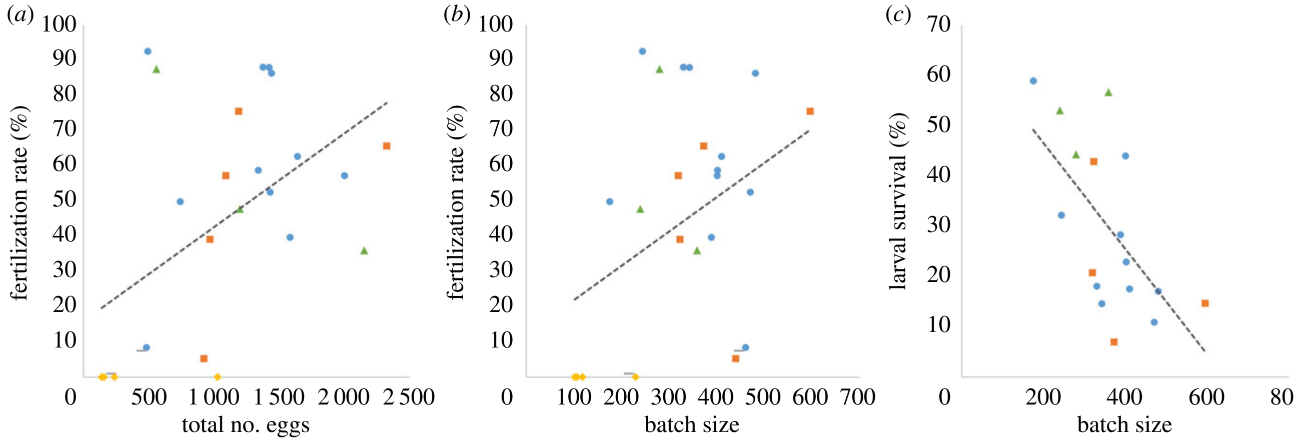


Figure 3. Relationships between reproductive traits of PAA females over all crosses. (*a*) Variation in average fertilization rate with total number of eggs, (*b*) variation in average fertilization rate with average batch size, and (*c*) variation in larval survival with average batch size. Markers for male genomotypes are as follows: PA, circles; PAA, squares; PPA, dotted lines; PPAA, triangles; PP, diamonds. (Online version in colour.)

PAA females laid fewer eggs with PP males than with the remaining. This may, at least partially, explain the lack of PA offspring when females had free access to males. In these circumstances, it appears that *S. alburnoides* females may invest more in hybrid 'conspecific' males, belonging to the allopolyploid complex itself.

Multiple factors may be involved in shaping mate choice. PAA females may identify some bad quality indicators in PP males and allocate their eggs accordingly. Indeed, in directional crosses, the total number of eggs was positively associated with fertilization success, and PP males seemed unable to fertilize the eggs of PPA females. Moreover, egg allocation was independent of male length, suggesting that size plays a minor role in defining male quality for females. However, it is also possible that male mate choice is also at play. PP males may be less available to mate with hybrid females and invest less in each spawning event than other more willing males, for example, by displaying less vigorous courtships or releasing insufficient amounts of sperm to fertilize the eggs. Note that PP males were significantly bigger than *S. alburnoides* male genomotypes and, thus, theoretically able to produce more sperm. Although both male and female mate choices could influence each other, this is unlikely to be the case here. Indeed, PP males were the second favourites of PAA females in previous affiliation trials [14], suggesting there was probably a low interest of PP males in mating with PAA females and not the opposite. Differential fertilization rates among male genomotypes have been reported for other polyploid fish [39–41], and, in certain fish species, non-spermiating males were found to exhibit courtship behaviours and induce spawning in mature females [42]. Although we cannot exclude the hypothesis that the null fertilization found for PP males in directional crosses was due to the lack of adequate substrate for preparing spawning pits [19], this seems unlikely, because, in the artificial pond, PP males did not produce any offspring, neither with PP nor PAA females, despite adequate substrate being available. Note that *S. alburnoides* and *S. pyrenaicus* specimens display external fertilization and probably share, to some extent, the spawning habitats in the wild, which may result in accidental intercrosses by sperm dispersal, that possibly contribute to the maintenance of some PA fish frequency.

Offspring survival may also play an important role in shaping the genomotype composition of *S. alburnoides* populations. Overall, offspring survival was higher in smaller egg batches, probably reflecting the influence of egg size on larval survival. Although no data on egg size was gathered for PAA females herein, females producing larger eggs are generally constrained to lay fewer eggs than females producing smaller ones [28]. Ultimately, this may lead to a higher survival of the offspring produced by the former females, because larvae hatching from larger eggs tend to be larger and more capable of resisting starvation and other environmental constraints [28].

Offspring produced in directional crosses involving PPAA males showed the highest survival rate. This was consistent with PAA females favouring PPAA males in affiliation trials [14] and with the results of paternity analysis of the offspring produced in the artificial pond. Excluding the PA male progenitor that produced the most offspring, the PPAA male fathered a higher proportion of offspring than all the other PA males. Taken together, these results suggest that a 'good genes' type of mate choice seems to be occurring in *S. alburnoides*, with females showing a preference towards the male genomotype with which they produce higher quality offspring (i.e. with higher survival). Similar mate choice trends have been documented for other organisms (e.g. [43,44]), including cyprinids with external fertilization and other freshwater fishes (e.g. [36,45]). Moreover, it is possible that *S. alburnoides* mate choice may also be upheld by a heterozygosity-based component [46]. Indeed, among all male genomotypes, PPAA tetraploids are the only male hybrids undergoing regular meiosis (producing PA sperm), thus contributing to a higher genetic variability of the offspring, which ultimately may contribute to its higher survival rate.

In conclusion, multiple mechanisms may be involved in shaping the genomotype composition of natural *S. alburnoides* populations. Besides the variation in mate choice, egg allocation, fertilization rate, and offspring survival among genomotypes, individual variation in reproductive success within genomotypes may also be important. The production of the PAA genomotype seems to be favoured by higher egg allocation, fertilization rate and offspring survival in crosses involving PAA females and PA or PPAA males, whereas the production of the PA genomotype seems to be hampered by lower allocation and fertilization of eggs produced by mating pairs involving PP males. Therefore, it appears that natural selection acting early on spawning and larval developmental stages may strongly contribute to structure the genomotype composition of populations. These findings open a path for future research on the ecology and evolutionary biology of *S. alburnoides*, namely, on the actual relevance of the sympatric *Squalius* bisexual species in the reproductive dynamics, and on the way the breeding net among genomotypes may lead to the tetraploidization of populations observed in some northern rivers [2] and, consequently, to a possible event of hybrid speciation. Altogether, these findings add substantially to knowledge on the influence of reproductive dynamics in driving allopolyploid populations through their multiplicity of possible evolutionary pathways.

Ethics. Field and laboratorial procedures followed the ethical guidelines [47] and conformed to the Portuguese legislation regarding animal capture, manipulation, and experimentation for scientific purposes. Fish sampling was conducted with the permission of the Instituto da Conservação da Natureza e das Florestas (permits 140/2012/CAPT and 239/2013/CAPT). Because studied taxa are listed as threatened [32], sample sizes were small to avoid natural stock depletion. All efforts were made to minimize accidental deaths and stress on fish throughout the study. At the end of the study, parental fish were in good condition and returned to the Ocreza River, whereas larvae and YOYs were kept in captivity for further research.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. Conception and design: M.M.S., L.V., and M.J.C.P. Acquisition of data: M.M.S. and S.C. Analysis and interpretation of data: M.M.S., S.C., M.F.M., and M.J.C.P. Drafting the article: M.M.S. Revising the article critically: M.F.M., S.C., L.V., and M.J.C.P. The final approval of the version to be published: all authors.

Competing interests. We have no competing interests.

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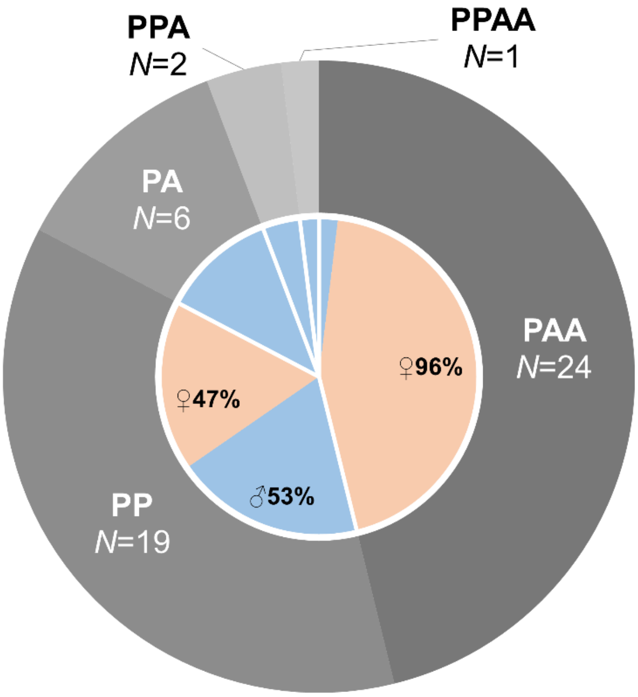
3.7 | *Supplementary material*



Supplementary Figure 1 | Composition of the experimental population maintained in the artificial pond to assess offspring production and paternity when PAA females had free access to mates of various genomotypes. Outer pie chart: number of individuals of each genomotype. Inner pie chart: proportion of sexes in each genomotype, with blue and pink representing males and females, respectively.

Supplementary Table 1 | Details on the ten microsatellites used for paternity analysis, including species of origin. Microsatellites marked with an asterisk (*) were amplified using primers with a M13 tail, as described in [26].

Loci	**Species**	**Primers (5' → 3')**	**PCR conditions**	**Ref.**
LCO1	*Luxilus cornutus*	CACGGGACAATTTGGATGTTTTAT AGGGGGCAGCATACAAGAGACAAC	**$MgCl_2$:** 1,00 mM **Annealing:** 51 °C (34 cycles)	[23]
LCO3	*Luxilus cornutus*	GCAGGAGCGAAACCATAAAT AAACAGGCAGGACACAAAGG	**$MgCl_2$:** 1,50 mM **Annealing:** 48 °C (28 cycles)	[23]
LCO4	*Luxilus cornutus*	ATCAGGTCAGGGGTGTCACG TGTTTATTTGGGGTCTGTGT	**$MgCl_2$:** 1,30 mM **Annealing:** 60 °C (31 cycles)	[23]
LC27*	*Leuciscus cephalus*	TCCAGTTCTTCCTTCCTAATT GCGGAGGGAGAGTATGTCAA	**$MgCl_2$:** 1,00 mM **Annealing:** 53 °C (23 cycles), 51 °C (10 cycles)	[25]

LC288*	*Leuciscus cephalus*	AAGAGCAGAGGAGAGCAGGG TACCTGCAGGGGCATAGGC	**$MgCl_2$:** 1,25 mM **Annealing:** 53 °C (23 cycles), 51 °C (15 cycles)	[25]
LC290*	*Leuciscus cephalus*	CCCTAATGGCCCTCAATACA ACTTCGCTGGCTTGACAAAT	**$MgCl_2$:** 1,25 mM **Annealing:** 54 °C (25 cycles), 53 °C (10 cycles)	[25]
Lsou05*	*Leuciscus souffia*	CTGAAGAAGACCCTGGTTCG CCCACATCTGCTGACTCTGAC	**$MgCl_2$:** 1,25 mM **Annealing:** 55 °C (25 cycles), 53 °C (12 cycles)	[24]
Lsou08*	*Leuciscus souffia*	GCGGTGAACAGGCTTAACTC TAGGAACGAAGAGCCTGTGG	**MgCl2:** 1,25 mM **Annealing:** 55 °C (25 cycles), 53 °C (12 cycles)	[24]
Lsou34*	*Leuciscus souffia*	CCAGACAGGGTGATGATTCC GTAGCGACGTTCAGGTCTCG	**$MgCl_2$:** 1,50 mM **Annealing:** 55 °C (25 cycles), 53 °C (8 cycles)	[24]
SpyrAMT*	*Squalius pyrenaicus*	GAAGAAAGTCTCATTGCTCTGC GAGGTCATCACCCACACCTT	**$MgCl_2$:** 1,25 mM **Annealing:** 55 °C (27 cycles), 53 °C (8 cycles)	

Supplementary Table 2 | Raw data on free-access crosses.

Descendant	Parental male genomotype	Parental female genomotype	Parental male individual	Parental female individual
#1	PA	PAA	e	n
#2	PA	PAA	c	g
#3	PA	PAA	e	c
#4	PA	PAA	e	c
#5	PA	PAA	e	c
#6	PA	PAA	b	ND
#7	PA	PAA	e	j
#8	PA	PAA	e	k
#9	PA	PAA	e	c
#10	PA	PAA	e	f
#11	PA	PAA	e	i
#12	PA	PAA	e	f
#13	PA	PAA	b	d
#14	PA	PAA	e	a
#15	PA	PAA	e	c
#16	PA	PAA	e	i
#17	PA	PAA	e	j
#18	PA	PAA	e	h

#19	PA	PAA	e	c
#20	PA	PAA	e	a
#21	PA	PAA	e	n
#22	PA	PAA	e	c
#23	PA	PAA	e	h
#24	PA	PAA	e	o
#25	PA	PAA	e	c
#26	PA	PAA	e	l
#27	PA	PAA	e	g
#28	PA	PAA	c	h
#29	PA	PAA	e	h
#30	PPAA	PAA	a	e
#31	PA	PAA	e	j
#32	PA	PAA	e	a
#33	PA	PAA	e	ND
#34	PA	PAA	e	n
#35	PA	PAA	e	j
#36	PA	PAA	e	j
#37	PA	PAA	f	b
#38	PPAA	PAA	a	e
#39	PA	PAA	e	f
#40	PA	PAA	b	a
#41	PA	PAA	e	ND
#42	PA	PAA	e	n
#43	PA	PAA	e	i
#44	PA	PAA	e	j
#45	PA	PAA	e	ND
#46	PA	PAA	e	a
#47	PA	PAA	e	g
#48	PA	PAA	e	c
#49	PA	PAA	e	j
#50	PPAA	PAA	a	l
#51	PPAA	PAA	a	ND
#52	PA	PAA	e	i
#53	PA	PAA	c	ND
#54	PA	PAA	e	ND

#55	PA	PAA	c	g
#56	PA	PAA	b	l
#57	PA	PAA	e	j
#58	PPAA	PAA	a	e
#59	PA	PAA	e	l
#60	PA	PAA	e	j
#61	PPAA	PAA	a	e
#62	PA	PAA	e	i
#63	PA	PAA	e	j
#64	PA	PAA	e	i
#65	PA	PAA	e	ND
#66	PA	PAA	e	f
#67	PA	PAA	e	j
#68	PA	PAA	d	j
#69	PA	PAA	e	d
#70	PA	PAA	e	j
#71	PA	PAA	e	g
#72	PPAA	PAA	a	d
#73	PA	PAA	e	k
#74	PA	PAA	e	i
#75	PPAA	PAA	a	c
#76	PA	PAA	e	i
#77	PA	PAA	e	j
#78	PA	PAA	e	j
#79	PA	PAA	e	d
#80	PA	PAA	e	n
#81	PA	PAA	e	j
#82	PPAA	PAA	a	n
#83	PA	PAA	e	j
#84	PA	PAA	e	d
#85	PA	PAA	e	m
#86	PA	PAA	e	i
#87	PA	PAA	c	g
#88	PA	PAA	e	j
#89	PA	PAA	e	c
#90	PA	PAA	e	j

#91	PA	PAA	e	j
#92	PA	PAA	e	i
#93	PA	PAA	e	j
#94	PA	PAA	e	j
#95	PA	PAA	g	ND
#96	PA	PAA	e	j
#97	PPAA	PAA	a	e
#98	PPAA	PAA	a	b
#99	PA	PAA	e	j
#100	PA	PAA	e	l

Supplementary Table 3 | Raw data on directional crosses.

Mating pair	Female type	Male type	Offspring type	Number of laid batches	Total number of eggs laid	Average batch size	Fertilization rate (%)	Survival rate (%)
#1	PAA	PA	PAA	4	1582	388	39.8	28.4
#2	PAA	PA	PAA	2	493	244	92.7	32.3
#3	PAA	PA	PAA	5	2000	400	57.3	44.0
#4	PAA	PA	PAA	1	483	460	8.5	-
#5	PAA	PA	PAA	4	742	175	50.0	59.0
#6	PAA	PA	PAA	3	1340	401	58.8	23.0
#7	PAA	PA	PAA	1	347	347	-	-
#8	PAA	PA	PAA	3	1431	470	52.7	11.0
#9	PAA	PA	PAA	4	1376	330	88.2	18.2
#10	PAA	PA	PAA	4	1639	410	62.9	17.6
#11	PAA	PA	PAA	4	1422	343	88.1	14.6
#12	PAA	PA	PAA	3	1441	480	86.5	17.1
#13	PAA	PAA	PAAA	2	923	439	5.3	-
#14	PAA	PAA	PAAA	3	968	323	39.3	42.9
#15	PAA	PAA	PAAA	2	1190	595	75.6	14.7
#16	PAA	PAA	PAAA	6	2324	372	65.7	7.0
#17	PAA	PAA	PAAA	3	1091	319	57.3	20.8
#18	PAA	PPA	PPAA	1	216	216	1.0	-
#19	PAA	PPA	PPAA	1	446	446	7.5	-
#20	PAA	PPAA	PAA	0	0	-	-	-

#21	PAA	PPAA	PAA	6	2152	359	36.1	56.7
#22	PAA	PPAA	PAA	2	559	280	87.7	44.3
#23	PAA	PPAA	PAA	5	1198	240	47.9	53.1
#24	PAA	PP	PA	0	5	-	-	-
#25	PAA	PP	PA	1	158	107	0.0	-
#26	PAA	PP	PA	1	238	118	0.0	-
#27	PAA	PP	PA	0	8	-	-	-
#28	PAA	PP	PA	1	139	102	0.0	-
#29	PAA	PP	PA	4	1029	230	0.0	-

CHAPTER 4

Mate choice and introgression: an interplay within a hybrid complex

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Chapter 4 | Mate choice and introgression: an interplay within a hybrid complex

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4.1 | ABSTRACT | Hybrid complexes lacking typical reproductive isolation and showing multiple mating options among hybrids and parental species are excellent models to study mate choice in relation to genetics. The allopolyploid fish complex *Squalius alburnoides* includes multiple fertile male and female genomotypes that reproduce among each other and with the sympatric bisexual species of the *Squalius* genus. Here, combining behavioural and genomic tools, we used this hybrid complex to study the relationship between mate choice and introgression, focusing in a population whose mating options include two sympatric bisexual *Squalius* species, diverging in times of coexistence with *S. alburnoides*. The preference of *S. alburnoides* hybrid females towards multiple male genomotypes was assessed in conventional affiliation trials, and introgression levels of the genomes of bisexual *Squalius* species were measured using next-generation genotyping for both hybrids and non-hybrids. Only one of the genomes was introgressed within the other, suggesting that one of the parental species is more prone to interspecific crosses. Female mate preferences were related to the levels of male and female introgression, with females showing higher preference towards introgressed males and towards males with introgression levels similar to their own. This trend favours crosses between the hybrids and the most recent bisexual *Squalius* species in the drainage, promoting the introgression of its genome into the hybrid complex. These findings highlight an intricate interplay between mate choice and introgression, which may be directly related to genetic benefits and significantly shape the genetic content of hybrid populations.

KEYWORDS | Intersexual selection; Nonsexual; Hybridogenetic complex; Next-generation genotyping; Cyprinidae; *Squalius alburnoides*

4.2 | *Introduction*

In animals, the persistence of fertile and stable hybrid populations has countered the view that hybrid organisms are evolutionary dead-ends (*e.g.* Neaves and Baumann 2011; Collares-Pereira *et al.* 2013), a preconception that arose mainly because most hybrid complexes developed nonsexual reproduction (Bullini 1994; Dowling and Secor 1997; Schlupp 2005; Lamatsch and Stöck 2009) and are sexual parasites of sympatric species (Lampert 2009; Schlupp 2010; Lehtonen *et al.* 2013). Thus, the reproductive dynamics of hybrid complexes may include not only the hybrids but also the parental bisexual species. Hybrid organisms benefit from this variety of mating options, either to attain population stability over time or to evolve towards new species through hybrid speciation (Seehausen 2004; Mavárez *et al.* 2006; Mallet 2007; Mavárez and Linares 2008; Seehausen *et al.* 2008; Abbott *et al.* 2013; Morgado-Santos *et al.* 2015).

In most hybrid complexes dominated by female clonal lineages (*i.e.* gynogenetic) (Bullini 1994; Dowling and Secor 1997; Schlupp 2005; Lamatsch and Stöck 2009), there is little to no evolutionary pressure for nonsexual females to be choosy in relation to mates (Joachim and Schlupp 2012) because the sperm of the sexual host is only needed to trigger embryogenesis, with all male genes being discarded after fertilization. For this reason, studies on mate choice among hybrid complexes seldom focus on hybrid females but rather address mate choice by males of the parental bisexual species (Gabor and Aspbury 2008; Mee and Otto 2010; Schlupp *et al.* 2009, 2010; Barbiano *et al.* 2012; Mee *et al.* 2013; Barron *et al.* 2016), which can greatly benefit from avoiding to mate with hybrids, since the offspring do not inherit any genes from the father. However, in hybridogenetic systems, the male genome is incorporated within the offspring and is only discarded after one or two generations, with direct descendants benefiting from genes of both mother (hybrid) and father (non-hybrid). Thus, mate choice by hybrid females of hybridogenetic complexes should not be ignored because there is an evolutionary advantage for hybrid females to be choosy. Indeed, mate choice may represent a key evolutionary force to hybrids, promoting genetic variability (*e.g.* Brown 1997; Kempenaers 2007), perpetuating the hybridization cycle (Abt and Reyer 1993; Engeler and Reyer 2001), preserving population stability (Morgado-Santos *et al.* 2015), shaping population dynamics (Morgado-Santos *et al.* 2016) and/or directly routing populations towards hybrid speciation (Stöck 1998; Holloway *et al.* 2006; Melo *et al.* 2009; Tucker and Gerhardt 2012; Morgado-Santos *et al.* 2015). However, empirical evidence on the role of mate choice in the triangle hybridization-introgression-speciation (Dowling and Secor 1997) remains scarce.

Squalius alburnoides is an Iberian fish complex with origin in unidirectional hybridization between *S. pyrenaicus* females (P genome) and males of an extinct species belonging to the *Anaecypris hispanica* lineage (A genome) (reviewed in Collares-Pereira *et al.* 2013). These intergeneric crosses led to the production of fertile hybrids, whose crosses and backcrosses created a hybridogenetic complex of organisms with distinct ploidies and combinations of the parental genomes (= genomotypes) (2n=50, PA; 3n=75, PAA/PPA; and 4n=100, PPAA), highly biased towards females, from which the once lost AA individuals were reconstituted as an all-male lineage with P mitochondrial DNA (Collares-Pereira *et al.* 2013). The persistence of most *S. alburnoides* populations is, however, dependent on crosses with the sympatric bisexual species of the *Squalius* genus, namely with *S. carolitertii* (C genome) in northern drainages, *S. pyrenaicus* (P genome) in central and southern drainages, and *S. aradensis* (Q genome) in a southwestern Portuguese drainage (Supplementary Fig. 1a). Over time, these crosses have resulted in the substitution of the ancestral P maternal nuclear genome of the complex by C, P or Q extant genomes, and produced considerable variation in genomotype composition among drainages (reviewed in Collares-Pereira *et al.* 2013).

