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Chromosomal manipulation in Senegalese sole (*Solea senegalensis* Kaup, 1858): induction of triploidy and gynogenesis

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Abstract In this study we have developed protocols for induced triploidy and gynogenesis of Senegalese sole (*Solea senegalensis*), a promising flatfish species for marine aquaculture, in order to: 1) identify the sex-determination mechanism; and 2) to improve its production by generating a) sterile fish, avoiding problems related with sexual maturation, and b) all-female stocks, of higher growth rate. Triploidy was induced by means of a cold shock. Gynogenesis was induced by activating eggs with UV-irradiated sperm, and to prompt diploid gynogenesis, a cold-shock step was also used. Ploidy of putative triploid larvae and gynogenetic embryos were determined by means of karyotyping and microsatellite analysis. Haploid gynogenetic embryos showed the typical "haploid syndrome". As expected, triploid and gynogenetic groups showed lower fertilization, hatching, and survival rates than in the diploid control group. Survival rate, calculated 49 days after hatching, for haploid and diploid gynogenetic groups was similar to those observed in other fish species (0 % and 62.5 %, respectively), whereas triploids showed worse values (45 %). Sex was determined macroscopically and by histological procedures, revealing that all the diploid gynogenetic individuals were females. In conclusion, we have successfully applied chromosomal-manipulation techniques in the flatfish species Senegalese sole in order to produce triploid, haploid, and diploid gynogenetic progenies.

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Introduction

Senegalese sole (*Solea senegalensis*) is a flatfish species that in recent years has aroused great interest in the marine aquaculture in Spain and Portugal because of its relatively rapid growth rate and high price in the market, and because it could diversify traditional fish cultures, currently composed mainly of sea bass, sea bream, and turbot. However, the production of this species has several problems, some related to its reproduction in captivity. Firstly, sexual maturation (which occurs in males earlier than in females) happens usually prior to reaching market size (Zanuy et al. 2001). Gonad development during sexual maturation results in major growth reduction, increased susceptibility to diseases (in this period mortality increases) and changes in the organoleptic properties of the edible parts (Felip et al. 2006). On the other hand, as in other cultured flatfishes, such as turbot (*Scophthalmus maximus*) or Atlantic halibut (*Hippoglossus hippoglossus*), females have higher growth rates than do males (Piferrer et al. 2009), making it necessary to handle the fish in order to segregate them according to their size, with the problems that this involves. In *S. senegalensis*, these problems are aggravated because of the lack of knowledge concerning sexing. Overall, all these phenomena result in serious economic losses during marketing.

However, despite the economic importance of this species and the problems in its farming, few genetic studies have sought to improve culturing, most efforts focusing on the development of molecular markers designed to marker-assisted selection or for QTLs. All these aspects underline the need for applied chromosomal manipulation techniques, including those aimed at inducing triploidy and gynogenesis,

both naturally occurring in fish (Thorgaard 1983). Triploidy can contribute to the production of sterile specimens without the problems related to sexual maturation and in some fish species with a higher growth rate than the diploid ones (Felip et al. 2001). However, there are exceptions, such as the triploids of Siberian sturgeon, some of which can be fertile (Havelka et al. 2014) or some fish species or hybrids of Acipenseridae where the triploid condition is not associated with an increase in growth (Fopp-Bayat et al. 2007). On the other hand, producing gynogenetic individuals (both haploid or diploid) can help elucidate the basis of the sex determinism, to assign molecular markers (microsatellites loci and/or AFLPs) to linkage groups, to determine the distance to the centromeres of these markers, and even to produce all-female or all-male populations (Piferrer et al. 2009).

As in the case of other Teleostei, flatfish triploids are produced by means of temperature shock in order to retain the second polar body normally extruded after fertilization. Gynogenesis is usually induced by egg fertilization with genetically inactivated sperm using radiation or chemical treatments (to produce gynogenetic haploids), followed by heat shock (to produce gynogenetic diploids).