The reproductive dynamics of the *S. alburnoides* hybrid complex has been extensively studied assuming bisexual *Squalius* species were allopatric, and, thus, only a single parental species coexisted with the complex in each drainage (*e.g.* Alves *et al.* 2001; Cunha *et al.* 2004; Pala and Coelho 2005; Collares-Pereira *et al.* 2013; Morgado-Santos *et al.* 2015, 2016). However, *S. alburnoides* was recently found to coexist not only with *S. aradensis* but also with *S. pyrenaicus* in a tributary of the Quarteira drainage (Supplementary Figs. 1b and 1c). The origin of *S. pyrenaicus* in this southwestern Portuguese drainage is more recent than that of *S. aradensis*, and may be associated with an ancient Pleistocenic migration from the nearby Guadiana drainage (Sousa-Santos *et al.* 2006b, 2007) or with a contemporary human-mediated introduction, undetected in previous fish surveys (Cunha *et al.* 2004; Mesquita *et al.* 2005; Sousa-Santos *et al.* 2006b, 2007). Whatever the ultimate cause, hybrids between *S. aradensis* and *S. pyrenaicus* were already found (PQ individuals), with males producing recombined haploid sperm (Morgado-Santos and Collares-Pereira, unpublished data), which opens a wide path to gene flow and introgression between both species. Moreover, the sympatry of *S. alburnoides* with both *S. aradensis* and *S. pyrenaicus* parental species doubles the number of possible crosses and offspring outcomes in the reproductive dynamics of the hybrid complex (Fig. 1), a reproductive network that becomes even more intricate if introgression between parental species is taken into account. Since *S. alburnoides* shows differential female mate preferences towards distinct genomotypes (Sousa-Santos *et al.* 2006a; Morgado-Santos *et al.* 2015, 2016) and can reproduce and receive genes from all bisexual *Squalius* species (Sousa-Santos 2007; Collares-Pereira *et al.* 2013), this population poses as an excellent template to study the interplay between mate choice and introgression.

In this study, we aimed at: a) analysing if and how mate choice by *S. alburnoides* females promotes the introgression of genes from *S. aradensis* (Q genome) and *S. pyrenaicus* (P genome) into the hybrid complex; and b) assessing whether mate choice itself is influenced by the identity of the genomes in both males and females. Specifically, we conducted a set of experimental mate choice affiliation trials to assess the preference of hybrid females towards males of *S. alburnoides*, *S. aradensis* and *S. pyrenaicus*. Introgression levels of males and females were estimated using Genotyping-by-Sequencing (GBS), and the data were crossed with mate choices. The observed patterns were used to explore the potential genetic benefits associated with the mate choice/introgression interplay, and its putative relevance in behavioural and evolutionary contexts.

4.3 | *Materials and methods*

4.3.1 | *Fish collection and maintenance*

Prior to fish sampling for affiliation trials, we established the zone of sympatry among *S. alburnoides*, *S. aradensis* and *S. pyrenaicus* from pilot surveys conducted across the stream where *S. pyrenaicus* has been found. Surveyed sites were selected in the field, based on accessibility and representativeness, and provided a thorough coverage of the stream. Each site was electrofished using short pulses and moderate voltage (300 V, 2-4 A) to avoid accidental deaths, and fish were identified to species based on morphology (Coelho *et al.* 1998; Sousa-Santos 2007) and returned to the water.

To maximize the likelihood of collecting individuals with genomes with and without introgression, fish for affiliation trials were then captured in one sampling site inside and another outside the zone of sympatry. To guarantee data independence, selected sites were 5.5 km apart from each other and at more than 2.0 km from the edges of the sympatry zone. Each site was electrofished as described above, covering microhabitat diversity to maximize the likelihood of capturing multiple *S. alburnoides* genomotypes (Martins *et al.* 1998). Fish sampling was conducted in April 2012, early in the fish reproductive season (Ribeiro *et al.* 2003; Sousa-Santos *et al.* 2014; Morgado-Santos *et al.* 2016), when individuals can be sexed by applying a gentle abdominal pressure to emit a few eggs or sperm. This was necessary because *Squalius* species lack evident sexual dimorphism, and only *S. alburnoides* nuclear non-hybrid AA males can be easily recognized. Overall, *S. alburnoides* females and *S. aradensis* males were caught in both sites, but *S. alburnoides* nuclear non-hybrid AA males and *S. pyrenaicus* males were only caught inside the sympatry zone. No *S. alburnoides* hybrid males were found. Immature individuals were returned to the water, and mature individuals without evidence of physiological stress or injuries, were transported to the laboratory in separate aerated vats.

Overall, 100 individuals were selected for affiliation trials, including 56 *S. alburnoides* females, 14 *S. alburnoides* nuclear non-hybrid AA males, 18 *S. aradensis* males (QQ) and 12 *S. pyrenaicus* males (PP). Only males caught inside the sympatry zone were selected to exclude potentially confounding effects associated with sampling site, given the lack of AA and PP males outside the sympatry zone. Selected fish were anesthetized (0.1 g/L MS-222, 0.2 g/L $NaHCO_3$), measured for standard length (SL, mm), photographed on both sides for individual recognition (Morgado-Santos *et al.* 2010) and then acclimated for two weeks in an in-house tank (150 cm × 40 cm × 40 cm; 240 L), at temperature (22 °C) and photoperiod (13 h light cycle) similar to those prevailing in the wild throughout the reproductive season.

4.3.2 | *Mate choice affiliation trials*

Mate preferences of *S. alburnoides* females for males of *S. alburnoides* (AA), *S. aradensis* (QQ) and *S. pyrenaicus* (PP) were assessed in 56 affiliation trials, without prior knowledge of individual ploidy, genomotype and putative introgression. Affiliation trials were performed in a tree-branched tank (Supplementary Fig. 2), previously used to test mate preferences in *S. alburnoides* (Morgado-Santos *et al.* 2015). In each trial, one female was allocated in the central neutral area of the tank, and AA, QQ and PP males were randomly allocated to isolated compartments, at the end of each branch. Compartments were delimited by transparent perforated acrylic plates, allowing the passage of all types of stimuli (*e.g.* visual, chemical and acoustic) among individuals.

Overall, each female was used in only one trial, but each male was used more than once. This was inevitable because large samples of males are difficult to obtain in the highly female-biased populations of *S. alburnoides* (see Collares-Pereira *et al.* 2013), and *S. pyrenaicus* and *S. aradensis* are listed as Endangered and Critically Endangered, respectively (Cabral *et al.* 2005). On average, each AA male was used in 4.0 trials (±1.0, 3-7), each QQ male was used in 3.1 trials (±1.8, 1-6), and each PP male was used in 4.7 trials (±2.9, 2-12). However, combinations of the same males were never repeated to minimize confounding effects associated with individual dependence. Between trials, the experimental tank was emptied, dried and the water replaced to remove vestiges of previous fish.

Before trials began, fish were allowed 30 min to acclimate to the tank. During each trial, fish were videotaped from the top of the tank using a digital camera, the tank was surrounded by an opaque curtain, and there was no one in the room. Each trial lasted 1 h, during which females swam freely and visited males. For each female, the time spent in the neutral area (7.2±8.9%) was ignored, and the proportion of time spent near each male was used to index its mate preference.

4.3.3 | *Assessments of ploidy, genomotype and introgression*

After the affiliation trials, fish were assessed for ploidy, genomotype and introgression. Each individual was anesthetized (0.1 g/L MS-222, 0.2 g/L $NaHCO_3$), and a small clip of the caudal fin was collected for ploidy assessment (Lamatsch *et al.* 2000) and for DNA extraction following an adapted phenol-chloroform protocol (Miller *et al.* 1988). The genomotype of AA males was only confirmed through sequencing of the β-actin gene (Sousa-Santos *et al.* 2005), whereas the genomotype of the remaining individuals, due to the possibility of introgression between Q or P genomes, was assessed through Genotyping-by-Sequencing (GBS) (Elshire *et al.* 2011), aimed at SNP discovery (Narum *et al.* 2013), besides β-actin sequencing.

To serve as templates for SNP identification, GBS sequencing also included DNA samples of 5 *S. alburnoides* males (AA) from other sites in the Quarteira drainage, 5 *S. aradensis* (QQ) from the Arade drainage and 4 *S. pyrenaicus* (PP) from the Almargem drainage (see Supplementary Fig. 1b for drainage locations). These additional samples of allopatric *S. aradensis* and *S. pyrenaicus* were used to guarantee pure genomes with no introgression and to allow the identification of reliable diagnostic SNP's for each species.

GBS procedures were performed in outsourcing at Beijing Genomics Institute (BGI, www.bgi.com). Following manufacturer instructions, DNA samples were sent to BGI mixed with DNAstable Plus (Biomatrica), allowing DNA preservation during shipment at room temperature. After library construction (see Elshire *et al.* 2011 for detailed procedures), samples were sequenced in Illumina HiSeq 2000 system. The raw 91-nt paired-end reads were cleaned at BGI's facility, prior to SNP calling analysis.

The discovered loci were filtered, and the ones sequenced on at least half of each parental "pure" individuals were kept, *i.e.* 3 AA's (60%), 3 allopatric QQ's (60%) and 2 allopatric PP's (50%). Then, these loci were filtered in order to find diagnostic SNP's for each parental genome (A, Q and P). Specifically, four groups of SNP's were used: a) $A \neq Q = P$ SNP's, distinguishing the A genome from the other genomes, were used to determine the proportion of A genome in all individuals; b) $Q \neq A = P$ SNP's, distinguishing the Q genome from the other genomes, were used to determine the proportion of Q genome in *S. alburnoides* females; c) P≠A=Q SNP's, distinguishing the P genome from the other genomes, were used to determine the proportion of P genome in *S. alburnoides* females; and d) Q≠ P SNP's, distinguishing the Q and P genomes, were used to determine the proportion of Q and P genomes in *S. aradensis* and *S. pyrenaicus* males, which have no A genome. The number of reads sequenced for these SNP's were used to index the proportion of each genome in each individual and thereby assess introgression and confirm genomotypes.

Finally, based on both genotyping procedures (*β-actin* sequences and GBS data), individuals were categorized in two genome groups, as containing mostly Q or P genomes, regardless of the presence or absence of A genome (*e.g.* PA, PAA and PPA females were grouped together into the P genome group).

4.3.4 | *Ethics statement*

Field and laboratorial procedures followed the recommended ethical guidelines (ASAB 2015) and the Portuguese legislation regarding animal capture, manipulation and experimentation for scientific purposes (ICNF, permit number 140/2012/CAPT). All efforts were made to minimize accidental deaths and stress on fish throughout the study. After analyses, individuals in good condition were returned to the sites of collection in the Quarteira drainage.

4.3.5 | *Data analyses*

Analyses of female mate preferences involved a three-stage procedure, focused in assessing variation in the proportion of time each female spent towards each male genomotype in affiliation trials. We started by quantifying mate preference in relation to male size, using linear regression analysis. Then, we analysed mate preferences in relation to male and female genome groups, using repeated-measures or one-way ANOVA, when data were dependent or independent, respectively. Finally, we used a moving average approach to identify underlying trends in mate preferences in relation to male and female introgression levels. Prior to analyses, variables were assessed for normality, sphericity and outliers using the Shapiro-Wilk's, Mauchly's and Grubbs' tests, respectively. Whenever data were skewed, the angular transformation was used to approach normality, and, in cases of no sphericity, the Greenhouse-Geisser correction was used to reduce the degrees of freedom associated with the *F* value. All analyses were performed in Statistica 13 software (StatSoft 2013).

Female mate preferences in relation to male SL (mm) were tested for all males and separately for AA, QQ and PP males (A, Q and P genome groups, respectively), regardless of female genome group. Since males were used in multiple trials, analysis focused on the average proportion of the time females spent with each male among trials.

To assess female mate preferences in relation to male genome group (A, Q and P), hybrid females were grouped into Q or P genome groups based only on the dominant component of their heterospecific genome (given they all share the A genome), irrespective of introgression levels.

The isolated effect of male introgression level on female mate preferences was tested using only females of the Q genome group, which showed no introgression of P genome, to exclude potentially confounding effects of female introgression level (see Results for further details). *Squalius pyrenaicus* (PP) males (the only showing evidence of introgression) were ordered based on introgression level and moving averages of the proportion of time spent by females towards each male type were determined for sets of 4 individuals.

The effect of female introgression on mate preferences was tested only in females of the P genome group, since, as previously referred, females of the Q genome group were not introgressed (see Results for further details). Two separate analyses were conducted, based on the introgression level of Q genome of each female and on the absolute difference in introgression level of Q genome between female and PP male pairs. In both cases, moving averages of the proportion of time spent by females towards each male type were determined for sets of 8 ordered females. These analyses allowed the assessment of the effects of female introgression on mate preferences, but also of eventual interactions between male and female introgression.

4.4 | *Results*

4.4.1 | *Ploidy, genomotype and introgression among individuals*

Flow cytometry confirmed the diploidy of all males (*S. alburnoides*, *S. aradensis* and *S. pyrenaicus*) and revealed the existence of diploid and triploid *S. alburnoides* females. Sequences of the *β-actin* gene were obtained for all individuals used in affiliation trials (*N*=100), but quality GBS data were only obtained for 84 individuals. For genomotype assessment, GBS data prevailed over *β-actin* sequences, which were used solely for the individuals without GBS data (*N*=16). Detailed results on fish ploidy, genomotype and introgression are presented in the supplementary material (Supplementary Document 1).

The genomotypes of all males of *S. alburnoides* and *S. aradensis* males were confirmed as AA (*N*=14; 4.0±0.4 cm, 3.5-4.7) and QQ (*N*=18; 6.5±0.9 cm, 5.2-8.7), respectively (Supplementary Fig. 3). However, one male morphologically identified as *S. pyrenaicus* (8.4 cm) was a PQ hybrid, being, thus, excluded from further analysis. All other *S. pyrenaicus* males were confirmed as PP (*N*=11; 7.4±1.5 cm, 5.9-10.7) (Supplementary Fig. 3). *Squalius alburnoides* diploid females included QA (*N*=1; 5.5 cm) and PA (*N*=11; 6.5±0.8 cm, 5.1-7.9) genomotypes, and triploid females included QAA (*N*=27; 6.7±1.0 cm, 5.4-9.5), PAA (*N*=15; 6.0±0.5 cm, 5.1-6.9) and PPA (*N*=1; 5.5 cm) genomotypes (Supplementary Fig. 4). The heterospecific genome of another triploid female (5.9 cm) was a mixture of Q and P genomes (17.7% and 12.8%, respectively, which translate into standardized percentages of 58.1% and 41.9%, excluding the two A genomes from the calculations), not belonging to either P or Q genome groups, and, thus, this female was excluded from analyses discriminating genome groups. Altogether, 50.0% of the females analysed belonged to the Q genome group (QA+QAA) and 48.2% belonged to the P genome group (PA+PAA+PPA). However, female genomotype composition varied between sites, with all females from outside the sympatry zone belonging to the Q genome group and 84.4% of the females from inside the sympatry zone belonging to the P genome group.

GBS data revealed that the Q genome was not introgressed with the P genome, neither in *S. alburnoides* females nor in *S. aradensis* males, showing only vestigial percentages of P-specific reads (*N*=46; 0.6±0.6%, 0.0-2.9), which fall within the technics' error range. However, P genome showed introgression of Q genome, with variable percentages of Q-specific reads in *S. alburnoides* females (*N*=27; 3.0±3.6%, 0.1-12.5) and in *S. pyrenaicus* males (*N*=11; 3.5±1.9%, 1.3-7.9).

4.4.2 | *Mate preferences in relation to male size and male and female genome group*

Mate preferences were derived from trials including all individuals and from trials including only the individuals whose genomotype was confirmed through GBS. Moreover, besides the trials involving the PQ male mentioned above, a trial involving a QAA female spending 98.3%, 0.8% and 0.0% of time interacting with AA, PP and QQ males, respectively, was also excluded since it was identified as an extreme outlier by

Grubbs' test ($G=5.3$, $p<0.001$). Raw data on all trials conducted are presented in the supplementary material (Supplementary Table 1).

Male genomotypes varied significantly in size ($F_{2,40}=44.0$, $p<0.001$), also when considering only the males whose genomotype was confirmed through GBS ($F_{2,38}=45.8$, $p<0.001$). Generally, PP males were the largest and AA males the smallest. Female mate preference was positively associated with male size, considering all individuals ($N=43$; $R^2=0.32$, $F_{1,41}=19.4$, $p<0.001$) and also only those with confirmed genomotype ($N=41$; $R^2=0.32$, $F_{1,39}=19.6$, $p<0.001$) (Fig. 2). However, no significant relationships were found when male genomotypes were analysed separately, irrespective of whether all individuals (AA: $N=14$, $R^2=0.00$, $F_{1,12}=0.0$, $p=0.923$; QQ: $N=18$, $R^2=0.01$, $F_{1,16}=0.2$, $p=0.687$; PP: $N=11$, $R^2=0.08$, $F_{1,9}=0.8$, $p=0.391$) or only those analysed through GBS were considered (AA: $N=14$ $R^2=0.01$, $F_{1,12}=0.2$, $p=0.682$; QQ: $N=17$, $R^2=0.01$, $F_{1,15}=0.2$, $p=0.702$; PP: $N=10$, $R^2=0.04$, $F_{1,8}=0.4$, $p=0.565$).

Overall, female mate preferences varied among male genomotypes, either considering all males ($F_{2,40}=13.0$, $p<0.001$) or only the ones whose genomotype was confirmed through GBS ($F_{2,38}=13.8$, $p<0.001$). In general, PP males (50.2±16.2%, 28.2-79.1; GBS only: 52.8±17.1%, 28.2-79.1) were favoured over AA males (12.2±6.2%, 2.1-27.4; GBS only: 11.3±5.6%, 2.1-23.6), and QQ males were intermediate (39.8±27.0%, 1.2-95.0; GBS only: 39.1±27.8%, 1.2-95.0) (Fig. 2). Similar patterns were found for the females of Q genome group ($N=27$; $F_{1.3,34.5}=9.1$, $p<0.001$; GBS only: $N=13$; $F_{1.3,15.2}=3.4$, $p=0.076$), as well as for the females of the P genome group ($N=24$; $F_{1.3,29.3}=9.1$, $p=0.003$; GBS only: $N=20$; $F_{1.2,22.8}=8.6$, $p=0.005$) (Fig. 3).

4.4.3 | *Mate preferences in relation to male and female introgression levels*

Mate preferences of females of the Q genome group, which showed no introgression of P genome, varied considerably depending on the introgression level of Q genome within PP males (Fig. 4). Although there was some variability, females generally showed a higher preference towards QQ males when tested against PP males with low introgression levels, but shifted towards PP males when these had higher introgression levels of Q genome (Fig. 4).

Females with P genome and with variable levels of introgression of Q genome, showed a higher mate preference towards PP males, with AA males being the least favoured and QQ males intermediate (Fig. 5). Specifically, variation of mate preferences towards PP males varied harmonically with female introgression level of Q genome (Fig. 5a), with oscillations differing in size among females. Instead, when mate preferences were analysed against the difference between female and male introgression levels, a linear relation was found (Fig. 5b), indicating that P females showed a higher preference towards PP males with similar introgression levels.

4.5 | *Discussion*

Our study of mate choice trends in an allopolyploid complex with a wide range of mating options unveiled that mating preferences of females are influenced by male size and genomotype and by male and female introgression levels. Hybrid females showed higher preference towards introgressed males and towards males with introgression levels similar to their own, a mate choice trend that favours crosses with the most recent bisexual species in the drainage, thus promoting the introgression of its genome into the hybrid complex.

Although one may question whether the low introgression levels (mostly <10%) here retrieved may be simple technique noise, we truly believe they represent true levels of introgression based on the following aspects: a) GBS sequencing error level is much smaller than the introgression levels obtained for most introgressed individuals (averaging 3.2%), as evidenced by the extremely low proportion of A-specific reads sequenced in QQ, PP and PQ individuals (averaging 0.2%); b) the introgression levels were unidirectional (Q within P: average of 3.2%; P within Q: average of 0.6%), which would be extremely unlikely to occur under any random effect such as technique noise; and c) the mate preferences of females were related to such levels of introgression, which, once again, would be extremely unlikely to result from random effects.

The mate preferences of *S. alburnoides* females were positively associated with male size, indicating a higher affiliation towards larger males (Fig. 2). Large body size is routinely considered advantageous for males, since it may relate to higher fitness, either through intrasexual (male-male competition) or intersexual selections (female mate choice) (Andersson 1994). On the one hand, large body size normally leads to higher competitive abilities (*e.g.* Jenkins 1969; Smith and Parker 1976; Johnsson *et al.* 1999), with larger males being normally dominant (Keenleyside *et al.* 1985; Magnhagen and Kvarnemo 1989; Candolin and Voigt 2001; Mills and Reynolds 2003; Wacker *et al.* 2012), particularly at low density (Mills and Reynolds 2003; Reichard *et al.* 2004a,b). On the other hand, females often choose larger males to mate (Keenleyside *et al.* 1985; Magnhagen and Kvarnemo 1989; Rosenthal and Evans 1998; Fagundes *et al.* 2007; Passos *et al.* 2014), probably because such trait is identified as a signal of better reproductive quality and consequent higher offspring fitness and success (Gonçalves *et al.* 2002; Rasotto *et al.* 2010; Pizzolon *et al.* 2012). In this context, male body size may be of relevance among *Squalius* species, in which spawning territory preparation and defence have already been observed, namely in *S. pyrenaicus* (Sousa-Santos *et al.* 2014). Moreover, in an allopolyploid complex such as *S. alburnoides*, which includes genomotypes with variable sizes, the association of female mate preference with male size will inevitably affect the probability of male genomotypes being chosen and, thus, will influence the genetic structure and dynamics of populations.

Females of *S. alburnoides* displayed intersexual selection, showing higher mating preference towards *S. pyrenaicus* males (PP) than towards *S. alburnoides* nuclear non-hybrid males (AA) (Figs. 2 and 3). This is consistent with the perceived patterns of mate choice based on male size, as *S. pyrenaicus* males (PP) and *S. alburnoides* nuclear non-hybrid males (AA) were the biggest and the smallest tested male genomotypes, respectively. The preferences of *S. alburnoides* females for males of other species over their own may, thus, lead to "heterospecific" crosses, a trend already reported for other fishes (Ryan and Wagner 1987; Hankison and Morris 2002, 2003). Unfortunately, it is not possible to ascertain whether the observed female mate preferences are related to the male genomotypes, to their sizes or to a combination of the two, since the variation in body size among males of *S. alburnoides* and of other *Squalius* species is substantial, and genomotypes show little to no overlap in size ranges (Sousa-Santos 2007; Cunha *et al.* 2009; present study). However, previous studies have suggested that females select males based on genomotype rather than size (Sousa-Santos *et al.* 2006a; Morgado-Santos *et al.* 2015, 2016), with particular genomotypes being favoured even when available mates have similar sizes (Morgado-Santos *et al.* 2015). This could also be the case here, given no relationships between female mate preference and male size were observed within each male genomotype (Fig. 2), but further research is needed to clarify this issue. Notwithstanding, the lack of female preference towards AA males, which are crucial for the persistence of southern populations, strengthens previous evidence suggesting that these males may display sneaking behaviour (Sousa-Santos 2007). This is a typical alternative strategy of smaller submissive males when females favour bigger males to breed (Fitzpatrick *et al.* 2016), that may result in a reproductive success similar to that of dominant males (*e.g.* through sperm competition), and even represent an advantage to females (Kanoh 1996; Fu *et al.* 2001; Smith and Reichard 2005). But why would *S. alburnoides* females prefer the males of the sympatric *Squalius* species over their own? Probably because, by parasitizing the reproduction of parental species, females directly produce nuclear hybrid offspring, perpetuating the hybridization cycle and the persistence of the complex, a route that can easily be attained through mate choice and that was already reported for other vertebrate hybrid complexes (Abt and Reyer 1993; Engeler and Reyer 2001).

While individuals of the Q genome group (QQ males and QAA females) were not introgressed with P genome, individuals of the P genome group (PP males and PA/PAA females) showed introgression with Q genome (Supplementary Figs. 3 and 4). The existence of introgressed individuals indicates that direct hybridization between *S. aradensis* (QQ) and *S. pyrenaicus* (PP) also occurs, although it may not be a common event, since only one PQ individual was found. Moreover, the variable levels of introgression of Q genome found in individuals of the P genome group strongly suggest that P and Q genomes undergo regular meiosis with standard crossing over in PQ hybrids. This hypothesis is supported by evidence that spermatozoa of the single PQ male found were haploid and showed Q and P *β-actin* haplotypes (Morgado-Santos and Collares-Pereira, unpublished data), and by the occurrence of one *S. alburnoides* triploid female whose heterospecific (single) genome was nearly 50% Q and 50% P. However, the unidirectionality found in introgression, with Q within P genome but not P within Q genome, suggests that crosses between PP individuals and PQ hybrids are more common and/or successful than crosses between QQ individuals and PQ hybrids, whatever the underlying cause (*e.g.* assortative mating strength, differential offspring viability, etc.). Nevertheless, direct hybridization threatens genome and species integrity, which may hamper the already Critically Endangered *S. aradensis* (Cabral *et al.* 2005) through hybridization and introgression, as reported for other freshwater fishes (Allendorf and Leary 1988; Leary *et al.* 1995).

Females of the Q genome group exhibited assortative mating, choosing males of their own genome group (QQ males), but shifted their preference towards PP males (disassortative mate choice) as Q genome introgression increased in the latter (Fig. 4). These results illustrate perfectly the known dilemma between mate choice based on heterozygosity *versus* genetic compatibility (Tregenza and Wedell 2000; Mays and Hill 2004). This well-discussed paradox implies that females that benefit from higher genetic variability for their offspring by mating with dissimilar males (mate choice based on heterozygosity) also risk genetic incompatibility, and, consequently, seek for an optimal level of dissimilarity. Although the *S. alburnoides* hybrid complex lacks strict reproductive isolation and can reproduce with all the sympatric species of the *Squalius* genus without risking genetic incompatibility, assortative or disassortative mating based on genetics may still be acting on hybrids, since they may hold the genes responsible for those traits in parental species. Herein, females of the Q genome group seemed to have found a balanced solution by avoiding the risk of reproducing with excessively dissimilar males (*i.e.* “pure” PP males), but mating with PP males introgressed with Q genome, that pose less risk of genetic incompatibility while benefiting from disassortative mating. This kind of genomic-based disassortative mate choice may arise accidentally and have harsh consequences for the populations (D'Amore *et al.* 2009), but it often brings about fitness advantages for individuals (Tregenza and Wedell 2000). Genetic-based disassortative mate choice is well-documented regarding the major histocompatibility complex, MHC (*e.g.* Reusch *et al.* 2001; Milinski 2003, 2006; Forsberg *et al.* 2007; Eizaguirre *et al.* 2009), with species seeking for heterozygosity in partners, but avoiding too dissimilar mates (Bonneaud *et al.* 2006; Hoffman *et al.* 2007). Disassortative mating may promote intrapopulation diversity (Takahashi and Hori 2008), the production of superior offspring with higher genetic variability and all the consequent genetic benefits related to higher heterozygosity (Brown 1997; reviewed in Kempenaers 2007), which are particularly important for nonsexual organisms that are normally linked to genetic uniformity and decreased variability due to the lack of meiotic recombination in most hybrid forms (Maynard Smith 1986; Kondrashov 1993; de Visser & Elena 2007). Genetic variability may be advantageous in heterogeneous and unstable environments, such as Mediterranean rivers (Gasith and Resh 1999), especially given the reduced genetic variability of the Q genome (Mesquita *et al.* 2005). Genetic benefits through mate choice can be easily accomplished by the *S. alburnoides* hybrid complex, which may bring such advantages to an interspecific level by reproducing with multiple sympatric species. Given *S. alburnoides* is a multiple spawner (Ribeiro *et al.* 2003; Morgado-Santos *et al.* 2016), females can potentiate even more their fitness by reproducing with multiple males in a single reproductive season, thus decreasing the risk of reproduction failure (Jennions and Petrie 2000).

Females of the P genome group also exhibited assortative mating, choosing males of their own genome group (PP males), and strengthening their preference towards these males when they had introgression levels of Q genome similar to their own (Fig. 5). The higher preference of P females towards PP males with similar levels of introgression suggests there is some sort of assortative mating based on hybrid heterozygosity when females are also introgressed. Assortative mating directly linked to genetics has already been reported for other species (Nevo and Heth 1976; Navas-García *et al.* 2009), including humans (Roberts *et al.* 2005). Unfortunately, QQ males were not introgressed with P genome, and, thus, it was not possible to assess if females of the P genome group would shift their mate preference towards QQ males if they were introgressed, similarly to what was found for females of the Q genome group towards PP males.

The overall mate choice patterns uncovered in this study tend to promote the introgression of the P genome into the local population of *S. alburnoides* complex, and may consequently lead to its spread across the drainage and shape the genetic content of a putative species arising from hybrid speciation. This flow of P genome into the hybrid complex is apparently consistent with patterns of fish abundance perceived in the sympatry zone, where *S. aradensis* (QQ) and *S. pyrenaicus* (PP) coexisted and where most *S. alburnoides* hybrids (>75%) belonged to the P genome group (see Supplementary Fig. 4). However, further studies are needed, including other variables, such as the relative frequency of parental species in the sympatry zone. In the case the sympatry area increases over time, this trend may lead to the replacement of the nuclear Q genome within *S. alburnoides* population by the P genome. Genomic replacements occurred in *S. alburnoides* populations throughout the history of the complex (Collares-Pereira *et al.* 2013), resulting in the complete substitution of the P ancestral maternal genome by the genomes of the sympatric *Squalius* species living in each drainage, including in the Quarteira drainage (Sousa-Santos *et al.* 2007). Although the nuclear genomic introgressions may bring about multiple genetic benefits as discussed above, they are unlikely to promote major changes in the reproductive dynamics of the hybrid complex, since reproductive mechanisms are apparently constant for each genomotype/ploidy independently of the particular genomes they comprise. For instance, QAA and PAA females both reproduce through meiotic hybridogenesis, producing mostly *a* oocytes (Collares-Pereira *et al.* 2013). However, introgressions are of key relevance from an evolutionary perspective, since, similarly to other hybrid systems (Seehausen 2004; Mallet 2007), *S. alburnoides* populations pose as templates for hybrid speciation (Morgado-Santos *et al.* 2015), with some of them being already on the verge of evolving towards new independent bisexual species (Cunha *et al.* 2008), whose genetic content would be entirely dependent on the genomes of the hybrids from which they evolved.

Taken together, our results indicate that mate choice and introgression may play an intricate game in hybrid organisms. While mate choice may promote introgression of new genes into the hybrid complexes, introgression itself can also influence mate choice, a cycle from which hybrids take advantage to the fullest, not only by enjoying genetic benefits, but also by accumulating an advantageous genetic baggage to the forthcoming species potentially arising from hybrid speciation. This underlines the role of mate choice as a key evolutionary mechanism for animal hybrid complexes, highlighting its importance in understanding the population dynamics and evolutionary pathways of allopolyploids.

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DATA ACCESSIBILITY | Analyses reported in this article can be reproduced using the data provided by the authors, including raw data on mate choice (Supplementary Table 1) uploaded to Dryad data depository. GenBank accession numbers of DNA sequences: a) known *β-actin* Q haplotypes from Quarteira drainage, DQ150267, DQ150321-2, DQ150353-4 (Sousa-Santos *et al.* 2007); b) known *β-actin* A haplotypes from Quarteira drainage, DQ350252, DQ150335-6, DQ150352 (Sousa-Santos *et al.* 2007); c) *β-actin* P haplotype found herein in Quarteira drainage, XXXXXXXX; d) *β-actin* sequence of sperm sample of PQ hybrid male, XXXXXXXX. Illumina reads are deposited in the NCBI Sequence Read Archive (SRA), under accession number XXXXXXXX.

4.6 | *References*

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4.7 | *Figures*

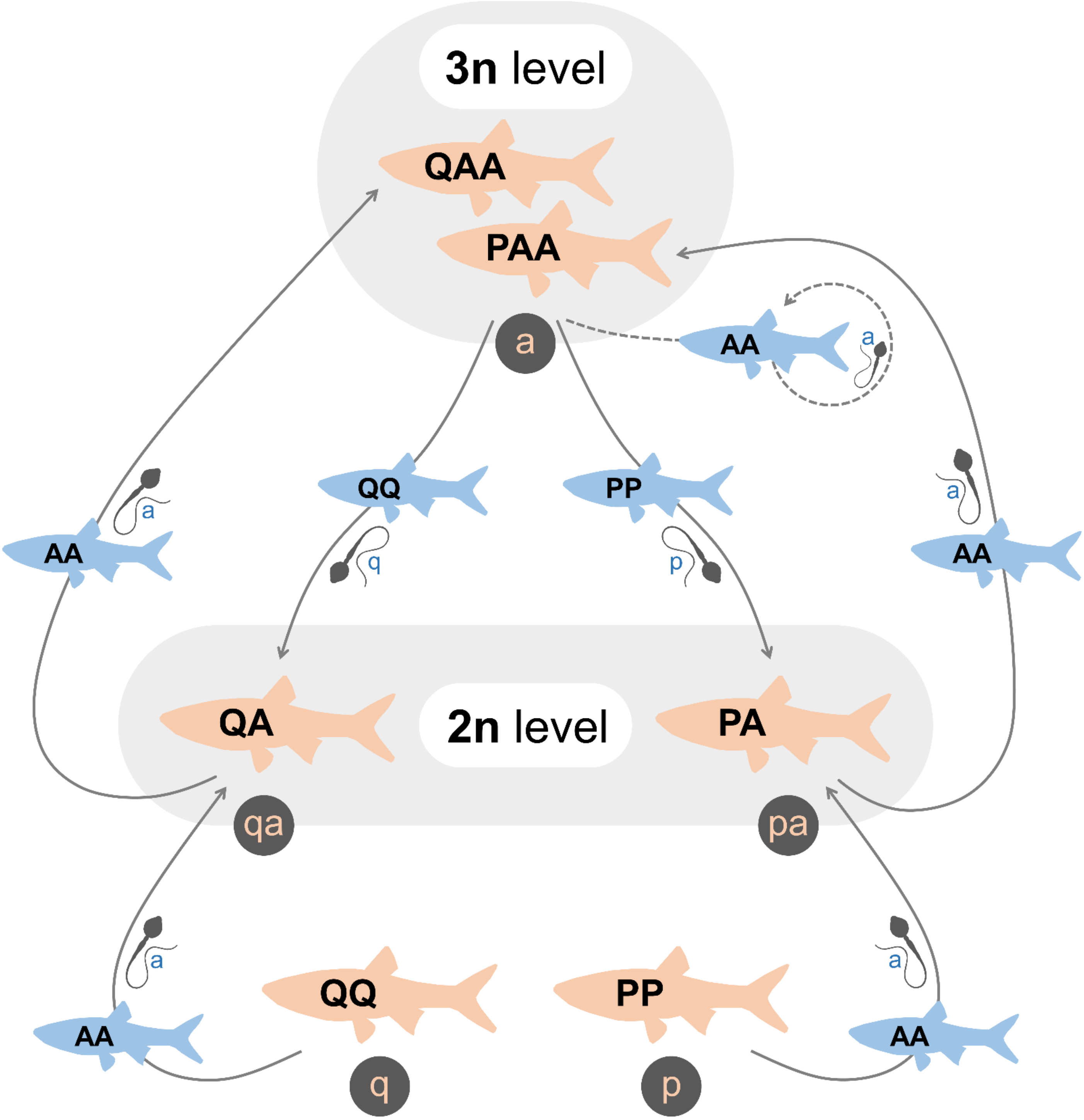


Figure 1 | Core of the reproductive framework of *S. alburnoides* allopolyploid complex in the zone of sympatry with *S. aradensis* and *S. pyrenaicus* in the Quarteira drainage, showing the main crosses that may uphold the persistence of hybrid populations, including the genomotypes and sexes recorded in this and previous studies (Mesquita *et*

al. 2005; Sousa-Santos *et al.* 2006b, 2007). The diagram highlights that, similarly to all other southern populations (Collares-Pereira *et al.* 2013), *S. alburnoides* triploid and diploid females in the Quarteira drainage are unable to directly produce offspring of their own kind, being, thus, interdependent and showing cyclic obligatory shifts. Females are presented in light red and males in blue. Capital letters refer to fish genomotypes, and small letters to gamete genomes: A,a from the *Anaecypris*-like paternal ancestor of the complex; Q,q from the *S. aradensis* bisexual species; and P,p from the *S. pyrenaicus* bisexual species. Reproductive mechanisms include i) meiotic hybridogenesis in PAA and QAA females, in which the heterospecific P and Q genomes are discarded, respectively, and the remaining AA genomes undergo regular meiosis, producing haploid *a* oocytes; ii) regular meiosis in AA, PP and QQ males, which produce haploid *a*, *p* and *q* sperm, respectively; and iii) clonal oogenesis in PA and QA females, in which diploid *pa* and *qa* oocytes are produced, respectively (see Collares-Pereira *et al.* 2013 for further details). No *S. alburnoides* males other than AA's were ever found in this population.

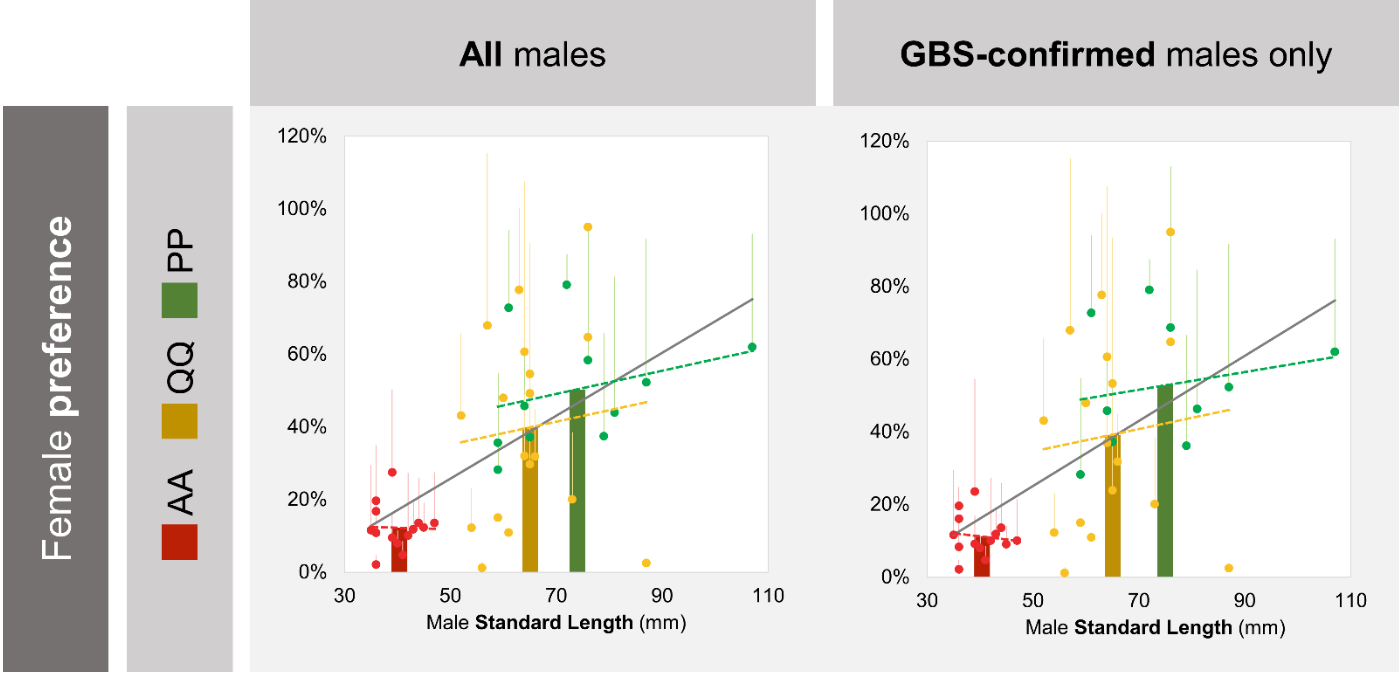


Figure 2 | Variation in female mate preferences in relation to male size, according to male genomotype, namely AA (red), QQ (yellow) and PP (green). Dots and error bars for each male represent the average and standard deviation of preferences by females, respectively, regardless of female genomotype. Lines represent the trends found for all male genomotypes (black) and for each male genomotype separately (AA, red; QQ, yellow; PP, green). Analyses were performed using all trials and using only the ones with

QQ and PP males whose genomotype was confirmed through GBS data (see Materials and Methods for further details).

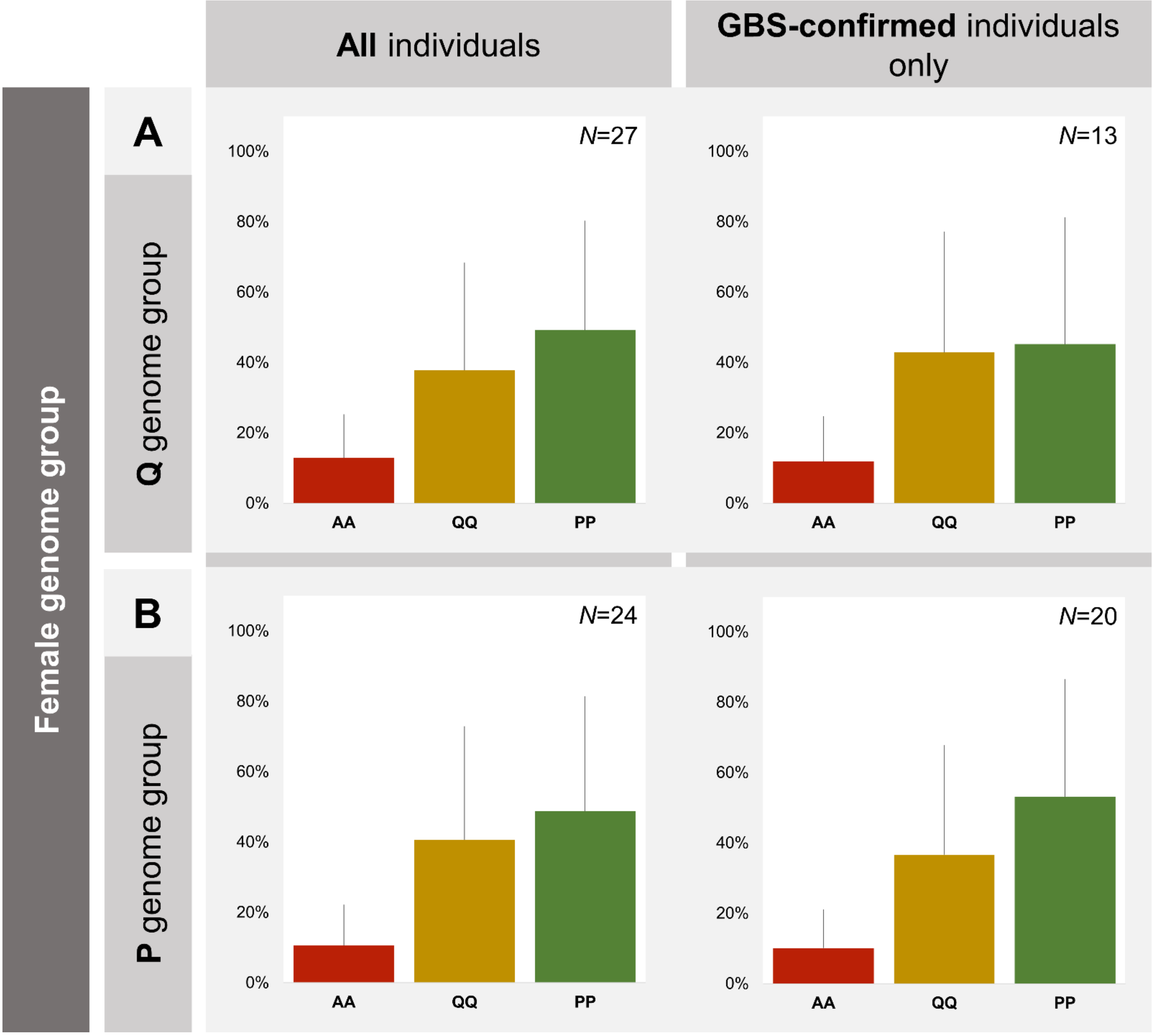


Figure 3 | Variation in female mate preferences in relation to male genomotype, according to female genome group (Q and P; **A** and **B** panels, respectively). Coloured bars and error bars for each male genomotype represent the average and standard deviation of preferences by females, respectively. Analyses were performed using all individuals and using only the individuals whose genomotype was confirmed through GBS data (see Materials and Methods for further details).