At present, triploids have been produced in a number of flatfish species, including Atlantic halibut *Hippoglossus* (Holmefjord and Refstie 1997), Japanese flounder or hirame *Paralichthys olivaceus* (Arai 2001), turbot *Scophthalmus maximus* (Piferrer et al. 2000; 2003), flounder *Paralichthys flexus*, plaice *Pleuronectes platessa*, and their hybrids (Purdom 1972).

On the other hand, gynogenetic offspring have been produced in hirame (Tabata 1991; Yamamoto 1992), common sole (Howell et al. 1995), southern flounder *Paralichthys lethostigma* (Luckenbach et al. 2004), turbot (Cal et al. 2006a; 2006b; Piferrer et al. 2004) and Atlantic halibut (Tvedt et al. 2006). In general, diploid gynogenetic offspring are viable in flatfish but, depending on the species, differences arise in the proportion of sexes in the progeny, indicating the presence within the group of sex-determining mechanisms both with homogametic females (XX-XY) as homogametic males (ZZ-ZW) (Cal et al. 2006b; Purdom 1972; Tabata 1991; Howell et al. 1995; Tvedt et al. 2006; Chen et al. 2009).

The aim of this study was to produce modified chromosomal progeny by inducing artificial triploidy and gynogenesis in Senegalese sole, useful for developing genetic maps, for identifying the sex-determination mechanism and its comparison with other flatfish, and for improving the production of this species.

Materials and methods

Trials were conducted at the facilities of the IFAPA Centre *Agua del Pino*, an experimental fish farm located in Huelva (south-western Spain). The experimental design is presented in Table 1. Wild Senegalese sole spawners were captured from a natural population of Huelva and stocked in batches.

Three trials were made, using different breeding pairs, in each case producing four experimental groups: haploid and diploid gynogenetic individuals, triploid individuals, and

Table 1 Treatments and results for each of the four groups of fishes

		Group			
Treatment		Gynogenetic haploid	Gynogenetic diploid	Triploid	Diploids control
Sperm treatment (UV irradiation) Egg treatment (cold shock)	Male x Female	UV (32 mJ*cm^{-2}) Untreated	UV (32 mJ*cm^{-2}) 0ºC 25 min	Untreated 0ºC 25 min	Untreated Untreated
Results					
Initial buoyant eggs	E27C x E945	150	23,000	6,400	6,400
	CF83 x DD53	700	22,500	11,250	12,750
	0B8E x D7BB	350	18,750	9,400	11,250
Fertilization rate*	E27C x E945	99 %	11 %	<1 %	100 %
	CF83 x DD53	93 %	49 %	64 %	98 %
	0B8E x D7BB	70 %	53 %	91 %	94 %
Hatching rate**	E27C x E945	61 %	71 %	<1 %	92 %
	CF83 xDD53	45 %	12 %	3 %	97 %
	0B8E x D7BB	44 %	69 %	49 %	95 %
Survival 49 DAH ***	0B8E x D7BB	0 %	62.5 %	45 %	82.5 %

Percentage of: *Embryonic buoyant eggs after 12 h/initial buoyant eggs; **Larvae after 48 h/embryonic buoyant eggs after 12 h; ***Live larvae 49d/initial hatched larvae

diploid controls. Eggs and sperm from a single female and male, respectively, were obtained in each case by stripping. Sperm was diluted 1:100 in Ringer solution (Rana and McAndrew 1989) without DMSO and kept on ice until fertilization. The eggs removed by stripping were mixed with sperm ($5 \cdot 10^3$ motile spermatozoa per egg) and were immediately activated by the addition of two volumes of seawater at 20ºC. To induce triploidy, 6.5 min after fertilization, the eggs were subjected to cold shock (0 to −1ºC) for 25 min. Then, they were incubated at 20ºC. The procedure followed to induce gynogenesis was basically as described by Cal et al. (2002), Castro et al. (2003) and (Piferrer et al. 2004) in turbot (*Scophthalmus maximus*) with some modifications. For haploid gynogenesis, eggs were fertilized with UV-irradiated sperm (32 mJ*cm^{-2}). The UV dose was adjusted previously to ensure sperm motility close to 25–50 % after irradiation. Sperm diluted in Ringer solution was irradiated on an ice plate with a CL1000 Crosslinker UVP. To induce diploid gynogenesis, eggs were fertilized with UV-irradiated sperm followed by a cold shock similar to those used to obtain triploids. Finally, a batch of eggs fertilized with unirradiated sperm and without cold shock was used as control in order to check egg viability. All batches were incubated at 20° for 48 h in filtered seawater (1 μm) and UV sterilized. Hatching typically took place over 2 days after fertilization. Initial buoyant eggs, embryonic eggs after 12 h and larvae after 48 h were counted in order to estimate the fertilization rate (embryonic buoyant eggs after 12 h/initial buoyant eggs) and hatching rate (larvae after 48 h/embryonic buoyant eggs after 12 h). After hatching, larvae were cultured at 20±1ºC and salinity of 35±2‰ under the feeding regime L100I50 described by Cañavate and Fernández-Díaz (1999). Survival was calculated 49 days after hatching as the percentage of live larvae with respect to the number of initial hatched larvae.

Ploidy was determined in embryos and larvae of the four experimental groups by means of cytogenetic (karyotyping) and molecular (microsatellite genotyping) techniques. For chromosome counting, samples of 20 embryos or larvae per group were kept in a solution of 0.005 % colchicine for 6 h. Metaphase spreads were prepared following the technique of Garrido-Ramos et al. (1994) and, slides were stained with Giemsa solution. Microsatellite analysis was performed using the markers (GATA 67, CA28, GATA 9, Mss28, Mss1, and Mss11) and conditions described by Funes et al. (2004) and De la Herrán et al. (2008). Within each experimental group, the genotypes of at least 20 embryos or larvae and their parents were determined. The ploidy condition was established by the number and the origin (paternal or maternal) of the alleles for each microsatellite locus. Thus, diploids were expected to have two alleles (one from each parent), triploids two maternal alleles and one paternal allele, gynogenetic haploids only a single allele from the mother, and gynogenetic diploids the two maternal alleles.

The sex for the diploid and gynogenetic diploid batches was determined after the first six months of culture. The sex was macroscopically recognized by dissection and also, when necessary because of the small gonad size, using standard histological procedures for light microscopy (Hendry et al. 2003).

Results and discussion

Numerous trials were conducted to produce enough viable gametes for the experiments. Most of the attempts failed due to the difficulty of obtaining eggs in their optimum ripeness or sperm mobile enough to be subjected to UV treatment. Three trials were successful. The results for each experimental group are summarized in Table 1.

Samples from each larval group or embryos were used for chromosome counting (Fig. 1). In all cases, the results were corroborated by means of six microsatellite analysis. Some examples of these checking are shown in Fig. 2a (gynogenetic haploids), Fig. 2b (gynogenetic diploids), and Fig. 2c (triploid larvae).

Most of the sampled fish showed the expected results: 100 % of the diploid controls showed two alleles, one from each parent; (79.8–83.9)% of the presumed gynogenetic haploids showed only a single allele from the female, (90.8–96)% of the presumed gynogenetic diploids consistently showed alleles from the female, i.e., one or two alleles if the mother was homozygous or heterozygous, respectively; and finally, 100 % of the triploids showed the two female alleles and one of the male alleles. In the cases of gynogenetic haploids and gynogenetic diploids the remaining percentages (20.2–16.1)%

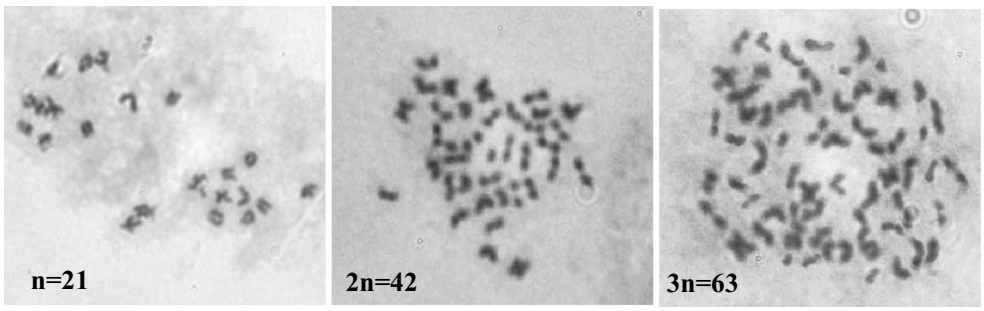

Fig. 1 Chromosome spreads at metaphase in haploid, diploid, and triploid Senegalese sole