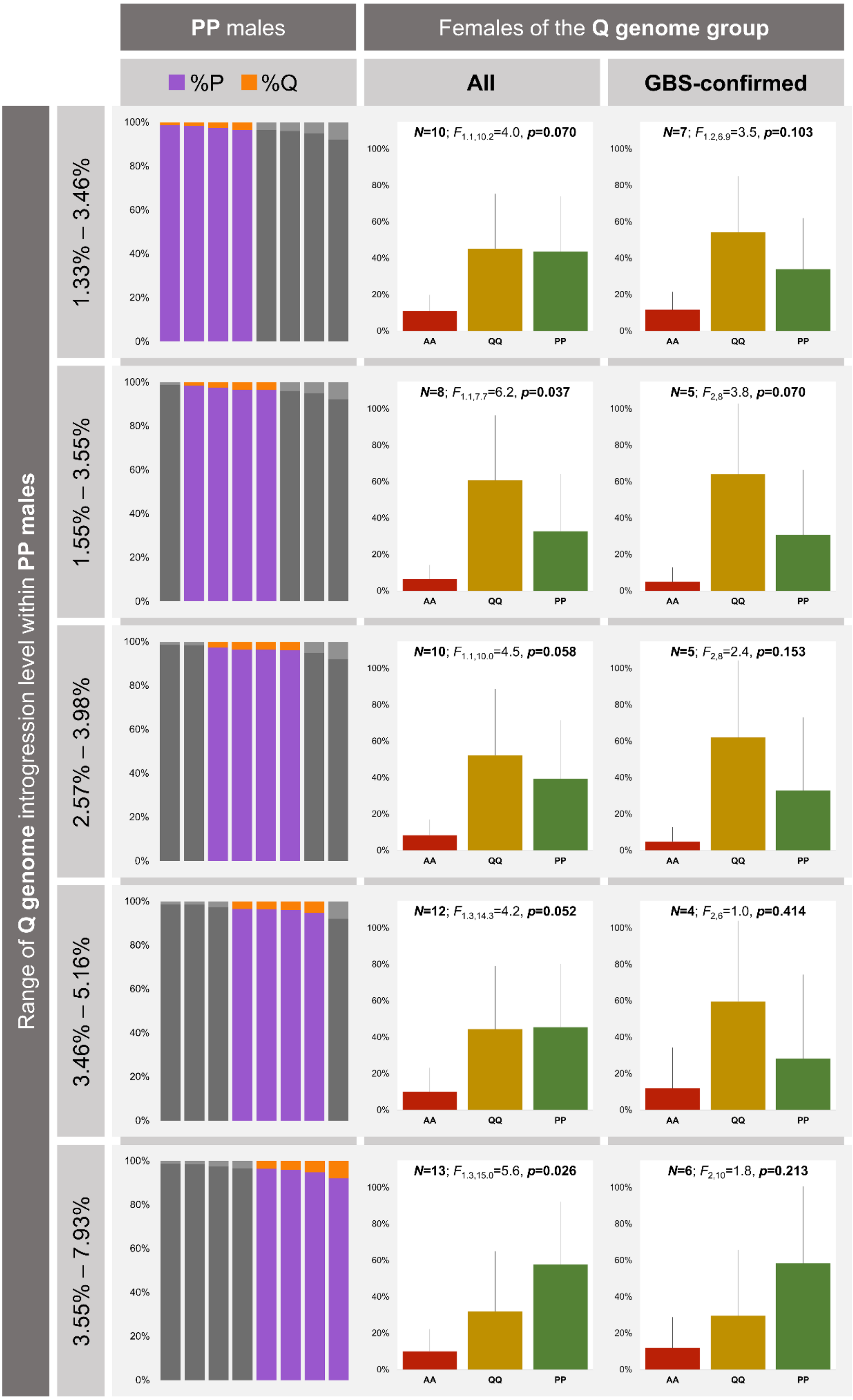


Figure 4. Variation in mate preferences of the females of the Q genome group in relation

to male genomotype, according to the introgression level of Q genome within PP males (see Materials and Methods for further details). The first column of bar plots shows the proportion of Q (orange) and P (purple) genomes in PP males, separated in groups of 4 individuals according to introgression level, with ranges shown on the left. The mean with error bars plots represent female preferences towards each of these groups of PP males relative to AA and QQ males. Error bars are the standard deviation of preferences by females, and sample sizes and repeated-measures ANOVA's results are shown for each analysis. Analyses were performed using all females or only the ones whose genomotype was confirmed through GBS.

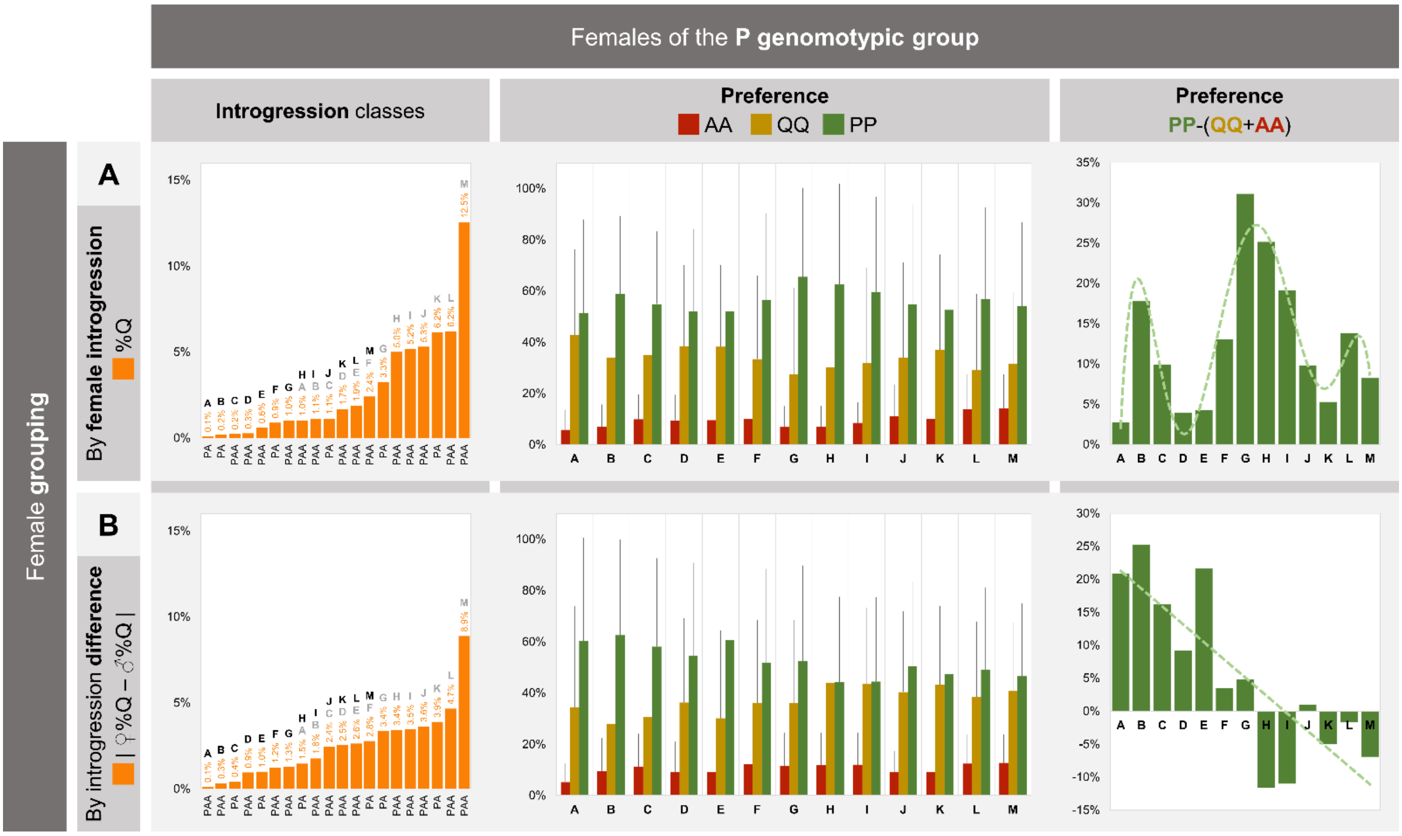
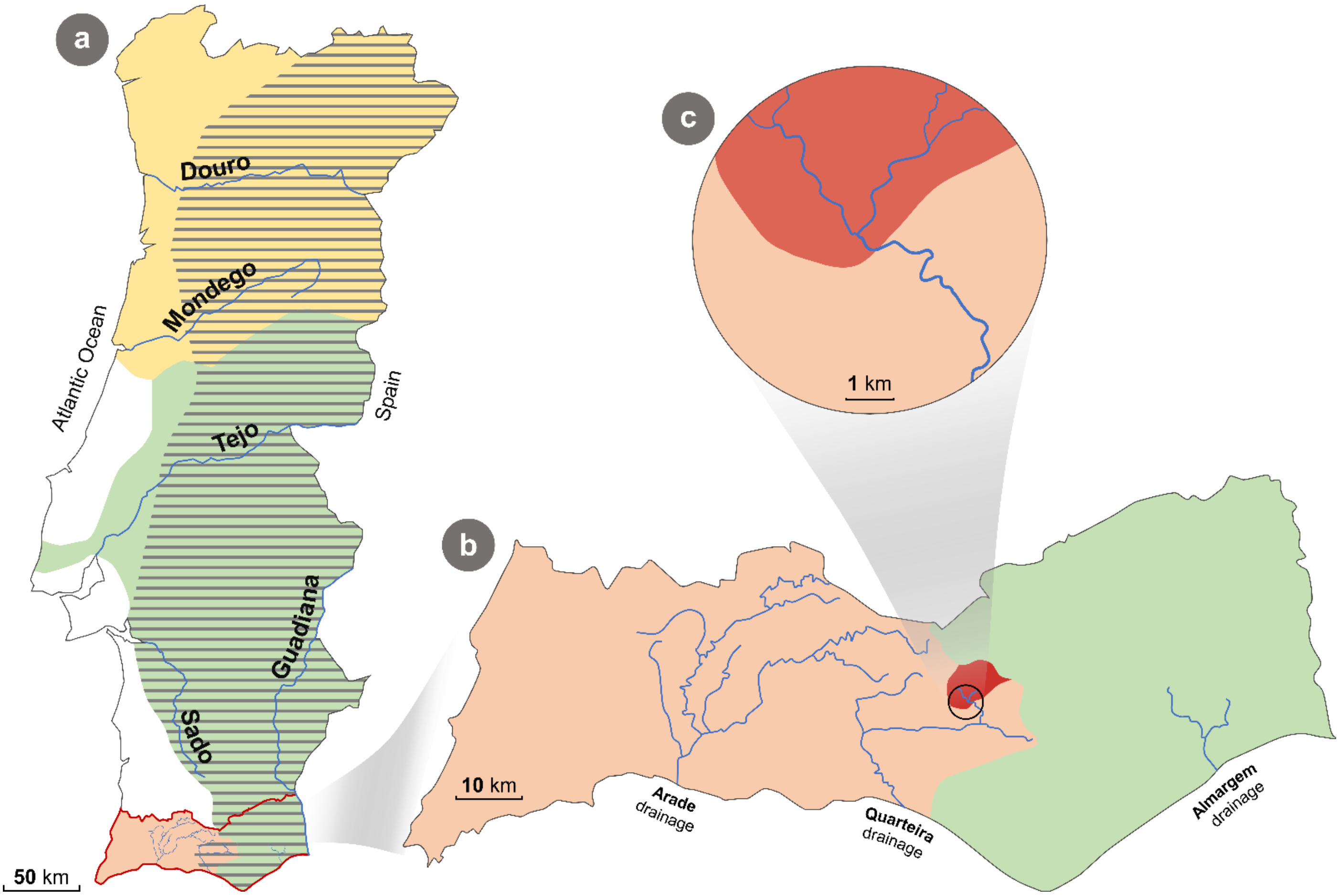


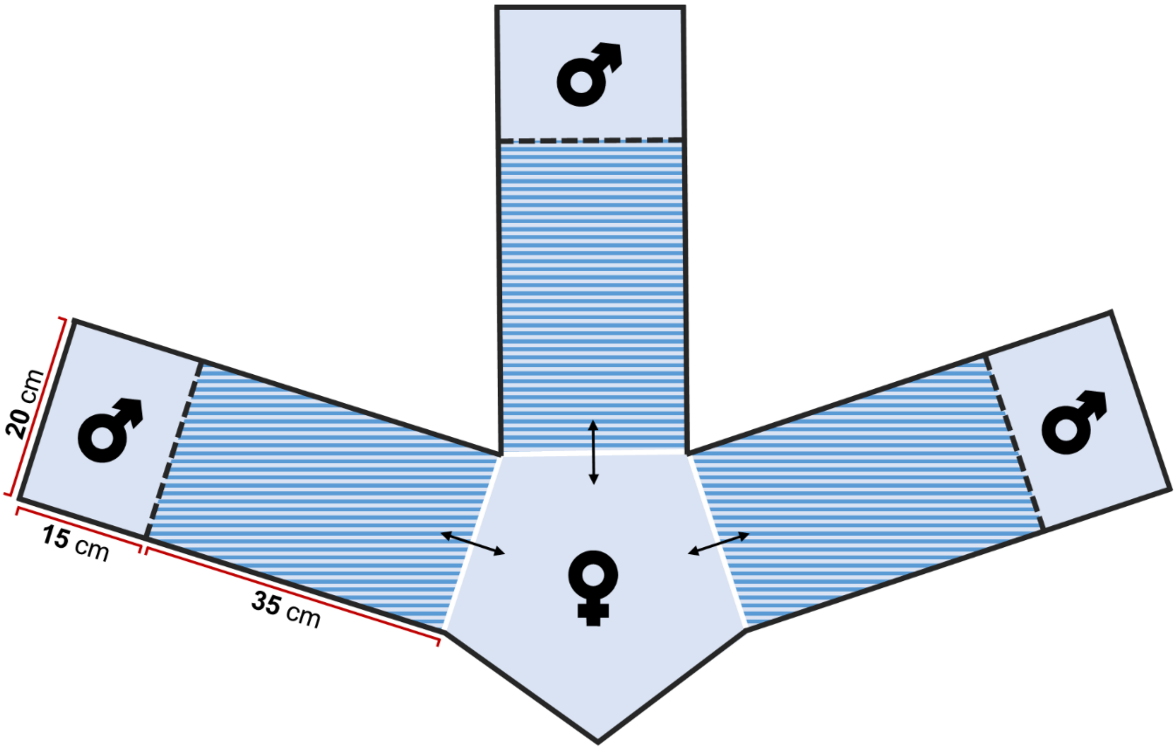
Figure 5. Variation in mate preferences of the females of the P genome group in relation to male genomotype, according to the introgression level of Q genome within males and females (see Materials and Methods for further details). In the first column, bar plots show **(A)** the proportion of Q genome for each individual female (*i.e.* their introgression level); and **(B)** the absolute difference in introgression level between each female and PP male in the same trial. Females were separated in groups of 8 individuals (A to M), whose introgression level is shown over each orange bar, with black and grey letters indicating the minimum and maximum values for each group, respectively. In the second column, mean and error bar plots represent the average mate preferences (and standard deviations) of females in each introgression group towards AA (red), QQ (yellow) and PP

(green) males. In the last column, bar plots represent the difference in female preference towards PP males and the remaining males (PP-QQ-AA), with the trends uncovered shown with a light green line. Analyses were performed using only individuals whose introgression level was assessed through GBS analysis. For statistics on all the comparisons performed, please see Supplementary Table 2.

4.8 | *Supplementary material*



Supplementary Figure 1 | Distribution ranges of *S. alburnoides* and of the bisexual *Squalius* species that contribute to the reproductive dynamics of the complex. **(a)** Distribution of *Squalius* species across the main Portuguese drainages. Coloured areas represent the distribution ranges of *S. carolitertii* (yellow), *S. pyrenaicus* (green) and *S. aradensis* (pink); the superimposed dashed area shows the distribution range of the *S. alburnoides* hybrid complex, which is sympatric with each of the three bisexual *Squalius* species represented. **(b)** Detailed distribution of *S. aradensis* (pink) and *S. pyrenaicus* (green) across the Algarve region (southern Portugal), showing the Quarteira drainage, where the two species coexist, and also the Arade and Almargem drainages, harbouring allopatric *S. aradensis* and *S. pyrenaicus*, respectively, and that provided samples to serve as templates for SNP identification (see Materials and Methods for further details). **(c)** Area of sympatry among *S. aradensis*, *S. pyrenaicus* and *S. alburnoides* in the Quarteira drainage, represented in red. The coordinates of the sites sampled inside and outside the sympatry zone are 37°13'35.9"N 8°01'54.9"W and 37°11'47.4"N 8°00'22.6"W, respectively.



Supplementary Figure 2 | Experimental tank used in affiliation trials to assess mate preferences of *S. alburnoides* females towards AA, QQ and PP males. The pentagonal area at the centre corresponds to the neutral "no choice" zone. The proportion of time spent by each female in the dashed areas of each branch was used to index its preference towards each male genomotype, isolated in the peripheral compartments by perforated transparent acrylic plates.

Supplementary Document 1 | Detailed results on fish ploidy, genomotype and introgression.

FLOW CYTOMETRY AND **B***-ACTIN SEQUENCING*

Flow cytometry and sequencing of the *β*-actin gene confirmed the genomotype of all *S. alburnoides* AA males (*N*=14; 4.0±0.4 cm, 3.5-4.7) and *S. aradensis* males (QQ) (*N*=18; 6.5±0.9 cm, 5.2-8.7) used in affiliation trials, but indicated that two males morphologically identified as *S. pyrenaicus* could be PQ hybrids (8.2±0.4 cm, 7.9-8.4), while the remaining were confirmed as PP (*N*=10; 7.3±1.5 cm, 5.9-10.7). The analyses of *S. alburnoides* females (*N*=56; 6.4±0.9 cm, 5.1-9.5) varied between sampling sites, with all females captured outside the sympatry zone (*N*=24; 6.5±1.0 cm, 5.4-9.5) being triploid with QAA genomotype, while, in the sympatry zone, diploid (*N*=12; 6.4±0.8 cm, 5.1-7.9) and triploid (*N*=20; 6.3±0.9 cm, 5.1-8.6) females were found, with QA (*N*=1; 5.5 cm), PA (*N*=11; 6.5±0.8 cm, 5.1-7.9), QAA (*N*=4; 7.5±1.3 cm, 5.7-8.6), PAA (*N*=15; 6.0±0.5 cm, 5.1-6.9) and PPA (*N*=1; 5.5 cm) genomotypes, revealing that a) 100% of the *S. alburnoides* nuclear hybrid females from outside the sympatric zone belonged to the Q genome group (QAA); and b) 84.4% of the *S. alburnoides* nuclear hybrid females from inside the sympatry zone belonged to the P genome group (PA+PAA+PPA).

GENOTYPING-BY-SEQUENCING (GBS)

Quality GBS data were obtained for 84 individuals, with an average of 3,910,003 reads per individual (±1,644,805, 1,045,608-9,121,408). No valid data were obtained for the remaining 16 individuals, thus their genomotype, assessed through *β-actin* sequencing, could not be confirmed and their introgression levels could not be determined. These included 1 QA female, 1 PPA female, 2 PA females, 1 PAA female, 9 QAA females, 1 QQ male and 1 PP male. Overall, 427,624 loci were discovered, from which genome-specific diagnostic SNP's were identified, including A≠Q=P (*N*=2233), Q≠A=P (*N*=709), P≠A=Q (*N*=383) and Q≠ P (*N*=1143).

a) Analysis of A≠Q=P SNP's

The results of the reads of SNP's distinguishing the A genome from Q and P genomes were consistent with the proportion of A genome obtained from the *β-actin* sequences for the 42 *S. alburnoides* females analysed with both methods. The analysis of the A≠Q=P SNP's sequenced for these females (1,857.0±202.0, 1,249-2,149), based on an average of 10,129.0 reads (±4,188.1, 3,781.-20,609), confirmed that PA diploids (*N*=9; 6.5±0.9 cm, 5.1-7.9) were close to 50% of A-specific reads (49.4±0.6%, 47.9-50.3) and PAA/QAA triploids (*N*=33; 6.4±0.9 cm, 5.1-8.6) were close to 67% of A-specific reads (65.8±0.7%, 64.6-67.4), as expected.

Likewise, the GBS data obtained for males of the bisexual *Squalius* species (*N*=28) indicated no significant percentage of A genome. The analysis of the A≠Q=P SNP's sequenced for these males (1,870.0±239.2, 1,263-2,164), based on an average of 10,893.2 reads (±4,694.9, 3,647-24,068), confirmed that QQ, PP and PQ males were close to 0% of A-specific reads (0.2±0.1%, 0.0-0.6), as expected.

b) Analysis of Q≠A=P and P≠A=Q SNP's

The results of the reads of SNP's diagnostic for P and Q genomes together with the A genome were consistent with the genomotype of most *S. alburnoides* females obtained from the *β-actin* sequences (Fig. 2), with only two exceptions. The heterospecific genome of a triploid female (5.9 cm), identified as PAA, showed a mixture of 17.7% P and 12.8% Q genomes, which translate into standardized percentages of 58.1% and 41.9%, respectively, excluding the A genome from the calculations. Given this female could not be considered neither as PAA or QAA nor included in P or Q genome groups, it was excluded from further analysis. A second triploid female (5.7 cm), identified as QAA, was instead a PAA. This female was, thus, included in the P genome group in the subsequent analyses.

In the females of the Q genome group (*N*=18), the analysis of the Q≠A=P SNP's (587.6±52.9, 487-667), based on an average of 3,032.1 reads (±1,078.2, 1,683-5,235), confirmed that QAA triploids (6.7±1.0 cm, 5.4-8.6) were close to 33% of Q-specific reads (34.0±1.7%, 31.5-37.1), meaning all *S. alburnoides* nuclear hybrids from outside the sympatry zone belonged to the Q genome group. In turn, the analysis of the P≠A=Q SNP's sequenced for these females (314.3±27.4, 274-372), based on an average of 1,615.8 reads (±556.1, 913-2,889) revealed no significant levels of P-specific reads (0.3%±0.3%, 0.1-1.0), meaning their Q genome was not introgressed with P genome (Sup. Fig. 4).