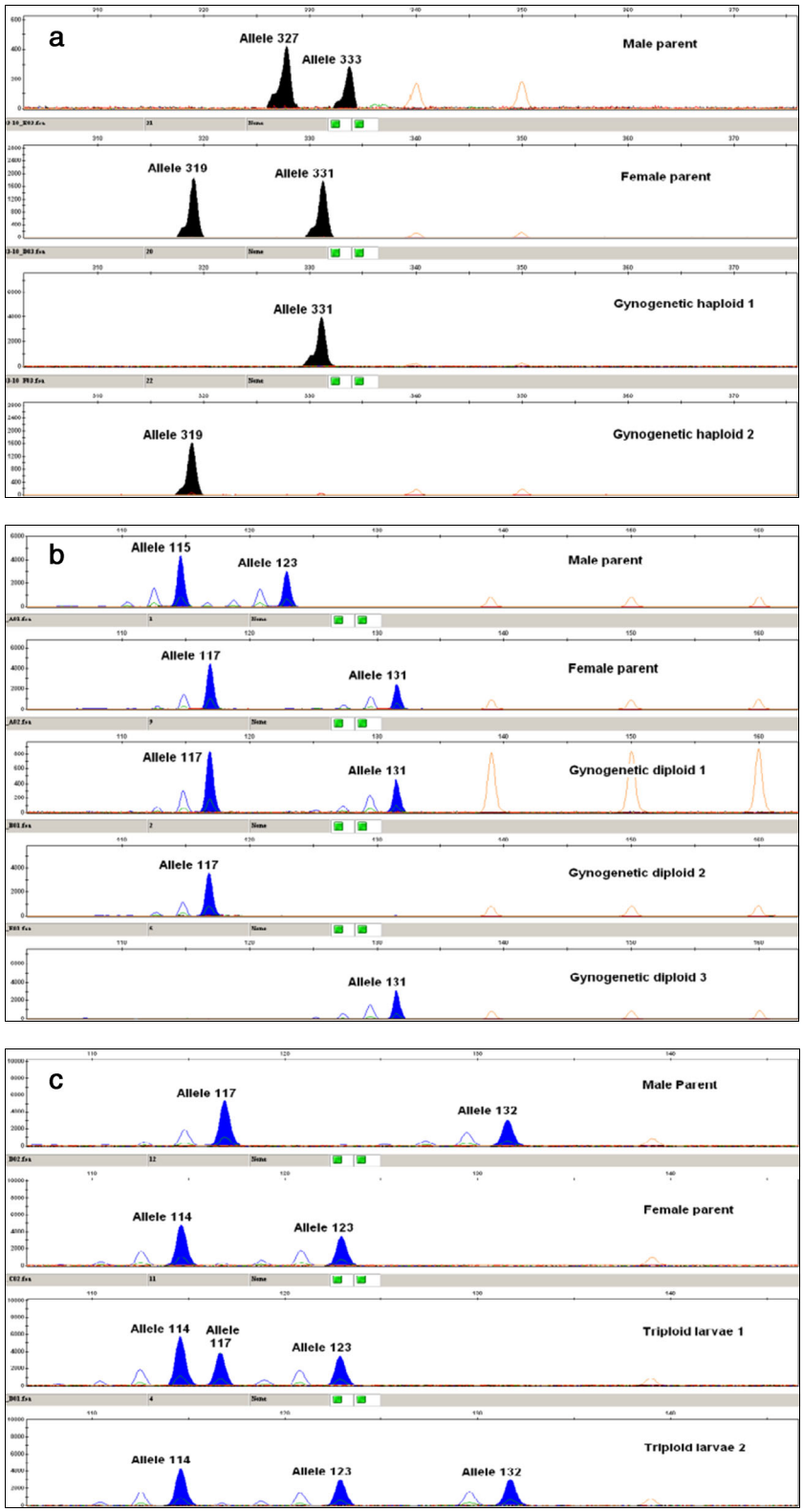

Fig. 2 **a** Chromatograms of microsatellite-DNA marker illustrating parental and filial genotypes in gynogenetic diploids Senegalese sole (checked with locus GATA67) showing only one of two allele from the female. **b** Chromatograms of microsatellite-DNA marker illustrating parental and filial genotypes in gynogenetic haploids Senegalese sole (locus checked with GATA9) showing one or two alleles from the female. **c** Chromatograms of microsatellite-DNA marker illustrating parental and filial genotypes in triploid larvae Senegalese sole (checked with locus Mss1), showing the three alleles present, two from the female and one from the male

and (9.2–4)%, respectively, showed diploid condition after analyses (Table 2). The treatment success was, therefore, similar to that reported in other flatfish. Thus, the gynogenetic diploids were detected in a mean of 94 %. These values are consistent with those of Piferrer et al. (2004) in turbot (100 %), Tvedt et al. (2006) in halibut (85 %), and Howell et al. (1995) in common sole (86 %). In relation to the latter case, our study constitutes the first time that irradiated sperm from donor

Table 2 Summary of genetic analysis carried out in individuals from the three cross

Group				
	Gynogenetic haploid (n)	Gynogenetic diploid (2n)	Triploid (3n)	Diploids control (2n)
Sex percentage	ND	100 % females (*N*=27)	ND	70 % females,30 % males (*N*=10)
Expected karyotype	100 % (*N*=20)	100 % (*N*=20)	100 % (*N*=20)	100 % (*N*=10)
Samples with expected SSR alleles	(79.8–83.9)% (*N*=235)	(90.8–96)% (*N*=75)	100 % (*N*=20)	100 % (*N*=8)

species has been used within the Soleidae family, because sperm from halibut has been used in the experiments in *Solea solea*. This hereby confirms the efficiency of UV treatment to inactivate the sperm in Senegalese sole, with results slightly better than for common sole (*S. solea*).

In this study, 100 % triploid progenies in Senegalese sole were produced using cold-shock treatment. Similar percentages of success have been reported in other flatfish species using similar protocols: plaice (*Pleuronectes platessa*), with 100 % triploid progeny (Purdom 1972); turbot, 90–100 % triploidy (Piferrer et al. 2004) and halibut, 84–95 % (Holmefjord and Refstie 1997). However, in a species as close to Senegalese sole as common sole, it was not possible to produce triploids by pressure-shock treatment (Howell et al. 1995).

The control group showed high fertilization and hatching rates (with means of 97.3 %, 94.6 %, respectively), and high survival rate in the crossing (0B8ExD7BB) in which it was calculated (82.5 %). However, the other three groups, corresponding to the progeny from the egg and/or sperm treatment (gynogenetic haploids and diploids, and triploids) showed generally lower rates than for the control group (Table 1). Thus, haploid gynogenesis showed a high fertilization rate (mean=87.3 %) but, as expected, all individuals died quickly, within 12 h after hatching. On the contrary, diploid gynogenesis registered the lowest fertilization rate (means=37.6 %) and the highest survival rate (62.5 %) within the non-control groups. On the other hand, triploids were intermediate between the two gynogenetic groups for fertilization and survival rates, but the hatching rate was low for this group (mean=17.6 %).

The survival rate (see Table 1) in the gynogenetic diploid group of Senegalese sole was basically similar to those reported in other fish species (Piferrer et al. 2009).

The fertilization and hatching rates were highly variable between the different trials in the groups treated by cold shock, gynogenetic diploids (11 to 53 % and 12 to 71 %, respectively), and triploid (<1 to 91 % and <1 to 49 %, respectively), indicating that this is a critical step for the success of manipulation techniques in Senegalese sole (Table 1).