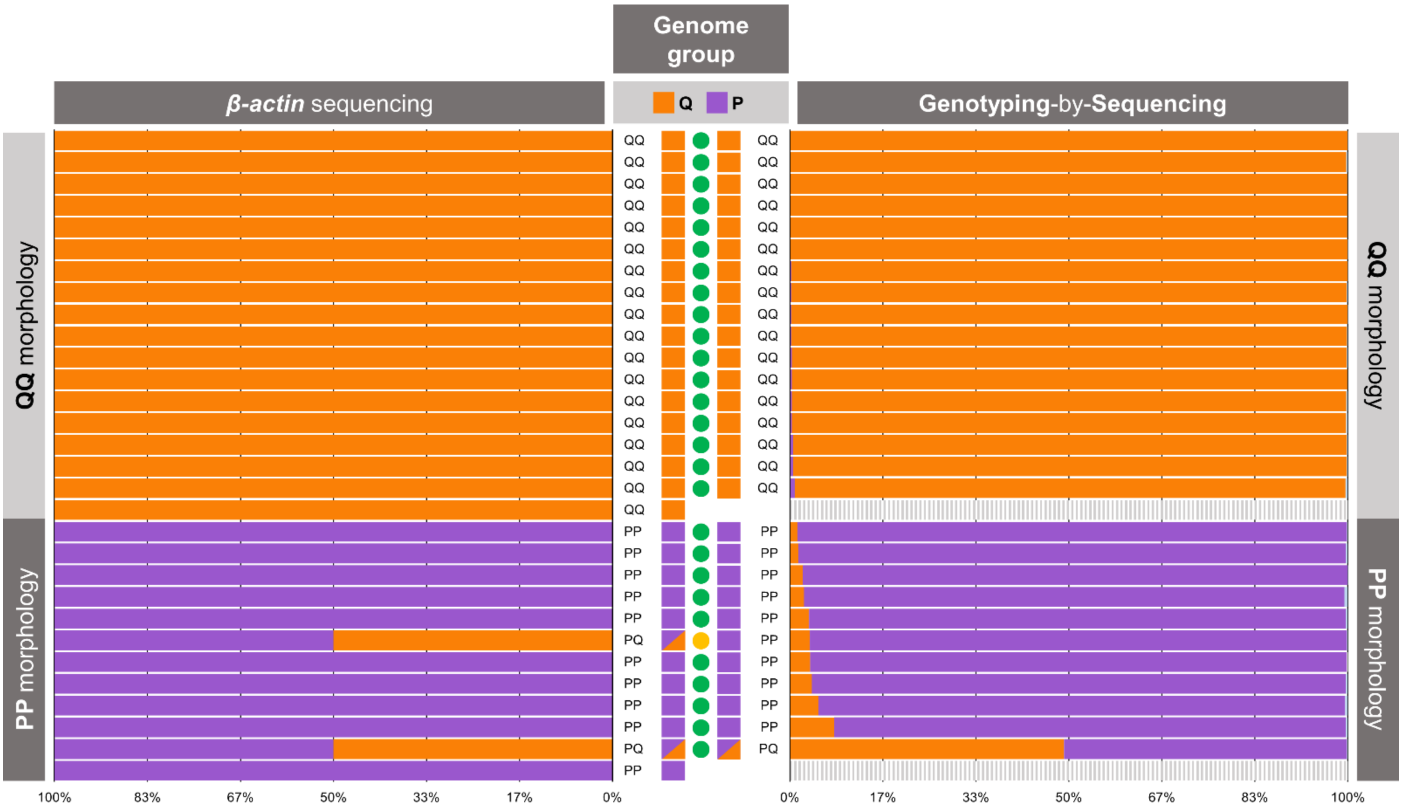
In the females of P genome group (*N*=23), the analysis of the P≠A=Q SNP's (315.8±33.7, 228-365), based on an average of 1,746.6 reads (±785.4, 626-3,466), confirmed that PA diploids (*N*=9; 6.5±0.9 cm, 5.1-7.9) were close to 50% of P-specific reads (46.9±3.1%, 39.8-51.3) and PAA triploids (*N*=14; 5.9±0.5 cm, 5.1-6.9) were close to 33% of P-specific reads (31.4±1.8%, 28.1-33.5), as expected. These results indicate that 85.2% of the *S. alburnoides* nuclear hybrids from inside the sympatry zone belonged to the

P genome group (PA+PAA). In turn, the analysis of the Q≠A=P SNP's sequenced for these females (596.0±64.8, 421-687), based on an average of 3,293.2 reads (±1,464.1, 1,258-6,337) revealed that some females of the P genome group were introgressed with Q genome, contrary to what was found in the females of the Q genome group. Introgression levels varied from 0.0% to 5.5%, which translates into a standardized introgression level average of 3.0% (±3.6%, 0.1-12.5) within their P genome, excluding the A genome from the calculations (Sup. Fig. 4).

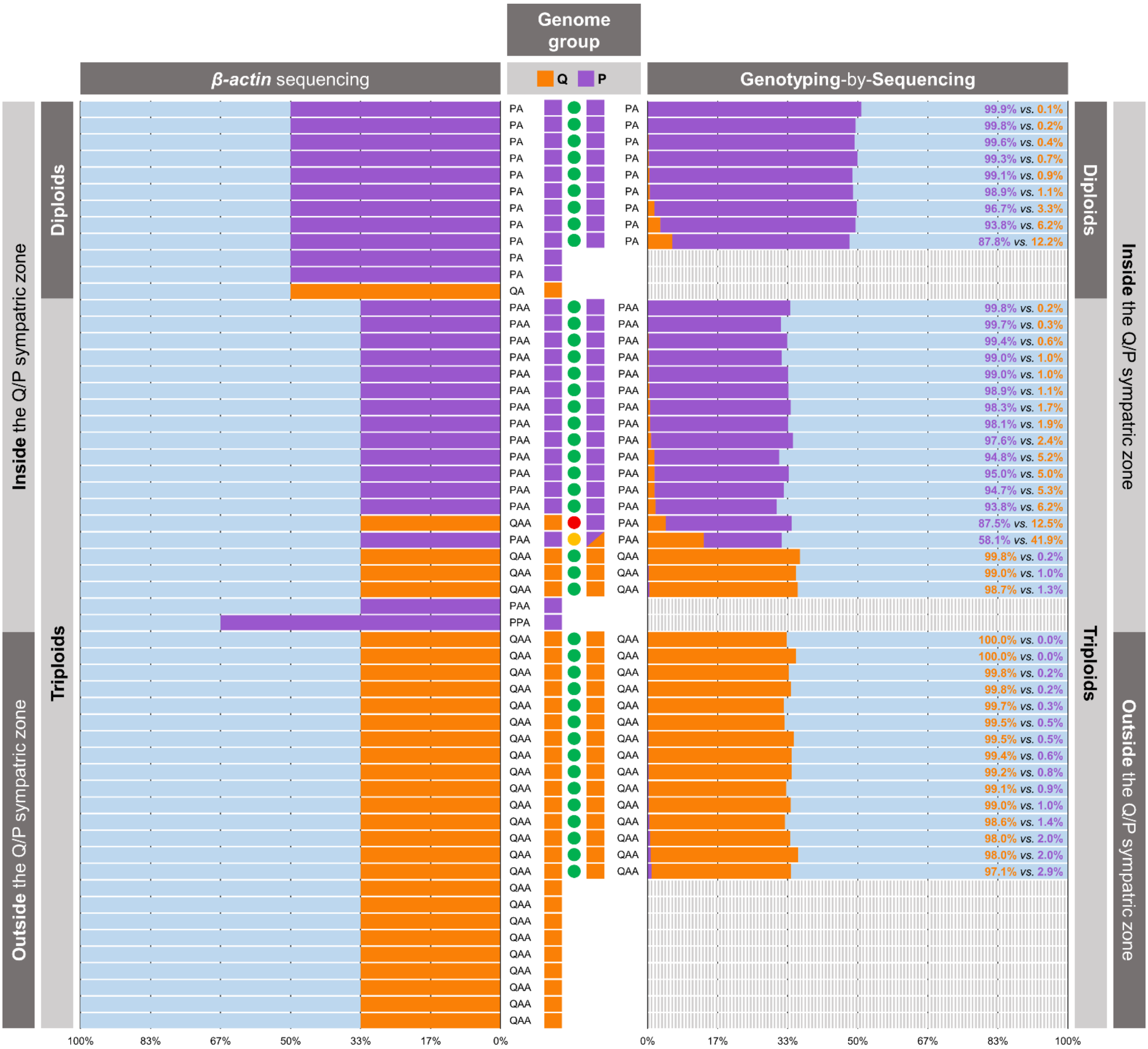
c) *Analysis of P≠ Q SNP's*

The analyses of the reads of SNP's diagnostic for P and Q genomes regardless of the A genome were consistent with the *β-actin* sequences for all *S. aradensis* and *S. pyrenaicus* (Fig. 3). Regarding the two PQ hybrids, one turned out to be a PP individual (7.9 cm), but the other was confirmed as PQ (PP morphology; 8.4 cm) by GBS data (49.2% Q, 50.8% P). This individual was considered a hybrid between *S. aradensis* and *S. pyrenaicus*, and was, thus, excluded from further analyses.

In the *S. aradensis* males (QQ) (*N*=17; 6.5±0.9 cm, 5.2-8.7), the analysis of the SNP's distinguishing the P and Q genomes (971.8±132.6, 660-1111), based on an average of 5,960.9 reads (±2,591.3, 1,782-12,171), confirmed that all were close to 100% of Q-specific reads (99.7±0.2%, 99.1-99.9), showing only vestigial percentages of P-specific reads (0.3±0.2%, 0.1-0.9) (Fig. 3). In turn, in the *S. pyrenaicus* males (PP) (*N*=10; 7.5±1.5 cm, 5.9-10.7), the analysis of these SNP's (937.8±91.0, 768-1072), based on an average of 4,751.3 reads (±1,713.2, 2,537-8,571), revealed they were less pure than QQ males, showing significant Q-specific reads (3.5±1.9%, 1.3-7.9), although the majority of the sequenced reads were P-specific (96.5±1.9%, 92.1-98.7) (Sup. Fig. 3).



Supplementary Figure 3 | Genomotype composition of *S. aradensis* and *S. pyrenaicus* used in affiliation trials, assessed using *β-actin* sequencing and Genotyping-by-Sequencing (GBS). The horizontal bars represent the proportion of A (blue), Q (orange) and P (purple) genomes for each individual. Grey dashed bars indicate the individuals for which no quality GBS data were obtained. The vertical bars at the centre indicate the genome group of each female, determined from the majority of their genome as obtained from *β-actin* and GBS analyses, and the circles at the centre indicate if these methods agreed (green) or disagreed (red) in genomotype identification.



Supplementary Figure 4 | Genomotype composition of diploid and triploid *S. alburnoides* females used in affiliation trials, assessed using *β-actin* sequencing and Genotyping-by-Sequencing (GBS). The horizontal bars represent the proportion of A (blue), Q (orange) and P (purple) genomes for each individual. Grey dashed bars indicate the individuals for which no quality GBS data were obtained. The percentages discriminated on the right represent the standardized proportion of Q and P genomes within each individual, excluding the A genome from the calculations. The vertical bars at the centre indicate the genome group of each female, determined from the majority of their genome as obtained from β-*actin* and GBS analyses, and the circles at the centre indicate if these methods agreed (green) or disagreed (red) in genomotype identification.

Supplementary Table 1 | Raw data on mate choice by *S. alburnoides* females towards AA, QQ and PP males obtained in affiliation trials. Highlighted in colour are the 4 trials that were excluded from all analyses, which included a PQ male that was used as PP (*N*=3; orange) and an extreme outlier female (*N*=1; blue).

Trial	♀ zone	♀ SL (cm)	♀ ploidy	♀ genome group	♀ type	AA ♂ SL (cm)	♀ pref. for AA male	QQ ♂ SL (cm)	♀ pref. for QQ male	PP ♂ SL (cm)	♀ pref. for PP male
#1	Inside	5.9	2n	P	PA	4.4	19.8%	6.0	38.8%	10.7	22.3%
#2	Inside	5.9	2n	P	PA	4.7	21.5%	5.2	20.7%	8.1	52.1%
#3	Inside	6.2	3n	P	PAA	4.7	1.7%	5.4	0.9%	8.1	96.7%
#4	Inside	5.8	2n	P	PA	4.2	25.6%	6.0	28.1%	8.4	41.3%
#5	Inside	5.9	2n	P	PA	3.6	0.0%	6.5	82.6%	8.1	17.4%
#6	Inside	5.4	3n	P	PAA	4.7	5.4%	5.2	65.5%	7.9	26.7%
#7	Inside	5.8	3n	P	PAA	4.0	6.6%	6.3	86.8%	8.1	3.3%
#8	Inside	8.6	3n	Q	QAA	3.5	2.5%	6.1	10.7%	8.1	85.1%
#9	Inside	6.7	2n	P	PA	3.6	15.7%	6.6	45.5%	7.6	36.4%
#10	Inside	5.1	2n	P	PA	4.0	5.8%	5.7	87.6%	8.1	0.8%
#11	Inside	5.9	3n	PQ	P/QAA	4.7	23.9%	6.4	24.8%	7.9	41.7%
#12	Inside	5.1	3n	P	PAA	4.2	2.0%	6.3	88.3%	8.1	6.1%
#13	Inside	5.5	2n	Q	QA	4.4	0.0%	5.7	96.7%	7.9	2.5%
#14	Inside	5.7	3n	P	PAA	3.6	3.3%	6.6	20.7%	10.7	71.9%
#15	Inside	7.1	2n	P	PA	3.5	0.0%	5.7	13.2%	7.9	86.0%
#16	Inside	5.5	3n	P	PPA	3.6	0.0%	7.6	93.4%	8.1	5.0%
#17	Inside	5.5	3n	P	PAA	4.4	14.1%	5.4	19.6%	7.9	53.1%
#18	Inside	5.7	3n	P	PAA	4.2	1.4%	5.6	2.3%	8.1	95.1%
#19	Inside	6.3	3n	P	PAA	4.0	11.1%	5.9	12.6%	8.1	60.2%
#20	Inside	6.4	3n	P	PAA	4.7	2.2%	6.3	50.9%	8.1	45.6%
#21	Inside	8.3	3n	Q	QAA	4.4	98.3%	7.6	0.0%	7.9	0.8%
#22	Inside	6.4	2n	P	PA	4.7	31.4%	6.5	17.4%	7.9	42.1%
#23	Inside	5.4	3n	P	PAA	3.5	27.0%	6.6	22.8%	8.1	33.9%
#24	Inside	7.4	3n	Q	QAA	4.2	0.8%	6.4	87.6%	7.9	5.0%

#25	Inside	6.0	3n	P	PAA	4.2	30.9%	5.2	34.5%	7.9	20.4%
#26	Inside	6.9	3n	P	PAA	4.0	5.2%	5.4	12.6%	10.7	77.1%
#27	Inside	7.1	2n	P	PA	3.5	9.9%	5.6	35.5%	8.4	51.2%
#28	Inside	7.4	2n	P	PA	3.6	33.9%	6.4	39.7%	8.4	9.9%
#29	Inside	6.5	3n	P	PAA	4.7	1.6%	8.7	2.5%	10.7	93.8%
#30	Inside	7.9	2n	P	PA	3.6	0.0%	5.6	0.0%	7.6	99.2%
#31	Inside	5.9	3n	P	PAA	3.6	4.8%	7.6	61.3%	10.7	28.6%
#32	Inside	6.7	3n	P	PAA	3.6	11.6%	6.5	32.2%	7.6	26.4%
#33	Outside	6.5	3n	Q	QAA	4.5	17.4%	6.5	54.5%	5.9	19.8%
#34	Outside	6.0	3n	Q	QAA	4.3	6.6%	6.5	12.4%	7.2	50.4%
#35	Outside	5.8	3n	Q	QAA	3.9	1.7%	6.5	4.1%	6.4	93.4%
#36	Outside	5.6	3n	Q	QAA	3.9	5.0%	6.4	57.9%	8.7	33.9%
#37	Outside	9.5	3n	Q	QAA	4.3	12.4%	7.3	12.4%	7.2	68.6%
#38	Outside	6.4	3n	Q	QAA	4.5	10.7%	6.4	22.3%	6.1	52.1%
#39	Outside	6.9	3n	Q	QAA	3.6	24.8%	6.4	29.8%	6.4	43.8%
#40	Outside	6.5	3n	Q	QAA	3.9	14.9%	6.4	28.1%	6.5	38.8%
#41	Outside	5.9	3n	Q	QAA	3.9	9.9%	6.5	56.2%	5.9	26.4%
#42	Outside	5.4	3n	Q	QAA	3.6	7.4%	7.3	33.9%	6.5	57.9%
#43	Outside	7.1	3n	Q	QAA	4.1	1.7%	6.5	0.8%	6.1	93.4%
#44	Outside	5.4	3n	Q	QAA	4.3	3.3%	6.5	83.5%	5.9	9.1%
#45	Outside	5.6	3n	Q	QAA	4.5	5.0%	6.5	14.0%	7.2	72.7%
#46	Outside	6.0	3n	Q	QAA	3.9	31.4%	6.4	6.6%	5.9	51.2%
#47	Outside	8.0	3n	Q	QAA	3.6	0.0%	6.5	93.4%	6.5	5.8%
#48	Outside	6.6	3n	Q	QAA	4.1	3.3%	7.3	16.5%	8.7	78.5%
#49	Outside	5.6	3n	Q	QAA	4.1	9.1%	7.3	0.0%	7.2	90.9%
#50	Outside	7.8	3n	Q	QAA	3.6	18.2%	6.4	33.1%	5.9	45.5%
#51	Outside	7.1	3n	Q	QAA	3.9	45.5%	7.3	49.6%	8.7	5.0%
#52	Outside	6.7	3n	Q	QAA	4.3	19.0%	6.5	17.4%	6.1	53.7%
#53	Outside	6.2	3n	Q	QAA	3.6	14.0%	6.5	57.9%	6.4	22.3%
#54	Outside	7.3	3n	Q	QAA	3.9	4.1%	7.3	6.6%	8.7	89.3%
#55	Outside	5.9	3n	Q	QAA	3.6	38.8%	6.5	21.5%	5.9	32.2%
#56	Outside	6.8	3n	Q	QAA	3.6	9.1%	6.5	32.2%	6.4	10.7%

Supplementary Table 2 | Results from repeated-measures ANOVA comparisons performed to assess the influence of female introgression over mate preferences.

	Class	*N*	ANOVA	*p*	
Female grouping by female introgression	**A**	8	$F_{1.1,7.6}=4.0$	0.080	*
	B	8	$F_{1.2,8.3}=7.1$	0.024	**
	C	8	$F_{1.2,8.7}=5.5$	0.040	**
	D	8	$F_{1.2,8.5}=3.7$	0.084	*
	E	8	$F_{1.2,8.4}=3.6$	0.090	*
	F	8	$F_{1.2,8.2}=3.8$	0.081	*
	G	8	$F_{1.1,7.9}=5.9$	0.039	**
	H	8	$F_{1.1,7.7}=4.0$	0.080	*
	I	8	$F_{1.1,7.8}=3.5$	0.098	*
	J	8	$F_{1.2,8.2}=2.5$	0.151	
	K	8	$F_{1.1,8.0}=2.4$	0.160	
	L	8	$F_{1.2,8.4}=3.2$	0.104	
	M	8	$F_{2,14}=3.2$	0.074	*
Female grouping by introgression difference	**A**	8	$F_{1.1,7.5}=3.7$	0.091	*
	B	8	$F_{1.2,8.1}=4.2$	0.070	*
	C	8	$F_{1.2,8.4}=3.6$	0.079	*
	D	8	$F_{1.2,8.1}=3.2$	0.109	
	E	8	$F_{1.1,7.9}=3.8$	0.086	*
	F	8	$F_{1.2,8.2}=2.5$	0.151	
	G	8	$F_{1.2,8.2}=2.6$	0.142	
	H	8	$F_{1.2,8.6}=2.5$	0.148	
	I	8	$F_{2,14}=2.6$	0.112	
	J	8	$F_{1.1,7.7}=3.5$	0.100	
	K	8	$F_{1.1,7.7}=3.4$	0.103	
	L	8	$F_{1.2,8.4}=2.8$	0.128	
	M	8	$F_{2,14}=3.2$	0.073	*

***** $p<0.10$

****** $p<0.05$

CHAPTER 5

First empirical evidence of naturally occurring androgenesis in vertebrates

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First empirical evidence of naturally occurring androgenesis in vertebrates

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Androgenesis among vertebrates is considered a rare phenomenon, with some cases reported so far, but linked to experiments involving gamete manipulation (artificial androgenesis). Herein, we report the first empirical evidence of the natural occurrence of spontaneous androgenesis in a vertebrate, the *Squalius alburnoides* allopolyploid complex. A genetically screened random sample of a natural population was allowed to reproduce in an isolated pond without any human interference, and the viable offspring obtained was later analysed for paternity. Both nuclear and mitochondrial markers showed that the only allodiploid fish found among all the allotriploid offspring was androgenetically produced by an allodiploid male. This specimen had no female nuclear genomic input, and the sequence of the mitochondrial fragment examined differed from that of the male progenitor, matching one of the parental females available in the pond, probably the mother. The possible role of androgenesis in the reproductive dynamics of this highly successful vertebrate complex is discussed.

1. Introduction

Androgenesis is a reproductive mode in which the offspring produced lack maternal nuclear genomic contribution, i.e. all the genetic content of the progeny is inherited from the father (reviewed in [1–3]). It is considered a quasi-sexual form of reproduction [1], since, conversely to parthenogenesis and similarly to gynogenesis [4], it requires fertilization and syngamy, in which the oocytes serve solely as involucres to host the genetic nuclear content of the spermatozoa, via multiple mechanisms [1], being, thus, considered a form of sexual parasitism [5].

Summarizing the literature on the subject, androgenesis may be divided into two types, according to its form of occurrence: artificial androgenesis versus natural androgenesis (reviewed in [1]). Artificial androgenesis occurs when gametes are manipulated in the laboratory (a procedure used in animals with external fertilization as fish and molluscs) to produce viable androgenetic offspring, for instance by fragmenting the pronucleus of oocytes (female genome) prior to fertilization or blocking the first mitotic division of the egg. Natural androgenesis occurs in natural contexts, without any kind of manipulative intervention on animals' reproduction. Individuals derived from natural androgenesis, with a 'paternal monopolization of parenthood' [6], may become clones of their father after the extrusion of the maternal nuclear genome post-fertilization, typically through the polar bodies, though they normally retain the cytoplasm, mitochondria and other organelles from the oocyte [2]. In turn, two sub-types of natural androgenesis can be considered: (i) obligate androgenesis, which is an integrant part of the reproductive dynamics of certain organisms, being the main reproductive strategy of some natural populations only producing androgenetic offspring; and (ii) spontaneous androgenesis, which occurs when parents from species that reproduce sexually unexpectedly yield a certain proportion, typically low, of descendants only inheriting the paternal nuclear genome among their mainly sexually derived offspring (reviewed in [1,2]).

Natural androgenesis is considered to be a rare phenomenon, which may or may not be related to its actual incidence in wild organisms. On the one hand, with the exception of haplodiploid systems, androgenetic offspring is considered unviable in the vast majority of cases, namely due to the abnormalities associated with the 'haploid syndrome', being, thus, necessary that the zygote comprises more than one set of parental (in this case, paternal) chromosomes in order to be successful. This can be accomplished through paternal genome duplication (e.g. diploidization by cell fusion during the first egg division or by polyspermic fertilization) or through the production of non-haploid spermatozoa (unreduced gametes), as typically occurs in fertile hybrids or in polyploid organisms (see [1]). Indeed, most known cases of natural androgenesis involve hybridization and/or polyploidy [1,2,6]. However, on the other hand, the rarity of reports on natural androgenesis may be related to the difficulty in identifying androgens in natural populations, namely in hybrid complexes and in those cases arising from spontaneous androgenesis, since detection procedures require in-depth parentage analyses. Specifically, it is necessary to confirm a totally unique sperm-derived inheritance in the progeny, using genetic and/or cytological genomic markers for both maternal and paternal gene pools. Similarly to other quasi-sexual reproductive modes [7,8], the actual evolutionary impact of spontaneous androgenesis in wild populations has been overlooked, due to the higher extinction risk of male-cloning systems, and is poorly understood also due to the lack of data [1,2,5,9].

In animals, only a few cases of natural androgenesis have been reported (in arthropods and molluscs) [1,3], and no cases are known among vertebrates. The *Hypseleotris* carp gudgeons, recently included in a review about natural androgenesis [1], are actually a case of hybridogenesis, as clearly stated by the authors. Only two cases of spontaneous androgenesis in vertebrates have been described so far, also in fishes [10,11], but, since they involved artificial strains and/or the use of fertilization techniques, they do not represent true cases of natural androgenesis, which by definition occurs in natural contexts and in wild populations. These two cases were excluded from that recent review on natural androgenesis [1], since they are more correctly assigned to artificial androgenesis. The first case of naturally occurring spontaneous androgenesis *sensu stricto* in vertebrates is here presented and documented; it was recently found in the allopolyploid fish complex *Squalius alburnoides* in the frame of a specific study aiming to compare the reproductive success of distinct genomotypes [12].

This hybrid complex had its origin in intergeneric crosses between *Squalius pyrenaicus* females (P genome) and males from an extinct species belonging to the *Anaecypris hispanica* lineage (A genome). The hybridization event produced fertile PA hybrids, which, through crosses among themselves and backcrosses, led to the arising of an allopolyploid complex, composed by diploid ($2n = 50$), triploid ($3n = 75$) and tetraploid ($4n = 100$) males and females with distinct proportions of the parental genomes (=genomotypes) (reviewed in [13]). In the breeding system of this fish complex, though natural populations are highly female-biased, there is a clear sperm-dependency (*sensu* [14]), with hybrid individuals reproducing either sexually or nonsexually (*sensu* [13]). All known genomotypes are fertile, exhibiting a wide range of reproductive modes, including regular (sexual) meiosis, meiotic hybridogenesis and clonal gametogenesis (reviewed in [13]). Some allotriploid females can produce both reduced and unreduced gametes simultaneously [15], and males and females of the same genomotype may have distinct reproductive modes [16,17]. Moreover, sex ratios vary among genomotypes (e.g. allotriploids are mostly females) and geographical areas (allodiploids are mostly all male in northern populations, but all female in southern populations) (see [13]).

The variety of reproductive strategies leads to changes in ploidy level through an intricate reproductive dynamics between genomotypes and illustrates well the occurrence of sexual parasitism

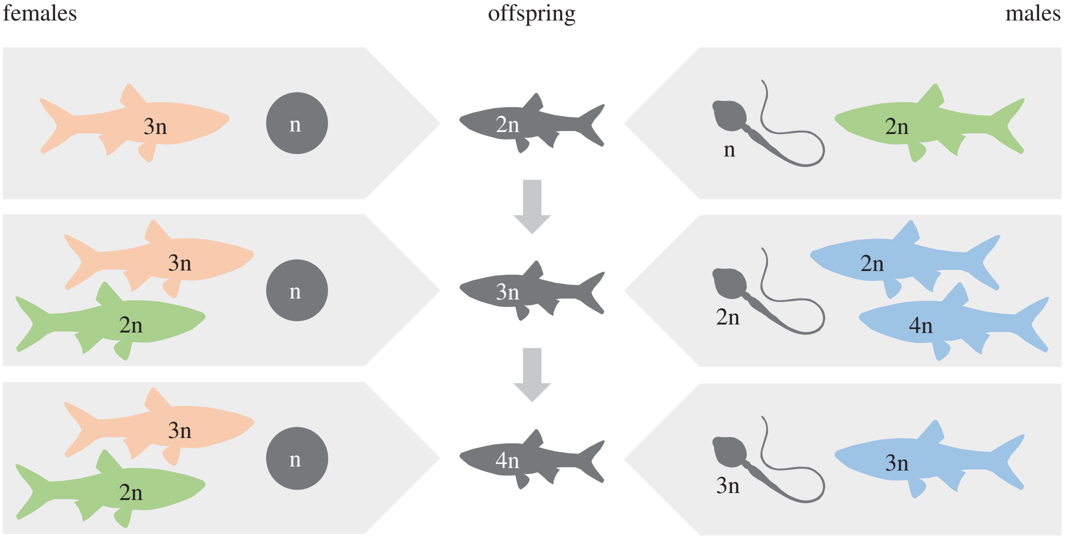

Figure 1. Ploidy level cascade of the reproductive framework of the *S. alburnoides* allopolyploid complex in the studied stretch of the Ocreza River (Tagus drainage, Central Portugal), illustrating the typical reproductive dynamics of northern populations dominated by triploid females. Hybrid males and females are represented in blue and pink, respectively, and males and females of the bisexual sympatric *Squalius* species are represented in green. Reproductive modes include (i) regular meiosis in *S. pyrenaicus* males and females (producing haploid P gametes) and in balanced tetraploids (producing diploid PA gametes); (ii) meiotic hybridogenesis in triploid females (producing haploid A oocytes); and (iii) clonal spermatogenesis in diploid and triploid males (producing diploid PA and triploid PAA or PPA spermatozoa, respectively). Diploid nuclear non-hybrid males (AA) are absent in the studied population (as in all northern populations) and were, thus, not included in the diagram. Both oocytes and sperm are represented in grey. 5n (and higher) offspring are unviable. The diagram illustrates well the dependence of the hybrid complex on the sympatric bisexual *Squalius* species, through the production of allodiploids, essential to the progression of the ploidy level cascade. Note that, since allotetraploids also produce diploid gametes, they could eventually replace allodiploids in the ploidy level cascade, but they are extremely rare in the vast majority of populations (see [13]).

(reviewed in [5,13]). Most genomotypes are reproductively interdependent, meaning their persistence in natural populations depends on crosses involving other genomotypes (figure 1). Moreover, the production of allodiploids is entirely dependent on crosses with bisexual species of the *Squalius* genus. Since allodiploids are crucial for the persistence of natural populations, being indispensable for the continuity of the ploidy level cascade (figure 1) and responsible for the production of allotriploids (the most common genomotype in natural populations), the persistence of *S. alburnoides* complex as a unit is, in turn, entirely dependent on the sympatric bisexual *Squalius* species.

As explained above, the production of unreduced gametes by hybrid organisms increases the likelihood of the occurrence of quasi-sexual successful reproduction, such as gynogenesis and androgenesis, which turns *S. alburnoides* complex into an excellent model to look for the existence of these unorthodox reproductive modes in the context of wild populations. Herein, using the same dataset previously published [12], which was obtained from an empirical study of a random sample from a natural population that was transferred to a pond and allowed to reproduce without any human interference, a new reproductive mode for *S. alburnoides* complex was discovered—spontaneous androgenesis—representing the first documented report of its natural occurrence among vertebrates.

2. Material and methods

2.1. Fish sampling and laboratory procedures

A random sample of *S. alburnoides* ($N = 33$) and *S. pyrenaicus* ($N = 19$) was captured in Ocreza River (Tagus drainage, Central Portugal) with short pulse and moderate voltage electrofishing (300 V, 2–4 A), during the reproductive season (April 2010), when mature individuals could be easily sexed by applying a mild pressure on the abdomen and observing the discharge of gametes. Because most *S. alburnoides* genomotypes are morphologically similar, the ploidy and genome combination of each individual were assessed in the laboratory. Individuals were anaesthetized ($0.1\,\mathrm{g\,l^{-1}}$ MS-222, $0.2\,\mathrm{g\,l^{-1}}$ $NaHCO_3$) and photographed on the left and right sides to be individually recognized when needed [18]. Small clips of the caudal fin were collected for genomotype assessment through flow cytometry [19] and Sanger sequencing of the β-actin gene (PCR conditions: 35 cycles of 94°C, 30 s; 55°C, 40 s; 72°C, 90 s) [20]. DNA extraction followed an adapted phenol-chloroform protocol [21].

All sampled fishes (*S. alburnoides* hybrids and *S. pyrenaicus,* PP; $N = 52$), composed of PAA ($N = 23$) and PP ($N = 9$) females, and by PA ($N = 6$), PAA ($N = 1$), PPA ($N = 2$), PPAA ($N = 1$) and PP ($N = 10$) males, were translocated to an exterior pond, under natural light and temperature conditions, in January 2011. This pond had a volume of 4200 l [300 cm length × 200 cm width × 50 cm mean depth (25–90 cm)] and was enhanced with macrophytes and with a bottom cover of small and large cobbles (2–15 cm), to provide adequate habitat conditions for the fish [22]. Two pumps and a UV lamp were used to prevent water stagnation and quality deterioration throughout the study period. Overall, habitat conditions in the pond were close to those found in Iberian rivers during seasonal drought, when fish concentrate in isolated pools [23]. Fish were fed twice a day with commercial flakes during the first month to prevent eventual lows in prey availability and facilitate adaptation to the pond conditions. The pond was monitored weekly for water pH (7–10) and inspected for dead fish (never detected) and larvae (first spotted in April). In October, parental fish and offspring were captured using electrofishing and transported to the laboratory in aerated vats. The pond was emptied to assure complete fish collection.

In the laboratory, a sample of 100 youngs-of-the-year (YOYs) was randomly selected for sex determination and paternity assessment, sacrificed with an overdose of anaesthetics (MS-222) and dissected for gonad examination, as described in [24]. Paternity was assessed through microsatellite genotyping, using nine microsatellites with high variability among cyprinids [25–27]. An extra microsatellite was haphazardly found after sequencing a genomic fragment containing the intron region of the *aminomethyltransferase* gene (AMT) (MM Coelho *et al*. 2013, unpublished data), from which the primers were designed [12]. Excepting LCO1, LCO3 and LCO4, all microsatellites were genotyped using primers with an M13 tail, as described in [28]. Complete information on the ten microsatellites used is shown in electronic supplementary material, table S1. Moreover, a mitochondrial fragment of the D-loop/control region [29] was amplified (PCR conditions: 35 cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 90 s) and sequenced. Sequences were analysed in software MEGA6 [30].

3. Results and discussion

All 261 YOYs obtained in the pond were morphologically identified as *S. alburnoides*, with no *S. pyrenaicus* (PP) found. Flow cytometry revealed that only one of the YOYs randomly sampled ($N = 100$) was diploid, with all the others being triploid (for more results and details, see [12]), and the sequence of the *β-actin* gene revealed that the diploid individual, with 5.1 cm of standard length, was an allodiploid (PA genomotype). Further flow cytometry analyses of all the remaining YOYs ($N = 161$) revealed only triploid individuals. According to present knowledge, the only way to obtain allodiploid offspring in populations where AA males are absent (i.e. all northern populations) is through crosses between allotriploid females (which generally produce haploid A oocytes by meiotic hybridogenesis) and males of the sympatric *Squalius* species (which produce haploid sperm by regular meiosis) (figure 1), emphasizing the reproductive dependence of the hybrid complex towards the sympatric bisexual species of the *Squalius* genus. However, paternity assessment using microsatellites revealed that the nuclear P genome present in the only PA YOY found was not inherited from a PP individual (table 1). Instead, its nuclear PA genome was an exact copy of one of the *S. alburnoides* allodiploid male progenitors (PA genomotype) present in the original random sample of the natural population transferred to the pond, with all alleles from all microsatellites being a match (named PA6 in table 1). Indeed, the allodiploid YOY was male, which is consistent with an androgenetic origin.

However, the referred allodiploid YOY did not share the mitochondrial DNA with his father. All SNPs present in the sequenced fragment matched one of the parental allotriploid females in the pond, probably its mother (named PAA♀1 in figure 2). It is important to note that this pair of parental fish (PAA female × PA male) produced more offspring that followed the expected reproductive modes (figure 1). They were all allotriploids (PAA), resulting from haploid oocytes (A) fertilized by unreduced spermatozoa (PA) [12]. As a side outcome from mitochondrial DNA analysis, haplotypes from *S. alburnoides* and *S. pyrenaicus* showed marked differences (figure 2), suggesting that, currently, PP

Table 1. Allele comparison between the PA YOY and all PP ($N = 10$), PA ($N = 6$) and PPAA ($N = 1$) parental males present in the pond. Highlighted alleles in the list correspond to PA YOY alleles, and matching alleles with each possible parental male are shown in green. Match percentage represent the proportion of microsatellites sharing alleles between the PA YOY and each possible parental male. Male reproductive modes were taken into account when calculating match percentages: (i) PP males produce haploid sperm (P genome), meaning they would only pass half of their genome (one allele per microsatellite) to the descendant; (ii) PA males produce unreduced clonal diploid sperm (PA genome), meaning they would pass their entire genome (two alleles per microsatellite) to the descendant; and (iii) PPAA males produce reduced diploid sperm (PA genome), meaning they would pass half of their genome (two alleles per microsatellite) to the descendant.

parental males		PP										PA						PPAA
		1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	1
LCO1	253	▨																
	257												▨					
	261					▨												
	265	▨			▨	▨		▨		▨	▨			▨	▨	▨		
	269		▨	▨			▨											▨
	273		▨				▨											
	290									▨		▨						
	309												▨					
	311			▨					▨		▨							
	315																🟩	
	318													▨				
	334											🟩			🟩		🟩	
	346															▨		▨
	356																	▨
LCO3	239	▨										▨						
	241	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩		🟩	🟩	🟩	🟩	🟩	🟩
	249											🟩	🟩	🟩	🟩	🟩	🟩	🟩
LCO4	236	▨	▨		▨		▨	▨		▨		▨		▨	▨			▨
	238				▨	▨				▨	▨							
	240	▨		▨					▨							▨		
	242						▨						▨					
	244			🟩		🟩					🟩						🟩	
	268														▨			▨
	271											🟩		🟩		🟩	🟩	🟩
	274												▨					
LC27	173																	▨
	186			▨		▨		▨										
	190	🟩		🟩	🟩	🟩	🟩		🟩	🟩	🟩	🟩	🟩		🟩		🟩	🟩
	209	▨	▨		▨		▨	▨	▨					▨		▨		
	214		▨								▨		▨	▨	▨			
	226																🟩	
	244											▨				▨		
	252																	▨
LC288	218									▨								
	235	🟩	🟩	🟩	🟩	🟩		🟩	🟩		🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	254		🟩				🟩			🟩			🟩				🟩	
	269										▨	▨						
LC290	200												▨		▨			
	208											🟩		🟩		🟩	🟩	🟩
	261	▨			▨	▨					▨							
	269		▨			▨	▨						▨		▨	▨		
	281		🟩	🟩			🟩		🟩	🟩	🟩	🟩		🟩			🟩	
	305	▨						▨										▨
Lsou5	**193**	🟩	🟩			🟩	🟩	🟩			🟩	🟩		🟩			🟩	
	195	▨	▨	▨	▨	▨	▨	▨	▨	▨			▨		▨	▨		▨
	197															▨		
	199											🟩		🟩	🟩		🟩	🟩
	203												▨					▨
Lsou8	**202**	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩			🟩	🟩	🟩	▨
	204							▨	▨	▨			▨	▨				
	208											🟩	🟩	🟩		🟩	🟩	🟩
	210														▨			▨
Lsou34	241	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	244	▨																
	253													▨	▨			
	257											🟩	🟩				🟩	
	263															▨		▨
AMT	242												▨	▨				▨
	244											🟩			🟩	🟩	🟩	🟩
	253		▨	▨				▨	▨							▨		
	255					▨	▨			▨	▨	▨	▨	▨				▨
	257																	
	259	🟩			🟩	🟩		🟩	🟩	🟩					🟩		🟩	
	269		▨	▨			▨				▨							
match (%)		70	60	70	60	80	70	60	70	70	80	40	30	30	20	20	100	90

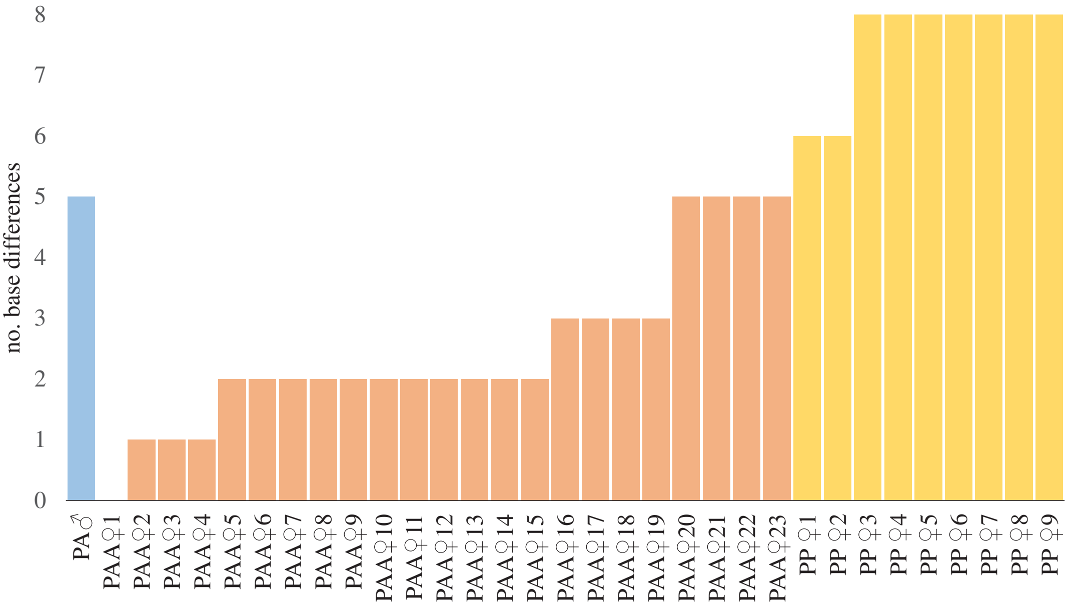

Figure 2. The number of base differences between the mitochondrial sequence of the androgenetic PA YOY and the mitochondrial sequences of all possible parental PAA (PAA♀1–PAA♀23; pink bars) and PP (PP♀1–PP♀9; yellow bars) females and of the PA male progenitor (PA♂; blue bar). Females were ordered according to the number of base differences towards the androgenetic PA YOY (see text for further details).

females tend not to cross with hybrid males in the studied population, thus hampering mitochondrial gene flow into the hybrid complex.

To our knowledge, this is the first report of naturally occurring spontaneous androgenesis among vertebrates. Similarly to this case, both descriptions of androgenesis in vertebrates, though occurring in artificial contexts, concerned teleost fishes [10,11]. They involved hybridization and/or genome polyploidization, which facilitate the (artificial) development of androgenesis, and the observed frequency of androgenetic offspring was very low (1% and 5% [10,11]). Regarding our data, the single androgenetic individual found represented 1.0% of the total offspring genetically screened, 1.3% of the offspring of its male progenitor, 4.2% of the offspring of its female progenitor and 4.3% of the offspring of its male and female progenitors' pair (for data on other crosses, see [12]).

Although *S. alburnoides*, similarly to other hybrid complexes, undergo significant population variations regarding sex ratios, ploidy and genomotype composition, the vast majority of natural populations share their dependence on the bisexual *Squalius* species to persist. Hybrids sexually parasitize these sympatric species to produce allodiploids, being, thus, able to proceed with the ploidy level cascade to form allotriploids (figure 1) [13], the genomotype dominating most natural populations. However, recent data showed that the occurrence of natural crosses between *S. alburnoides* and *S. pyrenaicus* individuals does not seem as likely as expected, either in free-access or directional crosses [12], despite offspring production being viable in forced (totally artificial) experimental crosses (e.g. [16]). Herein, our findings report, for the first time, a route to produce allodiploid males without the involvement of the sympatric bisexual *Squalius* species, i.e. via androgenesis. Though rare, this alternative reproductive mode may guarantee the production of a sufficient proportion of allodiploid males to assure the persistence of northern populations (where most allodiploids are males; see [13]), since even a low frequency of allodiploid males seems enough to stabilize genomotype composition at an equilibrium [31]. Moreover, the androgenetic male was produced by a particular allodiploid male showing an astonishingly high reproductive success (fathering 77% of the total offspring analysed; see [12] for further details), meaning this 'super-male' produced a copy of himself. Being a clone, the androgen probably shared the same reproductive traits leading to the high fitness of his father, meaning that spontaneous androgenesis, even occurring at a low frequency in natural populations, may lead to the emergence of extremely successful lineages of males. This finding highlights the relevance that single individuals may have to the overall dynamics of an entire population, and challenges the view that spontaneous androgenesis, due to its low incidence, is probably insignificant to the whole reproductive dynamics of natural populations.

Regarding *S. alburnoides* hybrid complex, our findings raise the question of how frequent is this quasi-sexual reproductive mode in northern populations, which depend mainly on allodiploid males to persist. On the other hand, in most southern populations, where all allodiploids found so far are females (see [13]), the putative incidence of gynogenesis (rarely observed in artificial crosses [13]) should also be investigated, since this equivalent quasi-sexual reproductive mode for females would also make these populations become independent from the sympatric bisexual *Squalius* species. Through a combination of sexual and quasi-sexual reproductive modes, *S. alburnoides* complex would become an autonomous evolutionary unit, independent from any parental species, being able to still keep its hybrid profile and to maintain a high genetic variability.

In hybrid complexes, a remarkable diversity of reproductive strategies that overcome meiotic constraints may well be the rule and not the exception, and, thus, all such truly 'open-systems' pose as excellent models to study unusual reproductive systems [6,13,32]. Whenever organisms are known to produce unreduced gametes in natural populations [33], an opportunity for the emergence of quasi-sexual reproduction is settled, since the offspring may directly get the minimum double genome (diploid condition) required in the absence of the genome of the other parent (gamete). Therefore, such organisms represent valuable windows-of-opportunity to reassess the actual expression of quasi-sexual reproduction, such as spontaneous androgenesis [1,3] in natural populations, especially in taxa in which a high incidence of natural hybridization is well known and typified.

Ethics. Both field and laboratorial procedures followed the recommended ethical guidelines [34] and conformed to the Portuguese legislation regarding animal capture, manipulation and experimentation for scientific purposes. Fish sampling was carried out with the permission of the Instituto da Conservação da Natureza e das Florestas (ICNF; permit numbers 140/2012/CAPT and 239/2013/CAPT). All efforts were made to minimize accidental deaths and stress on fish throughout the study. At the end of the study, parental fish were in good condition to be returned to the same stretch of the Ocreza River, whereas YOYs were kept in captivity for further research.

Data accessibility. GenBank accession numbers of DNA sequences: (i) β-actin P haplotype from Ocreza River, Tagus drainage, EF459400 [35]; (ii) β-actin A haplotype from Ocreza River, Tagus drainage, EF459401 [35]; (iii) mitochondrial D-loop of androgenetic PA YOY, KX910704; (iv) mitochondrial D-loop of androgenetic PA YOY father, KX910705; (v) mitochondrial D-loop of androgenetic PA YOY possible PAA mothers, KX910706–KX910728; and (vi) mitochondrial D-loop of androgenetic PA YOY possible PP mothers, KX910729–KX910737.

Authors' contributions. Conception and design: M.M.-S., L.V. and M.J.C.-P. Acquisition of data: M.M.-S. and S.C. Analysis and interpretation of data: M.M.-S., S.C. and M.J.C.-P. Drafting the article: M.M.-S. Revising the article critically: S.C. and M.J.C.-P. Final approval of the version to be published: M.M.-S., S.C., L.V. and M.J.C.-P.

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5.6 | *Supplementary material*

Supplementary Table 1 | Details on the ten microsatellites used for paternity analysis, including species of origin. Microsatellites marked with an asterisk (*) were amplified using primers with a M13 tail, as described in [28].