Gynogenetic haploids were easily recognizable by the haploid syndrome (Fig. 3): short thick body with poorly developed tails and small underdeveloped eyes (Purdom 1969). These haploid progenies showed null viability after hatching. Diploid gynogenetic progenies have high levels of homozygosity (Leary et al. 1985; Don and Avtalion 1988), being more susceptible to recessive mutations; this, together with the manipulations needed for their generation (stripping, sperm UV treatment, artificial fertilization, cold shock), seriously affected their viability, with survival values of less than 10 % of control (Piferrer 2007). However, despite the low survival rate of gynogenetic individuals, they are useful in breeding. Thus, the diploid gynogenetic individuals can be used as broodstocks in species where the female is the homogametic sex, in order to produce all-female populations. Additionally, being highly homozygotic, they are useful in other genetic and genomic applications for producing cloned fish lines (Thorgaard et al. 2002) or in developing genetic maps to calculate the distance of the markers to the centromere by half tetrad analyses, for example in flatfishes such as turbot (Martínez et al. 2008), halibut (Reid et al. 2007), tongue solea (Ji et al. 2009), and Senegalese sole (Molina-Luzón et al. 2014). In the same sense, although they are not viable, the haploid gynogenetic embryos are used to estimate the distance between molecular markers in these types of studies in turbot (Bouza et al. 2007, 2012) and Senegalese sole (Molina-Luzón et al. 2014).

The survival rates found in this study for triploids larvae after hatching of Senegalese sole were highly variable (<1 to 49 %), being in the best of cases appreciably lower than reported in other flatfish species; for example, survival in turbot triploids matched the 60–80 % found in the diploid fish (Piferrer et al. 2000; 2003), whereas these values were 20–82 % for halibut (Holmefjord and Refstie 1997). In our case, although mortality was high in two cases, it was the first time that triploid progeny have been induced in a *Solea* species and, at a percentage of 100 %. As described in Materials and methods, we followed the protocol developed in turbot. These results, with high success but high mortality, suggest that the method for obtaining Senegalese sole triploids should be adapted in the future for this species, in order to raise survival rates. Finally, sex determination by means of dissection and histological analysis revealed that all the diploid gynogenetic individuals sampled were female, indicating that the male is probably the heterogametic sex. This result is similar to that found in other flatfishes such as Japanese flounder (Yamamoto 1999) and Atlantic halibut (Tvedt *et al.* 2006). However, a study made with a most closely related species,

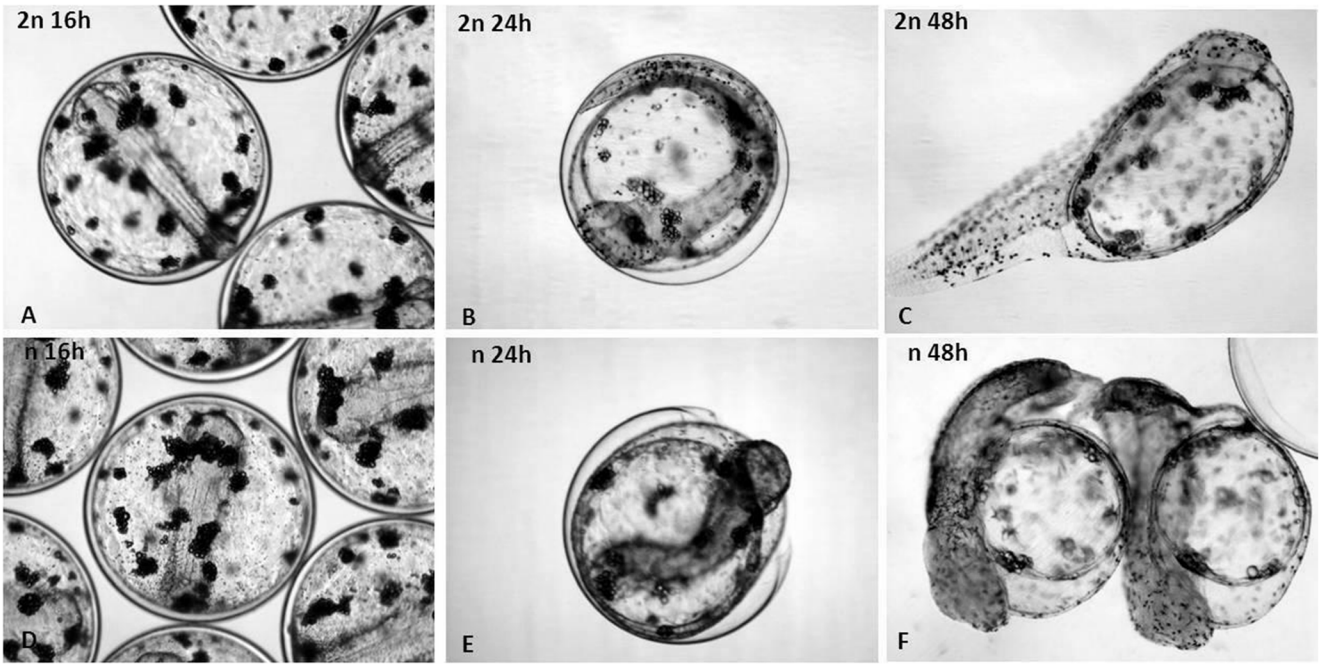

Fig. 3 Appearance of Senegalese sole eggs and fry from the diploid control and haploid gynogenetic fish groups. The haploid embryos showed the typical syndrome, characterized by abnormal body shortening and underdevelopment of the head

common sole (*S. solea*), showed that gynogenetic progeny were composed of both sexes, 60 % males and 40 % females (Howell et al. 1995), whereas in tongue sole (*Cynoglossus semilaevis*) the female has been demonstrated to be the heterogametic sex (Chen et al. 2009), showing a ZW sex-determination system. Meanwhile, in the case of turbot, contradictory results have been found by different authors, some indicating that females are the homogametic sex (Cal et al. 2006b) and others indicating that males are (Haffray et al. 2009). These results suggest that sex determination in these flatfish species could not be explained only by a simple XX-XY system but rather also involves alternative systems and genetic or environmental factors, such autosomal genes or temperature (Baynes et al. 2004; Piferrer et al. 2009, 2012). However, segregation patterns, of a microsatellite marker of LG5 and associated with the sex-determination region in turbot, support a ZZ/ZW mechanism in some of the families analyzed (Martínez et al. 2009).

The main advantage of producing triploid fishes lies in that they are sterile specimens and therefore do not present the problems related to sexual maturation and have a higher growth level than the diploid ones; this is true of species such as the turbot and the flounder (*Platichthys flesus*). However, in the present experiment, hybrid triploids (flounder x plaice), grew less than diploids (Lincoln 1981; Felip et al. 2001). At the moment, it is unknown whether this is the case for Senegalese sole, although this situation could be checked at one time to note improvement of the survival rates of the triploids in this species.

Producing gynogenetic progeny (both haploids and diploids) constitutes a useful tool for developing linkage maps, allowing us to calculate the distance between markers (haploids) and to the centromere of the markers by half-tetrad analysis (diploids). In fact, using both types of offspring produced here, our group is developing the first linkage map for Senegalese sole (Molina-Luzón et al. 2014). Such maps are essential, for example, for the development of molecular-assisted breeding programs, and have been used in other flatfish species such as turbot (Bouza et al. 2007). The use of diploid gynogenesis also offers promising options to improve production, as for example the production of "neomales", genetic female fish that produce sperm instead of viable eggs after being hormonally masculinized. In species in which the female is the homogametic sex (as appears to be the case of the Senegalese sole) all the gynogenetic diploids are female that can be converted into neomales and used as broodstock. The goal would be to produce all-female populations, these being more economically profitable. This approach has been successfully used in Japanese flounder (Yamamoto 1999) and Atlantic halibut (Hendry et al. 2003; Tvedt et al. 2006), together with other non-flatfish species. Studies by our group in the near future will determine whether these techniques can be easily applied in order to improve Senegalese sole production.

In conclusion, protocols for inducing artificial triploidy and gynogenesis have been developed for the first time in Senegalese sole, making it feasible to develop genetic tools such as linkage maps and to produce sterile and/or all-female stocks in

the near future. The results suggest that in this species, the female is the homogametic sex.

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