Loci	**Species**	**Primers (5' → 3')**	**PCR conditions**	**Ref.**
LCO1	*Luxilus cornutus*	CACGGGACAATTTGGATGTTTTAT AGGGGGCAGCATACAAGAGACAAC	**$MgCl_2$:** 1,00 mM **Annealing:** 51 °C (34 cycles)	[25]
LCO3	*Luxilus cornutus*	GCAGGAGCGAAACCATAAAT AAACAGGCAGGACACAAAGG	**$MgCl_2$:** 1,50 mM **Annealing:** 48 °C (28 cycles)	[25]
LCO4	*Luxilus cornutus*	ATCAGGTCAGGGGTGTCACG TGTTTATTTGGGGTCTGTGT	**$MgCl_2$:** 1,30 mM **Annealing:** 60 °C (31 cycles)	[25]
LC27*	*Leuciscus cephalus*	TCCAGTTCTTCCTTCCTAATT GCGGAGGGAGAGTATGTCAA	**$MgCl_2$:** 1,00 mM **Annealing:** 53 °C (23 cycles), 51 °C (10 cycles)	[27]
LC288*	*Leuciscus cephalus*	AAGAGCAGAGGAGAGCAGGG TACCTGCAGGGGCATAGGC	**$MgCl_2$:** 1,25 mM **Annealing:** 53 °C (23 cycles), 51 °C (15 cycles)	[27]
LC290*	*Leuciscus cephalus*	CCCTAATGGCCCTCAATACA ACTTCGCTGGCTTGACAAAT	**$MgCl_2$:** 1,25 mM **Annealing:** 54 °C (25 cycles), 53 °C (10 cycles)	[27]
Lsou05*	*Leuciscus souffia*	CTGAAGAAGACCCTGGTTCG CCCACATCTGCTGACTCTGAC	**$MgCl_2$:** 1,25 mM **Annealing:** 55 °C (25 cycles), 53 °C (12 cycles)	[26]
Lsou08*	*Leuciscus souffia*	GCGGTGAACAGGCTTAACTC TAGGAACGAAGAGCCTGTGG	**MgCl2:** 1,25 mM **Annealing:** 55 °C (25 cycles), 53 °C (12 cycles)	[26]
Lsou34*	*Leuciscus souffia*	CCAGACAGGGTGATGATTCC GTAGCGACGTTCAGGTCTCG	**$MgCl_2$:** 1,50 mM **Annealing:** 55 °C (25 cycles), 53 °C (8 cycles)	[26]
SpyrAMT*	*Squalius pyrenaicus*	GAAGAAAGTCTCATTGCTCTGC GAGGTCATCACCCACACCTT	**$MgCl_2$:** 1,25 mM **Annealing:** 55 °C (27 cycles), 53 °C (8 cycles)	[12]

CHAPTER 6

GENERAL DISCUSSION

Chapter 6 | GENERAL DISCUSSION

Mate choice plays a key role in hybrid asexual complexes [1-3] via male [4-25] and female [26-41] differential mate preferences, either in gynogenetic or hybridogenetic systems, allowing the coexistence of sexual and asexual lineages under the genetic constraints of clonality [42-56] and the ecological constraints of sexual parasitism [57]. Bringing polyploidy to this intricate mix of reproductive peculiarities [58-62], as occurs in many asexual systems [56,63-68], may add extra relevance to mate choice [69-74] because individuals have a wider range of mating options, including several hybrid forms and non-hybrid sexual hosts. In addition to upholding the maintenance of populations in their hybrid state, such complex gameplay of mating options may also route them towards hybrid speciation [75-95], an evolutionary pathway in which mate choice may also play a role via assortative mating [69-74,96-98], by favouring crosses among hybrids over backcrosses with parental species and, thus, directly contributing to the reproductive autonomy of the hybrids, whenever possible.

Squalius alburnoides complex brings together in a single biological entity all the complexities inherent to asexual systems, being a hybridogenetic polyploid sexual parasite with a vast range of mating options (via sexual and asexual mechanisms) among multiple host species and forms of fertile male and female hybrids, with most natural populations showing a high variability of hybrid forms, some being already on the verge of hybrid speciation [68]. Two main types of *S. alburnoides* populations may be considered according to their composition of genomotypes and consequent reproductive dynamics: triploid- and tetraploid-dominated populations. As the names suggest, triploid- and tetraploid-dominated populations are mainly composed by triploid and tetraploid genomotypes, respectively. The vast majority of wild populations are triploid-dominated, with only three populations dominated by tetraploids reported so far. While triploid-dominated populations are strictly sexual parasitic, whose reproduction is dependent on the bisexual sympatric species of the *Squalius* genus [68] (although putatively not exclusively, but further studies are needed [99]), tetraploid-dominated populations have an autonomous reproductive dynamics that dispenses the involvement of any other hybrids or sexual hosts [100,101]. Thus, *S. alburnoides* populations persisting in their hybrid state are dominated by the triploid genomotype, while those on the verge of hybrid speciation are dominated by the tetraploid genomotype. Among all these peculiarities and putative evolutionary pathways, mate choice is likely to play a major role.

6.1 | *The role of mate choice in maintaining* S. alburnoides *hybrid state*

6.1.1 | *Maintaining genetic variability*

Keeping the asexual hybrid state over evolutionary timeframes has putative genetic consequences, such as the accumulation of deleterious mutations and the reduction of genetic variability **[42-56]**. Therefore, how does *S. alburnoides* complex surpass these long-term genetic constraints, given most populations are maintained in their hybrid state, *i.e.* are triploid-dominated?

Gathering sexual and asexual reproduction through clonal, hemiclonal and non-clonal reproductive mechanisms, *S. alburnoides* is far from being a typical asexual complex. Indeed, among all its genomotypes, only allotriploid males and allodiploid males and females have lost sexuality over clonal gametogenesis, with all other genomotypes having reductional reproductive mechanisms with recombination. While triploid females undergo meiotic hybridogenesis, balanced tetraploids and nuclear non-hybrid AA males kept sexuality, performing standard meiosis as sexuals do, given the homologous character of their genomes. Thus, apart from sexual parasitism, *S. alburnoides* has few similarities with the majority of asexual complexes, characterized by strict clonality (*e.g.* parthenogenetic and gynogenetic systems), hardly fitting into the most conservative concept of asexual complexes (see **Box 1** in **Chapter 1**) **[102]**. The variable reproductive mechanisms of *S. alburnoides*, most including meiotic recombination, offer a way to avoid the genetic constraints associated with typical asexual reproduction. Moreover, *S. alburnoides* incorporates new genetic material from multiple host species into the hybrids on a generational basis **[103]**, as often occurs in hybridogenetic systems **[1,68,104-111]**, which, together with polyploidy, buffers even more long-term genetic disadvantages. However, it is arguable that *S. alburnoides* is still more prone to genetic constraints than a standard sexual species, given some genomotypes that actively participate in the reproductive dynamics of the hybrid complex are clonal, namely allodiploids. So, it is expected that natural selection would act to guarantee healthy long-term genetics in such an unquestionable successful hybrid complex as *S. alburnoides*, including via mate choice, which, for example, may strongly contribute to increase the genetic variability of the offspring **[112,113]**.

Indeed, triploid females showed a higher mate preference towards the balanced tetraploid than towards the allodiploid genomotype (**Chapter 2**), even though the resulting genomotype of the offspring in both cross types would be identical, given both these males produce the same type of gametes (*i.e.* allodiploid sperm). The difference between balanced tetraploid and allodiploid males resides on their reproductive mechanisms: while the gametogenesis of the former males includes standard meiosis with recombination, the latter undergo clonal gametogenesis. Thus, the genetic variability of the offspring produced by balanced tetraploid males would likely be significantly higher than that of allodiploid males. Mate choice based on heterozygosity may, indeed, be useful for *S. alburnoides*, not only due to the genetic constraints associated with its level of clonality as discussed above, but also because the complex inhabits Mediterranean-type rivers, characterized by a seasonally changing environment **[114]**, where genetic variability may be directly linked to higher fitness and success. Indeed, the balanced tetraploid male genomotype generally had higher reproductive success than other males when females were allowed free access to mates, and balanced tetraploid males fathered offspring with higher survival in directional crosses (**Chapter 3**). Thus, mate choice by triploid females towards balanced tetraploid males seems to guarantee genetic benefits and more successful offspring.

Mate choice by *S. alburnoides* females linked to genetic variability seems to be also based on other aspects, rather than just on the reproductive mechanisms of the males. Hybrid females coexisting simultaneously with two sympatric *Squalius* hosts (*i.e. S. aradensis* and *S. pyrenaicus*) in a tributary of the Quarteira drainage showed a deeper influence of male genetics on mate choice (**Chapter 4**). The presence of *S. pyrenaicus* in the Quarteira drainage, where only *S. aradensis* and *S. alburnoides* were thought to occur, was unexpected and was only spotted over the course of this thesis. Previous surveys on the drainage had not find the species, either because it was still absent or because samplings missed its limited distribution area. The confinement of *S. pyrenaicus* to a single tributary of the Quarteira River, suggests that its origin in this drainage is recent and likely related to a contemporary human-mediated introduction. However, an ancient migration from a nearby drainage cannot be currently discarded. One study hypothesized that *S. alburnoides* migrated from the Guadiana to the Quarteira drainage during the Upper Pleistocene, after the establishment of *S. aradensis*, when the Guadiana acquired a southward draining pattern that may have allowed drainage connections **[103]**. Thus, it is possible that *S. pyrenaicus* may have dispersed into the Quarteira drainage the same way. Irrespective of the process behind the origin of *S. pyrenaicus* in the Quarteira drainage, the presence of two bisexual *Squalius* species therein led to the production of fertile hybrids between *S. aradensis* and *S. pyrenaicus* (PQ genomotype), opening a path for gene flow and introgression between the genomes of both species (QQ and PP, respectively) and increasing considerably the range of hosts for *S. alburnoides* to sexually parasitize, among hybrids and non-hybrids. This is particularly important because the genetics of the individual males available seems highly influential of the mate preference of *S. alburnoides* females (**Chapter 4**).

Independently of their genomotype, hybrid females favoured *S. pyrenaicus* over other available males (*i.e. S. aradensis* and *S. alburnoides* nuclear non-hybrid AA males) (**Chapter 4**). Indeed, in the sympatry zone with *S. aradensis* and *S. pyrenaicus*, the majority of *S. alburnoides* hybrids (>75%) had the genome of the latter. However, further studies are needed to clarify the mechanisms behind this composition apart from mate choice by hybrid females, including quantifying the abundance of both *Squalius* bisexual species and assessing the willingness of both host male types to participate in interspecific crosses. Nevertheless, the general mate choice trend of hybrid females favouring *S. pyrenaicus* males may have different interpretations depending on the putative origin of *S. pyrenaicus* in the Quarteira drainage. In the one hand, if *S. pyrenaicus* has dispersed during the Upper Pleistocene from the Guadiana drainage together with *S. alburnoides*, hybrid females may maintain the preference towards that species, in some sort of "genomic-based assortative mate choice", in which females select males with which they share the same heterospecific genome (*e.g.* PAA females preferring PP males over QQ males), thus allowing the persistence of an independent and isolated P-based reproductive dynamics in a foreign drainage. On the other hand, if *S. pyrenaicus* was recently introduced in the Quarteira drainage via human mediation, the observed trend of hybrid females selecting these new males may indicate a tendency to reproduce with unfamiliar males, thereby increasing genetic variability. As *S. alburnoides* is a hybrid sexual parasite, it seems plausible that natural selection may have settled mate choice based on genetic dissimilarity, especially if accounting for the putative genetic constraints associated with clonal systems, as discussed above, and the fact that *S. aradensis* has a low genetic variability [115]. Indeed, *S. alburnoides* females have been shown to successfully reproduce with males of an exotic species, namely *Alburnus alburnus* [99], evidencing the capacity to successfully produce offspring in intergeneric crosses. In fact, reproducing with multiple host species has been recognized as one of the main sources to introduce genetic variability into clonal systems [1,68,104-111].

In addition, the maintenance of genetic variability via female mate choice in *S. alburnoides* may go further than choosing mates based on reproductive mechanisms and genetic dissimilarity. Indeed, mate choice by hybrid females was highly influenced by the genome purity of individual male hosts (**Chapter 4**). As stated above, in the Quarteira drainage, the coexistence of *S. aradensis* (QQ) and *S. pyrenaicus* (PP) lead to successful mating between these two species and to the production of fertile hybrids (PQ), which, through backcrosses, allowed the introgression of genetic portions, in variable levels, between the two species. Actually, gene flow was not bidirectional, with genetic portions of Q genome found within P genome, but not the reverse. This unidirectionality suggests that *S. pyrenaicus* may be more prone to crosses with hybrids than *S. aradensis*. This is also consistent with the *S. alburnoides* genomotype composition in the zone of sympatry, where the population mainly included hybrids with P genome instead of Q. Notwithstanding, the introgression of Q genetic portions into *S. pyrenaicus* increases the range of mate types available for *S. alburnoides* females, which may choose not only among *S. aradensis* and *S. alburnoides* AA males, but also among several *S. pyrenaicus* males with variable levels of Q introgression. Indeed, mate choice patterns varied according to such introgression levels (**Chapter 4**). *Squalius alburnoides* females with Q heterospecific genome showed a higher preference towards QQ males than towards PP males, but shifted their preference towards PP males if these were introgressed with Q genome. This mate choice pattern whereby females prefer unfamiliar highly heterozygotic males but avoid excessively dissimilar mates (choosing PP males with Q portions, but not pure PP males) seems likely to increase the genetic variability of the offspring, but aiming at an optimal level of genetic dissimilarity instead of increasing it blindly, thus avoiding putative genetic incompatibilities. Although *S. alburnoides* lacks strict reproductive isolation and can successfully reproduce with multiple species, it may still hold the genes responsible for avoiding genetic incompatibility in the parental species, thus possibly influencing mate preferences to some degree when choosing a mate among several genetic types.

Given the P genome was introgressed with Q genes, when *S. alburnoides* hybrids are produced from crosses with PP males, their heterospecific P genome may also hold Q genetic portions. Thus, hybrid females containing P genome also had their mate preferences influenced by their own levels of Q introgression (**Chapter 4**). Indeed, females with P genome showed a higher preference towards PP males, but that preference increased when these males had similar Q introgression levels than the choosy females. Once again, this mate choice pattern may reflect a tendency to optimize genetic variability among offspring, with descendants benefitting from high hybrid heterozygosity, while simultaneously avoiding too dissimilar mates, in some sort of “genome purity-based assortative mating”.

6.1.2 | *Upholding reproductive dynamics*

Most known *S. alburnoides* populations are triploid-dominated, characterized by a highly female-biased sex ratio **[68,116]**. Triploids depend on the sympatric bisexual *Squalius* species and on the other hybrid genomotypes to reproduce, given the reproductive interdependency among the genomotypes of the complex. Thus, the persistence of triploid-dominated populations requires a high variability of genomotypes, constantly shifting between diploid and triploid ploidy levels **[116]**. Taken into account that triploids abound in natural populations but diploids are much less common **[68]**, it may look virtually impossible to sustain such an equilibrium between diploid and triploid genomotypes in triploid-dominated populations, with this intricacy likely requiring additional mechanisms to be maintained. Mate choice seems to be one of these.

Triploid-dominated populations are maintained by a mandatorily low frequency of crosses between the dominant triploid females and males of the sympatric *Squalius* species (**Chapter 2**). Although this finding seems counterintuitive given *S. alburnoides* sexual parasitism, it is consistent with the reproductive dynamics of these populations. Contrary to the large majority of asexual complexes **[1,57,64,117-121]**, *S. alburnoides* is characterized by a multitude of reproductively interconnected and interdependent genomotypes, and, thus, triploid-dominated populations cannot rely exclusively on crosses with host species. Indeed, crosses between triploid females and males of the sympatric bisexual species lead to the production of allodiploid offspring, meaning it is virtually impossible to sustain a population dominated by the triploid genomotype if triploid females cross mainly with males of the sympatric host species. Lowering the frequency of crosses between *S. alburnoides* females and males of the sympatric host species is easily uphold by natural selection. As recurrently reported for asexual complexes **[4-6,8,9,11,13-16,19,22]**, males of the host species commonly avoid to mate with asexual females, given their null reproductive success when mating with females of lineages that do not incorporate the genes of the father into the offspring (gynogenesis) or discard them after one or two generations (hybridogenesis). If males of the *Squalius* bisexual species have evolved a mating avoidance towards *S. alburnoides* females, this may contribute to maintain triploid-dominated populations, by reducing the frequency of crosses between triploid females and male hosts, as required for population persistence (**Chapter 2**). Although mate choice by male hosts was not tackled herein as an isolated variable, our results (**Chapter 3**) suggest that these males may avoid reproducing with *S. alburnoides* females. In the one hand, directional crosses involving hybrid females and *S. pyrenaicus* males lead to unfertilized eggs, suggesting that host males prime little to no sperm in spawning events involving hybrid females, as observed in other asexual complexes **[4,5,13,14,16,22]**. Indeed, in certain fish species, non-spermiating males were found to exhibit courtship behaviours and induce spawning in mature females **[122]**. On the other hand, the absence of *S. pyrenaicus* brood when individuals had free access to hybrid and non-hybrid mates (*i.e. S. alburnoides* and *S. pyrenaicus* males and females) (**Chapter 3**) suggests that *S. pyrenaicus* males and/or females avoid reproducing in the presence of the sexual parasites. Although it cannot be discarded that *S. pyrenaicus* missed the optimal habitat conditions to mate in the tanks of directional crosses, given the absence of adequate substrate required for their optimal reproduction **[123]**, the same seems unlikely to apply to the exterior pond, where adequate substrate was available (**Chapter 3**).

Hereupon, too much crosses between *S. alburnoides* hybrid females and male hosts lead to the production of too much diploids, disrupting the dynamics of triploid-dominated populations and impairing their persistence. So, what crosses uphold the maintenance of the common populations, dominated by triploid females? Although one may think that assortative mating based on ploidy level/genomotype should stabilize these populations, as observed in other polyploid organisms **[69-74]**, it would actually shortly lead to the extinction of triploid-dominated populations, given the interdependent reproductive character of genomotypes. Apart from balanced tetraploids, which will be discussed later, no other genomotype can produce offspring of their own type by reproducing with similar partners. If, in triploid-dominated populations, the dominant triploid females mated preferentially with triploid males, it would lead to the production of unbalanced tetraploids, given the specific reproductive mechanisms of triploid males and females (*e.g.* PAA females, *a* oocytes × PAA males, *paa* sperm = PAAA offspring). In addition to these unbalanced tetraploids being extremely rare in natural populations, such mating trend would shortly disrupt the typical reproductive dynamics and genomotype composition of triploid-dominated populations. Thus, typical assortative mating, *i.e.* females mating with similar males to produce similar offspring, cannot rule triploid-dominated populations, but rather a modified version of it, in which hybrid females need to mate with dissimilar males to produce offspring of their own type, in some sort of assortative-disassortative mating (**Chapter 2**). Nevertheless, in some populations, triploid females cannot directly restock their own genomotype, since the males with which they produce triploid offspring may be absent, a reproductive constraint brought by the marked differences in genomotype composition among populations.

6.1.3 | *Triploid-dominated populations via allodiploid males*

In populations in which allodiploid males are present (Douro, Mondego and Tagus drainages), triploid females can easily and directly restock their own genomotype by reproducing with them (*e.g.* PAA females, *a* oocytes × PA males, *pa* sperm = PAA offspring). However, a very strong tendency to reproduce with these hybrid males would also imperil triploid-dominated populations, given a population only constituted by triploid individuals would not persist (**Chapter 2**), once again due to the mandatory interdependency of genomotypes. Thus, in triploid-dominated populations via allodiploid males, mate preferences must be varied, rather than favouring just a single genomotype over the others. Specifically, the only way to maintain a triploid-dominated genomotype composition in such populations, as vastly found in the wild **[68]**, is if crosses between triploid females and hybrid males (*i.e.* allodiploids and balanced tetraploids) are favoured over others, but, simultaneously, crosses with the sympatric *Squalius* hosts are not neglected (**Chapter 2**). This mate choice pattern would allow a high proportion of the triploid genomotype, while maintaining a low proportion of diploid hybrid males, necessary for the continuity of the ploidy level shifts between diploids and triploids. Indeed, this theoretical prediction was corroborated by empirical evidence, with triploid females showing a higher affiliation tendency towards hybrid males with which they produce triploid offspring, but showing also a lower, moderate preference towards the males of *Squalius* bisexual species (**Chapter 2**). Also as expected, triploid males were the last mate choice of triploid females. Moreover, these affiliation tendencies translated into variation in reproductive success in directional crosses, with triploid females laying less eggs with male sexual hosts than with hybrid males (**Chapter 3**). This trend may be explained, at least partially, by the putative low interest and consequent low fertilization rate of sexual *Squalius* males towards hybrid females, as discussed above, in a gameplay between male and female mate choices, likely drawing a positive link between egg allocation and fertilization rate.

6.1.4 | *Triploid-dominated populations via allodiploid females*

In contrast to northern and central triploid-dominated populations, triploid females to the south of the Tagus River (Sado, Guadiana, Quarteira and Almargem drainages) cannot produce their own genomotype as easily. In the one hand, the allodiploid genomotype is only constituted by females, with no hybrid diploid males ever found in a vast set of surveys across multiple drainages [68]. On the other hand, the alternative of triploid females to restock their own genomotype, *i.e.* by reproducing with balanced tetraploid males, is also difficult to accomplish, since such genomotype is very seldom found in southern populations due to the absence of allodiploid males (*e.g.* PA females, *pa* oocytes × PA males, *pa* sperm = PPAA offspring). Other indirect ways to form balanced tetraploids are also unlikely in these populations (as discussed below). Thus, the reproductive dynamics upholding the dominance of the triploid genomotype observed in all southern populations follows alternative routes. In the impossibility of producing their own genomotype, it would be expected that triploid females mated mainly with the males of the sympatric *Squalius* species, given such crosses would produce allodiploid females, which, in turn, can restock the abundant triploid females by mating with nuclear non-hybrid AA males, only present in southern populations, as previously mentioned. Although this reproductive dynamics is much more intricate than that in which allodiploid males play a role, it appears to succeed, since all southern *S. alburnoides* populations known to date are triploid-dominated. Indeed, the mate choice pattern of triploid females in that southern population where two distinct *Squalius* hosts coexisted (detailed above) revealed that *Squalius* host males were favoured over *S. alburnoides* nuclear non-hybrid AA males (**Chapter 4**). However, all females followed the same trend, even allodiploid females, which were expected to show a higher preference towards nuclear non-hybrid AA males, thus sustaining the dominance of the triploid genomotype (*e.g.* PA females, *pa* oocytes × AA males, *a* sperm = PAA offspring). These females seemed to ignore AA males similarly to all other females, suggesting that the reproductive success of these males is likely uphold by other reproductive strategies, such as sneaking behaviour, previously documented **[102]**. If AA males parasitize crosses of *Squalius* individuals, hybrids or non-hybrids, they may be able to produce *S. alburnoides* offspring with any genomotype, namely: a) AA offspring with triploid females (*e.g.* PAA females, *a* oocytes × AA males, *a* sperm = AA offspring); b) allodiploid offspring with *Squalius* host females (*e.g.* PP females, *p* oocytes × AA males, *a* sperm = PA offspring); and c) triploid offspring with allodiploid females (*e.g.* PA females, *pa* oocytes × AA males, *a* sperm = PAA offspring). Indeed, this hypothesis is consistent with previous studies reporting introgression of mitochondrial DNA from *Squalius* bisexual species into the hybrid complex only in populations in which AA males are present **[103,124]**, suggesting that these males play a major role in the reproductive dynamics of southern triploid-dominated populations, namely by parasitizing *Squalius* host females.

6.2 | *The role of mate choice in* S. alburnoides *tetraploidization and hybrid speciation*

Hybrid speciation is often a possibility among asexual organisms with males and females, since, in such situations, asexual complexes may putatively abandon sexual parasitism and establish their own independent reproductive dynamics via a multitude of ways **[100,125-129]**. This often occurs among hybrids with an even number of chromosomes, which may eventually regain homology and, consequently, bring back sexual reproduction (standard meiosis with recombination) **[100,130,131]**, thus paving the way to the arising of new bisexual species. Indeed, several extant bisexual species are likely derived from past hybrid organisms via this evolutionary route, a list that is likely underestimated given the difficulty in identifying this process if already concluded **[78]**. With a high variability of male and female genomotypes, some already including gametogenesis with standard reductional meiosis, *S. alburnoides* is undoubtedly a possible template for hybrid speciation. As discussed above, mate choice may uphold the persistence of *S. alburnoides* populations in their hybrid state, not only establishing a stable and intricate reproductive dynamics among sexual and asexual interdependent genomotypes and non-hybrid hosts, but also allowing the maintenance of a high genetic variability while countering eventual long-term genetic constraints. Overall, mate choice seems to contribute actively to avoid the extinction of *S. alburnoides*, until it eventually strides towards hybrid speciation. But does mate choice also play a role in routing such evolutionary pathway? It surely does.

Abandoning sexual parasitism, the first step towards hybrid speciation, is not a straightforward route in *S. alburnoides* triploid-dominated populations, since allodiploids, which play a key role in the reproductive dynamics of these populations (see above), can only be produced through crosses with host species. Thus, leaving sexual parasitism behind in triploid-dominated populations would imply the development of an alternative way to produce allodiploids. This could be attained via the clonal reproduction of the allodiploid genomotype through gynogenesis or androgenesis, with unreduced gametes (*e.g.* PA) developing into new individuals without incorporation of genetic material of one of the parents, which could also be a hybrid. Indeed, natural androgenesis was found in an allodiploid male of *S. alburnoides*, being the first description of this asexual reproductive mode among vertebrates in a non-manipulative approach (**Chapter 5**). In a free-access cross with a triploid hybrid female, one allodiploid male produced one descendant androgenetically (1.3% of the offspring it fathered). Although this low occurrence rate may seem insignificant, acknowledging that this allodiploid progenitor had an extremely high reproductive success, fathering 77% of the offspring analysed with distinct females (**Chapter 3**), may put the finding into another perspective. If highly successful lineages of allodiploid males develop spontaneous androgenesis, even a low proportion of androgens may be sufficient to sustain the reproductive dynamics of triploid-dominated populations via allodiploid males (**Chapter 2**). If the allodiploid males produced via androgenesis maintain the high reproductive success of their fathers and the capacity to undergo androgenesis, selection will shortly act to increase the incidence of this reproductive mode in natural populations, given such allodiploid males will produce more offspring than other males. Possibly, this process may be currently starting, with these populations being perhaps on the verge on abandoning sexual parasitism. Indeed, the emancipation of hybrids from parental species has been reported in other asexual complexes, which may be able to sustain their whole reproductive dynamics through the production of an apparently insignificant proportion of males recreated from the hybrids themselves [125,129].

Notwithstanding, the main route for hybrid speciation in *S. alburnoides* is likely via tetraploidization, since this genomotype brings together all the hard-to-combine requisites for such evolutionary pathway, namely: a) an even number of homologous chromosomes (*e.g.* PPAA); b) males and females with a balanced sex ratio; c) sexual reproduction (*e.g.* *pa* sperm and oocytes); and d) an independent reproductive dynamics excluding sexual parasitism (*e.g.* PPAA females, *pa* oocytes × PPAA males, *pa* sperm = PPAA offspring). Indeed, three *S. alburnoides* populations mainly composed by symmetrical tetraploids with balanced sex ratio have already been found in northern rivers (Lodeiro and Paiva Rivers, Douro drainage; CCAA genomotype) and in a Spanish cave (Peal del Becerro Cave, Guadalquivir drainage; PPAA genomotype), being certainly on the verge of hybrid (allopolyploid) speciation **[100,101]**. However, these populations only account for a small fraction of the *S. alburnoides* populations, with the large majority still being dominated by the triploid genomotype, even when theoretical modelling based on genomotype frequency and female mate preferences indicates a wider range of conditions allowing tetraploidization than allowing triploidization (**Chapter 2**). Thus, what may cause this discrepancy in wild populations? Several aspects seem to uphold the rarity of tetraploid-dominated populations and the abundance of triploid-dominated ones:

a. Lack of enough evolutionary time for such transition to occur, that is, the steps towards hybrid speciation may yet not have been fulfilled in most populations;

b. Although both triploid and balanced tetraploid females restock their own genomotype by reproducing with allodiploid or balanced tetraploid males, triploids are in advantage because they are highly female-biased, a leverage that leads to a higher population growth rate among the triploid genomotype;

c. The transition from triploid-dominated to tetraploid-dominated genomotype composition is not equally straightforward in all populations, depending on their reproductive dynamics. In northern populations, there is a direct route to tetraploidization via reproduction between allodiploid males and females (*e.g.* CA females, *ca* oocytes × CA males, *ca* sperm = CCAA offspring), but the lack of allodiploid males in southern populations jeopardizes this direct route, with tetraploidization requiring extra far-fetched steps. In southern populations, balanced tetraploids can only be produced via triploid males (*e.g.* PAA females, *a* oocytes × PPA males, *ppa* sperm = PPAA offspring; or PPA/PP females, *p* oocytes × PAA males, *paa* sperm = PPAA offspring), which are generally rare, thereby decreasing expressively the likelihood of tetraploidization in these populations.

According to theoretical predictions (**Chapter 2**), the occurrence of tetraploidization seems limited to triploid-dominated populations in which the sympatric

Squalius species become more frequent and/or in which triploid females show a higher preference towards the males of bisexual species than towards hybrid males. These conditions may start a sequence of events in populations with allodiploid males, ending in tetraploidization and hybrid speciation:

i. Triploid females start reproducing more with *Squalius* sexual hosts, increasing the proportion of allodiploids in the population (*e.g.* PAA females, *a* oocytes × PP males, *p* sperm = PA offspring);

ii. Consequently, there is an increase in the frequency of crosses between allodiploid males and females (*e.g.* PA females, *pa* oocytes × PA males, *pa* sperm = PPAA offspring), a trend than can ultimately be boosted by assortative mating based on ploidy level/genomotype, as observed in other polyploid complexes [69-74];

iii. Balanced tetraploids that were initially in a small frequency (**Chapter 2**), outcompete the triploid genomotype over time through assortative mating (*e.g.* PA/PPAA females, *pa* oocytes × PA/PPAA males, *pa* sperm = PPAA offspring), buffering the leverage of the female-biased sex ratio of triploids, whose females continue to mate preferentially with *Squalius* sexual hosts. The advantage of the tetraploid genomotype may be further enhanced by the putative higher survival of balanced tetraploid offspring [132] or of the offspring fathered by balanced tetraploid males (**Chapter 3**);

iv. Maintaining an independent reproductive dynamics, the balanced tetraploid genomotype increases continually in frequency until it competitively excludes other genomotypes, dominating populations and paving the way to hybrid speciation.

Thus, hybrid speciation in *S. alburnoides* seems more prone to occur in northern than in southern populations, given the genomotype composition, sex ratios and consequent reproductive dynamics in the current populations of these geographical regions.

6.3 | *Concluding remarks, critics and future research*

With a high variability of fertile male and female genomotypes reproducing through sexual and asexual reproductive modes, *S. alburnoides* allopolyploid complex poses as a template for multiple potential evolutionary pathways. Through a variety of genetic, ecological and behavioural mechanisms, this complex may persist over time in its hybrid state or evolve towards the emergence of new species trough hybrid speciation (see **Appendix 1**). The success and evolutionary plasticity of *S. alburnoides* hybrid complex is well illustrated by the variety of thriving populations found in the wild, with diverse reproductive dynamics and genomotype compositions, some already independent from sexual hosts and others maintaining their hybrid state by parasitizing multiple sympatric species. Though ignored among most hybrid asexual complexes, given the clonal nature of their reproduction, mate choice by hybrid females seems to play a key role in organizing all the reproductive intricacies of *S. alburnoides*, whatever its current population composition and evolutionary stage.

Through mate choice, females may maintain the hybrid state of the population by showing a particularly varied mate choice pattern, distributing their preference in variable degrees towards distinct genomotypes, rather than favouring a single male genomotype. This mate choice trend allows the maintenance of a high variability of genomotypes, which is essential for the persistence of the populations, given the reproductive interdependency of genomotypes. The putative long-term genetic disadvantages of maintaining the hybrid asexual state over evolutionary timeframes, namely offspring genetic uniformity and accumulation of deleterious mutations, are also buffered by mate choice. In the one hand, hybrid females showed recurrently a high preference towards males reproducing sexually, likely promoting an increase in the genetic variability of the offspring. On the other hand, hybrid females showed a mate tendency towards unfamiliar males or towards those with more heterozygotic genomes, likely evidencing a deep influence of male genetics on mate choice, while avoiding genetic incompatibility.

Mate choice by hybrid females seems, thus, to uphold the persistence of *S. alburnoides* populations in their hybrid state, not only assuring the reproductive dynamics among hybrids and non-hybrids, but also the long-term genetic health of populations, until they eventually stride towards hybrid speciation. *Squalius alburnoides* poses as an excellent template for this evolutionary route, since it accomplishes all the hard-to-combine requisites, namely the presence of hybrid males and females and genomotypes with autonomous sexual reproductive dynamics. Wild populations composed exclusively by balanced tetraploid males and females are *per se* the clear empirical evidence that the evolutionary route towards hybrid speciation is a reality for *S. alburnoides*. Mate choice by hybrid females may lead populations to tetraploidization via assortative mating, if tetraploid males and females preferentially mate with each other, with our theoretical and empirical evidence supporting this view.

In this PhD thesis, we assessed the effect of mate choice and reproductive traits on the dynamics of *S. alburnoides* allopolyploid complex. However, we acknowledge that such factors only account for a fraction of the mechanisms shaping *S. alburnoides* putative evolutionary pathways, and that other factors, even within the sphere of reproduction (*e.g.* intrasexual selection, sperm competition, etc.), are likely to share a role on the trends here reported. Thus, such issues should not be ignored in the future (see below).

Squalius alburnoides, *S. pyrenaicus* and *S. aradensis* are listed as threatened (Red Book of Portuguese Vertebrates: Vulnerable, Endangered and Critically Endangered, respectively; IUCN Red List of Threatened Species: Vulnerable, Not Evaluated and Vulnerable, respectively) **[133-135]**. This poses ethical and legal constraints to the capture of larger numbers of live specimens, thus leading to the small sample sizes used in some of the experiments. Moreover, due to the lack of sexual dimorphism, the sexing of these fishes requires direct observation of forcedly-extruded gametes, which also jeopardizes sample sizes, since both individual sexing and reproductive experiments must be performed when fish are mature, within the same period of reproductive season (March-July). This logistical issue gains further significance because a) distinct genomotypes and sympatric *Squalius* species may have slightly different peaks of reproduction over the same season, which may bias mate choice results if the experiments take place in a short period; and b) most *S. alburnoides* genomotypes are undistinguishable to the naked eye, and, therefore, specimens must be analysed in the laboratory prior to experiments, a procedure that must be performed several times before a representative sample is obtained, after multiple cycles of field sampling and laboratory analyses. All these procedures steal time to the reproductive experiments, which must be performed during the reproductive season, when fish are still able and willing to mate.

When dealing with small sample sizes, one must acknowledge that individual effects gain strength and may mask or bias the results obtained. Indeed, as evidenced in the present dissertation (**Chapter 3**), individual males may show marked differences in reproductive success even within the same genomotype, highlighting the need of a representative number of replicates to dilute such individual effects. Male samples used in our mate choice trials were smaller than the ideal, not only due to the constraints described above, but also due to the highly female-biased sex ratio of *S. alburnoides*, which further complicates the capture of larger samples of males. Small male samples meant stimulus males had to be repeatedly used in some experimental tests, which is not an ideal design, since it steals independence among trials, in addition to the risk of significant individual effects over the results. However, we tried to weaken such jeopardizing effects by reducing the putative interactions brought by individual repetitions, avoiding to use the same combinations of males whenever possible.

Moreover, replicates in experimental mate choice affiliation trials were the female individuals, and these were never used more than once.

The experimental design was also affected by difficulties in capturing balanced samples of all available female genomotypes in the studied populations. Given the dominance of the triploids, the number of allodiploid females tested (whenever present) was always smaller than that of triploid females, leading to an unbalanced experimental design. Once again, this is an unsurpassable logistical constraint associated with the dynamics of *S. alburnoides*, namely with the uneven genomotype composition of natural populations. However, our experimental designs always privileged triploid females, which dominate most natural populations and, consequently, play the biggest role in *S. alburnoides* reproductive dynamics.

Apart from genomotype composition of natural populations, other particular traits of *S. alburnoides* have posed as challenges to a balanced experimental design, such as the association between individual size and genomotype. As previously mentioned, *S. alburnoides* genomotypes and *Squalius* hosts vary significantly in size, which precludes a clear and easy discrimination between the effects of these two variables over mate preferences. Although some of our data suggest females choose based on male genomotype rather than size (see **Chapter 2**), other results were affected by this association between size and genomotype (see **Chapter 4**), being impossible to discriminate between the effects of the two. This is again an unsurpassable logistical issue inherent to *S. alburnoides* characteristics. Notwithstanding, this issue only clouds our understanding on the causes of the mate patterns retrieved, rather than on the consequences, since the outcome of females choosing males based on size or genomotype is the same (*e.g.* the effect of triploid females preferring PP males is the same regarding population dynamics, either if this choice is made based on genomotype or larger size).

Despite the hard-to-surpass logistical constraints to an ideal experimental design, we believe that the results here obtained as well as the conclusions taken in the context of this PhD thesis likely represent the reality. Moreover, such findings can serve as a solid template for future studies, namely:

a. A potentially interesting topic within the context of *S. alburnoides* mate choice would regard the type of stimuli (*i.e.* visual, chemical and/or acoustical) used by individuals, namely by hybrid females, when choosing mates, especially after the discovery of a wild population inhabiting a Spanish cave. The collected individuals presented eye atrophy and were all balanced tetraploids, being on the verge of hybrid speciation. Thus, assortative mating occurring among this genomotype seems to be based on other types of stimuli rather than visual;

b. The maintenance of *S. alburnoides* triploid-dominated populations seems to be uphold through mate choice by hybrid females, but it is likely that a major contribution of male mate choice is also at play, namely by males of the host *Squalius* species, as occurs in many hybrid asexual complexes. Mate choice by male hosts and mate choice by hybrid females are likely interacting forces driving the coexistence of *S. alburnoides* and the sympatric bisexual *Squalius* species and, consequently, the persistence of the hybrid complex. Thus, bridging together knowledge about both male and female mate processes would certainly be relevant to further understanding the dynamics of *S. alburnoides*;

c. Still under the sphere of male mate choice, future studies should also focus on the behaviour of *S. alburnoides* nuclear non-hybrid AA males. As previously referred, these males may play a central role in southern triploid-dominated populations by producing all the genomotypes commonly found in southern rivers. Thus, it is likely that the behaviour of these males contributes actively to the dynamics of such populations, either directly through mate choice or through alternative reproductive strategies, such as the already observed sneaking behaviour;

d. Long-term monitoring of the Quarteira population would be of interest to assess if the *S. pyrenaicus* genome is spreading across the drainage through *S. alburnoides* hybrid complex, following our results regarding mate choice by hybrid females. Moreover, monitoring the incidence of hybrids between *S. aradensis* and *S. pyrenaicus* would also be relevant, especially regarding the persistence of the Critically Endangered *S. aradensis*, since hybridization may imperil species and push them to extinction, as observed in other cases **[136-142]**;

e. Still focusing on the Quarteira population, deeper introgression assessments should also be performed, aimed at determining the genes responsible for the mate choice patterns observed (*e.g.* MHC complex). Furthermore, future studies should evaluate whether those genes may flow between both bisexual species and into the hybrids, and what are the consequences of the introgression of such specific genes over mate choice;

f. Although the empirical evidence retrieved herein focused solely on *S. alburnoides* triploid-dominated populations and specifically on mate choice and reproductive traits of the dominant triploid females, further work concerning the balanced tetraploids should also be performed in the future. The mate choice pattern of this genomotype should bring further light into the route towards hybrid speciation, since the settlement of assortative mating among these tetraploids should be one of the major steps. However, this mate choice pattern by balanced tetraploids is still to confirm;

g. The extent of the incidence of androgenesis in natural populations should also be assessed, especially given its potential in the independence of the hybrid complex and, consequently, in the abandonment of sexual parasitism, also a major step towards hybrid speciation. Detecting such asexual reproductive modes in the wild requires comprehensive and strategic genetic analyses, which further strengthens the need of a directional in-depth study to address this question, with a design focusing solely on the subject.

In nature, it is hard to find a biological entity with such varied on-going evolutionary pathways as *S. alburnoides*, even within the highly diverse context of hybrid asexual complexes, shredding preconceptions about their long-term viability and evolutionary potential. *Squalius alburnoides*' hard-to-combine known features challenge even the most conservative views about the role of hybridization in the diversification of species. Studying this exceptionally curious allopolyploid complex is like looking at a snapshot of evolution and, undoubtedly, a privilege to every passionate evolutionary biologist.

6.5 | *References*

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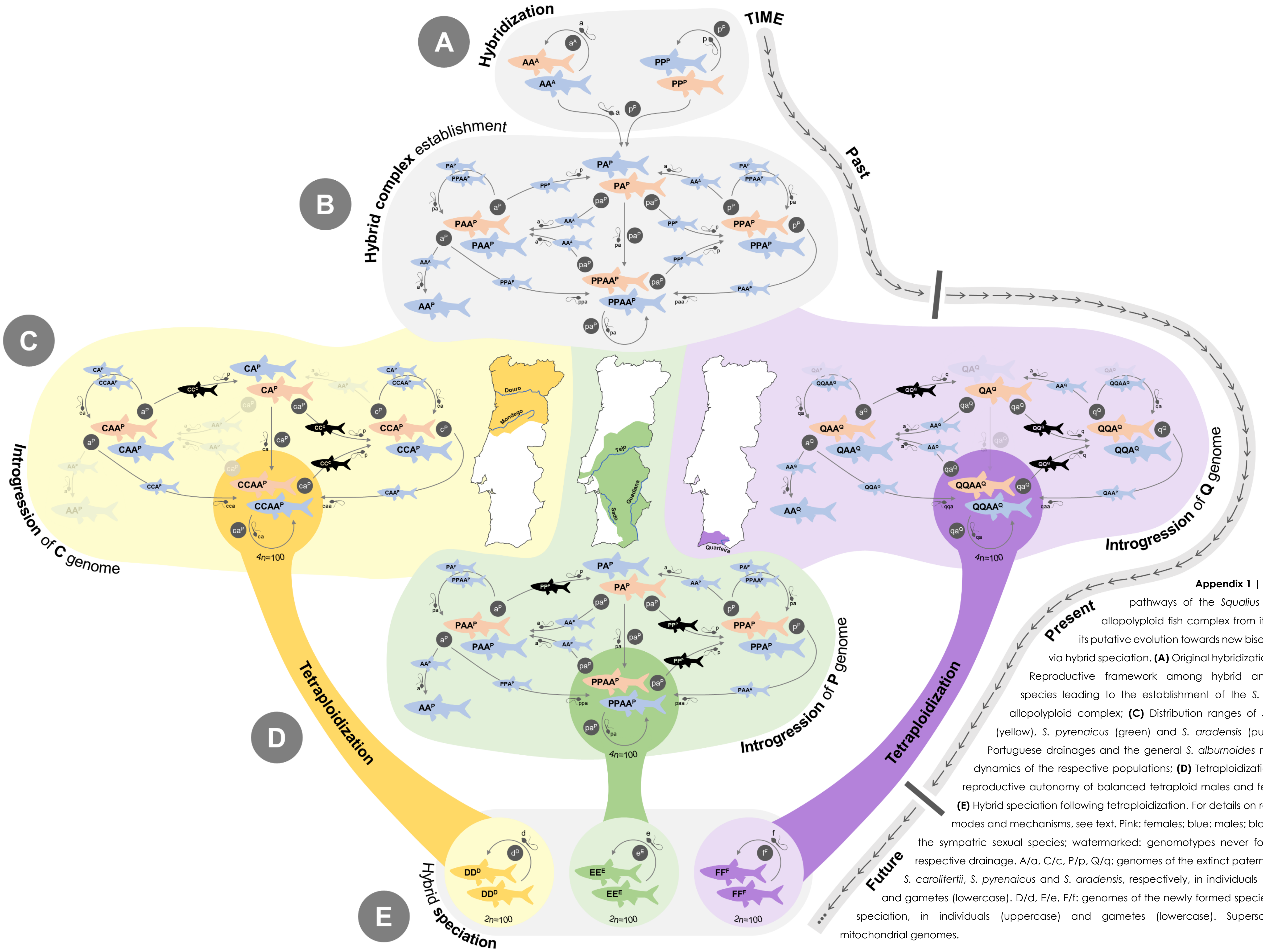
APPENDIX 1

Evolutionary pathways of the *Squalius alburnoides* allopolyploid fish complex from its origin until its putative evolution towards new bisexual species



Appendix 1 | Evolutionary pathways of the *Squalius alburnoides* allopolyploid fish complex from its origin until its putative evolution towards new bisexual species via hybrid speciation. **(A)** Original hybridization event; **(B)** Reproductive framework among hybrid and parental species leading to the establishment of the *S. alburnoides* allopolyploid complex; **(C)** Distribution ranges of *S. carolitertii* (yellow), *S. pyrenaicus* (green) and *S. aradensis* (purple) across Portuguese drainages and the general *S. alburnoides* reproductive dynamics of the respective populations; **(D)** Tetraploidization following reproductive autonomy of balanced tetraploid males and females; and **(E)** Hybrid speciation following tetraploidization. For details on reproductive modes and mechanisms, see text. Pink: females; blue: males; black: males of the sympatric sexual species; watermarked: genomotypes never found on the respective drainage. A/a, C/c, P/p, Q/q: genomes of the extinct paternal ancestor, *S. carolitertii*, *S. pyrenaicus* and *S. aradensis*, respectively, in individuals (uppercase) and gametes (lowercase). D/d, E/e, F/f: genomes of the newly formed species via hybrid speciation, in individuals (uppercase) and gametes (lowercase). Superscript letters: mitochondrial genomes.